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Development and validation of a liquid chromatographic/electrospray ionization mass spectrometric method for the determination of benazepril, benazeprilat and hydrochlorothiazide in human plasma

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A new method was developed and fully validated for the quantitation of benazepril, benazeprilat and hydrochlorothiazide in human plasma. Sample pretreatment was achieved by solid-phase extraction (SPE) using Oasis HLB cartridges. The extracts were analysed by high-performance liquid chromatography (HPLC) coupled to a single-quadrupole mass spectrometer (MS) with an electrospray ionization interface. The MS system was operated in selected ion monitoring (SIM) modes. HPLC was performed isocratically on a reversed-phase porous graphitized carbon (PGC) analytical column (2.1 × 125.0 mm i.d., particle size 5 μm). The mobile phase consisted of 55% acetonitrile in water containing 0.3% v/v formic acid and pumped at a flow rate of 0.15 ml min^{-1}. Chlorthalidone was used as the internal standard (IS) for quantitation. The assay was linear over a concentration range of 5.0–500 ng ml^{-1} for all the compounds analysed, with a limit of quantitation of 5 ng ml^{-1} for all the compounds. Quality control (QC) samples (5, 10, 100 and 500 ng ml^{-1}) in five replicates from three different runs of analyses demonstrated intra-assay precision (coefficient of variation (CV) ≤14.6%), inter-assay precision (CV ≤ 5.6%) and overall accuracy (relative error less than −8.0%). The method can be used to quantify benazepril, benazeprilat and hydrochlorothiazide in human plasma, covering a variety of pharmacokinetic or bioequivalence studies. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: liquid chromatography/mass spectrometry; benazepril; benazeprilat; hydrochlorothiazide; chlorthalidone; human plasma.

INTRODUCTION

Benazepril hydrochloride, (3-[1-(ethoxycarbonyl)-3-phenyl-(1S)-propylamino]-2,3,4,5-tetrahydro-2-oxo-1H-1-(3S)-benzazepine-1-acetic acid monohydrochloride), is a prodrug type angiotensin-converting enzyme (ACE) inhibitor that is widely used in the management of essential hypertension, stable chronic heart failure, myocardial infarction and diabetic nephropathy.[1–4] Its main metabolic route involves the hydrolytic cleavage of the ester linkage leading to its active carboxylic acid metabolite, benazeprilat, (3-[1-(carbonyl)-3-phenyl-(1S)-propylamino]-2,3,4,5-tetrahydro-2-oxo-1H-1-(3S)-benzazepine-1-acetic acid).[5–7] Hydrochlorothiazide, (6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulfonamide-1,1-dioxide), is a diuretic and anti-hypertensive agent that reduces plasma volume by increasing the excretion of sodium, chloride and water and, to a lesser extend, that of potassium ion as well.[8,9] Combinations of benazepril and hydrochlorothiazide achieved a greater lowering of blood pressure than benazepril alone, and this approach maybe suitable for patients with severe hypertension.[10,11] After a single oral administration of 20 mg of benazepril hydrochloride, it is absorbed rapidly (T_{max} 0.5–1.0 hr) and converted to the active metabolite benazeprilat (T_{max} 2.0–4.0 hr). Peak plasma concentration levels, C_{max}, for benazepril and benazeprilat are 271 ng ml^{-1} and 379 ng ml^{-1}, respectively. Hydrochlorothiazide is not metabolized; a peak plasma concentration of 130 ng ml^{-1} is reached within 1.5–2.0 h of a single oral administration of 25 mg of the compound.[12]

A variety of methods exist that focus on the detection and determination of benazepril and its metabolite, benazeprilat, in biological matrices. Gas chromatographic (GC)[13–16] and liquid chromatographic (LC)[17,18] procedures coupled to electrospray mass spectrometric detection have also been

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applied for the sensitive and selective determination of benazepril and benazeprilat in plasma and urine. A microbore high-performance liquid chromatographic (HPLC) method has been developed to investigate the kinetics of the chemical and enzymatic hydrolysis of benazepril.[19]

Pharmacokinetic behaviour of hydrochlorothiazide has been studied by a variety of HPLC procedures.[20–22] Liquid chromatographic tandem mass spectrometric methods have been developed for the screening[23] and quantitation[24,25] of hydrochlorothiazide in human plasma. Mass spectrometric behaviour of thiazidic diuretics, including hydrochlorothiazide, after electrospray ionization and collision-induced dissociation has been thoroughly investigated.[26,27]

Although the above described methods include several successful approaches, no report has been mentioned in the literature for the simultaneous determination of benazepril, benazeprilat and hydrochlorothiazide in human plasma by LC/electrospray ionization mass spectrometry.

The coupling of LC to mass spectrometry has provided a useful and rugged technique for the analysis of drug compounds as an alternative to gas chromatography/mass spectrometry (GC/MS) in which some compounds thermally decompose.[28,29] The high sensitivity and selectivity that are attained by the use of mass spectrometry (MS) detection techniques make this suitable for the analysis of biological samples in which the matrix is complex.[30–33]

The purpose of the present work is to develop and validate a rapid, sensitive and selective liquid chromatographic mass spectrometric method for the determination of benazepril, its metabolite (benazeprilat) and hydrochlorothiazide in human plasma. As there are no reports on the mass spectrometric behaviour of benazepril and benazeprilat in an electrospray ionization (ESI) single-quadrupole MS, an investigation on the fragmentation behaviour of these compounds was performed.

EXPERIMENTAL

Chemicals and reagents

Acetonitrile and formic acid were of HPLC grade and purchased from Merck, Darmstadt, Germany. Water was deionized and further purified by means of a Milli-Q Plus water purification system (Millipore Ltd.) and was filtered through a 0.22 μm filter prior to liquid chromatographic procedure. Benazepril and benazeprilat of pharmaceutical purity grade were kindly provided by Novartis Pharma AG (Basel, Switzerland). Hydrochlorothiazide and the internal standard (IS), chlorthalidone, of pharmaceutical purity grade were purchased from Sigma-Aldrich (Seelze, Germany). Oasis HLB cartridges 30 mg/1ml were purchased from Waters (Milford, MA).

Liquid chromatographic and mass spectrometric conditions

The HPLC system included a SpectraSeries Model P100 isocratic pump (SP ThermoSeparation products) and a Rheodyne model 7725i injector with a 20 μl loop. The analytical column was a porous graphitized carbon (PGC) column, 2.1×125.0 mm, particle size 5 μm (Thermo, USA). A prevail C18 guard column (Alltech Associates, Inc., Deerfield, IL, USA) was used to prolong column lifetime. The mobile phase consisted of 55% acetonitrile in water containing 0.3% v/v formic acid, and pumped at a flow rate of 0.15 ml min^{-1}. Chromatography was performed at 25 ± 2 °C with a chromatographic run time of 13 min; a 60 μl volume was injected into a 20 μl loop.

Mass spectrometry was performed using a Finnigan AQA single-quadrupole MS (ThermoQuest, Manchester, UK) equipped with an ESI source. The detection of benazepril, benazeprilat and chlorthalidone was performed in electrospray positive ion mode, while the detection of hydrochlorothiazide was performed in electrospray negative ion mode. The ESI probe temperature was set at 230 °C, the cone voltage (AQA_{max}) was set at 20.0 V and the capillary voltage was set at 4.0 and 3.5 kV for ESI positive and ESI negative, respectively. Data acquisition and analysis were performed using the Xcalibur (v. 1.2) IBM data system running on Windows NT (v. 4.0) on an IBM Pentium III computer. The SIM mode was chosen for the quantitative determination of the analytes. Different time windows presented in Table 1 were used to monitor the dominant mass peaks of the analytes.

Ion trap mass measurements were performed on a Finnigan LCQ advantage ion trap MS (San Jose, CA, USA) with the associated Xcalibur (v. 1.2) IBM data system (ThermoQuest, Manchester, UK). The standard Finnigan electrospray interface was operated in the positive ion mode. Operating conditions for the mass measurements were: capillary temperature, 230 °C; capillary voltage, 30.0 V; ion spray voltage, 5.0 kV; sheath gas flow rate, 45 arbitrary units and auxiliary gas flow rate, 15 arbitrary units. MS/MS scans were performed at a collision energy of 100% arbitrary units.

Table 1. Instrument method in SIM mode for the quantitative determination of benazepril, benazeprilat and hydrochlorothiazide by LC-ESI/MS using chlorthalidone as IS

Time window (min)	Analyte	Polarity	Capillary voltage (kV)	Cone voltage (V)	Selected ions (m/z)	Mass Span
3.0–8.0	Benazeprilat	+ve	4.0	20	397.0	0.30
–	Chlorthalidone	+ve	4.0	20	321.1	0.60
–	Benazepril	+ve	4.0	20	425.0	0.30
8.0–13.0	Hydrochlorothiazide	−ve	3.5	20	295.9	0.30

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Stock and working standard solutions

Stock standard solutions of benazepril, benazeprilat, hydrochlorothiazide and chlorthalidone, 1.0 mg ml^{-1}, were prepared by dissolving the appropriate amounts of the compounds in acetonitrile-water (10 : 90, v/v). These solutions when stored in the dark and under refrigeration (4 °C) were found to be stable for 4 weeks.

A series of aqueous mixed working standard solutions of benazepril, benazeprilat and hydrochlorothiazide were prepared by subsequent dilution of the stock standard solutions in acetonitrile-water (10 : 90, v/v) to reach concentration ranges of 50.0–5000 ng ml^{-1} for all of the analytes. A working standard solution of the IS, chlorthalidone, 10.00 μg ml^{-1} was also prepared in acetonitrile-water (10 : 90, v/v). The working standard solution was freshly prepared every week and stored in the dark and under refrigeration.

Calibration spiked plasma standards and quality control samples

Drug-free human plasma was screened prior to use to ensure that it was free of endogenous interference at the retention times of the analytes. Calibration spiked plasma standards were prepared freshly every day for the concentration range of 5, 10, 20, 25, 50, 100, 200, 300, 400 and 500 ng ml^{-1} for each one of the analytes by appropriate dilutions of the above mentioned mixed working standard solution in 1.0 ml of human plasma. Each calibration sample contained 1000 ng ml^{-1} of the IS by the addition of 100 μl of the 10.0 μg ml^{-1} chlorthalidone working standard solution. Quality control (QC) samples were prepared in human plasma at four concentration levels (5, 10, 100 and 500 ng ml^{-1}). All the QC samples were freshly prepared for each run. Separate analyte stock solutions were used for the preparation of calibration standard solutions and QC samples.

Solid-phase extraction procedure (SPE)

Extraction and cleanup of human plasma samples was carried out by solid-phase extraction (SPE) according to the following procedure. The cartridges were pretreated with 1 × 1.0 ml of methanol and 1 × 1.0 ml of water. The spiked plasma samples were vortex mixed for 1 min along with 100 μl of H_3PO_4 10% v/v and applied to the HLB Oasis cartridges. Following sample application, the columns were washed with 2 × 1.0 ml of water containing 2.0% formic acid. After drying, the analyte was eluted with 1 × 0.8 ml of methanol and the eluate was then subjected to LC/MS procedure.

Validation procedures

Spiked plasma calibration standards at ten different concentration levels, ranging from 5 to 1000 ng ml^{-1} for each analyte, were prepared and analysed in duplicate in three different analytical runs. Calibration curves, on the basis of peak area ratio of each analyte to that of the IS multiplied by an appropriate factor *versus* the theoretical concentration, were prepared for each run. Weighted ($1/y^2$) least-squares linear regressions were used to obtain the equation of the calibration curves. QC samples were processed in five replicates at each concentration (5, 10, 100, 500 ng ml^{-1}) for three different analytical runs to evaluate the intra- and inter-assay accuracy and precision.

The recovery of the SPE procedure was evaluated at three concentration levels (10, 100 and 500 ng ml^{-1}) for benazepril, benazeprilat and hydrochlorothiazide and at 1000 ng ml^{-1} for the IS. It was determined by comparing the peak areas obtained from the QC samples after SPE procedure to the peak areas obtained from the analysis of methanolic standard solutions in equivalent concentrations.

The stability of benazepril, benazeprilat and hydrochlorothiazide in spiked human plasma samples was investigated under various storage conditions. In particular, plasma samples were spiked at two concentrations (25 and 200 ng ml^{-1}) of the analytes and were stored at ambient temperature for 6 h and at −20 °C for 3 weeks. Freeze–thaw stability was also evaluated by successive cycles of freezing and thawing; three complete freeze–thaw cycles were performed with the samples frozen at −20 °C for 7 days per cycle and thawed (without warming) at room temperature. Absolute peak area measurements obtained from the analysis of the stored samples were compared with the absolute peak area measurements those were obtained from the analysis of freshly prepared spiked plasma samples. The analyte was considered stable in the biological matrix when 80–120% of the initial concentration was found.

RESULTS AND DISCUSSION

Optimization of mass spectrometric conditions

Mass spectral fragmentation reactions of ACE inhibitors[34–36] and a series of thiazide diuretics[26,27] have been thoroughly investigated in the reports of various studies, using electrospray ionization and several types of mass analysers. However, in none of these reports were benazepril and its metabolite included among the ACE inhibitors in the studies. In the present work, studies on the electrospray ionization mass spectral behaviour of the analysed compounds were performed. The aim of these fragmentation studies was to optimize the mass spectrometric conditions for quantitation purposes and to add a useful insight to the ionization behaviour of these compounds.

Acquisition parameters were determined by direct infusion into the MS of a 5000 ng ml^{-1} solution (in mobile phase) of each one of the compounds at a flow rate of 20 μl min^{-1}. Variable mass spectrometric conditions (ESI probe temperature, capillary voltage and cone voltage) were investigated. ESI probe temperature was set at the minimum acceptable value (230 °C) with regard to the mobile phase flow rate (0.15 ml min^{-1}) to avoid thermal degradation of the compounds. Capillary voltage was kept at 4.0 kV for the detection of benazepril, benazeprilat and chlorthalidone in ESI positive ion mode, and at 3.5 kV for the detection of hydrochlorothiazide in ESI negative ion mode.

The electrospray ionization mass spectral behaviour of benazepril and benazeprilat was thoroughly investigated by cone voltage fragmentation in the single-quadrupole MS. Mass spectra of benazepril and benazeprilat obtained in ESI positive ion mode and under variable cone voltage conditions are presented in Figs 1 and 2, respectively. In the

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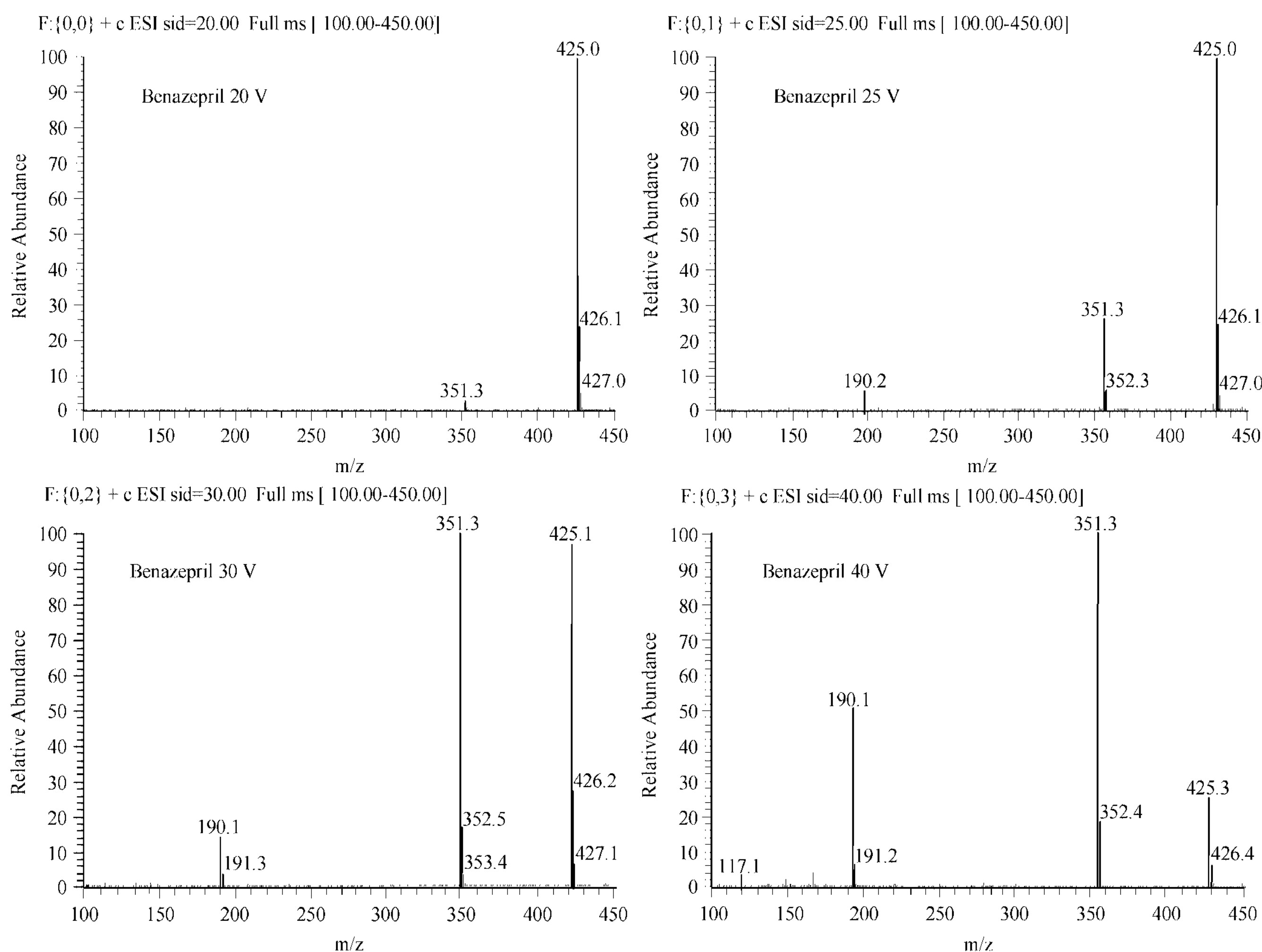

Figure 1. ESI electrospray mass spectra of a 5000 ng ml^{-1} benazepril standard solution in a mixture of 55% acetonitrile in water containing 0.3% v/v formic acid under variable cone voltage conditions that ranged from 20.0 to 40.0 V in ESI positive ion mode. MS conditions: positive ESI mode; ESI probe temperature, 230 °C; capillary voltage, 4.0 kV; AQA flow rate, 20 µl min^{-1}.

case of benazepril, the mass spectrum gave the protonated species, $[M + H]^+$, at m/z 425.0 after ionization in the electrospray source using a cone voltage of 20.0 V. An increase in the cone voltage causes concomitant increase of the fragmentation process of benazepril molecule. Thus, the resulting protonated species at m/z 425.0 loses a propanoic acid molecule (74 u) to give a species at m/z 351.3. The m/z 190.1 signal in the spectrum of benazepril presumably results via the losses of 3-phenylpropylideneamine (133 u) and carbon monoxide (28 u) in a one-step rearrangement. The ESI spectrum of benazeprilat is dominated by the peak at m/z 397.0 owing to the $[M + H]^+$ ion using a cone voltage of 20.0 V. Then, an increase in the cone voltage causes the loss of a formic acid molecule (46 u) to give a species at m/z 351.1. A species, similar to that obtained from the fragmentation of benazepril, is then observed at m/z 190.1, which is caused by losses of 3-phenylpropylideneamine (133 u) and carbon monoxide (28 u) in a one-step rearrangement. The proposed fragmentation patterns for benazepril and benazeprilat presented in Scheme 1 were further supported by MS/MS experiments. Ion trap MS^2 measurements were performed by direct infusion into the ion trap MS of a 20 000 ng ml^{-1} solution (in mobile phase) of each one of the compounds at a flow rate of 20 µlmin^{-1}. The full-scan spectrum of benazepril (Fig. 3(A)) is dominated by the protonated molecule at m/z 425.1, while fragments at m/z 351.1 and 190.1 are also present to a lesser extend. The product ion spectrum of the molecular ion at m/z 425.1 yields the fragment ions at m/z 351.1 and 190.1 (Fig. 3(B)). Isolation of the fragment ion at m/z 351.1 in the ion trap and subsequent fragmentation produces the MS^2 spectrum displayed in Fig. 3(C) where the fragment at m/z 190.1 is abundantly generated. The full-scan spectrum of benazeprilat (Fig. 4(A)) yields the molecular ion at m/z 397.1 along with the fragment ions at m/z 351.2 and 190.1. The product ion spectrum of the molecular ion at m/z 397.1 yields the fragment ions at m/z 351.1 and 190.1 (Fig. 4(B)). The fragment ion at m/z 351.1 was trapped and its fragmentation resulted in a mass spectrum shown in Fig. 4(B) where the fragment at m/z 190.1 is abundantly generated.

Electrospray ionization in positive ion mode was chosen for the detection of chlorthalidone (IS) as it was eluted in the same retention window with benazepril and benazeprilat. The protonated molecular ion $[M + H]^+$ of chlorthalidone at m/z 339.1 loses a water molecule (18 u) to give a species at m/z 321.1 that is abundantly generated under variable cone voltage conditions (Fig. 5).

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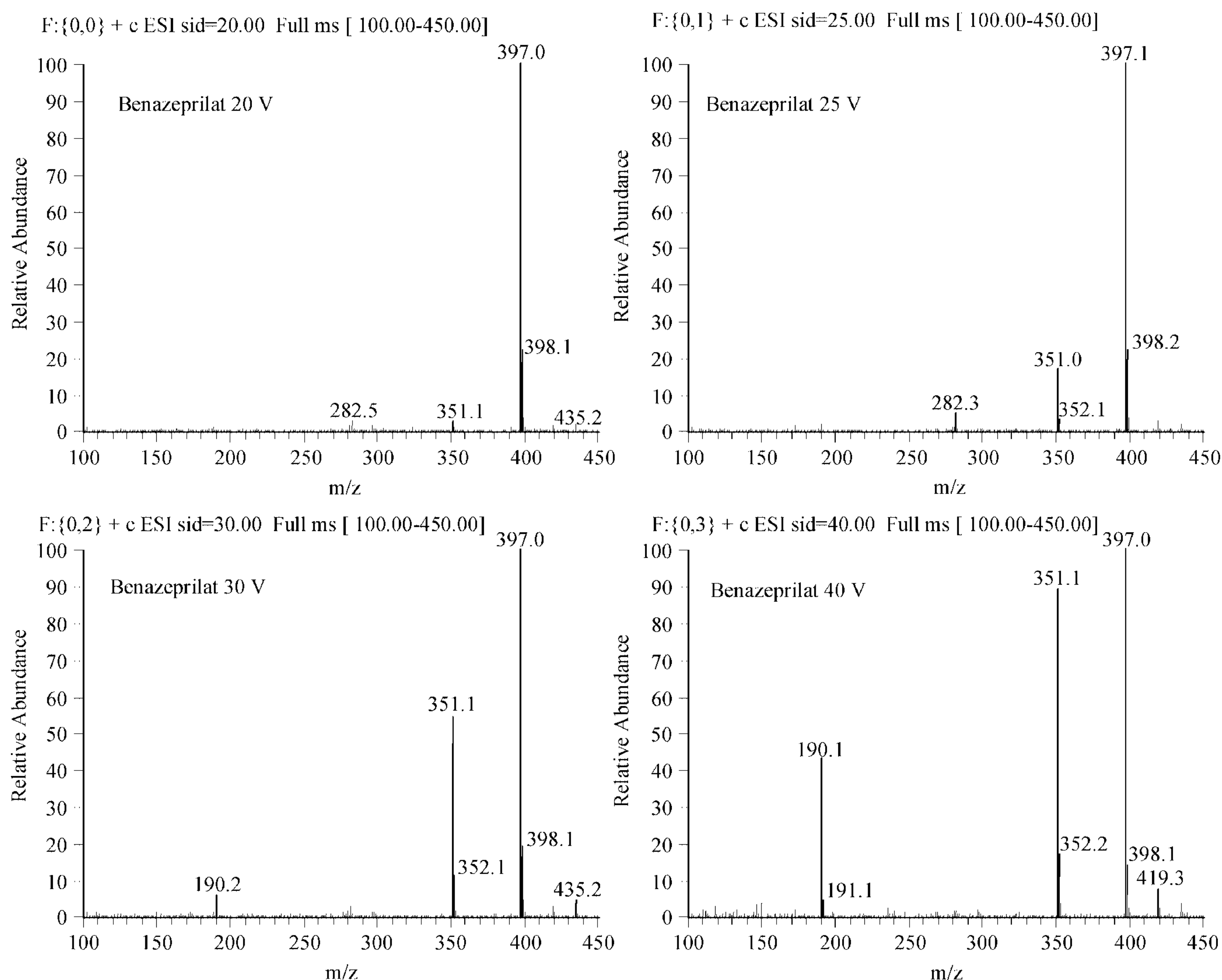

Figure 2. ESI electrospray mass spectra of a 5000 ng ml^{-1} benazeprilat standard solution in a mixture of 55% acetonitrile in water containing 0.3% v/v formic acid under variable cone voltage conditions that ranged from 20.0 to 40.0 V. MS conditions: positive ESI mode; ESI probe temperature, 230 °C; capillary voltage, 4.0 kV; AQA flow rate, 20 µl min^{-1}.

The mass spectrum of hydrochlorothiazide obtained in ESI negative ion mode under variable cone voltage conditions is presented in Fig. 6. Under electrospray conditions in the negative ion mode, the deprotonated molecular ion $[M-H]^-$ of hydrochlorothiazide at m/z 295.9 is abundantly generated. An increase in the cone voltage entailed an extensive fragmentation of the diuretic. Thus, the loss of an HCN portion (27 u) gives a species at m/z 269.0, which then by the loss of a $-SO_2$ portion (64 u) leads to a species at m/z 205.2. Data on ESI-MS studies of hydrochlorothiazide are in accordance with the studies that have already been published in the literature.[26,27]

The monitoring of all analytes in SIM mode by their dominant mass peaks was found to be crucial as it increases significantly the signal-to-noise ratio of the analyte's peaks in the ion chromatograms. Thus, a cone voltage value of 20.0 V was chosen as the optimum to obtain the maximum abundance of the molecular ions. Instrument method in SIM mode is presented in Table 1. In particular, benazepril and benazeprilat were evidenced in an ESI positive ion mode by the protonated molecular ions at m/z 425.0 and 397.0, respectively. Hydrochlorothiazide was evidenced by its deprotonated molecular ion at m/z 295.9 in an ESI negative ion mode. The IS was monitored by the most abundant species at m/z 321.1.

Chromatography

The chromatography of the analytes was confronted with several analytical problems. In particular, benazeprilat and hydrochlorothiazide are highly polar compounds that were eluted close to the solvent front with the classical stationary phases that were tested (C_{18} and C_8). Moreover, because hydrochlorothiazide gave an excellent electrospray response in the negative ion mode, it had to be completely resolved from the other analytes (benazepril and benazeprilat) that exhibited better response in the positive ion mode. By the use of a PGC column the analytes were adequately retained, well separated and the detection could be performed in separate time windows (Table 1). Various combinations of acetonitrile and water with changed contents of each component and a number of acidic modifiers (trifluoroacetic acid, formic acid and glacial acetic acid) were investigated to identify the

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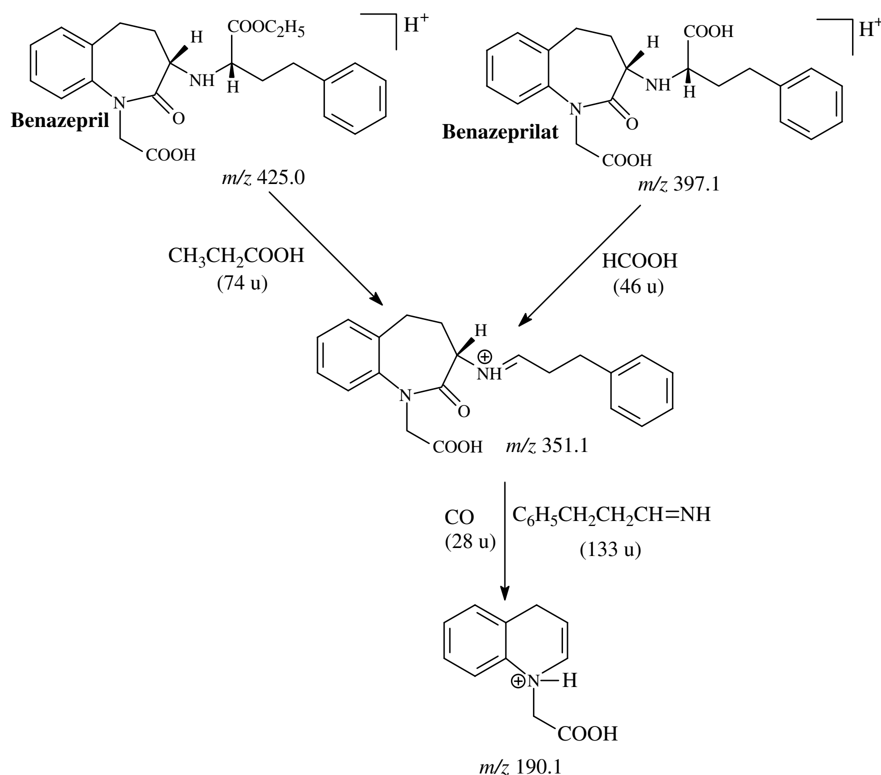

Scheme 1. Proposed ESI-MS fragmentation processes for benazepril and benazeprilat in positive ion mode.

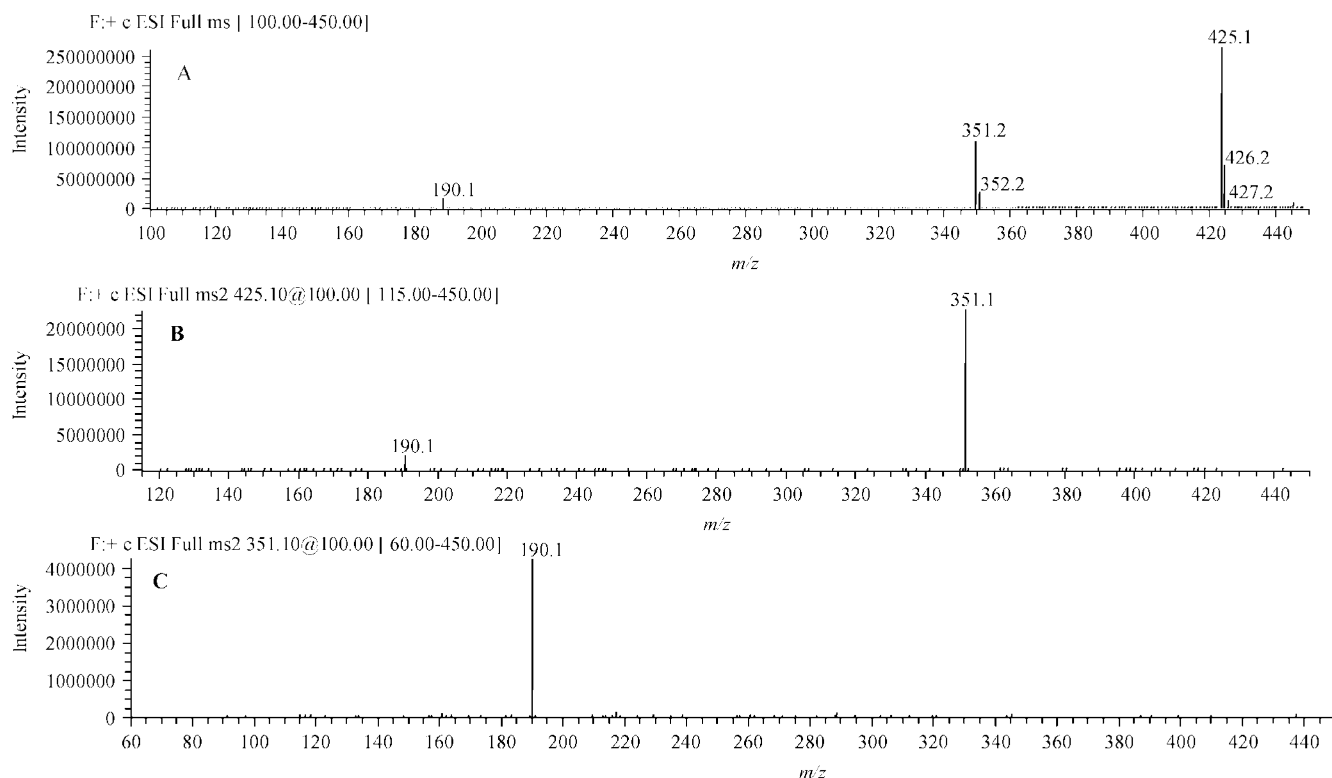

Figure 3. (A) Full-scan ESI/MS spectrum of a 20 000 ng ml^{-1} benazepril standard solution in a mixture of 55% acetonitrile in water containing 0.3% v/v formic acid, (B) ESI/MS/MS product ion spectrum of the protonated molecular ion $[M + H]^+$ at m/z 425.1 and (C) ESI/MS/MS product ion spectrum of the fragment ion at m/z 351.1. MS conditions: positive ESI mode; temperature settings, 230 °C; capillary voltage, 30.0 V; ion spray voltage, 5.0 kV; sheath gas flow rate, 45 arbitrary units and auxiliary gas flow rate, 10 arbitrary units; collision energy, 100%.

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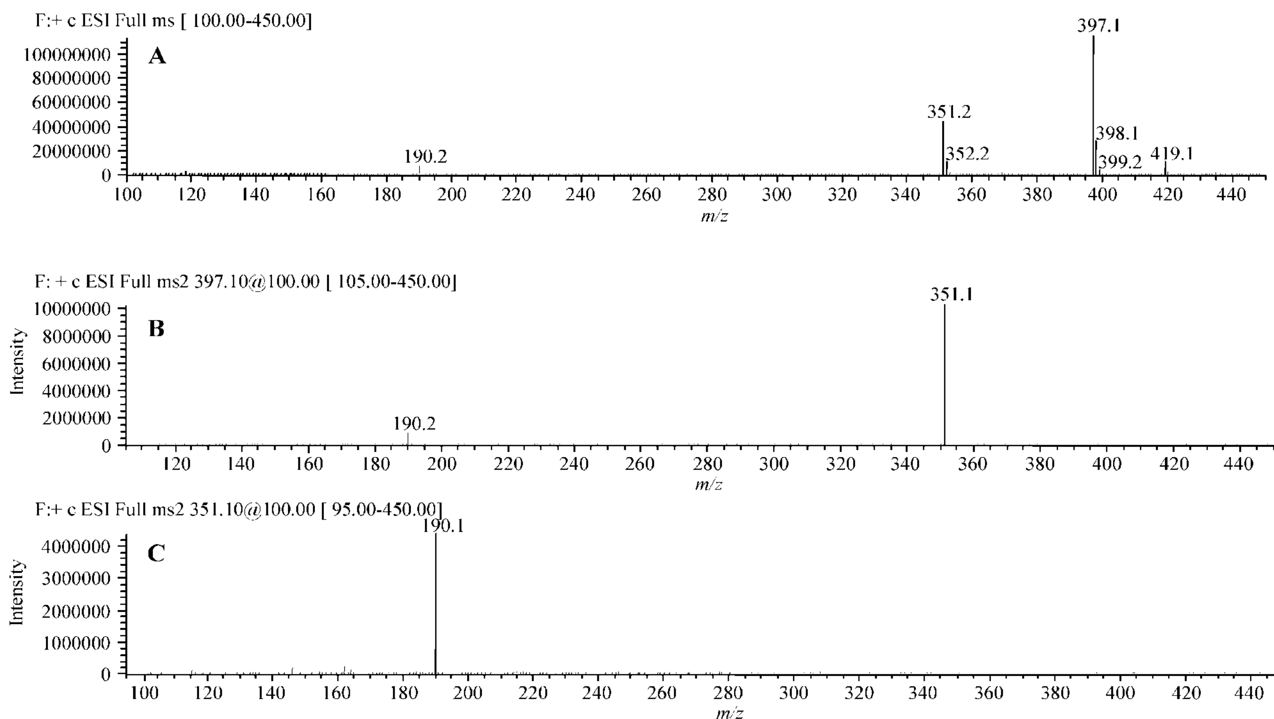

Figure 4. (A) Full-scan ESI/MS spectrum of a 20 000 ng ml^{-1} benazeprilat standard solution in a mixture of 55% acetonitrile in water containing 0.3% v/v formic acid, (B) ESI/MS/MS product ion spectrum of the protonated molecular ion $[M + H]^+$ at *m/z* 397.1 and (C) ESI/MS/MS product ion spectrum of the fragment ion at *m/z* 351.1. MS conditions: positive ESI mode; temperature settings, 230 °C; capillary voltage, 30.0 V; ion spray voltage, 5.0 kV; sheath gas flow rate, 45 arbitrary units and auxiliary gas flow rate, 10 arbitrary units; collision energy, 100%.

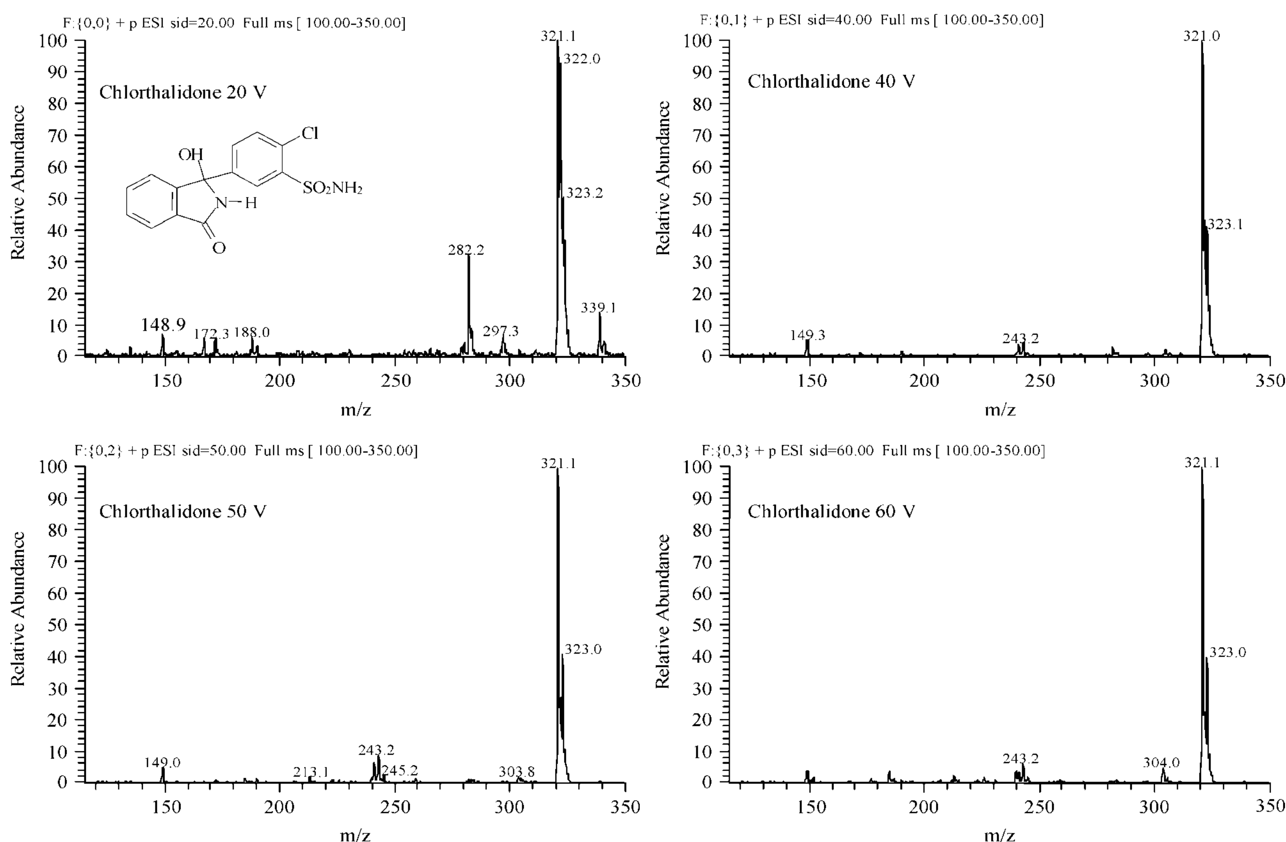

Figure 5. ESI electrospray mass spectra of a 5000 ng ml^{-1} chlorthalidone standard solution in a mixture of 55% acetonitrile in water containing 0.3% v/v formic acid under variable cone voltage conditions ranging from 20.0 to 60.0 V. MS conditions: positive ESI mode; ESI probe temperature, 230 °C; capillary voltage, 4.0 kV; AQA flow rate, 20 µl min^{-1}.

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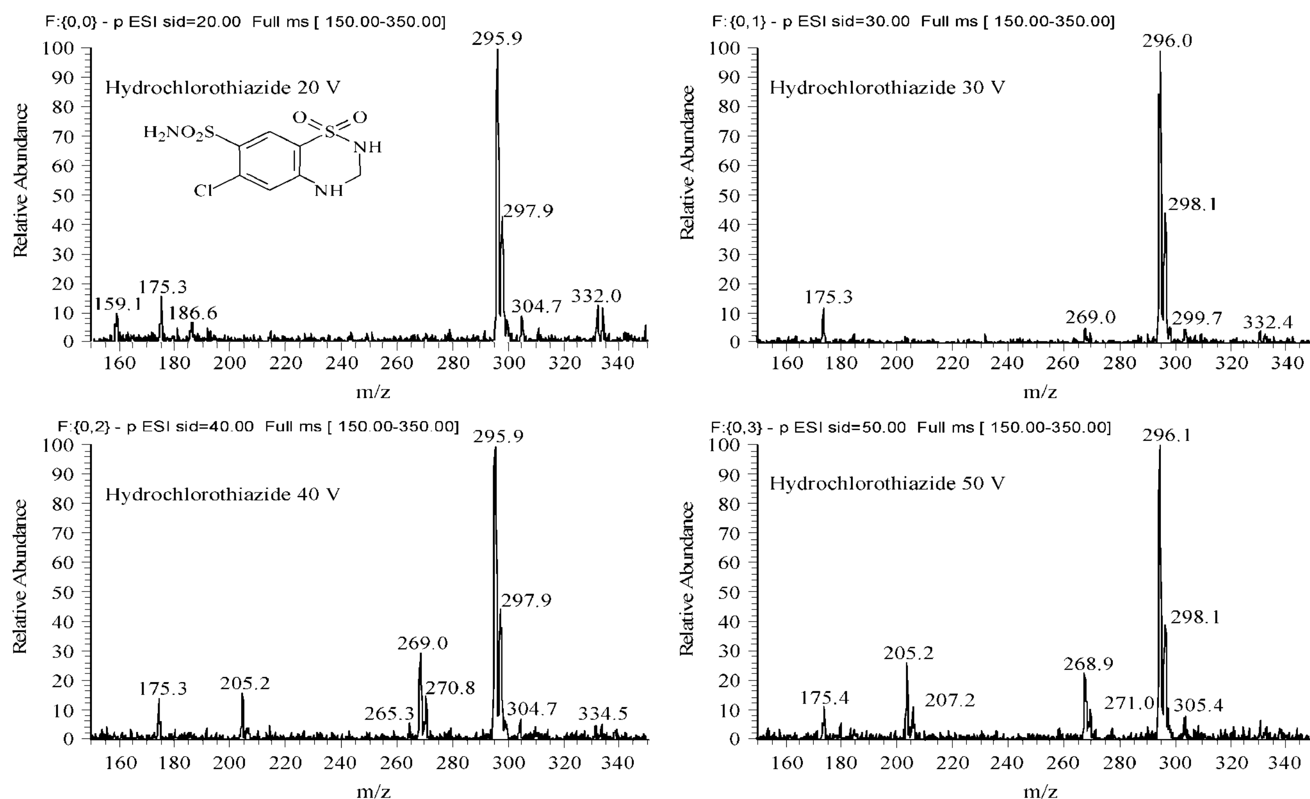

Figure 6. ESI electrospray mass spectra of a 5000 ng ml^{-1} hydrochlorothiazide standard solution in a mixture of 55% acetonitrile in water containing 0.3% v/v formic acid under variable cone voltage conditions ranging from 20.0 to 50.0 V. MS conditions: negative ESI mode; ESI probe temperature, 230 °C; capillary voltage, 3.5 kV; AQA flow rate, 20 µl min^{-1}.

optimal mobile phase composition that produced the best sensitivity and peak shape for all the analytes. It was found that an increase in the content of acetonitrile as the organic modifier in the mobile phase could improve the peak shape, whereas an increase in the water content broadened the peak. The use of formic acid as an acidic modifier in the mobile phase gave excellent positive ion electrospray response for benazepril and benazeprilat and adequate negative ion electrospray response for hydrochlorothiazide. A mobile phase consisting of 55% acetonitrile in water containing 0.3% formic acid was finally used. Each chromatographic run was completed within 13.0 min.

A representative MS chromatogram obtained from the analysis of a blank plasma sample along with a chromatogram obtained from the analysis of a sample spiked with 100.0 ng ml^{-1} of each one of the analytes are presented in Fig. 7(A) and (B), respectively. Under the current chromatographic conditions benazeprilat, chlorthalidone, benazepril and hydrochlorothiazide were eluted at 4.09, 5.00, 5.90 and 10.02 min, respectively.

Statistical analysis of data

Spiked plasma calibration standards of the benazepril, benazeprilat and hydrochlorothiazide were analysed in duplicate in three analytical runs for the calibration procedure. Ratios of the peak area signals of benazepril and benazeprilat to that of the IS were multiplied by a factor of 0.1, while ratios of peak area signals of hydrochlorothiazide to that of the IS were multiplied by a factor of 10. Linear relationships between the multiplied ratios of the peak area signals of the analytes to that of the IS and the corresponding concentrations were observed using a weighting factor of $1/y^2$, as shown by the equations presented in Table 2. In all the cases, back-calculated concentrations in the calibration curves were within 15% of the nominal values, which are in agreement with international guidelines[37] and indicate that the linear model acceptably describes the relationship between concentration and response. The average regression equations of the three calibration curves over a period of one month, the standard deviation values of the slopes and intercepts along with the correlation coefficients are also presented in Table 2.

The limits of detection (LOD) for the analytes were calculated using the equation LOD $= (3.3\,S_a)/b$ (where b is the slope and S_a is the standard deviation of the intercept of the regression line), and they were found to be at the levels of 1.45, 1.51 and 1.47 ng ml^{-1} for benazepril, benazeprilat and hydrochlorothiazide, respectively. The limits of quantitation, (LOQ) were also attained using the equation LOQ $= (10\,S_a)/b$ (where b is the slope and S_a is the standard deviation of the intercept of the regression line). The LOQ were found to be 4.4, 4.6 and 4.5 ng ml^{-1} for benazepril, benazeprilat and hydrochlorothiazide, respectively.

The selectivity towards endogenous plasma compounds was tested in six different lots of drug-free human plasma by analysing blanks (non-spiked plasma samples) and plasma samples spiked with benazepril, benazeprilat and hydrochlorothiazide at the LOQ level (5.0 ng ml^{-1}) and IS. Mass chromatograms of six batches of the drug-free plasma contained no co-eluting peaks greater than 20%

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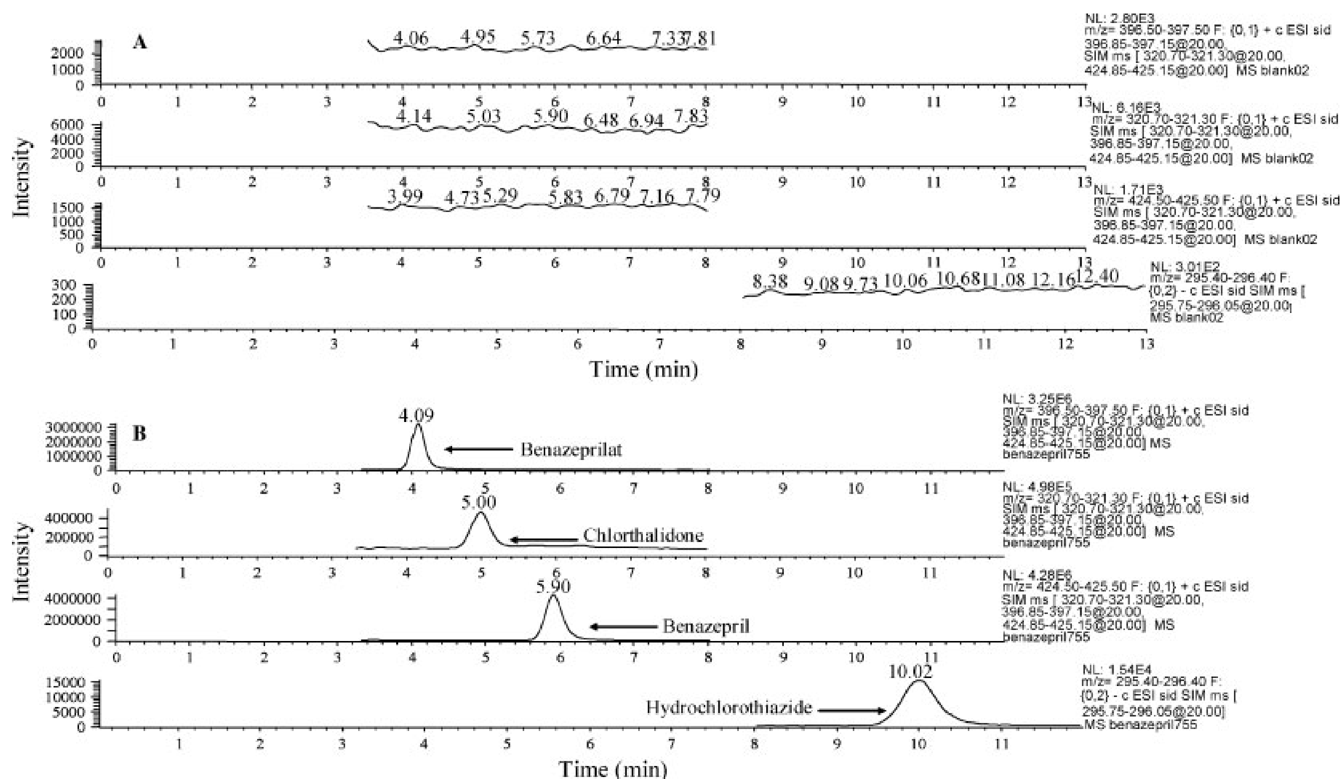

Figure 7. Smoothed and normalized mass chromatogram (SIM mode) of a blank plasma extract (top), along with a smoothed and normalized mass chromatogram (SIM mode) of a calibration plasma sample spiked with 100.0 ng ml^{-1} of benazepril, 100.0 ng ml^{-1} of benazeprilat, 100.0 ng ml^{-1} of hydrochlorothiazide and 1000.0 ng ml^{-1} of the IS; the retention times of benazeprilat, chlorthalidone, benazepril and hydrochlorothiazide are 4.09, 5.00, 5.90 and 10.02 min, respectively.

Table 2. Analytical concentration parameters of the calibration equations for the determination of benazepril, benazeprilat and hydrochlorothiazide

Compound	Concentration range (ng ml^{-1})	Regression equations[a]	r[b]	Standard deviation		S_r[c]
				Slope	Intercept	
Representative calibration data						
For one of the runs						
Benazepril	5–500	$R_{Bz} = 0.00525 \times C_{Bz} + 0.0116$	0.998	1.0×10^{-4}	0.0023	0.047
Benazeprilat	5–500	$R_{Bzlat} = 0.00327 \times C_{Bzlat} + 0.0096$	0.998	5.8×10^{-5}	0.0015	0.042
Hydrochlorothiazide	5–500	$R_{Hct} = 0.00336 \times C_{Hct} + 0.0012$	0.9996	3.6×10^{-5}	0.0011	0.025
Mean of three calibration curves over a period of 1 month						
Benazepril	5–500	$R_{Bz} = 0.00547 \times C_{Bz} + 0.0157$	≥ 0.998	2.5×10^{-4}	0.0046	<0.053
Benazeprilat	5–500	$R_{Bzlat} = 0.00279 \times C_{Bzlat} + 0.0173$	≥ 0.998	4.5×10^{-4}	0.011	<0.051
Hydrochlorothiazide	5–500	$R_{Hct} = 0.00351 \times C_{Hct} - 0.0017$	≥ 0.9994	3.6×10^{-4}	0.0034	<0.039

[a] Ratios of the peak areas signals of benazepril and benazeprilat to that of the IS were multiplied by a factor of 0.1; ratios of peak area signals of hydrochlorothiazide to that of the IS were multiplied by a factor of 10.
[b] Correlation coefficient.
[c] Standard error of the estimate.

of the area of each one of the analytes at the LOQ level, and no co-eluting peaks greater than 5% of the area of the IS chlorthalidone. The concentrations of the analytes obtained after the analysis of the six different lots of human plasma were found to be 5.21 ± 0.65, 5.41 ± 0.81 and 5.33 ± 0.71 ng ml^{-1} for benazepril, benazeprilat and hydrochlorothiazide, respectively, and indicate that the matrix effect does not cause any inaccuracy for the assay.

To evaluate the ion suppression effect, six samples of the drug-free human plasma were processed according to the sample preparation procedure (SPE) and then spiked with each analyte at medium QC concentration (100.0 ng ml^{-1}) and IS at 1000.0 ng ml^{-1}. The corresponding peak areas were then compared to those of methanolic standard solutions in equivalent concentration. Ion suppression recoveries were found to be $97.9 \pm 4.9\%$, $104.9 \pm 3.5\%$, $99.6 \pm 3.3\%$ and $104.3 \pm 1.0\%$, for benazepril, benazeprilat,

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Table 3. Accuracy and precision evaluation of QC samples for benazepril, benazeprilat and hydrochlorothiazide ($n = 3$ runs, five replicates per run)

	Concentration (ng ml^{-1})			
Compound	5	10	100	500
Benazepril				
Run 1 (mean ± SD)	5.06 ± 0.74	10.39 ± 0.25	101.0 ± 3.4	501.7 ± 9.6
Run 2 (mean ± SD)	4.52 ± 0.49	9.73 ± 0.29	103.7 ± 1.2	510.4 ± 7.0
Run 3 (mean ± SD)	5.08 ± 0.76	10.24 ± 0.33	97.5 ± 4.0	487.4 ± 9.2
Overall mean	4.85	10.12	100.7	499.8
Intra-assay CV (%)[a]	14.6	2.9	3.1	1.7
Inter-assay CV (%)[a]	2.0	3.2	2.8	2.2
Overall accuracy % E_r[b]	−3.0	1.2	0.7	−0.04
Benazeprilat				
Run 1 (mean ± SD)	4.72 ± 0.40	10.53 ± 0.33	99.2 ± 3.0	498.1 ± 9.2
Run 2 (mean ± SD)	4.76 ± 0.48	10.15 ± 0.25	94.0 ± 2.1	529.9 ± 7.3
Run 3 (mean ± SD)	5.18 ± 0.72	9.91 ± 0.40	102.8 ± 2.7	473.5 ± 8.6
Overall mean	4.89	10.20	98.7	500.5
Intra-assay CV (%)[a]	11.6	3.2	2.7	1.7
Inter-assay CV (%)[a]	1.4	2.7	4.3	5.6
Overall accuracy % E_r[b]	−2.2	2.0	−1.3	0.1
Hydrochlorothiazide				
Run 1 (mean ± SD)	4.34 ± 0.15	9.36 ± 0.22	97.3 ± 3.7	475.2 ± 8.2
Run 2 (mean ± SD)	4.72 ± 0.69	9.66 ± 0.19	101.8 ± 3.5	503.6 ± 8.9
Run 3 (mean ± SD)	4.74 ± 0.50	10.13 ± 0.33	103.2 ± 1.9	529.4 ± 4.9
Overall mean	4.60	9.71	100.7	502.7
Intra-assay CV (%)[a]	11.0	2.6	3.1	1.5
Inter-assay CV (%)[a]	0.7	3.8	2.7	5.3
Overall accuracy % E_r[b]	−8.0	−2.9	0.7	0.6

[a] Coefficient of variation; intra- and inter-assay CVs were calculated by ANOVA.
[b] Relative percentage error = [(overall mean assayed concentration − added concentration)/(added concentration) × 100].

hydrochlorothiazide and chlorthalidone, respectively, and indicated that there was no appreciable matrix suppression.

One-way analysis of variance (ANOVA) was used to evaluate the intra- and inter-assay precision. Results presented in Table 3 indicate that intra-assay coefficients of variations, % coefficient of variations (CV), were between 1.7 and 14.6% for benazepril, between 1.7 and 11.6% for benazeprilat and between 1.5 and 11.0% for hydrochlorothiazide. The inter-assay % CVs were lower than 3.2% for benazepril, lower than 5.6% for benazeprilat, while for hydrochlorothiazide it was lower than 5.3%. The overall accuracy was assessed by the relative percentage error, absolute % E_r, which ranged from −3.0 to 1.2% for benazepril, from −2.2 to 2.0% for benazeprilat, and from −8.0 to 0.7% for hydrochlorothiazide.

The recoveries of the proposed SPE procedure were determined by calculating the ratio of the peak areas of extracted spiked plasma samples to the area of methanolic standard solutions in equivalent concentrations. Recovery data presented in Table 4 indicated average recoveries of 97.8%, 97.5% and 96.5% for benazepril, benazeprilat and hydrochlorothiazide, respectively.

The stability results presented in Table 5 indicate that the analytes can be considered stable under the various conditions investigated. In particular, benazepril and benazeprilat concentrations deviate by no more than −6.1% relative to the reference, hydrochlorothiazide concentrations deviate by no more than −2.8% relative to the reference, while no degradation products were observed for any of the tests. Calibration plasma samples containing benazepril, its metabolite (benazeprilat) and hydrochlorothiazide may

Table 4. Recovery data of the SPE procedure for benazepril, benazeprilat and hydrochlorothiazide

Compound	Concentration levels (ng ml^{-1})	% Recovery (mean ± SD, $n = 6$)
Benazepril	10	97.7 ± 1.4
	100	97.1 ± 1.7
	500	98.7 ± 1.6
Benazeprilat	10	98.5 ± 1.4
	100	98.2 ± 1.0
	500	95.8 ± 8.7
Hydrochlorothiazide	10	97.9 ± 2.1
	100	97.6 ± 4.7
	1000	95.0 ± 4.5
Chlorthalidone	1000	97.0 ± 2.2

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Table 5. Stability data for benazepril, benazeprilat and hydrochlorothiazide in human plasma under various storage conditions

Storage conditions/time	Concentration levels (ng mL^{-1})	Calculated concentration (ng mL^{-1}) (mean ± SD, $n = 5$)			% E_r [a]		
		Benazepril	Benazeprilat	Hydrochloro-thiazide	Benazepril	Benazeprilat	Hydrochloro-thiazide
Ambient temperature/6 h	25	25.05 ± 0.49	23.62 ± 0.45	24.3 ± 1.2	0.2	−5.5	−2.8
	200	198.6 ± 5.3	198.7 ± 5.1	198.2 ± 3.0	−0.7	−0.7	−0.9
−20 °C/7 days	25	23.47 ± 0.35	23.92 ± 0.54	25.00 ± 0.51	−6.1	−4.3	0.0
	200	199.8 ± 1.8	195.3 ± 1.8	200.1 ± 7.0	−0.1	−2.3	0.0
−20 °C/14 days	25	24.97 ± 0.69	25.37 ± 0.94	24.73 ± 0.42	−0.1	1.5	−1.1
	200	200.6 ± 2.6	197.0 ± 2.2	198.7 ± 5.2	0.3	−1.5	−0.7
−20 °C/21 days	25	23.79 ± 0.36	23.98 ± 0.23	25.12 ± 0.44	−4.8	−4.1	0.5
	200	202.6 ± 3.3	194.2 ± 2.8	199.5 ± 4.9	1.3	−2.9	−0.3
−20 °C/2 freeze-thaw cycles	25	24.83 ± 0.19	24.93 ± 0.29	24.62 ± 0.38	−0.7	−0.3	−1.5
	200	200.0 ± 6.0	187.8 ± 1.3	199.9 ± 4.7	0.0	−6.1	0.0
−20 °C/3 freeze-thaw cycles	25	24.97 ± 0.69	25.37 ± 0.94	24.73 ± 0.42	−0.1	1.5	−1.1
	200	200.6 ± 2.6	197.0 ± 2.2	198.7 ± 5.2	0.3	−1.5	−0.7

[a] Relative percentage error = [(overall mean assayed concentration – added concentration)/(added concentration) × 100].

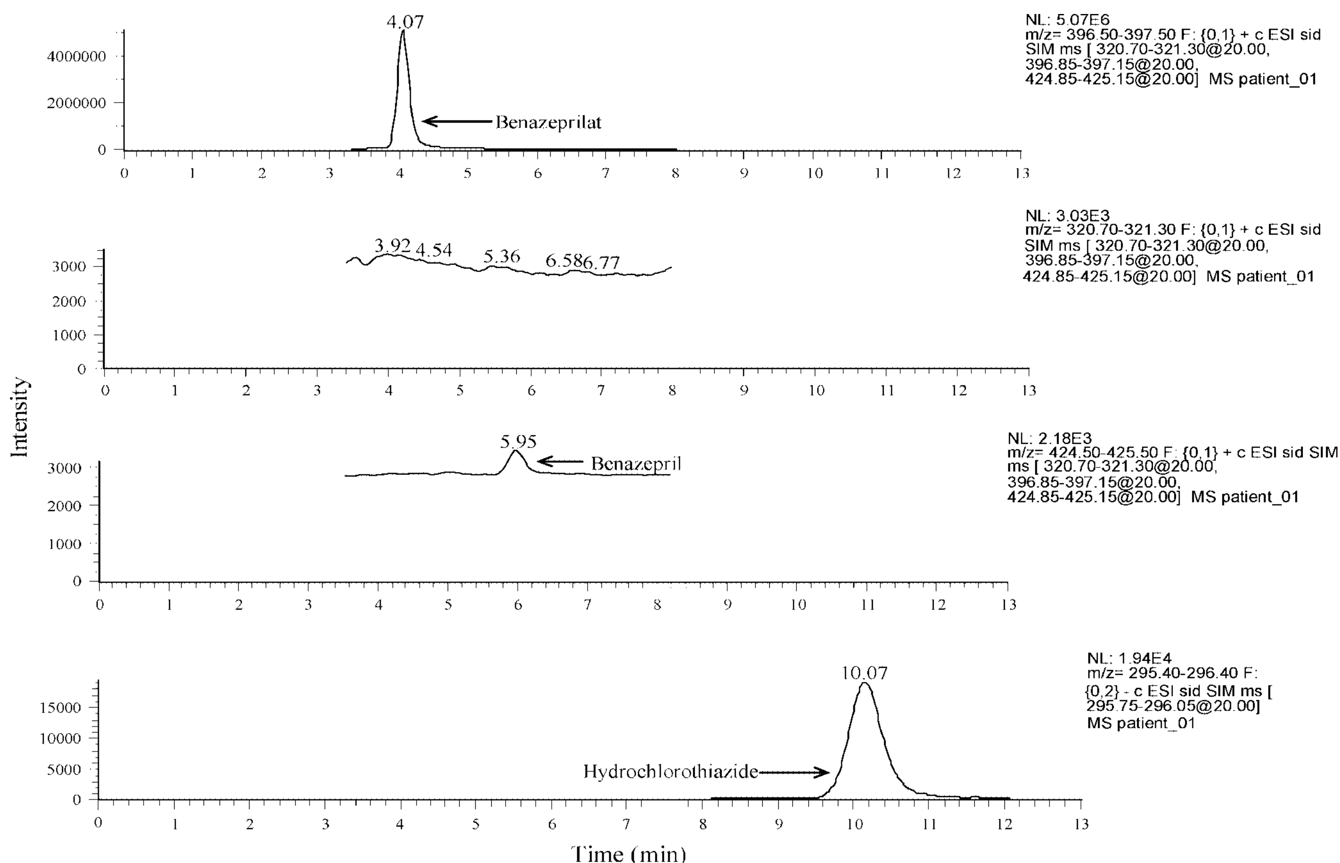

Figure 8. Smoothed and normalized mass chromatogram (SIM mode) obtained from the analysis of a patients plasma sample containing benazepril (below LOQ level), benazeprilat and hydrochlorothiazide without IS addition. Benazeprilat, benazepril and hydrochlorothiazide are eluted at 4.07, 5.95 and 10.07 min, respectively.

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therefore be kept for up to 6 h at ambient temperature, for 21 days at −20 °C, and after 3 freeze-thaw cycles (7 days per cycle) at −20 °C without any significant degradation.

Actual measurements of the analytes in human plasma after oral administration

The proposed methodology was also applied to the analysis of a plasma sample obtained after oral administration of a benazepril/hydrochlorothiazide combination to a patient. In particular, the patient in the study was a 62-year-old male who had been receiving 20 mg of benazepril combined with 25 mg of hydrochlorothiazide once daily in the morning (Cibadrex® 20/25, Novartis Pharma) for a long-term treatment. During the period of sample collection, the patient also received 4 mg glimepiride, 15 mg pioglitazone, 500 mg diltiazem, 40 mg fluvastatin and 0.4 mg tamsulosine once daily, as well as 850 mg gliclazide, 80 mg theophyllinate choline, 500 mg cefuroxime and 150 mg ranitidine twice daily (morning and night). A combination of 0.020 mg salbutamol and 0.120 mg ipratropium was also administered to the patient by inhalation once daily. Blood sample was collected, 5 h after dosing of benazepril/hydrochlorothiazide combination, in a Venoject® tube containing lithium heparin as anticoagulant. Immediately after drawing, the sample was shaken gently and centrifuged at 4000 rpm for 10 min at 4 °C. The plasma sample was analysed one day after storage at −20 °C. Three aliquots (1000 μl) of this plasma sample were analysed according to the sample preparation procedure (IS was added) to calculate the concentration of the analytes. The concentration of benazepril in the sample could not be quantified as it was found to be below 5 ng ml^{-1} (LOQ limit). Benazeprilat and hydrochlorothiazide concentrations were found to be 193.5 ± 5.0 and 103.5 ± 2.6 ng ml^{-1}, respectively. Figure 8 illustrates a typical MS chromatogram obtained from the analysis of the patient's plasma sample without IS addition to demonstrate that there is no interference in the retention time of chlorthalidone (IS).

CONCLUSIONS

The proposed LC/ESI-MS method enables a rapid, accurate and selective assay for the determination of benazepril, its metabolite (benazeprilat) and hydrochlorothiazide in human plasma with a run time lower than 13.0 min. The method consists of a simple SPE procedure for sample pretreatment and a reversed-phase liquid chromatographic procedure. Mass spectrometric detection increases the sensitivity and selectivity of the proposed method. The proposed method, with an LOQ of 5.0 ng ml^{-1} for all of the analytes, is suitable to support a wide range of pharmacokinetic or bioequivalence studies.

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