Analysis of Dihydropyridine Calcium Channel Blockers Using Negative Ion Photoionization Mass Spectrometry

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Abstract

The fragmentation of dihydropyridine calcium-channel antagonists are compared by electrospray ionization (ESI) and atmospheric pressure photonization (APPI). The results demonstrate that in ESI the preferred ionization process is in positive mode, with the mass spectra of $[M+H]^+$ showing base peak ions probably formed by loss of alcohols from carboxyl groups. Conversely, in APPI, a high intense peak is observed in negative mode due to deprotonated molecule $[M–H]^–$ after two serial 1, 2-hydride shifts leading to a rearranged deprotonated molecule $[M–H]^–$. These ions undergo another 1,2-hydride shifts to produce a nitro-phenyl product ion of *m/z* 122. The APPI is also used to develop a method for the quantitation of dihydropyridines (e.g., nifedipine) in human plasma.

Introduction

Dihydropyridine (Figure 1) type calcium-channel antagonists are essential in the treatment of systemic sclerosis because they decrease vasospastic propensity. In other diseases, these drugs are suspected to have anti-oxidant properties (1,2). Several analytical methods based on mass spectrometry have been used for the quantitation of different dihydropyridines in complicated matrices such as plasma. These include high resolution gas chromatography coupled to electron impact mass spectrometry (3–5), high-performance liquid chromatography (HPLC) coupled to electrospray ionization mass spectrometry (HPLC–ESI–MS) (6), and HPLC coupled to ESI tandem MS (HPLC–ESI–MS–MS) (7–8) .

MS is preferred as a detector in pharmaceutical analysis due to its high specificity, sensitivity, ruggedness, versatility, and ease of use. (9). Regarding HPLC, ESI and atmospheric pressure chemical ionization (APCI) have by far been the most popular interfaces coupled with MS. In ESI, gas-phase ions of the analytes are formed by using a high electric field. Efficacious ionization is achieved when the analytes are already charged in solution; therefore, ESI is best suited for the analysis of polar and ionic compounds. In APCI, the liquid sample is first evaporated, after which charged plasma is formed by using a corona discharge, and the analytes are produced by gas-phase reactions. The polarity of the analytes can be somewhat lower with APCI than with ESI, but completely non-polar analytes cannot be ionized by either of the two ionization methods. In order to address this gap, the atmospheric pressure photoionization (APPI) was introduced (10) as an alternative ionization method for HPLC–MS. The ionization process in APPI is initiated by 10 eV photons emitted by a krypton discharge lamp. The photons can ionize compounds that possess ionization energies below their energy (10 eV), which includes larger molecules, but omits most of the typically used gases and solvents. However, as the ionization of the analytes is dependent on the ionization energy of the analyte rather than its proton affinity as with ESI and APCI, the ionization of molecules of low polarity is also possible.

In the present work, different dihydropyridines were analyzed by ESI and APPI, and the mechanisms of fragmentations were compared.

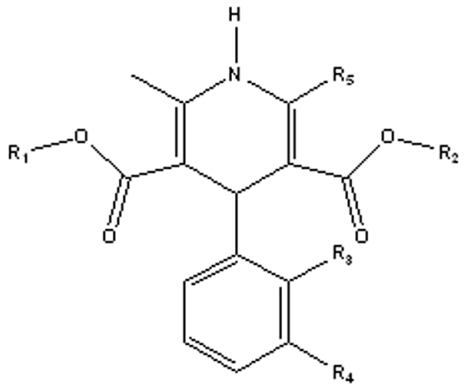

Figure 1. Structure of the studied dihydropyridines. Amlodipine: $R_1 = CH_3$, $R_2 = CH_2CH_3$, $R_3 = Cl$, $R_4 = H$, and $R_5 = CH_2OCH_2CH_2NH_2$; Felodipine: $R_1 = CH_3$, $R_2 = CH_2CH_3$, $R_3 = Cl$, $R_4 = Cl$, and $R_5 = CH_3$; Nimodipine: $R_1 = CHCH_3CH_3$, $R_2 = CH_2CH_3OCH_3$, $R_3 = H$, $R_4 = NO_2$, and $R_5 = CH_3$; and Nifedipine: $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = NO_2$ $R_4 = H$, and $R_5 = CH_3$.

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Experimental

ESI

The experimental results in ESI mode were recorded on a Sciex API 4000 triple stage quadrupole MS (Applied Biosystems, Foster City, CA) equipped with an API electrospray source operating in positive mode (ES+). The source block temperature was set at 650°C and the electrospray capillary voltage to 5.5 kV. Nitrogen was used as a collision gas. The ions were monitored in multiple reaction monitoring (MRM), and the conditions are described in Table I. The HPLC system (Agilent, Palo Alto, CA, Model 1100) consisting of a binary pump (G1312A) was used for all of the analyses. The chromatographic system consisted of a C8 analytical column (100 mm × 4.6 mm i.d., 3 µm film thickness) and an isocratic mobile phase of acetonitrile–water (80:20, v/v, with 10mM of formic acid) at a flow rate of 800 µL/min. The column operated at room temperature and presented a void time of 1.0 min. The temperature of the autosampler (CTC Analytics, Zwingen, Switzerland, HTS PAL) was maintained at 6.5°C and was devised to make 40-µL sample injections every 5.0 min. Data were acquired by Analyst software (1.4, Applied Biosystems).

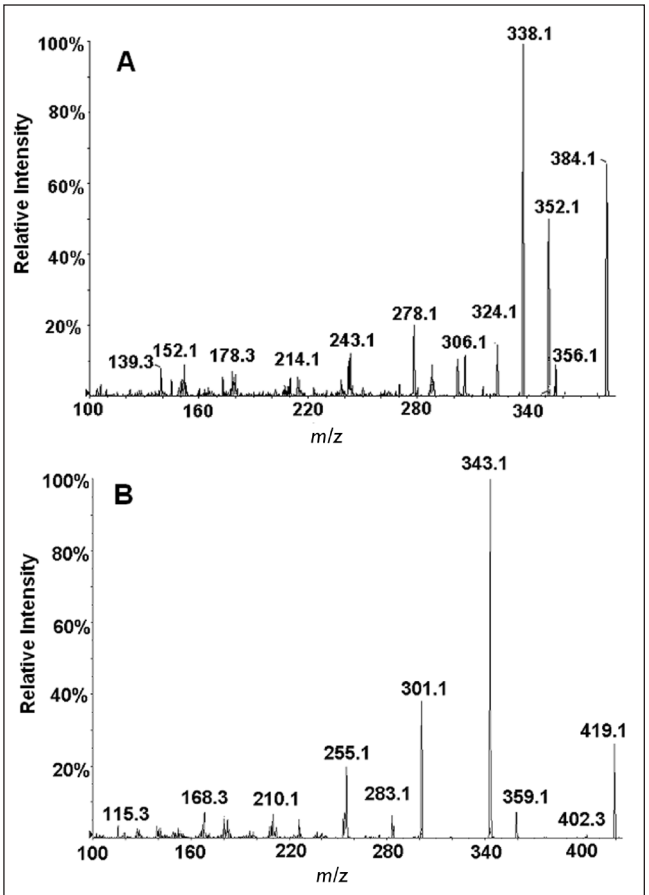


Figure 2. MS–MS mass spectra in ESI mode. Felodipine (A) and Nimodipine (B).

Table I. Conditions of MRM in the Analysis of Dihydropiridines

Compound	Transition (*m/z*)	DP* (V)	FP† (V)	CE‡ (eV)	CXP§ (V)
Electrospray ionization					
Felodipine	383.9 → 352.1	56	–	17	16
Amlodipine	409.1 → 238.0	36	–	13	14
Nimodipine	419.1 → 343.1	36	–	13	20
Atmospheric pressure photoionization					
Nifedipine	344.9 → 222.0	–16	–130	–16	–7
Nimodipine	417.1 → 122.0	–31	–200	–30	–7
Felodipine	382.0 → 144.8	–26	–110	–18	–9
Amlodipine	407.0 → 295.1	–25	–140	–17	–8

* Declustering potential
† FP = Focusing potential
‡ CE = Collision Energy
§ CXP potential

APPI

The experimental results in APPI mode were recorded on a Sciex API 3000 triple stage quadrupole MS (Applied Biosystems) equipped with an APPI source operating in negative mode (PS–). The source block temperature was set at 400ºC, the photoionization capillary voltage at 1.2 kV, and nitrogen was used as the collision gas. The ions were monitored in MRM, and the conditions are described in Table I. An HPLC system (LC10ADvp, Shimadzu, Japan) consisting of a pump and an autosampler was used for all of the analyses. The chromatographic system consisted of a C8 Genesis analytical column (100 mm × 2.1-mm i.d., 4-µm film thickness) and the mobile phase was a mixture of acetonitrile–water–acetone (80:15:5, v/v/v, with 20mM of ammonium acetate and 0.1% of acetic acid) at a flow rate of 350 µL/min. The total run time was set for 2.0 min. The column operated at room temperature and presented a void time of 0.8 min. The temperature of the autosampler was maintained at 8.0°C and was set up to make a 40 µL sample injection. Data were acquired by Analyst software (1.4, Applied Biosystems).

Sample preparation

Aliquots (0.50 or 0.20 mL) of human plasma were submitted to liquid–liquid extraction after addition of internal standard (IS) solution (50 µL of the working standard solution). The tubes were vortex mixed for 20 s and allowed to stand at room temperature for 2 min. Four milliliters of diethyl ether–hexane (80:20, v/v) were added and samples were vortexed for 40 s. The upper layers were transferred to clean tubes and evaporated under N_2 (40°C). Dry residues were dissolved with 200 µL of mobile phase acetonitrile–water (50:50 v/v), transferred into glass microvials, capped, and placed in the autosampler.

Specificity/selectivity

Each blank sample of five different pools of plasma, including a pool of lipemic and another of haemolyzed, were tested for interference using the proposed extraction procedure and analytical conditions.

Recovery and matrix effect

The recovery was evaluated by calculating the mean response of each concentration and dividing the extracted sample mean by the

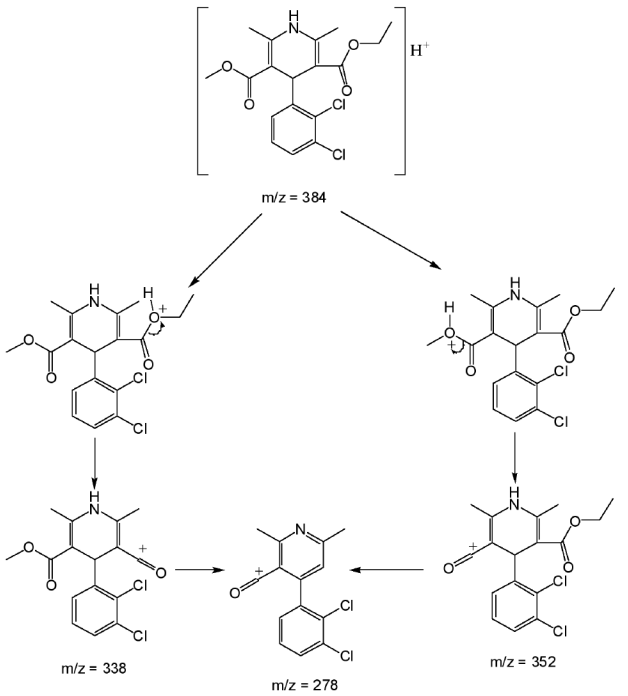


Figure 3. Proposed mass fragmentation pathways for dihydropiridines by ESI (Felodipine case).

unextracted (spiked blank-plasma extract) sample mean of the corresponding concentration. A comparison with the unextracted samples, spiked on plasma residues, was performed in order to eliminate the matrix effect. Experiments were carried out using the ratio between spiked mobile phase solution and unextracted samples, spiked on plasma residues. The matrix effect was determined by dividing the mean of the blank-plasma samples set by the mobile-phase samples set.

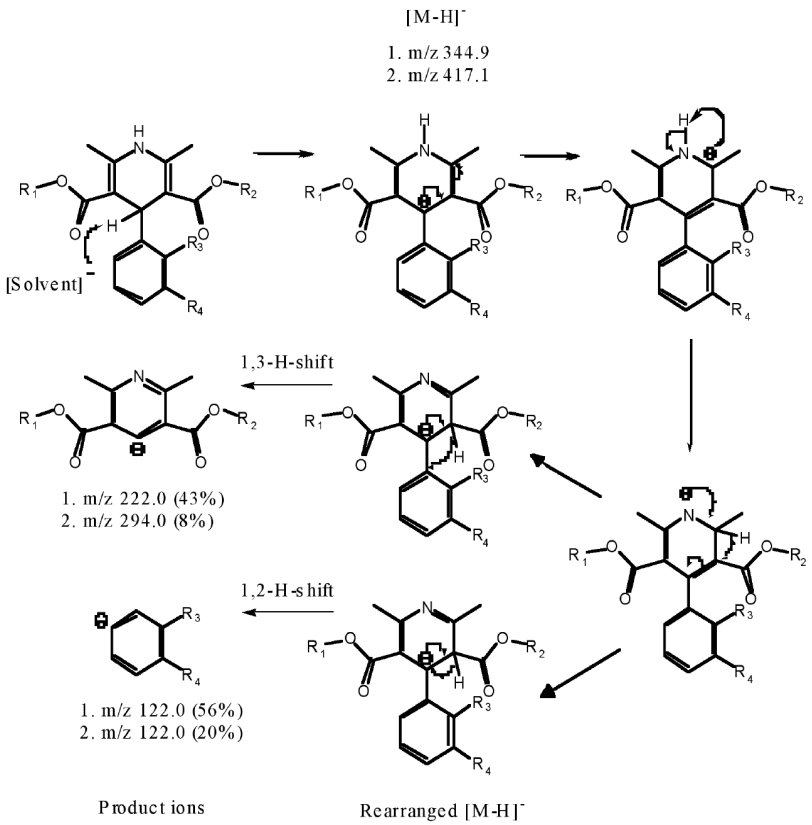
Stability

Quality control samples prepared to test stability were subjected to short-term (6 h) room temperature, three freeze-thaw cycles, and 24 h long-term autosampler (8°C) stability tests. Subsequently, the analyte concentrations were measured and compared with freshly prepared samples.

Precision and accuracy

To assess the precision and accuracy of the developed analytical method, three distinct concentrations in the range of expected concentrations were evaluated using eight determinations per concentration.

Precision and accuracy were assessed on a within-day basis (intra-batch) during a single analytical run and on a between-day basis (inter-batch), measuring the between day variability, possibly involving different analysts, reagents, etc.



1. Nifedipine: $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = NO_2$, $R_4 = H$
2. Nimodipine: $R_1 = (CH_3)_2CH$, $R_2 = CH_2CH_2OCH_3$, $R_3 = H$, $R_4 = NO_2$

Figure 4. Proposed mass fragmentation pathways for dihydropiridines by APPI.

Results and Discussion

The use of APPI for LC–MS analysis has grown considerably. Photospray ionization is widely used as an efficient ionization mode for many different classes of substances, including polycyclic aromatic compounds. An advantage of this ionization system is that the ionization of the analyte is soft and produces minimal fragmentation and predominant molecular ion signals (11–13).

However, in the case of dihydropyridines, our studies have demonstrated that the ionization processes in ESI and APPI follow different pathways. With ESI, the ionization in positive mode is favored, while with APPI, the ionization predominantly occurs in the negative mode.

The ionization process in ESI can occur by different mechanisms (14–17). The analyte ions may be formed by electrolytic oxidation or reduction, from acid/base reactions within the electrospray droplets, or from gas phase proton transfer reactions that take place among ions that have been released from electrospray droplets. In APPI, the ionization process is initiated by 10 eV photons emitted by a krypton discharge lamp. The initial reaction is the formation of a radical ion of the dopant, whose ionization energy must be lower than the energy of the photons. The basic mechanism of photoionization is $M + h\upsilon \rightarrow M^+ + e^-$ (10). Consequently, toluene is normally

used as dopant. Finally, the dopant radical ions ionize the analytes through charge exchange (10–12,18–19).

Dihydropyridines in ESI

As previously published (8), the electrospray positive mass-spectrum for both dihydropyridines analyzed (felodipine, and nimodipine) showed the same fragmentation pattern (Figure 2) with the base peak ions at *m/z* 338 for felodipine and *m/z* 343 for nimodipine. In the cases felodipine and nimodipine, the MS–MS product ion spectra of $[M+H]^+$ showed the same kind of base peak ions (Figure 2), probably formed by loss of alcohols from carboxyl groups (Figure 3). In felodipine, the loss of ethyl alcohol is energetically favored in relation to the methyl alcohol, and in nimodipine the loss of 2-methoxy-ethyl alcohol is energetically favored in relation to the 1-methylethyl alcohol (Figures 2 and 3). Due to the presence of the 2-aminoethoxymethyl group in the amlodipine molecule, a more complex fragmentation is produced which will not be discussed in this paper.

Dihydropyridines in APPI

There are several possible ionization mechanisms in APPI in negative mode: interactions between electron and molecule, charge exchange, proton transfer, and association and displacement reactions. In the present case, as shown in Figure 4, the proposed ionization mechanism is initiated by reacting with charged solvent molecules $[S–H]^–$; both nimodipine and nifedipine are ionized through proton transfer, forming deprotonated molecule $[M–H]^–$ of *m/z* 344.9 and 417.1, respectively. These, after two serial 1,2-hydride shifts leading to a rearranged of the deprotonated molecule $[M–H]^–$, undergo either another 1,2-hydride shift to produce a nitro-phenyl product ion of *m/z* 122 from both nimodipine and nifedipine, or a 1,3-hydride shift to provide the aromatization of the substituted dihydropyridine ring producing ions of *m/z* 222 and *m/z* 294 from nimodipine and nifedipine, respectively.

A comparison of Figures 2 and 5 shows a considerable reduction in complexity of the mass spectra by APPI compared to ESI. Minimal fragmentation during the ionization process is one of the advantages APPI in relation to ESI, increasing the selectivity of the analysis.

During the experiments, it was observed that the use of acetate buffer in the mobile phase increases the intensity of the deprotonated molecule by approximately a factor of 10 times. The literature shows that different mobile phases containing acetate buffers results in the formation of abundant $CH_3COO^–$ ion by the APPI (18).

Table II. Quality Controls Validating Results of the Accuracy and Precision

Nifedipine	Parameter	Nominal concentration (ng/mL) 0.100	0.30	50	400
Intra-batch	Mean found (*n* = 8) (ng/mL)	0.102	0.274	49.2	380
	Precision (%)	8.8	4.1	2.2	2.7
	Accuracy (%)	101.7	91.4	98.4	95.0
Inter-batch	Mean found (*n* =3) (ng/mL)	0.104	0.271	50.3	380
	Precision (%)	12.9	7.4	2.4	2.9
	Accuracy (%)	103.9	90.3	100.5	95.0
Felodipine	**Parameter**	**Nominal concentration (ng/mL) 0.020**	**0.060**	**50**	**400**
Intra-batch	Mean found (*n* = 8) (ng/mL)	0.102	0.274	49.2	380
	Precision (%)	8.8	4.1	2.2	2.7
	Accuracy (%)	101.7	91.4	98.4	95.0
Inter-batch	Mean found (*n* = 3) (ng/mL)	0.104	0.271	50.3	380
	Precision (%)	12.9	7.4	2.4	2.9
	Accuracy (%)	103.9	90.3	100.5	95.0
Amlodipine	**Parameter**	**Nominal concentration (ng/mL) 0.100**	**0.300**	**3.0**	**45.0**
Intra-batch	Mean found (*n* = 8) (ng/mL)	0.102	0.274	49.2	380
	Precision (%)	8.8	4.1	2.2	2.7
	Accuracy (%)	101.7	91.4	98.4	95.0
Inter-batch	Mean found (*n* = 3) (ng/mL)	0.104	0.271	50.3	380
	Precision (%)	12.9	7.4	2.4	2.9
	Accuracy (%)	103.9	90.3	100.5	95.0

Dopant effect in APPI in negative mode

Toluene is a dopant commonly used due to its ionization potential of 8.83 eV, as well as its potentially high purity grade and low toxicity. Acetone (ionization potential of 9.70 eV) is another substance that can be used as dopant. However, the toluene and acetone were compared in the analysis of free anabolic steroids, and with toluene, approximately 20–50% higher sensitivity was achieved (20). The literature shows that formed reaction ions depend not only on the mobile phase but also on the dopant used, as the proton transfer between the dopant molecular ion and the solvent (mobile phase) is the same and depends on the proton affinities of the solvent and the dopant (21). In our laboratory, acetone has been used routinely as a dopant in several methods using positive or negative ionization mode in the analysis of different substances (e.g., isosorbide 5-mononitrate) (22). Although the literature indicates the best results using toluene as compared to acetone, the use of acetone (5% of the mobile phase volume) as a dopant has the practical advantage in that it can be used in the mobile phase without HPLC column damage (we have columns with more than 5000 analysis without loss of performance). Adding the dopant to the mobile phase affords higher throughput (more than 200 samples/day can be analyzed) and ruggedness than the more common procedure of adding it post-column, because dopant and mobile phase flows cannot vary in relation to each

other. Consequently, one does not experience variation in sensitivity. The use of acetone also shows a high sensitivity (in the case of nifedipine, and the use of APPI permits a limit of quantitation of the 0.1 ng/mL of nifedipine in plasma).

Ion suppression in ESI and APPI

In the analysis of biological samples (e.g., plasma extracts) one important factor that can affect the quantitative performance of a mass detector is ion suppression. Sample matrix, coeluting compounds, nonvolatile materials, can contribute to this effect. Ion suppression can have potentially deleterious effects on both ESI and APCI. Ionization effects can theoretically occur in either the solution phase or the gas phase. Experiments involving ESI of biological extracts have shown that the main cause of ion suppression is a change in the spray-droplet solution properties caused by the presence of nonvolatile or less volatile solutes. These nonvolatile materials (e.g., salts, ionpairing agents, endogenous compounds, drugs/metabolites) change the efficiency of droplet formation or droplet evaporation, which in turn affects the amount of charged ion in the gas phase that ultimately reaches the detector (23). To evaluate the ion suppression effect in APPI as ESI ionization, the assay proposed by Bonfiglio et al. (24) was used, which is based on post-column mixing of the analyte of interest with the eluate of a column in which a blank sample is injected. Figure 6 shows that in both ionization processes (APPI and ESI), the ion suppression for the dihydropyridines (example of nimodipine) is similar, and therefore in the present case there is no advantage in using APPI.

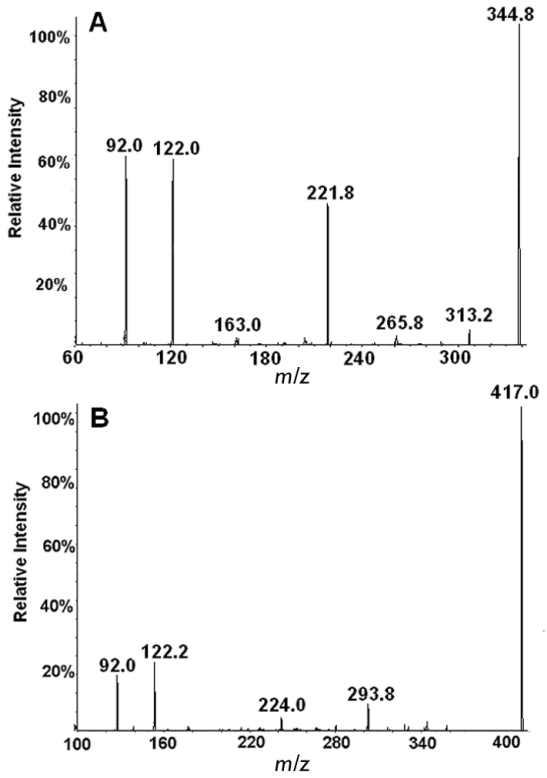


Figure 5. Full scan mass spectra in APPI mode. Nifedipine (A) and Nimodipine (B).

APPI assay performance for dihydropyridines in human plasma

The methods were validated (results later) and applied to evaluate the bioequivalence studies (the pharmacokinetic data were not discussed in this paper).

The optimized method was validated by assessment of recovery, linearity, quantitation limit, precision, and accuracy. Coefficients of variation (CV) and relative errors of less than 15% were considered acceptable, except for the quantitation limit (LOQ), whose values were extended to 20%, as recommended by Shah et al. (25) and Bressole et al. (26) for the analysis of biological samples for pharmacokinetic studies. The method was linear for nifedipine from 0.1 to 50 ng/mL ($r^2 > 0.9990$).

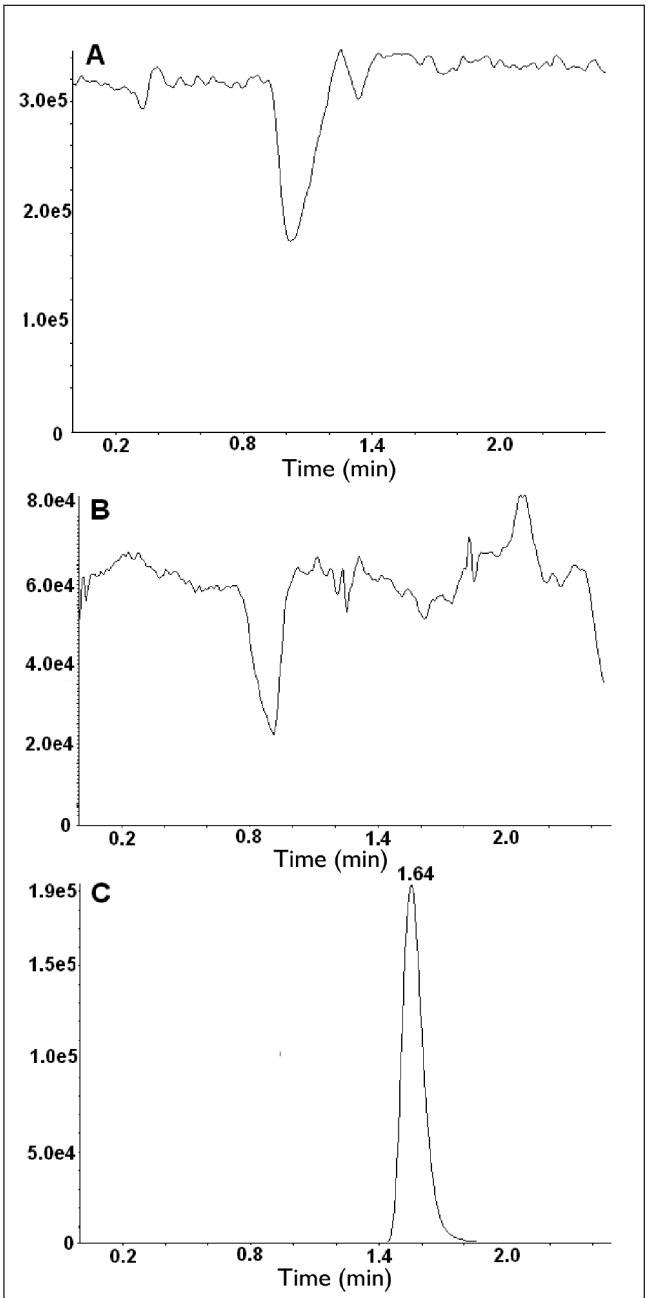


Figure 6. Ion suppression result for nimodipine in APPI (A); ion suppression result for nimodipine in ESI (B); and typical MRM chromatogram in APPI mode of nimonipine at 33 ng/mL in human plasma (C).

Nifedipine validation data

The recovery of nifedipine was 104.9% (CV 2.6%); 109.0% (CV 3.9%); and 109.5% (CV 5.9%) for the 1.5, 50, and 400 ng/mL standard concentrations, respectively. The recovery of the internal standard (nimodipine) was 103.1% (CV 1.9%); 100.3% (CV 3.2%), and 101.2% (CV 1.4%) for the 1.5, 50, and 400 ng/mL standard concentrations, respectively. Stability tests performed indicated no significant degradation under the conditions described previously, including in the freeze and thaw test, short-term room temperature test and long-term test (27 days). The human plasma spiked at final concentrations of 1.5, 50, and 400 ng/mL (n = 5 for each concentration). In the latter case (long-term test) a variation of –8.5%, –7.2%, and –6.5%, respectively, was determined relative to freshly spiked samples.

Amlodipine validation data

The recovery of amlodipine was 70.5% (CV 6.3%); 71.9% (CV 13.3%), and 68.5% (CV 4.0%) for the 0.3, 3.0, and 45 ng/mL standard concentrations, respectively. The recovery of the internal standard (nimodipine) was 88.3% (CV 7.3%); 95.2% (CV 11.0%); and 96.4% (CV 7.2%) for the 1.5, 50, and 400 ng/mL standard concentrations, respectively. Stability tests performed indicated no significant degradation under the conditions described earlier, including in the freeze and thaw test, short-term room temperature test, and long-term test (370 days). The human plasma spiked at final concentrations of 0.3, 3.0, and 45 ng/mL (n = 5 for each concentration). In the latter case (long-term test) a variation of –10.5%, –15.0%, and –13.2%, respectively, were determined relative to freshly spiked samples.

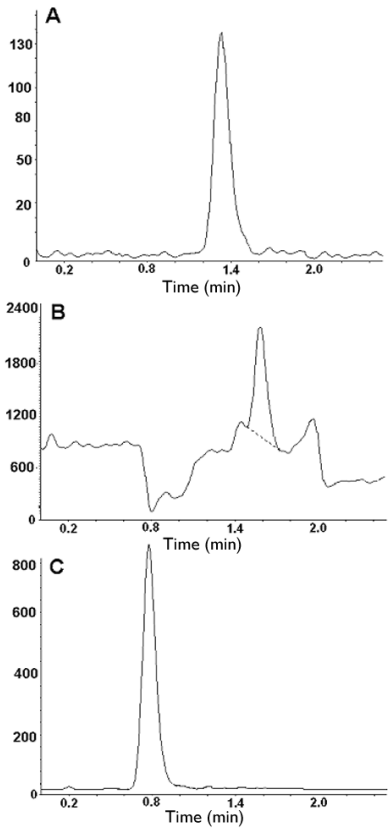


Figure 7. The representative MRM Chromatograms at LOQ concentration: felodipine at 20 pg/mL (A), nifedipine at 100 pg/mL (B), and amlodipine at 100 pg/mL (C).

Felodipine validation data

The recovery of felodipine was 83.0% (CV 5.9%), 83.1% (CV 2.1%), and 84.7% (CV 1.2%) for the 0.6, 1.2, and 9.0 ng/mL standard concentrations, respectively. The recovery of the internal standard (nimodipine) was 90.3% (CV 9.4%), 86.1% (CV 4.5%), and 86.7% (CV 1.7%) for the 0.6, 1.2, and 9.0 ng/mL standard concentrations, respectively. Stability tests performed indicated no significant degradation under the conditions described earlier, including the freeze and thaw test, short-term room temperature test, and long-term test (17 days). The human plasma spiked at final concentrations of 0.6, 1.2, and 9.0 ng/mL (n = 5 for each concentration). In the latter case (long-term test), variations of –1.2%, +3.6%, and –7.0%, respectively, were determined relative to freshly spiked samples.

Between- and within-run accuracy and precision as summarized in Table II meet the requirements for biological procedures as laid down in the international guidelines (27).

The HPLC–MS–MS method described here for nifedipine, felodipine, and amlodipine quantitation in human plasma was developed to analytical runs up until 66 h, 127 h, and 24 h, respectively, as was observed in the post processing tests and the total run time in all cases was the 2.5 min, permitting a high throughput. Figure 7 shows the typical chromatograms at LOQ for nifedipine, felodipine, and amlodipine.

Conclusions

Different mass fragmentation patterns were provided by APPI and ESI for the dihydropyridines felodipine, nifedipine, nimodipine, and amlodipine. The negative ionization mode was favored with APPI whereas with ESI the positive mode was favored.

We believe such a difference would be explained by the reaction (charge transfer) of analyte molecules with negatively charged acetonitrile (solvent) clusters only in APPI. Contrasted with toluene, the use of acetone as dopant made it possible for the dopant to become a mobile phase component, increasing throughput without damaging the HPLC column. LC–MS–MS using APPI provided an efficient, precise, and accurate method for the determination of dihydropyridines in human plasma.

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