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Development of a liquid chromatography–mass spectrometry method for monitoring the angiotensin-converting enzyme inhibitor lisinopril in serum

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Abstract

In this study, a sensitive, specific, precise and accurate method for lisonopril quantitative determination in human serum was developed and validated. The method comprises lisinopril isolation from serum by means of solid-phase extraction followed by its quantification by liquid chromatography–mass spectrometry. Chromatographic separation was performed at 55 °C on Kromasil C_{18} 5 μm 250×3.2 mm HPLC column with mobile phase composed of 50 m*M* ammonium formate buffer (pH 3)–acetonirile–methanol (72:7:21, v/v/v). A Finnigan AQA benchtop mass spectrometer with a pneumatically assisted electrospray (ES) interface and a single quadrupole mass filter was used to detect and quantify lisinopril in column effluent. Ion signals were acquired by selected ion monitoring of the protonated lisinopril ion $m/z=406.5$ (M+1). The detector response was linear with $r>0.9993$ in the investigated concentration range 6–150 ng/ml. The mean recovery of lisinopril from serum samples was 88%. The limit of quantitation for lisinopril was 6 ng/ml with a signal-to-noise ratio at this concentration level $S/N=34.75\pm3.9$ ($n=4$).

Keywords: Lisinopril

1. Introduction

Lisinopril is an effective drug used for the treatment of hypertension and heart failure [1–3]. It acts as inhibitor of the enzyme angiotensin convertase (ACE inhibitor). The literature presents few methods for determination of lisinopril in biological fluids. In particular, the levels of lisinopril in plasma/serum can be determined either indirectly, by ACE activity inhibition assays [3], or by radioimmunoassay [4,5]. Radioimmunoassay offers desired sensitivity (LOD 0.2–0.4 ng/ml) but requires radiolabels and antilisinopril antiserum which renders this method not readily available to all researchers. Recently, an assay based on gas chromatography–negative ion chemical ionization mass spectrometry was described [6,7]. According to the authors the applied two-step derivatization allowed conversion of the thermolabile and ionic lisinopril into a derivative suitable for gas chromatography analysis. The method was success-

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fully applied to the measurements of lisinopril in human plasma with the limit of quantification found to be 0.5 ng/ml plasma.

High-performance liquid chromatographic methods were also described for the detection of lisinopril, but only for analysis of solid dosage formulations and for the measurements of lisinopril in urine [8], where the drug concentrations are rather high. The low electromagnetic absorbances of lisinopril [3,9] together with low peak plasma concentration found after therapeutic dosage (<100 ng/ml [10]) render the conventional HPLC method with UV detection unsuitable for lisinopril monitoring in human plasma/serum. The use of liquid chromatography in combination with mass spectrometry offers substantially improved capabilities for drug discovery and monitoring. Here, we now report a sensitive method for lisinopril quantification in serum. The method comprises lisinopril isolation from serum by means of solid-phase extraction followed by its quantification by liquid chromatography–mass spectrometry.

2. Experimental

2.1. Reagents and chemicals

Lisinopril dihydrate and hyoscyamine were supplied by Pharmathen (Athens, Greece). Ammonium formate (Sigma–Aldrich, Steinheim, Germany), formic acid, hydrochloric acid, chloroform and methanol (Merck, Darmstadt, Germany) were analytical grade reagents. For liquid chromatography HPLC grade acetonitrile (Riedel-deHaen, Sigma–Aldrich, Germany) and methanol (J.T. Baker, Deventer, The Netherlands) were used. Type I reagent grade water with resistivity up to 18.3 MΩ/cm and organic content <5 ppb was produced by passing deionized water through Barnstead EASYpure RF water purification system and was subsequently used for buffer and standards preparations. All HPLC solvents were filtered through a 0.2-μm filter prior to use.

Lyphochek drug-free serum (Bio-Rad, Munich, Germany) was used for the preparation of matrix calibrators, quality controls and method validation standards.

2.2. Stock solutions

Two lisinopril 1 mg/ml primary stock solutions A and B were prepared by dissolution of lisinopril in 50 m*M* ammonium formate buffer (pH 3) and stored at +4 °C. Primary stock solution A was used for the preparation of matrix calibrators and quality controls (QC), while primary stock solution B was used for the preparation method validation standards (MV). Stock solution A was diluted daily with water to prepare 10 μg/ml and 1 μg/ml solutions, which were subsequently used for the preparation of serum matrix calibrators. Quality controls and method validation standards were prepared in a blank matrix at the beginning of the experiment, aliquoted and stored at −4 °C. Hyoscyamine was used for the monitoring of mass spectrometer sensitivity. Hyoscyamine 1 mg/ml primary stock solution was prepared by dissolution of hyoscyamine in acetonitrile. The hyoscyamine working solution of 0.2 μg/ml was prepared daily by the corresponding dilution of primary stock solution with acetonitrile.

2.3. Sample preparation

The method applied for lisinopril extraction from serum was similar to a SPE method described by Leis and co-workers [6,7].The procedure was as follows: 3-ml Extract-Clean C_{18} SPE cartridges with 200 mg sorbent bed (Alltech Associates, USA) were employed for lisinopril isolation from serum samples. The cartridges were conditioned by passing through them successively 5 ml of methanol, 3 ml of water and 3 ml of 0.1 *M* HCl.

A 1-ml volume of lisinopril serum standard or sample was diluted with 2 ml of 0.1 *M* HCl and vortexed. The diluted samples were then applied on previously conditioned SPE cartridges and passed through under gravity. The cartridges were subsequently washed with 3 ml of 0.1 *M* HCl and 3 ml of chloroform. After washing, a low vacuum was applied to remove the traces of chloroform before elution of lisinopril with 2 ml of methanol. Methanol was evaporated at 40 °C and under nitrogen flow. Samples were reconstituted in 1 ml of 50 m*M* ammonium formate buffer (pH 3) and 25 μl of hyoscyamine 0.2 μg/ml solution were added under

weight control. Reconstituted samples were filtered through a 0.2-μm nylon syringe filter (Alltech Associates) into the autosampler vials and submitted for LC–MS analysis.

2.4. LC–MS instrumentation and analysis

Liquid chromatography was carried out using Thermoquest (Manchester, UK) HPLC system consisting of a P4000 quaternary HPLC pump, SCM 3000 vacuum degasser, AS3000 auto sampler with column oven and Reodyne 7725i injector with 100-μl sample loop. A mobile phase of 50 m*M* ammonium formate buffer (pH 3)–acetonirile–methanol (72:7:21, v/v) was pumped isocratically at 0.6 ml/min through Kromasil C_{18} 5μm 250×3.2 mm HPLC column (Alltech Associates) thermostatted at 55 °C. A 100-μl volume of sample was injected in the mobile phase flow.

A Finnigan AQA benchtop mass spectrometer (Thermoquest), comprising a pneumatically assisted ES interface and a single quadrupole mass filter, was used to detect and quantify lisinopril in column effluent. The probe was maintained at 450 °C and 3.5 kV voltage in positive ionization mode. Ion signals were acquired in time scheduled selected ion monitoring (SIM) mode with ion $m/z=406.5$ (lisinopril monocation) registered in the time window 0–5 min and ion $m/z=290.2$ (hyoscyamine monocation) in the time window 5–6 min. The cone voltage was maintained at 28 eV.

The mass spectrometer was daily tuned by the use of freshly prepared lisinopril water standard 10 μg/ml.

The XCALIBER data system (Thermoquest) was employed for data acquisition and processing.

3. Results

3.1. Method development

As an amphoteric compound, lisinopril can produce both negative and positive ions at different pH values and correspondingly the signal can be acquired in positive or negative ES mode. Taking into consideration the better stability of bonded phase packing at moderately acid pH the positive ESI mode was preferred for lisinopril detection. Lisinopril (M_w 405.5 amu) was detected as monocation $[M+H]^+$ with $m/z=406.5$. The fractional concentration of monocation was calculated as a function of pH and on the basis of these calculations pH 3 was selected as optimum, ensuring the maximum concentration of lisinopril monocation in solution. Volatile ammonium formate buffer with concentration 50 m*M* was used.

The buffer concentration was selected as an optimum balance between the chromatographic separation efficiency and MS sensitivity (Fig. 1). It is known that too high a buffer concentration results in the suppression of analyte signal, whilst too low a concentration results in poor peak shape and efficiency for most basic analytes. Our preliminary investigations demonstrated that the best lisinopril peak shape (Fig. 3) could be achieved with 50 m*M* ammonium formate buffer (pH 3) without substantial loss in the detector sensitivity (Fig. 1).

Chromatographic analysis of lisinopril has some peculiarities due to the fact that at room temperature it exists as two *cis–trans* isomers. The *cis–trans* isomerisation of the lisinopril is the consequence of the partial double bond character of the proline peptide bond, which restricts the free rotation of pyrrolidine ring (Fig. 2) [11,12]. *Cis*- and *trans*-isomers differ in their hydrophobicity and so chemi-

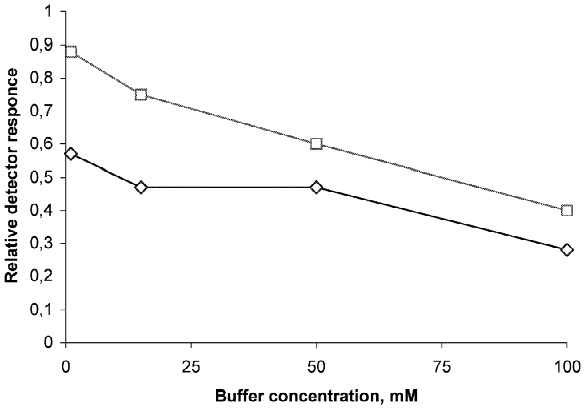

Fig. 1. Variations in MS detector response for lisinopril as a function of ammonium formate buffer concentrations and organic modifier content; □, buffer–acetonitrile (80:20); ◇, buffer–acetonitrile (90:10).

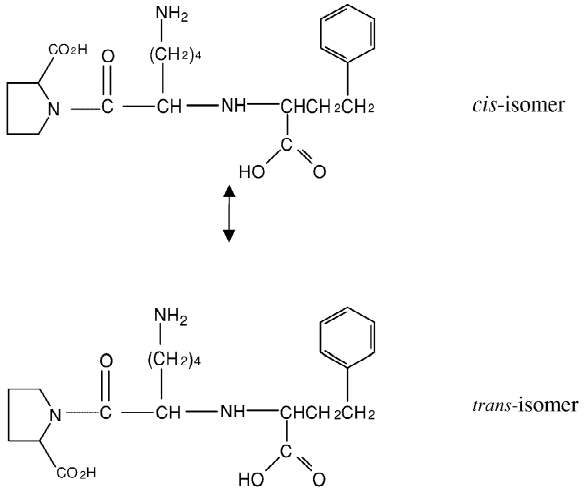

Fig. 2. Lisinopril rotation isomers.

cally pure lisinopril can give rise to either two separate chromatographic peaks or one good or bad shaped peak depending on the column chemistry and mobile phase composition (Fig. 3).

Fig. 3 shows that the mobile phase composition can be adjusted so that the two isomers co-elute and under these conditions the method can be applied for the analysis of bulk drug and solid dosage formulations. Unfortunately, it could not provide desirable lisinopril selectivity in case of plasma/serum samples due to matrix interferences. In this case column temperature as another parameter of chromatographic separation can be involved. The rate of isomerization, which influences the peak shape, increases with temperature so that single sharp lisinopril peak can be detected at elevated (50–60 °C) column temperature [11]. In our case optimum separation of lisinopril from serum endogenous peaks was achieved at 55 °C utilizing a mobile phase of 50 m*M* ammonium formate buffer (pH 3)–acetonitrile–methanol (72:7:21, v/v) (Fig. 4).

The stability of bonded-phase packings tends to deteriorate at elevated temperatures [14]. However, our investigation demonstrated adequate stability of selected Kromasil C_{18} column at 55 °C so that it is efficiency remained satisfactory after about 400 injections. The column lifetime was maintained by flushing it overnight with acetonitrile–water (60:40) at 0.3 ml/min and ambient temperature.

Lisinopril was extracted from serum sample by the SPE extraction method similar to that introduced by Leis and co-workers [6,7]. A better recovery (88%) was achieved in this study in comparison to the results of Leis et al. (72% [6]). This may be a consequence of the better performance of the SPE cartridge used here. We experienced substantial variations in lisinopril recovery for SPE cartridges of the same chemistry but from the different suppliers.

The lisinopril quantification was conducted by the method of external standard. Hyoscyamine was added for the monitoring of the MS sensitivity so as to avoid false zero results in case of occasional loss of MS sensitivity, e.g. as the result of ion source flooding.

3.2. Method validation

The method was validated on the basis of five analytical runs—one run a day. Each analytical run comprised three sets of analytical sub runs, which included blank matrix and matrix calibrators, blank solvent to detect carry over effect, quality control (QC) samples and method validation (MV) samples, recovery spikes. The method performance parameters assessed during the method validation are discussed below.

3.2.1. Specificity

Specificity was determined by analyzing drug free serum from different lot numbers. No endogenous peaks that interfered with the quantification of lisinopril were detected. A representative chromatogram of extracted blank serum is shown in Fig. 4. Carryover effects in injections of blank serum after a high calibrator were not observed.

3.2.2. Calibration curve linearity

The calibration curves were generated to investigate the linear relationship between the peak area of lisinopril and its concentration in the sample. Lisinopril was added to drug free serum to yield final concentrations of 6, 18, 60, 120 and 150 ng/ml. The matrix calibrators were extracted as described above (Section 2.2) and analyzed in triplicate—at the

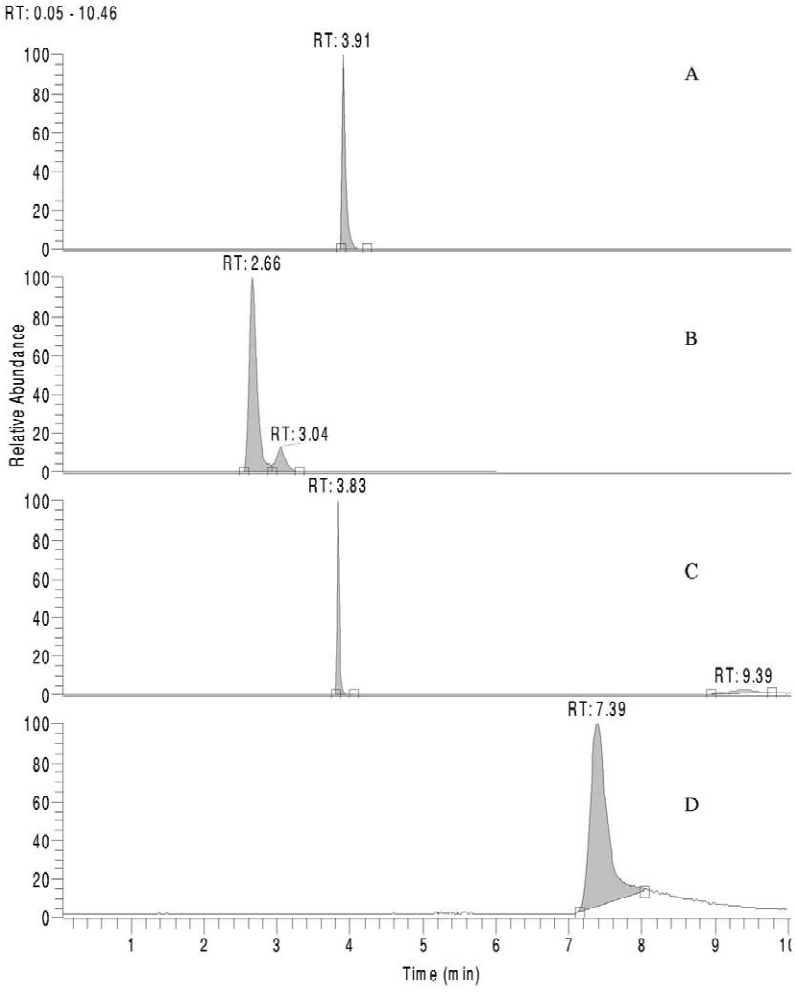

Fig. 3. Chromatograms of lisinopril water standard (1μg/ml) at room temperature and different compositions of mobile phase: (A) 50 m*M* ammonium formate buffer (pH 3)–acetonitrile (80:20); (B) 15 m*M* ammonium formate buffer (pH 3)–acetonitrile (80:20); (C) 50 m*M* ammonium formate buffer (pH 3)–acetonitrile (90:10); (D) 15 m*M* ammonium formate buffer (pH 3)–acetonitrile (90:10). Column Inertsil 250×4.6, flow-rate 1 ml/min, SIM of $[M+H]^+$ with m/z 406.5.

beginning, in the middle and at the end of each analytical run/day. After analysis a standard calibration curve for lisinopril was constructed by quadratic regression analysis. The regression analysis results are summarized in Table 1. Excellent linearity of calibration curves was observed in the investigated range of lisinopril concentrations from 6 to 150 ng/ml. Correlation coefficients for all runs were substantially greater than acceptance criteria ($R>$ 0.95 [13]) with the mean value 0.9997 and sufficiently reproducible with a C.V. of 0.026%.

3.2.3. Sensitivity

The LOQ for lisinopril was 6 ng/ml with a signal-to-noise ratio of 34.75±3.9 ($n=4$). The LOD was approximated at 0.5 ng/ml based on the ratio $S/N=$ 3.

3.2.4. Precision and accuracy

3.2.4.1. Intra-day precision and accuracy. The intra-day precision for lisinopril was evaluated by analysis

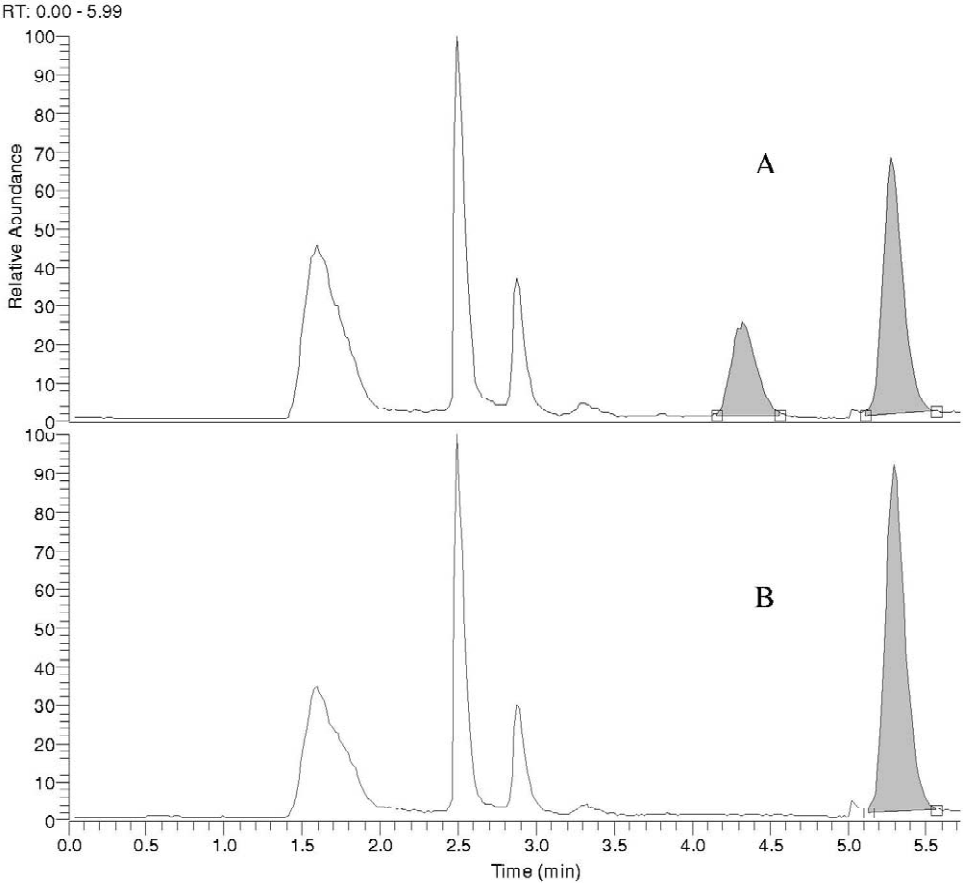

Fig. 4. Chromatograms of lisinopril fortified serum 30 ng/ml (a) and blank serum (b). Peaks: lisinopril $t_R = 4.33$ min, hyoscyamine $t_R = 5.29$ min.

Table 1
Regression analysis results

Curve	Least squares regression: $y = ax + b$			
	R^2	R	a	b
1	0.9991	0.9995	0.0127	−0.0043
2	1	1	0.0108	−0.3321
3	0.9987	0.9993	0.011	−0.7079
4	0.9998	0.9999	0.0129	−0.3708
5	0.9997	0.9998	0.0154	0.8242
Mean	0.99946	0.9997	0.01256	0.02925
SD	0.00048	0.00026	0.00166	N/A
C.V. (%)	0.04844	0.02608	13.1945	N/A

of method validation standards ($n = 6$) bracketed between three independent calibration curves.

The MV samples were prepared at four levels: LOQ (6 ng/ml), 3LOQ (18 ng/ml), at the medium level (60 ng/ml) and at 80% of the highest calibrator (120 ng/ml). The results are summarized in Table 2 demonstrate that the assay fulfils the demands for analytical method for human studies [13]: accuracy is substantially lower $\leq \pm 20\%$ at LOQ level (6 ng/ml) and $\leq \pm 15\%$ for all the other levels. Assay demonstrated good precision with C.V.% ranged from 2.59 to 7.91% ($\leq 15\%$).

3.2.4.2. Inter-day precision and accuracy. As can be judged from Table 2 the inter-day precision of the

Table 2
Inter- and intra-assay precision and accuracy of lisinopril quantification in serum

Target concentration (ng/ml)	Found concentration			Accuracy (%)	Bias (%)
	Mean	SD	C.V.%		
Intra-day ($n=6$)					
6	5.95	0.28	4.67	99.2	−0.8
18	16.55	0.43	2.59	91.94	−8.06
60	57.41	4.74	8.26	95.68	−4.32
120	121.11	9.58	7.91	100.92	0.92
Inter-day ($n=5$)					
6	5.87	0.55	9.39	97.84	−2.16
18	17.38	1.1	6.32	96.55	−3.45
60	54.67	5.36	9.81	91.12	−8.88
120	111.98	10.82	9.67	93.32	−6.68

assay was satisfactory with C.V. values ranging from 6.32 to 9.81% (≤15%) and 9.39% at LOQ (≤20%).

The biases ranged from −8.88 to −2.16% and were within the required limit—≤±15%.

3.2.5. Recovery

Lisinopril extraction recovery was calculated by comparing the peak areas of extracted serum samples to the corresponding peak areas of unextracted water samples of identical concentration. Recovery was estimated for the standards of 6, 75, 120 ng/ml measured in triplicate for each concentration. Table 3 shows that the recovery ranged from 85.08 to 89.47% with a mean value 87.88%.

3.2.6. Stability

3.2.6.1. Short-term stability. Three aliquots of spiked serum samples at concentrations 18 and 120 ng/ml were thawed at room temperature and kept for 4 h at this temperature. Freshly prepared samples at the same concentrations were extracted simultaneously with the aged one and analyzed. The differences between freshly prepared and aged samples were −1.62 and 8.85% for low and high concentration stability samples correspondingly, which are within the method acceptance criteria ≤±10% [13] and demonstrate the bench top stability of lisinopril in human serum.

3.2.6.2. Long-term stability. To evaluate the stability of lisinopril in human serum under samples storage conditions (freezer at −20 °C) the three aliquots of low (18 ng/ml) and high concentration (120 ng/ml) were prepared and stored under above described conditions. After 2 weeks the samples were thawed, extracted simultaneously with the freshly prepared stability samples of the same concentrations and analyzed. The differences between the concentrations detected in fresh and aged standard solutions were 1.30 and 2.98% for low and high concentration stability samples correspondingly, which demon-

Table 3
Recovery of lisinopril from human serum

	Peak area					
	6 ng/ml		75 ng/ml		120 ng/ml	
	Nonextracted	Extracted	Nonextracted	Extracted	Nonextracted	Extracted
Mean ($n=3$)	649.22	580.89	7255.11	6462.00	11 059.44	9409.89
SD	30.98	64.38	645.41	724.20	1330.91	965.67
RSD (%)	4.77	11.08	8.90	11.21	12.03	10.26
Recovery	89.47		89.07		85.08	
Mean recovery	87.88					

strates that lisinopril was stable under the described storage conditions.

4. Conclusions

The introduced LC–MS method for lisinopril quantification in human serum is accurate, precise and selective. The method meets the acceptance criteria for analytical methods used for human studies [13] and can be applied for pharmacokinetics and bioavailability studies. To the best of our knowledge this is the first LC–MS assay for lisinopril quantification in serum. The method was successfully employed in a bioequivalence study of lisinopril tablet formulations and a corresponding publication is now in press [15].

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