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Determination of Captopril in Blood and Urine by High-Performance Liquid Chromatography

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A new procedure has been established for the determination of captopril [1-(D-3-mercapto-2-methyl-1-oxopropyl)-L-proline] in biological fluids. Captopril was trapped with *p*-bromophenacyl bromide or N-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM), then the addition products were separated and determined by high-performance liquid chromatography (HPLC) on a reversed-phase column. The disulfides, metabolic products derived from captopril and excreted in the urine, were reduced with tributylphosphine to captopril which in turn was trapped with DDPM. These adducts were separated and determined by HPLC. The method provided quantitative results for captopril levels of 5—2000 ng/ml of whole blood and 0.1—100 μg/ml of urine with satisfactory accuracy and precision.

Keywords——captopril; *p*-bromophenacyl bromide; N-(4-dimethylamino-3,5-dinitrophenyl)maleimide; high-performance liquid chromatography; derivatization for mercapto group; cleavage of disulfide linkage with tributylphosphine; whole blood and urine

Captopril [1-(D-3-mercapto-2-methyl-1-oxopropyl)-L-proline] is a potent and specific angiotensin-converting enzyme inhibitor,[1—4] and has been tested clinically for the treatment of hypertension.[5] However, in spite of its clinical use, its pharmacological action is not yet completely understood, because captopril is rapidly converted in living animals into metabolites such as disulfides.[6] Sensitive and selective methods for monitoring the levels of captopril in the body fluids are essential for a clear understanding of the relationship between the pharmacokinetics and drug responses in the body. Radiochemical,[7] gas chromatographic[8] and gas chromatography-mass spectrometric methods[9] for the determination of captopril in biological fluids have been reported. In order to develop a highly sensitive determination method for captopril, we have attempted to utilize high-performance liquid chromatography (HPLC). This paper describes HPLC methods for the determination of captopril in the non-protein fraction of whole blood and in urine of humans and dogs. The method is as follows: captopril was trapped with *p*-bromophenacyl bromide (p·BPB) or N-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM),[10] which have been used as coupling reagents for the mercapto group, then the addition products were separated by HPLC, with a UV-detector. In addition to the clinical assay of captopril, this method is also suitable for studies of the pharmacokinetic behavior of the drug.

Experimental

Apparatus and Chromatographic Conditions——A liquid chromatograph from Waters Associates (Milford, Mass.) equipped with an injector (U6K), pump (6000A), detector (440) and μBondapak C_{18} column was used. The mobile phases consist of acetonitrile–water–acetic acid (48:51.5:0.5 for blood and 46.5:53.0:0.5 for urine sample assay). The solvents were degassed by ultrasonication before use. The column effluent was monitored by UV-absorption measurement at 254 nm. Chromatograms were traced on a chart recorder and peak height measurements were used for quantitation.

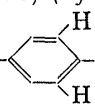
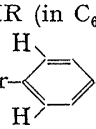
Chemicals——p·BPB was purchased from Tokyo Kasei Chemicals (Tokyo, Japan) and purified by column chromatography on silica gel with dichloromethane, followed by recrystallization from ethanol. DDPM was obtained from Aldrich Chemical Co. Captopril and oxidized captopril (dimer of captopril;

SQ 14551) were obtained from E.R. Squibb and Sons, Inc. Other chemicals utilized in this work were of guaranteed-reagent grade.

GC/MS, NMR and UV Spectrometry——Mass spectra were measured on a Hitachi RMU-6MG machine equipped with a direct inlet under the following conditions: ionization voltage, 20 eV; ion accelerating voltage, 3.1 kV. GC conditions were as follows: column, 2% OV-17 on Chromosorb 750; carrier gas, He 40 ml/min; separator temp., 250°; injection temp., 255°. NMR spectra were taken on a Varian T-60 spectrometer in C_6D_6 for captopril-p·BPB adduct, with tetramethylsilane as an internal standard. UV spectra were taken on a Hitachi 200-20 spectrometer.

Preparation of Standard Compounds for Assay

(1) **Preparation of Captopril-p·BPB Adduct**——To a solution of p·BPB (2.4 mmol) and captopril (2.0 mmol) in 40 ml of methanol, 1.0 N NaOH was added dropwise to adjust pH 7.0 at room temp. After being stirred for about 10 min, the resulting solution was evaporated to dryness *in vacuo*. A solution of the residue in 0.05 M phosphate buffer (pH 7.0, 40 ml) was washed twice with 20 ml each of ethyl ether, adjusted to pH 2 by addition of diluted hydrochloric acid and extracted with 40 ml of ethyl acetate. Removal of the solvent by evaporation provided the captopril-p·BPB adduct as a colorless oil. The homogeneity of the product was confirmed by TLC with benzene–acetic acid (3: 1) (*Rf* 0.5) and also by HPLC. NMR (in C_6D_6) ppm:

10.78 (bs. 1H, –COOH), 7.51 (d, $J=9$ Hz, 2H, Br–  –CO), 7.17 (d, $J=9$ Hz, 2H, Br–  –CO).

(2) **Preparation of Captopril-DDPM Adduct**——Captopril (651 mg) and DDPM (1.1 g) were dissolved in 50 ml of acetone and the whole was kept for 30 min at pH 7.0 by dropwise addition of 2 N NaOH. After removal of the solvent by evaporation, the residue was suspended in 0.02 M phosphate buffer (pH 7.0, 100 ml) and washed with hexane. Next, 2 N hydrochloric acid was added dropwise to the mixture with stirring to make pH 2.0 and the solution was extracted with ethyl acetate. After removal of the solvent, the residue was dissolved in benzene and diluted with hexane to give the adduct as reddish-yellow crystals. The product was homogeneous as judged by TLC with benzene–acetic acid (3: 1) (*Rf* 0.32).

(3) **Preparation of Internal Standard**——A procedure similar to the method used for preparation of the captopril-p·BPB adduct was employed: 2.4 mmol each of p·BPB and thiosalicyclic acid were dissolved in 40 ml of methanol, then the solution was adjusted to pH 7 by dropwise addition of 1.0 N NaOH and allowed to stand for 10 min at room temp. After being washed with hexane in the manner described in (1), the ethyl acetate extract was evaporated by dryness under reduced pressure. Recrystallization of the residue from benzene gave the internal standard as pale yellow plates.

Assay Procedure for Captopril in Whole Blood

A 3 ml portion of the freshly drawn blood was transferred into a glass tube containing 1.5 ml of 0.5% p·BPB in acetone and immediately vortex-mixed for about 30 sec. The mixture was allowed to stand for 5 min to ensure complete adduct formation of captopril, made acidic by addition of 0.3 N hydrochloric acid and stored in a freezer (below −15°). The mixture was extracted successively with 16 ml and 8 ml of benzene. The organic layers were combined and evaporated to dryness *in vacuo*. Four milliliters of 0.05 M phosphate buffer, pH 7, and 20 ml of hexane were added to the residue and the whole was mixed in an ultrasonicator. The mixed solution was then washed with 6 ml of hexane, and the organic layer was discarded. The remaining aqueous layer was acidified with 0.1 ml of 2 N hydrochloric acid and extracted successively with 6 ml and 2 ml of benzene. The benzene extract was combined and 0.5 μg of the internal standard (thiosalicyclic acid-p·BPB adduct) was added. The benzene extract was carefully evaporated to dryness in the manner described above. The final residue was dissolved in 200 μl of acetonitrile and injected into the HPLC. Sample volumes ranging from 5 to 25 μl were used. Calibration was achieved by loading the blood of nontreated subjects with increasing amounts of captopril and a fixed amount of internal standard.

Assay Procedure for Captopril and Oxidized Captopril in Urine

Urine (5 ml) was placed in a test tube containing 2.0 ml of 0.5 M phosphate buffer (pH 7.0) and 0.5 ml of p·BPB solution (20 mg/ml in methanol) was added to the test tube with vigorous shaking. One ml of this solution was transferred into another tube, diluted with 1.5 ml of water and extracted with 6 ml of hexane. To the aqueous layer (2 ml), 0.1 ml of 2% solution of tributylphosphine in methanol was added, and the mixture was heated for 30 min at 50°. Washing with 6 ml of hexane was required to remove excess reductant. The aqueous layer was mixed with 0.2 ml of 0.2% DDPM in acetone and allowed to stand for 5 min at room temp. The reagent was removed by hexane extraction, and the aqueous layer was acidified with 2 N hydrochloric acid (to make pH 1.5; about 0.2 ml) then extracted twice with 6 ml each of benzene. The organic layer was transferred to another tube containing 10 μg of thiosalicyclic acid-p·BPB adduct as an internal standard and evaporated to dryness *in vacuo*. The residue was taken up in 200 μl of methanol and aliquots (5—20 μl) of this solution were injected into the chromatograph.

Calculations

A calibration curve was constructed by plotting the ratio of the peak height of captopril to that of the internal standard against the amount of captopril. The unknown blood or urine concentrations were calculated from the calibration curves thus obtained.

Results and Discussion

Derivatization of Captopril for HPLC

In HPLC, derivatization has been used as a means of improving chromatographic properties, selectivity and sensitivity. p·BPB is a commonly used derivatizing agent for carboxylic acids[11,12] and halocarbonyl compounds such as ICH_2COOH, ICH_2CONH_2, $BrCH_2COOH$.[13] They have been applied widely as alkylating agents for mercapto groups. Our initial effort was therefore directed towards derivatization of a mercapto group of captopril to prevent its oxidation and to provide UV-absorbing properties.

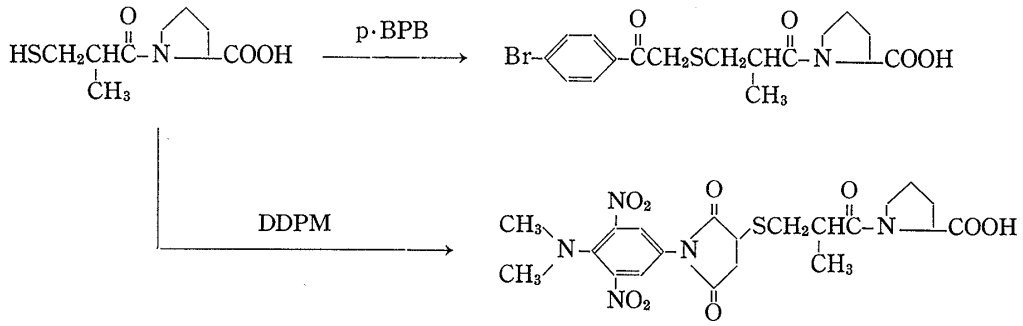


Fig. 1. Derivatization into the Adducts with *p*-Bromophenacyl Bromide and DDPM

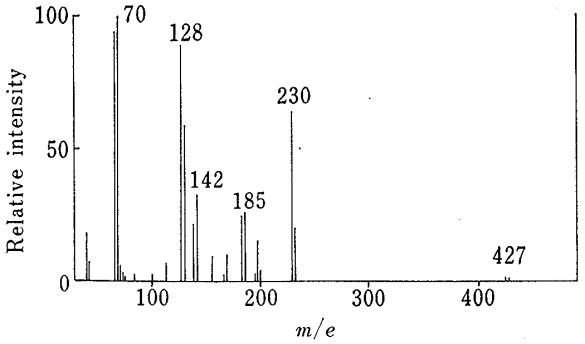


Fig. 2. Mass Spectrum of the Captopril-p·BPB Adduct

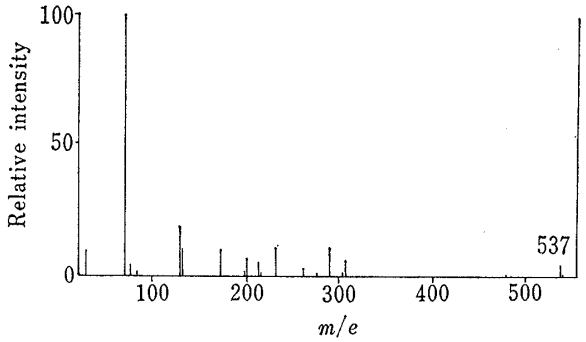


Fig. 3. Mass Spectrum of the Captopril–DDPM Adduct

(1) **Derivatization of Captopril**——The formation of p·BPB and DDPM adducts from captopril is shown in Fig. 1. The derivatives were prepared as described in "Experimental." The structure of captopril-p·BPB adduct was characterized by gas chromatography-mass spectrometry as shown in Fig. 2. The molecular ion peak, $[M]^+$, and fragment ion peak, $[M-197]^+$, formed with the loss of the *p*-bromophenacyl residue appeared at *m/e* 427 and 230, respectively. The base peak at *m/e* 128 was assigned as the methyl ester of proline produced by the fission of an amide linkage. These results, together with the NMR spectral data, indicated that p·BPB alkylated a mercapto group but did not esterify a carboxyl group of captopril under these experimental conditions. The structure of captopril-DDPM adduct was confirmed by gas chromatography–mass spectrometry (Fig. 3). The molecular ion peak, $[M]^+$, appeared at *m/e* 537. This result indicated an addition product structure involving the maleimide moiety in DDPM, as predicted in the literature.[8,9]

(2) **Optimum Conditions for Derivatization of Captopril**——On the basis of the reaction rate studies, a solution containing 10 μg of captopril in 1.0 ml of 0.05 M phosphate buffer, pH 7.0, was used. The reaction was initiated by addition of 50 μl of 0.5% p·BPB solution in acetone at 24° and 4°. Aliquots of the reaction mixture were withdrawn at selected times, and

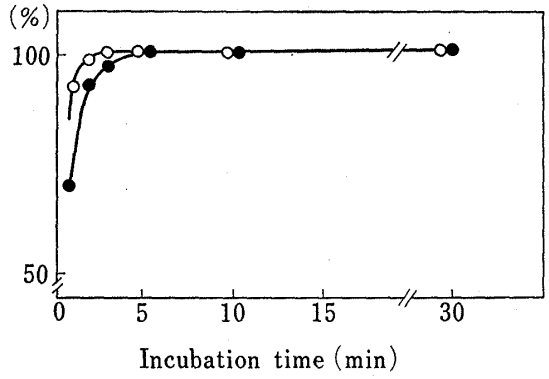


Fig. 4. Time Course of Adduct Formation from Captopril with p·PBB

—●—: incubation at 4°,
—○—: incubation at 24°.
The reaction was initiated by addition of 50 μl of p·BPB solution (5 mg/ml in acetone) to 1.0 ml of reaction mixture containing 10 μg/ml of captopril in 0.05 M phosphate buffer (pH 7.0).

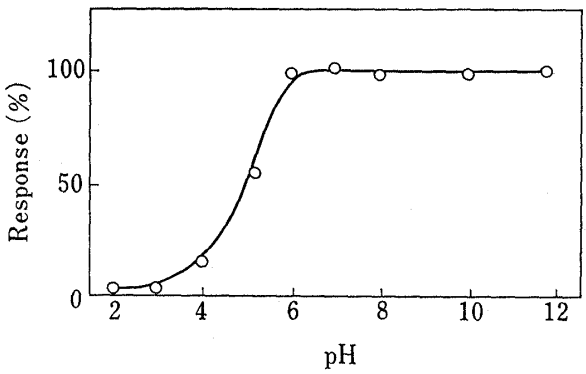


Fig. 5. Effect of pH on the Reaction of Captopril with p·BPB

Reactions were carried out by addition of 50 μl of p·BPB solution (5 mg/ml in acetone) to 1.0 ml of solution containing 10 μg/ml of captopril in Britton–Robinson buffer of various pHs.

the yield was determined by measurement of the peak height of captopril-p·BPB. Fig. 4 shows the amount of captopril-p·BPB adduct formed versus reaction time under two different conditions. The reaction was completed in 5 min. Therefore, the present derivatization procedure with p·BPB was found to be suitable for quantitation purposes. The choice of pH in the derivatization was also investigated. The results indicated that the reaction was essentially complete in the pH range of 6 to 12 (Fig. 5). The pH-dependence of the reaction for captopril with DDPM was studied at 24° for 10 min with captopril (20 μg/ml) and DDPM (50 μg/ml) in Britton–Robinson buffer ranging from pH 2 to 11 (Fig. 6). DDPM reacted rapidly with captopril in aqueous solution at pH 6—8, whereas hydrolysis of the reagent or captopril-DDPM adduct may occur over pH 8. The addition reaction was slowed below pH 6. From these results it was concluded that optimal pH for the addition reaction was 6 to 8.

(3) **UV-absorption Spectrum Studies**—UV-absorption spectra of captopril-p·BPB and captopril-DDPM adducts obtained in 0.02 M phosphate buffer, pH 7.0, are illustrated in Fig. 7. UV monitoring of captopril at 220 nm or below appeared to be promising, but was

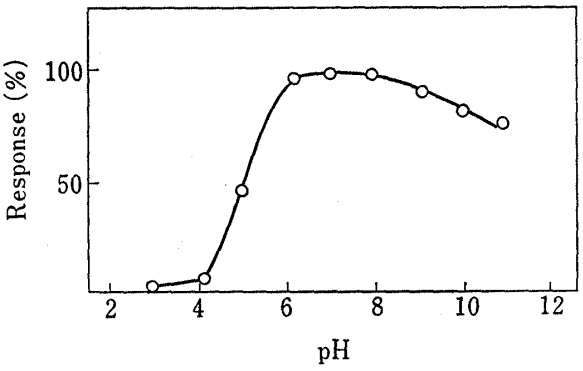


Fig. 6. Effect of pH on the Reaction of Captopril with DDPM

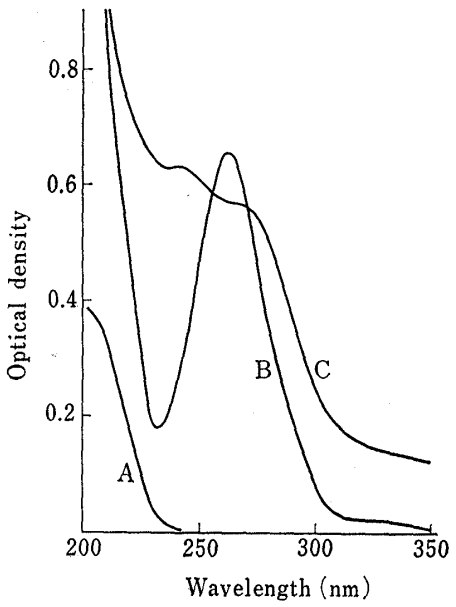


Fig. 7. UV Spectra of Captopril Adducts with p BPB and DDPM

A: captopril,
B: captopril-p·BPB adduct,
C: captopril-DDPM adduct.
Each sample was dissolved in 0.05 M phosphate buffer (pH 7.0) at a concentration of 4.27×10^{-5} M.

not applicable to biological samples due to the low concentration. These adducts of captopril showed ε values of 13860 for p·BPB and 14130 for DDPM at 254 nm, which provided a detection limit of approximately 0.2 ng.

Stability of Captopril in Solutions and Biological Samples

Since thiols are readily oxidized,[13] the stability of captopril in solutions was studied in order to find suitable conditions for the preservation of samples before analysis. Portions of each solution were stored at room temperature (24°) and at 4°. Table I shows the stability of captopril (10 μg/ml) in Britton–Robinson buffer solutions of various pHs stored at room temperature. Similar experiments (Table II) with non-treated human whole blood and plasma indicated that these blood samples should be kept as cold as possible and also should be acidified immediately, because captopril is unstable in these media. To avoid significant loss of captopril by oxidation, captopril in these media should be stabilized immediately after the collection of blood. The stability of captopril in human and dog urine was also studied. The results indicated that urine specimens should be derivatized or acidified immediately after collection in order to avoid significant loss of captopril, because the pH of urine was variable. Figure. 8 shows that human urine samples should also be stabilized immediately.

Table I. Stability of Captopril in Solutions of Various pHs at Room Temperature (Percent Remaining)

	Incubation time (hr)									Rate constant
pH	0.5	1	2	4	8	24	48	72	96	k (hr^{-1})
1.0	—	—	—	—	—	100.4	100.9	100.2	98.5	—
2.1	—	—	—	—	—	98.8	99.5	96.6	99.3	—
3.0	—	—	—	—	—	76.9	55.7	44.1	32.5	0.0118
4.0	—	—	—	—	—	77.9	51.4	44.1	27.3	0.0122
5.0	—	—	—	—	—	68.4	50.4	41.3	25.3	0.0143
6.0	—	—	—	—	—	68.1	51.6	35.4	24.4	0.0147
7.0	—	—	—	80.5	57.5	30.6	8.5	—	—	0.0499
7.9	98.2	87.1	74.2	55.8	33.3	—	—	—	—	0.146
9.2	96.2	81.7	60.7	45.8	21.7	—	—	—	—	0.195
9.7	82.4	66.4	44.0	18.7	—	—	—	—	—	0.425

Table II. Stability of Catopril in Biological Fluids (Percent Remaining)

Biological fluids		Incubation time (min)							Rate constant k (hr^{-1})
		5	15	30	60	120	180	240	
Human plasma	24°	90.4	67.8	50.0	25.3	7.2			1.26
	4°	99.4	94.7	88.3	79.8	64.8	48.2	44.9	0.18
Human whole blood	24°	89.5	77.9	54.7	36.0	17.5	—	—	1.10
	4°	98.6	95.7	90.3	79.5	74.2	51.3	47.2	0.18
Dog whole blood	24°	88.1	77.5	56.3	33.1	11.5	—	—	1.08
	4°	100.6	94.2	84.0	73.8	64.9	—	—	0.24
Human urine	24°	—	—	—	94.6	87.0	81.0	75.0	0.072
(pH 6.1)	4°	—	—	—	98.2	99.0	97.7	98.5	—
Dog urine	24°	—	—	—	47.6	23.4	11.0	3.8	0.759
(pH 7.5)	4°	—	—	—	97.6	94.0	91.2	87.3	0.033

Captopril in Human Whole Blood

The proposed method is described in "Experimental." Suitable conditions for extraction were examined with regard to agitation time, solvent and solvent volume. Of the solvents

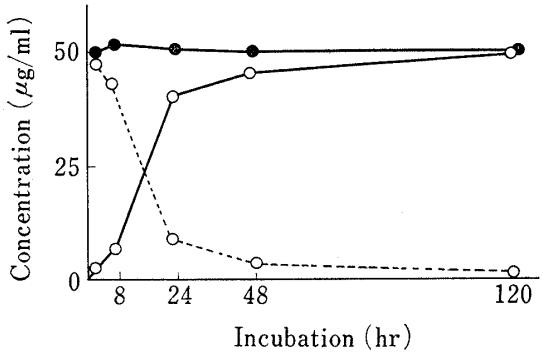


Fig. 8. Time Course of the Changes of Captopril and Oxidized Captopril in Human Urine at Room Temperature

···○···: captopril,
—○—: oxidized captopril,
—●—: sum of free captopril and oxidized captopril.

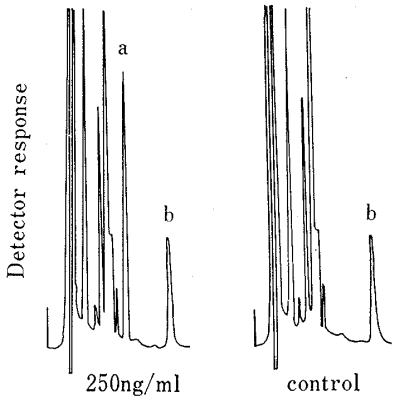


Fig. 9. HPLC Chromatograms of Extracts from Human Whole Blood

Portions (3 ml) of human whole blood containing 250 ng/ml of added captopril or without the drug were treated according to the assay procedure.
a: captopril, b: internal standard.

tested (ethyl acetate, benzene, methylene chloride, ethyl ether and hexane), benzene gave the best result. A good base line was obtained when washing was carried out with hexane in the neutral pH media. Representative chromatograms of the extract from human whole blood to which 250 ng/ml had been added and control blood extract are shown in Fig. 9. Under the chromatographic conditions described, no peak corresponding to captopril was observed on the control chromatogram.

Captopril in Human Urine

The procedure for total captopril assay gives the sum of the free captopril and its oxidized products. Captopril is stabilized in the form of the adduct with p·BPB, as described in the case of blood, and the oxidized captopril is converted to free captopril and also stabilized with DDPM. These two adducts separated and determined by HPLC. The assay procedure for the oxidized form is based on the quantitative reduction of the oxidized forms with tributyl-

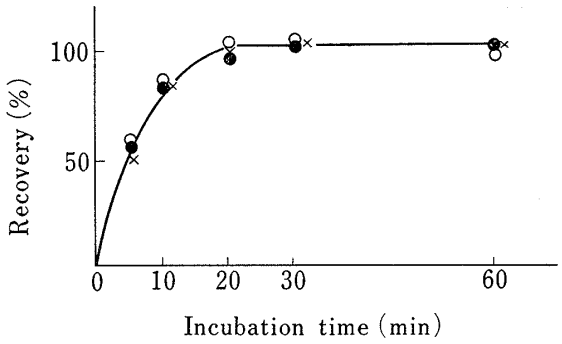


Fig. 10. Effect of the Incubation Time on the Reduction of Oxidized Captopril with Tributylphosphine

●: in H_2O, ○: in human urine, ×: in dog urine.

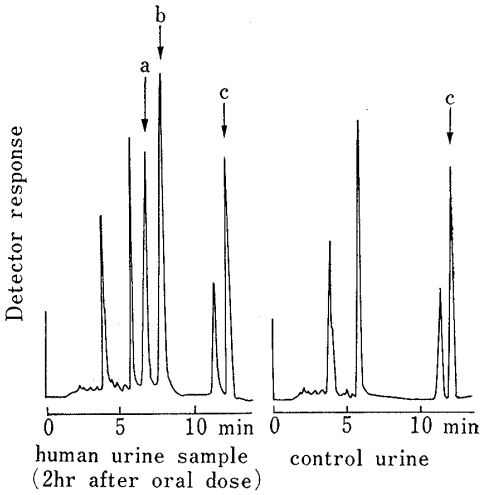


Fig. 11. HPLC Chromatograms of Extracts from Human Urine with or without Added Captopril

a: captopril, b: oxidized captopril,
c: internal standard.
Urine sample of a patient who had received 50 mg of captopril orally (2 hr after administration).

phosphine. Several methods for reduction of disulfides were studied. Attempts to achieve the chemical reduction of oxidized captopril with mercapto compounds such as dithiothreitol, cysteine, mercaptoethanol and sulfide met with failure. The use of phosphines for reduction of disulfides, which has been reported by Overman[14] and Humphrey,[15] was found to be applicable to oxidized captopril, and the reaction was completed quantitatively within 30 min at 50°, as shown in Fig. 10. Quantitative reduction of oxidized captopril with tributylphosphine was effective between pH 7—11. Captopril-p·BPB adduct formed from free captopril was stable against reduction with tributylphosphine. The percent recoveries of oxidized captopril added to human urine were 95.2±4.4 (2.5 μg/ml), 97.0±4.6 (5.0 μg/ml), 100.5±3.8 (10 μg/ml) and 101.7±4.7 (20 μg/ml), respectively. Representative HPLC chro-

Table III. Recovery of Captopril Added to Human Whole Blood

Added μg/ml	Captopril	Found μg/ml
0.25		0.230
		0.250
		0.235
		0.245
		0.255
	Mean±SD	0.243±0.005
0.50		0.525
		0.530
		0.470
		0.475
		0.470
	Mean±SD	0.496±0.013
1.00		1.080
		0.965
		0.940
		1.045
		1.005
	Mean±SD	1.007±0.026

Table IV. Recovery of Free and Oxidized Captopril added to Human Urine

Free captopril		Oxidized captopril	
Added, μg/ml	Found, μg/ml	Added, μg/ml	Found, μg/ml
2.50	2.49	2.50	2.54
	2.50		2.36
	2.50		2.41
	2.52		2.34
	2.70		2.23
Mean±SD	2.54±0.09	Mean±SD	2.38±0.11
5.00	5.35	5.00	4.87
	5.20		5.01
	5.20		4.86
	5.25		5.12
	5.35		4.71
Mean±SD	5.25±0.07	Mean±SD	4.85±0.23
10.0	9.20	10.00	10.42
	10.00		10.32
	9.50		10.01
	10.00		10.00
	8.60		9.47
Mean±SD	9.50±0.60	Mean±SD	10.05±0.38

matograms of extracts from urine of a patient who had received 50 mg of captopril orally and from control urine are shown in Fig. 11.

Precision and Sensitivity

In order to check the validity of the proposed method, a known amount of captopril was added to human whole blood and the recovery was determined. The precision of the method was examined by comparing the results of five replicate assays at each concentration level, as shown in Table III. The mean observed values were 243, 496 and 1007 ng/ml for human whole blood samples to which 250, 500 and 1000 ng/ml had been added, respectively. The coefficient of variation was below 6.0%. In the case of urine, as shown in Table IV, the mean observed values were 2.54, 5.25 and 9.50 μg/ml of free captopril, or 2.38, 4.85 and 10.05 μg/ml of oxidized captopril at concentrations of 2.5, 5.0 and 10.0 μg/ml added to human urine samples, respectively. Good linearity was obtained in the range of 5 ng/ml to 2 μg/ml captopril for whole blood and 100 ng/ml to 20 μg/ml captopril in urine.

The proposed method for the determination of captopril in human biological fluids such as whole blood and urine is satisfactory with respect to sensitivity, rapidity, accuracy and precision. It may also be applicable to the determination of captopril in dog blood and urine.

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References and Notes

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