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Determination of captopril and its disulphides in whole human blood and urine by high-performance liquid chromatography with ultraviolet detection and precolumn derivatization

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Abstract

An assay that measures the total, reduced, and protein-bound captopril, the orally active antihypertensive drug, in whole human blood and urine has been developed. The procedure involves a precolumn derivatization of the drug via its sulfhydryl group with 1-benzyl-2-chloropyridinium bromide followed by solid-phase extraction and reversed-phase high-performance liquid chromatography separation with ultraviolet detection at 314 nm. Oxidized and protein-bound captopril is converted to reduced form by the use of triphenylphosphine and derivatized and quantified in the same manner.

The proposed method offers the possibility of determining the *in vivo* redox status of captopril in blood of patients orally given a standard dose of at least 12.5 mg of captopril as part of the treatment of hypertensive disease and/or congestive heart failure. In the recommended procedure the sulfhydryl form of captopril is trapped with minimal oxidation by derivatizing blood samples at the time of collection. This is attained by drawing blood directly into tubes containing solutions of 1-benzyl-2-chloropyridinium bromide. The method enables also the determination of urinary excretion of captopril and its disulphides after oral administration of the drug. Accurate determinations are possible over a concentration range of 10 to 500 ng/ml captopril in blood, 50 to 1200 ng/ml captopril in urine and 10 to 1000 ng/ml captopril disulphide and 50 to 3000 ng/ml captopril disulphide in blood and urine, respectively. The detection and quantification limits for both blood and urine are 0.3 and 10 ng/ml, respectively.

Keywords: Captopril

1. Introduction

The angiotensin converting enzyme inhibitor, captopril, has been used in the treatment of essential hypertension and heart failure for more than ten years [1]. Methods so far available for identification and quantification, cited in our earlier report [2], are varied and complex, ranging from titrimetry via radioimmunoassay to gas chromatography–mass spectrometry and high-performance liquid chroma-

tography. Though many methods for the determination of captopril have been published, clinical studies are mostly not supported by blood and urine level measurements which mirrors the problems of assay development in case of an unstable and difficult to detect aliphatic mercapto-compound like captopril. Both *in vitro* and *in vivo* experiments have revealed that captopril is readily converted into disulphides [3–5]. The easy oxidation of the captopril sulfhydryl group results in a variety of disulphides *in vivo*. These include the captopril dimer and mixed captopril disulphides with endogenous

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low-molecular-mass compounds, e.g., cysteine, glutathione and homocysteine. Captopril also forms mixed disulphides with proteins, and in blood, a major fraction of these compounds is associated with albumin [6–8].

It has been shown by us that good HPLC properties can be obtained for this drug by derivatizing it with 1-benzyl-2-chloropyridinium bromide (BCPB) [2] to form a S-pyridinium derivative of captopril. The pyridinium moiety is an excellent chromophore and results in a high molar absorption coefficient at 314 nm. The derivatization reagent itself shows very weak absorption in this ultraviolet region.

In this communication we describe a technique for the determination of the *in vivo* redox status of captopril in human blood. This technique, which is a further development of a previously elaborated method [2] for the measurement of various forms of captopril in plasma, is based on the derivatization of the free thiol function of captopril with BCPB, and uses the ability of triphenylphosphine (TPP) to reduce free and protein-bound disulphides. The determination of the status of captopril in blood is particularly challenging since redox conditions change rapidly after blood collection and pharmacological activity shows predominantly reduced captopril.

The performance of this analytical method was assessed in human blood and urine.

2. Experimental

2.1. Materials and reagents

Captopril was obtained from Pharmaceutical Works 'Jelfa' S.A., Jelenia Góra, Poland. Acetonitrile, methanol, sodium octanesulphonate, citric acid, ascorbic acid, perchloric acid, EDTA, TRIS and potassium dihydrogenphosphate were from J.T. Baker (Deventer, Netherlands). Triphenylphosphine was from Aldrich (Steinheim, Germany) and 2-chloro-4-methylpyridine from Loba-Chemie (Wien, Austria). Benzyl bromide and 2-chloropyridine were from Sigma (St. Louis, MO, USA).

The derivatization reagent, 1-benzyl-2-chloropyridinium bromide (BCPB), and internal standard, 1-benzyl-2-chloro-4-methylpyridinium bromide

(BCMPB)–captopril derivative, were prepared in this laboratory according to a procedure described in our previous report [2]. All other chemicals used were of analytical-reagent grade.

Triple distilled water was used throughout the experiments. All liquids used for HPLC system were filtered over 0.2 μm membranes.

2.2. Instrumentation

Two solvent delivery systems (a Hewlett-Packard Series 1050 pump and a Laboratorni Pistroje, Praha, Czech Republic, pump) controlled by a gradient programmer (Laboratorni Pistroje) were used. The detector was a Hewlett-Packard Series 1050 variable-wavelength UV–VIS spectrophotometer. Samples were injected through a Rheodyne Model 7125 valve equipped with a 20 μl loop. Plotting and integration of peaks were performed by the use of a line recorder Model TZ 4620 and a computing integrator Model CI 100 A (both Laboratorni Pistroje). The Bakerbound SPE disposable extraction columns and vacuum manifold SPE-12G were supplied by J.T. Baker (Deventer, Netherlands).

2.3. High-performance liquid chromatography

Gradient separation was performed on an octadecylsilane modified silica column (Separon SGX C18, 150 \times 3.3 mm I.D., Struženi, Praha Czech Republic) packed with 7 μm particles and fitted with a Hypersil (5 μm , 20 \times 2.1, I.D.) precolumn. The temperature was 50°C and the flow-rate was 0.5 ml/min. Elution solvent A consisted of 20 mM sodium octanesulphonate in pH 2.5 0.1 M citric buffer and acetonitrile in ratio 3:1 (v/v). Solvent B was an 1:1 mixture of methanol and acetonitrile (v/v). The elution profile was as follows: 0–10 min, 0% B; 10.1–20 min, 0–20% B; 20.1–25 min, 20% B; 25.1–30 min, 20–40% B; post-run, 7 min, 40–0% B. The mobile phase was continuously purged with helium and maintained under positive helium pressure to remove dissolved oxygen. The retention time for the BCPB derivative of captopril and the detector analytical wavelength were 23 min and 314 nm, respectively.

2.4. Sample collection and processing

Blood

A volume of 3 ml of blood is collected into a 10-ml tube containing 100 μl of 0.1 M EDTA solution, 100 μl of 0.2 M ascorbic acid solution, 2 ml of pH 8.2 1 M TRIS buffer solution, 200 μl of the internal standard solution (3 $\mu\text{g}/\text{ml}$) and 100 μl of 20 $\mu\text{g}/\text{ml}$ BCPB solution. The tube is shaken and vortex mixed for 15 min and centrifuged for 10 min (3000 g) to remove cells and platelets. From this plasma preparation aliquots are taken for further treatment as described below in order to determine captopril, protein-bound captopril and total captopril.

(a) *Captopril*. A volume of 1 ml of BCPB-treated plasma is deproteinized with 400 μl of 3 M perchloric acid solution and centrifuged for 15 min followed by rinsing the precipitated proteins with three 500 μl portions of water. Combined supernatant and washings are adjusted to pH 2.5–3.0 (indicator paper) with 0.1 M sodium hydroxide and passed through the conditioned C_{18} extraction column [2]. The column is rinsed with 1 ml of water and dried by means of suction the air for 10 min. The analyte is then eluted with one 200- μl portion of a mixture methanol–acetic acid and two 200- μl portions of a mixture methanol–water (both 4:1, v/v), evaporated to dryness at 60°C, reconstituted with 50 μl of water and injected (20 μl) into the liquid chromatograph.

(b) *Protein-conjugated captopril*. The precipitated protein pellet (from procedure a) is resuspended with 2 ml of 0.1 M perchloric acid solution followed by adding 100 μl of 0.2 M EDTA solution, 100 μl of 0.2 M ascorbic acid solution, 70 μl of the internal standard solution, 100 μl of TPP solution in acetonitrile (40 mg/ml) and heated on a water bath at 50°C for 40 min with occasional shaking. After cooling the sample is deproteinized with 400 μl of 3 M perchloric acid solution and centrifuged for 15 min. The precipitated proteins are separated from the supernatant and washed with three 500 μl portions of water. The supernatant and washings are combined together, neutralized (indicator paper) with 1 M sodium hydroxide and treated with 50 μl of BCPB solution in the presence of 3 ml of pH 8.2 1 M TRIS buffer. After 15 min the mixture is acidified by adding 4 M phosphoric acid solution (pH 2.5–3.0,

indicator paper) and centrifuged again. The combined supernatant and washings are extracted and processed further as in procedure a.

(c) *Total captopril*. To 1 ml of BCPB-treated plasma are added 2 ml of 0.1 M perchloric acid solution and 100 μl of TPP solution in acetonitrile, and the mixture is incubated on a water bath at 50°C for 40 min with occasional shaking. After cooling and deproteinizing the sample is processed further as in procedure b.

Urine.

Urine is analysed for captopril and total captopril.

(a) *Captopril*. To 0.5 ml of urine, placed in 10 ml centrifuge tube, are added 100 μl of 0.2 M EDTA solution, 100 μl of 0.2 M ascorbic acid solution, 3 ml of pH 8.2 1 M TRIS buffer, 100 μl of BCPB solution and 200 μl of internal standard solution. After 15 min the mixture is acidified with 4 M phosphoric acid to pH 2.5–3.0, extracted and processed further as in the procedure a (Section 2.4.1).

(b) *Total captopril*. To 0.5 ml of urine are added 2 ml of 0.1 M perchloric acid solution, 100 μl of 0.2 M EDTA solution, 100 μl of 0.2 M ascorbic acid solution, 200 μl of internal standard solution and 100 μl of TPP solution and the mixture is incubated on a water bath at 50°C for 40 min with occasional shaking. After cooling the sample is derivatized and processed further as in the procedure b (Section 2.4.1).

2.5. Calibration

Standard curves are prepared by adding known amounts of captopril and captopril disulphide to captopril free blood and urine sample and assaying in the manner described in Section 2.4. The peak surface ratio of captopril–BCPB derivative to that of internal standard is plotted against blood and urine concentration of captopril.

2.6. Detection and quantification limits

The detection and quantification limits are estimated for the analyte by analysis of standard solutions of decreasing concentration. They are established as the concentrations required to generate a signal-to-noise ratios of 3 and 10, respectively. The

values obtained are confirmed by analysis of spiked blood and urine samples at concentrations equivalent to the estimated limits.

2.7. Stability of captopril in blood

Blood for the captopril decay study is routinely collected from a patient, orally given drug, into a tube containing heparin as an anticoagulant with no addition of BCPB solution and derivatized after time delay 15, 30, 45, 60 and 120 min. For the time 0 blood is derivatized during collection according to the procedure described in Section 2.4.1.

2.8. Patient study

Captopril blood levels and urinary excretion studies are performed with patients treated for hypertension for prolonged time with an oral dose of 12.5 mg or 50 mg twice a day. Blood samples were drawn at 1.5 h after a dose and analysed for captopril, protein-conjugated captopril and total captopril. Urine was analysed for captopril and total captopril.

3. Results

3.1. Stability of captopril in human blood

Significant alterations of captopril level (thiol form) occur when blood samples are derivatized with delay after collections. Within 30 min after collection its concentration dropped to 25% of initial value.

3.2. Chromatographic separations

Under the chromatographic conditions used in this study, the captopril-BCPB derivative is eluted after 23 min. Typical chromatograms for captopril human blood and urine can be seen in Fig. 1 and Fig. 2, respectively. The peak of captopril derivative is separated from other peaks, including that of internal standard, and plasma thiols. Chromatographic conditions were altered as compared with our previous investigations [2]. We have changed from isocratic to gradient elution what resulted in better resolution and simplification of solid-phase extraction of samples

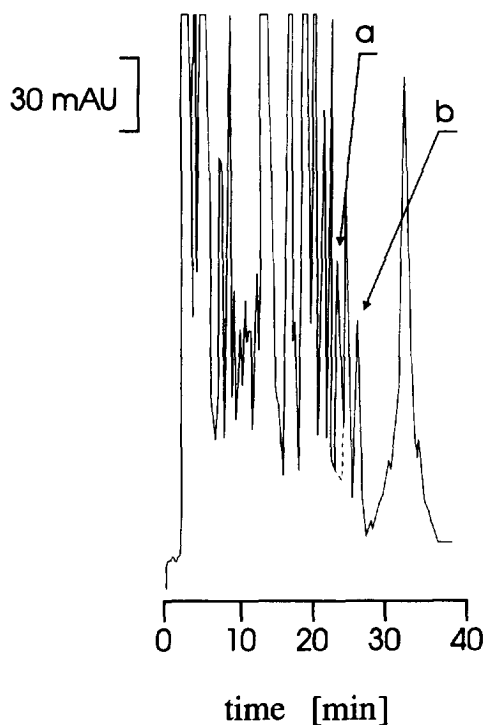


Fig. 1. Typical chromatogram of captopril (50 mg oral dose) in human blood as BCPB derivative. Peaks: a=captopril, b=internal standard. The broken line represents the background from control blood. Chromatographic conditions are described under Experimental.

before HPLC analysis. However, we could not quit addition of ion-pair reagent (sodium octanosulphonate) to the mobile phase.

3.3. Calibration curves

The calibration graphs (peak surface ratio, y , versus concentration, x) for captopril and captopril disulphides are as follows: for blood $y=0.0102x+0.09796$ ($r=0.9995$) and $y=0.0123x-0.0508$ ($r=0.9998$), respectively; for urine $y=0.0043x-0.0249$ ($r=0.9997$) and $y=0.0046x-0.0132$ ($r=0.9998$), respectively. In all cases a good linearity over the working concentration intervals was observed. The detection and quantification limits of captopril for bath blood and urine are 0.3 and 10 ng/ml, respectively. The validation data are given in Table 1 and Table 2.

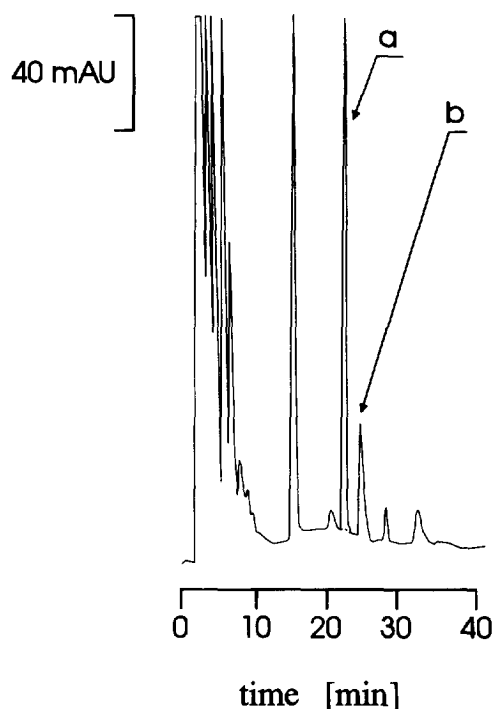


Fig. 2. Typical chromatogram of captopril in human urine as BCPB derivative. Peaks: a=captopril, b=internal standard.

certain antihypertensive drugs, the doses must be carefully tailored to each individual and it is recommended that a temporary follow-up of the blood concentration is carried out. In the case of captopril the assay is complicated by the susceptibility of the sulfhydryl function to oxidation resulting in a variety of sulphide forms. Since redox conditions change dramatically after blood collection the determination of the *in vivo* captopril redox status is particularly important. The procedure described herein allows analysis of the *in vivo* redox status of captopril in blood before significant oxidation or redistribution occurs. This is obtained by derivatization of the captopril sulfhydryl groups at the time of blood collection. This method is based on a previously elaborated assay designed for the determination of the reduced, oxidized and protein-bound forms of captopril in human plasma [2]. In the former report [2], the principles and motives for the choice of the reagents, as well as derivatization reaction and solid-phase extraction condition were evaluated and are not described here. Now the method is extended to include determination of captopril and captopril disulphides in urine.

4. Discussions

The aim of an antihypertensive treatment is to attain a blood pressure level within the limits. To achieve this, an uninterrupted long time, sometimes life-long, treatment with antihypertensive drugs is necessary. In view of the even slight toxicity of

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Table 1
Validation data for captopril and captopril disulphide added to captopril-free blood

Taken (ng/ml)	Captopril, <i>n</i> =5		Captopril disulphide, <i>n</i> =5	
	Found±SD (ng/ml)	Recovery±RSD (%)	Found±SD (ng/ml)	Recovery±RSD (%)
10	10.2±0.61	102.2±6.10	9.82±0.94	98.2±9.37
50	48.9±1.81	97.7±3.61	53.16±4.01	106.3±8.01
100	97.9±2.49	97.9±2.49	96.89±3.51	96.9±3.51
200	193.5±9.50	96.7±4.75	194.62±5.52	97.3±2.76
500	521.6±27.10	104.3±5.24	488.04±26.08	97.6±5.22
1000			1016.5±33.84	101.7±3.38

Table 2
Validation data for captopril and captopril disulphide added to captopril-free urine

Taken (ng/ml)	Captopril, <i>n</i> =5		Captopril disulphide, <i>n</i> =5	
	Found±SD (ng/ml)	Recovery±RSD (%)	Found±SD (ng/ml)	Recovery±RSD (%)
50	48.9±1.22	97.8±2.43	49.9±2.59	99.7±5.18
100	103.3±3.51	97.7±5.86	97.2±3.20	97.2±6.40
200	97.9±2.49	97.9±2.49	206.3±5.62	103.2±2.81
500	491±18.70	98.4±3.74	494.7±20.13	98.5±4.03
1200	1117.2±68.66	93.1±5.72	1212.1±53.07	101.0±4.42
3000			3042.3±98.91	101.4±3.30

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