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Determination of captopril in plasma by high-performance liquid chromatography for pharmacokinetic studies

C. Arroyo[a], C. López-Calull[a,*], L. García-Capdevila[a], I. Gich[b], M. Barbanoj[b], J. Bonal[a]

[a]*Hospital Sta. Creu i St. Pau, Pharmacy Department, Pharmacokinetic Laboratory, Avda. Sant Antoni M[a] Claret 167 08025 Barcelona, Spain*

[b]*Hospital Sta. Creu i St. Pau, Pharmacology Department, Barcelona, Spain*

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Abstract

A high-performance liquid chromatographic method is described for the determination of free captopril in human plasma. *N*-Acetyl-L-cysteine (NAC) was used as an internal standard. Plasma samples were immediately derivatized with *N*-(1-pyrenyl)maleimide (NPM) and stabilized with 11 *M* HCl. The drug of interest was isolated using a liquid–liquid extraction with ethyl acetate and separation was obtained using a reversed-phase column under isocratic conditions with fluorescence detection. The sample volume was 150 μl plasma. The intra- and inter-day accuracy and precision, determined as relative error and coefficient of variation respectively, were less than 10%. The lower limit of quantitation, based on standards with acceptable coefficients of variation, was 25 ng/ml. No endogenous compounds were found to interfere. The linearity was assessed in the range of 25–600 ng/ml. This method has been demonstrated to be suitable for pharmacokinetic studies in humans.

Keywords: Captopril

1. Introduction

Captopril, 1-[(2*S*)-3-mercapto-2-methylpropionyl)](*S*)-proline, is an angiotensin-converting enzyme inhibitor used in the treatment of hypertension and congestive heart failure in adults and children.

To measure total or free captopril in blood or plasma, several methods have been developed [1,2], including high-performance liquid chromatography (HPLC) with UV and fluorescence detection, gas chromatography–mass spectrometry (GC–MS), radioimmunoassay (RIA) and enzyme immunoassay. These methods are either cumbersome, involve specialized and expensive equipment not usually available in a clinical setting, or required high quantities of plasma which is not easily available in all populations, such as children and critically ill patients.

Captopril has a limited stability in biological fluids because the thiol group is readily oxidized to disulfide dimer [3]. To measure free or unchanged captopril concentrations, a chemical stabilizer must be added to the biological samples to prevent the formation of the disulfides [4]. In addition, captopril has no prominent absorption properties in the UV spectrum. The use of a derivatization reagent having functions which are reactive towards the thiol group

*Corresponding author.

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and high absorption in the UV spectrum would thus seem suitable.

Some authors [5,6] formed a captopril adduct with *p*-bromophenacyl bromide (*p*-BPB) which showed UV-absorbing properties to enhance sensitivity. Pereira et al. [7] and Jarrot et al. [4] used maleimide derivatives to stabilize the thiol group and provide a suitable fluorophore. These previously reported methods [4,7] required 1 and 0.5 ml of plasma, and 3 and 6 ml of organic solvent, respectively. In addition, the method of Pereira et al. was not automatized, was not fully validated and insufficient information about the stability of captopril in plasma was given.

In this paper we report a simple accurate HPLC method to determine the plasma concentration of free captopril with fluorescence detection. The method uses NPM as a stabilizing agent and the adduct formed shows fluorescence properties. Blood samples are collected immediately into tubes containing EDTA and ascorbic acid as stabilizer. The method presented here can be performed on 0.150 ml of plasma in similar chromatographic conditions to Pereira et al. [7], but only requires 1 ml of organic solvent. This method is completely validated and provides information about stability of captopril in plasma and stability of captopril-adduct in the autosampler, which is a clear advantage for determining a large number of plasma samples for pharmacokinetic and bioavailability studies in patients and healthy volunteers.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Waters (Milford, MA, USA) instrument equipped with computer system for acquisition and integration of data (Maxima 820 Chromatography Data Station), a 510 pump, a 470 variable-wavelength fluorescence detector and a 715 autosampler Ultrawisp.

2.2. Reagents

Captopril in its free form was received from Squibb (Madrid). *N*-(1-Pyrenyl)maleimide (NPM), a derivatizing agent, was provided by Fluka (Switzerland). Acetonitrile for HPLC was obtained from Carlo Erba Reagenti (Italy). *N*-Acetyl-L-cysteine (NAC), used as internal standard, was supplied by Janssen Chimica (Belgium). All other chemicals used were of analytical-reagent grade.

2.3. Chromatographic conditions

The mobile phase was acetonitrile–acetic acid 100%, pH 2.30 (42:58, v/v), delivered at a flow-rate of 1.5 ml/min. Separation was accomplished at room temperature on a Nova-Pak C_{18} column (4 μm, 150×3.9 mm I.D.). The fluorescence detector was set to E_{ex}=340 nm and E_{em}=389 nm.

2.4. Derivatization of captopril

Venous blood samples (5 ml) were withdrawn to the heparinized Vacutainers tubes [3] containing 250 μl 0.2 *M* EDTA–0.2 *M* ascorbic acid (50:50). Blood samples were at once centrifuged at 2500 rpm for 10 min at 4°C, and 150 μl of plasma was immediately added to a tube containing 600 μl phosphate buffer (pH 7.0) and 90 μl of a solution of the derivatizing agent NPM. The internal standard (10 μl) was added. This mixture was carefully vortex-mixed and left protected from the light at room temperature for 20 min to complete the process of derivatization. Resulting captopril adduct was stabilized by adding 30 μl of HCl (11 *M*). All samples were prepared in duplicate and stored at −40°C until analysis.

To check the stability of captopril in plasma before derivatization, replicates of plasma samples were treated with NPM buffer solution at 0, 30 and 60 min after sampling [8]. Owing to the great number of plasma samples needed for pharmacokinetic studies, suitable storage conditions are required. However, as there is considerable controversy regarding stability of captopril adduct in plasma samples [2,6,7], we performed a study to establish appropriate storage conditions. The stability study of captopril adduct in plasma was carried out on three different standards in two independent replicates (25, 350, 750 ng/ml). The plasma samples were analyzed on days 0, 1, 2 and 3.

2.5. Extraction procedure

Extraction was made by adding 1.5 ml ethyl acetate to the tubes and shaking for 5 min. After centrifugation at 3 rpm for 5 min, the organic layer was evaporated to dryness under the stream of nitrogen and reconstituted in 150 μl of methanol. A 2-μl sample was injected into the HPLC system. The stability of captopril adduct in the autosampler was checked after 2, 4, 7 and 24 h at room temperature, for three different standards in plasma (10, 100 and 1000 ng/ml). For all concentrations, no significant difference appeared between $t=0$, $t=2$, $t=4$, $t=7$ and $t=24$ h.

2.6. Drug standards

Working stock solutions for captopril were prepared each day as required in water at a concentration of 1 mg/ml and stored at 4°C. Plasma standards from the stock solutions were prepared using drug-free plasma from healthy volunteers (25, 75, 150, 300, 600 ng/ml). Working stock solution for NPM was prepared in acetonitrile at a concentration of 1 mg/ml. *N*-Acetyl-L-cysteine (NAC), internal standard, was used as a 12.75 μg/ml solution in methanol. Both stock solutions were prepared at the beginning of study and were stored at −40°C for two months.

2.7. Analytical variables

Absolute extraction recoveries of captopril adduct and NAC adduct from human plasma were estimated using standard samples at concentrations ranging from 25 to 600 ng/ml of captopril and a constant amount (850 ng/ml) of the internal standard by comparing the peak heights from processed plasma standard samples to those from a calibration curve prepared from analytes in water. Plasma standard samples (25, 75, 150, 300, 600 ng/ml) were analyzed in sextuplicate on three separate days during method validation. Revalidation was assessed from the duplicate standard curves made on days when volunteers' samples were analyzed. The peak-height ratio of captopril to internal standard was plotted against the concentration of captopril. Linearity of standard curves, intra- and inter-assay precision and accuracy were determined from these data. The limits of detection (LOD) and quantitation (LOQ) of captopril were determined from the peak and the standard deviation of the noise level, *S/N*. The LOD and LOQ were defined as the sample concentration of captopril resulting in a peak height of 3 and 10 times *S/N*, respectively.

2.8. Application

The assay has been applied in pharmacokinetic studies. Samples from healthy volunteers were taken

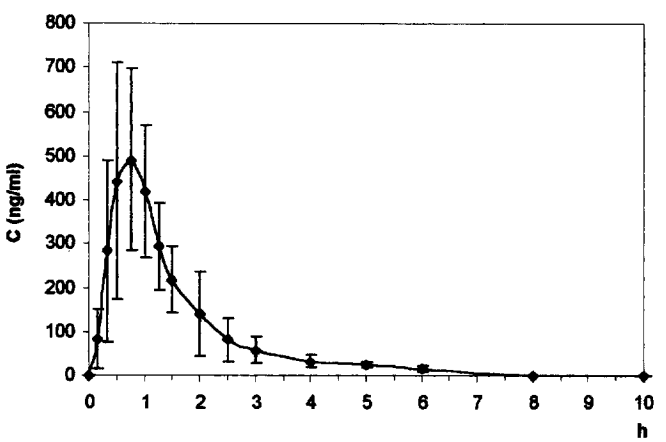

Fig. 1. Mean plasma captopril concentration vs. time profiles in 24 healthy volunteers after administration of a single 100-mg oral dose.

prior to the dose and again at 10, 20, 30, 45, 60, 75, 90, 120 and 150 min and 3, 4, 5, 6, 8, 10 and 24 h after drug ingestion. Comparison of peak-height ratios from the unknown samples to those from the calibration curve permitted quantitation of the assayed samples. Concentrations of captopril adduct measured in plasma samples obtained from twenty-four healthy volunteers given a single oral 100-mg dose of captopril (Capoten® from Squibb) are shown in Fig. 1. Samples that exceeded the highest standard concentration were diluted (1:2) and reprocessed.

3. Results and discussion

Fig. 2 illustrates a representative chromatogram of blank plasma (a), plasma with 300 ng/ml of captopril and 850 ng/ml of internal standard (b) and plasma sample from a volunteer at 20 min after drug administration (c). Drug-free pooled human plasma yielded relatively clean chromatograms with no significant interfering peaks. Derivatization with NPM led to the formation of two diastereoisomers, separable by HPLC [8]. The two peaks corresponding to the two diastereomers of the captopril were not seen when the drug was chromatographed using the previously mentioned mobile phase [4]. Retention times of NAC and captopril were 2.05 and 3.61 min respectively.

The calibration curves were made on three different days (validation) and duplicate standard curves were generated daily to determine the sample concentrations (revalidation). Linearity of the standard curves was found in the range 25–600 ng/ml and was statistically confirmed (F test for lack of fit) [9]. The determination coefficient (r^2) and the y-intercept for the straight lines were 0.9915 and $-8.107\times10^{-4}\pm5.49\times10^{-3}$ respectively for the validation and 0.9953 and $4.524\times10^{-3}\pm3.76\times10^{-3}$ respectively for the revalidation. Results are shown in Table 1. The limit of detection of captopril was 10 ng/ml, whereas the limit of quantitation was 25 ng/ml. Although other assays have similar detection limits [2,5–7], our method required only 150 μl plasma. We carried out an extensive validation and revalidation study with a great number of replicates that provides extensive information about the concentration range studied.

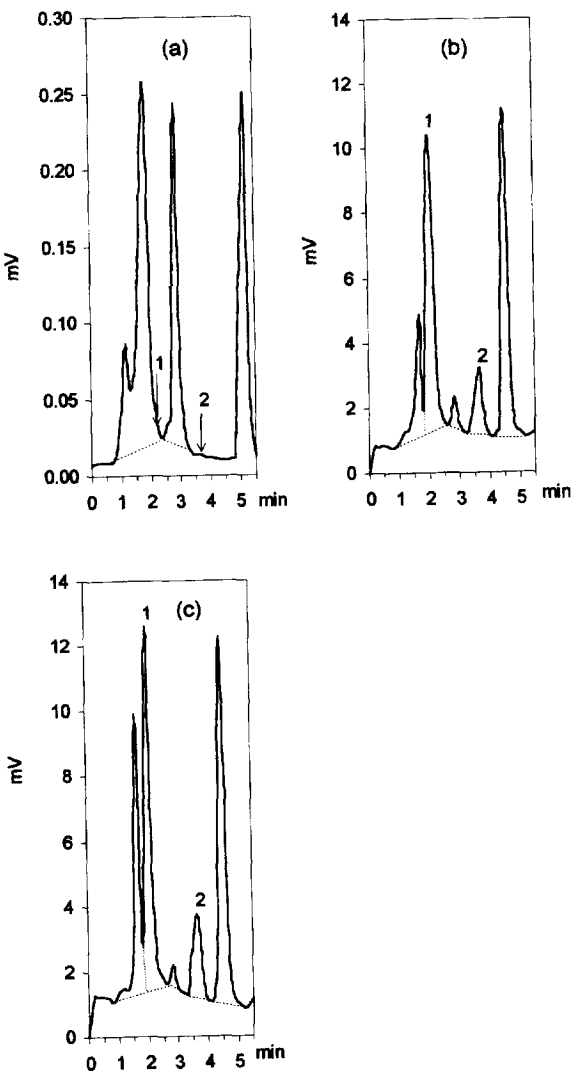

Fig. 2. (a) Chromatogram of blank pooled human plasma. (b) Chromatogram of pooled human plasma spiked with captopril concentration of 300 ng/ml (peaks: 1=NAC internal standard, 2=captopril). (c) Chromatogram of plasma sample from a single volunteer at 20 min after dosing (peaks: 1=NAC internal standard, 2=captopril).

Precision of the assay, calculated as a coefficient of variation for intra-assay variability ranged from 8.2% for 600 ng/ml to 15.3% for 25 ng/ml. Inter-assay validation ranged from 6.4% for 600 ng/ml to 18.8% for 25 ng/ml. For revalidation, precision ranged from 2.8% for 600 ng/ml to 8.3% for 75 ng/ml and at 25 ng/ml we observed good accuracy

Table 1
Results of linearity

	Mean	S.D.	C.V. (%)
Validation (n=3)			
r^2	0.9915	0.00234	0.24
a	-8.1066×10^{-1}	5.4881×10^{-3}	677
b	4.333×10^{-2}	2.4090×10^{-3}	5.55
Revalidation (n=26)			
r^2	0.9953	0.0024	0.24
a	4.524×10^{-3}	3.76×10^{-3}	83.1
b	4.399×10^{-2}	4.382×10^{-2}	9.96

S.D.=standard deviation, C.V.=coefficient of variation, a=y-intercept, b=curve slope.

(4.36%) but precision, however, was 31.9%. Pereira et al. [7] obtained a lower coefficient of variation, but only an intra-assay with four replicates is reported.

Extraction recovery of captopril adduct from human plasma was estimated using triplicate standard samples at concentrations ranging from 25 to 600 ng/ml by comparing the peak heights from processed plasma standard samples to those from a calibration curve prepared from analytes in water. In the range of calibration standards, the mean recovery of captopril was 93.5% in plasma, whereas mean recovery for internal standard at concentration 850 ng/ml was 88.6% (n=6). The results of method validation and revalidation are summarized in Table 2 and Table 3.

Using p-bromophenacyl bromide (p-BPB) as a derivatizing agent other authors acidified the captopril adduct with 1 *M* HCl and preserved stability at −20°C for six weeks [6]. According to our results, captopril adduct was not stable in plasma in the frozen state (−40°C) for more than three days even though 11 *M* HCl was added. As of this time, the remaining captopril was less than 90% of the initial concentration. Furthermore, the rapid derivatization of captopril to a suitable adduct immediately after sample collection is necessary.

This analytical method has been applied to more than 800 clinical samples in pharmacokinetic studies. The C.V. (%) are deemed acceptable when they are

Table 2
Validation of analytical method

Concentration added (ng/ml)	Concentration found (mean±S.D.) (ng/ml)	C.V. (%)	Accuracy (%)
Intra-assay (n=6)			
25	30.86±4.72	15.31	23.44
75	69.21±10.80	15.61	−7.72
150	151.43±17.70	11.69	0.95
300	303.82±11.94	3.93	1.27
600	594.17±48.82	8.18	−0.97
Inter-assay (n=18)			
25	29.05±5.45	18.76	16.20
75	71.94±7.89	10.97	−4.08
150	152.93±13.13	8.59	1.95
300	295.04±15.38	5.21	−1.69
600	598±38.44	6.42	−0.18

S.D.=standard deviation, C.V.=coefficient of variation.

Table 3
Revalidation of analytical method

Concentration added (ng/ml)	Concentration found (mean±S.D., n=52) (ng/ml)	C.V. (%)	Accuracy (%)
25	26.09±8.32	31.9	4.36
75	76.54±6.35	8.30	2.05
150	148.7±12.67	8.52	0.87
300	300.8±22.88	7.61	0.27
600	598.0±16.77	2.80	−0.33

S.D.=standard deviation, C.V.=coefficient of variation.

about 10–20% [10]. Mean plasma captopril concentrations vs. time profiles in 24 healthy volunteers are illustrated in Fig. 1. Captopril was not detected in the samples taken at 8, 10 and 24 h. In the last part of terminal profiles the increase in drug concentrations can sometimes be observed. This phenomenon may be caused by conversion of captopril in blood into dimer, as well as re-conversion to free captopril in time [6].

This paper describes a sensitive, specific, rapid and robust reversed-phase HPLC method with fluorescence detection for the measurement of captopril in small volumes of human plasma. The method has been demonstrated to be suitable for use in pharmacokinetic studies of captopril in humans.

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