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Lisinopril quantification in human plasma by liquid chromatography–electrospray tandem mass spectrometry

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Abstract

An analytical method based on liquid chromatography with positive ion electrospray ionization (ESI) coupled to tandem mass spectrometry detection was developed for the determination of Lisinopril in human plasma using Enalaprilat as internal standard. The analyte and internal standard were extracted from the plasma samples by solid-phase extraction using Waters HLB Oasis® SPE cartridges and chromatographed on a C_8 analytical column. The mobile phase consisted of acetonitrile/water (60:40, v/v) + 20 mM acetic acid + 4.3 mM of triethylamine. The method had a chromatographic total run-time of 6.5 min and was linear within the range 2.00–200 ng/ml. Detection was carried out on a Micromass triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM). The precision (CV%) and accuracy, calculated from limit of quantification (LOQ) samples ($n = 8$), were 8.9 and 98.9%, respectively. The method herein described was employed in a bioequivalence study of two tablet formulations of Lisinopril 20 mg.

Keyword: Lisinopril

1. Introduction

Lisinopril (*N*-[1-carboxy-3-phenylpropyl-L-lysil]proline), the third ACE inhibitor approved for use in the United States, is the lysine analog of Enalaprilat. Unlike enalapril, Lisinopril itself is active [1]. It has been demonstrated to be effetive in the treatment of heart failure, hypertension and acute myocardial infarction [2].

Lisinopril has been measured by several techniques, such as bioassay (through inhibition of ACE [3]), radioimmunoassay [4–8], fluoroimmunoassay [9].

Recently, an assay based on gas chromatography-negative ion chemical ionization mass spectrometry (GC–MS) [10,11] and liquid chromatography coupled with mass spectrometry (LC–MS) [12] were described. Both methods were successfully applied to the measurements of Lisinopril in human plasma/serum.

Here we present a fast, sensitive and selective method for measuring plasma Lisinopril using liquid chromatography coupled to tandem mass spectrometry (LC–MS–MS). This method was applied to a bioequivalence study in healthy volunteers.

2. Experimental

2.1. Chemicals and reagents

Lisinopril was kindly provided by EMS, Brazil, lot number 00109987. Enalaprilat was obtained from United States Pharmacopeia (USP), lot number I. Waters Oasis® HLB cartridges (30 mg, 1 cm^3) for solid phase extraction was purchased from Waters Co. (Milford, MA, USA). Acetonitrile and methanol (HPLC-grade), fuming hydrochloric acid (37%) and glacial acetic acid (analysis grade) were purchased from Mallinckrodt (Paris, ST, USA). Ultra pure water

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was obtained from an Elga UHQ system (Elga, UK). Blank human blood was collected from healthy, drug-free volunteers. Plasma was obtained by centrifugation of blood treated with the anticoagulant sodium heparin. Pooled plasma was prepared and stored at approximately −20 °C until needed. All HPLC solvents were filtered through a 0.45 μm filter prior to use.

2.2. Calibration standards and quality control

Stock solutions of Lisinopril and internal standard (Enalaprilat) were prepared in methanol–water (50:50, v/v) at concentrations of 1 mg/mL. Calibration curves of Lisinopril were prepared by spiking blank plasma at concentrations of 2.00, 5.00, 10.0, 20.0, 50.0, 100 and 200 ng/mL and the analysis was carried out in duplicate for each concentration. The quality control samples were prepared in blank plasma at concentrations of 6.00, 30.0 and 150 ng/mL (QCA, QCB, and QCC, respectively). The spiked plasma samples (standards and quality controls) were extracted on each analytical batch along with the unknown samples.

2.3. Sample preparation

All frozen human plasma samples were previously thawed at ambient temperature and centrifuged at 2550 × *g* for 5 min at 4 °C to precipitate solids. Four hundred microliters of aqueous hydrochloric acid solution (10 mM) were dispensed into appropriate glass tubes and then 500 μL of sample human plasma was added. The tubes were briefly vortex-mixed (10 s) and 50 μL of I.S. (2.0 μg/mL Enalaprilat in aqueous solution) were dispensed into the tubes. The tubes were briefly vortex-mixed (10 s) and stood at room temperature for 5 min. Sufficient previously unused Waters HLB Oasis® SPE cartridges for the assay were pre-conditioned by washing first with 2 mL methanol and then 1 mL of hydrochloric acid solution (10 mM). Each wash solution was drawn through the columns under light vacuum. All of each plasma samples were applied to the individual Waters Oasis® SPE cartridges. Using light vacuum the samples were slowly drawn through the cartridges. The cartridges were washed for five times with 1 mL of aqueous hydrochloric acid solution (10 mM). The wash was drawn through the columns under light vacuum. The Waters Oasis® SPE cartridges were placed into 12 mm × 120 mm appropriately numbered glass tubes. Cartridges were then eluted with 0.5 mL of methanol, which were drawn through the cartridge slowly and were collected into the glass tubes by applying a light positive pressure using an nitrogen flow. The solvent was evaporated by using a flow of nitrogen at 37 °C. This process was conducted in a fume cupboard.

The dry residues were reconstituted with 200 μL of acetonitrile/water (80:20; v/v) + 10 mM acetic acid solution and vortex-mixed for 15 s. The solutions were then transferred to the auto-injector microvials.

2.4. Chromatographic conditions

An aliquot (30 μL) of each plasma extract was injected into a Genesis C_8 analytical column, (150 mm × 4.6 mm i.d.) operating at 40 °C. The compounds were eluted by pumping the mobile phase (acetonitrile/water (60/40; v/v) containing 20 mM acetic acid and 4.3 mM of triethylamine) at a flow-rate of 0.5 ml/min. Under these conditions, typical standard retention times were 4.06 min for Lisinopril and 4.17 min for Enalaprilat, and back-pressure values of approximately 60–100 bar were observed.

A split of the column eluant of approximately 1:10 was included so that only 50 μL/min entered the mass spectrometer. The temperature of the auto-sampler was kept at 5 °C and the run-time was 6.5 min.

2.5. Mass-spectrometric conditions

The mass spectrometer (Micromass model Quattro II) equipped with an electrospray source using a crossflow counter electrode run in positive mode (ES+), was set up in Multiple Reaction Monitoring (MRM), monitoring the transitions 406.3 > 84.3 and 349.1 > 206.1, for Lisinopril and IS, respectively. Fig. 1 shows the full scan spectra (upper trace) and the product ion spectra (lower trace) obtained for Lisinopril (panel A) and Enalaprilat (painel B). In Fig. 2, we proposed an unusual fragmentation mechanism for the Lisinopril. Firstly, the protonated molecule (*m/z*, 406) loss a proline residue generating the fragment *m/z* 263. Secondly, a cyclisation process is proposed in order to get the unsaturated nitrogen-six-member ring (*m/z*, 84). The proposed fragmentation route for the internal standard, Enalaprilat, is also shown in Fig. 2.

In order to optimize all the MS parameters, a mix standard solution (10 μg/mL) of the analyte and I.S. was infused into the mass spectrometer. For both Lisinopril and Enalaprilat, the following optimized parameters were obtained: for Lisinopril the dwell time, the cone voltage and the gas pressure (Helium) were 0.8 s, 25 V and 1.3×10^{-3} mBar. For Enalaprilat the dwell time, the cone voltage and the gas pressure (Helium) were 0.8 s, 20 V and 1.3×10^{-3} mBar. The collision energy was 20 eV for Lisinopril and 15 eV for Enalaprilat. Data acquisition and analysis were performed using the software MassLynx (v 3.2) running under Windows NT (v 4.0) on a Digital Celebris GL 6200 PC.

2.6. Stability

Stability quality control plasma samples (3.00, 30.0 and 150 ng/mL) were subjected to short-term (6 h) room temperature, three freeze/thaw (−20 to 25 °C) cycles and long-term stability 148 days tests. For the 56 h-autosampler stability (5 °C) the quality control samples used were 6.00, 30.0 and 150 ng/ml. Subsequently, the Lisinopril concentrations were measured compared to freshly prepared samples.

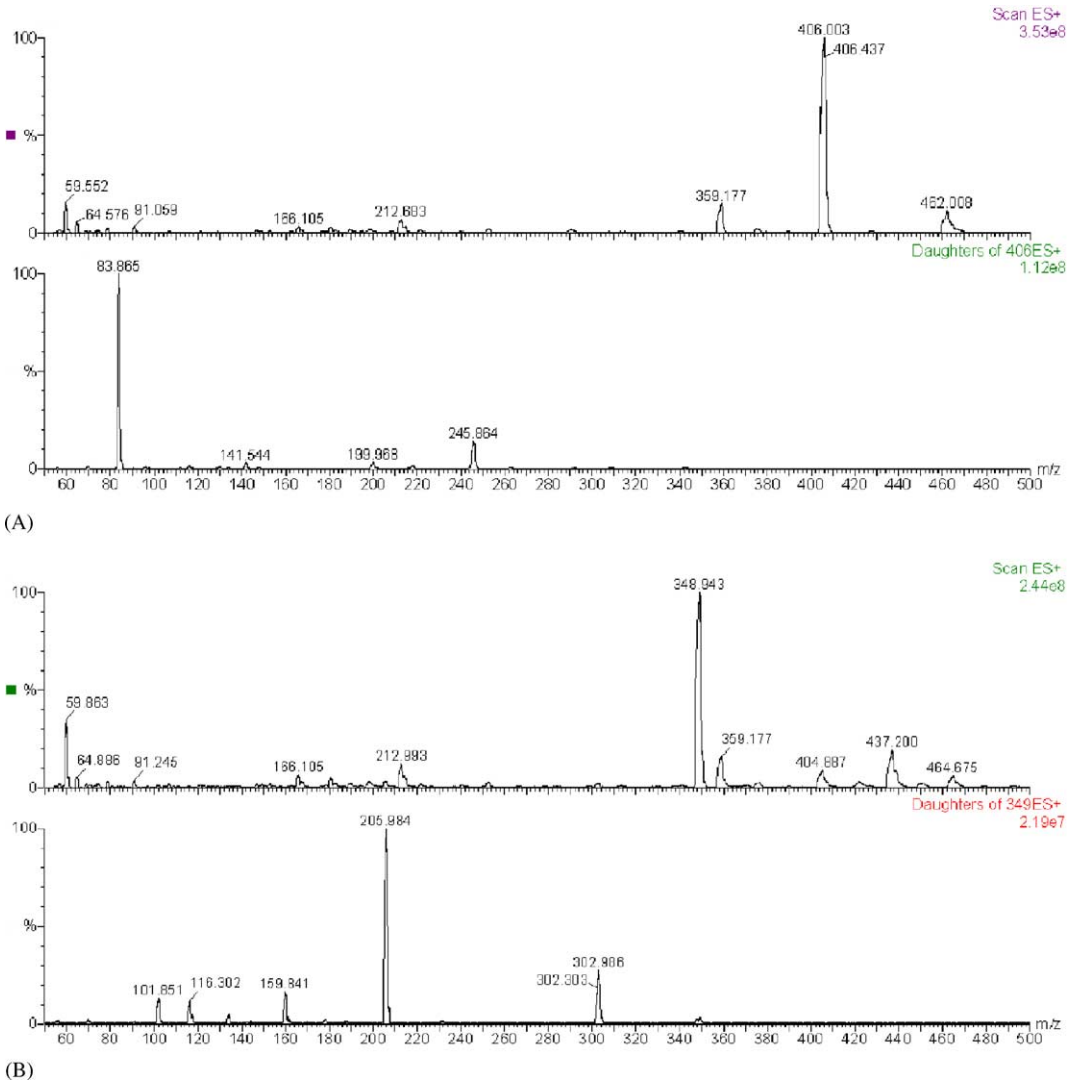

Fig. 1. Full scan mass spectra in upper trace and product ion spectra in lower trace of (panel A) Lisinopril and (panel B) Enalaprilat.

2.7. Recovery

The recovery was evaluated by calculating the mean of the response of each concentration and dividing the extracted sample mean by the unextracted (spiked blank plasma extract) sample mean of the corresponding concentration. Comparison with the unextracted samples, spiked on plasma residues, was done in order to eliminate matrix effects, giving a true recovery. The matrix effect experiments were carried out using the ratio between spiked mobile phase solutions and unextracted samples, spiked on plasma residues.

2.8. Ion supression

Suppression of the MS signal ("ion suppression") can be caused by contaminants (e.g. salts) in the LC eluant entering the MS. Thus, a non-specific extraction procedure may produce ion suppression that could interfere with the analysis of the samples. The effects of the sample preparation method (for the matrix that is being analyzed) on the variability of the electrospray ionization (ESI) response could be determined.

To assess the effect of ion suppression on the MS/MS signal of the analyte, Lisinopril, and the internal standard, Enalaprilat, the extraction procedure described in item 2.3 was evaluated. The experimental set-up consisted of an infusion pump connected to the system by a "zero volume tee" before the split and the HPLC system pumping the mobile phase, which was the same as that used in the routine analysis of Lisinopril, i.e. acetonitrile/water (60/40; v/v) + 20 mM acetic acid + 4.3 mM of triethylamine at 0.5 mL/min. The infusion pump was set to transfer (50 μL/min) of a mixture of analyte and internal standard in mobile phase (both 50 μg/mL). A sample of human pooled blank plasma was extracted by the extraction procedure. The reconstituted extract was injected into the HPLC system while the standard mixture was being infused. In this system any ion

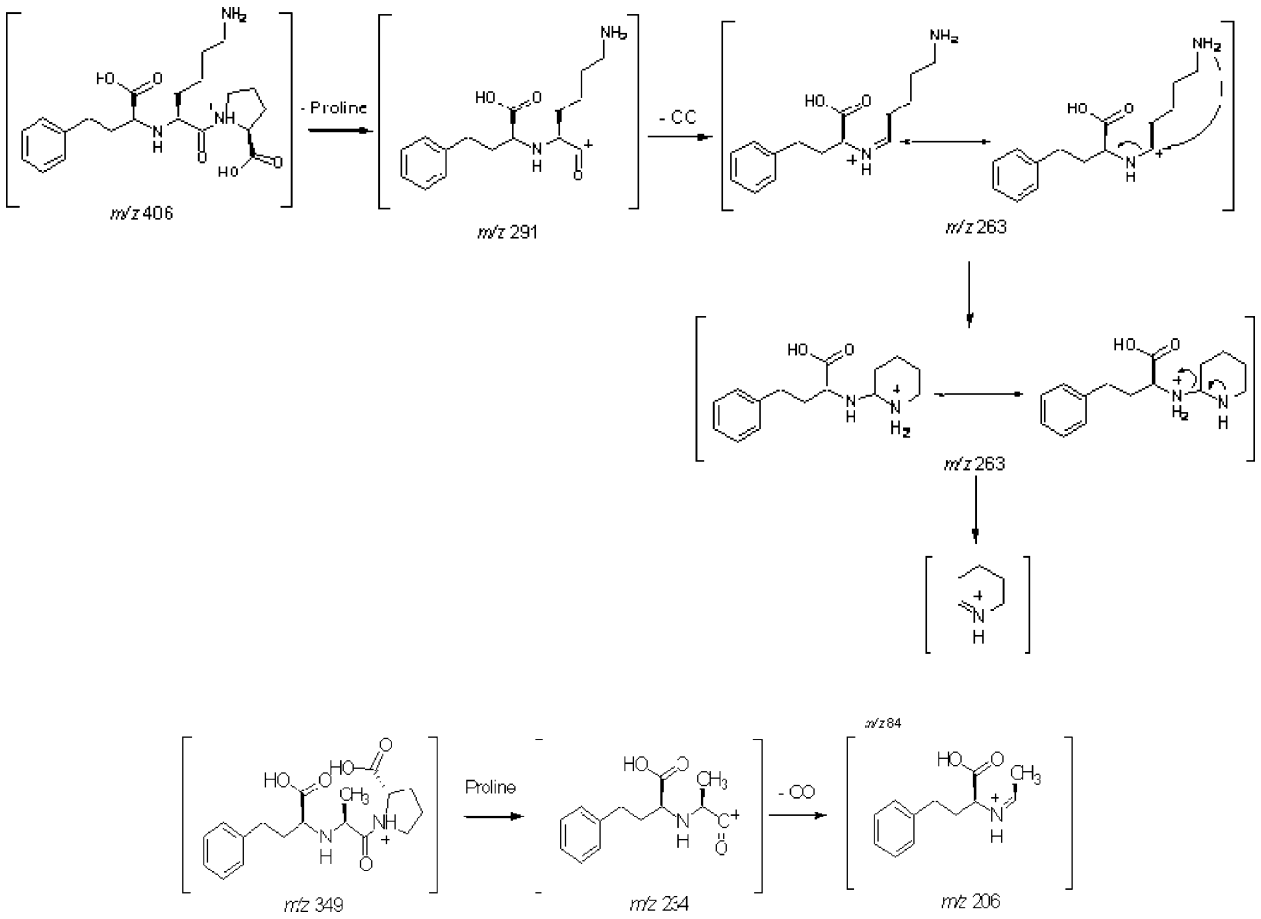

Fig. 2. Proposed fragmentation pathways for the Lisinopril (*m/z*, 406) and Enalaprilat (*m/z*, 349).

suppression would be observed as a depression of the MS signal.

2.9. Bioequivalence study

The method was applied to evaluate the bioequivalence of two tablet formulations of Lisinopril 20 mg in healthy volunteers: Lisinopril (test formulation from Medley S/A Indústria Farmacêutica, Brazil; lot no. LIC 06/01-1, expiry date June 2003) and Zestril® (standard reference formulation from Astra Zeneca; lot no. A03534, expiry date October 2004).

Twenty-six healthy volunteers of both sexes were selected for the study. The study was a single dose, two-way randomized crossover design with a 2-week washout period between the doses. Blood samples were collected before and 1, 2, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 10, 12, 24 and 48 h post-dosing.

The bioequivalence between the two formulations was assessed according to US-FDA methodology [13].

3. Results

As show in Fig. 3A, no endogenous peak was observed in the mass chromatogram of blank plasma. The chromatogram

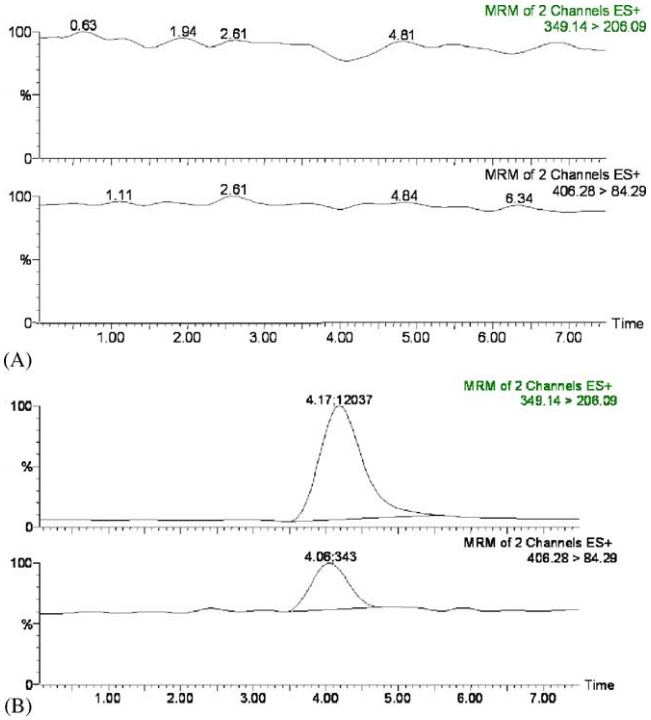

Fig. 3. MRM chromatograms of blank (panel A) and LOQ 2.0 ng/mL (panel B) samples for Lisinopril and the internal standard.

Table 1
Data for quantified concentration (ng/mL) of individual QC samples for intra-batch and inter-batch validation

	Nominal concentration (ng/mL)		
	6	30	150
Intra-batch ($n = 8$)			
Accuracy (%)	99.7	95.0	109.1
Precision (%)	6.2	8.2	7.2
Inter-batch ($n = 3$)			
Accuracy (%)	94.3	94.0	98.6
Precision (%)	5.0	4.9	10.0

for the standard LOQ sample is shown in Fig. 3B, in which the retention times for Lisinopril and I.S. were 4.06 and 4.17 min, respectively.

Linearity, precision and accuracy were determined to assess the performance of the method. A linear least-squares regression with a weighting index of $1/x$ was carried out on the peak area ratios of Lisinopril and I.S. versus Lisinopril concentrations of the 7 human plasma standards (in duplicate) to generate a calibration curve. The calibration curves showed good linearity within the range 2.00–200 ng/mL. Table 1 shows the between-run calibration quality report for the Quality Control samples (QC).

The recoveries observed (value ± CV%, $n = 5$) were 79.2 ± 5.9%, 80.3 ± 3.4% and 82.5 ± 3.9% (3.0, 30.0 and 150.0 ng/mL, respectively) for Lisinopril, and 77.7 ± 7.2% for I.S. (250 ng/mL).

In the Fig. 5, it is shown the ion suppression experiment. In the case of Lisinopril and its internal standard, Enalaprilat, there was no significant ion suppression in the region where the analyte and internal standard were eluted as shown in Fig. 4.

The lower limit of quantification (LOQ), defined as the lowest concentration at which both precision and accuracy were less than or equal to 20%, was 2.00 ng/mL.

Stability analysis was carried out with plasma quality control. All samples showed no significant degradation under the conditions previously described in Section 2, item 2.6.

The geometric mean and respective 90% confidence interval (CI) of Lisinopril/Zestril® percent ratios were

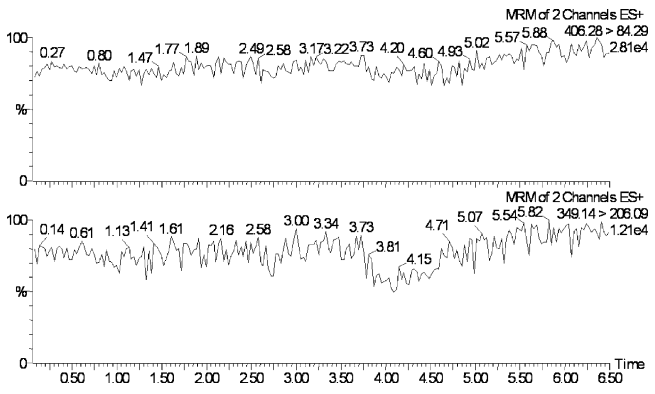

Fig. 4. MRM chromatogram of ion supression test.

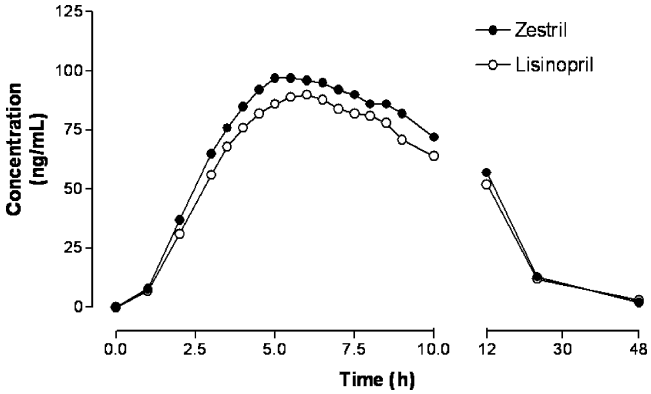

Fig. 5. Mean plasma concentrations vs. time curve for two Lisinopril tablet formulations ($n = 26$).

95.5% (85.0–107.4%) for AUC_{last}, 94.6% (85.0–105.4%) for $AUC_{0\text{-inf}}$ and 92.6% (81.7–105.1%) for C_{max}. T_{max} was statistically analysed and the point estimate for individual differences (Lisinopril/Zestril®) was 0.06 h (90% confidence interval of −0.4 to 0.5 h).

4. Discussion

Measurement of ACE activity as an indicator of serum concentration of ACE inhibitor has a major drawback in that the degree of enzyme inhibition does not parallel the drug levels [14]. Radioimmunoassay is a very sensitive technique (0.2–0.4 ng/mL), however, the antisera and/or tracers are generally not commercially available, making difficult its use for researchers. High-performance liquid chromatography methods were also described for the determination of Lisinopril, but only in bulk drug analysis and for analysis of solid dosages formulations [15,16], where the drug concentrations are rather high. An assay using high-performance liquid chromatography was described and used for the measurement of Lisinopril in urine [17]. However, the lower limit of quantification (LOQ) was reported to be 0.5 μg/mL, a concentration far beyond the plasma concentration found after therapeutic dosage (<100 ng/mL). Therefore, up to now, HPLC does not meet the requirements for pharmacokinetic plasma level determinations.

This is the first report on using LC–MS/MS to quantify Lisinopril in human plasma samples. In the literature, an LC–MS method for lisinopril in serum has been reported [12]. The main advantages of this method are the inherent higher selectivity (due to the MS/MS sytem) and sensitivity observed (2.00 ng/mL as compared to the 6.0 ng/mL); lower amount of sample (0.5 mL versus 1.0 mL) that allows to collect less sample per volunteer and the shorter total run-time which allows a larger sample throughput.

Other techniques using MS detection were also reported, such as GC–MS [11]. However, this analytical method involves also SPE extraction followed by a derivatisation process which is time consuming and more complicated than the extraction protocol herein discussed (Fig. 5).

After the oral administration of the Lisinopril tablets to the volunteers, the observed Lisinopril peak plasma concentration (C_{max}) values and the time values taken to be achieved (t_{max}) were similar to those reported in the literature [2,5,18] and equivalent between the formulations (Fig. 6). In addition, the calculated 90% Cl for mean C_{max}, AUC_{last} and AUC_{inf} Lisinopril/Zestril® individual ratios were within the 80–125% interval defined by the US Food and Drug Administration [13].

5. Conclusion

A fast and sensitive LC–MS–MS method for the quantification of Lisinopril in human plasma was developed and validated. The method satisfied the requirements of high sensitivity, specificity and rapid sample throughput required for pharmacokinetic studies.

References

[1] E.K. Jackson, Renin and Angiotensin, in: Goodman, Gilman (Eds.), The Pharmacological Basis of Therapeutics, 10 ed., The McGraw-Hill Companies, 2002, pp. 809–841.
[2] PDR (2003) 692.
[3] D. Sáenz-Campos, M.C. Bayés, E. Masana, S. Martín, M. Barbanoj, F. Jané, Meth. Find Exp. Clin. Pharmacol. 18 (8) (1996) 533.
[4] P.J. Worland, B. Jarrot, J. Pharm. Sci. 75 (1986) 512.
[5] S.G. Lancaster, P.A. Todd, Drugs 35 (1988) 646.
[6] E. Bellissant, N.P. Chau, J.F. Giudicelli, J. Cardiovasc. Pharmacol. 28 (1996) 470.
[7] D. Johnston, D. Duffin, Am. J. Cardiol. 70 (1992) 151C.
[8] M.J. Vandenburg, F. Morris, C. Marks, J.G. Kelly, I.M. Dews, J.D. Stephens, Xenobiotica 18 (10) (1988) 1179.
[9] A.S. Yuan, J.D. Gilbert, J. Pharm. Biomed. Anal. 14 (1996) 773.
[10] H.J. Leis, G. Fauler, G. Raspoting, W. Windischholer, Rapid Commun. Mass Spectrom. 12 (1998) 1591.
[11] H.J. Leis, G. Raspoting, W. Windischholer, Rapid Commun. Mass Spectrom. 13 (1999) 650.
[12] A. Tsakalof, K. Bairachtari, M. Georgarakis, J. Chromatogr. B 783 (2003) 425.
[13] Food and Drug Administration, Fed. Reg. 63 (1998) 64222.
[14] W. Ribeiro, M.N. Muscará, A.R. Martins, H. Moreno, G.B. Mendes, G. Nucci, Eur. J. Clin. Pharmacol. 50 (1996) 399.
[15] A. El-Gindy, A. Ashour, L.A. Abdel-Fattah, M.M. Shabana, J. Pharm. Biomed. Anal. 25 (2001) 913.
[16] D. Bonazzi, R. Gotti, V. Andrisano, V. Cavrini, J. Pharm. Biomed. Anal. 16 (1997) 431.
[17] Y.C. Wong, B.G. Charles, J. Chromatogr. B 673 (1995) 306.
[18] S.C. Sweetman (Ed.), Martindale—The Complete Drug Reference, 33rd ed., Pharmaceutical Press, 2002, p. 921.