DETERMINATION OF CAPTOPRIL BY HPLC-TANDEM MASS SPECTROMETRY: APPLICATION IN A BIOEQUIVALENCE STUDY

Determinação de captopril por HPLC acoplado a espectômetro de massa: aplicação em estudo de bioequivalência

Original Article

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ABSTRACT

Objective: *To assess three different captopril tablet formulations of 25mg for their bioavailability (Capoten® as the reference formulation and Captopril from FURP and Farmanguinhos as the test formulations) in 24 healthy volunteers of both sexes.* **Methods:** *The volunteers were free from serious disease, as assessed by physical and psychiatric examination, EKG, and laboratory tests. The study was open, with a three-period crossover design and a five-day washout period. Plasma samples were obtained over a 24-hour interval. Captopril concentrations were determined by reversed phase liquid chromatography tandem mass spectrometry (LC-MS-MS).* **Results:** *The geometric mean for Capoten® /Captopril-FURP 25 mg was 96.9 % for AUC_{0-24}, 95.58 % for $AUC_{0-\infty}$, and 98.17% for C_{max}. The 90% confidence intervals (CI) were 84.8-100.65%, 88.5-109.42% and 82.52-116.8%, respectively. The geometric mean for Capoten®/Captopril-Farmanguinhos 25 mg was 99.63 % for AUC_{last}, 98.52% for $AUC_{0-\infty}$, and 95.52 for C_{max}. The 90% CI were 87.23-113.8%, 86.06-112.79% and 80.29-113.64%, respectively. Therefore, the 90% CI for C_{max}, AUC_{last}, $AUC_{0-\infty}$ were within the 80-125% interval proposed by the Food and Drug Administration.* **Conclusion:** *Captopril-FURP and Captopril-Farmanguinhos 25 mg tablets were bioequivalent to Capoten® 25 mg, according to both the rate and extent of absorption.*

Descriptors: *Therapeutic Equivalency; Captopril; Chromatography, High Pressure Liquid; Pharmacokinetics.*

RESUMO

Objetivo: Avaliar a bioequivalência de três diferentes formulações de captopril 25mg (Capoten® como formulação de referência e Captopril produzido pela FURP e Farmanguinhos como formulações testes) em 24 voluntários saudáveis de ambos os sexos. **Métodos:** Os voluntários selecionados eram livres de doenças, como confirmado pelo exame físico, psiquiátrico, ECG e exames laboratoriais. O estudo foi do tipo aberto, cruzado, em três períodos com 5 dias de intervalo entre eles. As amostras plasmáticas foram obtidas num intervalo de 24 horas e as concentrações de Captopril foram determinadas por cromatografia líquida de fase reversa acoplada à espectrometria de massa *(LC-MS-MS)*. **Resultados:** A média geométrica para Capoten®/Captopril-FURP 25mg foi 96.9% para AUC_{0-24}, 95.58 % para $AUC_{0-\infty}$, e 98.17% for C_{max}. O intervalo de confiança (IC) de 90% foi de 84.8-100.65%, 88.5-109.42% e 82.52-116.8%, respectivamente. A média geométrica para Capoten®/ Captopril-Farmanguinhos 25mg foi 99.63 % para AUC_{last}, 98.52% para $AUC_{0-\infty}$, e 95.52 para C_{max}. O IC de 90% foi de 87.23-113.8%, 86.06-112.79% e 80.29-113.64%, respectivamente. Portanto, os IC de 90% para C_{max}, AUC_{last}, $AUC_{0-\infty}$ estavam dentro da variação de 80-125% proposta pelo Food and Drug Administration. **Conclusão:** Os comprimidos de 25mg Captopril-FURP e Captopril-Farmanguinhos foram bioequivalentes ao Capoten® 25mg em sua taxa e extensão de absorção.

Descritores: Equivalência Terapêutica; Captopril; Cromatografia Líquida de Alta Pressão; Farmacocinética.

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INTRODUCTION

Captopril, an oral antihypertensive drug 1-[(2S)-3-mercapto-2-methylpropionyl]-L-proline, is an angiotensin- converting enzyme inhibitor used in the treatment of hypertension and congestive heart failure[1]. It is commercially available as 12.5, 25 and 50mg tablets. Captopril has been determined in blood by several methods, including gas chromatography (GC)[2], gas chromatography-mass spectrometry (GC-MS)[3,4] and high-performance liquid chromatography (HPLC)[5-7].

Oral captopril is absorbed (68-76%) from the gastrointestinal tract[7,8] and the bioavailability of unchanged captopril is 62-65%[9]. A peak plasma concentration is observed generally about one hour after drug administration[9]. It is readily converted to its disulfide dimers and forms disulfide conjugates with endogenous thiol compounds[10]. Only free captopril is pharmacologically active; however, the formation of the inactive disulfide is reversible. The disulfide derivative may act as a reservoir for free captopril and contribute to a longer duration of action than predicted by the blood concentrations of free captopril[11].

Elimination is mainly by renal excretion[9]. Renal handling includes glomerular filtration and tubular secretion. It is rapidly and extensively metabolized in reactions involving its sulfhydryl group. The major metabolic pathway for captopril involves not only the formation of its disulfide dimer but also of mixed conjugates with endogenous thiol-containing compounds and plasma proteins as well[12]. The disulfide metabolites of captopril are inactive and there is evidence for their conversion back to the active form in vivo[13]. This observation could explain the lack of concentration-effect relationship for captopril metabolites.

The objective of this study was to evaluate, in healthy volunteers, the bioequivalence of two captopril test formulations, 25 mg tablets, manufactured by FURP (SP) and Farmanguinhos (RJ), and a commercial formulation of 25 mg (tablets) captopril (Capoten®) made by Bristol-Myers Squibb Brazil S.A. used as the reference formulation. The end of these tests allows the sale of two formulations with proven quality and at low prices for the treatment of a chronic disease like hypertension.

METHODS

Clinical protocol

Twenty-four volunteers of both sexes (12 males and 12 females) were enrolled in the study. They aged between 18 and 44 years old (mean ± SD: 23.87 ± 4.2 years) with a height between 150 cm and 183.5 cm (164.96± 6.58), weighing between 49 kg and 96.5 kg (64.63± 8.99), and within 15% of their ideal body weight. All subjects gave their written informed consent prior to participating in the study[14], and the Ethics Committee for Clinical Investigation of the University Hospital (Federal University of Ceará) approved the clinical protocol. The study was conducted during the years 2000 and 2001.

The volunteers were free from notable cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological diseases, as assessed by physical and psychiatric examination, EKG, and the following laboratory tests: blood glucose, urea, creatinine, AST, ALT, alkaline phosphatase, γ-GT, total bilirrubin, albumin, total protein, triglycerides, total cholesterol, hemoglobin, hematocrit, total and differential white cell counts, erythrocyte sedimentation rate and routine urinalysis. All subjects were negative for HIV, hepatitis B and C. In female volunteers, pregnancy was ruled out by a urine assay for β-human chorionic gonadotrophin.

The study was conducted in an open, randomized, three-period crossover design with a five-day washout interval. During each period, the volunteers were hospitalized at 9:00 p.m., had a normal evening meal, and fasted overnight. The following morning at 7:00 a.m., the subjects received a single 25mg dose of either the test captopril formulation, manufactured by FURP (batch Nº 9030) and Farmanguinhos (batch Nº 623) or the reference formulation (Capoten®, batch Nº 135716) along with 200mL of tap water. Food was withheld for 2 hours following drug administration, after which a light breakfast was provided; a standard lunch and evening meal were consumed at 4h and 10h, respectively, after dosing. No other food was permitted during hospital stay. Liquid consumption was permitted *ad libitum* after lunch, but xanthine-containing drinks including tea, coffee, and cola were avoided.

At each blood sampling time, systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer) and heart rate were recorded.

Drug analysis

Blood samples (9mL) from a suitable antecubital vein were collected into heparin-containing tubes before and at 0.25, 0.5, 0.75, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 12 and 24h after the administration of each captopril formulation. The blood samples were centrifuged at 3,500 rpm for 12 min at room temperature and the plasma recanted and stored at –20ºC until assayed for captopril content. All samples from a single volunteer were analyzed in duplicate on the same day in order to avoid intra-assay variation. Plasma captopril concentrations were determined in duplicate by

combined reversed phase liquid chromatography-tandem mass spectrometry with positive ion electrospray ionization using selected daughter ion monitoring (MRM).

Briefly, 4mL diethyl ether/dichloromethane (70/30) were added to 0.5mL plasma with 0.04mL dithiothreitol containing internal standard (100mg/mL enalapril maleate), and the samples were vortex-mixed for 30 to 40 seconds. The tubes were then centrifuged at 3500rpm for 5min at 4ºC. The upper organic layer was carefully removed, transferred to new tubes, and evaporated to dryness with a gentle stream of nitrogen in a dry bath at 37ºC. A 200µL aliquot of mobile phase, comprised of acetonitrile (70%), water (30%) and trifluoroacetic acid (0.1%), was added to the tubes, which were then vortex-mixed for 15 seconds to reconstitute the residue. The solutions were transferred to microvials, which were then capped and placed in an HP 1100 autosampler rack; 80µL were injected automatically into the liquid chromatography system.

Chromatography was performed on a Genesis C8 column, 150 mm x 4.6 mm, at a temperature of 25ºC. The temperature of the autosampler was maintained at 4ºC and the injection volume was 80 µL. The mobile phase was delivered at a flow rate of 0.6mL/min. A split of the column eluent of approximately 1:10 was included so that only approximately 60mL/min entered the mass spectrometer.

Mass spectrometry conditions

Mass spectrometry was performed using a Quatro II triple stage quadruple mass spectrometer with an electrospray ionization (EIS) source. The EIS was operated in a positive ionization mode (ES+), and MRM, m/z 218.1 $\rightarrow$ 115.9 and m/z 377.4 $\rightarrow$ 234.2, was used to monitor for captopril and enalapril maleate, respectively. The dwell time, cone voltage and collision energy were 0.5s, 23.2V and 15eV for captopril and 0.5s, 28.2V and 20eV for enalapril maleate, respectively.

Precision and accuracy

Between-run accuracy and precision were calculated for both the calibration and quality control samples. Within-run precision was calculated for the quality control samples. Calibration curve experiments were performed in duplicate. Each quality control sample was analyzed three or four times every assay.

Pharmacokinetics and statistical analysis

The first-order terminal elimination rate constant (Ke) was estimated by linear regression from the points describing the elimination phase on a log-linear plot. The elimination half-life ($t_{1/2}$) was derived from this rate constant ($t_{1/2}$ = ln(2)/Ke). The maximum observed plasma concentration (C_{max}) and the time taken to achieve this maximum level (T_{max}) were obtained directly from the data. The areas under the curve for captopril plasma concentration versus time for 0-24h (AUC_{0-24h}) were calculated by applying the linear trapezoidal method to the observed data versus time profiles. Extrapolation of this area to infinity ($AUC_{0-\infty}$) was done by adding the value C_{24}/Ke to the calculated AUC_{0-24}) (C_{24} = plasma concentration calculated from the log-linear regression equation obtained for the estimation of Ke 24 h after dosing).

Bioequivalence, among the three formulations, was assessed by calculating C_{max}, $AUC_{0-\infty}$) and AUC_{0-24} mean ratios and their 90% confidence interval (90 % CI) on log-transformed data. The inclusion of the 90% CI for the ratio in the 80 – 125% bioequivalence interval, and that of the zero value in the 90 % CI for the differences were analyzed using a parametric test (ANOVA) for all pharmacokinetics parameters except T_{max}, for which a non parametric test (Wilcoxon) was used.

Software used was WinNonlin Professional Network Edition, version 1.5 (Pharsight Corporation, Mountain View, CA, USA), Microsoft Excel version 7 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism version 3.00 (GraphPad Software, Inc. San Diego, CA, USA).

RESULTS

The three captopril formulations were well tolerated at the administered doses. The subjects' biochemical parameters were unchanged or within the reference range at the end of the study, and the following adverse events were reported: eight volunteers reported headache, three reported nausea, one cough and another drowsiness. The most common symptoms were mild and possibly related to captopril administration. Nausea was described as moderate, and one volunteer needed to take acetaminophen for headache. All the volunteers completed the three periods of the study.

The collision-induced dissociation (CID) of captopril (m/z 218.1) showed a 115.9 daughter dissociated molecular form and the CID of enalapril (m/z 377.4) showed a 234.2 daughter. Figure 1 shows the typical mass chromatogram obtained with a human plasma sample spiked with 500ng/mL captopril (retention time was 3.5 min) and enalapril (retention time was 3.4 min).

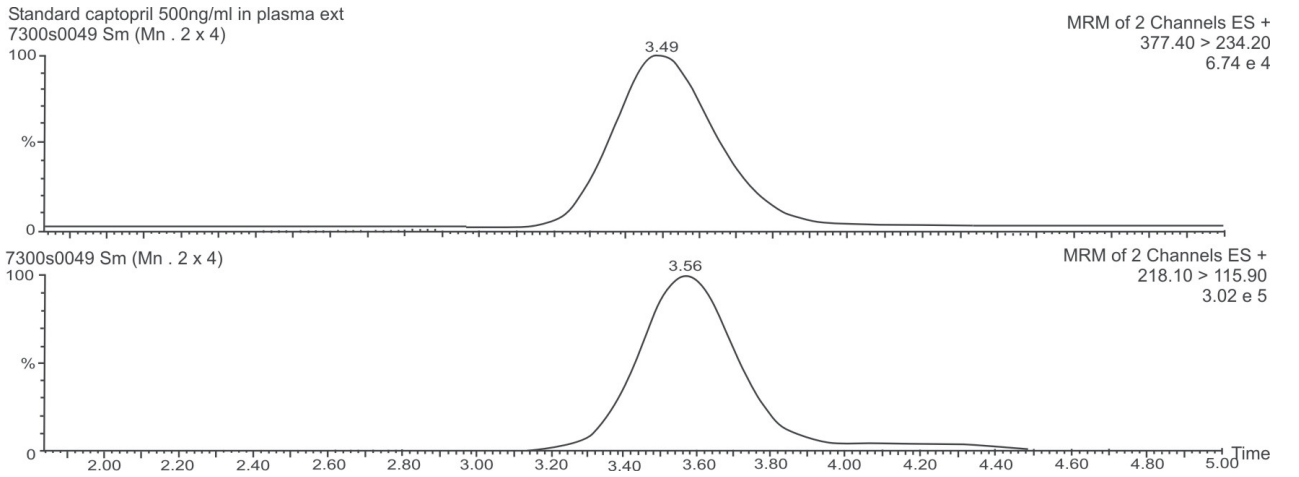

Figure 1 - Selected ion chromatograms showing retention times (min) and integrated area. A) Captopril, B) Enalapril.

The calibration curve was linear over a range of 2 to 4000 ng/mL, with a regression coefficient equal to or higher than 0.996, with intercepts not significantly different from zero.

The lower limith of quantification (LLOQ) was defined as the lowest point on the standard curve for which precision and accuracy were ≤ 20%. The current assay had a LOQ of 2.0 ng/mL in plasma for captopril based on 0.2 mL aliquots of plasma.

Five quality controls (2.0, 3, 90, 600, 3000 ng/mL) were chosen. The intra-day variability was 5.85, 4.86, 7.43, 6.21, 1.77 %, respectively.

The mean plasma captopril concentration versus time profiles obtained after a single oral administration of each 25-mg captopril formulation are shown in Figure 2.

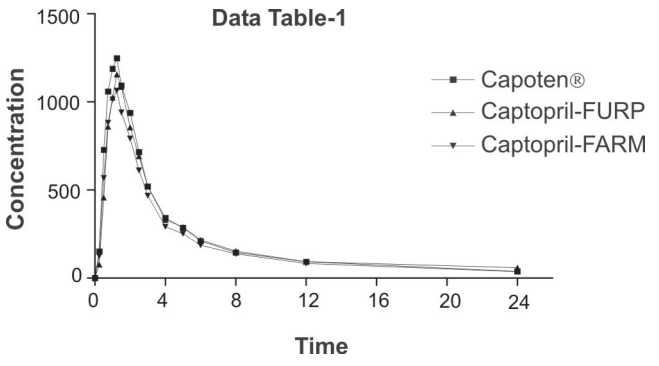

Figure 2 - Captopril plasma mean concentrations versus time profile obtained after a single oral administration of 50mg of the Captopril tablet formulations. Each curve represents the mean of 24 volunteers.

Table I shows the mean pharmacokinetic parameters (AUC_{0-24}, $AUC_{0-\infty}$, C_{max}, Ke, $T_{1/2}$, and T_{max}) obtained from 24 health volunteers after oral administration of each 25-mg captopril formulation. The relationship between AUC_{0-24} and $AUC_{0-\infty}$ was found to be 93.50, 94.55 and 94.98 for

Table I - Mean pharmacokinetic parameters obtained from 24 volunteers after administration of each captopril tablet formulation (25 mg).

Parameter	Captopril		
	Capoten®	FURP	Farmanguinhos
AUC_{0-24} (ng/h mL)			
Geom. mean	4749	4650	4916
SD	2654	1898	2886
$AUC_{0-\infty}$ (ng/h mL)			
Geom. mean	5065	4918	5159
SD	2876	2074	3008
C_{max} (ng/mL)			
Geom. mean	1327	1376	1422
SD	722	706	872
$T_{1/2}$ (h)			
Geom. mean	6.81	6.02	5.84
Range	(1.95-17.58)	(1.20-11.25)	(1.83-11.93)
ke (h^{-1})			
Geom. mean	0.12	0.14	0.14
Range	(0.04-0.35)	(0.06-0.58)	(0.06-0.38)
T_{max}			
Median	1.00	1.00	1.25
Range	(0.5-2.0)	(0.75-3.0)	(0.5-2.0)

Capoten®, captopril FURP and captopril Farmanguinhos, respectively.

Bioequivalence analysis by parametric and non-parametric treatment of individual ratios and differences is described in Table II and III.

Table II - Statistical analysis for Captopril FURP versus Capoten® formulation in regard to individual AUC_{0-48h}, $AUC_{0-\infty}$ and C_{max}.

Statistical analysis	Statistical analysis			
	Parametric analysis		Non-parametric analysis	
	Geometric mean	90% CI	Point Estimate	90% CI
AUC0-24 (ng/h mL)	96.9 %	84.8-100.65%	99.8%	86.3114.8%
AUC0- (ng/h mL)	95.58 %	88.5-109.42%	100.1%	89.5-115.2%
Cmax (ng/mL)	98.17%	82.52-116.8%	104.3%	92.1-117.4%

AUC_{0-24h}: areas under the curve for captopril plasma concentration versus time for 0-24h,
$AUC_{0-\infty}$: areas under the curve for captopril plasma concentration versus time for 0-Infinity,
C_{max}: maximum observed plasma concentration.

Table III - Statistical analysis for Captopril Farmanguinhos versus Capoten® formulations in regard to individual AUC_{0-24h}, $AUC_{0-\infty}$ and C_{max}.

Statistical analysis	Statistical analysis			
	Parametric analysis		Non-parametric analysis	
	Geometric mean	90% CI	Point Estimate	90% CI
AUC_{0-24} (ng/h mL)	99.63%	87.23-113.8%	99.1	89.4-110-8%
$AUC_{0-\infty}$ (ng/h mL)	98.52%	86.06-112.79%	97.9 %	87.4-110.6%
Cmax (ng/mL)	95.52%	80.29-113.64%	104.9%	91.5-117.9%

AUC0-24h: areas under the curve for captopril plasma concentration versus time for 0-24h,
$AUC_{0-\infty}$: areas under the curve for captopril plasma concentration versus time for 0-Infinity,
C_{max}: maximum observed plasma concentration.

DISCUSSION

Captopril is an unstable compound *in vivo*. The formation of disulfide dimers and mixed conjugates occurs rapidly and complicates the measurement of drug levels[15]. In our method, dithiothreitol (DTT) was added to the plasma samples to reconstitute captopril that had been converted to disulfides.

In addition, the drug and internal standard used in our method were extracted from biological matrices by a single liquid-liquid extraction (diethyl ether/hexane). The validation results indicated that the method is robust, precise and accurate and is suitable for the routine determination of captopril in human plasma[16].

No significant differences were observed in the mean AUC_{0-24}, $AUC_{0-\infty}$, C_{max}, Ke and T_{max} values obtained for each formulation. When captopril concentration-time curves were examined to assess bioequivalence[17,18] no significant difference in the extent and rate of absorption was observed among the three formulations (assessed by inclusion of the 90% confidence interval for individual AUC_{0-24} and C_{max} ratios in the bioequivalence range of 80-125%).

According to the literature, the $t_{1/2}$ of captopril is approximately 1-2 h[19]. Our results demonstrated, however, that the mean half-life of captopril for the three formulations tested was 6.8 h. Such a discrepancy could be due to the greater sensitivity of our method, whose limit of detection was as low as 2 ng/mL while values found in previous studies were in the range of 10 to 50 ng/mL[20,21]. This improved sensitivity allowed the determination of plasma drug concentrations up to 24 h after drug administration, which was not possible in other studies that allowed the

measurement of plasma drug levels only up to 8 h after drug administration.

The method described in this study has proved to be simple, and confirmed that LC/MS/MS shows adequate sensitivity and specificity for the determination of captopril in human plasma. It was therefore possible to determine captopril with precision and accuracy down to a level of 2 ng/mL, thereby making it unnecessary to expose volunteers to high doses of captopril of as much as 50 and 100 mg as reported in other studies[22,23] reducing the possibility of adverse effects, since these are in the majority of cases dose-dependent.

For sampling time, the agency rules required that not more than 20% should be extrapolated in the relationship AUC_{last} (AUC from zero to infinity). In this study we demonstrated that a period of 24 hours post-dosing satisfied the proposed rules. A Mass spectrometry method quantified and analyzed lower drug levels extending the sampling time period, and has been recently used in other bioequivalence trials.

In conclusion, the application of either parametric and non parametric statistics reveals the presence of bioequivalence between the three captopril formulations with regard to the rate and extent of absorption as required by the Food and Drug Administration regulations and the three products can be considered interchangeable in medical practice.

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