Journal of Chromatography A, 1218 (2011) 3089–3094

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Ultra-thin-layer chromatography mass spectrometry and thin-layer chromatography mass spectrometry of single peptides of angiotensin-converting enzyme inhibitors

Irena Vovk[a,b,*], Gordana Popović[c], Breda Simonovska[a], Alen Albreht[a], Danica Agbaba[d]

[a] National Institute of Chemistry, Laboratory for Food Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia
[b] EN-FIST Centre of Excellence, Dunajska 156, SI-1000 Ljubljana, Slovenia
[c] University of Belgrade, Faculty of Pharmacy, Department of General and Inorganic Chemistry, Vojvode Stepe 450, P.O.B. 146, 11000 Belgrade, Serbia
[d] University of Belgrade, Faculty of Pharmacy, Department of Pharmaceutical Chemistry, Vojvode Stepe 450, P.O.B. 146, 11000 Belgrade, Serbia

ARTICLE INFO

Article history:
Received 20 December 2010
Received in revised form 14 March 2011
Accepted 16 March 2011
Available online 13 April 2011

Keywords:
UTLC
UTLC–MS
TLC
TLC–MS
Densitometry
Image analysis
Prils
Prilates

ABSTRACT

The separation of structurally related angiotensin-converting enzyme (ACE) inhibitors lisinopril, cilazapril, ramipril and quinapril and their corresponding active diacid forms (prilates) by conventional TLC silica gel 60 plates was contrasted with that afforded by monolithic ultra-thin-layer chromatographic (UTLC) plates. For the use of UTLC plates technical modifications of the commercially available equipments for the sample application, development and detection were made. Plates were developed in modified horizontal developing chamber using ethyl acetate–acetone–acetic acid–water (4:1:0.25:0.5, v/v). Detection of the separated compounds was performed densitometrically in absorption/reflectance mode at 220 nm and after exposure to iodine also by image analysis. The obtained results showed that monolithic layer is more efficient for the separation of structurally similar polar compounds, such as *prilates* than conventional silica layers. Identification of the compounds was confirmed by ESI-MS after their on-line extraction from the UTLC and TLC plates by means of Camag TLC–MS interface.

1. Introduction

Recent investigations in planar chromatography and other chromatographic techniques have been focused primarily on miniaturization and their use with mass spectrometry (MS). Since ultrathin-layer chromatographic (UTLC) plates were introduced in 2001 by Merck many different new monolithic and nanostructured stationary phases have been reported. Coupling of TLC and HPTLC with mass spectrometry [1–5] has been described, but further development in the field of hyphenation of UTLC with mass spectrometry is demanded. The implementation of these new developments is currently limited and requires the development of new devices for sample application, development and detection.

The commercially available UTLC layers have a 10 μm thick monolithic structure based on a silica gel matrix formed by the hydrolytic polycondensation of a liquid film of an alkoxysiloxane on a glass plate. A number of UTLC applications including separation of amino acids [6], pesticides [7], steroids [7,8], active ingredients in pharmaceuticals [8], phenols [8], plasticisers [8], lipophilic dyestuffs [7,9] and flavanols [10] have been reported and recently, UTLC coupled with atmospheric pressure matrix assisted laser desorption/ionization (UTLC–AP-MALDI-MS) has been used for the analyses of small molecules such as different derivatives of triazoles, midazolam, verapamil and metoprolol [11] and benzodiazepines [12]. Desorption electrospray ionization mass spectrometry has been applied to *in situ* identification of several structural classes of drugs of different polarities, such as highly polar acetylcholine and less polar such as, steroids, benzodiazepines or verapamil [13].

To further explore the field of UTLC coupled with mass spectrometry in pharmaceutical analysis we used single peptides such as ACE inhibitors as model compounds. ACE inhibitors familiar as *prils* are widely used as antihypertensive drugs. The active forms of these drugs called *prilates* are polar, typically dicarboxylic acids. In order to be absorbed they are used as their more lipophilic ethyl esters [14]. The free diacid forms cilazaprilat, ramiprilat and quinaprilat are present as impurities in the bulk ethyl ester drugs and their dosage forms. They are the active forms in biological fluids and methods for their analysis are also required. Different

* Corresponding author at: National Institute of Chemistry, Hajdrihova 19, SI-1000 Ljubljana, Slovenia. Tel.: +386 1 4760 341; fax: +386 1 4760 300.
E-mail address: irena.vovk@ki.si (I. Vovk).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.03.039

analytical methods have been developed for determination of ACE inhibitors alone or in a mixture, in either dosage forms or biological samples. Capillary electrophoresis (CE) has been employed for the separation of eight of the ACE inhibitors enalapril, lisinopril, quinapril, fosinopril, perindopril, ramipril, benazepril and cilazapril with either a free solution electrophoresis system [15] or alkylsulphonates as ion-pairing agents [16]. In both cases a combination of two systems is necessary for the selective identifications of the eight ACE inhibitors. CE was used to separate four ACE inhibitors (lisinopril, ramipril, benazepril and quinapril) and ramipril and benazepril were quantitatively evaluated [17]. Chromatographic methods such as gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometric detection, have been widely used for determination of ACE inhibitors and their active forms in biological fluids [18]. As a result of the stringent regulatory guidelines outlined by the International Conference on Harmonization (ICH) and published by the FDA [19], when identification is required LC–MS has become a versatile tool for the determination of the molecular mass of impurities in drugs as well as for profiling the impurities in pharmaceuticals [20,21]. The identification and isolation of an unknown impurity present in quinapril were accomplished using HPLC/UV, preparative HPLC and structural characterization by LC–MS–MS and ^{1}H and ^{13}C NMR spectroscopy [22]. LC–MS has been used for the evaluation of stability of aqueous solutions of enalapril and perindopril at different pH values [23]. The retention behaviors of different ACE inhibitors have been determined with different TLC and HPTLC sorbents [24–28] and HPTLC plates have been used for quantitative evaluation of selected ACE inhibitors in pharmaceuticals [29,30].

No attempt has been made, however, to investigate the use of UTLC plates in methods that are fast, miniaturized and combined with MS for the identification and detection of structurally related ACE inhibitors. This is the main goal of our research and was achieved by the introduction of some technical innovations regarding the application, development and detection. We compared the separation power of conventional TLC and UTLC and also the differences when coupled with MS.

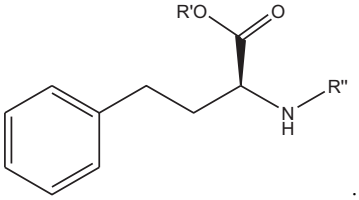
2. Experimental

2.1. Materials

Lisinopril (1), L-proline, N2-[(1S)-1-carboxy-3-phenylpropyl]-L-lysyl was obtained from Sigma (St. Louis, MO, USA); cilazapril (2), 6H-pyridazino[1,2-a][1,2]diazepine-1-carboxylic acid, 9-[[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]octahydro-10-oxo-(1S,9S) and cilazaprilat (3), 6H-pyridazino[1,2-a][1,2]diazepine-1-carboxylic acid, 9-[[(1S)-1-carboxy-3-phenylpropyl]amino]octahydro-10-oxo-(1S,9S) were obtained from Ranbaxy (Gurgaon, India). Ramipril (4), cyclopenta[b]pyrrole-2-carboxylic acid, 1-[(2S)-2-[[(1S)-1-(ethoxycarbonyl)-3-phenylpropyl]amino]1-oxopropyl]octahydro-(2S, 3aS, 6aS) and ramiprilat (5), cyclopenta[b]pyrrole-2-carboxylic acid, 1-[(2S)-2-[[(1S)-1-carboxy-3-phenylpropyl]amino]1-oxopropyl]octahydro-(2S, 3aS, 6aS) were obtained from Dr. Reddy's Laboratories (Bollaram, India); quinapril (6), 3-isoquinolinecarboxylic acid, 2-[(2S)-2-[[(1S]-1-(ethoxycarbonyl)-3-phenylpropyl]amino]-1-oxopropyl]-1,2,3,4-tetrahydro, (3S) and quinaprilat (7), 3-isoquinolinecarboxylic acid, 2-[(2S)-2-[[(1S]-1-carboxy -3-phenylpropyl]amino]-1-oxopropyl]-1,2,3,4-tetrahydro, (3S) were obtained from Cipla Ltd. (Mumbai, India). Chemical structures of the studied compounds are presented in Table 1.

Ethyl acetate, acetone, acetic acid and methanol were obtained from Merck (Darmstadt, Germany). All chemicals and solvents were

Table 1
Chemical structures of investigated *prils* and *prilates*.



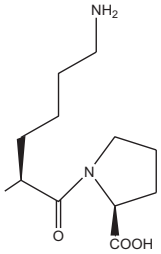
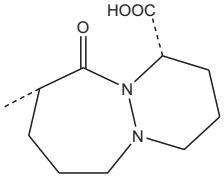
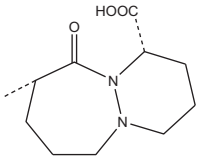
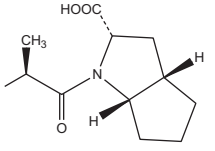
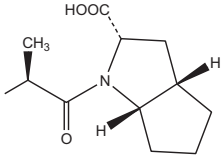
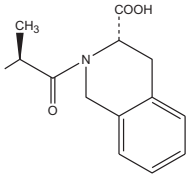
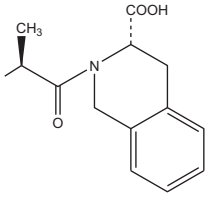
Compound	R′	R″
1. Lisinopril	H	
2. Cilazapril	C_2H_5	
3. Cilazaprilat	H	
4. Ramipril	C_2H_5	
5. Ramiprilat	H	
6. Quinapril	C_2H_5	
7. Quinaprilat	H	

Table 2
Chromatographic parameters.

	UTLC		TLC
Plates	UTLC silica gel (monolithic), 6 cm × 3.6 cm, (Merck)		TLC silica gel 60 F_{254}, 10 cm × 10 cm (Merck)
Developing solvent	Ethyl acetate–acetone–acetic acid–water (4:1:0.25:0.5, v/v)		Ethyl acetate–acetone–acetic acid–water (4:1:0.25:0.5, v/v)
Volume of developing solvent	4 mL		4 mL
Application volume	0.10 μL (1 mm bands)	0.02 μL (spots)	1 μL (spots)
Application position	5 mm		10 mm
Developing distance	28 mm		90 mm
Migration time	6.5 min		30 min
Chamber	Horizontal (10 cm × 10 cm)		Horizontal (10 cm × 10 cm)
Wavelength	220 nm and 435 nm		220 nm and 435 nm

of analytical reagent grade. Stock solutions of all tested compounds were prepared in methanol (ca. 3 mg/mL).

2.2. TLC and UTLC

The samples were applied by means of Automatic TLC Sampler 4 (ATS 4, Camag) equipped with a 10 μL syringe or manually by 1 μL syringe. Evaluation of the developed plates was performed densitometrically with Camag TLC Scanner3 in absorption/reflectance mode and by image analysis with the DigiStore 2 Documentation System using white light illumination. Both instruments were controlled by winCATS programme (Version 1.4.1.8154), while image analysis after detection with iodine vapor was performed using additional software VideoScan V1.02. Details of the chromatographic conditions are presented in Table 2.

2.3. TLC–MS and UTLC–MS transfer and identification of compounds

A TLC–MS interface (Camag) was used for the elution of compounds from the TLC and the UTLC plates into the LCQ (Thermo Finnigan, San Jose, CA, USA) system. The eluent used was methanol and its flow rate was set to 0.5 mL/min in the case of TLC plates and 0.3 mL/min in the case of UTLC plates; 0.2% acetic acid (in methanol) at 0.1 mL/min was added to the methanol effluent prior to injecting the solution into the LCQ system.

- Electrospray ionization (ESI) source in positive mode was employed for the identification of compounds.
- The spray voltage was set to 6 kV and the capillary temperature to 250 °C.
- The sheath and auxiliary gas flow rates were 80 and 10 a.u. (arbitrary units), respectively.

3. Results and discussion

We compared the separation, detection and identification of structurally related ACE inhibitors and their active diacid forms on conventional TLC silica gel 60 plates (250 μm layer) and monolithic ultra-thin-layer chromatographic plates (UTLC, 10 μm layer). For a successful evaluation of the compounds on the UTLC plates it was necessary to solve several technical problems concerning the application, development and detection.

The problems with the application of small volumes (0.02–0.1 μL) of the analytes are associated mainly with the thickness and the size of the UTLC plates; when applying the samples on these plates with automatic TLC sampler ATS 4 this inconvenience was overcome by fixation of the UTLC plate with additional TLC plates.

An ascending development mode has been generally used for separation on UTLC plates [6–10] although there is a recent report on the use of a horizontal separation chamber configured for miniaturized glancing angle deposition (GLAD) UTLC plates [31]. Due to the lack of proper devices we firstly applied ascending development mode, which, however, in the UTLC mode required a three times longer developing time (20 min for the ascending mode vs. 6.5 min the horizontal mode) with the final developing solvent. Using the ascending development mode, peak tailing was observed with all the investigated compounds, which most probably happened due to the improper developing chamber and very thin 10 μm layers. The long developing time can cause the well-known problems with changing composition of the developing solvent due to different evaporation of its components from the plate during the development, sorption and desorption phenomena, etc. Horizontal developing chambers for UTLC plates are not commercially available, and thus we constructed a simple but very practical adapter for the existing Camag horizontal developing chambers with dimensions 10 cm × 10 cm and 10 cm × 20 cm.

The retention behavior using single non-polar (cyclohexane, toluene) and polar (methanol, isopropanol, water) solvents was investigated. Polar solvents are required in order to suppress strong intermolecular interaction between polar groups of the investigated compounds and the silanol groups of the layers. It was observed that different applied alcohols caused very high tailing of the bands and for this reason, they were replaced with a mixture of acetic acid and water, which resulted in more compact chromatographic zones of all the investigated compounds. In order to obtain satisfactory resolution, the ternary mixtures were prepared containing different organic modifiers (chloroform, methyl ethyl ketone, ethyl acetate, butyl acetate, acetone), but an improved resolution was obtained using ethyl acetate and butyl acetate only. Ethyl acetate in a mixture with acetic acid and water provides satisfactory retention and resolution of all the investigated compounds on the TLC and UTLC layers. In order to prepare a better miscible organic phase with water and to avoid the turbidity of the ternary mixture, acetone was added. The best resolution was obtained with the quaternary system composed of ethyl acetate–acetone–acetic acid–water (4:1:0.25:0.5, v/v), which was finally used as developing solvent. No significant differences between ascending and horizontal development were observed on the TLC plates using the final developing solvent.

Fast visual detection of the separated compounds at UV (254 nm) is not feasible, therefore densitometric scanning of *prils* and *prilates* on TLC (Figs. 1 and 2) and UTLC (Fig. 3) plates was performed in absorption/reflectance mode at 220 nm. Alternatively, the plates were exposed to iodine vapor and documented as images and video densitograms by the image analyzing system (Fig. 4).

Chromatographic data and the migration distances of the compounds are presented in Tables 2 and 3. Considering the application volume (of the same solution) of 1 μL in conventional TLC vs. 0.1 and 0.02 μL in UTLC the latter is found to be more sensitive. Application of the same developing solvent in horizontal developing mode provides 5 times faster separations with UTLC plates. The monolithic UTLC layer was more efficient than conventional TLC silica layer (Fig. 2) for the separation of *prilates* (Fig. 3). Under the same

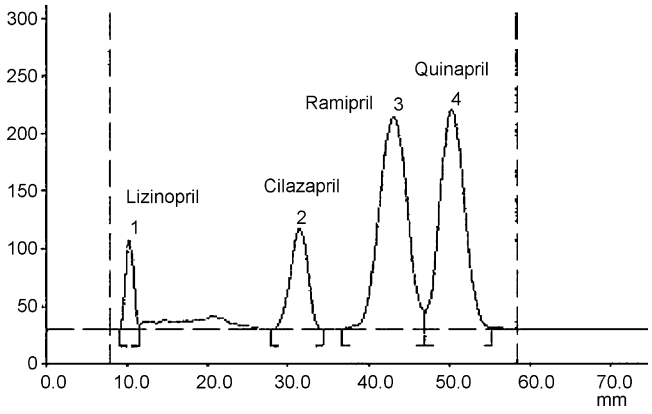


Fig. 1. TLC densitogram of *prils*.

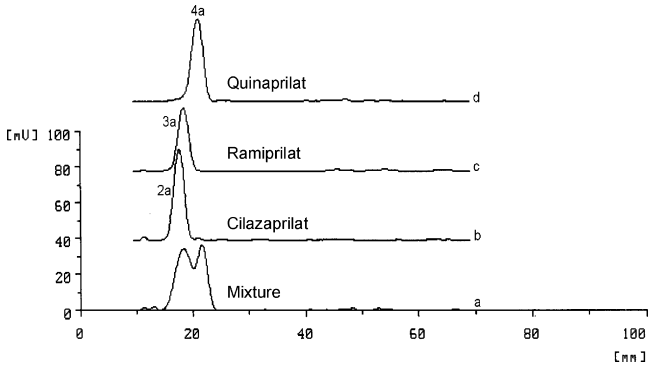


Fig. 2. TLC densitograms of *prilates*.

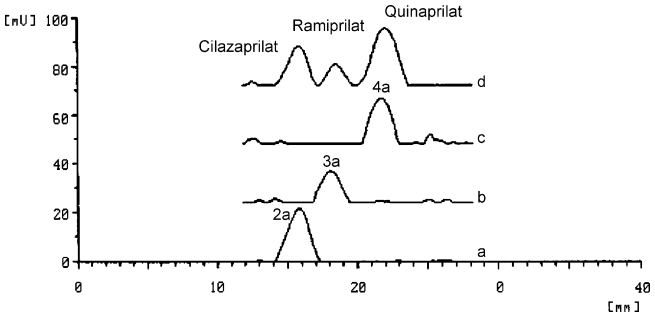


Fig. 3. UTLC densitograms of *prilates*.

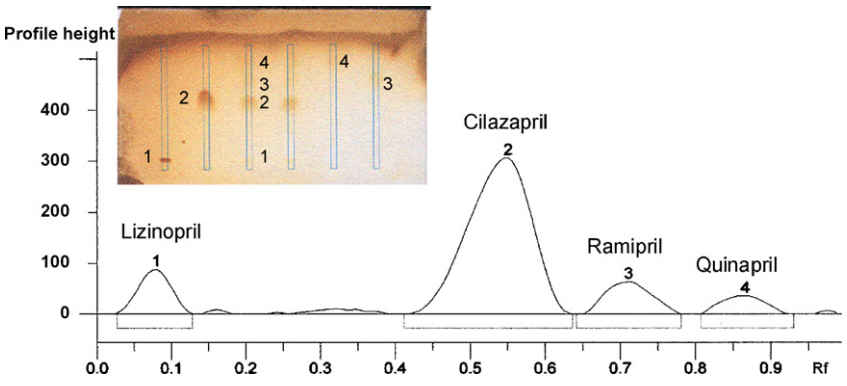


Fig. 4. UTLC image and video densitogram of *prils*.

Table 3
Migration distances of the studied compounds.

Compound	UTLC Migration distances (mm)	TLC
Lizinopril	6.0	10.7
Cilazapril	17.5	32.0
Cilzaprilat	16.3	17.2
Ramipril	21.6	43.9
Ramiprilat	18.8	18.5
Quinapril	25.1	50.8
Quinaprilat	22.5	21.3

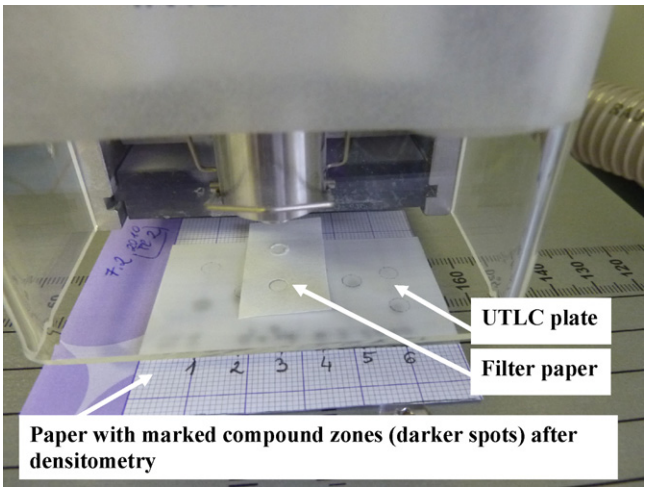


Fig. 5. UTLC–MS by modified use of Camag TLC–MS interface.

chromatographic conditions as in the UTLC analysis the conventional TLC silica layer provided good resolution for *prils* (Fig. 1) but unsatisfactory resolution for *prilates* (Fig. 2). As can be seen from the migration distances presented in Table 3, *prilates* had shorter elution distance than the corresponding *prils* indicative of their stronger affinity for the polar UTLC surface.

Coupling of UTLC and TLC with mass spectrometric detection of the studied compounds by means of Camag TLC–MS interface was studied. Densitometrically obtained start and end points of the spots were used for marking the compound zones in all the tracks as the dark spots on the millimeter paper with marked plate size (Fig. 5), which was placed under the UTLC plate and served for easier and proper manual positioning of the UTLC plate under the piston of the TLC–MS interface. On the TLC plates the compound zones in all the tracks were marked from all the sides by a pencil directly on the silica gel layer. There are no published reports of coupling UTLC–ESI-MS or UTLC–APCI-MS (APCI – atmospheric pressure

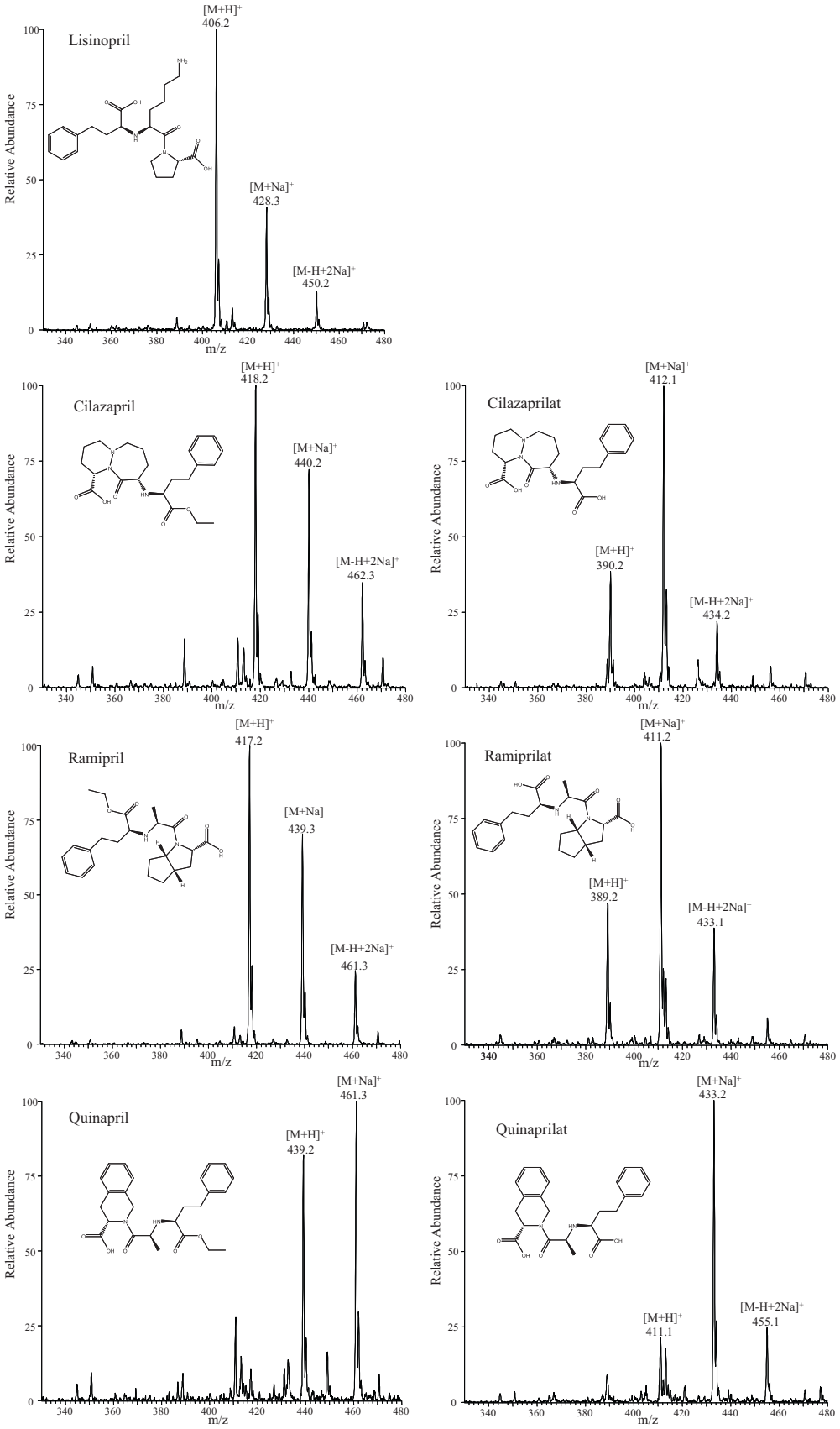


Fig. 6. Mass spectra of *prils* and *prilates* obtained by UTLC–ESI-MS.

Table 4
Assignment of mass spectral peaks.

	m/z values		
	$[M+H]^+$	$[M+Na]^+$	$[M-H+2Na]^+$
Lisinopril	406	428	450
Cilazapril	418	440	462
Cilazaprilat	390	412	434
Ramipril	417	439	461
Ramiprilat	389	411	433
Quinapril	439	461	–
Quinaprilat	411	433	455

chemical ionization). We experienced no problems in the case of TLC–ESI-MS with Camag TLC–MS interface. In the case of UTLC–ESI-MS however we experienced difficulties due to the "flooding" of the plate resulting from insufficient sealing of the zones. This problem was solved by placing a piece of filter paper, washed with methanol, on the corresponding zone on the UTLC plates, which enabled the automatic piston of the TLC–MS interface to pressure seal the chromatographic zone (Fig. 5).

The TLC–MS interface was employed for the elution of compounds from the plates which were then injected into the MS. The assignment of the main peaks in the mass spectra is given in Table 4. All analyzed compounds show a substantial affinity for alkali metals; this can be seen as a high relative abundance of sodium adduct ions in the MS spectra. The sodium probably originates from the binder (TLC plates) or the glass support of the stationary phase since the sodium adducts were not detected when standards were injected directly into the MS for tuning purposes. The mass spectra are presented in Fig. 6.

4. Conclusions

To the best of our knowledge UTLC–ESI-MS coupling has not been reported previously. UTLC is more efficient than conventional TLC for the separation of single peptides, lisinopril, cilazapril, ramipril and quinapril from their corresponding *in vivo* active forms or *in vitro* potential degradation products, cilazaprilate, ramiprilate and quinaprilate. UTLC is faster, has diminished costs and waste, but its combination with MS is necessary for compound identification. It is expected that in the era of miniaturization the development of new technical solutions in the field of instrumentation will finally simultaneously follow the developments in the field of stationary phases.

Acknowledgements

This study was carried out with financial support from the Slovenian Research Agency, EN→FIST Centre of Excellence (Dunajska 156, SI-1000 Ljubljana, Slovenia), Ministry for Science and Technological Development of the Republic of Serbia (contract #172033) and partly by bilateral project BI – RS/08-09-025.

References

[1] K.L. Busch, in: J. Sherma, B. Fried (Eds.), Handbook of Thin-Layer Chromatography, 3rd ed., Marcel Dekker, New York, 2003, p. 239.
[2] G. Morlock, W. Schwack, Trends Anal. Chem. 29 (2010) 1157.
[3] G. Morlock, W. Schwack, J. Chromatogr. A 1217 (2010) 6600.
[4] S.P. Pasilis, G.J. Van Berkel, J. Chromatogr. A 1217 (2010) 3955.
[5] S.-C. Cheng, M.-Z. Huang, J. Shiea, J. Chromatogr. A (2011) 077, doi:10.1016/j.chroma.2011.01.
[6] H.E. Hauck, M. Schulz, J. Chromatogr. Sci. 40 (2002) 550.
[7] H.E. Hauck, O. Bund, W. Fischer, M. Schulz, J. Planar Chromatogr. 14 (2001) 234.
[8] H.E. Hauck, M. Schulz, Chromatographia 57 (2003) S313.
[9] G.E. Morlock, C. Oellig, L.W. Bezuidenhout, M.J. Brett, W. Schwack, Anal. Chem. 82 (2010) 2940.
[10] M. Krauze-Baranowska, I. Malinowska, D. Głód, M. Majdan, A. Wilczańska, J. Planar Chromatogr. 22 (2009) 385.
[11] P.K. Salo, H. Salomies, K. Harju, R.A. Ketola, T. Kotiaho, J. Yli-Kauhaluoma, R. Kostiainen, J. Am. Soc. Mass Spectrom. 16 (2005) 906.
[12] P.K. Salo, S. Vilmunen, H. Salomies, R.A. Ketola, R. Kostiainen, Anal. Chem. 79 (2007) 2101.
[13] T.J. Kauppila, N. Talaty, P.K. Salo, T. Kotiaho, R. Kostiainen, R.G. Cooks, Rapid Commun. Mass Spectrom. 20 (2006) 2143.
[14] M. Harrold, in: T.L. Lemke, D.A. Williams (Eds.), Foye's Principles of Medicinal Chemistry, 6th ed., Lippincott Williams & Wilkins, Philadelphia, 2008, p. 738.
[15] S. Hillaert, W. Van den Bossche, J. Chromatogr. A 895 (2000) 33.
[16] S. Hillaert, Y. Vander Heyden, W. Van den Bossche, J. Chromatogr. A 978 (2002) 231.
[17] R. Gotti, V. Andrisano, V. Cavrini, C. Bertucci, S. Furlanetto, J. Pharm. Biomed. Anal. 22 (2000) 423.
[18] P. Rudzki, K. Bus, H. Ksycinska, K. Kobylinska, J. Pharm. Biomed. Anal. 44 (2007) 356.
[19] ICH Q3A(R): Impurities in New Drug Substances (Revised Guideline), Fed. Regist. 68 (2003) 6924.
[20] T. McGovern, D. Jackobson-Kram, Trends Anal. Chem. 25 (2006) 790.
[21] S. Gorog, Trends Anal. Chem. 25 (2006) 755.
[22] V. Shinde, A. Trivedi, P.R. Upadhayay, N.L. Gupta, D.G. Kanase, R.C. Chikate, Rapid Commun. Mass Spectrom. 21 (2007) 3156.
[23] R. Roškar, Z. Simončić, A. Gartner, V. Kmetec, J. Pharm. Biomed. Anal. 49 (2009) 295.
[24] M. Aleksić, D. Agbaba, R. Baošić, D. Milojković-Opsenica, Ž. Tešić, J. Serb. Chem. Soc. 66 (2001) 39.
[25] N.U. Perišić–Janjić, D. Agbaba, J. Planar Chromatogr. 25 (2002) 210.
[26] M.B. Aleksić, D. Agbaba, D. Milojković-Opsenica, Ž. Tešić, Chromatographia 53 (2001) S442.
[27] J. Odović, B. Stojimirović, M. Aleksić, D. Milojlović-Opsenica, Ž. Tešić, J. Planar Chromatogr. 18 (2005) 98.
[28] A. Gumieniczek, L. Przyborowski, Acta Pol. Pharm. 54 (1997) 13.
[29] K. Czerwinska, E. Wyszomirska, T. Kaniewska, Acta Pol. Pharm. 58 (2001) 331.
[30] D. Kowalczuk, R. Pietras, H. Hopkata, Chromatographia 60 (2004) 245.
[31] S.R. Jim, M.T. Taschuk, G.E. Morlock, L.W. Bezuidenhout, W. Schwack, M.J. Brett, Anal. Chem. 82 (2010) 5349.