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Development and validation of reversed phase high performance liquid chromatography (RP-HPLC) for quantification of captopril in rabbit plasma

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ABSTRACT

Th accurate rapid, simple and selective reversed phase high performance liquid chromatography (RP-HPLC) has been established and validated for the determination of captopril (CAP). Chromatographic separation was accomplished using prepacked ODSI C18 column (250 mm × 4.6 mm with 5 µm particle size) in isocratic mode, with mobile phase consisting of water: acetonitrile (60:40 v/v), pH adjusted to 2.5 by using 85% orthophosphoric acid at a flow rate of 1 mL/min and UV detection was performed at 203 nm. RP-HPLC method used for the analysis of CAP in mobile phase and rabbit plasma was established and validated as per ICH-guidelines. It was carried out on a well-defined chromatographic peak of CAP was established with a retention time of 4.9 min and tailing factor of 1.871. The liquid–liquid extraction method was used for extraction of CAP from the plasma. Excellent linearity ($R^2 = 0.999$) was shown over range 3.125–100 µg/mL with mean percentage recoveries ranges from 97 to 100.6%. Parameters of precision and accuracy of the developed method meet the established criteria. Intra and inter-day precision (% relative standard deviation) study was also performed which was less than 2% which indicate good reproducibility of the method. The limit of detection (LOD) and quantification for the CAP in plasma were 3.10 and 9.13 ng/mL respectively. The method was suitably validated and successfully applied to the determination of CAP in rabbit plasma samples.

KEYWORDS

CAP, RP-HPLC, validation, plasma, system suitability

INTRODUCTION

Experimental drug

Captopril (CAP), chemically it is known as (2S)-three-mercapto-2-methyl-1-oxo-propionyl-L-proline shown in Fig. 1. It is used in the treatment of high blood pressure and congestive heart failure both in adults and youngsters. It blocks the conversion of angiotensin I to angiotensin II through inhibiting the angiotensin converting enzyme and inactivates

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Fig.1. Chemical structure of CAP

bradykinin, a powerful vasodilator. The hypotensive effect of CAP may results from the both inhibitory action on renin angiotensin system and simulating action on kallikrein kinin system [1]. It differs from different ACE inhibitors through the presence of a sulfhydryl group [2]. CAP is a white to off-white powder which is soluble in water (4.60 mg/mL), methanol and ethanol. It has practically highly solubility in water and its bioavailability is 70%. Thus, it belongs to class 1 of the Biopharmaceutical Classification System (BCS) [3]. CAP has a short half-life of approximately 2 h. [4]. CAP melting point is 104–108 °C with molecular weight of 217.29. Its pK_a value is 3.70 and $\log p_{a,w}$ (biphasic system of octanol and water) is 0.34 [5].

Several methods were reported for determination of CAP in rabbit plasma which includes gas chromatography (GC) [6], gas chromatography-mass spectrometry (GS-MS) [7, 8], high performance liquid chromatography (HPLC) [9–15] and liquid chromatography-mass spectrometry (LC-MS) [16] low-injection-analysis, capillary electrophoresis methods with laser-induced fluorescence detection (LIFD) [17]. However, most of compound do not exhibit native fluorescence and decomposed and this method is highly suitable with compounds contain thiol group [18]. The GC technique is restricted through sensitivity and the GC-MS or LC-MS methods may not be extensively used and these techniques require specific detectors, and fluorometer and others require benzene for washing. Radioimmunoassay (RIA) and enzyme immunoassay methods are complicated, and involve specialized and expensive equipment that are not generally available in all scientific settings and required high quantity of plasma which is not easily available in all populations. Among these methods, reversed phase high performance liquid chromatography (RP-HPLC) is the mostly used because of high sensitivity and selectivity and economical [19–21].

This method was very economical and reliable for the determination and validation of drug in rabbit plasma and pharmaceutical dosage form [22]. Literature survey showed that other methods presented many drawbacks including use of maximum solvents and consumption of time. Furthermore, the sensitivity is not high sufficient for the determination of CAP in plasma samples. We developed sensitive and accurate validated HPLC assay with ultraviolet detection for determination of CAP in rabbit plasma. But this method,

in comparison to already reported method required a shorter analysis time (10 min) and relatively low cost instrumentation needed for analysis. [23–25]. There is a need to develop novel inexpensive economical method for the quantification of CAP both in mobile phase and plasma which was not previously reported.

The objective of this study aimed to generate reliable, accurate and precise data regardless its acceptance. Therefore chief purpose of this work is to develop a simple, precise, accurate and sensitive RP-HPLC method established for the determination of CAP. This method will provide other alternatives for already existing HPLC method with an advantage of higher resolution, shorter run-time, economical, and using low flow rate.

EXPERIMENTAL

Instrumentation

An isocratic High-Performance Liquid Chromatographic System (Agilent 1100 series Germany) equipped with auto-sampler, Diode detector, binary pump, column oven, vacuum degasser quaternary solvent manager. Welch ODSI C18 column (250 mm × 4.6 mm with 5 µm particle size) was utilized for detection of analyte. The computer running software Agilent chemstation 32 (HPLC software) was used for data acquisition and processing. Other instruments such as Centrifuge machine (Hettich Zentrifugen Germany), Vortex mixer (Scilogex MX-S Germany), pH meter (OHAUS Corporation USA), Ultrasonic cleaner (FSF-010S by Huanghua Instrument), Electric Balance (OHAUS Corporation USA), Syringe filter (0.22 µm ALLPURE Nylon), membrane filter (0.22 µm, Germany), Vacuum pump (Model AS-20 Germany), Filtration assembly (Pyrex France), and Micro pipette were used.

Drug sample and chemicals

Pure sample of CAP was obtained as a gift from Valor Pharmaceuticals, Islamabad, Pakistan. HPLC grade organic solvents such as acetonitrile, methanol and orthophosphoric acid (85%) were obtained from Sigma Aldrich, Germany. Deionized water was obtained from drug testing laboratory, Multan from their in-house purification system. All chemicals and reagents were of HPLC grade.

Chromatographic conditions

The mobile phase consisted of water and acetonitrile in the ratio of 60:40 (v/v) pH 2.5 adjusted with orthophosphoric acid (85%) was pumped through the column at a constant flow rate of 1 mL/min at ambient temperature with injection volume 20 µL and quantification was achieved at 203 nm.

Selection and optimization of mobile phase

A solvent system of HPLC grade acetonitrile and water was selected as mobile phase for good analyte solubility and stability of drug. To attain adequate resolution different

ratios of mobile phase had been tried for mobile phase optimization. In preliminary studies trials had been made with water: acetonitrile (pH 2.5 adjusted with orthophosphoric acid 85%) in various proportions, like 70:30, 80:20, 50:50 but 60:40 (v/v) showed maximum separation, better peak resolution and sensitivity.

Drug-stock solution

The mobile phase comprising of water and acetonitrile in ratio of 60:40 (v/v) pH 2.5 adjusted with orthophosphoric acid (85%) was prepared. The mobile phase was sonicated, filtered through 0.22 μm filter paper and then degassed for 10 min. Drug stock solution of 100 $\mu\text{g/mL}$ was prepared in 100 mL volumetric flask using mobile phase as diluents. Further dilutions of 100, 50, 25 12.5, 6.125, and 3.125 $\mu\text{g/mL}$ drug were prepared under the range of 100–3.125 $\mu\text{g/mL}$ with mobile phase as solvent.

Drug-plasma solution

Blood samples have been collected from healthy rabbits in citrated tubes (bolton scientific restrained) and immediately centrifuged at 4,500 rpm for 20 min. The supernatant liquid was collected and saved at -20°C . The study was performed with the approval of ethical committee for utilization of laboratory animals, Bahauddin Zakariya University Multan Pakistan. After thawing the plasma samples were spiked with above mentioned drug concentration under the range of a 100–3.125 $\mu\text{g/mL}$.

Drug extraction from plasma

Drug was extracted from plasma samples by using liquid–liquid extraction technique. Two milliliter of acetonitrile as deproteinizer was added in equal volume of rabbit plasma vortexed for 20 min at 2,000 rpm and centrifuged for 20 min at 4,000 rpm. Supernatant layer was removed and transferred to another test tube and evaporated to complete dryness at 40°C . Then 100 μL mobile phase was added and again vortexed for 10 min at 2,000 rpm. Sample was filtered through 0.22 μm membrane filter and then 20 μL of sample was injected to HPLC system for analysis and detection was done at 203 nm.

Construction of calibration graphs

Aliquots ranging from 3.125 to 100 $\mu\text{g/mL}$ were transferred from their stock solution into volumetric flasks. Chromatographic conditions were applied in triplicate for these solutions and chromatograms were recorded. Preparation of calibration curves of CAP was done by plotting the average peak area verses corresponding concentrations at 203 nm and regression equations were calculated.

Method validation

Validation of proposed HPLC method was accomplished according to guidelines of the International conference on the Harmonization of Technical Requirements for the

Registration of Pharmaceuticals for Human Use (ICH) [26]. Various parameters like, selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, precision and robustness of drug in plasma were assessed.

Selectivity

The selectivity of developed method was performed by evaluating blank as well as CAP samples. The RT was determined under similar chromatographic conditions. Selectivity was calculated by matching the chromatograms of six repeat injections of each blank with the standard drug [27].

Linearity

The linearity of an analytical technique is the functionality to bring out results that are directly proportional to the concentration of analyte in sample within a given range and is evaluated by means of regression analysis. The linearity of the technique was evaluated with repeatedly injecting different concentrations of the standard solution of the captopril. Calibration curve was constructed by plotting sample peak area (mean) against concentration and regression equation was computed.

Limits of detection and quantification

LOD is the lowest amount of analyte in a sample that can be detected however not quantified as an exact value while the LOQ is the lowest quantity of analyte in a sample that may be quantitatively determined with suitable precision and accuracy [3]. The LOD and LOQ were calculated were determined based on calibration curve using Eqs. (1) and (2) respectively [26].

$$\text{LOD} = \frac{3.3 \times \sigma}{S} \quad (1)$$

$$\text{LOQ} = \frac{10 \times \sigma}{S} \quad (2)$$

wherein, σ is the standard deviation of y -intercept of calibration curve and S is the slope of calibration curve.

Accuracy and precision

Accuracy of an analytical technique that measures the closeness of measured values to the authentic or true values. accuracy was determined by estimating the % recovery of known concentration of CAP spiked in three different working standards (50, 25, and 12.5 $\mu\text{g/mL}$). It was evaluated as percentage relative standard deviation (RSD) among the measured values and true values. Minimal of three concentration levels had been selected and five runs had been performed for each concentration and then peak area was calculated as given in Table 2. Precision of a method is a measure of the ability to create reproducible results. Precision was determined by calculating % RSD of peak area of three different concentrations (50, 25, and 12.5 $\mu\text{g/mL}$) each

in triplicate within day over the entire calibration curve range.

Robustness

The robustness of method was evaluated by deliberate variation in chromatographic conditions including analytical instrument, wavelength, pH, mobile phase composition, column oven temperature and flow rate [28]. The robustness of the developed technique was evaluated by altering mobile phase ratio and flow rate.

System suitability

In order to verify the suitability of chromatographic system for intended analysis, system suitability test was performed by six replicate injections of standard solution of CAP (50 µg/mL) and determining % RSD between obtained retention time, tailing factor (10%), number of theoretical plates.

Drug stability studies

The drug stability in a biological fluid is a function of the storage conditions as well as chemical properties of CAP was studied in rabbit plasma using freeze-thawing method. The samples of drug were freeze-dried at -20°C for a period of 24 h. Samples were removed after 24 h from refrigerator and allowed to de-freeze then injected into HPLC system for analysis.

Application to plasma samples

The assay was applied to an open-label, single-dose (50 mg CAP) pharmacokinetic study in 6 healthy rabbits. Albino rabbits of weight about 2.0–2.5 kg obtained from animal house of Pharmacology Laboratory, Faculty of Pharmacy; Bahauddin Zakariya University Multan, Punjab (Pakistan). The research protocols were studied and approved by the departmental animal ethics committee. All rabbits were housed in well maintained separate chamber with proper temperature conditions ($25 \pm 1^{\circ}\text{C}$). Rabbits which have already received any drug or those whose weight was less than 2 kg were excluded from study. After drug administration, blood samples for analytical determinations were collected in heparinized tubes. Liquid–liquid extraction method was used for the extraction of CAP from rabbit plasma samples. Within 30 min after blood collection, blood plasma was separated. To 1 mL plasma samples, acetonitrile in equal volume was added. The tubes were vortex-mixed at 2,000 rpm for 2 min and was centrifuged at 2,000 rpm for 20 min. The supernatant portion was removed and transferred to another test tube and evaporated to complete dryness at 40°C for a period of 30 min, then stored at -20°C until analysis after filtering through a 0.22-µm filter paper.

RESULTS AND DISCUSSION

Developing a sensitive, simple and accurate stability indicating RP-HPLC method for the determination of CAP both

Table 1. Results of system suitability study

Parameters	Mean ($n = 6$)	% RSD	Limits
Retention time (min)	4.9	0.0277	–
Peak area	759337.1667	0.851	<2
Tailing factor	1.72	1.871	<2
Theoretical plates	5,681	0.016	–

in mobile phase and rabbit plasma with satisfactory precision for good analytical practice was the main goal of this work. To optimize the separation and highest analytical sensitivity for drug, different mobile phase compositions have been checked. Such as ratios (80:20, 70:30, 50:50 v/v) of mobile phase were tested as trial solvent for system suitability approach. The variation within the mobile phase leads to considerable modifications within the chromatographic parameters like peak symmetry and retention time. But, the ratio of (60:40 v/v) yields the best consequences in terms of peak resolution and retention time. The pH impact confirmed the optimized conditions when pH value is 2.5 generating properly resolved and sharp peaks for the drug assay at wavelength 203 nm.

System suitability

The HPLC system became equilibrated with the preliminary mobile phase composition, followed by six injections of the same standard. These six consecutive injections were used to assess the system suitability on each day of method validation. Parameters of system suitability were peak symmetry (tailing element), area, theoretical plates and determination of retention time had been summarized in Table 1. The capacity factor was in the within in range as reported [29].

Linearity

To establish linearity of the proposed method six different concentration of drug solution were prepared and analyzed. Standard curve was generated at concentrations of 100, 50, 25, 12.5, 6.125, and 3.125 µg/mL for CAP. The calibration curve was shown to be linear in the above mentioned range in rabbit plasma. Curve was obtained by plotting peak area against concentrations of the drug. Linear calibration curve was generated by using linear regression analysis and obtained over the respective standard concentrations range. The curve, slope, intercept and the correlation coefficient were determined. It was found that regression coefficient and regression equation had been within limits as proven in Fig. 2.

LOD and LOQ

LOD and LOQ values were calculated from the known quantities of CAP in rabbit plasma. The LOD and LOQ values were found to be 3.10 and 9.13 ng/mL respectively.

Recovery (%)

The accuracy was measured at five concentration levels (50, 25, 12.5, 6.25, and 3.125%) by using known quantities of

Fig. 2. Representative calibration curve of CAP

Table 2. Percentage recoveries of CAP in plasma

Concentration (µg/mL)	Concentration found (mean ± SD)	% RSD	% Recovery
50	50.01 ± 0.0195	0.039	100.02
25	25.15 ± 0.019	0.075	100.6
12.5	12.15 ± 0.0629	0.51	97.2
6.25	6.22 ± 0.056	0.90	99.52
3.125	3.03 ± 0.0345	1.1	97

spiking drug analytes. Three injections of each concentration were injected into system and % recoveries have been calculated as shown in Table 2.

Accuracy and precision

In intra-day and inter-day precision and accuracy assay were performed. Slight variations in retention times were observed using the mobile phases that had been prepared on different days. Five replicates of every concentration have been injected in to system on two alternate days in each case and % RSD and % accuracy and precision were calculated. The outcomes of this studies predicted that compound was not affected only slight changes in retention time was observed. Intra-assay and inter-assay precision and accuracy of the purposed method is illustrated in Tables 3 and 4 respectively.

Robustness

Robustness was determined by small changes in the composition of mobile ratio and flow rates. Peak area of the drug was measured in every step and % RSD was calculated

Table 3. Intra-day precision for determination of CAP

Added concentration (µg/mL)	Measured ± SD	% RSD
50	50 ± 0.41	0.82
25	25.1 ± 0.21	0.83
12.5	12.18 ± 0.052	0.426
6.25	6.20 ± 0.0321	0.51
3.125	3.01 ± 0.025	0.83

Table 4. Inter-day precision for determination of CAP

Added concentration (µg/mL)	Measured ± SD	% RSD
50	49.98 ± 0.47	0.94
25	25.01 ± 0.29	1.15
12.5	12.46 ± 0.099	0.792
6.25	6.23 ± 0.0401	0.641
3.125	3.11 ± 0.0287	0.922

Table 5. Robustness results of different conditions

Conditions	Added conc. (µg/mL)	Measured conc. (µg/mL)	% RSD
Mobile phase composition	50	46.15 ± 0.954	2.067
Water:Acetonitrile (70:30)	25	22.29 ± 1.13	5.069
Mobile phase composition	12.5	11.21 ± 0.404	3.603
Water:Acetonitrile (80:20)	50	45.15 ± 0.90	1.993
Changes in flow rate (1.2 mL/min)	25	22.35 ± 0.916	4.098
	12.5	11.30 ± 0.175	1.548
Changes in flow rate (0.8 mL/min)	50	46.12 ± 0.952	2.056
	25	22.27 ± 1.12	5.068
	12.5	11.20 ± 0.403	3.602
	50	45.12 ± 0.89	1.991
	25	22.33 ± 0.905	4.097
	12.5	11.29 ± 0.172	1.547

which was given in Table 5. These values have been within limits which imply that the advanced technique turned into robust [7].

Specificity and selectivity

The chromatograph of CAP demonstrated absence of interference of any other peaks during analysis. Resolution between and release media peak was found to be 3.59 ± 0.26 which confirmed the specificity of developed RP-HPLC method for CAP. No peak was observed at the retention time of analyte in blank sample indicated in Fig. 3 that showed proposed method is highly specific for CAP analysis. Fig. 3 represents the chromatograms of CAP in mobile phase, solvents, rabbit plasma spiked with CAP and blank plasma. No change was found within the chromatogram of CAP both in mobile phase and rabbit plasma extraction. That's why, this method was accurate and precise for the drug [30].

The stability of CAP was determined by diluting samples in mobile phase. The samples were checked after 3 consecutive days of storage. The observed data was compared with fresh prepared samples. In each analysis RSD (%) values were determined which found to be less than 3%. This indicates that CAP is stable in mobile phase for at least 3 days. The stability of CAP in plasma was determined by periodic analysis of spiked samples. Results indicated no degradation

Fig. 3. chromatograms of (A) Drug solution in Mobile phase. (B) Mobile phase (solvent). (C) Drug in rabbit Plasma. (D) Blank plasma

was observed after 3 success which showed high stability of CAP at -20° . Furthermore, CAP was stable through at least for two freeze–thaw cycles.

Pharmacokinetic analysis

The developed method was applied to the determination of CAP in plasma samples. Plasma samples were periodically collected up to 10 h after oral administration of CAP tablets to six healthy rabbits. Fig. 4 illustrates the mean plasma drug concentration vs time profile of CAP following an oral dose containing 50.0 mg. The plasma level of CAP reached a maximum at almost 0.75 h after the administration. From the results presented in Fig. 4 it can be concluded that maximum level of drug concentration was attained in a sample taken after approximate 1 h of oral administration of CAP powder. Hence time to reach maximum concentration ($t_{\max}$) was 1 h due to its immediate dissolution in biological fluids. In short duration of time maximum concentration of CAP was achieved in blood. Mean plasma concentration of

Fig. 4. Mean plasma concentration of CAP after single dose representing mean $\pm$ SD ($n = 2$)

CAP after single dose was 800 ng/mL within 1 h was achieved. Similar pharmacokinetic profiles that were obtained correspond to pharmacokinetic profiles that were previously reported in the literature [31, 32].

CONCLUSION

The proposed method is sensitive, selective, economical and specific for the determination of CAP in rabbit plasma. Percent RSD was less than 2.0%, which indicates developed method is highly precise. The run time of 4.9 min allowed analysis of large number of samples in shorter time duration. The method was validated over a concentration range of 3.125–50 µg/mL ($r^2 = 999$) and possessed good accuracy and precision for monitoring the pharmacokinetics of CAP in rabbits. Consequently, this approach has significance for the evaluation of CAP in pharmaceutical novel formulations such as microgels, multi drug pharmacokinetics and human plasma. Furthermore, this method can be useful in routine quality control analysis.

Conflict of interest: The authors declared there is no conflict of interest, financial or otherwise.

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