Biochemical Pharmacology, Vol. 32, No. 10, pp. 1563–1568. 1983.
Printed in Great Britain.

0006–2952/83 $3.00 + .00

TISSUE DISTRIBUTION OF CAPTOPRIL, REDUCIBLE CAPTOPRIL CONJUGATES AND *S*-METHYLCAPTOPRIL IN THE RAT

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(*Received* 15 *September* 1982; *accepted* 3 *December* 1982)

Abstract—The tissue distribution of captopril, an antihypertensive drug possessing a free sulfhydryl group, and its sulfur-conjugated metabolites was studied in rats by gas chromatography–mass spectrometry at 15, 30 and 60 min following a single 10 mg/kg oral dose of captopril. It was found that tissue accumulation of captopril was rapid with both free and oxidized-forms already present at 15 min post-dose. A maximum concentration of captopril was achieved at 30 min in tissues studied, being substantially higher in kidney (14.2 μg/g), with lesser amounts occurring in liver, lung, heart, blood cells, spleen and plasma in that order. Oxidized disulfide forms of captopril were usually present in the same or slightly higher proportion than free captopril except for liver which only contained detectable disulfides at 15 min after oral dosing. *S*-methylcaptopril was also present at 30 min in all tissues examined with highest levels occurring in liver and kidney (1.05 μg/g) followed by plasma, lung, heart, spleen and blood cells.

Captopril [1-(D-3-mercapto-2-methylpropanoyl)-L-proline (*S,S*)] is a sulfhydryl compound that is a potent and selective inhibitor of angiotensin I converting enzyme both *in vitro* and *in vivo* [1]. Captopril is being increasingly used as an antihypertensive drug in man [2] and side effects such as membranous glomerulopathy, neutropenia, proteinuria and taste loss have been reported [2, 3]. One group [3] have suggested that metabolites of captopril may play a role in determining the nature and time course of adverse reactions to captopril in man. Thus, it becomes important to study the tissue accumulation of captopril and its metabolites in order to define the basis of any adverse reactions to captopril.

Metabolic studies in other laboratories have utilized radio-isotopically labeled captopril [4–6] and have shown that biotransformations occur through the sulfur moiety by forming disulfide conjugates [4, 5] possibly spontaneously [4]. However, there is a need for more specific non-isotopic techniques for studying captopril metabolism and our laboratory have recently identified an *S*-methyl metabolite of captopril in the urine of man by gas chromatography–mass spectrometry [7]. We now report on the modification of this method using selected ion monitoring for increased sensitivity which allowed us to determine the tissue distribution of captopril, total captopril disulfide conjugates and *S*-methylcaptopril in rats following a 10 mg/kg dose of captopril by gavage.

METHODS

Preparation of tissue homogenates. Male Sprague–Dawley rats (160–280 g) were given a 10 mg/kg dose of captopril by gavage of a 2 mg/ml solution. At given times rats were anaesthetized with halothane and various tissues excised including heart, lung, spleen, kidney and liver. Blood was also collected into heparin-tubes and centrifuged at 2000 *g* for 10 min at 4° to separate plasma from the red and white blood cells. Blood cells were resuspended in 5 volumes of 10 mM potassium phosphate buffer, pH 7.4 containing 1 mM EDTA† for assay. Tissues were briefly rinsed free of blood with ice-cold normal saline and homogenized for 60 sec with a Polytron PT-10 homogenizer in 5 volumes of the ice-cold potassium phosphate buffer. The homogenate was finally filtered through two layers of cheese cloth and an aliquot assayed for captopril and metabolites.

Measurement of free captopril and S-methylcaptopril by GC–MS. Free tissue captopril was measured with modification of the plasma captopril assay described by Drummer *et al.* [8] using gas chromatography–mass spectrometry (GC–MS). Tissue homogenates, plasma or resuspended blood cells (200 μl) were added to glass extraction tubes containing 1 mg *N*-ethylmaleimide (10 mg/ml solution in water). Internal standard, YS-980 [3-(3-mercapto-2-methylpropanoyl)-4-thiazolidine carboxylic acid], 2 μg (1 mg/ml solution in acetone), was added and allowed to stand for 15 min. Sodium chloride, *ca.* 2 g and 500 μl 2 M hydrochloric acid were

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† Abbreviations used: EDTA, ethylenediamine-tetraacetic acid; GC–MS, gas chromatography–mass spectrometry; SAM, *S*-adenosylmethionine.

added and vortex mixed for 5 sec. Redistilled ethyl acetate (10 ml) was then added to the tube which was then mixed for 10 min on a reciprocating shaker. Following centrifugation an aliquot of the organic layer (8 ml) was transferred to a fresh glass extraction tube and then evaporated to about 1 ml under a gentle stream of nitrogen. The remaining ethyl acetate was transferred to a glass mini-vial and finally evaporated to dryness. The residue was treated with 50 μl 1,1,1,3,3,3-hexafluoropropan-2-ol and 50 μl perfluorobutyric anhydride for 15 min at 60°. Excess reagents were removed by evaporation under nitrogen and the residue reconstituted with 50 μl dry ethyl acetate. An aliquot (usually 1–2 μl) was then injected into the gas-chromatograph. Derivatives were stable for up to 1 week when stored at 4°.

Measurement of total captopril. Tissue homogenates or plasma (200 μl), or resuspended blood cells (100 μl) were added to glass extraction tubes containing 1 mg dithioerythritol in 2 ml of Tris buffer, 50 mM, pH 9.0. Standards were prepared by adding 0.55, 1.10 μg and 4.40 μg captopril disulfide dimer (solution in methanol) to 200 μl Tris buffer. These tubes were treated as for unknowns. After a 60 min incubation at room temperature the pH was adjusted to 7.0 (range 6.5–7.5) with dilute hydrochloric acid and 2 mg sodium meta-arsenite added (4 mg/ml solution in water). Five minutes later 1 mg *N*-ethylmaleimide and 2 μg internal standard, YS-980 were added. The assay then proceeded in an identical manner to that described for free captopril.

Gas chromatography conditions. For measurement of free captopril and *S*-methylcaptopril a 2 m long × 2 mm i.d. glass column packed with 3% SP-2100 was temperature-programmed from 150° to 290° at a rate of 10°/min following an initial hold time of 1 min. Injector and detector zone temperatures were 280°. Helium (ultra high purity grade) 30 ml/min was the carrier gas.

The gas chromatography conditions for measurement of total captopril were identical to that used for free captopril except that the column was held isothermally at 245°. The run lasted five minutes.

Mass spectrometry conditions. A Finnigan model 4021 combined gas chromatograph–mass spectrometer was used in these studies interfaced by a glass jet separator held at 280°. Electron-impact ionization was used at a source temperature of 300° (corrected). Filament current was 0.25 mA, and the electron energy was 70 eV.

The INCOS software package of the mass spectrometer controlled data acquisition and samples were routinely analysed by selected ion monitoring. The ions 264.1, 334.1, 366.1 and 282.1 were monitored at an acquisition rate of 1.169 scans/sec (Table 1).

Materials. Sources of chemicals were: *N*-ethylmaleimide, Sigma Chemical Co. (St. Louis, MO); dithioerythritol, Calbiochem (San Diego, CA); sodium meta-arsenite, Merck (Darmstadt, Germany); 1,1,1,3,3,3-hexafluoropropan-2-ol, Fluka (Basle, Switzerland) and perfluorobutyric anhydride, Pierce (Rockford, IL.). All other chemicals and reagents were analytical reagent grade. Captopril and captopril disulfide dimer were obtained from Squibb Institute (New Brunswick, NJ) and YS-980 ((4R)-3-((2S)-3-mercapto-2-methylpropanoyl)-4-thiazolidine carboxylic acid) was a generous gift of Dr. J. Iwao, Santen Pharmaceutical (Osaka, Japan). *S*-Methylcaptopril sodium salt was prepared as described previously [8].

RESULTS

Assay specifications

The measurement of captopril using the described GC–MS procedure was relatively simple, specific and highly sensitive. The limit of detection for captopril using selected ion monitoring was 1 ng, and the coefficient of variation was 4.9% at 1.1 μg/ml. The error of measurement was greatly reduced by incorporating an internal standard structurally similar to captopril (YS-980). A chromatogram of a 5 min isothermal run of a blank tissue extract and a tissue extract containing captopril, both with internal standard is shown in Fig. 1. As can be seen the two peaks are well separated. No other observable peaks are present and in this respect chromatograms of tissue extracts and biological fluids like plasma and blood cells are indistinguishable. The short run-time of the chromatogram allows as many as forty samples to be processed in eight hours. Oxidation of captopril to other products was not observed once *N*-ethylmaleimide had been added to tissue homogenates.

The use of temperature-programmed gas chromatography allowed measurement of both the *S*-methylcaptopril metabolite and disulfide oxidation dimer of captopril in one run [8]. However, significant amounts of the disulfide dimer of captopril were not evident in the tissues examined in the present study.

The use of dithioerythritol at pH 9.0 as a reducing agent was both reproducible and quantitative and was used to measure forms of captopril that existed

Table 1. Summary of gas chromatographic and mass spectrometric parameters for hexafluoroisopropanol esters of captopril analogs

Analyte	Retention time	Distribution of ions			
		264	334	366	282
S-methylcaptopril	3.4 mins	100	30	10	0
Captopril*	9.8 mins	100	8	75	0
Captopril disulfide dimer	12.6 mins	75	52	100	0
YS-980	10.6 mins	0	0	0	100

* As *N*-ethylmaleimido adduct with sulfur.

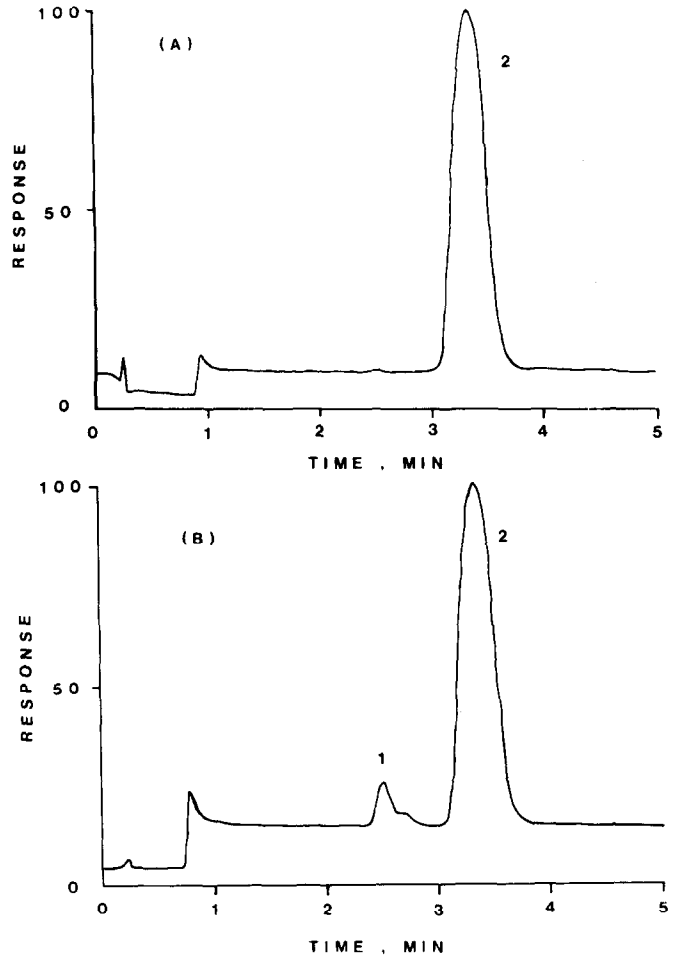

Fig. 1. Chromatograms derived from selected ion monitoring of the ions with m/z 264, 334, 366 and 282 of (a) a blank tissue extract and (b) an extract obtained from a liver homogenate of a rat given captopril 10 mg/kg p.o. at 30 min post dose. Tracings show elution of internal standard (2) and captopril (1) at 3.2 and 2.3 min, respectively. The column effluent was diverted away from the mass spectrometer during the first minute. Typical calibration line for captopril was $y = 0.251x + 0.0002$.

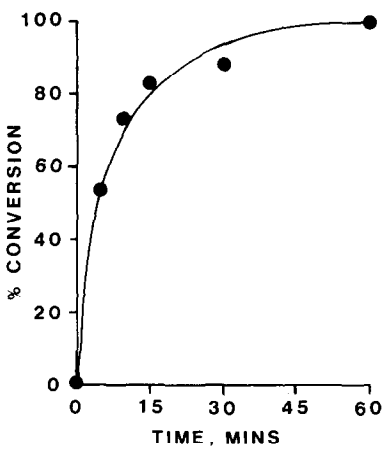

Fig. 2. Time course of the appearance of captopril from captopril disulfide dimer (2.2 μg in plasma) by reduction with dithioerythritol at room temperature (21°). Mean of three experiments.

as disulfide conjugates. The measurement of total captopril, therefore, refers to both free and combined forms of captopril present in tissue homogenates or biological fluids. However, *S*-methylcaptopril is not reducible under these conditions. Assessment of the rate of reduction was accomplished by measuring captopril formation from the disulfide dimer of captopril following various incubation times with dithioerythritol (Fig. 2). Although all apparent oxidized captopril was converted to captopril in 30 min, a 60 min reaction time was employed to give sufficient time for other less readily reducible forms of oxidized captopril to be converted. Known low molecular weight conjugates such as the cysteine and glutathione conjugates were also completely reduced back to captopril in 60 min.

Blood levels of captopril

Blood levels of captopril following a single oral dose of captopril (gavage) to rats were measured at 15, 30 and 60 min (Fig. 3). Free levels of captopril were present in significant amounts in both blood

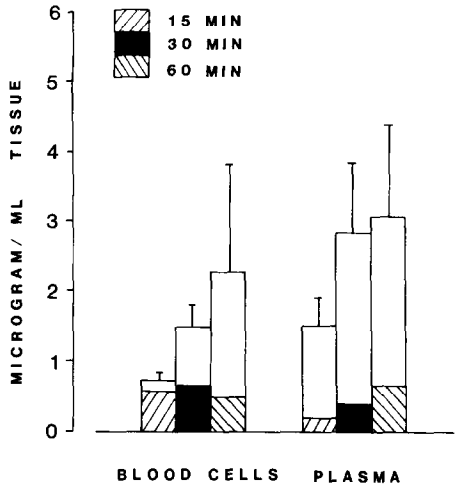

Fig. 3. Concentration of captopril and total captopril disulfide conjugates in blood cells and rat plasma at 15, 30 and 60 min following a single oral 10 mg/kg dose of captopril in the rat. Mean ± S.E.M. of four experiments for 15 min, six to eight experiments for 30 min, three to four experiments for 60 min. Hatched bars refer to free captopril and open bars refer to disulfide conjugated forms of captopril. Each experiment refers to a separate rat. Standard error bars for free captopril which were similar in magnitude to those for total captopril were omitted for clarity.

cells and plasma. At 15 min, captopril in blood cells was 3 times that of plasma, but this ratio had declined to 0.7 at 60 min suggesting that blood cells are freely permeable to captopril. The proportion of captopril present in the form of oxidized metabolites increased steadily over the first hour post dose, as free levels remained steady or declined.

Tissue levels of captopril

The tissue distribution of captopril demonstrated a marked variation with consistently greater amounts present in kidney (Fig. 4). The peak level occurred at 30 min. Liver contents were intermediate with a maximum level of 2.28 μg/g also occurring at 30 min. Heart, lung and spleen although showing significant levels were much lower than for kidney (Fig. 4). A trend indicating a peak of free captopril at 30 min is apparent in all tissues studied with the exception of the spleen.

The amount of free captopril present as oxidized captopril at the different time intervals in the tissues studied was variable with greater amounts present at 60 min than at the earlier times. Heart, lung and spleen contained the highest proportion of conjugated captopril whereas in kidney less than 50% occurred as combined captopril and in the liver where a negligible amount of captopril was found to be conjugated.

When the total tissue amounts of captopril and reducible captopril in the tissues are calculated at the time of maximum free levels (30 min), 2% of the dose administered was present as the free form while only 0.18% was present as disulfide conjugates (Table 2). Calculation of the plasma and blood volume in these rats [9] estimated a total amount of captopril in all measured tissues at 2.4% for free captopril, and 1.51% for disulfide conjugates.

Tissue levels of S-methylcaptopril

Tissue levels of *S*-methylcaptopril were measured in homogenates at 30 min post dose since this time represented the maximum tissue levels of captopril (Table 3). The tissues with the highest content of *S*-methylcaptopril were liver and kidney both with 1.05 μg/g wet weight although a wider variability of

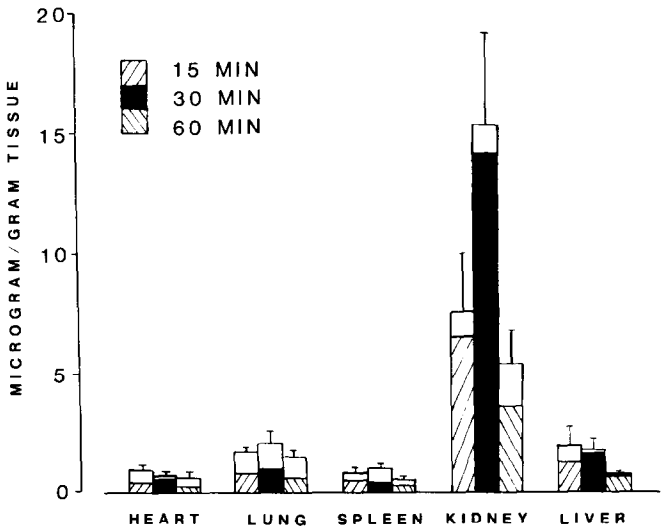

Fig. 4. Concentration of captopril and total captopril disulfide conjugates in heart, lung, spleen, kidney and liver homogenates at 15, 30 and 60 min following a single oral 10 mg/kg dose of captopril in the rat. Mean ± S.E.M. (μg/g wet weight) of four experiments for 15 min, six to eight experiments for 30 min and three to four experiments for 60 min. Hatched bars refer to free captopril and open bars refer to disulfide conjugated forms of captopril. Each experiment refers to a separate rat. Standard error bars for free captopril which were similar in magnitude to those for total captopril were omitted for clarity.

Table 2. Tissue content of captopril, reducible captopril and *S*-methylcaptopril at 30 min post dose

Tissue	Content (μg) Captopril	Reducible captopril	*S*-methylcaptopril
Heart	0.70	0.11	0.17
Lung	1.24	1.42	0.27
Spleen	0.34	0.44	0.07
Kidney	24.28	2.31	1.8
Liver	20.41	0	9.39
Total, percentage of dose	2.0	0.18	0.13

tissue levels was observed in liver. Plasma also contained appreciable amounts of this metabolite (0.47 μg/ml). All other tissues contained measurable levels which were significantly ($P < 0.05$ unpaired *t*-test) less than kidney. Heart, lung, spleen and blood cells contained the lowest amounts of this metabolite which were not significantly different from one another ($P > 0.05$). The total amount of *S*-methylcaptopril in tissues was 0.13% of the dose at 30 min (Table 2) which was only marginally less than reducible conjugates of captopril when the contribution of plasma and blood cells were taken into consideration [9]. 0.71% of the captopril dose given was present as *S*-methylcaptopril in the tissues studied. The concentration of this metabolite in plasma at 60 min had declined to 0.27 ± 0.12 ng/ml ($n = 4$) suggesting that peak levels of *S*-methylcaptopril were occurring at approximately 30 min post dose.

DISCUSSION

Metabolic and disposition studies of captopril, although limited have shown a rapid elimination of unchanged captopril in a number of species including man [4, 7] rat [6, 8], dog and monkey [11]. Metabolites of captopril are known and are exclusively based on conjugates of the free sulfhydryl group [5, 6]. These are either low molecular weight conjugates with endogenous thiols such as cysteine, glutathione and *N*-acetylcysteine or unidentified high molecular weight conjugates with peptides or proteins. More recently, an *S*-methyl conjugate of captopril has been described in the urine of man [7]. We have previously found the rat to rapidly eliminate free and conjugated forms of captopril and *S*-methylcaptopril in the urine which were comparable to the urinary excretion of captopril found in man [7].

Since local tissue levels of these metabolites or of the parent drug are more likely to reflect a drug-induced toxicity in a particular organ, it was decided to develop a specific method to allow measurement of all known forms of captopril. It was clearly not possible to measure every disulfide conjugate, especially since unknown protein-bound metabolites are present. Hence, a biochemical reduction using dithioerythritol was developed in our laboratory and used in conjunction with our previously reported GC–MS assay [8] for captopril, *S*-methylcaptopril and the disulfide dimer of captopril. This allowed measurement of total reducible captopril, i.e. captopril combined with other thiol compounds as disulfides which can be reduced back to free captopril. *S*-methylcaptopril is not a reducible form of captopril and can be measured separately from captopril and the reducible forms.

Following the oral administration of captopril at a dose comparable with other studies, the tissue distribution of captopril and reducible captopril were widespread but extremely variable, with extensive accumulation of both forms occurring in the kidneys. At 30 min (peak concentration) the level of the captopril in the kidney was 6 fold higher than the liver and 32 fold higher than the spleen. The order of tissue distribution for captopril at 30 min was kidney >> liver > lung > heart > spleen. Plasma levels were moderate but still much lower than kidney.

The rapid tissue distribution of captopril would be consistent with a rapid absorption process. This is indicated from pharmacokinetic studies of captopril in man which have shown early peak plasma levels (30 min) using both HPLC [10] and thin layer radiochromatographic techniques [4].

The peak rat blood cell level was also obtained at 30 min and it was interesting to note that the partition ratio of captopril in blood cells and plasma declined from a ratio of 3.1 at 15 min to 0.70 at 60 min. Blood cells appear therefore to be readily permeable to captopril as shown by the very early peak levels in this tissue (15 min), possibly reflecting an active uptake process in blood cells for captopril similar to that known for glutathione [12]. Variation in the activity of this uptake mechanism may explain the variation with time in the partition ratio of captopril between blood cells and plasma. These data therefore suggest that during the dynamic absorption

Table 3. Distribution of *S*-methylcaptopril in rat tissues at 30 min following 10 mg/kg p.o. captopril

Tissue	*S*-methylcaptopril content*, μg/g (wet weight)
Heart	0.20 ± 0.06
Lung	0.22 ± 0.15
Spleen	0.085 ± 0.018
Kidney	1.05 ± 0.18
Liver	1.05 ± 0.60
Blood cells	0.076 ± 0.028
Plasma	0.47 ± 0.23

* Mean of 4–5 determinations ± S.E.M.

phase the plasma level of drug does not reflect the level in erythrocytes.

The tissue distribution of reducible captopril, i.e. disulfides was also widespread and followed the distribution of captopril fairly closely. Relatively high levels of captopril disulfide conjugates were most evident in heart, lung and spleen and particularly in plasma and blood cells where total captopril was 2–3 times that of free captopril. There was a relatively lower proportion of reducible captopril in kidney which most likely results from saturation of conjugating reactions caused by the high captopril accumulation in this organ. The lack of significant disulfide levels in liver was surprising considering the very high oxidative activity in this tissue. However, a likely explanation is that high endogenous glutathione reductase levels act to keep captopril in a reduced state similar to that observed for maintenance of reduced glutathione levels in cells [12].

It was of interest that when captopril and disulfide conjugates were expressed as a total amount per tissue only 8.2% of the total captopril seen in the solid tissues was present as disulfide conjugates, whereas 77% was present in the blood suggesting that oxidation of captopril may be more important in blood, particularly plasma, than in tissues like kidney, lung and liver.

The known high metabolic activity in liver could account for the relatively high tissue levels of the *S*-methyl metabolite of captopril. This metabolite was present in all tissues examined with distribution at 30 min post dose being liver = kidney > plasma > lung > heart > spleen > blood cells confirming the significant contribution *S*-methylation has on the metabolism of captopril.

The tissue levels of this metabolite did not parallel tissue captopril levels. This was not surprising since formation of *S*-methylcaptopril will be dependent on the activity of thiol methyltransferase enzymes and also on the concentration of captopril as well as the availability of *S*-adenosylmethionine in each tissue. As peak levels of unmetabolised drug were maximal at 30 min it is possible that delayed tissue formation of the *S*-methyl metabolite can occur after 30 min.

Liver is particularly rich in thiol methyltransferase activity [13] and this would account for the relatively high levels of *S*-methylcaptopril in this tissue. Availability of the methyl donor *S*-adenosylmethionine (SAM) is also essential for biological *S*-methylation. Tissue levels of SAM show a relatively small variation in most tissues studied with levels ranging from 37.8 μg/g in liver [14] to 13.8 μg/g in heart [15]. In contrast, blood cells have quite low concentrations of SAM (3.2 μg/ml) [16] and this would explain the low concentration of the *S*-methyl metabolite in erythrocytes at a time when the captopril content is higher than a number of other tissues examined.

The presence of captopril and metabolites in other tissues that may be important in the mechanism of action of captopril such as skin and brain were not examined here. It has previously been shown that captopril is absent in brain [17] and more recently both captopril and reducible captopril conjugates have been shown to be absent in brain of rats given captopril 20 mg/kg i.v. at times ranging from 5 to 120 min post dose [Jarrott, unpublished observations].

The present study has demonstrated rapid accumulation and metabolism of captopril in peripheral tissues following a single oral dose. Maximal effects in this study were evident at 30 mins post dose. The high concentration of captopril and indeed other sulfur-conjugated metabolites, particularly *S*-methylcaptopril in kidney may account in part for certain pathological findings such as membranous glomerulopathy and for proteinuria [3, 18] in subjects receiving captopril. However, it is yet to be proven whether any metabolite of captopril *per se* is directly responsible for onset of adverse reactions during captopril therapy or whether this is a direct consequence of inhibition of converting enzyme activity.

Acknowledgements—The authors wish to thank the National Health and Medical Research Council of Australia and the National Heart Foundation of Australia for their financial assistance and the Squibb institute for supplies of captopril and disulfide conjugates.

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