Gas chromatography/mass spectrometric study of non-commercial C-4-substituted 1,4-dihydropyridines and their oxidized derivatives

C. López-Alarcón[1], J. A. Squella[1], Luis J. Núñez-Vergara[1]*, H. Baez[2] and Cristián Camargo[2]

[1]Laboratory of Bioelectrochemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, P.O. Box 233, Santiago, Chile
[2]Laboratory of Antidoping and Abuse Drug Control, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, P.O. Box 233, Santiago, Chile

A gas chromatography/mass spectrometry (GC/MS) method for the qualitative and quantitative determination of the calcium-channel antagonists C-4-substituted 1,4-dihydropyridines, and their corresponding N-ethyl derivatives, is presented. Also, the electrochemical oxidation and the reactivity of the compounds with alkyl radicals derived from 2,2′-azobis-(2-amidinopropane) were monitored by GC/MS. Mass spectral fragmentation patterns for the C-4-substituted 1,4-dihydropyridine parent drugs were significantly different from those of their oxidation products, generated either by electrochemical oxidation or by reaction with alkyl radicals. However, for *N*-ethyl-1,4-dihydropyridine compounds it was not possible to detect the final products (pyridinium salts) using these experimental conditions.

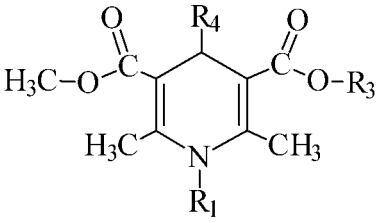
The discovery that the 1,4-dihydropyridine class of calcium-channel antagonists inhibits Ca^{2+} influx represented a major therapeutic advance in the treatment of cardiovascular diseases such as hypertension, angina pectoris, and other spastic smooth muscle disorders.[1] The dihydropyridine class of compounds, of which nifedipine is the prototype, has been the subject of many structure-activity relationship studies.[2,3] Chemically, this family contains as the structural basis a tetrasubstituted 1,4-dihydropyridine (1,4-DHP) ring, often with a substituted phenyl group at the 4-position. The 1,4-DHP moiety is essential for the pharmacological activity on the cardiovascular system.[1,4] The calcium antagonists are extensively metabolized in the liver prior to excretion; the main metabolic pathway is oxidation of the dihydropyridine ring to the pyridine analogue. None of the metabolites is pharmacologically active.[5]

Second-generation analogues of nifedipine with improved bioavailability, a slower onset and longer duration of action, and amenable to a once-a-day dosage regimen, are being actively investigated. Changes in the substitution pattern at the C-3, C-4 and C-5 positions of nifedipine alter activity and tissue selectivity.[6,7] These compounds are of interest also because of the similarity in properties between 1,4-dihydropyridines and biologically important compounds such as NADH and NADPH. The electrochemical oxidations of 1,4-dihydropyridines, NADH, and related compounds have also received attention.[8] 1,4-Dihydropyridines have been demonstrated to possess antioxidant effects.[9,10] It has been clearly demonstrated that this effect is not due to the Ca^{2+} channel antagonist effect, but rather is related to the reactivity of these structures towards various radical species.[11]

In addition to the well-known cardiovascular properties, there seems to be a relationship between hypertension and free radicals,[12] as well as between antihypertensive agents and antioxidants. A major goal of our research has been to prepare C-4-substituted 1,4-dihydropyridines with activity equal to or greater than that of currently used therapeutic agents, such as nifedipine. Consequently, we have explored structural changes in C-4 substituents that are electron-rich (Fig. 1, *p*-methoxyophenyl-1, 4DHP), electron-poor aryl rings (Fig. 1, *p*-nitrophenyl-1,4-DHP), or simply an alkyl group (Fig. 1, 4-methyl-1,4-DHP). Furthermore, the effect of N-alkylation on both the electrochemical oxidation and reactivity with alkyl radicals derived from 2,2′-azobis-(2-amidinopropane) (ABAP), of C-4-substituted 1,4-dihydropyridines, was also studied.

In the present paper, a gas chromatography/mass spectrometry (GC/MS) method for the simultaneous determination of some non-commercial C-4-substituted 1,4-dihydropyridines, and their corresponding N-ethyl analogues, is reported. Also, the identification and quantification of the products generated during the time-course of electrolysis, and from the reaction with ABAP-derived alkyl radicals, have been investigated.

**Correspondence to*: L. J. Núñez-Vergara, Laboratory of Bioelectrochemistry, Faculty of Chemical and Pharmaceutical Sciences, University of Chile, P.O. Box 233, Santiago, Chile.
E-mail: lnunezv@ciq.uchile.cl



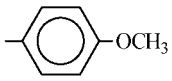
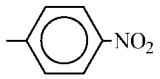
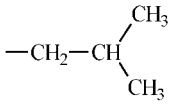
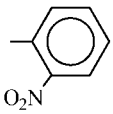
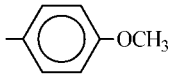
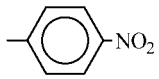
Compound	R_1	R_3	R_4
4-methyl-1,4-DHP	−H	$-CH_3$	$-CH_3$
p-methoxyphenyl-1,4-DHP	−H	$-CH_3$	
p-nitrophenyl-1,4-DHP	−H	$-CH_3$	
Nisoldipine	−H		
N-ethyl-4-methyl-1,4-DHP	$-CH_2CH_3$	$-CH_3$	$-CH_3$
N-ethyl-p-methoxyphenyl-1,4-DHP	$-CH_2CH_3$	$-CH_3$	
N-ethyl-p-nitrophenyl-1,4-DHP	$-CH_2CH_3$	$-CH_3$	

Figure 1. Chemical structures of C-4-substituted 1,4-dihydropyridines.

EXPERIMENTAL

Compounds

All 1,4-dihydropyridine derivatives (with the exception of nisoldipine, Fig. 1) were synthesized in our laboratory, according to previously published methods.[13,14] Melting points (uncorrected) were obtained using a Buchi 530 apparatus after introducing the sample into the bath at a temperature 10 °C lower than the melting point. Compounds were routinely checked by infrared spectroscopy (FT-IR); ^{1}H- and ^{13}C-nuclear magnetic resonance spectra were recorded at 300 and 75 MHz, using a Bruker Advance DRX 300 spectrometer.

Synthesis of 1,4-dihydropyridines (1,4-DHP)

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 (e.g. for 4-methyl-1,4DHP, acetaldehyde was used; for *p*-methoxyphenyl-1,4-DHP, 4-methoxybenzaldehyde; for *p*-nitrophenyl-1,4-DHP, 4-nitrobenzaldehyde was used), plus 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 from ethyl alcohol. The yields were in the range 80–90% depending on the derivative. The following compounds were synthesized with the following specific characteristics:

4-Methyl-2, 6-dimethyl-3, 5-dimethoxycarbonyl-1,4-dihydropyridine (4-methyl-1,4-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, J = 6.5Hz, >CH-CH_3), 2.29 (s, 6H, -CH_3), 3.73 (s, 6H, -O-CH_3), 3.83 (q, 1H, J = 6.5Hz, >CH-CH_3), 5.73 (s, 1H, -NH-). ^{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; m.p.:147–149 °C.

4-(4-Methoxyphenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-1,4-dihydropyridine (p-methoxyphenyl-1,4-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, J = 8.6Hz, 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. m.p.: 181–183 °C.

4-(4-Nitrophenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-1,4-dihydropyridine (p-nitrophenyl-1,4-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, -CH_3), 3.66 (s, 6H, -O-CH_3), 5.12 (s, 1H, Ar-CH<), 5.85 (s, 1H, -NH-), 7.46 (d, 2H, J = 8.8Hz, 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; m.p.:165–168 °C.

Nisoldipine [4-(2-nitrophenyl)-2,6-dimethyl-3-carbomethoxy-5-carbisobutoxy-1,4-dihydropyridine] was supplied by Sanitas Laboratories (Santiago, Chile). This drug was used without additional purification.

Synthesis of N-*ethyl-1,4-dihydropyridines*
This synthesis was performed by N-alkylation with ethyl iodide of each C-4-substituted 1,4-dihydropyridine. The general procedure was as follows: 0.004, 0.003 and 0.0029 mol of the compounds 4-methyl-1,4-DHP, *p*-methoxyphenyl-1,4-DHP and *p*-nitrophenyl-1,4-DHP, respectively, were mixed with 0.0125 mol of sodium hydride and 0.0124 mol of ethyl iodide in 20 mL tetrahydrofuran. Argon was bubbled through the solutions for 15 min which were then incubated at room temperature under constant agitation for 10 h. The resulting solutions were filtered under vacuum and the liquid was extracted with chloroform three or four times. The chloroform extracts were evaporated and the solid products were recrystallized from ethyl alcohol. The yields were in the range 30–40%. The following compounds were prepared using this general procedure:

*4-Methyl-2,6-dimethyl-3,5-dimethoxycarbonyl-*N-*ethyl-1,4-dihydropyridine (*N-*ethyl-4-methyl-1,4-DHP)*
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, >$CHCH_3$), 1.16 (t, 3H, J = 7.1 Hz N-CH_2CH_3), 2.4 (s, 6H, -CH_3), 3.7 (q, 2H, J = 7.1 Hz N-CH_2CH_3), 3.72 (s, 6H, -OCH_3), 3.77 (q, 1H, J = 6.6 Hz, >$CHCH_3$). ^{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; m.p.: 78–80 °C.

*4-(4-Methoxyphenyl)-2,6-dimethyl-3,5-dimethoxycarbonyl-*N-*ethyl-1,4-dihydropyridine (*N-*ethyl-p-methoxyphenyl-1,4-DHP)*
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, NCH_2-CH_3), 2.47 (s, 6H, -CH_3), 3.67 (q, 2H, J = 5.9, -N-CH_2CH_3), 3.7 (s, 6H, -OCH_3), 3.75 (s, 3H, Ar-OCH_3), 5.04 (s, 1H, Ar-CH<), 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; m.p.: 106–108 °C.

*4-(4-Nitrophenyl)-2, 6-dimethyl-3, 5-dimethoxycarbonyl-*N-*ethyl-1, 4-dihydropyridine (*N-*ethyl-p-nitrophenyl-1, 4-DHP)*
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_2CH_3), 2.5 (s, 6H, -CH_3), 3.72 (s, 6H, -OCH_3), 3.72 (q, 2H, J = 7.1 Hz, N-CH_2-CH_3), 5.19 (s, 1H, Ar-CH<), 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, m.p.: 156–157 °C.

2,2′-Azobis(2-methylpropionamidine) dihydrochloride (ABAP) and tetrabutylammonium hexafluorophosphate (TBAHFP) were supplied by Aldrich Chemical Company. Potassium chloride (KCl) was supplied by Merck.

Equipment
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. A Hewlett Packard data system based on a Pentium II processor printer was used to control instrumentation and for data processing. The *m/z* range monitored was 45–550 with a scan rate of 1 scan/s; the nominal electron energy was 70 eV.

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

Chromatographic conditions
Detector temperature, 300 °C; injector temperature, 250 °C; split ratio, 1:10; pressure, 13 psi; purge flow, 40 mL/min; purge time, 0.5 mL/min. Temperature program: the oven temperature was programmed from 130 to 305 °C (hold for 5 min) at 15 °C/min; run time was 16.67 min. Helium was used as 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).

Formation of the oxidized derivatives from the synthesized 1,4-dihydropyridines
The formation of the oxidized derivatives used two different experimental procedures: (a) the reaction of 1,4-DHP derivatives with ABAP-derived alkyl radicals, and (b) during the time-course of the controlled-potential electrolysis of the 1,4-DHP derivatives.

(a) Reaction with ABAP-derived alkyl radicals
The oxidized derivatives (pyridine or pyridinium salt derivatives) were formed during the time-course of the reaction between the alkyl radical generator ABAP and the respective 1,4-dihydropyridines. ABAP solutions (20 mM; Britton-Robinson buffer, pH 7.4) were incubated with 100 μM solutions of each substituted 1,4-dihydropyridine in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4) at

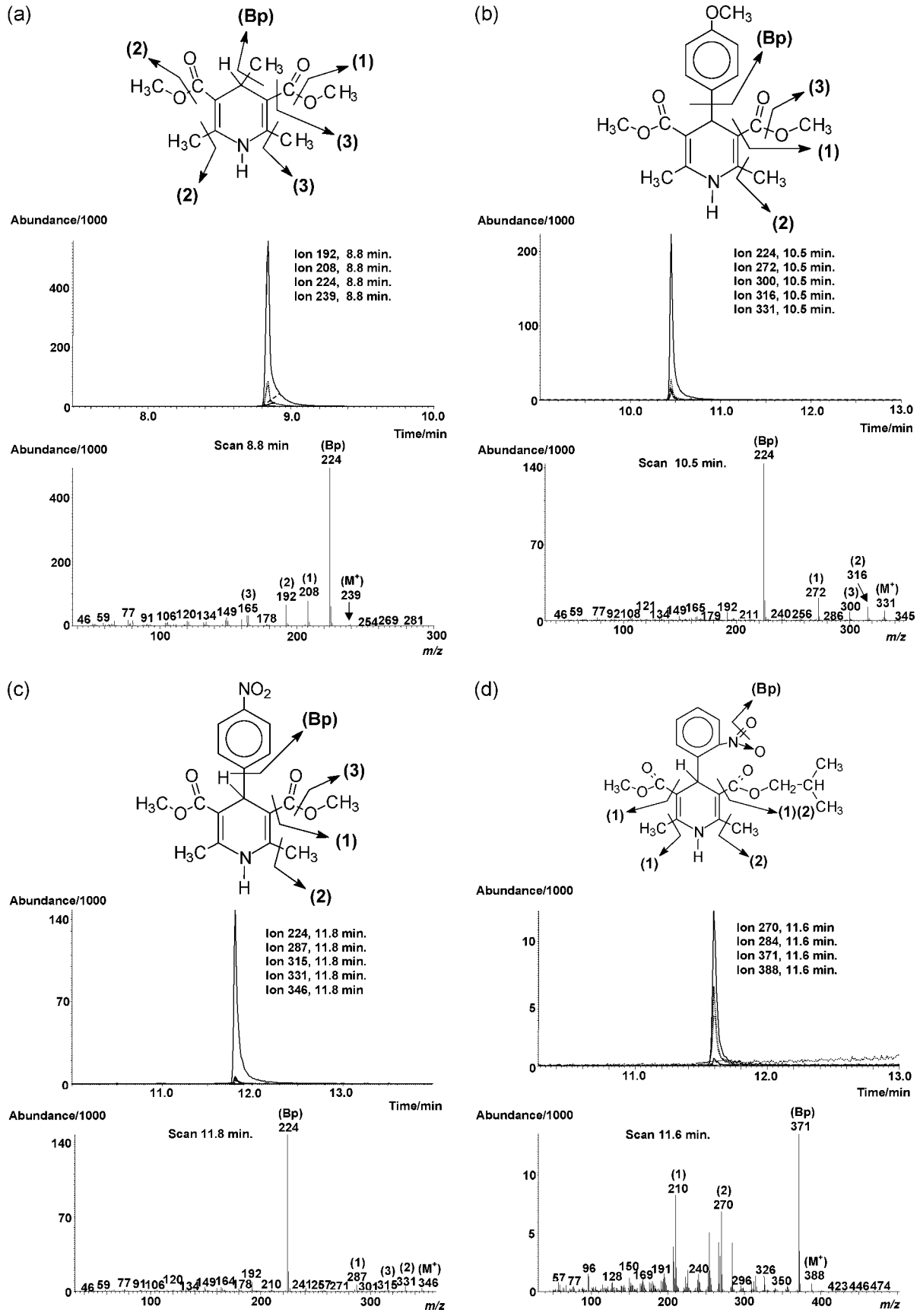


Figure 2. Extracted ion chromatograms and mass spectra of 100 μM parent drug 1,4-DHP derivatives (a)–(d) in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4).

Table 1. Base peaks (Bp) and the main fragment ions of parent 1,4-DHP compounds and of their corresponding oxidized derivatives obtained by controlled potential electrolysis in 0.04M Britton-Robinson buffer/ethanol (70:30, pH 7.4) at 25 °C

Derivative	Bp (100%)	Ion (1)	Ion (2)	Ion (3)	M^+
Parent drugs:					
4-methyl-1,4-DHP	224: M^+-CH_3	208 (12%): M^+-OCH_3	192 (10%): M^+-OCH_3-CH_3	165 (5%): M^+-$COOCH_3$-CH_3	239 (1%)
p-Methoxyphenyl-1,4-DHP	224: M^+-$\varnothing OCH_3$	272 (12%): M^+-$COOCH_3$	316 (7%): M^+-CH_3	300 (4%): M^+-OCH_3	331 (5%)
p-Nitrophenyl-1,4-DHP	224: M^+-$\varnothing NO_2$	287 (4%): M^+-$COOCH_3$	331 (3.5%): M^+-CH_3	315 (2.5%): M^+-OCH_3	346 (2%)
Nisoldipine	371: M^+-$O(NO_2)$	210 (56%): M^+-$COOCH_2$*i*Pr-$COOCH_3$-CH_3	270 (46%): M^+-$COOCH_2$*i*Pr-CH_3	–	388 (5%)
Oxidized derivatives:					
4-Methylpyridine	206: M^+-OCH_3	222 (61%): M^+-CH_3	178 (30%): M^+-$COOCH_3$	190 (16%): M^+-OCH_3-CH_3	237 (48%)
p-Methoxyphenylpyridine	329 (M^+)	266 (89%): M^+-OCH_3-OCH_3	298 (15%): M^+-OCH_3	–	–
p-Nitrophenylpyridine	313: M^+-OCH_3	327 (99%): M^+-$O(NO_2)$	297 (37%): M^+-NO_2	–	344 (71%)
Oxidized nisoldipine	284: M^+-$COOCH_2$*i*Pr	340 (42%): M^+-NO_2	236 (15%): M^+-$COOCH_2$*i*Pr-NO_2	313 (10%): M^+-OCH_2*i*Pr	–

37 °C for 48 h. Oxygen was removed by pre-saturation with 'pure and dry' nitrogen. The rate of alkyl-radical formation from this initiator is constant at a given temperature, and the radicals are capable of reacting directly with certain biological molecules.[15] Two types of control solutions were independently run: (a) solutions of 20 mM ABAP in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4) and (b) 100 μM solutions in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4) of each substituted 1,4-dihydropyridine. Results of these experiments did not reveal any changes in chromatograms. The time-course of the reaction of the synthesized 1,4-DHP derivatives with the generated alkyl radicals was followed using GC/MS.

(b) Controlled-potential electrolysis (CPE) of the 1,4-DHP derivatives

CPE experiments were performed using a platinum mesh electrode in anhydrous acetonitrile solutions (0.1 M) of TBAHFP, at +0.99, +1.06 and +1.20 V for 4-methyl-1,4-DHP, *p*-methoxyphenyl-1,4-DHP and *p*-nitrophenyl-1,4-DHP, respectively. Also, CPE experiments on Britton Robinson buffer/ethanol (70:30, pH 7.4) solutions containing KCl (0.1 M) were performed at +0.90, +0.95 and +1.050 V for 4-methyl-1,4-DHP, *p*-methoxyphenyl-1,4-DHP and *p*-nitrophenyl-1,4-DHP, respectively.

CPE experiments on *N*-ethyl-1,4-DHP derivatives were performed on anhydrous acetonitrile solutions (0.1 M) of TBAHFP at +1.0, +1.1 and +1.2 V for *N*-ethyl-4-methyl-1,4-DHP, *N*-ethyl-*p*-methoxyphenyl-1,4-DHP and *N*-ethyl-*p*-nitrophenyl-1,4-DHP, respectively. Also, CPE experiments on solutions in Britton Robinson buffer/ethanol (70:30, pH 7.4) containing KCl (0.1 M) were performed at +0.95, +0.97 and +1.05 V for *N*-ethyl-4-methyl-1,4-DHP, *N*-ethyl-*p*-methoxyphenyl-1,4-DHP and *N*-ethyl-*p*-nitrophenyl-1,4-DHP, respectively. Oxygen was removed by pre-saturation with 'pure and dry' nitrogen. A three-electrode circuit, with a Ag/AgCl electrode as reference and a platinum wire as a counter electrode, was used. A BAS-CV 50 power supply was used for the electrolysis experiments. At different time intervals, samples were taken from the electrolytic cell and analyzed by GC/MS.

RESULTS AND DISCUSSION

The main objective of this work was to study the pattern of ion fragmentation of a group of non-commercial 1,4-dihydropyridines and their corresponding N-ethyl derivatives (Fig. 1), modified using different experimental conditions. For comparative purposes, a commercial 1,4-dihydropyridine drug, nisoldipine, was used. The methodology presented here was developed as a requirement to study the electrochemical oxidation mechanism of the

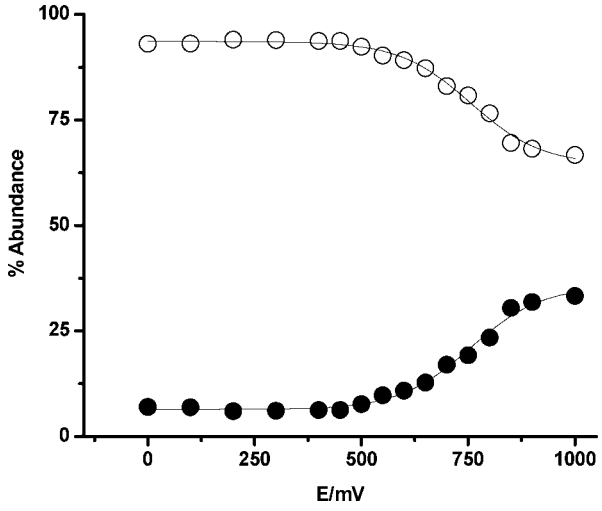


Figure 3. Relative intensities of extracted ion chromatograms of characteristics ions of 4-methyl-1,4-DHP in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4), *m/z* 224 (○), and of its oxidation product monitored at *m/z* 206 (●), plotted against the applied cell potential varied over 2 min.

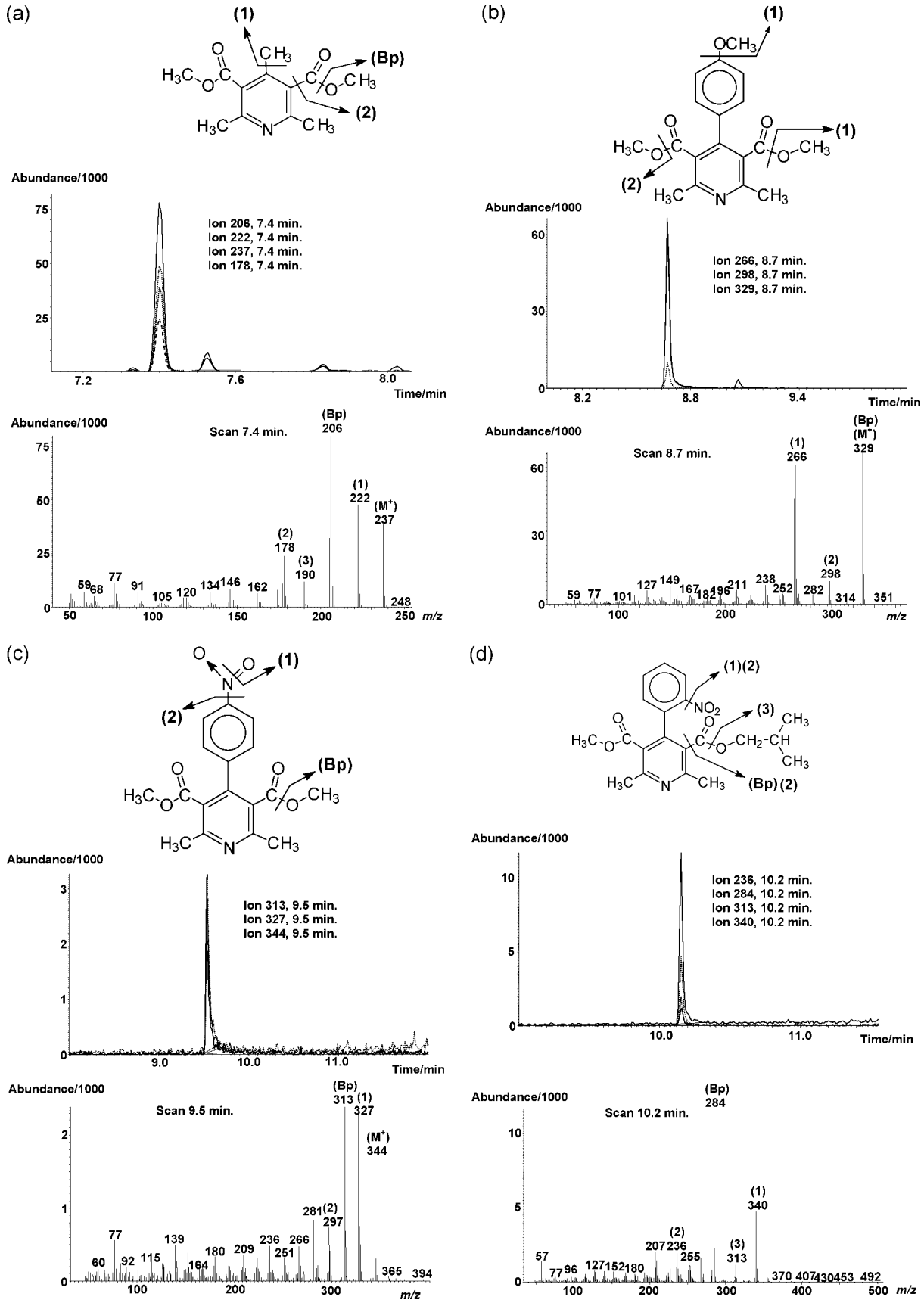


Figure 4. Extracted ion chromatograms and mass spectra of 100 μM solution of parent drug 1,4-DHP derivatives (a)–(d) oxidized by CPE in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4).

synthesized compounds, and their reactivity towards ABAP-derived radicals.

Parent drugs: C-4-substituted 1,4-DHP derivatives

Figure 2 shows typical extracted ion chromatograms and mass spectra corresponding to 1,4-DHP derivatives. Table 1 shows data on molecular ion abundances, and *m/z* values of base peaks, for all the derivatives. From these results it can be concluded that all the new synthesized 1,4-DHP analogues had the same base peak, corresponding to the complete loss of the C-4 substituent on the dihydropyridine ring (*m/z* 224). Apparently, this behavior strongly depends on the nature of the C-4 substituent, since nisoldipine did not exhibit the same behavior (Table 1). A second common characteristic of the EI fragmentation patterns of these compounds is the loss of the C-3 substituent ($COOCH_3$), giving $[M^+ - 59]$ fragment ions.

Oxidized derivatives

Aromatization of the 1,4-dihydropyridine rings to the corresponding pyridine derivatives is the most important reaction from the pharmacological point of view,[5] corresponding to the main metabolic pathway to give an inactive product lacking any biological activity. The electrochemical oxidation of compounds of this type also generates, as one of the major products, the pyridine derivatives.[6] Also, we have observed the formation of pyridine derivatives during the assessment of the potential antioxidant ability of 1,4-dihydropyridines.

In addition, some general conclusions on the GC/MS characteristics of these oxidation products can be summarized: (1) The mass spectral fragmentation pattern was different from that of the parent drug; in particular the substituents in the 4-position were not expelled, presumably a consequence of the aromatization of the 1,4-dihydropyridine rings. (2) In all the oxidized forms of all the new synthesized compounds (Fig. 1), the substituent in the 3-position was lost, i.e. an $-OCH_3$ group. (3) The molecular ion was one of the most important ions. (4) Retention times of the oxidized derivatives were lower than those of the parent drugs.

In addition, calibration curves were obtained for GC/MS quantitation of each oxidized 1,4-DHP derivative, using as internal standard the corresponding *N*-ethyl-1,4-DHP, for a range of concentrations between 15 and 3000 μg/L. Average correlation coefficients higher than 0.9998 were obtained for this linear range. Intra-day assays assessed for a 300 μg/L concentration of each of the oxidized compounds showed an average σ and CV of 8.8 and 2.91%, respectively.

Controlled-potential electrolysis (CPE)

The search for the optimal conditions of electrolysis in both acetonitrile and aqueous media was conducted using both the GC/MS technique and cyclic voltammetry (CV) on a glassy carbon electrode. Figure 3 shows the evolution of the oxidation product of 4-methyl-DHP derivative by CPE in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4). The onset of conversion of the parent compound (measured as *m/z* 224 in the GC/MS analysis) and the oxidized form (monitored as *m/z* 206) occurred at an applied potential of about 500 mV. Similar results were obtained for the other compounds in both media. Figure 4 shows a typical mass spectrum corresponding to the oxidized 1,4-DHP derivatives (Fig. 1) after CPE in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4). Consistent with the results summarized above, the base peak was the *m/z* 266 ion corresponding to the loss of the $-OCH_3$ fragment from the 3-position. GC/MS data for the oxidized compounds from CPE for the other compounds are shown in Table 1.

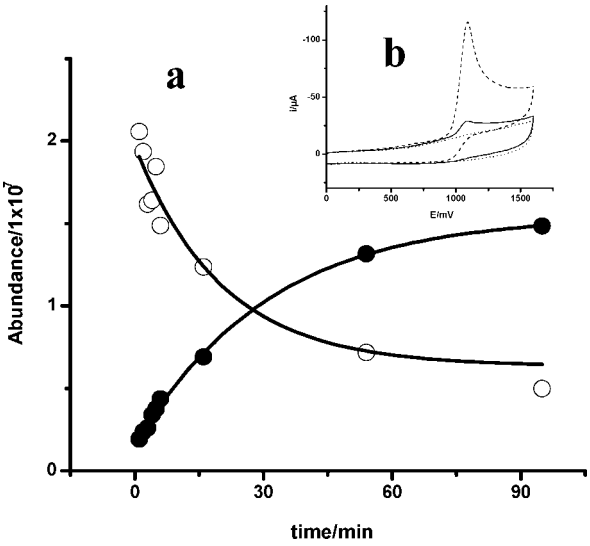


Figure 5. Time-course of intensity of *m/z* 224 (○) corresponding to the parent drug (*p*-methoxyphenyl-1,4-DHP), and of *m/z* 266 (●) corresponding to its pyridine analogue, after CPE at 950 mV in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4). Insert: Time-course of electrolysis of 4-methyl-1,4-DHP, followed by CV on platinum electrode in acetonitrile + 0.1M TBAHFF. (- -) 0 min, (—) 7 min, and (·····) 14 min.

A typical time-course of the CPE at 0.95 V in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4) for *p*-methoxyphenyl-1,4-DHP, monitored by both GC/MS and CV techniques, is shown in Fig. 5. From this figure it is clear that the application of the potential resulted in the abundances of the corresponding ions of the parent drug and its oxidized derivative to change significantly over 30 min of CPE. However, use of CV on glassy carbon is only capable of following the temporal decay of the parent drug, with no indication as to the formation of new products. Also, a clear difference in sensitivity of the two techniques is obtained in this type of experiment. Thus, after 14 min of electrolysis no signal was observed by CV while, in contrast, even after 90 min electrolysis, the GC/MS technique permitted the detection of signals due to the parent compound (Fig. 5).

Reaction between C-4-substituted 1,4-DHP and ABAP-derived alkyl radicals

From the studies on the reactivity between 1,4-dihydropyridines and ABAP-derived alkyl radicals, monitored by the

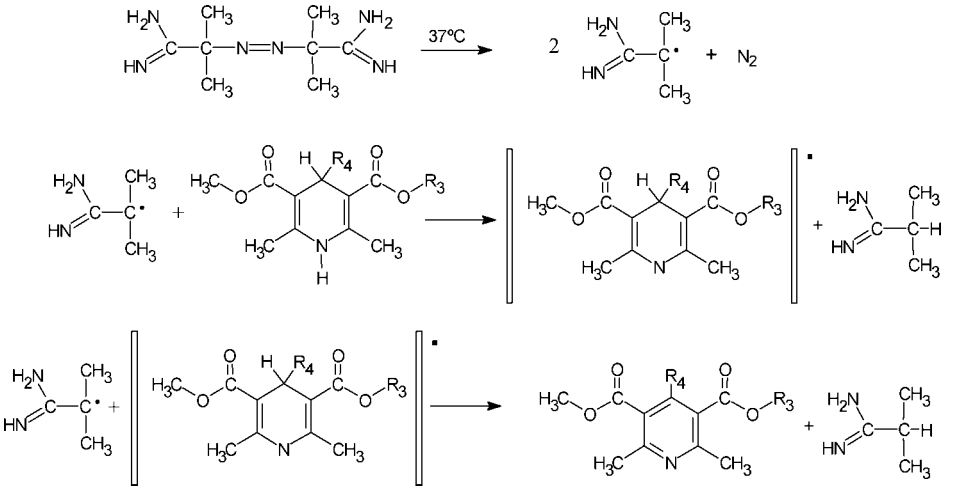


Figure 6. Scheme proposed for the reaction between C-4-substituted 1,4-dihydropyridines and ABAP-derived alkyl radicals in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4) at 37°C.

GC/MS technique, the following conclusions can be summarized: (1) 1,4-dihydropyridines, in reaction with alkyl radicals, undergo a dehydrogenation process yielding pyridine-based metabolites. This conclusion is supported by the respective retention times and the mass spectral fragmentation patterns for each compound, shown in Table 1. Clearly, the scavenging of the radicals involves an electron transfer reaction. (2) Retention times corresponding to the pyridine derivatives were lower than those of the corresponding parent drugs. (3) The tested 1,4-DHP derivatives reacted with alkyl free radicals, forming the pyridine derivative with yields ranging between 40 and 68%. Additionally, to document the proposed reaction mechanism between alkyl radicals and 1,4-dihydropyridines, the corresponding *N*-ethyl-1,4-DHP derivatives were assayed. In all cases these compounds did not react with alkyl free radicals, confirming the key role in the mechanism of the hydrogen atom in the 1-position of the 1,4-dihydropyrine ring (see below).

Based on results obtained by both the GC/MS and CV techniques, in connection with the reaction between the synthesized 1,4-dihydropyridines and ABAP-derived alkyl radicals, the reaction scheme shown in Fig. 6 can be proposed. Following the formation of the alkyl radicals, these react with the 1,4-DHP derivatives to give rise to an intermediate radical by hydrogen abstraction from the 1-position. Finally, a second hydrogen atom is lost from the 4-position by action of a second alkyl radical molecule to generate the oxidized 1,4-DHP, i.e. the pyridine derivative.

Parent drugs: *N*-ethyl-C-4-substituted 1,4-dihydropyridines

Table 2 lists the MS characteristics of the *N*-ethyl-1,4-DHP compounds. These compounds had the lowest retention times of all the compounds studied. Figure 7 shows the mass spectra of the *N*-ethyl-1,4-DHP derivatives with proposed structures for the main fragment ions. The base peak corresponds to the loss of the C-3 substituent ($COOCH_3$) to give $[M^+ - 59]$.

Concerning the mass spectral fragmentation pattern of these N-ethyl derivatives, the following characteristics can be summarized: (1) All the *N*-ethyl-1,4-DHP derivatives lost the C-4 substituent. (2) *N*-Ethyl-*p*-methoxyphenyl-1,4-DHP and *N*-ethyl-*p*-nitrophenyl-1,4-DHP had the same base peak, i.e. *m/z* 300 and 315, corresponding to the complete loss of the C-3 substituent. In contrast, *N*-ethyl-4-methyl-1,4-DHP lost the C-4 substituent. (3) Both *N*-ethyl-4-methyl-1,4-DHP and *N*-ethyl-*p*-methoxyphenyl-1,4-DHP lost the OCH_3 fragment from the C-3 position. (4) Loss of the nitro group from the C-4 substituents in *N*-ethyl-*p*-nitrophenyl-1,4-DHP was also observed.

Controlled-potential electrolysis (CPE)

The time-course of CPE of the N-ethyl derivatives, both in acetonitrile and aqueous media, as monitored by the GC/

Table 2. Base peaks (Bp) and the main fragment ions of *N*-ethyl-1,4-DHP compounds

Derivative	Bp (100%)	Ion (1)	Ion (2)	Ion (3)	M^+
N-Ethyl-4-methyl-1,4-DHP	252: M^+-CH_3	236 (16%): M^+-OCH_3	176 (15%): M^+-$COOCH_3$-OCH_3	192 (12%): M^+-$COOCH_3$-CH_3	267 (2%)
N-Ethyl-*p*-methoxyphenyl-1,4-DHP	300: M^+-$COOCH_3$	252 (35%): M^+-∅OCH_3	3328 (7%): M^+-OCH_3	–	359 (9%)
N-Ethyl-*p*-nitrophenyl-1,4-DHP	315: M^+-$COOCH_3$	252 (55%): M^+-∅NO_2	285 (24%): M^+-$COOCH_3$-CH_3-CH_3	269 (13%): M^+-$COOCH_3$-NO_2	374 (9%)

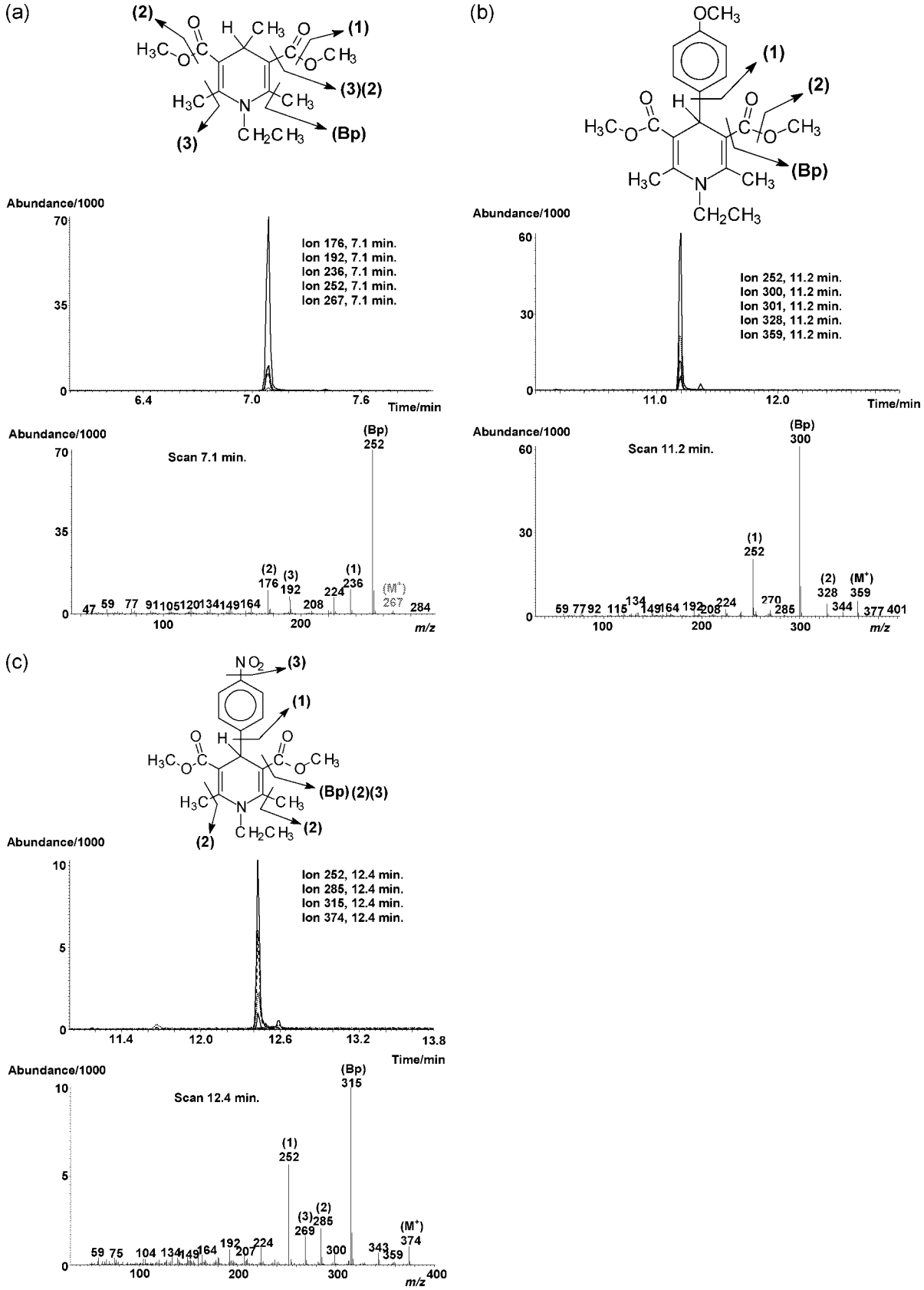


Figure 7. Mass spectra and proposed scheme for the mass spectral fragmentation of 100 μM parent drug *N*-ethyl-1,4-DHP derivatives (a)–(c) in 0.04 M Britton-Robinson buffer/ethanol (70:30, pH 7.4).

MS technique, did not reveal any new signals. In contrast, CV measurements showed a significant drop in the current corresponding to the parent drugs. The lack of new signal observed by the GC/MS technique can be explained based on the previous knowledge that electrochemical oxidation of N-substituted 1,4-DHP compounds

gives rise to pyridinium salts,[17,18] that are not amenable to GC analysis.

Reaction with ABAP-derived alkyl radicals

Results from these experiments on the N-ethyl derivatives indicated that, under the same experimental conditions as those used for the 1,4-DHP compounds, the N-ethyl-substituted 1,4-DHP compounds showed no apparent reaction, confirming the predictions from the mechanism outlined in Fig. 6.

CONCLUSIONS

1. The GC/MS procedure used to characterize the parent drugs, i.e. C-4-substituted 1,4-dihydropyridines and their corresponding N-ethyl derivatives, did not require derivatization.
2. Electrochemical oxidation of C-4-substituted 1,4-dihydropyridines, monitored by GC/MS, revealed the formation of the corresponding pyridine derivatives. In the case of the N-ethyl compounds, new signals were not detected by GC/MS, consistent with the expected formation of pyridinium salts.
3. Linear dependences of GC/MS peak area on a wide range of concentrations (15–3000 μg/L) were found for all the compounds.
4. C-4-substituted 1,4-dihydropyridines significantly reacted with ABAP-derived alkyl radicals generating the pyridine derivatives as final products. The corresponding N-ethyl compounds did not exhibit this reactivity, confirming the key role of the hydrogen atom on the nitrogen of the 1,4-dihydropyridine ring in the mechanism of scavenging of the alkyl radicals by the N-unsubstituted 1,4-dihydropyridines.
5. Results from this study revealed that the GC/MS procedure possessed enough sensitivity, under the conditions used here, to monitor both CPE and the reaction of the synthesized 1,4-DHP compounds with alkyl free radicals.

Acknowledgements

This work was partially supported by Grants from FONDECYT 8000016 and 2010044. Also, the support of DID (FPT-91) of University of Chile is acknowledged.

REFERENCES

1. Triggle D, Epstein GM (eds). Biochemical and Pharmacological Differences Among Calcium Channel Antagonists: Clinical Applications. In: *Calcium Antagonists in Clinical Medicine*. Hanley & Belfus: Philadelphia, 1992.
2. Langs DA, Strong PD, Trigle DJ. *J. Comput. Aided Mol. Des.* 1990; **4**: 215.
3. Mager PP, Coburn RA, Solo AJ, Triggle DJ, Rothe H. *Drug Des. Discov.* 1992; **8**: 273.
4. Kazda S. *Drugs* 1994; **48**: 32.
5. Lee CR, Bryson HM. *Drugs* 1994; **48**: 274.
6. Prabha PS, Das UN, Koratkar R, Sagar PS, Ramesh G. *Prostaglandin, Leukotrienes and Essential Fatty Acids* 1990; **41**: 27.
7. Spedding M, Fraser S, Clark B, Patmore L. *J. Neural Transm. (suppl.)* 1990; **31**: 5.
8. Blaedel WJ, Haas RG. *Anal. Chem.* 1970; **42**: 918.
9. Tong Mak I, Boehme P, Weglicki W. *Biochem. Pharmacol.* 1995; **50**: 1531.
10. Van Amsterdam FThM, Roveri A, Maiorino M, Ratti E, Ursini F. *Free Rad. Biol. Med.* 1992; **12**: 183.
11. Mason RP, Tong Mak I, Trumbore MW, Mason PE. *Am J. Cardiol.* 1999; **84**: 16L.
12. Vo D, Matowe WC, Ramesh M, Iqbal N, Wolowy MW, Howlett SE, Knaus EE. *J. Med. Chem.* 1995; **38**: 2851.
13. Berson J, Brown E. *J. Am. Chem. Soc.* 1955; **77**: 444.
14. Stout DM, Meyers AI. *Chem. Rev.* 1982; **82**: 223.
15. Haliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine*, Oxford University Press: New York, 2000.
16. Núñez-Vergara LJ, Sturm JC, Álvarez-Lueje Olea-Azar C, Sunkel C, Squella JA. *J. Electrochem. Soc.* 1999; **146**: 1478.
17. Ludvik J, Volke J, Klima J. *Electrochim. Acta* 1987; **32**: 1063.
18. Hurvois JP, Moinet C, Taillec A. *Electrochim. Acta* 1993; **38**: 1775.