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A selective and rapid method for the quantification of captopril in human plasma using liquid chromatography/selected reaction monitoring mass spectrometry

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Abstract

A specific hyphenated high performance liquid chromatography–mass spectrometric (LC–MS/MS) assay was developed for the determination of captopril in plasma. The drug was extracted from plasma using liquid–liquid extraction with a mixture of diethylether:dichloromethane. After the addition of the internal standard, samples were applied to a prepacked C_8 Waters Symmetry column. The ion trap MS/MS detector was equipped with electrospray ionization (ESI) source operating in the positive ion mode. Drug determination was accomplished monitoring captopril at molecular ion *m*/*z* 218 and MS/MS (daughter) at *m*/*z* 171.6. The method was applied to captopril determination in human plasma after the administration of captopril 50 mg tablets to healthy volunteers who have participated in a pharmacokinetic study.

The method was proved to be specific and precise by testing six different plasma batches. Linearity was established for the range of concentrations 25–3000 ng/ml with a regression factor of 0.9995. Intra-day accuracy ranged from 90.16 to 96.18%, while the intra-day precision ranged from 2.60 to 9.66% at the concentrations of 75, 1440 and 2500 ng/ml. Inter-day precision of the method ranged from 5.04 to 10.10%. This validated method of analysis was successfully applied to human plasma analyses after the administration of a single dose of 50 mg captopril tablets to healthy volunteers.

Keywords: Captopril; LC; MS/MS; Human plasma; Method validation

1. Introduction

Captopril, (*S*)-1-(3-mercapto-2-methyl-1-oxo propyl)-L-proline, is a specific competitive inhibitor of angiotensin-converting enzyme. Captopril is indicated for the treatment of hypertension, heart failure, and left ventricular dysfunction after myocardial infarction [1].

After oral administration of therapeutic-doses of captopril, rapid absorption occurs with peak blood levels at about 1 h with average minimal absorption of approximately 75%. Like other thiols, captopril undergoes rapid oxidation to disulphide metabolites both in vitro and in vivo [2]. Intracellularly, disulphide metabolites are reduced to the free thiol and as such they can act as a reservoir for free captopril [2]. Only the free captopril is pharmacologically active; however, the formation of the inactive disulphides is reversible; subsequently they may act as a reservoir of free captopril and contribute to a longer duration of action than predicted by the blood concentrations of free captopril (2).

Numerous methods of analysis have been cited in bibliography for captopril determination in plasma. These methods, radiochemical [3], gas chromatographic [4], GC–mass spectrometric [5–7] and HPLC [8–12], are in somehow burdensome and involve derivatization or/and specialized and expensive equipment. Up to our best knowledge there is no published LC–MS/MS method for captopril determination in human plasma. The aim of the present study was to develop and validate a robust and reproducible reversed-phase

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LC–MS/MS method for the determination of the total captopril in human plasma with a reliable quantification of at least 25 ng/ml. In order to enable the determination of the total captopril, a chemical stabilizer was to be used to revert the converted disulphides dimmer (or conjugates) into captopril as well as to prevent the formation of the disulphides dimmers during the samples preparation and analysis. At the same time, it was expected that this method of analysis would be efficient in analyzing large number of plasma samples supporting pharmacokinetic, bioavailability and bioequivalence studies.

2. Experimental

2.1. Reagents

The following chemicals and reagents were used: acetonitrile (HPLC grade, TEDIA, USA); methanol (HPLC grade, Acros, Belgium); diethylether (Panreac, Spain); dichloromethane and trifluoroacetic acid (Scharlau, Spain). The 1,4-dithio-DL-threitol (DTT) was supplied by Fluka (Switzerland). DTT is a widely used reagent for the purposes of reducing disulphide bonds and also to maintain monothiols in the reduced state. A Milli-Q® (Millipore, France) water purification system was used to obtain the purified water for the HPLC analysis. Six different batches of lithium heparin plasma of healthy volunteers were obtained from Ibn Al Haitham hospital (Jordan).

2.2. Preparation of stock solutions

All stock solutions were prepared daily under the light of a sodium lamp according to the below mentioned procedures.

Primary stock solutions of captopril for the preparation of standards and quality controls were prepared from separate weighing. The primary stock solutions (100.0 μg/ml) were prepared in methanol/water (50:50, v/v) and stored at −20 °C. Working solutions of captopril were prepared daily in methanol/water (50:50, v/v) by appropriate dilution at: 0.25, 0.50, 1.00, 3.00, 6.00, 10.00, 20.00 and 30.00 μg/ml.

The internal standard stock solution was prepared by dissolving 10.0 mg of enalapril in 100 ml 0.78 M trifluoroacetic acid solution to produce a concentration of (100.0 μg/ml). This solution was also stored at −20 °C. Working solutions of internal standard were prepared daily in 0.78 M trifluoroacetic acid solution to produce a final concentration of 100 ng/ml.

2.3. Calibration curves

Calibration curves were prepared by spiking different samples of 400 μl plasma each with 40 μl of one of the abovementioned working solutions to produce the calibration curve points equivalent to 25, 50, 100, 300, 600, 1000, 2000 and 3000 ng/ml of captopril. Forty microliters of 200 mM of 1,4-dithio-DL-threitol solution (excess) were added to each sample then the samples were vortexed and left for 10 min at room temperature. Afterward, each sample was spiked with 200 μl of internal standard working solution (100 ng/ml) to produce a final concentration equivalent to 50.0 ng/ml of the internal standard. Zero plasma samples used in each run were prepared containing 50.0 ng/ml of internal standard only. In each run, a plasma blank sample (no IS) was also analyzed.

2.4. Quality control samples

Quality control (QC) samples were prepared at three different levels, low limit (three times the lower limit of quantitation, LLOQ), middle level and a high level (80% of the upper limit of quantitation limit, ULOQ). QCs were prepared daily by spiking different samples of 400 μl plasma each with 40 μl of the corresponding captopril working standard solution to produce a final concentration equivalent to 75, 1440 and 2500 ng/ml of captopril. Forty microliters of 200 mM of 1,4-dithio-DL-threitol solution were added to each sample then the samples were vortexed and left for 10 min at room temperature. Samples were then spiked with 200 μl of the prepared IS working solution (100 ng/ml) to produce a final concentration equivalent to 50 ng/ml of IS.

2.5. Extraction

QC, calibration curve and blank plasma samples preparation was always done under the light of a sodium lamp. Drug was extracted from plasma samples using liquid–liquid extraction technique. Each human plasma sample (400 μl) was extracted with 6 ml of diethylether:dichloromethane (70:30), vortexed for 60 s and centrifuged for 5 min at 3200 × *g*. The organic layer was separated and evaporated under nitrogen stream at 40 °C. Finally, sample's residue was reconstituted with 50 μl of 0.78 M trifluoroacetic acid solution. Samples were transferred to prelabeled HPLC vials and kept at the autosampler at 4 °C. Only 10 μl were injected into the HPLC column.

2.6. HPLC conditions

Chromatography for separation and determination of the drug was carried out by applying the samples to a prepacked C_8 Waters Symmetry 5 μm, 150 mm × 4.6 mm. (Waters, Milford, MA, USA), using a Waters 2690 Alliance high performance liquid chromatograph (Waters, Milford, MA, USA). The analytical column was protected by a 4 × 2.0 mm i.d., Phenomenex C_{18} guard column (Phenomenex, USA). The combination of the mobile phase, prepared by mixing acetonitrile: water in a ratio of 70:30 (v/v) and 13 mM trifluoroacetic acid solution, and a flow rate of 1.5 ml/min (a splitter was used after the column resulting in a final flow of 0.25 ml/min into the detector), was found to be adequate for the samples analysis. Separations were performed at room temperature.

2.7. LC–MS/MS conditions

Drug monitoring and quantitation were done using a Finnigan LCQ^{DUO} quadrupole ion trap mass spectrometer (Finnigan ThermoQuest, USA) equipped with an ESI source (Finnigan) run by XCALIBUR® 1.2 software.

Operating conditions for the ESI source, used in the positive ionization mode, were optimized by constantly adding captopril in methanol (10 μg/ml) to the HPLC flow by a syringe pump via a T-connector in the infusion mode. The signal was optimized on the total ion current in MS mode, producing a transfer capillary temperature of 230 °C, a spray voltage of 4.5 kV, and a sheath gas flow of 73 units (units refer to arbitrary values set by the LCQ software). At the same time, the selection of ions and the collision voltages were optimized using LCQ software. In the MS/MS experiments, the protonated precursor molecular ions $[MH]^+$ of captopril (*m*/*z* 218) and the IS (*m*/*z* 377) were selected and fragmented by helium gas collision in the ion trap at a relative collision energy of 42%. The mass spectra resulting from these fragmentations were acquired in the SRM mode at *m*/*z* 171.6 for captopril and *m*/*z* 234 for IS. These product ions, *m*/*z* 171.6 for captopril and *m*/*z* 234 for the IS, were extracted for quantification.

Although the detection in MS/MS technique is highly specific and sensitive, nevertheless, endogenous substances can exist in much higher concentration than the analytes of interest and may co-elute with those affecting the ionization of the analytes leading to high imprecision and loss of sensitivity. In order to determine ion suppression matrix effect profiles, analytes were infused into the mobile phase through a T-connection between the column and the interface while injecting the extracted blank plasma samples. The purpose of this post-column infusion with the analytes is to raise the background level so the suppression matrix will appear as negative peaks.

2.8. Data treatment

The linearity of captopril method determination in human plasma was tested for the range of concentrations 25–3000 ng/ml. Calibration curves were prepared by determining the best-fit of peak area ratios (peak area analyte /peak area internal standard) vs. concentration, and fitted to the equation $y = bx + a$ by unweighted least-squares regression.

2.9. Application

The method was applied to analyze the samples of 26 healthy male adult volunteers who have participated in a phramcokinteic study. The study was planned and performed

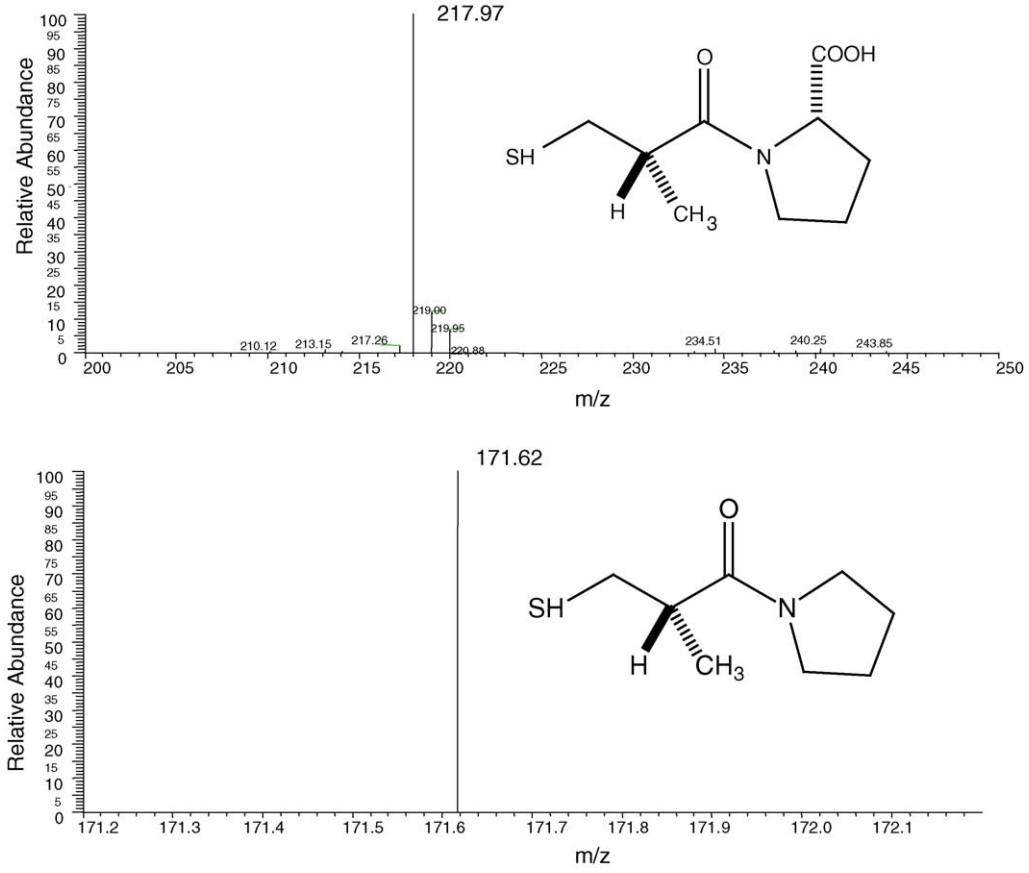

Fig. 1. Positive ion electrospray mass spectrum (top) and full scan product ion mass spectrum (bottom) used in SRM for captopril determination.

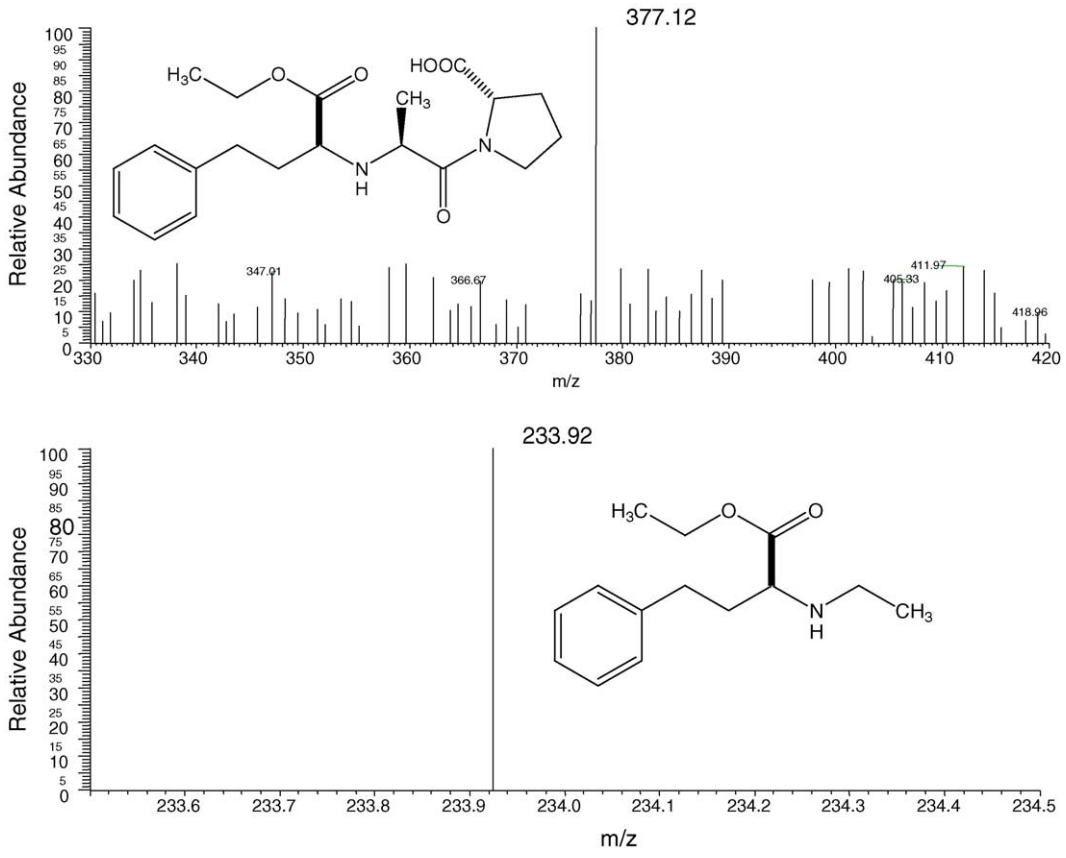

Fig. 2. Positive ion electrospray mass spectrum (top) and product ion mass spectrum (bottom) used in SRM for enalapril (internal standard) determination.

according to the current GCP guidance. Each volunteer was administered a single dose of 50 mg captopril tablet. Seven milliliters of venous blood were withdrawn from each volunteer. Blood samples were transferred immediately to heparinized tubes, centrifuged for 10 min at 3200 × *g* (at 4 °C) and the separated plasma transferred to Eppendorf tubes and stored at −70 °C until the day of analysis. Clinical samples (400 μl plasma) were spiked with 40 μl of 200 mM of 1,4-dithio-DL-threitol solution then the samples were vortexed and left for 10 min at room temperature. Afterward, 200 μl of solution containing 100 ng/ml of internal standard was added to each plasma sample. Forty microliter of methanol:water (50:50) solution were also added to each sample to compensate for the added volume during the preparation of calibration curve and QC samples. The samples were then extracted as described above.

3. Results and discussion

3.1. Separation and specificity

ESI is a "gentle" ionization technique that produces high mass-to-charge $[M + 1]^+$ precursor ions with minimal fragmentation of the analyte. Captopril and IS gave protonated precursor $[M + 1]^+$ in the MS mode. The major ions observed were *m/z* 218 for captopril (Fig. 1) and *m/z* 377 for the IS (Fig. 2). The most intense product ion observed in the MS/MS spectra was *m/z* 171.6 for captopril and *m/z* 234 for the IS. The corresponding spectra of captopril and the IS (enalapril) are shown in Figs. 1 and 2, respectively.

The combination of HPLC (under the isocratic conditions described) with ESI–MS/MS leads to short retention times and yields both high selectivity and sensitivity. The SRM chromatograms obtained from an extracted blank and 25 ng/ml spiked plasma samples are depicted in Fig. 3. As shown, the retention times of captopril and the IS (enalapril) were 1.2 and 1.0 min, respectively. No interferences of the analytes were observed because of the high selectivity of the MS/MS technique. Fig. 3 shows also an LC–MS/MS chromatogram of a blank plasma sample indicating no endogenous peaks at the retention times (t_R) of captopril or internal standard (enalapril). No ion suppression effects were observed.

The SRM chromatograms obtained from an extracted plasma sample of a healthy volunteer who participated in the pharmacokinetic study conducted on 26 volunteers, is depicted in Fig. 4 where captopril was unambiguously identified and was quantified as 70 ng/ml.

3.2. Method validation

In our laboratory, samples analysis is always carried out in a GLP-compliant manner and therefore the LC–MS/MS

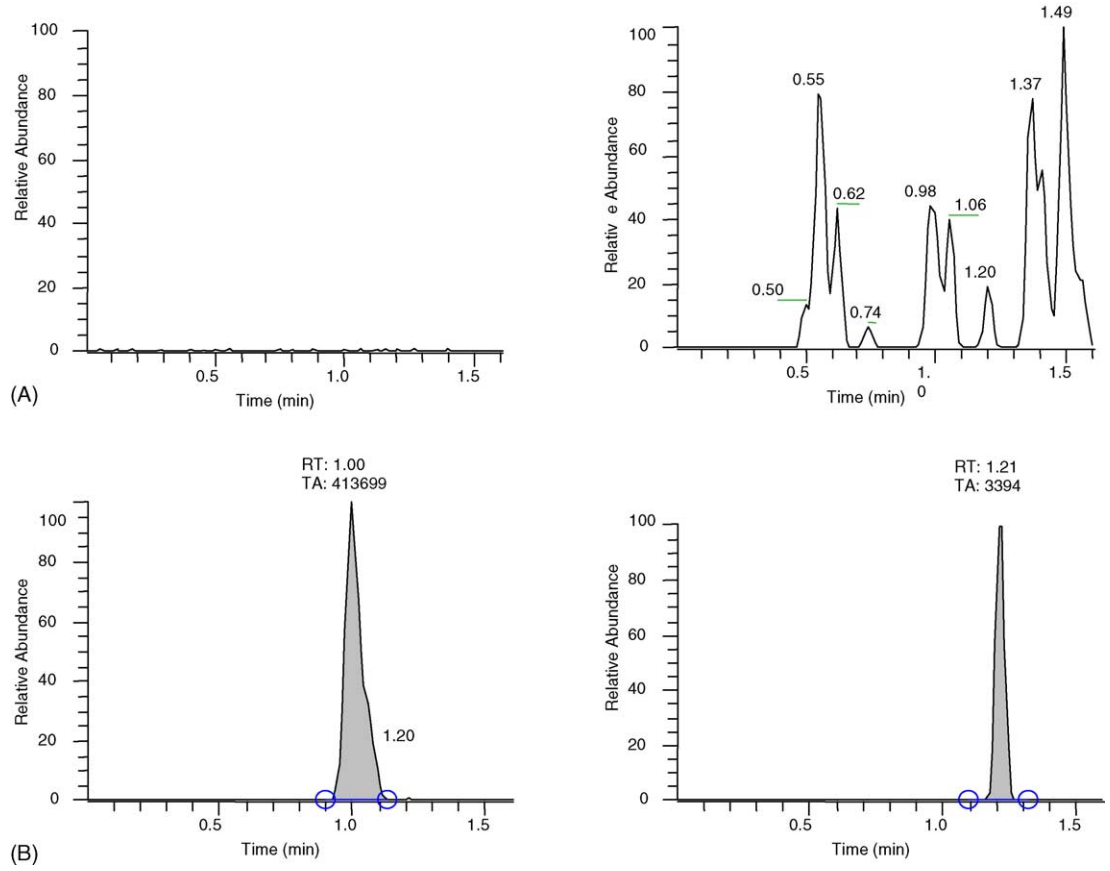

Fig. 3. LC–MS/MS chromatograms showing: (A) a blank human plasma sample and (B) human plasma sample spiked with 50.0 ng/ml internal standard (left) and 25 ng/ml captopril (right).

methods need to be validated according to currently accepted US Food and Drug Administration (FDA) Bioanalytical method validation guidance [13]. The following parameters were considered as described below.

To test the specificity, six batches of human plasma were tested. Each blank sample was tested for interference using the proposed extraction procedure and chromatographic/mass spectrometric conditions and compared with those obtained with an aqueous solution of the analyte at a concentration near to the LLOQ. No significant interference at the retention time of the drug or internal standard were found, as illustrated in the chromatograms presented in Fig. 3.

In order to test possible matrix effects, five different (0.4 ml) blank plasma samples were extracted and processed as abovementioned. Afterward, each one of the dried samples was spiked with captopril at the low QC level (75 ng/ml). The

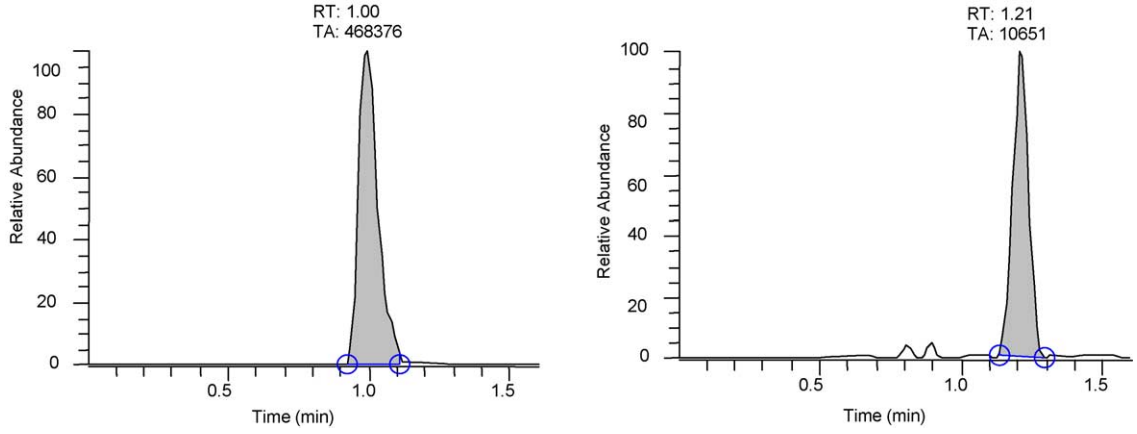

Fig. 4. LC–MS/MS chromatograms showing volunteer's plasma sample after the administration of an oral single dose of 50 mg tablets of captopril. The sample's concentration was 70 ng/ml.

Table 1
Inter-day accuracy, precision and relative error for captopril determination in spiked plasma samples

Day of analysis	Captopril concentration in human plasma		
	Low QC 75 ng/ml	Medium QC 1440 ng/ml	High QC 2500 ng/ml
Day 1	66.64	1249.24	2307.62
	67.65	1362.80	2330.30
	66.62	1592.41	2623.25
	66.53	1295.13	2172.96
	70.65	1425.47	2219.66
Day 2	69.10	1253.91	2424.59
	78.93	1327.73	2336.86
	68.32	1302.56	2177.87
	66.72	1402.20	2427.46
	64.94	1431.16	2410.80
Day 3	83.37	1246.32	2245.13
	76.47	1264.53	2340.95
	71.81	1250.95	2315.50
	84.25	1295.83	2213.96
	86.15	1244.35	2327.24
Mean	72.54	1329.64	2324.94
S.D.	7.32	98.13	117.15
Precision as CV%	10.10	7.38	5.04
Accuracy%	96.72	92.34	93.00
RE%	3.28	7.66	7.00

samples were vortexed and 10 μl were injected into the HPLC column. The matrix effect was calculated by comparing peak areas obtained for these samples with the unextracted pure authentic standard solution peak areas at the low QC level (75 ng/ml). Compared to the absolute recovery at the same low QC level (75 ng/ml), the results revealed 3.87% matrix effect.

Linearity was tested for the range of concentrations 25–3000 ng/ml, employing standard calibration curves of at least 8 points (non-zero standards). In addition, a blank (non-spiked sample) and a zero plasma samples (only spiked with IS) were also analyzed to confirm absence of interferences. These two samples were not used to construct the calibration function. The method exhibited a good linear response for the range of concentrations from 25 to 3000 ng/ml. Correlation coefficients ranged from 0.9988 to 0.9999 (CV 0.05%), while the calculated accuracy at the LLOQ ranged form 94.93 to 117.69 (CV 6.5%) and from 98.79 (CV 1.99%) to 100.23 (CV%) at the ULOQ. The obtained results were within the acceptance criteria of no more than 20% deviation at LLOQ and no more than 15% deviation for standards above this point (LLOQ). The acceptance criterion for correlation coefficient was 0.98 or more, otherwise the calibration curve should be rejected.

The intra-day precision and accuracy of the assay was measured by analyzing five spiked samples of captopril at each QC level (75, 1440 and 2500 ng/ml). Intra-day accuracy of the method for captopril ranged from 90.16 to 96.18%, while the intra-day precision (CV) ranged from 2.60 to 9.66% at the QC levels. The acceptance criteria for precision and accuracy deviation values was ±15% of the actual values.

The inter-day precision and accuracy was determined over three days by analyzing 45 QC samples. Data for the inter-day precision and accuracy are presented in Table 1. These results were within the acceptance criteria for precision and accuracy, which establish that the deviation values should be within ±15% of the actual values.

The absolute recoveries were evaluated for both captopril and IS by comparing peak areas of the extracted samples with

Table 2
Data showing short-term stability of captopril in human plasma at different QC levels

Run	Low QC recovery%	Medium QC recovery%	High QC recovery%
1	102.73	110.90	116.48
2	102.10	113.81	105.37
3	114.78	114.61	105.77
4	110.31	116.19	116.91
5	105.38	114.75	110.64
Mean	107.06	114.05	111.03
S.D.	5.39	1.96	5.57
CV%	5.04	1.72	5.02

Table 3
Data showing freeze and thaw stability of captopril in human plasma at different QC levels

Run	Low QC recovery%	Medium QC recovery%	High QC recovery%
1	108.05	92.66	99.19
2	107.92	96.11	89.41
3	111.26	110.94	91.74
4	119.70	120.14	91.43
5	111.98	125.15	101.44
Mean	111.78	109.00	94.64
S.D.	4.79	14.33	5.32
CV%	4.29	13.15	5.62

Table 4
Data showing long-term stability of captopril in human plasma at different QC levels

Run	Low QC recovery%	Medium QC recovery%	High QC recovery%
1	108.55	113.27	104.85
2	98.05	117.00	113.46
3	105.76	115.86	104.95
4	104.57	113.56	112.65
5	100.18	110.44	113.79
Mean	103.42	114.03	109.94
S.D.	4.26	2.54	4.62
CV%	4.12	2.23	4.20

the unextracted pure authentic standard solutions peak areas at three QC levels (75, 1440 and 2500 ng/ml). Results ranged from 87.47% (CV 7.49%) to 93.32% (CV 6.74%) at the three QC levels. Absolute analytical recovery of internal standard (enalapril) was 86.09% (CV 8.36%).

For sensitivity determination, the lowest standard concentration in the calibration curve was considered as the lower limit of quantitation. Although captopril's limit of detection could be brought down to 1 ng/ml, nevertheless, for the purposes of this study, the lower limit of quantitation for captopril was investigated and proved to be 25 ng/ml, with 99.35% accuracy and 8.48% precision. The LLOQ met the following criteria: LLOQ response was more than five times the response of the blank and the LLOQ response was identifiable, discrete and reproducible with precision of 20% and 80–120% accuracy.

The stability of the analytes in human plasma under different temperature and timing conditions, as well as the stability of the analytes in stock solution, was evaluated as follows:

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for a period of time exceeding that expected to be encountered

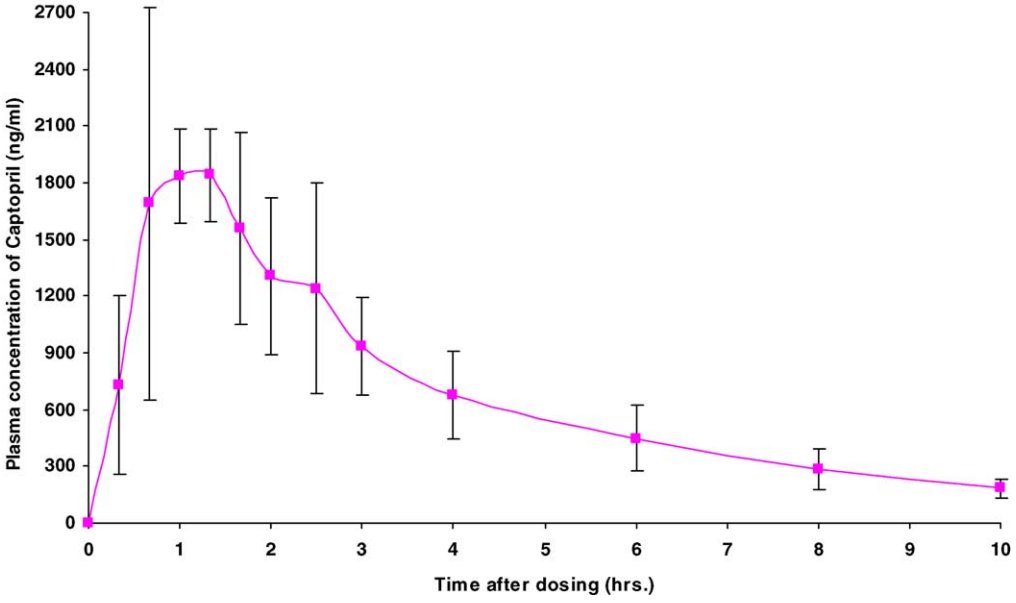

Fig. 5. Mean plasma concentration-time profiles of four healthy volunteers after the administration of an oral single dose of 50 mg tablet of captopril. The error bars represents ± standard deviation.

during the routine sample preparation (around 6 h). Samples were extracted and analyzed as abovementioned. Results are given below in Table 2. Short-term stability indicated reliable stability behavior under the experimental conditions of the regular runs.

The post-preparative stability of QC samples kept in the autosampler for 24 h at 4 °C, was also assessed. The mean recoveries of the low, mid and high QC levels ranged from 102.54% (CV 4.40%) to 110.43 (CV 5.12%). The results indicate that captopril and internal standard can remain at the autosampler temperature (4 °C) for at least 24 h, without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

The data that represent the stability of captopril plasma samples at three QC levels over three cycles of freeze (−70 °C) and thawing (room temperature) are given in Table 3. The performed tests indicate that the analyte is stable in human plasma for three cycles of freeze and thaw, when stored at −70 °C and thawed to room temperature.

Table 4 summarizes also the long-term stability data of captopril in plasma samples stored for a period of 3 weeks at −70 °C. The stability study of captopril in human plasma showed reliable stability behavior as the mean of the results of the tested samples were within the acceptance criteria of ±15% of the initial values of the controls. These findings indicated that storage of captopril plasma samples at −70 °C is adequate, and no stability-related problems would be expected during the samples routine analysis for pharmacokinetic studies.

The stability of the stock solutions was tested and established at room temperature for 6 h in absence of light. The recovery for captopril was 95.03% (CV 0.17%). Enalapril (IS) recovery was 100.27% (CV 0.10%). The results revealed optimum stability for the prepared stock solutions throughout the period intended for their daily use. Working solutions and serial dilution standard solutions were prepared freshly just before spiking for both the calibration curve and the QC samples and were not allowed to stand for a period of time more than that needed to complete spiking of plasma samples.

4. Application

This specific and precise method was applied to analyze plasma samples obtained after the administration of a single dose of 50 mg captopril tablets to 26 healthy volunteers in a pharmacokinetic study. The mean plasma concentration-time profile of four volunteers is represented in Fig. 5.

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