Journal of Chromatography, 647 (1993) 155–166
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2795

Investigation into lacidipine and related metabolites by high-performance liquid chromatography–mass spectrometry

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ABSTRACT

HPLC–MS methods were developed in order to characterize the main biotransformation products of lacidipine, a new antihypertensive drug. Thermospray and particle beam interfaces were used because of their complementary information. In fact, the former provided molecular mass indication, while PB allowed the acquisition of typical electron impact and chemical ionization spectra. Chemical ionization was performed with methane and isobutane as reagent gases. This paper describes the application of these techniques for the analysis of lacidipine and standard metabolites and their identification in biological fluids.

INTRODUCTION

Lacidipine (Fig. 1) is a dihydropyridine derivative [1] that has been developed as an antihypertensive drug [2]. As shown in Fig. 1, the main phase I metabolic routes consist of the oxidation of the dihydropyridine ring to pyridine (compounds **B**, **D**, **F**, **G** and **H**) and the hydrolysis of the ester moieties (compounds **C**, **D**, **E** and **F**). Further phase I metabolic pathways involve the hydroxylation of the methyl groups (compound **G**) and the formation of lactones (compound **H**).

Lacidipine is administered orally in relatively low doses (2–6 mg/day) [3] and, in order to study its metabolic profile, it has been necessary to develop a sensitive and specific method for the assay of the parent drug and its metabolites in biological fluids. Mass spectrometry plays an important role in the structural elucidation and determination of substituted dihydropyridines at a low level. Electron impact (EI) [4], chemical ionization (CI) [5] and fast atom bombardment (FAB) [6] were previously used to investigate lacidipine and related compounds. For other dihydropyridines, combined techniques (GC–MS [7–14] and LC–MS [15,16]) have also been used to analyse biological fluids. For lacidipine and its metabolites, HPLC–MS methods were developed by using thermospray (TSP) and particle beam (PB) interfaces. The characterization of the metabolites by TSP-MS and PB-MS is described, including an example of the application of TSP-MS for the identification of the main metabolite in rat plasma.

EXPERIMENTAL

The HPLC–MS system comprised a Model 1090A solvent-delivery system (Hewlett Packard, USA), a Model Uvidec-100 UV detector (Jasco, Japan) and a Model 5988A single quadrupole mass spectrometer (Hewlett-Packard), equipped with a thermospray or a Model 59980A particle beam interface (Hewlett-Packard).

Lacidipine and its standard metabolites were synthesized by Chemical Development, Glaxo Research Laboratories (Verona, Italy) [1]. Stock solutions of standard compounds in methanol were prepared at a concentration of about 1

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0021-9673/93/$06.00

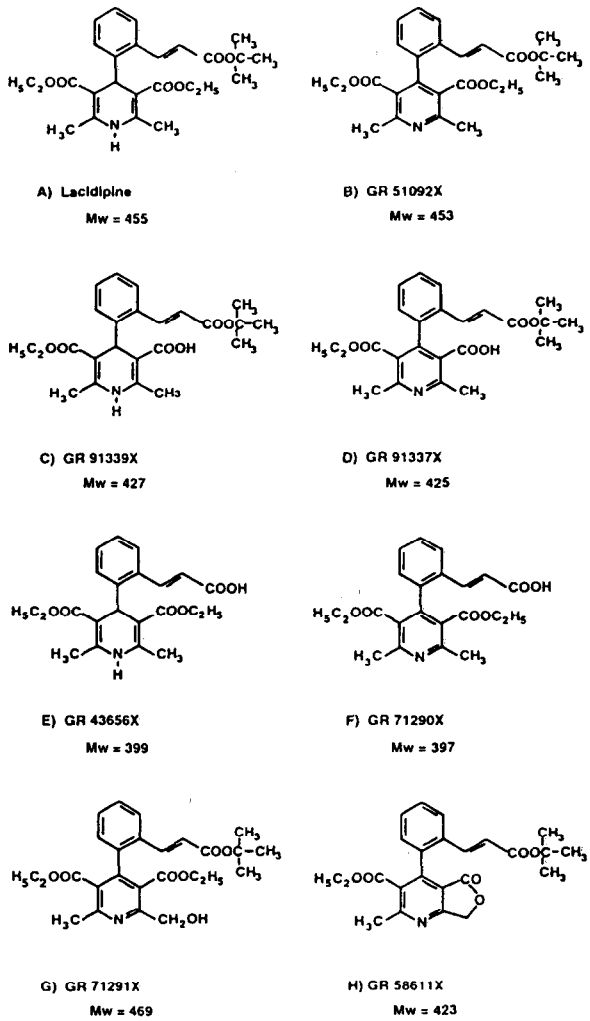

Fig. 1. Structures of lacidipine and investigated metabolites. Mw = Molecular mass.

mg/ml. They were diluted with the mobile phase just before injection.

HPLC–TSP-MS conditions for standard metabolites

Mobile phase for thermospray HPLC–MS consisted of 0.05 *M* ammonium acetate (pH 6.2)–methanol–acetonitrile (55:35:10, v/v/v). The flow-rate was 1.2 ml/min and the injection volume was 100 μl.

The thermospray interface was operated with probe temperatures set at 100°C (stem) and 162–164°C (tip). The source was maintained at 275°C, with the analyser manifold pressure of $2.6 \cdot 10^{-6}$ Torr (1 Torr = 133.322 Pa). The measurements were performed in the "filament-on" acquisition mode, in both positive- and negative-ion conditions. The electron multiplier was set at 2800 V and the scan range was 300–600 mass units.

HPLC–PB-MS conditions for standard metabolites

Mobile phase (flow-rate 0.4 ml/min) consisted of acetonitrile–water (75:25, v/v). The sample volume injected was 100 μl.

The particle beam interface was operated with the desolvation chamber temperature set at 70°C, the second momentum separator pressure at 0.6 Torr and the helium inlet pressure at 30 p.s.i. (1 p.s.i. = 6894.76 Pa).

For EI measurements the source temperature was maintained at 250°C, and for CI at 200°C. The reagent gases used for CI were methane and isobutane. Mass spectra were obtained in both positive- and negative-ion mode with source pressure of 0.3 Torr and manifold pressure of $1.1 \cdot 10^{-4}$ Torr when methane was used, while for isobutane the two pressures were 0.45 and $1.6 \cdot 10^{-4}$ Torr, respectively.

HPLC–TSP-MS conditions for plasma samples

A column-switching technique was used for the direct injections of rat plasma samples.

The loop of a Model 7125 manual injection valve (Rheodyne) was substituted by a 30 × 4.6 mm precolumn packed with Spherisorb RP-18 30–40 μm silica (Merck), which was conditioned with 3 ml of methanol and 3 ml of water before each injection.

Rat plasma (0.5–1.0 ml) was diluted before injection with an equal volume of 20% acetonitrile in water and 100 μl of concentrated formic acid. After each injection the precolumn was washed with 3 ml of water, then backflushed by valve switching into a 100 × 4.6 mm Novapack analytical column (Waters) maintained at 50°C.

Gradient elution was performed at 1.2 ml/min flow-rate with a mobile phase consisting of methanol, acetonitrile and aqueous ammonium acetate (0.05 *M*, pH 5), the compositon of which varied according to Table I.

The thermospray interface was operated with

TABLE I

CHROMATOGRAPHIC CONDITIONS FOR HPLC–MS ANALYSIS OF RAT PLASMA SAMPLES

Column, 100 × 4.6 mm Novapack (Waters); precolumn, 30 × 4.6 mm, Perisorb RP-18, 30–40 μm (Merck); mobile phase, Ammonium acetate (0.05 *M*, pH 5.0) in a mixture of methanol, acetonitrile and water; flow-rate, 1.2 ml/min; temperature, 50°C; UV detector wavelength, 282 nm; injection volume, 100 μl.

Time (min)	Methanol (%)	Acetonitrile (%)	Water (%)
0	35	10	55
7	35	10	55
30	60	10	30
35	80	10	10
45	80	10	10
70	35	10	55

the probe temperatures initially set at 102°C (stem) and 162–164°C (tip) for 7 min, then gradually changed according to a linear gradient up to 98°C (stem) and 150–152°C (tip) at 30 min. The mass spectrometer source was maintained at 275°C with the filament on and the measurements were performed in positive-ion conditions. The other MS conditions were the same as reported in the previous section.

RESULTS AND DISCUSSION

Thermospray

Positive and negative thermospray mass spectra of lacidipine are reported in Fig. 2. The base peak was assigned as the adduct ion $[M + NH_4]^+$, *m/z* 473, whereas a low-intensity peak was observed for the protonated molecular ion, *m/z* 456. The presence in the mobile phase of acetonitrile and ammonium acetate led to the formation of an adduct ion at *m/z* 514, ascribed to $[M + CH_3CN + NH_4]^+$, [17]. In the negative-ion spectra, molecular ion, *m/z* 455, and deprotonated $[M - H]^-$ ion, *m/z* 454, were both present. These ionic species are originated by two mechanisms —electron attachment and deprotonation— both present in thermospray ionization.

The molecular ions and the related adducts of metabolites in positive- and negative-ion spectra are reported in Table I. The base peak in the positive-ion TSP spectra of three pyridine derivatives was the protonated molecular ion, $[MH]^+$, whereas for the dihydropyridine it was the adduct ion $[M + NH_4]^+$. In negative-ion TSP mass spectra, the ionic species $[M + OAc]^-$ were assigned as the adducts with the acetate of the mobile phase.

The positive-ion TSP mass spectra of compounds **G** and **H**, the hydroxylated and the lactone metabolite, respectively, are reported in Fig. 3. For metabolite **H** the protonated molecular ion (*m/z* 424) and two adduct ions ($[M + NH_4]^+$ and $[M + CH_3CN + NH_4]^+$) were observed. The two fragment ions at *m/z* 368 and 385 can be ascribed to the cleavage of the *tert.*-butyl ester moiety (*m/z* 368 = $[MH - C(CH_3)_3 + H]^+$ and its ammonium adduct ion (*m/z* 385 = $[MH - C(CH_3)_3 + NH_4]^+$).

For metabolite **G**, the protonated molecular ion (*m/z* 470) and the adduct $[M + CH_3CN + NH_4]^+$ (*m/z* 528) were present. The other fragments corresponded to the ionic species previously described for the lactone metabolite **H** (*m/z* 368, 385, 424, 441 and 482). This was probably due to the condensation of side chains ($-COOC_2H_5$ and $-CH_2OH$) of metabolite **G** with loss of ethanol caused by the operating high temperatures.

Particle beam

Electron impact. Additional structural information was obtained from particle beam mass spectra. The electron impact mass spectrum of lacidipine is shown in Fig. 4(a). The molecular ion abundancy was very low (*m/z* 455, 2%), while the fragment ions related to cleavages of the side chains were observed. Radical losses of $\cdot C(CH_3)_3$, $\cdot OC(CH_3)_3$ and $\cdot COOC(CH_3)_3$ produced the ionic species at *m/z* 398, 382 and 354 already described [4]. The base peak, at *m/z* 252, is generated by elimination of *tert.*-butyl cinnamate (see structure in Fig. 4a).

The other compounds showed a similar behaviour and the mass spectra were in agreement with those previously obtained by direct probe introduction [4]. The base peaks included the

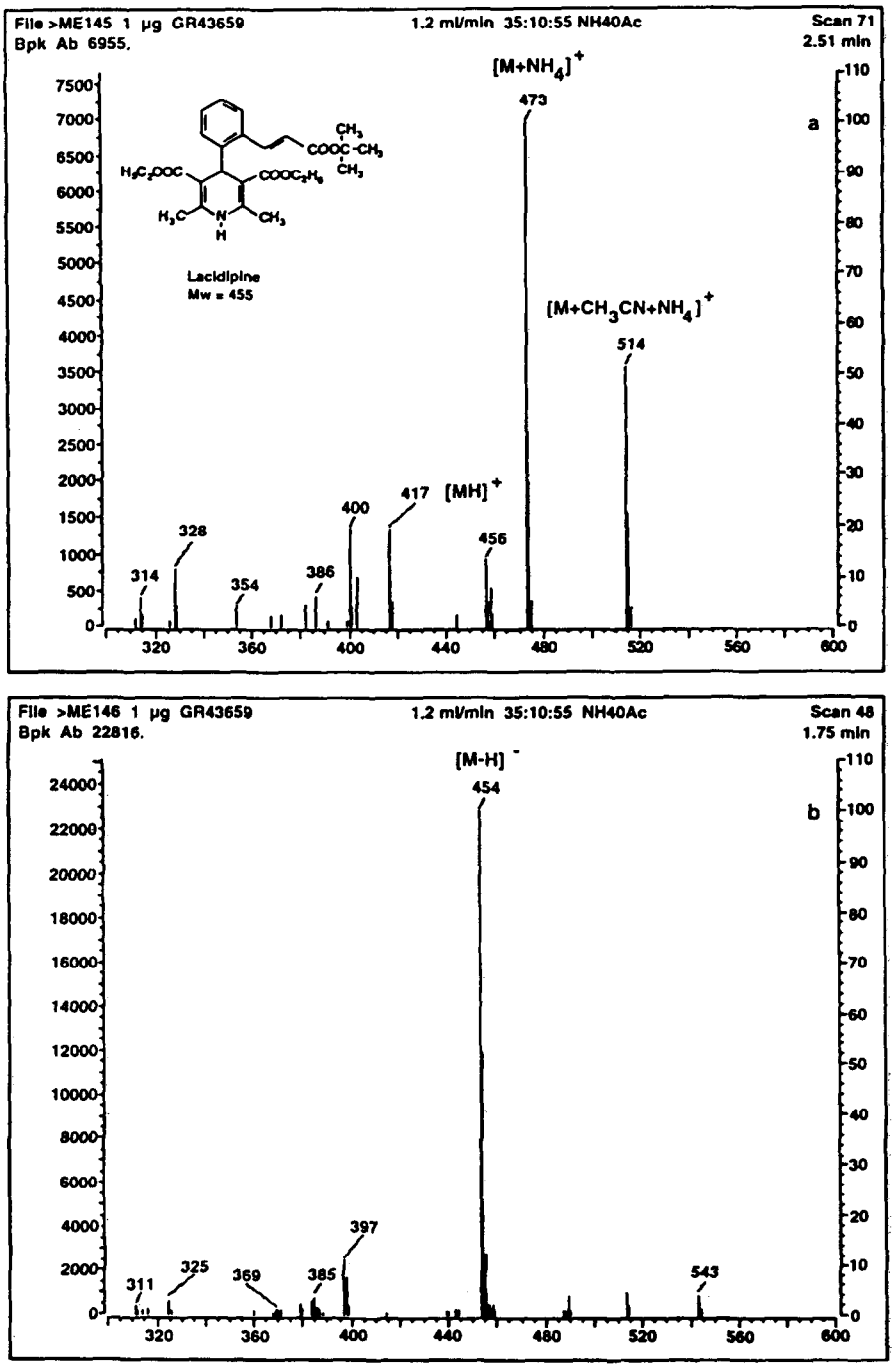

Fig. 2. Thermospray mass spectra of lacidipine: (a) positive-ion mode; (b) negative-ion mode.

pyridine ring but they were generated by different fragment ions.

It is noteworthy that the EI mass spectra of compounds **G** and **H** (Fig. 4b and c) were almost superimposable. For compound **G** the neutral loss of CH_3CH_2OH and the condensation to lactone was the primary reaction, followed by a fragmentation pathway similar to compound **H**. By PB-EI it was not possible to distinguish these two metabolites.

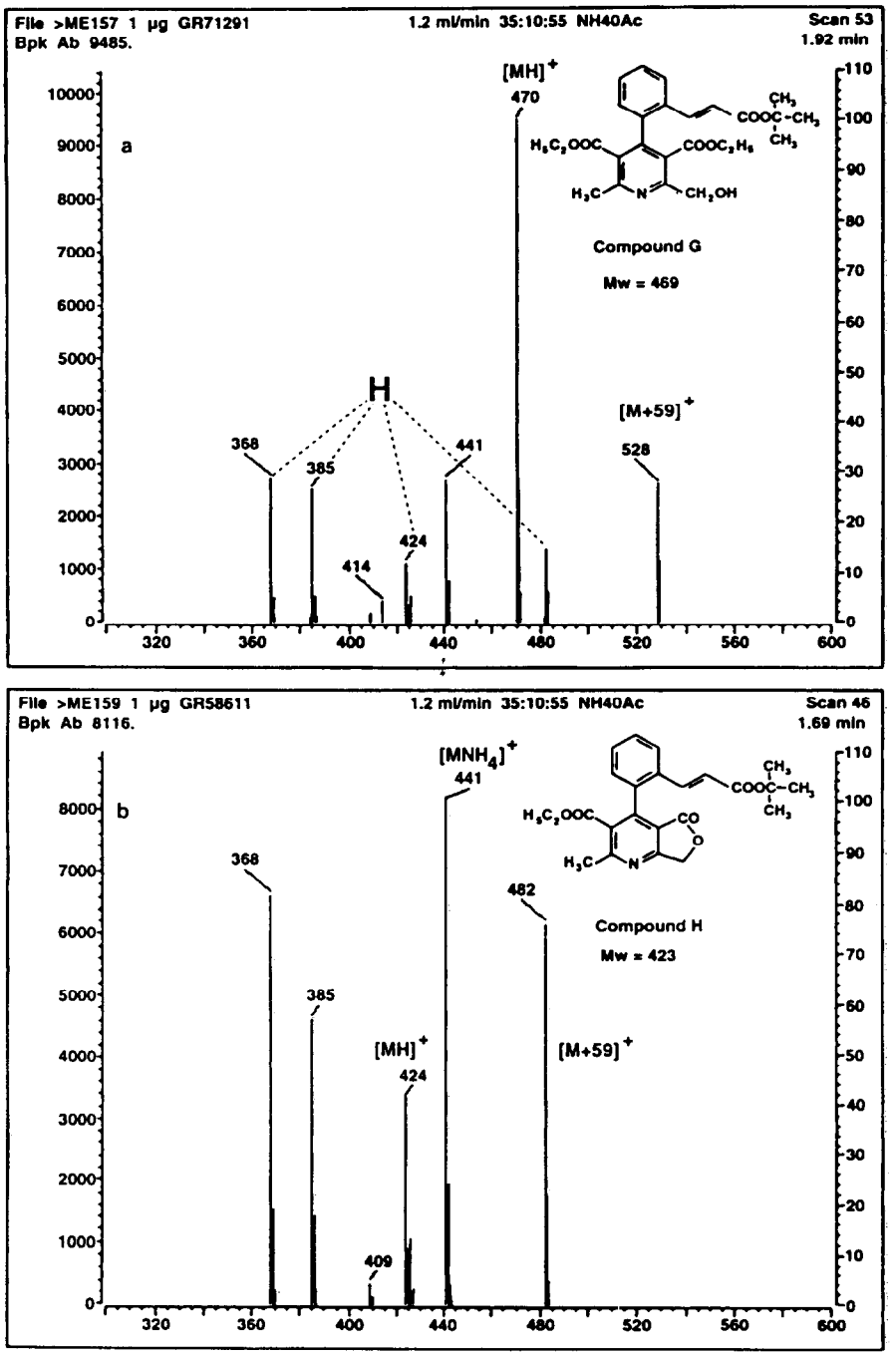

Fig. 3. Thermospray positive-ion mass spectra of metabolites **G** and **H**.

Chemical ionization. PB-CI-MS-measurements were performed using two reagent gases: methane and isobutane. The *methane* CI^+ mass spectra of lacidipine and metabolites **G** and **H** are shown in Fig. 5a, b and c. An intense fragmentation was observed for all the analysed compounds. In the lacidipine spectrum (Fig. 5a) the base peak was the fragment ion at m/z 354, which was assigned as $[MH - CH_2{=}C(CH_3)_2, -C_2H_5OH]^+$ and $[MH - HCOOC(CH_3)_3]^+$ ions.

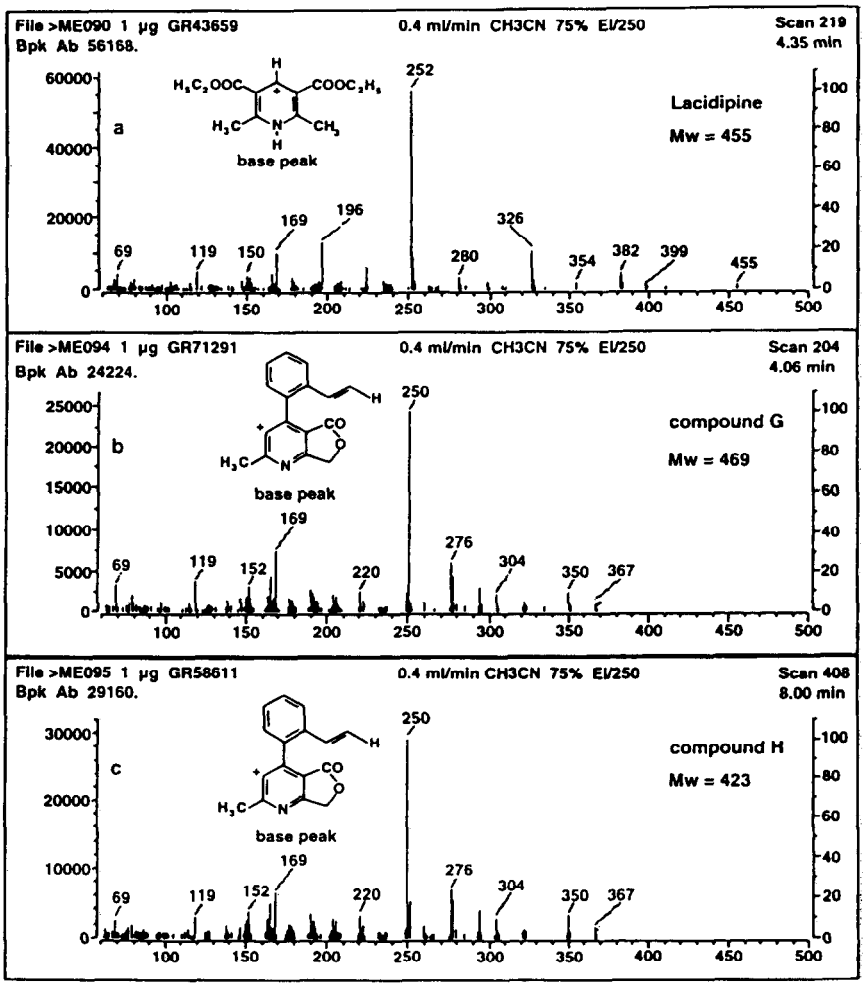

Fig. 4. PB-EI mass spectra of: (a) lacidipine; (b) compound **G**; (c) compound **H**.

Both processes were confirmed by exact mass measurements performed on a ZAB-2F double-focusing instrument [5]. The protonated molecular ion was observed at m/z 456 and the product of addition with reagent gas, $[M + C_2H_5]^+$, was at m/z 484.

For compound **G** (Fig. 5b) the protonated molecular ion was not detectable and, as already observed in the PB-EI mass spectrum, its cyclization to lactone was the preferred reaction. The mass spectra of the two compounds, **G** and **H**, were similar. The base peak at m/z 350 was attributed to $[MH - HOC(CH_3)_3]^+$ and/or $[MH - HCOOC_2H_5]^+$. The second most intense peak in both spectra at m/z 368 was assigned as the $[MH - C(CH_3)_3 + H]^+$ ion.

Methane negative-ion CI mass spectra of these compounds are shown in Fig. 6. The molecular ions of lacidipine and compound **H**, generated by electron attachment, were observed as base peaks at m/z 455 and 423, respectively. For compound **G** the molecular ion was observed at m/z 469, although at a very low relative abundance (1%). The other signals in the mass spectrum (Fig. 6b) were the same as for the lactone (Fig. 6c).

Isobutane positive-ion CI mass spectra of compounds **G** and **H** are reported in Fig. 7b and 7c.

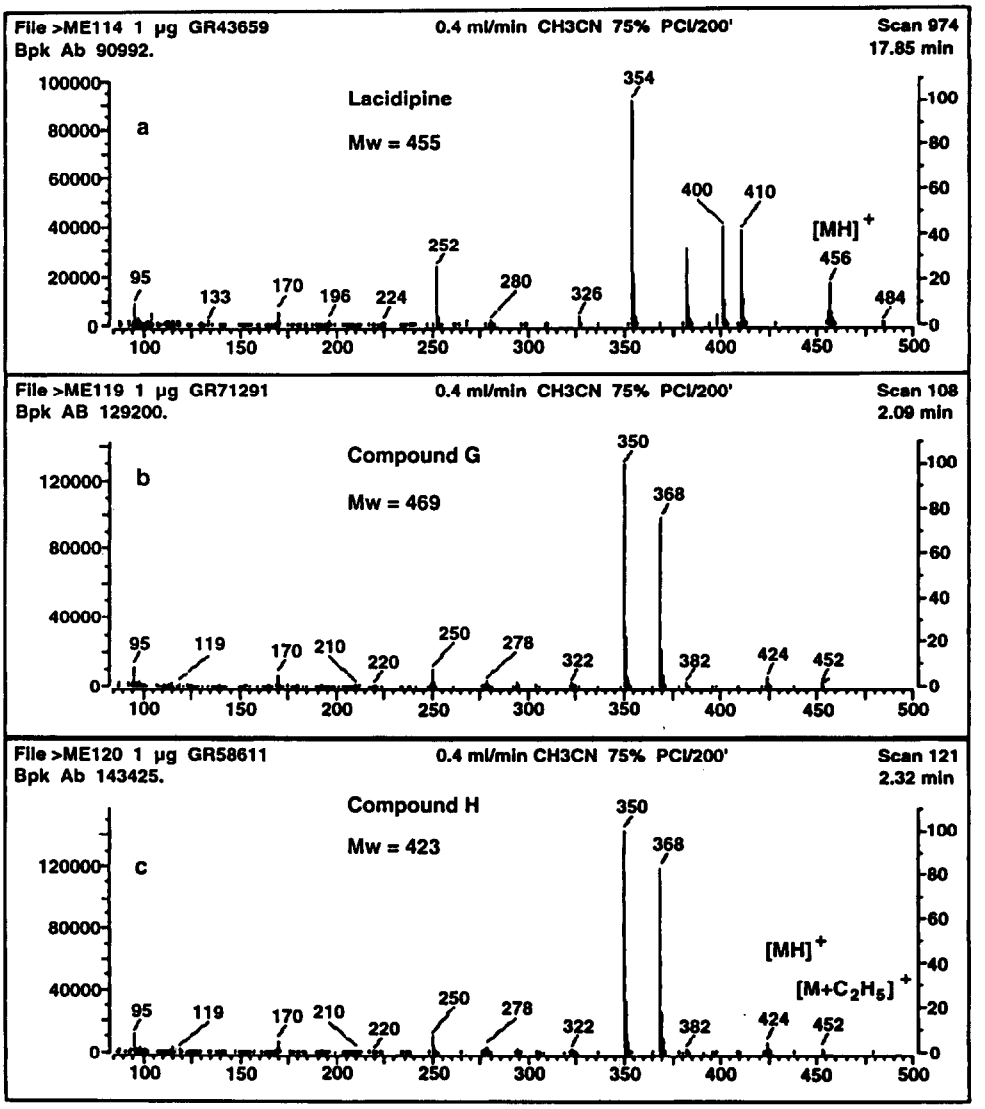

Fig. 5. PB-CI in methane, positive-ion mode, mass spectra: (a) lacidipine; (b) compound **G**; (c) compound **H**.

A very weak protonated molecular ion was present for compound **H** and the two more abundant peaks at *m/z* 350 and 368 were the same as PB-CI$^+$ spectra with methane (Fig. 5c). Also, for lacidipine (Fig. 7a) a close resemblance with the mass spectrum in methane was observed.

A similar behaviour as with methane was observed in the negative-ion mode CI with isobutane.

Identification of lacidipine metabolite in rat plasma

HPLC–TSP-MS was also used for the identification of a lacidipine metabolite in rat plasma. The profile of metabolites in the plasma of rats administered a 2.5 mg/kg oral dose of [2,6-^{14}C] lacidipine has already been described [18]. The metabolic pathways involve as a preliminary step the hydrolysis of ethyl and *tert.*-butyl ester moieties or, alternatively, the oxidation of the

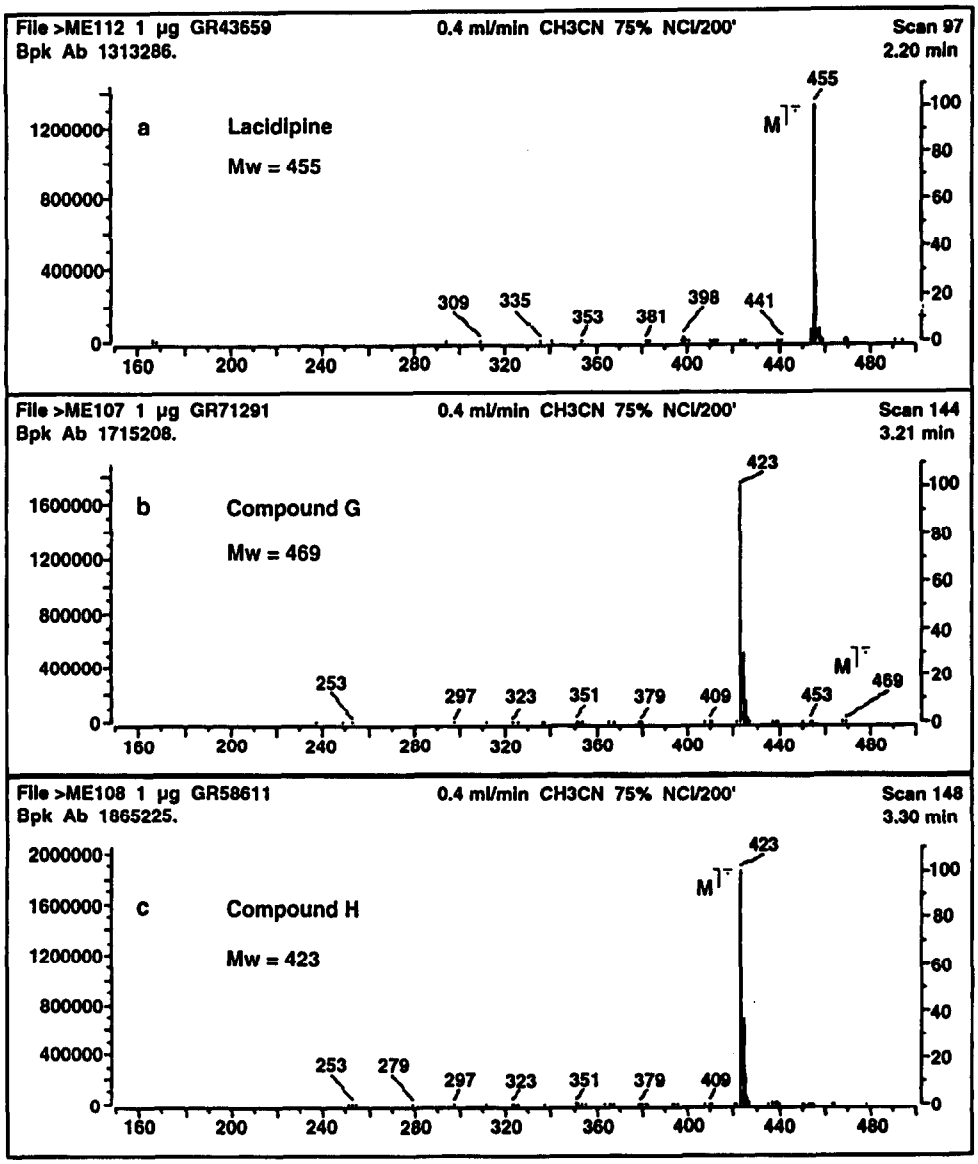

Fig. 6. PB-CI in methane, negative-ion mode, mass spectra: (a) lacidipine; (b) compound **G**; (c) compound **H**.

heterocyclic ring to pyridine. A full thermospray mass spectrum was obtained only for the main metabolite, which is formed by hydrolysis of the ethyl ester moiety and maintains an intact dihydropyridine ring. In Fig. 8 TSP-MS measurements of a rat plasma sample are reported. The total ion chromatogram (Fig. 8a) shows, in addition to the main metabolite peak, several other peaks related to plasma matrix compounds. The mass spectrum of the metabolite, reported in Fig. 8b, was in agreement with that of standard compound **C** (the relative abundances of its $[MH]^+$ species and the adducts ions are reported in Table II). The base peak at m/z 384 was ascribed to the decarboxylation reaction $[MH - CO_2]^+$. The main peaks in the spectra showed

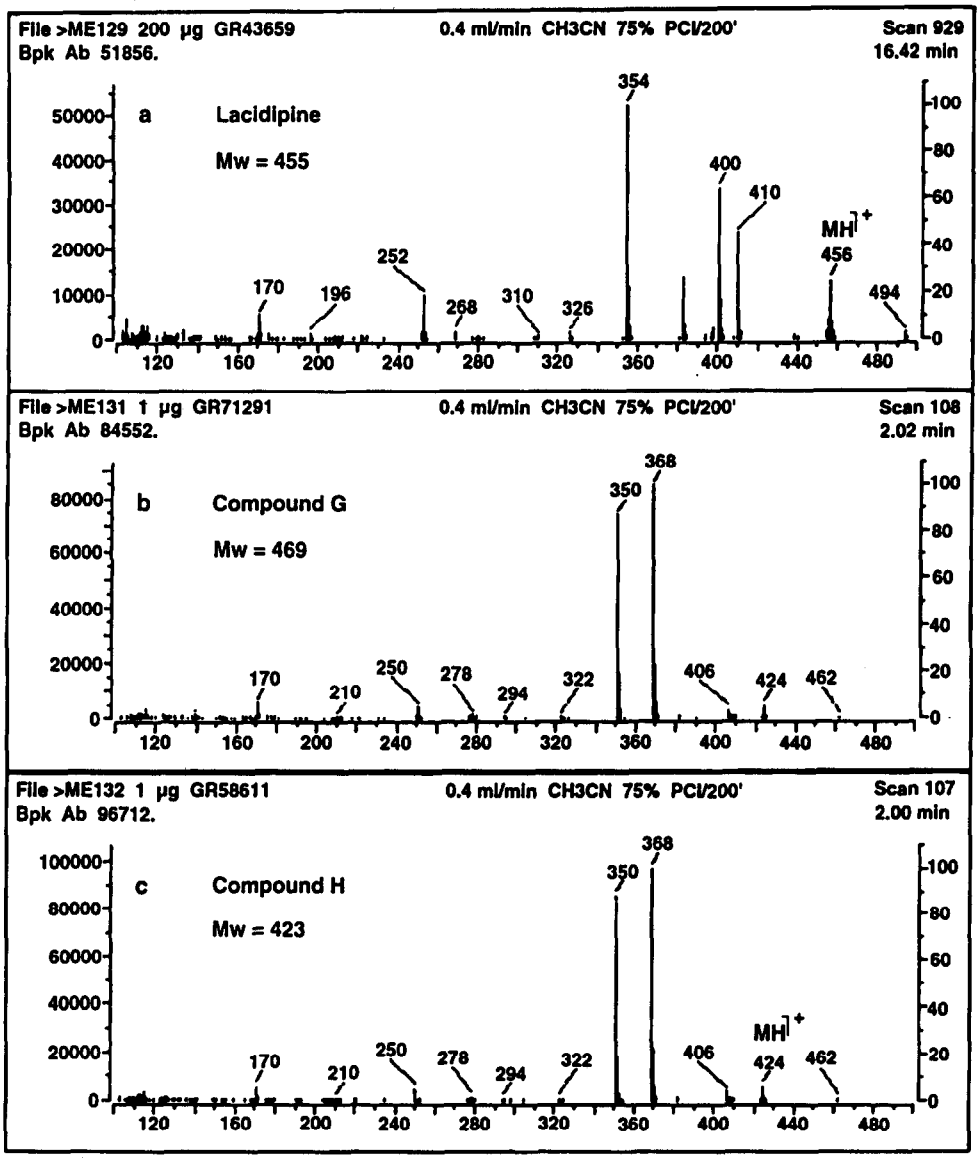

Fig. 7. PB-CI in isobutane, positive-ion mode, mass spectra: (a) lacidipine; (b) compound **G**; (c) compound **H**.

the isotopic cluster, due to ^{14}C-labelled lacidipine.

CONCLUSIONS

Mass spectra obtained by HPLC–MS with thermospray and particle beam interfaces and different ionization techniques (EI and CI) were compared to determine a system suitable for the analysis of lacidipine and its metabolites. This was performed by thermospray HPLC–MS, in fact particle beam mass spectra in either electron impact or chemical ionization mode of two metabolites (compounds **G** and **H**) were not distinguishable.

The thermospray interface was used for the

TABLE II

RELATIVE INTENSITIES OF (PSEUDO)MOLECULAR IONS AND ADDUCT IONS IN TSP (POSITIVE AND NEGATIVE ION MODE)

Ionic species (rel.ab.%)		Compound							
		A (lacidipine)	B	C	D	E	F	G	H
TSP^+	$[MH]^+$	456 (12%)	454 (100%)	428 (10%)[a]	426 (100%)	400 (28%)	398 (100%)	470 (100%)	424 (40%)
	$[M + NH_4]^+$	473 (100%)	–	445 (45%)	–	417 (100%)	–	–	441 (100%)
	$[M + CH_3CN + NH_4]^+$	514 (50%)	512 (30%)	486 (20%)	484 (30%)	458 (48%)	456 (16%)	528 (30%)	482 (78%)
TSP^-	$[M]^{-\cdot}$	455 (50%)	453 (100%)	427 (60%)	425 (100%)	399 (88%)	397 (80%)	469 (100%)	423 (100%)
	$[M - H]^-$	454 (100%)	–	426 (100%)	424 (78%)	398 (100%)	396 (100%)	–	–
	$[M + CH_3COO]^-$	–	–	486 (10%)	–	458 (22%)	456 (50%)	–	–

[a] Base peak in TSP^+ is the fragment ion at m/z 384, $[M - CO_2]^+$.

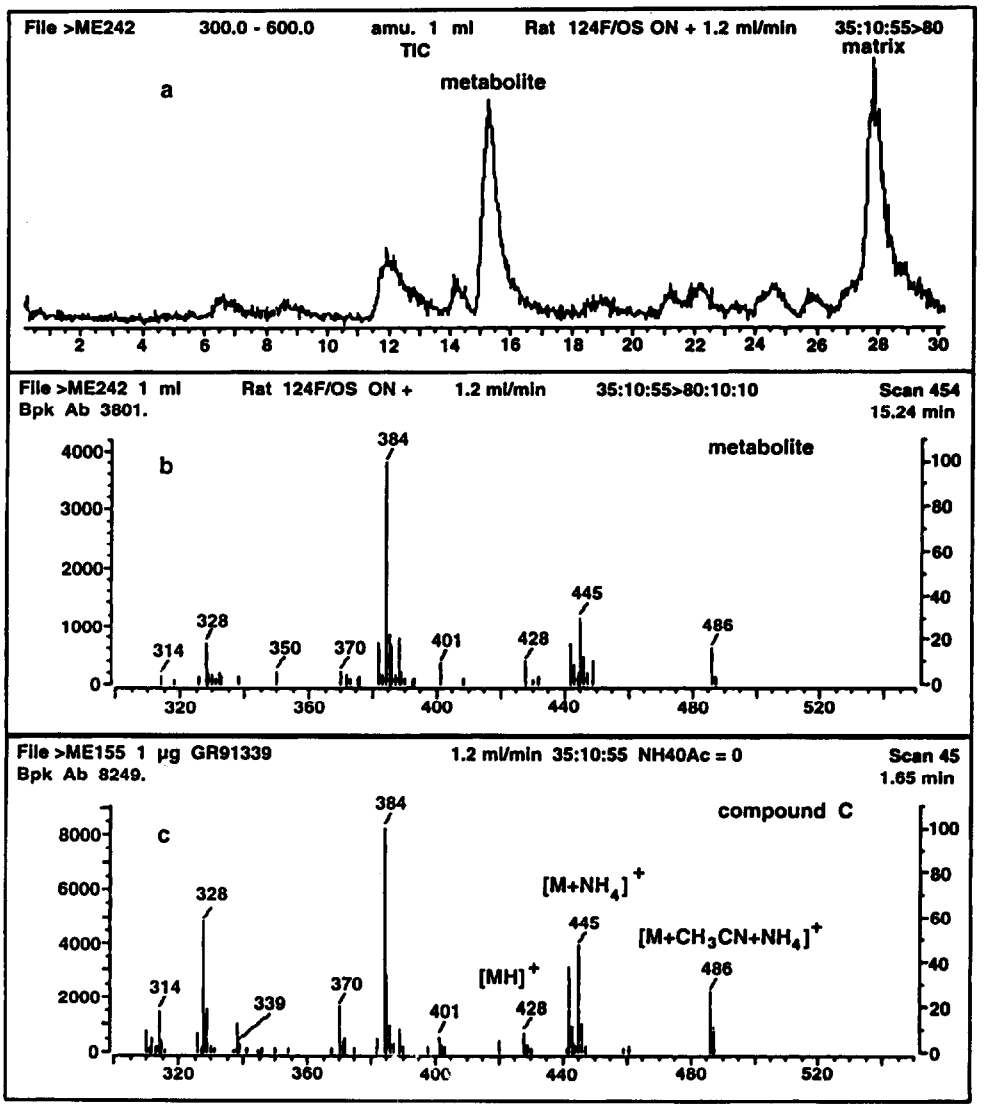

Fig. 8. HPLC–TSP-MS measurements of a rat plasma sample. (a) Total ion current (TIC) chromatogram. (b) Mass spectrum of the peak eluting at 15.2 min. (c) Mass spectrum of compound C standard.

identification of lacidipine metabolites in biological fluids as shown by the HPLC–TSP-MS analysis performed to elucidate the structure of the main metabolite in rat plasma.

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