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Electrochemical and EPR Characterization of 1,4-dihydropyridines. Reactivity Towards Alkyl Radicals

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This work reports the electrochemical oxidation of a series of three synthesized 4-substituted-1,4-dihydropyridine derivatives in different electrolytic media. Also, an EPR characterization of intermediates and the reactivity of derivatives towards ABAP-derived alkyl radicals are reported.

Dynamic, differential pulse and cyclic voltammetry studies on a glassy carbon electrode showed an irreversible single-peak due to the oxidation of the 1,4-dihydropyridine (1,4-DHP) ring via 2-electrons to the corresponding pyridine derivative.

Levich plots were linear in different media, indicating that the oxidation process is diffusion-controlled. Calculated diffusion coefficients did not exhibit significant differences between the derivatives in the same medium.

The oxidation mechanism follows the general pathway (electron, H^+, electron, H^+) with formation of an unstable pyridinium radical. One-electron oxidation intermediate was confirmed with controlled potential electrolysis (CPE) and EPR experiments. On applying *N-tert*-butyl-α-phenylnitrone (PBN) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) as the spin trap, these unstable radical intermediates from the oxidation of 1,4-DHP derivatives were intercepted. The final product of the CPE, i.e. pyridine derivative, was identified by GC–MS technique.

Direct reactivity of the synthesized compounds towards alkyl radicals was demonstrated by UV–Vis. spectroscopy and GC–MS technique. Results indicate that these derivatives significantly react with the radicals, even compared with a well-known antioxidant drug such as nisoldipine.

Keywords: 1,4-Dihydropyridines; Oxidation; EPR; Alkyl radicals

INTRODUCTION

4-Substituted Hantzsch 1,4-dihydropyridines (1,4-DHPs) of the PyH type, i.e. those having at least a single hydrogen atom at position 4, are very interesting compounds not only from the point of view of the heterocyclic chemistry, but also as pharmacologically active substances, as antioxidants[1,2] and, as NADH coenzyme analogues, which mediate the hydrogen transfer in biological systems.[3–5] More recently,[6–8] the oxidation of Hantzsch 1,4-DHPs has attracted more attention from chemists because of its reactivity with endobiotics such as nitric oxide, coincident with the discovery of its active roles in a wide range of human physiological processes.[9]

In humans, 1,4-DHP derivatives undergo rapid and extensive hepatic oxidative metabolism by cytochrome P450 enzymes, giving rise to pyridine analogues that are pharmacologically inactive and further biotransformation including ester cleavage.[10,11] Concerning the mechanism of 1,4-DHP electro-oxidation, a number of studies have been already published.[12–16] Ludvik *et al.*[17] studied the influence of substituents on the oxidation potentials and proposed a mechanism for the oxidation of 1,4-DHPs. On the other hand, Ludvik *et al.*[18] proved that 4-disubstituted 1,4-DHPs are electrochemically oxidized in acetonitrile in a process giving rise to 4-substituted pyridines or pyridinium cations.

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As above-mentioned, the antioxidant character of this type of molecules has been previously studied.[19] In particular, the antioxidant effects of 1,4-DHPs have been well recognized, including the protective effects in peroxidation of membranes induced by alkyl radicals.[20] As several properties of 1,4-DHP derivatives such as their pharmacological activity, photostability, biotransformation and reactivity with free radicals involve oxidation of the dihydropyridine moiety, a systematic voltammetric study on the oxidation of three newly synthesized 1,4-DHP derivatives in different media has been carried out. In order to study the influence of different substituents at the 4-position, we have synthesized compounds with alkyl or aryl substituents at this site (Fig. 1). In order to demonstrate that the radical intermediates are generated in the electro-oxidation process, spin trapping studies were done. Furthermore, the reactivity of the synthesized compounds towards ABAP-derived alkyl radicals is also reported.

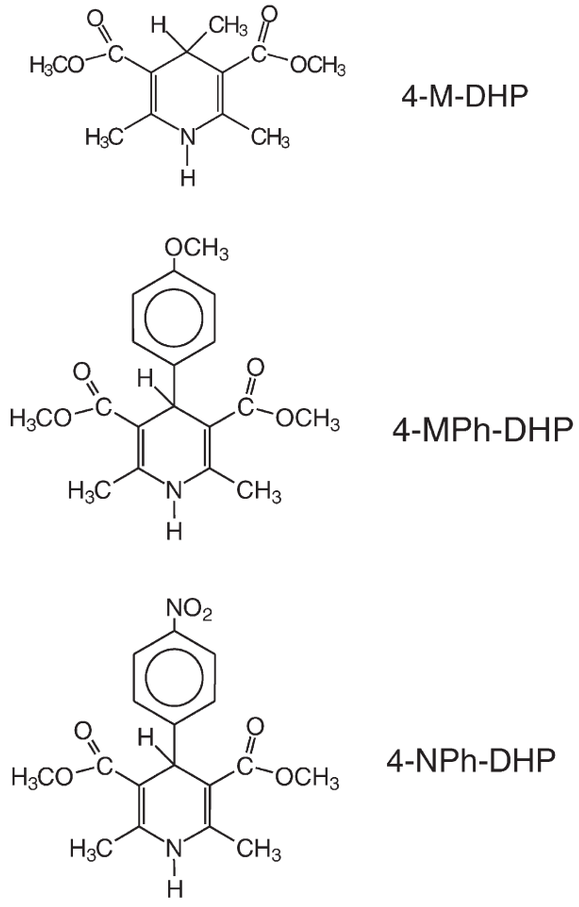


FIGURE 1 Chemical structures of the synthesized compounds. 4-M-DHP: 3,5-dimethoxycarbonyl-2,6-dimethyl-4-methyl-1,4-dyhydropyridine, 4-MPh-DHP: 3,5-dimethoxycarbonyl-2,6-dimethyl-4-(*p*-methoxy-phenyl)-1,4-dihydropyridine, 4-NPh-DHP: 3,5-dimethoxycarbonyl-2,6-dimethyl-4-(*p*-nitro-phenyl)-1,4-dihydropyridine.

EXPERIMENTAL

Chemicals

All solvents were of high-pressure liquid chromatography (HPLC) grade and all reagents were of analytical grade.

Compounds

All 1,4-DHP derivatives (Fig. 1) were synthesized in our laboratory according to previous works.[21,22]

General Procedure. A mixture of 0.079 mol of methyl acetoacetate and 10 ml of concentrated ammonium hydroxide in 20 ml of ethyl alcohol was heated under reflux for 2.5 h. The resulting clear solution was added to a mixture of 0.079 mol of methyl acetoacetate, 0.165 mol of aldehyde (4-M-DHP = acetaldehyde; 4MPh-DHP = 4-methoxybenzaldehyde; 4-NPh-DHP = 4-nitrobenzaldehyde), 25 ml of concentrated ammonium hydroxide and 20 ml of ethyl alcohol and maintained under reflux for 15 h. The crude solid product was filtered and recrystallized in ethyl alcohol. The yields were in the 80–90% range depending upon the derivative. The following compounds were prepared using this general procedure:

4-Methyl-2,6-dimethyl-3,5-dimethoxycarbonyl-1,4-dihydropyridine (4-M-DHP)

IR (KBr): ν_{max} *3342, 2950, 1680, 1650, 1435, 1351, 1226, 1056 cm*^{1} ^{1}*H NMR (300 MHz, CDCl*$_3$*):*δ *0.96 (d, 3H, J6.5 Hz, CH–CH*$_3$*), 2.29 (s, 6H, –CH*$_3$*), 3.73 (s, 6H, –O–CH*$_3$*), 3.83 (q, 1H, J6.5 Hz, CH–CH*$_3$*), 5.73 (s, 1H, –NH–)* ^{13}*C NMR (75 MHz, CDCl*$_3$*): 20.353, 23.195, 29.295, 51.924, 77.524, 77.948, 78.371, 105.267, 145.644, 169.191. Anal. Calcd. for* $C_{12}H_{17}NO_4$*: C, 60.25; H, 7.13; N, 5.86. Found: C, 60.27; H, 7.23; N, 5.87. m.p: 147–149°C.*

4-(4-Methoxyphenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-1,4-dihydropyridine (4-MPh–DHP)

IR (KBr): ν_{max} *3349, 2949, 1697, 1650, 1431, 1383, 1251, 1213, 1027 cm*^{1} ^{1}*H NMR (300 MHz, CDCl*$_3$*):* δ *2.33 (s, 6H, –CH*$_3$*), 3.65 (s, 6H, –O–CH*$_3$*), 3.75 (s, 3H, Ar–O–CH*$_3$*), 4.94 (s, 1H, Ar–CH), 5.76 (s, 1H, –NH–), 6.75 (d, 2H, J8.6 Hz, Ar–H), 7.2 (d, 2H, J8.6 Hz, Ar–H).* ^{13}*C NMR (75 MHz, CDCl*$_3$*): 19.513, 38.309, 50.937, 55.052, 76.540, 76.963, 77.387, 104.000, 113.300, 128.530, 139.862, 143.926, 157.852, 168.056. Anal. Calcd for*

$C_{18}H_{21}NO_5$: C, 65.24; H, 6.39; N, 4.23. Found: C, 65.00; H, 6.47; N, 4.36. m.p: 181–183°C.

4-(4-Nitrophenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-1,4-dihydropyridine (4-NPh–DHP)

IR (KBr): *ν_{max} 3343, 2948, 1703, 1655, 1518, 1434, 1384, 1347, 1218, 1020 cm^1 ^{1}H NMR (300 MHz, $CDCl_3$):* δ *2.38 (s, 6H, $-CH_3$), 3.66 (s, 6H, $-O-CH_3$), 5.12 (s, 1H, Ar–CH), 5.85 (s, 1H, –NH–), 7.46 (d, 2H, J8.8 Hz, Ar–H), 8.12 (d, 2H, J8.8 Hz, Ar–H). ^{13}C NMR (75 MHz, $CDCl_3$): 20.627, 40.729, 52.112, 77.515, 77.938, 78.362, 103.916, 124.388, 129.527, 145.811, 147.294, 155.643, 168.378. Anal. Calcd for $C_{17}H_{18}N_2O_6$: C, 58.96; H, 5.24; N. 8.09. Found: C, 58.76; H, 5.03; N, 8.25. m.p: 165–168°C.*

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

FT–IR: The IR spectra were recorded on a Bruker IFS 55 Equinox spectrometer.

Elemental analyses were performed on a Fisons Instrument equipment.

Electrolytic Media

In protic media, all compounds were dissolved in 30% ethanol and diluted with 0.04 M Britton–Robinson buffer solution to obtain final concentrations varying between 0.05 and 5 mM. The aprotic media consisted of a solution of tetrabutylammonium hexafluorophosphate (TBAHFP) in acetonitrile (0.1 M) for the voltammetric experiments. A routine concentration of 1,4-DHP derivatives of 100 μM was used. Solutions were bubbled with extra pure nitrogen (Indura, $H_2O < 3$ ppm, $O_2 < 2$ ppm $H_nH_m < 0.5$ ppm) before each determination.

Voltammetry

Differential Pulse (DPV), cyclic (CV), and linear sweep voltammetry (LSV) were performed with a BAS CV50 assembly. A glassy carbon stationary electrode as working electrode for DPV and CV experiments was used. For dynamic experiments, a glassy carbon rotating disk electrode was also employed. A platinum wire was used as a counter electrode, and all potentials were measured against an Ag/AgCl electrode.

Controlled Potential Electrolysis

CPE were carried out on a platinum mesh electrode in anhydrous acetonitrile + 0.1 M TBAHFP at +0.99, +1.06, and +1.20 V for 4-M-DHP, 4-MPh-DHP, and 4-NPh-DHP, respectively. Oxygen was removed with pure and dry pre-saturated nitrogen. A three-electrode circuit with an Ag/AgCl electrode was used as reference and a platinum wire as a counter electrode. A BAS-CV 50 assembly was used to electrolyze the different derivatives.

UV–Vis

The progress of the electrolysis or reactivity with free radicals was followed spectrophotometrically using an UNICAM UV-3 spectrophotometer. An electrolytic UV–Vis. cell of our own construction with a Pt foil as a working electrode was used for spectro-electrochemistry measurements.[23] UV–Vis. spectra were recorded in the 220–700 nm ranges at different intervals. Acquisition and data treatment were carried out with Vision 2.11 software.

Electrolysis-EPR

The EPR spectra were recorded *in situ* on a Bruker ECS 106 spectrometer with 100 kHz field modulation at X band (9.78 GHz) and room temperature. The hyperfine splitting constants were estimated to be accurate within 0.05 G. The electrolysis was performed in the EPR cell using a platinum mesh electrode and the conditions described above. The concentration of the 1,4-DHP derivatives was: 1 mM for 4-M-DHP derivative and 10 mM for 4-MPh-DHP and 4-NPh-DHP. The concentration of the spin trap, *N-tert*-butylamine-α-phenylnitrone (PBN) and 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) were at least one hundred times higher.

Coulometric Analysis

These studies were based on exhaustive electrolysis at constant electrode potential at +0.99, +1.06 and +1.20 V (vs. Ag/AgCl) for 4-M-DHP, 4-MPh-DHP and 4-NPh-DHP, respectively, on a glassy carbon coil electrode. The measurements were carried out on a BAS CV 50 analyzer using a two compartment electrolytic cell and the derivatives were dissolved in 0.04 M Britton–Robinson buffer/Ethanol 70/30 for the aqueous media and acetonitrile + 0.1 M TBAHFP for the non-aqueous media. The net charge was calculated by correction for the estimated background current.

Reactivity Towards Alkyl Radicals

ABAP (2,2′-azobis (2-amidinopropane) dihydrochloride, Aldrich Chemical Company) was used as an alkyl radical generator. Solutions of 20 mM ABAP in 0.04 M Britton–Robinson buffer pH 7.4 were incubated with 100 μM solutions of each 1,4-DHP or vitamin E at 37°C for 120 min with constant bubbling of nitrogen. The rate of alkyl radical formation from this initiator is constant at a given temperature.[24]

However, the rate of alkyl radical formation from ABAP will not be constant as it depends upon 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 will be approximately constant, i.e. 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.[25]

Control solutions containing either 1,4-DHPs or vitamin E solutions were run under the same conditions as the above mixtures. The time-course of reaction of the synthesized 1,4-DHP derivatives with the generated alkyl radicals was followed by UV–Vis. spectroscopy and GC–MS technique.

By UV–Vis. spectroscopy, the following bands were followed: 350 nm for 4-M-DHP, 4-MPh-DHP and nisoldipine; 290 nm for 4-NPh-DHP and 300 nm for vitamin E (Trolox), respectively. Concentrations of the drugs in 0.04 M Britton–Robinson buffer/Ethanol 70/30 at pH 7.4 were determined from the respective calibration curves (10–100 μM).

The reactivity towards alkyl radicals was expressed comparatively either with vitamin E or nisoldipine using the following ratio:

$$\text{Relative reactivity} : \text{Slope 1,4-DHP tested} / \text{Slope vitamin E or Nisoldipine}$$

where, slope 1,4-DHP and slope vitamin E or nisoldipine means slopes of concentration–time plots of both the tested 1,4-DHP derivatives and vitamin E or nisoldipine, respectively, in the presence of alkyl radicals.

Under the experimental conditions, photodecomposition of 1,4-DHPs was negligible.

GC–MS

A Gas Chromatograph/Mass Selective Hewlett Packard 5890/5972 Detector (Palo Alto, California, USA) and Hewlett Packard 7673 Auto sampler were used for these measurements. A Hewlett Packard Pentium II Data System-Laser Jet 4000 printer, controlled instrumentation and data handling was also used.

Chromatography column. Hewlett-Packard Ultra-1 column, 25 m × 0.2 mm i.d. × 0.11 film thickness (Little Falls, Wilmington, Delaware, USA).

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}.

Temperature program. The oven temperature was programmed from 130 to 305°C (1 min) at 15°C min^{-1}; run time, 16.67 min. Helium was used as carrier gas with an inlet pressure of 35 kPa. The mass range monitored was 45–550 amu with a scan rate of 1 scan/s and the ionization energy was set at 70 eV.

Peak identification relies upon full spectra comparison of the test samples with certified reference material analyzed within the same batch. There must be a complete agreement with both acceptable chromatography and mass spectrometry. After electrolysis and reactivity reaction, the final solutions were diluted and injected without any pre-treatment. No extraction or filtration procedures were necessary.

RESULTS AND DISCUSSION

In order to study the influence of 4-substitution in 1,4-DHP, we have synthesized three different compounds wherein alkyl or aryl substituents are interchanged in this position.

The oxidation of 1,4-DHP derivatives was studied using a glassy carbon electrode as a working electrode in different electrolytic media (aqueous and aprotic). Also, the reactivity of the synthesized 1,4-DHP derivatives towards alkyl radicals was assessed in aqueous media at pH 7.4.

Aqueous Media

Voltammetry

All compounds produced a very well-resolved voltammetric peak at all pHs examined.

Electro-oxidation of three derivatives was affected by pH changes. Thus, peak potential values were shifted towards less anodic potentials by increasing pH. In the alkaline range, oxidation was easier than at acidic pHs. Ep–pH plots corresponding to compounds exhibited two breaks (Fig. 2). As can be seen, at acidic pHs, potential peak values remain practically unchanged, suggesting that no proton transfer reactions are involved previously to the rate-determining step. However, at pH > 4, the peak potential results are pH-dependent consistent with proton transfer occurring prior to, or in, the rate determining step. In Table I, the effect of 4-substitution on the electro-oxidation at different pHs is shown. As can be seen from this table, 4-M-DHP and 4-MPh-DHP derivatives are more readily oxidized than 4-NPh-DHP. This is consistent with the electron-donating character of the methyl group and *p*-methoxy-phenyl groups when compared to the *p*-nitro phenyl group.

The above experiments show that 4-substitution strongly affects the oxidation of the DHP ring.

In order to check the voltammetric results at a different time scale of the experiments, CV was also tested (i.e. sweep rate of 20 mV/s DPV mode vs. 0.1–10 mV/s in CV).

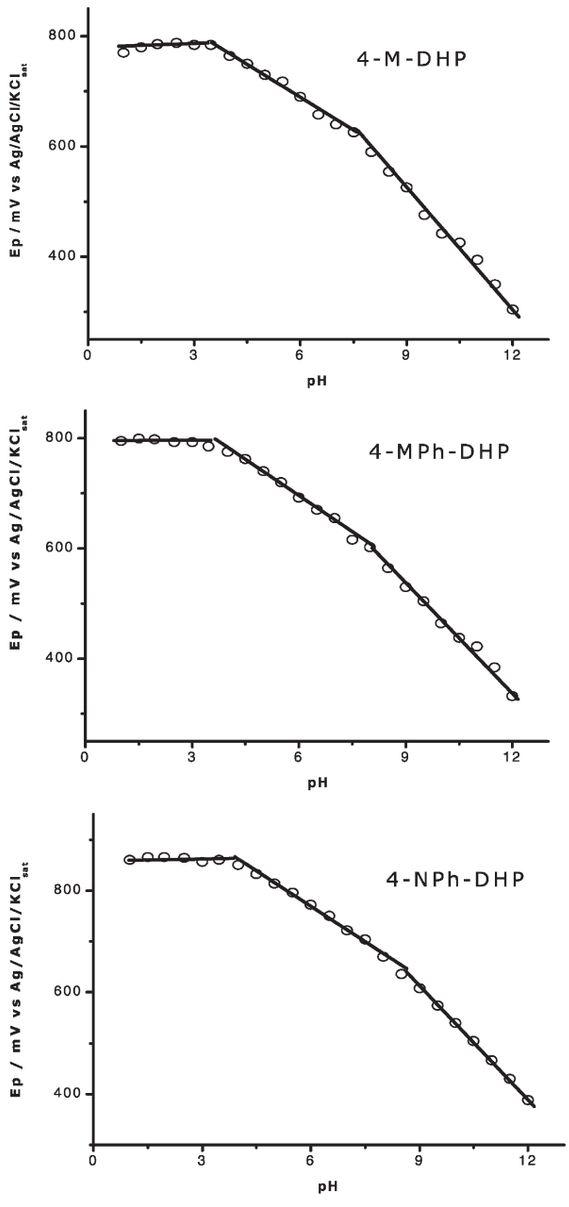


FIGURE 2 Dependence of peak potentials on pH for a 100 μM concentration of 1,4-dihydropyridines in protic media (0.04 M Britton–Robinson buffer + ethanol: 70/30).

From the studies at different sweep rates (0.1–10 V/s) and pHs (2.4, 7 and 12), for the three compounds, a single irreversible anodic signal was observed (Fig. 3). In all the cases, log *ip* vs. log *V* plots exhibited slopes close to 0.5, indicating the current is diffusion-controlled. Furthermore, the peak potential values (E_p) were dependent on the sweep rates, indicating the irreversible character of the process. The oxidation process did not show differences in the time scale of the DPV or CV experiments.

A study of the behavior of the limiting current was carried out by using dynamic voltammetry on a glassy carbon rotating disk electrode. As can be seen in Fig. 4, all the studied compounds show a very well-resolved voltammetric wave at the RDE. The $E_{1/2}$ values showed the same trend as peak potentials obtained by DPV. In Fig. 4, the behavior of the limiting current with pH at fixed rotating rate is shown. We can conclude that at acidic pH

TABLE I Comparison between the peak potentials values obtained by DPV on glassy carbon electrode at different pH

	E_p/mV		
Compound	pH 3.0	pH 7.4	pH 12.0
4-M-DHP	784	640	304
4-MPh-DHP	793	655	332
4-NPh-DHP	858	722	388

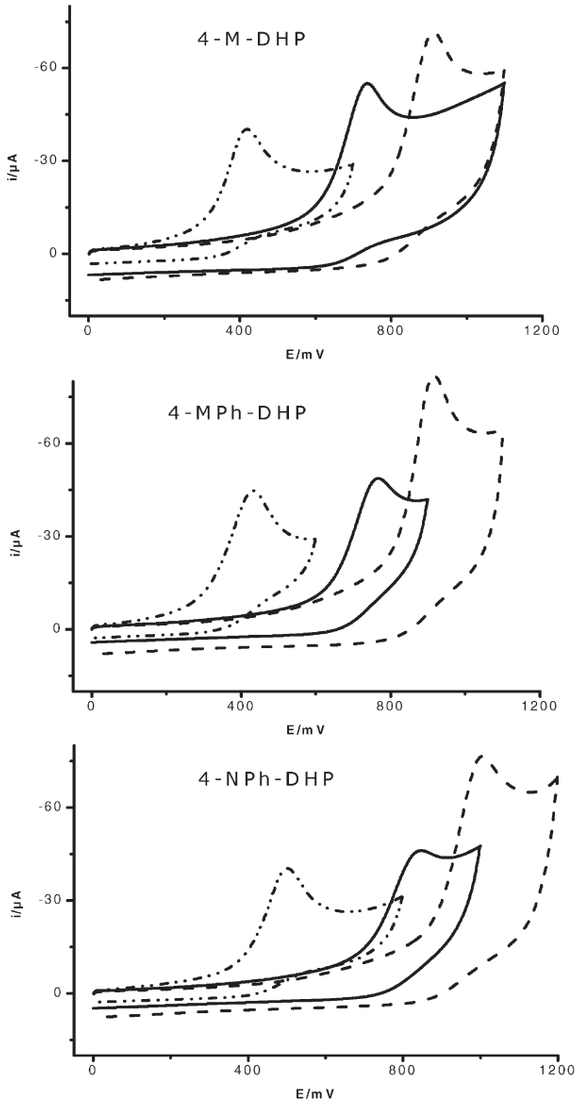


FIGURE 3 Effect of pH on the cyclic voltammograms for 1 mM solution of 1,4-dihydropyridines in protic media (0.04 M Britton–Robinson buffer + ethanol: 70/30). (--) pH 2.4 (—) pH 7 (--) pH 12. Sweep rate: 0.5 V/s.

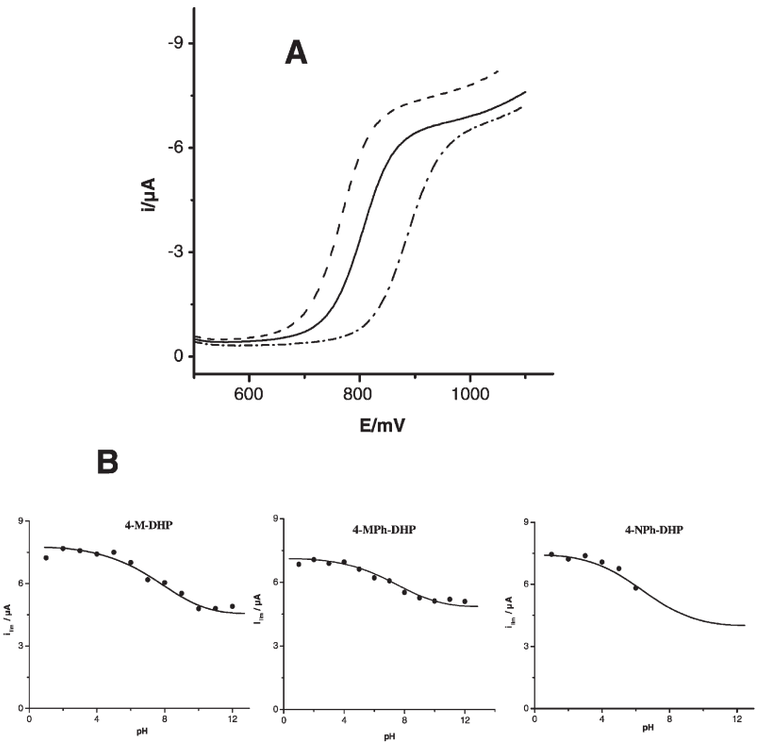


FIGURE 4 (A) Dynamic voltammograms on glassy carbon electrode in protic media at pH 5. (--) 4-M-DHP (—) 4-MPh-DHP (---) 4-NPh-DHP. (B) Dependence of limiting current with pH for a 100 μM concentration of 1,4-dihydropyridines in protic media (0.04 M Britton—Robinson buffer + ethanol: 70/30). Rotating rate 5.200 rpm.

the limiting current is practically constant, but above pH 5 the current decreased.

In order to obtain the diffusion coefficients, we have plotted the limiting current vs. the square root of the rotating rate. The result of this plot was a line (result not shown) according with the Levich equation.[26]

From the slopes of these lines in both aqueous and aprotic media, the calculation of the respective diffusion coefficients for the compounds was possible (Table II). As can be seen from the table, no significant differences between the diffusion coefficients in the same media were found. However, in aprotic media, the calculated diffusion coefficient values were significantly higher than the values in aqueous media. This result can probably be attributed to the different values of the cinematic viscosity of the solvents (0.004 stokes for acetonitrile vs. 0.01 stokes for an aqueous media).

TABLE II Diffusion coefficients* for the 4-substituted 1,4-DHP derivatives

Compound	Diffusion coefficient, $cm^2 s^{-1} \times 10^{-6}$
Aqueous media, pH 7.4	
4-M-DHP	2.0
4-MPh-DHP	2.2
4-NPh-DHP	1.1
Aprotic media	
4-M-DHP	15.5
4-MPh-DHP	15.6
4-NPh-DHP	16.3

*Obtained from Levich plots.

UV–Vis. Spectrophotometry

UV–Vis. spectra for each compound were recorded between 200 and 600 nm. All compounds exhibited two absorption bands at approximately 230 and 360 nm. 4-NPh-DHP showed other absorption band at approximately 290 nm (Table III). In all the studied compounds, these absorption bands were insensitive to pH changes and therefore, no apparent pK_a could be determined by means of this technique.

TABLE III Molar absorptivity and absorption bands of the synthesized 1,4-DHP derivatives

	Absorptivity/$M^{-1} cm^{-1}$		
Compound	λ230 nm	λ360 nm	λ290 nm
4-M-DHP	35,440	8,030	–
4-MPh-DHP	36,600	7,690	–
4-NPh-DHP	41,400	4,400	13,670

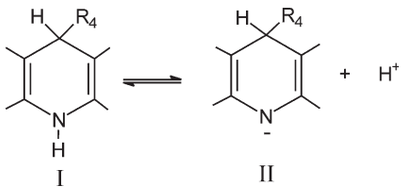
Aprotic Media

Voltammetry

In this medium, a single anodic signal for the three compounds was observed. From the peak potential values, the ease of oxidation is as follows: 4-M-DHP > 4-MPh-DHP > 4-NPh-DHP, and correlates directly with the electron-donor character of the substituent at the 4-position as occurred in aqueous media.

With the objective to study possible different acid–base species from the studied molecules, the addition of an acid (0.4 mM alcoholic $HClO_4$ solution) or a base (0.4 mM alcoholic NaOH solution) on the oxidation of the three derivatives was studied. Addition of 0.4 mM alcoholic NaOH solution resulted in the disappearance of the main signal (at approximately 1000 mV) in parallel with the appearance of a new signal at approximately −100 mV. Addition of 0.4 mM alcoholic $HClO_4$ solution to the above solution resulted in the reversal of the described phenomenon, i.e. the original signal was recovered and no signal at −100 mV was detected (Fig. 5). When 0.4 mM alcoholic $HClO_4$ solution was added first, no change was evidenced.

These results imply a change of the electroactive species as a consequence of adding base or acid suggesting dissociation equilibrium as described in Eq. 1:



The results obtained can be explained as follows, the original voltammetric signal (1000 mV) could correspond to the oxidation of the species I, with the unprotonated species II, being responsible for the signal appearing at −100 mV. This shift in oxidation potential can be readily explained by considering changes to the re-organisation energy component as described by Marcus theory.[27] In fact, probably species II could display ion–solvent interactions affecting the re-organisation energy producing lower free energy barrier and then a lower oxidation potential.

By C.V. at all sweep rates (0.1–10 V/s), 1,4-DHP derivatives exhibited a single anodic irreversible peak. Log *ip* vs. log *V* plots show slopes close to 0.5, indicating that currents were diffusion-controlled. Furthermore, the peak potential values were sweep rate-dependent, giving support for an irreversible oxidation mechanism in this medium.

UV–Vis. Spectrophotometry

In order to corroborate the existence of the equilibrium of Eq. 1 we have also used spectrophotometric measurements.

In Fig. 6, the changes in the UV–Vis absorption bands after the addition of 0.4 mM alcoholic NaOH solution are reported. These results can be summarized a follows. With 4-M-DHP and 4-MPh-DHP derivatives, concomitantly with the formation of a very deep yellow solution, a new absorption band at 450 nm was evidenced. 4-NPh-DHP gave rise to a brown solution, in parallel with the appearance of a new absorption band at 410 nm. These new absorption bands gradually disappeared after the addition of 0.2 mM $HClO_4$ solution, returning these solutions to their original colors (Fig. 6). These results

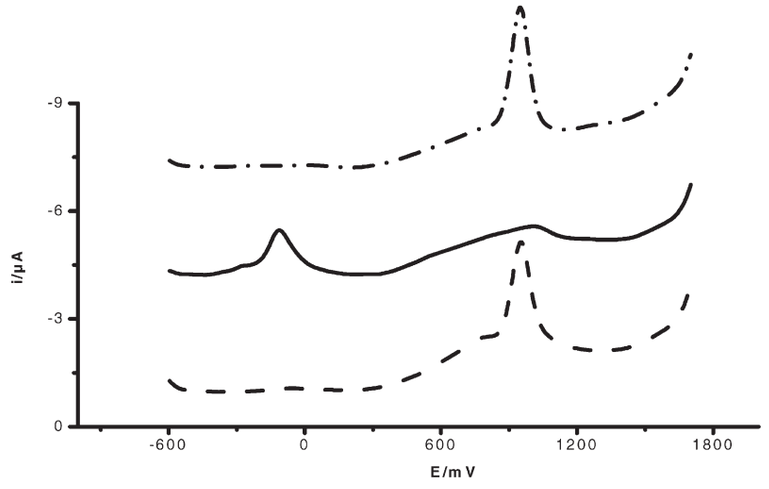


FIGURE 5 Differential pulse voltammograms of 100 μM concentration of 4-M-DHP in acetonitrile. (--) original signal, (—) in the presence of 0.4 mM alcoholic NaOH (---) solution with 0.4 mM NaOH in the presence of 0.4 mM $HClO_4$.

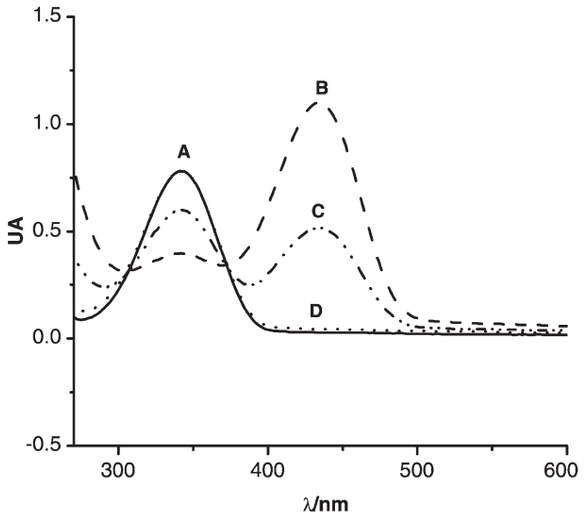


FIGURE 6 UV–Vis. spectra of a 100 μM concentration of 4-M-DHP in acetonitrile. A: (—) original spectrum; B: (– –) scan A + 400 μM alcoholic NaOH; C: (-·-·) scan B + 200 μM $HClO_4$; D: (·····) scan C + 200 μM $HClO_4$.

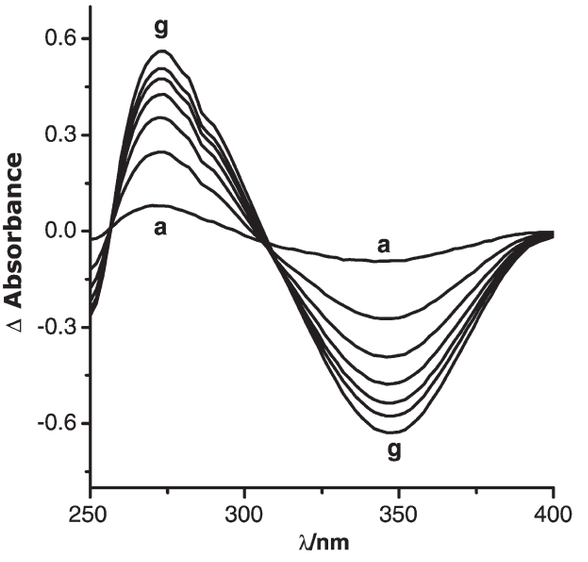


FIGURE 7 UV–Vis. differential spectra corresponding to CPE at 1060 mV of a 100 μM concentration of 4-MPh-DHP. a–g: 60 min.

can be explained on the basis of the acid–base equilibrium of the –NH group. In the presence of NaOH, the equilibrium is displaced towards the anionic form. The negative charge can be delocalized over the system up to the ester groups in 3- and 5-position, thus explaining the appearance of colored solutions in this medium.

Coulometric Analysis

In order to understand the mechanism of oxidation in greater detail and to determine the number of electrons involved in the oxidation process, coulometric studies were carried out in aqueous and in aprotic media. Solutions containing accurately weighed amount of the 1,4-DHP derivatives with TBAHFP were subjected to electrolysis at constant potential. The applied potentials were +0.99, +1.06 and +1.20 V for 4-M-DHP, 4-MPh-DHP and 4-NPh-DHP, respectively. Coulometric studies in nitrogen-saturated acetonitrile or aqueous media show values of 1.8 ± 0.15, 2.08 ± 0.2, 2.2 ± 0.17 for 4-M-DHP, 4-MPh-DHP and 4-NPh-DHP, respectively. These values are in accord with a two-electron oxidation mechanism for the oxidation of the DHP derivatives, i.e. the oxidation of the 1,4-DHP to the respective pyridine derivative.

Controlled Potential Electrolysis

1,4-DHP solutions were submitted to exhaustive electrolysis and then analyzed by UV–Vis., GC–MS and EPR techniques in order to identify the oxidation product(s).

UV–Vis

To follow the time-course of electrolysis, a wide range of wavelengths were recorded. In Fig. 7, both the original spectrum and the spectra after different times of electrolysis are shown. For the three studied compounds new absorption bands at approximately 270 nm appeared, which could correspond to the aromatized final product, i.e. the pyridine derivative.[28]

GC–MS

The formation of the above product was confirmed by GC–MS experiments, which provided data on retention time and mass fragmentation. These data support the formation of the oxidized derivative (Table VI).

EPR Spin Trapping Studies

In order to gather evidence for the mechanism of electrochemical oxidation proceeding via the formation of a radical, EPR experiments using the spin trap PBN were carried out. The spin trap was electrochemically tested for purity, as was the magnitude of the so-called potential window (the potential range within which the trap is electrochemically inactive). The oxidation peak potential of PBN in acetonitrile +0.1 M TBAHFP at a Pt electrode was +1.42 V vs. Ag/AgCl. Klima *et al.*,[29] previously reported a similar value for the oxidation of PBN under these experimental conditions. Electrolysis of 4-M-DHP (1 mM), 4-MPh-DHP and 4NPh-DHP at a 10 mM concentration, in the presence of PBN, gave spectra characteristic of a nitroxide spin adduct (Fig. 8). The EPR spectra have been assigned to a triplet from the nitrogen split into a doublet due to

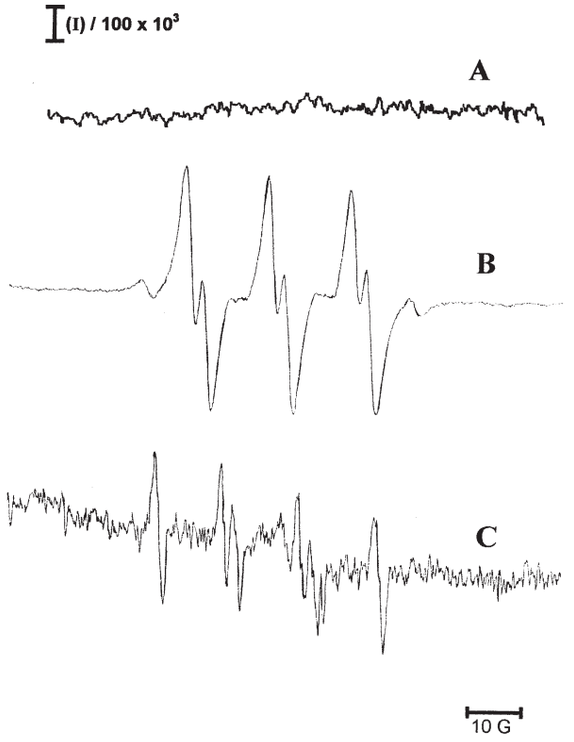


FIGURE 8 Experimental EPR spectra of adduct PBN-pyridinium radical and DMPO-pyridinium radical. Radical generated electrochemically from dihydropyridine solution in acetonitrile + 0.1 M TBAHFP of: A: base line, B: 1 mM 4MPh-DHP + PBN (70 scans) and C: 1 mM 4-M-DHP + DMPO (30 scans).

the presence of the adjacent hydrogen. a_N values around 14 G and a_H splitting values of 2–3 G were obtained for these spin adducts. These values are consistent with the trapping of carbon-centered radicals as reported for other DHP derivatives (National Institute of Environmental Health Sciences) and related compounds[30] with the same spin trap (Table IV). Furthermore, we have carried out spin trapping using DMPO and detected a six-line signal typical of C-centered radicals with a_N values around 14 G and a_H 21 G (Fig. 8). On the other hand, 1,4-DHP in acetonitrile in the absence of spin traps did not yield any spectra. The electro-oxidation

TABLE IV Comparison between the peak potentials values* on glassy carbon electrode and splitting constants of the resulting spin adducts of 4-substituted 1,4-DHP compounds

Derivative	+Ep/mV	a_N/Gauss	a_H/Gauss
4-M-DHP	990	14.0	3.4
4-MPh-DHP	1,060	13.9	2.8
4-NPh-DHP	1,200	13.8	2.5

* Values determined in acetonitrile containing 0.1 M TBAHFP.

of 1,4-DHP derivatives yields two types of radical intermediates: primarily the radical cation $PyH^{+\bullet}$ and after deprotonation, the neutral radical $Py^{\bullet}$. It seems probable that from the spectra obtained the dihydropyridyl radical $Py^{\bullet}$ is added to the spin trap. From quantum chemical calculations[31] it follows that the unsubstituted dihydropyridyl radical, $Py^{\bullet}$, has the unpaired electron localized prevalently in the 2,4 or 6 positions; position 4 is preferred. In addition, electronegative substituents at positions 3 and 5 increase spin density in the vicinal positions. In position 4, their effects are added; this leads to an even greater preference for this position. Consequently, one may assume that the radicals generated in our experiments are preferentially added to the spin trap via this reactive C-4 position. Based on our own results, and data from the literature,[17,18,23,28,30] it can be concluded that the trapped species could correspond to the pyridinium radical. According to the above evidences, i.e. 2 electrons transferred, pyridine derivative formation and trapping of the radical specie, we can propose the following mechanism:

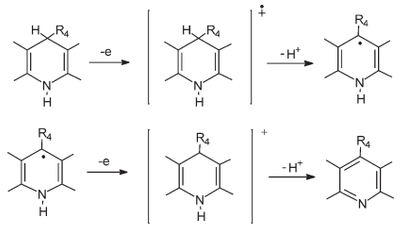


TABLE V Comparison between the peak potentials values* on glassy carbon electrode and the reactivity of 4-substituted 1,4-DHP derivatives towards ABAP-derived alkyl radicals

Derivative	$+E_p$/mV	Reactivity†	R/Nisoldipine‡	R/Vitamin E¶
Vitamin E (Trolox)	108	−36.4	—	1
4-M-DHP	640	−5.6	1.5	0.15
4-MPh-DHP	655	−3.2	0.8	0.09
4-NPh-DHP	722	−4.7	1.2	0.13
Nisoldipine	681	−3.8	1.0	0.10

* Aqueous Britton–Robinson buffer pH 7.4. † Slopes corresponding to the time-course of concentration–time plots of the derivatives in the presence of alkyl radicals. ‡ Ratio between slope of concentration–time plot of the tested 1,4-DHP derivatives/slope of concentration–time plots of Nisoldipine in the presence of alkyl radicals. ¶ Ratio between slope of concentration–time plot of the tested 1,4-DHP derivatives/slope of concentration–time plots of vitamin E (Trolox) in the presence of alkyl radicals.

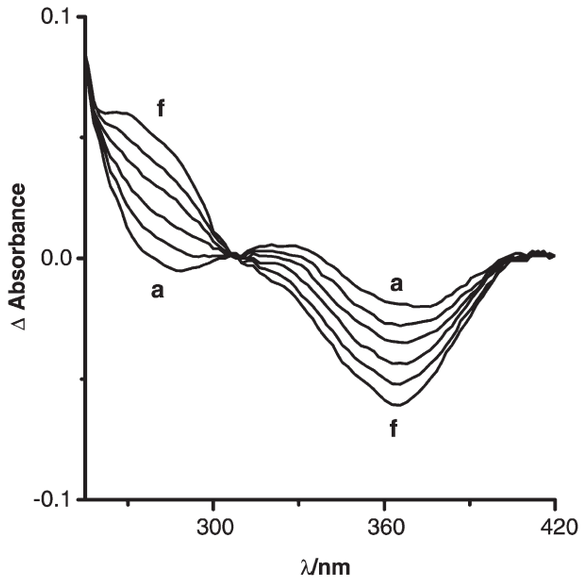


FIGURE 9 UV–Vis. differential spectra corresponding to reaction between a 100 μM concentration of 4-MPh-DHP and 20 mM ABAP-derived alkyl radicals. a–f: 120 min.

REACTIVITY OF THE 1,4-DHP DERIVATIVES TOWARDS ABAP-DERIVED ALKYL RADICALS IN AQUEOUS MEDIA

To follow the reactivity of 1,4-DHP derivatives both UV–Vis. spectroscopy and GC–MS technique were used.

UV–Vis. Spectroscopy

For these studies, the UV absorption bands at λ360 nm corresponding to the 1,4-DHP derivatives were followed with the exception of 4-NPh-DHP, which was followed at λ290 nm (Table III). The results revealed that after the addition of 1,4-DHP to an aqueous mixture containing alkyl radicals, the absorption band at λ360 nm or λ290 nm, decreased with time, in parallel with the appearance of a new band at 270 nm (Fig.9). This signal could correspond to the oxidized derivative, i.e. the pyridine derivative, which is consistent with previous observations.[28] To compare reactivity, slopes of concentration–time plots of the tested 1,4-DHP derivatives and slopes of the corresponding plots of the reference compounds (vitamin E or Nisoldipine) were used (Table V). Clearly, from the results summarized in this table, 1,4-DHP derivatives react with alkyl radicals at varying rates. Thus, when are compared with vitamin E, the antioxidant activity of the 1,4-DHP derivatives were lower than the vitamin E, with the most active being 4-M-DHP, i.e. the 4-methyl substituted derivative. In contrast, when the synthesized compounds were compared with a well-known antioxidant 1,4-DHP derivative, such as nisoldipine, a comparable antioxidant activity was found (Table V). Attempts to correlate oxidation peak potentials with antioxidant ability did not reveal a markedly tendency (Table V).

GC–MS

In order to identify the products of the reaction with ABAP, we have used GC–MS. In Table VI, the GC–MS data for both 1,4-DHP derivatives and the product obtained after 1,4-DHP was added to a solution containing ABAP are shown. The conclusions of these studies can be summarized as follows: (a) The GC–MS procedure used to characterize the parent 1,4-DHPs and its subsequent reactivity with radicals did not require a derivatization process. (b) 1,4-DHPs after the reaction with alkyl radicals suffered a dehydrogenation process yielding the pyridine-derived metabolites. This conclusion is supported by the respective retention times and the mass fragmentation pattern for each compound displayed in the table. Clearly, in the scavenging effect of the radicals, an electron transfer reaction is involved. (c) Comparison between the retention times of the pyridine derivatives and the parent compounds suggest that the former are lower. This result could be explained on the basis of the different affinity for the stationary phase, taking into account that pyridine derivatives have lost a hydrogen in 1-position in the dihydropyridine group and

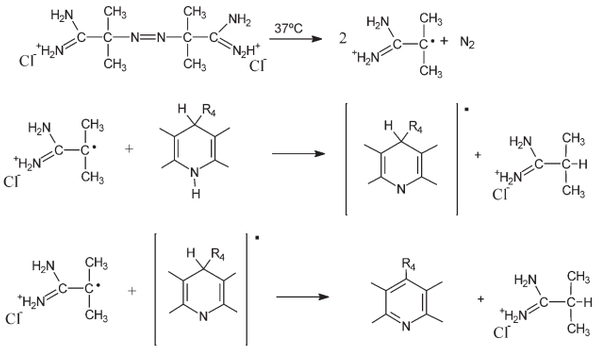
TABLE VI GC–MS data obtained from parent 1,4-DHP derivatives and after reaction with ABAP-derived alkyl radicals

Derivative	$(Rt/min)_{st}$*	Pbst†	PM_{st}‡	$(Rt/min)_{ox}$¶	Pb_{ox}§	PM_{ox}#	% Pyridine
4-M-DHP	8.84	224	239	7.42	206	237	56.0
4-MPh-DHP	10.46	224	331	8.68	266	329	67.5
4-NPh-DHP	11.81	224	346	9.55	327	344	40.0
Nisoldipine	11.63	210	388	10.19	284	386	51.0

*Retention time corresponding to the parent drug. †Peak base corresponding to the parent drug. ‡Molecular peak corresponding to the parent drug. ¶Retention time corresponding to the oxidized derivatives. §Peak base corresponding to the oxidized derivatives. #Molecular peak corresponding to the oxidized derivatives.

the loss of a second hydrogen by the formation of an aromatic ring. (d) The mass fragmentation patterns for the synthesized 1,4-DHP compounds are similar, with a peak base of 224.[32] (e) The mass fragmentation pattern of the aromatized derivatives shows a different peak base depending on 4-substitution on the 1,4-DHP ring. (f) The tested 1,4-DHP derivatives reacted with free radicals of alkyl type, forming as a final product the pyridine derivative with yields higher than 40% (Table VI). In Fig. 10, a typical GC–MS spectrum for the reaction between 4-NPh-DHP derivative and ABAP-derived alkyl radicals is displayed. As can be seen, both the peak base of 327 and the molecular peak of 344 document the pyridine derivative formation, confirming that after the reaction, an electron transfer reaction occurred.

Based on results obtained from UV–Vis. spectroscopy and GC–MS experiments, the following mechanism of scavenging can be proposed:



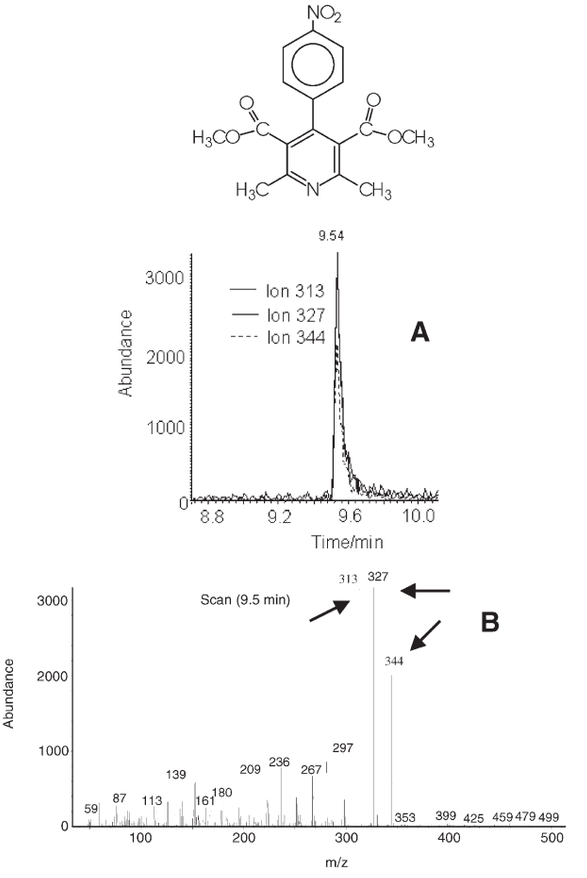


FIGURE 10 Ion chromatogram (A) and mass spectrum (B) corresponding to 4-NPh-DHP derivative after the reaction during 48 hr with ABAP-derived alkyl radicals.

more, the scavenging effect is comparable to that detected for the well-known antioxidant 1,4-DHP, nisoldipine.

CONCLUDING REMARKS

1. All the synthesized 1,4-DHP derivatives were electro-oxidizable in different electrolytic media with a mechanism involving a two-electron transfer to produce the pyridine derivative. Electrochemical, spectroscopy and GC–MS evidence supports this mechanism.
2. In aprotic media, it was possible to differentiate the equilibrium between the 1,4-DHP derivatives and its corresponding anion forms.
3. On applying PBN as spin trap, unstable radical intermediates arising from the oxidation of the synthesized derivatives were intercepted. Based on the observed splitting constants it can be concluded that PBN interacted with carbon-centered radicals.
4. Studies on the reactivity indicated a direct reaction between these novel 1,4-DHP derivatives and ABAP-derived alkyl radicals. Further-

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