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DETERMINATION OF CAPTOPRIL AND ITS MIXED DISULPHIDES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method has been developed which enables sensitive determination of captopril and its mixed disulphides in plasma and urine after oral administration of a new antihypertensive agent, 1-(D-3-acetylthio-2-methylpropanoyl)-L-prolyl-L-phenylalanine (DU-1219, I). Captopril is derivatized with a new reagent, N-(4-benzoylphenyl)maleimide and the derivative is extracted with chloroform and assayed using a liquid chromatograph equipped with an ultraviolet detector at 254 nm. Mixed disulphides of captopril with thiol compounds such as cysteine, glutathione and plasma proteins are reduced with tributylphosphine to form captopril, followed by derivatization with N-(4-benzoylphenyl)maleimide.

Accurate determinations are possible over a concentration range of 10—500 ng/ml captopril in plasma, and 100—2500 ng/ml captopril in urine. The coefficients of variation of captopril in plasma (200 ng/ml) and urine (500 ng/ml) are 3.7% and 2.6%, respectively, and those of mixed disulphides of captopril are similar to those of captopril. Plasma levels and urinary excretion of captopril and its mixed disulphides in healthy volunteers following single oral administration of I (50 mg) have also been determined.

INTRODUCTION

The compound 1-(D-3-acetylthio-2-methylpropanoyl)-L-prolyl-L-phenylalanine (DU-1219, I, Fig. 1) is an orally active antihypertensive agent, designed to inhibit angiotensin-converting enzyme in vivo [1, 2]. Disposition and metabolic studies of ^{14}C-labelled I in rats have revealed that I was considerably converted to an active metabolite, captopril, and captopril and its mixed disulphides were detected in plasma and urine after dosing of I [3]. In order to

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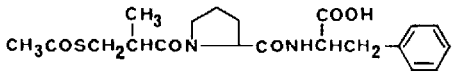
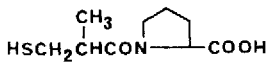
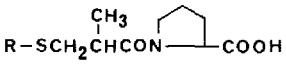


I

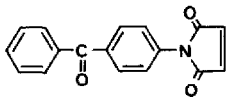


Captopril



Mixed disulphides

R = cysteine,
glutathione,
proteins,
captopril,
etc.



BPM

Fig. 1. Chemical structures of I, captopril and mixed disulphides of captopril and BPM.

study the pharmacokinetics of I in man, a sensitive and specific assay method for captopril and its mixed disulphides is necessary.

Captopril in blood or urine has been determined by gas chromatography (GC) [4], gas chromatography–mass spectrometry (GC—MS) [5], a radiochemical method [6] and high-performance liquid chromatography (HPLC) [7—9]. However, the GC method is limited by sensitivity, and the GC—MS method of Funke et al. [5] and the HPLC methods of Jarrott et al. [8] and Shimada et al. [9] require specific detectors.

In this paper we describe a HPLC method with ultraviolet detection at 254 nm. The method, based on extraction and derivatization using a new thiol derivatizing reagent, N-(4-benzoylphenyl)maleimide (BPM), is selective and sensitive for the determination of captopril and its mixed disulphides in plasma and urine.

EXPERIMENTAL

Materials

Captopril, I, 1,1′-[dithiobis(2-D-methyl-3-propanoyl)]-bis-L-proline (captopril dimer) and (4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid (II) were synthesized in our laboratories. Thiosalicylic acid and disodium EDTA were obtained from Wako (Osaka, Japan). The derivatizing reagent of thiol compounds, N-(4-benzoylphenyl)maleimide (BPM), was also synthesized in our laboratories [10] (Fig. 1). Human serum albumin conjugated captopril (HSA—captopril) was prepared by the methods of Harrap et al. [11] and Meredith [12], and stored in 0.01 *M* phosphate buffer (pH 7.4) (6 μg of captopril per 10 mg of protein per ml). Silicic acid (Kieselgel 60, 70—230 mesh; E. Merck, Darmstadt, F.R.G.) and silica gel 60 plates F_{254} (Merck) were used for column chromatography and thin-layer chromatography (TLC), respectively. All other chemicals were of analytical-reagent grade.

Instruments

HPLC was carried out using a Waters Model ALC/GPC 204 liquid chromato-

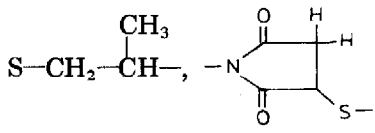
graph, equipped with a dual-delivery pump (Model 6000), an automatic sampler (Model 710B), a UV detector (Model 440) with a 254-nm filter and a recorder with integration (Data Module, Model 730) (Waters Assoc., MA, U.S.A.), and a Shimadzu Model LC-4A liquid chromatograph, with a Rheodyne 7125 sample loop injector and a recorder with Shimadzu Chromatopac C-R2A (Kyoto, Japan).

A stainless-steel column (30 × 0.4 cm I.D.) packed with μBondapak C_{18} (10 μm; Waters Assoc.) was used. The following mobile phases were used at a flow-rate of 1.0 ml/min: (1) acetonitrile—methanol—1% acetic acid (45:11:75) for captopril in plasma; (2) 42.5:11:74 for the mixed disulphides in plasma; (3) 42.5:8.2:47.3 for captopril in urine; and (4) acetonitrile—1% acetic acid (47:53) for the mixed disulphides in urine.

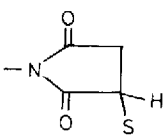
Nuclear magnetic resonance (NMR) spectra were recorded on a Varian FT-80A NMR spectrometer. Field desorption (FD) mass spectra were recorded on a JEOL Model JMS-D300 mass spectrometer.

Synthesis of captopril—BPM adduct

To 0.11 g of captopril in 2 ml of water were added 0.15 g of BPM in 2 ml of acetone and one drop of triethylamine. The mixture was allowed to stand at room temperature for 15 min and evaporated to dryness in vacuo. Oily residue was purified by silica gel chromatography (column, 15 × 0.9 cm I.D.). The eluate with chloroform—methanol (19:1, v/v) was evaporated to give 0.1 g of captopril—BPM adduct as a white amorphous solid. A TLC spot of captopril—BPM was obtained with R_F = 0.29 (chloroform—methanol—water, 100:10:0.1, v/v/v) detected by UV lamp at 254 nm and iodine vapour. ^{1}H-NMR (in deuterated dimethyl sulphoxide, DMSO-d_6): δ = 1.1 [d, J = 6 Hz, 3H, CH_3]; δ = 1.7—2.3 [m, 4H, proline 3-CH_2, 4-CH_2]; δ = 2.6—3.8 [m, 7H, proline 5-CH_2,



]; δ = 4.2 [m, 2H, proline 2-CH,



];

δ = 7.4—8.0 [m, 9H, aromatic]; δ = 12.3 [bs, 1H, COOH]. FD—MS: m/z 494 (M^+).

Thiosalicylic acid—BPM adduct (TLC: R_F = 0.52) and II—BPM adduct (TLC: R_F = 0.18), used as the internal standards, were prepared by the same method as described above.

Assay procedure for captopril and its mixed disulphides in plasma and urine

(a) Captopril in plasma. To 1 ml of plasma in a glass-stoppered 15-ml centrifuge tube were added 2 ml of 0.1 *M* phosphate buffer (pH 6.0) and 0.5 ml of 0.5% BPM acetone solution. The tube was vortex-mixed for 15 sec and allowed to stand at room temperature for 10 min. To the mixture 2 ml of 0.5 *M* phosphate buffer (pH 7.0) and 0.1 ml of internal standard solution A (4 μg of II—BPM adduct in 0.1 ml of acetone) were added. The mixture was washed twice

with 4 ml of diethyl ether, acidified with 0.5 ml of 6 *M* hydrochloric acid and extracted with 7 ml of chloroform. The organic layer was evaporated to dryness below 40°C under a gentle stream of air.

The clean-up procedure was carried out as follows. The residue was dissolved in 2 ml of 0.5 *M* phosphate buffer (pH 7.0) and washed twice with 4 ml of diethyl ether. After acidification with 6 *M* hydrochloric acid the aqueous layer was again extracted with 7 ml of chloroform. The organic layer was evaporated to dryness. The residue was dissolved in 100 μl of methanol and a 20-μl aliquot of solution was injected into the liquid chromatograph.

(b) Total captopril (captopril and its mixed disulphides) in plasma. To 1 ml of plasma sample in a glass-stoppered 15-ml centrifuge tube were added 2 ml of 0.1 *M* phosphate buffer (pH 6.75), 0.2 ml of 0.1 *M* EDTA solution and 0.2 ml of 0.8% tributylphosphine methanol solution. The mixture was incubated at 50°C for 60 min. After the reaction was stopped in the ice box for a few minutes, 1 ml of 0.5 *M* phosphate buffer (pH 7.0) and 0.1 ml of internal standard solution A were added, and the mixture was then washed with 4 ml of diethyl ether. To the aqueous layer was added 0.5 ml of 0.5% BPM acetone solution; the mixture was vortex-mixed for 30 sec, stood at room temperature for 30 min, and was then washed with 4 ml of diethyl ether. The aqueous layer was acidified and extracted with 7 ml of chloroform. The organic layer was evaporated to dryness and the residue was then treated by the clean-up procedure described above. The residue was dissolved in 100 μl of methanol and a 10-μl aliquot of the solution was injected into the liquid chromatograph.

(c) Protein-conjugated captopril in plasma. To 1 ml of plasma sample in a glass-stoppered 15-ml centrifuge tube was added 0.2 ml of 3 *M* perchloric acid; the tube was vigorously vortex-mixed for 30 sec and centrifuged at 700 *g* for 10 min. The supernatant was removed, and the precipitated pellet was then resuspended in 2 ml of water and neutralized with 1 *M* tripotassium phosphate. To the mixture were added 1 ml of 0.5 *M* phosphate buffer (pH 7.0), 0.2 ml of 0.1 *M* EDTA solution and 0.2 ml of 1% tributylphosphine solution. The mixture was incubated at 50°C for 60 min. Captopril in the mixture was determined as described in the above procedure (a).

(d) Captopril in urine. To 0.2 ml of urine sample in a glass-stoppered 15-ml centrifuge tube were added 0.2 ml of 0.5% BPM solution and 0.2 ml of 0.1 *M* phosphate buffer (pH 6.5), and the tube was allowed to stand at room temperature for 15 min. To the mixture were added 2.5 ml of 0.5 *M* phosphate buffer (pH 7.0) and the mixture was washed with 4 ml of diethyl ether. To the aqueous layer was added 0.1 ml of internal standard solution B (1 μg of thiosalicylic acid—BPM adduct in acetone). The mixture was acidified with 0.25 ml of 6 *M* hydrochloric acid, and extracted with 6 ml of chloroform. The organic layer was evaporated to dryness. The residue was dissolved in 200 μl of acetonitrile and a 20-μl aliquot of the solution was injected into the liquid chromatograph.

(e) Total captopril in urine. To 0.2 ml of urine sample in a glass-stoppered 15-ml centrifuge tube were added 2 ml of 0.1 *M* phosphate buffer (pH 7.0), 0.1 ml of 0.1 *M* EDTA solution and 0.1 ml of 2% tributylphosphine solution. The mixture was incubated at 50°C for 60 min and kept in the ice box for a few minutes. To the mixture was added 0.5 ml of 0.5% BPM solution. After

30 min the mixture was washed twice with 4 ml of diethyl ether. To the aqueous layer was added 0.2 ml (2 μg) of internal standard solution B. Captopril in the mixture was determined as described in the above procedure (d).

Calibration curves for captopril and its mixed disulphides in plasma and urine

Calibration curves were constructed by assaying plasma and urine spiked with known amounts of captopril, captopril dimer or HSA—captopril by the methods described above. The peak height ratios of captopril to the internal standard were plotted against the plasma or urine concentrations seeded.

Stability of captopril—BPM adduct

The stability of captopril—BPM in plasma and urine was determined. The derivatized captopril in plasma (0.1—0.5 μg/ml) or in urine (0.2—10 μg/ml) was stored in a freezer (about −20°C) for 30 days.

Human study

The study was carried out under the supervision of physicians. Four healthy volunteers fasted overnight, then orally received 50 mg of I. Blood was taken into a heparinized 10-ml tube (JMS plavetest, Japan Medical Supply, Tokyo, Japan) containing a solution of 0.1 *M* EDTA and 0.1 *M* ascorbic acid [8]. The samples were immediately centrifuged for 2 min. For the determination of captopril, plasma and urine samples were treated with BPM as described in the assay procedure and kept frozen until analysis.

RESULTS AND DISCUSSION

Derivatization of captopril

It is well known that thiol compounds are unstable in biological fluids and undergo rapid oxidation to form the disulphides and other unidentified products [6,13], indicating the necessity for the rapid derivatization of captopril to a suitable adduct immediately after sample collection. In addition, captopril has no prominent absorption properties in the UV spectrum. The use of a derivatization reagent having functions reactive toward the thiol group and high absorption in the UV spectrum would thus seem suitable. BPM was chosen from 30 maleimide compounds synthesized in our laboratories for this purpose [10].

When captopril was treated with BPM, captopril—BPM adduct was rapidly formed in phosphate buffer solution and showed a high ϵ value ($1.8 \cdot 10^4$ at 254 nm). The structure of the adduct was characterized by NMR and MS. Effects of time and pH on the reaction of captopril with BPM were examined. The derivatization of captopril was complete in 5 min at both 0°C and 24°C. The pH of the reaction was optimum at pH 4—7.5. Therefore, captopril was reacted with BPM at pH 6—7 at room temperature for 10—30 min.

Reduction of mixed disulphides of captopril

The mixed disulphides represent a fraction of captopril covalently binding to thiol compounds such as captopril itself, cysteine, glutathione and plasma

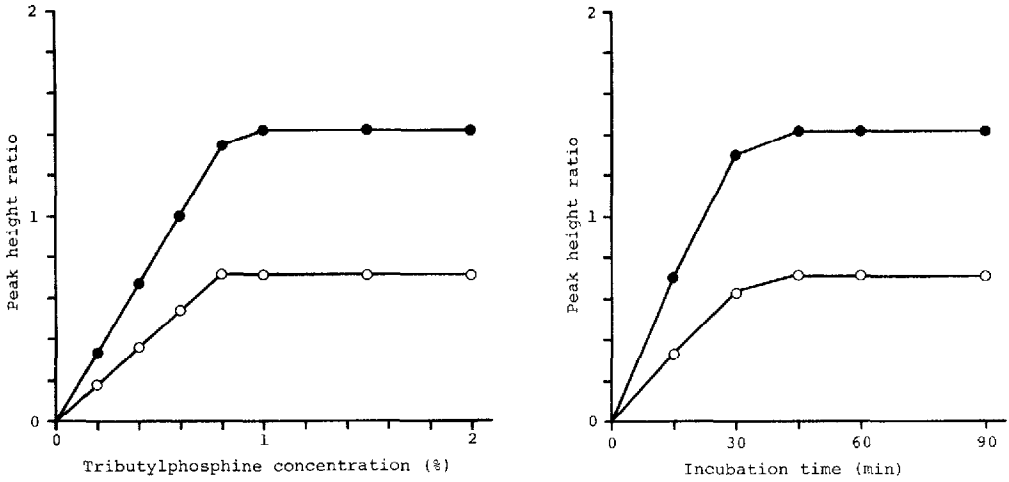


Fig. 2. Effect of tributylphosphine concentration on the reduction of captopril dimer at 50°C. The reaction mixture consisted of 1 ml of plasma, 0.2 ml of EDTA solution, 0.2 ml of tributylphosphine (0.2—2.0% in methanol) and 1 ml of 0.1 *M* phosphate buffer (pH 6.75) containing 2 μg (●) or 1 μg (○) of captopril dimer.

Fig. 3. Effect of incubation time on the reduction of captopril dimer at 50°C. Captopril dimer: (●), 2 μg; (○), 1 μg.

proteins [14—17]. These compounds were reduced to captopril with tributylphosphine [7,18]. The reduction of captopril dimer and HSA—captopril was complete in 45 min at 50°C when the concentration of tributylphosphine was 0.8—2%, as shown in Figs. 2 and 3. Dithiothreitol and sodium borohydride [12,19] were also used for the reduction of the disulphides in plasma. In this case we did not succeed in separating the peaks of captopril from those owing to plasma.

Determination of captopril and its mixed disulphides in plasma

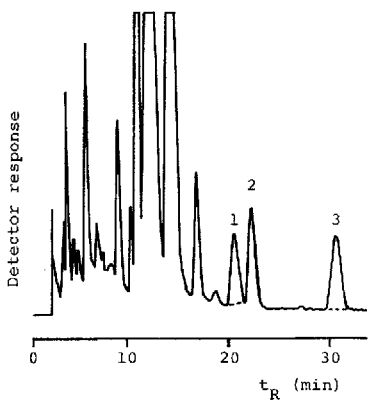
A typical chromatogram of plasma containing 200 ng/ml captopril is shown in Fig. 4 with the assay procedure a. The peaks of captopril and internal standard were separated from those of control plasma and solvent. The calibration curve obtained with 10—250 ng of captopril in 1 ml of plasma was rectilinear and passed through the origin. Quantitative reduction of captopril

TABLE I

ACCURACY AND PRECISION OF THE METHOD FOR DETERMINATION OF CAPTOPRIL AND ITS MIXED DISULPHIDES IN HUMAN PLASMA

Assay procedure a			Asay procedure b			Assay procedure c		
Captopril (ng/ml)		C.V. (%)	Captopril dimer (ng/ml)		C.V. (%)	HSA—captopril (ng/ml)		C.V. (%)
Added	Found ± S.D.*		Added	Found ± S.D.*		Added	Found ± S.D.*	
500	500.7 ± 26.7	5.3	500	498.7 ± 6.3	1.3	500	501.6 ± 14.1	2.8
200	200.3 ± 7.3	3.7	200	197.5 ± 8.7	4.4	200	196.7 ± 8.0	4.0
100	100.3 ± 2.8	2.8	100	98.1 ± 0.6	0.6	100	98.0 ± 1.0	1.0
50	48.3 ± 1.6	3.2	50	49.2 ± 5.1	10.2	50	50.1 ± 2.9	5.8
25	25.5 ± 1.9	7.6	25	23.4 ± 4.0	16.0	25	27.5 ± 5.4	21.6
10	12.4 ± 0.6	6.0						

*$n = 6$.



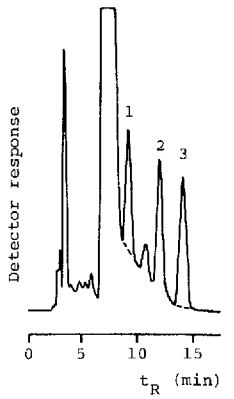


Fig. 4. Typical chromatogram of captopril in plasma (200 ng/ml). Broken lines represent the background from control plasma. HPLC conditions as described in the text. Peaks: 1 = captopril—BPM; 2 = BPM; 3 = internal standard.

Fig. 5. Typical chromatogram of captopril in urine (200 ng in 0.2 ml of urine). Peaks: 1 = captopril—BPM; 2 = BPM; 3 = internal standard.

dimer and HSA—captopril was observed in the range of 25—500 ng/ml as captopril.

Table I shows data on accuracy and precision of the assay. The coefficients of variation at the 200 ng/ml level of captopril and captopril dimer were 3.7% and 4.4%, respectively. The assay limit was 10 ng/ml captopril.

Determination of captopril and total captopril in urine

A typical chromatogram of urine containing captopril is shown in Fig. 5. Good linearity was obtained in the range 100—2500 ng/ml captopril and 250—5000 ng/ml captopril dimer. Accuracy and precision of this method are shown in Table II. The coefficients of variation at the 500 ng/ml level of captopril and captopril dimer were 2.6% and 8.2%, respectively. The minimum detectable concentration was 50 ng/ml captopril.

TABLE II

ACCURACY AND PRECISION OF THE METHOD FOR DETERMINATION OF CAPTOPRIL AND TOTAL CAPTOPRIL IN HUMAN URINE

Assay procedure d			Assay procedure e		
Captopril (ng/ml)		C.V. (%)	Captopril dimer (ng/ml)		C.V. (%)
Added	Found ± S.D.*		Added	Found ± S.D.*	
100	120 ± 4.0	4.0	250	234 ± 6.5	2.6
250	258 ± 11.9	4.8	500	553 ± 41.0	8.2
500	504 ± 12.7	2.6	1000	957 ± 85.0	8.5
1000	982 ± 31.2	3.1	2500	2503 ± 51.3	2.1
2500	2504 ± 107.6	4.3	5000	4984 ± 322.3	6.4

*n = 4.

Stability of captopril—BPM adduct in plasma and urine

Captopril—BPM in plasma and urine were stable for at least 30 days in a freezer.

Plasma levels and urinary excretion of captopril and its mixed disulphides in man receiving 50 mg of I

Plasma levels of captopril, protein-conjugated captopril and total captopril following oral administration of I are shown in Fig. 6. Plasma levels of captopril reached a maximum 1 h after dosing with a level of 246 ng/ml. Plasma levels of protein-conjugated captopril and total captopril were maximal 1 h after dosing with levels of 552 ng/ml and 932 ng/ml, respectively. Plasma levels of mixed disulphides of captopril were three to four times higher than those of free captopril.

Urinary excretion of captopril and total captopril for 24 h after dosing were 29.0% and 62.5% of the dose, respectively.

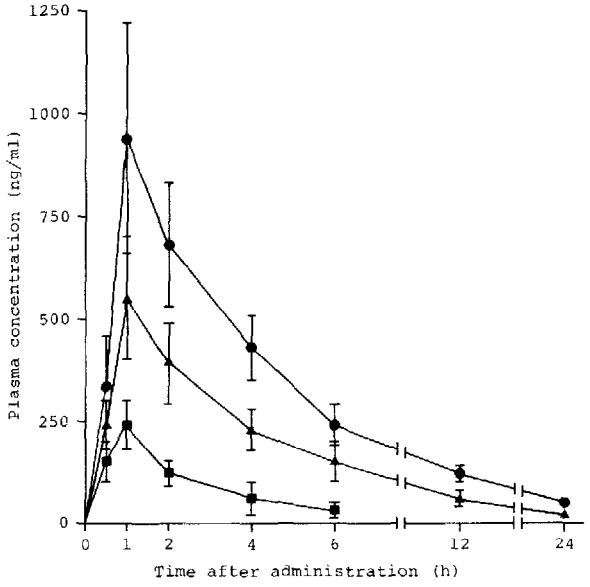


Fig. 6. Plasma levels of captopril (■), protein-conjugated captopril (▲) and total captopril (●) in healthy volunteers following oral administration of 50 mg of I. Data points are mean values ± S.E. from four subjects.

CONCLUSION

We have developed a selective and sensitive HPLC method for the determination of captopril and its mixed disulphides in plasma and urine. This method should permit pharmacokinetic studies in man.

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