Journal of Chromatography, 239 (1982) 585–594
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,545

DETERMINATION OF CAPTOPRIL AND ITS DISULPHIDE IN BIOLOGICAL FLUIDS

YASUHIKO MATSUKI*, TOMIHARU ITO, KATSUHARU FUKUHARA, TETSUYA NAKAMURA, MICHIKO KIMURA and HIROSHI ONO
Hatano Research Institute, Food and Drug Safety Centre, Hadano, Kanagawa (Japan)
and
TOSHIO NAMBARA
Pharmaceutical Institute, Tohoku University, Aobayama, Sendai (Japan)

SUMMARY

A gas chromatographic–mass spectrometric method for the simultaneous determination of captopril (SQ 14,225) and its disulphide (SQ 14,551) in biological fluids by means of selected ion monitoring is described. In order to prevent oxidative degradation, captopril was treated with N-ethylmaleimide (NEM). The captopril–NEM adduct and the disulphide were converted into the hexafluoroisopropyl esters, which were separated on a 10% Dexsil 300GC column and determined by employing the captopril–N-butylmaleimide adducts as an internal standard. The blood and urine levels of captopril and its disulphide in dogs to which captopril had been administered orally were measured by the proposed method. The urinary excretion of these two substances in rats was also determined in a similar manner.

INTRODUCTION

Captopril [1-(D-3-mercapto-2-methyl-1-oxopropyl)-L-proline] (SQ 14,225) is a potent inhibitor of angiotensin-converting enzyme, one of the most important components in the renin–angiotensin system[1–4]. It was demonstrated that the disulphide is a major urinary metabolite in living animals[5,6] and exhibits pharmacological activity in the dog[7]. Both *in vitro* and *in vivo* experiments revealed that captopril is readily converted into the disulphide and unknown sulphur-containing metabolites[8]. A simple method for the gas chromatographic determination of captopril in blood and urine using a flame photometric detector has previously been reported[9]. However, suitable conditions for quantitative derivatization of captopril into the N-ethylmaleimide (NEM) adduct still remain to be established. The methods so far available for the determination of captopril in biological fluids involve thin-layer chromatography (TLC) using a radioactive substrate[5,6,8] and gas chroamtography–mass spectrometry (GC–MS)[10]. These methods, however, are not necessarily satisfactory with respect to feasibility, reliability and sensitivity. An urgent need to investigate the metabolic fate and pharmacokinetics of captopril and its disulphide administered

0021-9673/82/0000–0000/$02.75

orally to dogs and rats prompted us to develop a method for their simultaneous determination.

This paper describes a GC–MS method for the determination of captopril and its disulphide in blood and urine by means of selected ion monitoring.

EXPERIMENTAL

Gas chromatography–mass spectrometry

A Shimadzu Model LKB-9000B gas chromatograph–mass spectrometer was used. A coiled glass column (1 m × 3 mm I.D.) was packed with 10% Dexsil 300GC on Gas-Chrom Q (80–100 mesh). The flow-rate of the carrier gas (helium) was 30 ml/min. The temperature of the column was 258°C, and the injection port and ion source were kept at 270°C. The accelerating voltage, ionization voltage and trap current were 3.5 kV, 70 eV and 60 μA, respectively.

Materials

Captopril (SQ 14,225) and its disulphide (SQ 14,551) were kindly donated by Sankyo Co. (Tokyo, Japan). Hexafluoroisopropanol (HFIP), trifluoroacetic anhydride (TFAA), N-ethylmaleimide (NEM) and other chemicals were of analytical-reagent grade. Piperidinohydroxypropyl Sephadex LH-20 (PHP-LH-20) was prepared in these laboratories by a previously reported method[11]. Amberlite XAD-2 resin was purified prior to use. The resin was stirred with 0.6% hydrochloric acid in 70% ethanol at 70°C for 24 h and washed successively with water, 0.6% sodium hydroxide in 70% ethanol, water, acetone and ethanol. The purified resin was further washed with water and stored in a refrigerator. When used for blood and plasma specimens, the resin should first be washed with ethanol.

Synthesis of captopril–N-butylmaleimide adduct

A solution of maleic anhydride (60 mmol) in chloroform (60 ml) was added dropwise to a stirred solution of *n*-butylamine (50 mmol) in chloroform (50 ml) at 0°C, and the resulting solution was evaporated to dryness. The residue was dissolved in acetic anhydride (125 ml) and the solution heated with anhydrous sodium acetate (6.0 g) at 100°C for 1 h and then at 120°C for 10 h. The reaction mixture was poured into ice–water to decompose the excess of the reagent, and the resulting solution was adjusted to pH 8–9 with 30% sodium hydroxide and 5% sodium hydrogen carbonate. After removal of the precipitate by filtration, the filtrate was extracted twice with 100-ml volumes of *n*-hexane. The organic layer was evaporated and the oily residue was chromatographed on silica gel (50 g) using *n*-hexane–benzene (9:1) as eluent. The eluate was subjected to preparative TLC using chloroform–benzene (9:1) as developing solvent. Elution of the area corresponding to the spot gave N-butylmaleimide (NBM) (1.2 g) as a colourless oil (Mass spectrum: *m/z* 153, $[M]^+$). The product was homogeneous as judged by TLC.

The captopril–NBM adduct was prepared from captopril and NBM as described in a previous paper[9]. The desired compound was not obtained in the crystalline state but was substantially homogeneous as judged by TLC; mass spectrum (HFIP derivative), *m/z* 520 ($[M]^+$); TLC [chloroform–methanol–acetic acid (30:8:0.5)], R_F 0.68.

Derivatization into hexafluoroisopropyl ester

Captopril and its disulphide were dissolved in HFIP (0.3 ml) and TFAA (0.6–60 μl) and the solution was heated at 50°C for 60 min. After removal of the excess of reagents with aid of a stream of nitrogen, the residue was dissolved in ethyl acetate (100 μl) and a 1-μl aliquot of this solution was subjected to GC–MS.

Assay of captopril and its disulphide in blood, plasma and urine

A 1-ml volume of blood was diluted with 0.5% NEM in 0.2 *M* phosphate buffer (2 ml) containing an internal standard and washed twice with 10-ml volumes of benzene. The aqueous layer was treated with 20 ml of ethanol for deproteinization. The precipitate was removed by centrifugation at 1600 *g* for 15 min and the supernatant was evaporated to dryness. The residue was dissolved in 5 ml of ethanol and percolated through an Amberlite XAD-2 resin column (15 cm × 10 mm I.D.). The desired fraction was obtained by elution with 45 ml of ethanol, and the eluate was evaporated to dryness. The residue was dissolved in 1 ml of 90% ethanol and an aliquot of the solution (0.8 ml) was applied gently to a PHP-LH-20 column (20 mm × 5 mm I.D.). After washing with 15 ml of 90% ethanol, captopril and its disulphide were eluted with 15 ml of 7% acetic acid in ethanol. The two compounds were converted into the hexafluoroisopropyl esters with HFIP (0.3 ml) and TFAA (0.03 ml) and then subjected to GC–MS. Clean-up of a plasma sample (0.5 ml) was carried out in a similar manner.

With urine, 0.1 ml of a specimen was treated with 0.5% NEM in 0.2 *M* phosphate buffer (2 ml) and washed twice with 5-ml volumes of *n*-hexane. After complete removal of *n*-hexane with the aid of a stream of nitrogen the aqueous phase was adjusted to pH < 2 with 0.5 *N* hydrochloric acid. The resulting solution was percolated through an Amberlite XAD-2 resin column (15 cm × 10 mm I.D.) for removal of water-soluble compounds. After washing with 200 ml of water, captopril and its disulphide were eluted with 50 ml of 90% ethanol, and the eluate was evaporated to dryness. The subsequent procedure was carried out as described above.

Stability of NEM in phosphate buffer solution

To a canine urine specimen (0.1 ml) was added 0.5% NEM in phosphate buffer (2 ml) which had previously been left at room temperature for 3 or 24 h. The determination of captopril and its disulphide was carried out according to the procedure described above.

Effect of the amount of NEM on the formation of captopril–NEM adduct

To a canine urine specimen (0.1 ml) was added freshly prepared 0.01–1.0% NEM in 0.1 *M* phosphate (2 ml). The determination of captopril and its disulphide was carried out according to the procedure described above.

Stability of captopril and its disulphide in blood, plasma and urine

A canine blood specimen was divided into two portions. The plasma was obtained from one of these by centrifugation at 1600 *g* for 10 min. To the blood specimen (1.0 ml) was added freshly prepared 0.5% NEM in 0.1 *M* phosphate buffer at intervals of 15, 30 or 60 min. To the plasma specimen was also added freshly prepared 0.5% NEM in 0.1 *M* phosphate buffer at intervals of 1 or 3 h. The determi-

nation of captopril and its disulphide was carried out according to the procedure described above.

Recovery tests on captopril and its disulphide added to blood, plasma and urine

The test samples were prepared by dissolving *ca* 100 ng of captopril–NEM, 1000 ng of the disulphide and 100 ng of internal standard in canine blood (1 ml), plasma (0.5 ml) and urine (0.1 ml). Each sample was diluted with 2 ml of 0.5% NEM in 0.2 *M* phosphate buffer. The determination of captopril and its disulphide was carried out according to the procedure described above.

Determination of captopril and its disulphide in blood and urine

Female CSK strain beagle dogs (body weight 11.2–12.3 kg) were orally given a single dose of 10 mg/kg of captopril in a gelatine capsule. The blood was withdrawn from a superficial vein, and urine specimens were collected at several intervals through a catheter inserted into the bladder. To the blood (1.0 ml) or urine (0.1 ml) specimen was added 2 ml of freshly prepared 0.5% NEM in 0.2 *M* phosphate buffer.

Sprague Dawley rats (body weight 277–318 g) were orally given through a catheter a single dose of 10 mg/kg each of captopril and its disulphide suspended with 1% carboxymethylcellulose solution. The urine was collected for 24 h throught the metabolic cage into the beaker containing 12.5 ml of 1% NEM in 0.2 *M* phosphate buffer. The determination of captopril and its disulphide was carried out according to the procedure described above.

RESULTS AND DISCUSSION

It has previously been demonstrated that significant amounts of oxidation products are formed from captopril in blood unless it is immediately derivatized[10]. Therefore, initial efforts were directed to establishing suitable conditions for derivatization into the captopril–NEM adduct in the assay procedure.

First, the stability of an NEM solution in phosphate buffer against oxidative decomposition was examined. The urine captopril and its disulphide in dogs administered captopril were determined. The use of NEM reagents that had been left at room temperature for 3 or 24 h after preparation gave the values corresponding to 89% and 84% of the control, respectively. In contrast, there was observed no substantial change in the value for the disulphide (Table I). The immediate treatment of a urine specimen with freshly prepared reagent containing NEM in the range 0.2–20 mg

TABLE I

STABILITY OF NEM IN PHOSPHATE BUFFER

A urine specimen from a dog administered captopril was used for the test.

Time after administration (h)	*Captopril (μg/ml)*	*Disulphide (μg/ml)*
0	175.34	332.44
3	152.68	340.03
24	148.39	342.62

TABLE II

STABILITY OF CAPTOPRIL AND ITS DISULPHIDE IN BLOOD AND PLASMA

Blood (1.0 ml) and plasma (0.5 ml) specimens from a dog administered captopril were used for the test.

Time after administration (min)	*Blood*		*Plasma*	
	Captopril (ng)	*Disulphide (ng)*	*Captopril (ng)*	*Disulphide (ng)*
0	943.8	222.8	–	–
15	825.7	190.6	654.5	428.7
30	684.6	194.9	381.9	475.6
60	587.4	207.0	144.7	443.5

gave substantially identical values for captopril. The use of NEM in these amounts was sufficient to provide the captopril–NEM adduct quantitatively.

The stability of captopril and its disulphide in blood, plasma and urine was also examined (Tables II and III). Employing freshly prepared NEM solution, captopril and its disulphide were determined in blood and plasma specimens left for 15, 30 and 60 min and urine pooled for 1 and 3 h at room temperature. It is evident from the results that a considerable decrease in the level of captopril in plasma was observed as compared with that in blood, although no plausible explanation can be given at present. On the other hand, there was no difference in the disulphide levels in blood and plasma. These results indicated that captopril underwent no oxidation into the disulphide in biological fluids.

TABLE III

STABILITY OF CAPTOPRIL AND ITS DISULPHIDE IN URINE

Urine specimens from a dog administered captopril were used for the test.

Time after administration (h)	*Captopril (μg/ml)*	*Disulphide (μg/ml)*
0	175.34	332.44
1	113.09	336.88
3	66.77	330.80

The utility of the hexafluoroisopropyl ester as a derivative for the GC of captopril has been demonstrated in a previous paper[9]. The disulphide and captopril–NEM were simultaneously derivatized with TFAA and HFIP in various proportions. When treated with these two reagents at 50°C for 60 min, the disulphide was converted into the bis(hexafluoroisopropyl) ester (Fig. 1). The esterification rate was plotted against the ratio of the two reagents in the range 0.002–0.2 as illustrated in Fig. 2. Based on these data, TFAA and HFIP in a ratio of 0.1 was used for derivatization.

The structure of the derivatized disulphide was characterized by GC–MS (Fig. 3). The molecular ion ($[M]^{+}$) and fragment ion ($[M-366]^{+}$) formed by cleavage of the disulfide bond appeared at m/z 732 and 366, respectively. The base peak at m/z

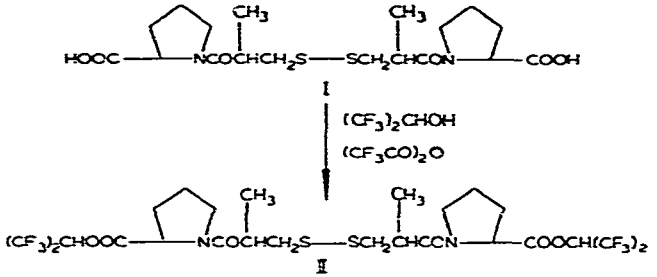

Fig. 1. Derivatization of the disulphide (I) into the bis(hexafluoroisopropyl) ester (II).

264 was assignable to the hexafluoroisopropyl ester of proline produced by the fission of the amide linkage[9]. Among the closely related compounds prepared in these laboratories, the captopril–NEM adduct was chosen as a pertinent internal standard. Dexsil 300GC was the most suitable stationary phase for the separation of captopril–NEM, the disulphide and internal standard. A typical chromatogram obtained by selected ion monitoring is illustrated in Fig. 4. These compounds gave a single peak of the correct theoretical shape.

Calibration graphs were constructed by plotting the ratio of the peak of captopril and its disulphide to that of the internal standard against the amount of the former two. Satisfactory linearity was observed in the range 2.5–10 ng of captopril and its disulphide (Fig. 5). The detection limits of these two were calculated to be 0.5 and 1 ng, respectively.

The utility of the present method for the quantitation of captopril and its disulphide in blood and urine was tested. Clean-up of a blood specimen was readily achieved by extraction with benzene for removal of lipids followed by deproteinization with ethanol. Without the extraction step, captopril and its disulphide were recovered at unsatisfactory levels. The separation of water-soluble compounds in blood, plasma and urine was effected by the use of Amberlite XAD-2 resin. Efficient purification of captopril–NEM and the disulphide was attained by chromatography on PHP-LH-20. Captopril–NEM, the disulphide and the internal standard were simultaneously eluted with 7% acetic acid in ethanol. In order to check the validity of the

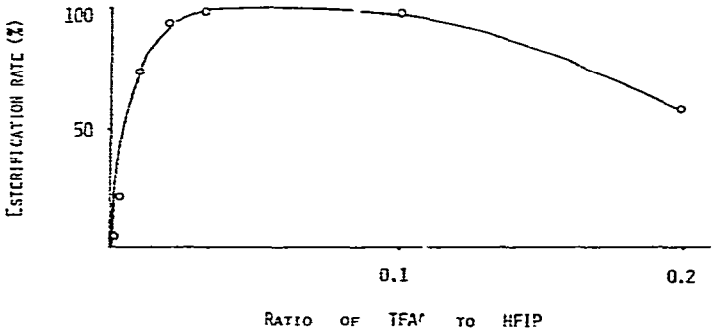

Fig. 2. Effect of TFAA on esterification of the disulphide. The reaction was carried out with hexafluoroisopropanol (0.3 ml) and trifluoroacetic anhydride (0.6–60 μl) at 50°C for 60 min.

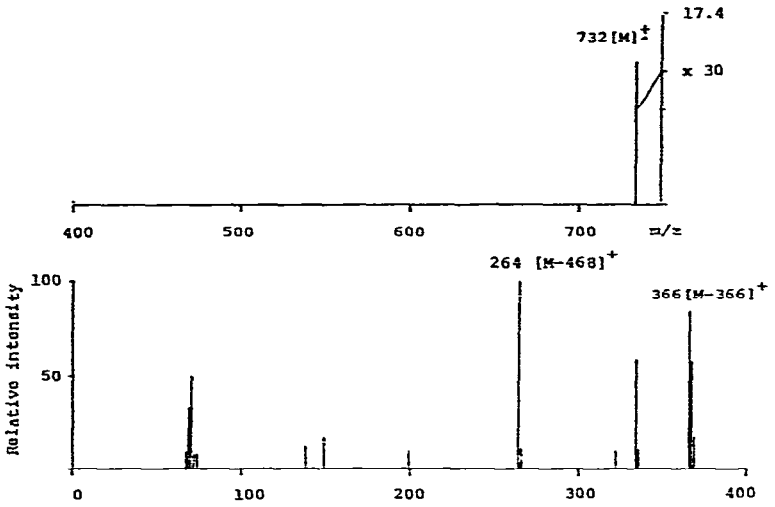

Fig. 3. Mass spectrum of the disulphide bis(hexafluoroisopropyl) ester.

proposed method, known amounts of captopril–NEM and the disulphide were added to biological fluids and their recovery rates were determined by the proposed method. The results obtained with blood, plasma and urine specimens are given in Table IV.

The blood and urine levels of captopril and its disulphide were determined after oral administration of captopril (10 mg/kg) to two dogs. The changes in blood levels of these two are illustrated in Fig. 6. The maximum blood levels of captopril and its disulphide were observed at 1 and 1.5 h, respectively. A significant difference in the change of the disulphide level was observed between the two dogs.

The data for cumulative urinary excretion in dogs are given in Table V. The

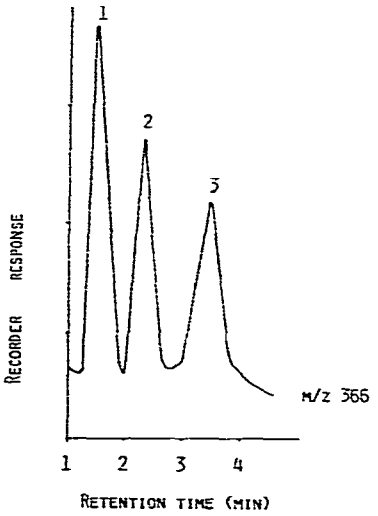

Fig. 4. Chromatogram of captopril (1), internal standard (2) and the disulphide (3) obtained by selected ion monitoring.

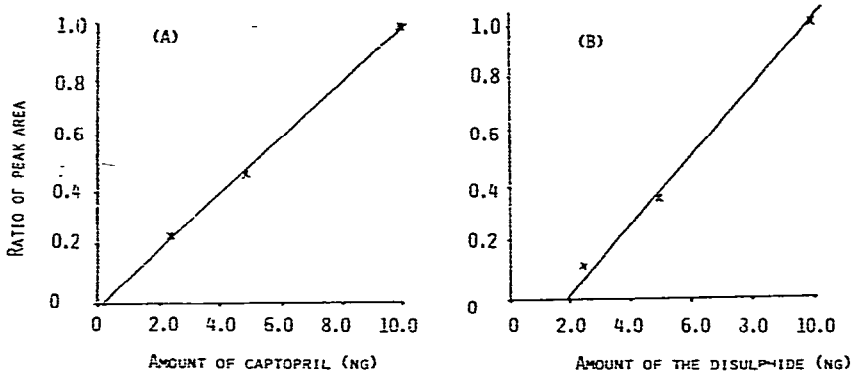

Fig. 5. Calibration graphs for captopril (A) and its disulphide (B).

TABLE IV

RECOVERY OF CAPTOPRIL AND ITS DISULPHIDE ADDED TO BLOOD, PLASMA AND URINE SPECIMENS FROM A DOG

Compound	*Added (μg)*	*Blood**		*Plasma***		*Urine***	
		Found (μg)	*Recovery (%)*	*Found (μg)*	*Recovery (%)*	*Found (μg)*	*Recovery (%)*
Captopril	0.10	0.098	98	0.086	86	0.092	92
Disulphide	1.00	0.85	85	0.88	88	0.87	87

* $n = 5$.
** $n = 6$.

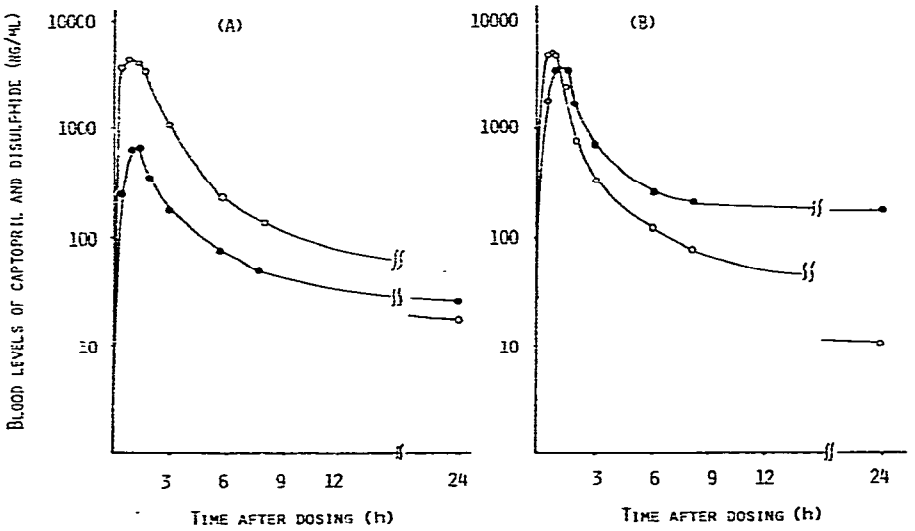

Fig. 6. Change in blood levels of captopril (○) and its disulphide (●) in dog 1 (A) and dog 2 (B). A single dose of captopril (10 mg/kg) was given orally to each dog.

TABLE V

CUMULATIVE URINARY EXCRETION OF CAPTOPRIL AND ITS DISULPHIDE IN DOGS

A single dose of captopril (10 mg/kg) was given orally to each dog.

Time after administration (h)	*Amount excreted (% of dose)*			
	Dog 1		*Dog 2*	
	Captopril	*Disulphide*	*Captopril*	*Disulphide*
0– 1	17.7	n.d.*	15.5	2.7
1– 2	7.4	n.d.*	19.9	3.7
2– 3	1.5	2.8	6.1	0.9
3– 6	0.7	2.9	5.1	0.7
6– 8	0.2	1.3	1.1	0.2
8–24	0.4	0.3	0.1	0.1
0–24 (total)	27.9	7.3	47.8	8.3

* Not detectable.

cumulative amounts of captopril and its disulphide excreted in 24 h were calculated to be 37.8% and 7.8% of the dose, respectively.

The data for urinary excretion of captopril and its disulphide in rats to which the compounds had been administered are given in Table VI. These results indicate that the disulphide was absorbed from the intestine to a lesser extent than captopril in the rat. The *in vivo* formation of captopril from the disulphide lent support to previous work[6]. The amount of captopril excreted in urine after administration of captopril was less than that reported by Ikeda *et al.*[5].

The proposed method for the determination of captopril and its disulfide in biological fluids is satisfactory with respect to sensitivity and reliability. It should be noted that solutions of NEM in phosphate buffer should be prepared freshly prior to use.

It is hoped that the availability of a satisfactory method for the determination of captopril and its disulphide will provide more precise knowledge of the relationship between the pharmacological activities and pharmacokinetics of these drugs. Further studies on the pharmacodynamics of captopril and its disulphide in man and experimental animals are being conducted and details will be reported elsewhere.

TABLE VI

URINARY EXCRETION OF CAPTOPRIL AND ITS DISULPHIDE IN RATS FOR 24 h

A single dose of 10 mg/kg of captopril and its disulphide was given orally to each of three rats. Results are means obtained with the three rats.

Drug	*Amount excreted (% of dose)*		
	Captopril	*Disulphide*	*Total*
Captopril	20.9	5.4	26.3
Disulphide	2.6	2.8	5.4

ACKNOWLEDGEMENTS

The authors are grateful to Dr. K. Hashimoto, Director of the Hatano Research Institute, for his support and encouragement throughout this work. They are indebted to Sankyo Co. Ltd. for the generous supply of captopril and its disulphide.

REFERENCES

1 R. R. Vollmer, J. A. Boccagno, D. N. Harris and V. S. Murthy, *Eur. J. Pharmacol.*, 51 (1978) 39.
2 B. Rubin, R. J. Laffan, D. G. Kotler, E. H. O.Keefe, D. A. DeMaio and M. E. Goldberg, *J. Pharmacol. Exp. Ther.*, 204 (1978) 271.
3 D. N. Harris, C. L. Heran, H. J. Goldberg, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 37 (1978) 718.
4 D. W. Cushman, H. S. Cheung, E. F. Sabo and M. A. Ondetti, *Biochemistry*, 16 (1977) 5484.
5 T. Ikeda, T. Komai, K. Kawai and H. Shindo, *Chem. Pharm. Bull.*, 29 (1981) 1416.
6 T. Komai, T. Ikeda, K. Kawai, E. Kameyama and H. Shindo, *J. Pharm. Dynam.*, 4 (1981) 677.
7 N. O'Hara, H. Ono and K. [illegible]shimoto, *Jap. J. Pharmacol.*, 29, Suppl. (1979) 102.
8 K. K. Wong and J. Dreyfuss, *Pharmacologist*, 20 (1978) 213.
9 Y. Matsuki, K. Fukuhara, T. Ito, H. Ono, N. O'hara, T. Yui and T. Nambara, *J. Chromatogr.*, 188 (1980) 177.
10 P. T. Funke, E. Ivashkive, M. F. Malley and A. I. Cohen, *Anal. Chem.*, 52 (1980) 1086.
11 J. Goto, M. Hasegawa, H. Kato and T. Nambara, *Clin. Chim. Acta*, 87 (1978) 141.