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# COMBINED GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC PROCEDURE FOR THE MEASUREMENT OF CAPTOPRIL AND SULFUR-CONJUGATED METABOLITES OF CAPTOPRIL IN PLASMA AND URINE

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## SUMMARY

A gas chromatographic—mass spectrometric method is described for the simultaneous measurement of the novel anti-hypertensive drug captopril, and the following metabolites: captopril disulfide dimer, S-methyl captopril, and S-methyl captopril sulfone. With this method all derivatives can be chromatographed using conventional gas chromatography of hexafluoroisopropyl esters in one temperature-programmed run and these can then be quantitated using selected-ion monitoring techniques.

Using urine or plasma, captopril, S-methyl captopril and the disulfide dimer of captopril in concentrations as low as 1 ng/ml, 10 ng/ml and 25 ng/ml, respectively can be detected. The reproducibility of the method is satisfactory both within-assay and inter-assay.

This technique has demonstrated that the pattern of urinary excretion of these compounds in both man and rat was similar. Excretion of unchanged captopril, S-methyl captopril and the disulfide dimer over 6 h in man given captopril (50 or 100 mg) chronically was 18.3%, 0.97% and 3.06%, respectively. Corresponding excretion of these three compounds in the rat following a single 10 mg/kg dose was 18.3%, 2.69% and 1.8%, respectively. A possible sulfone oxidation product of S-methyl captopril was not detected in the urine of either man or rat.

## INTRODUCTION

The angiotensin-converting enzyme inhibitor captopril {1-[(2D)-3-mercapto-2-methyl-1-oxopropyl]-L-proline} is a thiol-containing dipeptide based on proline [1]. This drug is now being widely used as an antihypertensive drug [2]. Its metabolism has been studied in both man [3, 4] and rat [5, 6] and has been shown to occur entirely by conjugation of the sulfur of captopril either as thiomethyl derivatives or disulfide conjugates with other thiol compounds

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such as cysteine, glutathione and even proteins. Methods used to quantitate captopril and these metabolites have relied largely on thin-layer chromatographic techniques [4] with radioactive captopril or high-performance liquid chromatographic (HPLC) methods [7—10], and gas—liquid chromatographic [11] and gas chromatographic—mass spectrometric (GC—MS) methods [12, 13] have also been reported for the measurement of captopril, and in some instances, the disulfide dimer of captopril [8, 12, 13]. GC methods have relied on the use of N-ethylmaleimide to derivatize the sulfur of captopril in blood at the time of collection to prevent formation of oxidized captopril species [11—13]. Derivatization of the carboxyl group of captopril has been accomplished using hexafluoroisopropanol esters [11, 13] or methyl esters [12]. The most sensitive and specific method appears to be GC—MS using selected-ion monitoring (SIM) [12, 13]. Recent attention has focused on an S-methyl metabolite of captopril as a possible inactivation product of captopril metabolism whose formation may be time-dependent [3]. We present in this paper a specific GC -MS method for measuring S-methyl captopril and the sulfone oxidation product of S-methyl captopril in addition to captopril and its dimer in plasma and urine. Data are also presented to show the excretion of these compounds in rat and human urine.

## EXPERIMENTAL

### *Reagents and chemicals*

N-Ethylmaleimide (NEM, Sigma), perfluorobutyric anhydride (HFB, Pierce), 1,1,1,3,3,3-hexafluoroisopropanol (HFI) dimethyl sulfate (Fluka, puriss grade), (4*R*)-3-((2*S*)-3-mercapto-2-methylpropanoyl)-4-thiazolidine carboxylic acid (YS-980, Santen Pharmaceutical), captopril and captopril disulfide dimer (Squibb) were used as obtained. All other chemicals were analytical reagent grade. Ethyl acetate (Merck) was purified over alumina and distilled prior to use.

### *GC conditions*

The stationary phase was 3% OV-101 coated on 100—120 Chromosorb W AW DMCS packed into a 2 m × 2 mm I.D. glass column. Helium (flow-rate 30 ml/min) was the carrier gas. Injector and detector zone temperatures were 280°C. Temperature program conditions were: initial temperature 150°C, initial hold time 1 min, rate of rise 10°C/min, final temperature 290°C.

### *MS conditions*

A Finnigan Model 4021 gas chromatograph—mass spectrometer equipped with an INCOS data system was used for the instrumental analyses. The ion source temperature was 250°C (corrected) and the electron energy was 70 eV for electron impact mass spectrometry (EIMS). When positive-ion chemical ionization mass spectrometry (CIMS) was used, isobutane (Matheson) was the reagent gas at a source pressure of 0.3 torr and source temperature of 150°C (corrected). Extractor voltage settings were 4 V for EIMS and 10 V for CIMS and these were varied by the data system as function of mass to compensate

for mass discrimination of the quadrapole mass filter. The filament current was kept constant at 0.25 mA.

*Sample preparation*

Urine or plasma (100 $\mu$l—1 ml), 1 ml of potassium phosphate buffer, pH 7.4 (0.1 *M*), 10 $\mu$l of YS-980 (1 mg/ml solution in acetone) and 100 $\mu$l of N-ethylmaleimide (NEM) (10 mg/ml solution in water) were added successively. After 10 min reaction time excess NEM was removed by extraction with 5 ml of purified ethyl acetate. The aqueous phase was then acidified with 500 $\mu$l of hydrochloric acid (2 *M*), saturated with sodium chloride (ca. 2 g) and finally extracted with 10 ml of purified ethyl acetate. The ethyl acetate (8 ml) was transferred to a clean reaction tube and evaporated to dryness under nitrogen. Traces of water were azeotroped out with dichloromethane and the dry extract reacted with 50 $\mu$l of both hexafluoroisopropanol (HFI) and perfluorobutyric anhydride (HFB). HFB was present as a transesterification agent to facilitate esterifications with HFI. Derivatization proceeded for 15 min at 60°C before finally removing all reagents by evaporation under nitrogen. The residue was reconstituted into dry purified ethyl acetate (100 $\mu$l).

*Synthesis of S-methyl captopril*

Captopril (100 mg) was dissolved in 20 ml barium hydroxide solution (0.2 *M*) and a 10% molar excess of dimethyl sulfate was then gradually added with regular mixing at ambient temperature. After 15 min the solution was acidified with sulfuric acid (2 *M*) and centrifuged to separate out the insoluble barium sulfate. The supernatant was saturated with sodium chloride and extracted with 3 × 10-ml volumes of purified ethyl acetate. The organic layer was then transferred to a clean tube and evaporated to dryness under nitrogen. The remaining oil was dissolved in a small volume of distilled water, neutralized with dilute sodium hydroxide (0.1 *M*) and lyophilized. The residue was treated with dichloromethane (1 ml) to azeotrope out any moisture and lyophilized. This last procedure was repeated twice and the hydroscopic crystals (sodium salt) obtained were stored in a vacuum desiccator. EI mass spectra of the HFI ester gave *m/z* 381 $(M^+)$, *m/z* 366 $(M-CH_3)^+$, *m/z* 334 $(M-SCH_3)^+$ and *m/z* 264 (proline moiety)$^+$ (Fig. 1a). CI mass spectra (isobutane) gave the expected ion $(MH)^+$ at *m/z* 382 (Fig. 2a). Thin-layer chromatography (TLC) (silica) with benzene—acetic acid (3:1) gave a single spot, $R_F$ 0.53.

*Synthesis of sulfone of S-methyl captopril*

S-methyl captopril (sodium salt) (10 mg) prepared as described above was dissolved in 2 ml of Tris buffer, pH 10 (100 m*M*) and treated with an excess of hydrogen peroxide (30%, v/v, 200 $\mu$l).

After 30 min the reaction mixture was acidified with 2 *M* hydrochloric acid, saturated with sodium chloride and extracted with 10 ml purified ethyl acetate. The ethyl acetate was evaporated to dryness under dry nitrogen to yield an oily residue which was not contaminated with S-methyl captopril as judged by GC—MS. EI mass spectra of the HFI ester gave *m/z* 413 $(M)^+$, *m/z* 334 $(M-CH_3 \cdot SO_2)^+$, *m/z* 264 (proline moiety)$^+$, and *m/z* 149 $(CH_3 \cdot SO_2 \cdot CH_2 \cdot CH(CH_3) \cdot CO)^+$ (Fig. 1b). CI mass spectra (isobutane) gave the expected ion

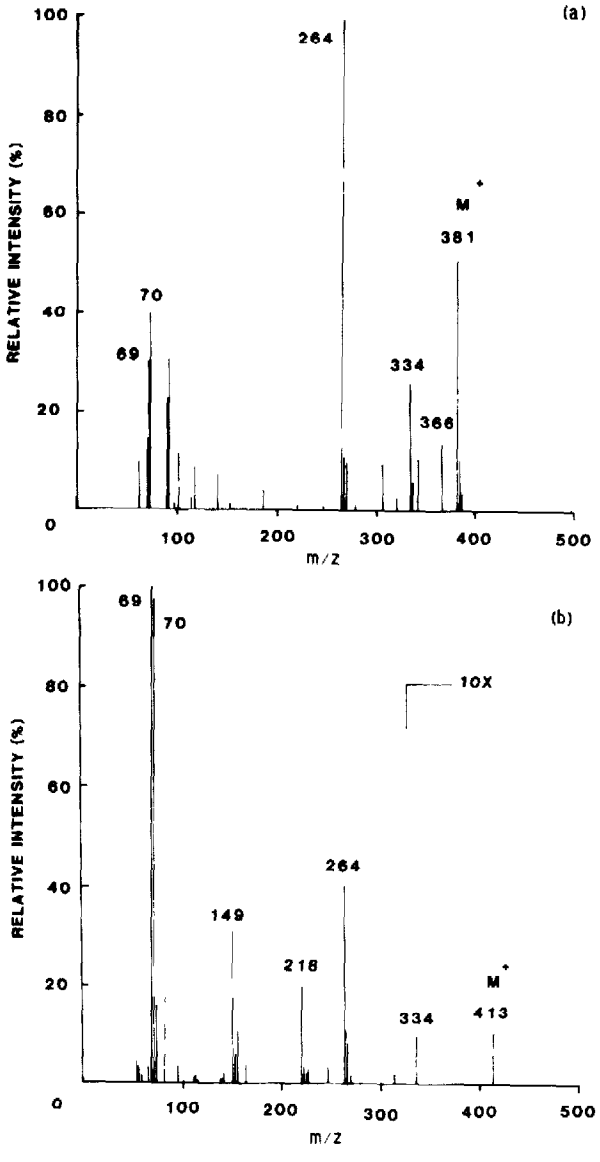


Fig. 1. EI mass spectra of (a) S-methyl captopril HFI ester and (b) S-methyl captopril sulfone HFI ester.

$(MH)^+$ at *m/z* 414 (Fig. 2b). TLC (silica) with benzene—acetic acid (3:1) gave a single spot, $R_F$ 0.30.

*Reproducibility studies*

Reproducibility studies were performed for captopril, the dimer and S-methyl captopril by doing replicate analyses of urine samples which had been spiked with a known amount of pure compound. These samples were assayed both within a run and after several runs over a period of one month.

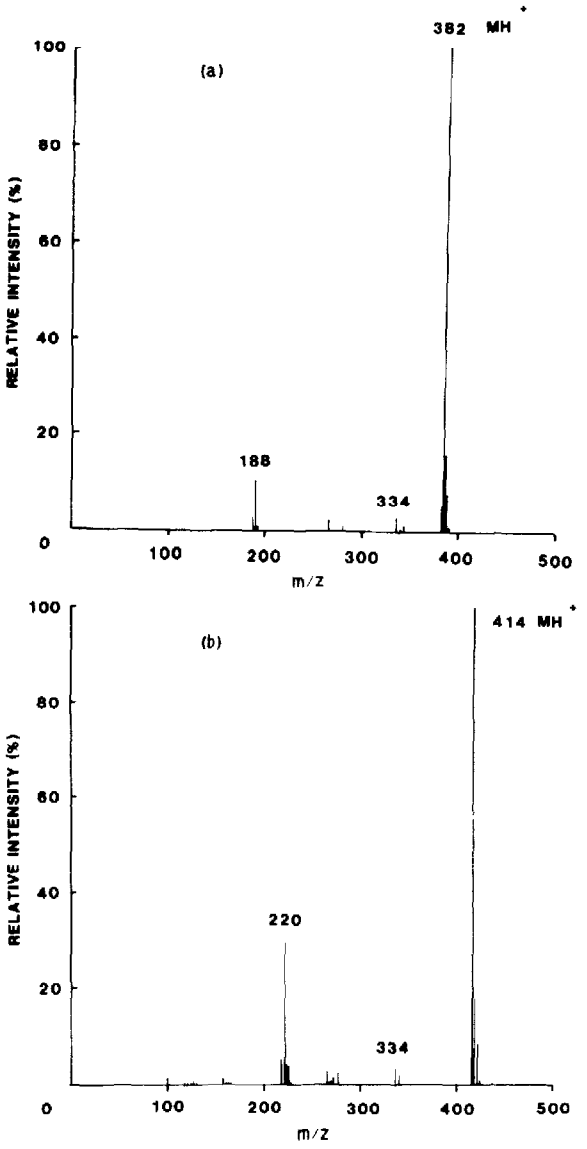


Fig. 2. Isobutane CI mass spectra of (a) S-methyl captopril HFI ester, and (b) S-methyl captopril sulfone HFI ester.

*Quantitation*

Blank urine (100 $\mu$l) or plasma (1 ml) were spiked with various amounts of captopril (10 ng to 10 $\mu$g), S-methyl captopril (25 ng to 2 $\mu$g), captopril disulfide dimer (25 ng to 5 $\mu$g) and treated as for the unknown samples. Quantitation was achieved using SIM of the fragment ions at *m/z* 264, 366, 334 and 282. Peak areas were evaluated using the INCOS data system. The areas of the peaks from ions *m/z* 264, 366 and 334 were expressed as a ratio of the area of the internal standard (YS-980) peak (*m/z* 282 ion). These ratios

were then related to those obtained from unknown samples containing the same amount of YS-980.

*Urine sampling procedures*

Eight patients (mean age 57.4, range 53—60 years) who had a history of essential hypertension with normal renal function and who were already receiving captopril (50—100 mg p.o., t.i.d.) as part of a comprehensive pharmacokinetic study of this drug were investigated. Patients had been on captopril for at least two weeks prior to collection of urine for this study. The bladders were emptied immediately prior to the dose and urine was collected over a 6-h period following the dose of captopril. Urine was immediately treated with NEM (10 mg/ml urine) on voiding to alkylate the sulfhydryl group and prevent spontaneous oxidation of captopril to disulfides [4]. Urine was then frozen in aliquots until assayed.

Rats of either sex (mean weight 274 g, range 250—325 g) were housed in metabolic cages and captopril (10 mg/kg) was given to rats by gavage of a fresh aqueous solution (1 mg/ml). Urine was collected over a period of 24 h into tubes containing 50 mg NEM. Urine was subsequently aliquoted out and frozen.

## RESULTS AND DISCUSSION

The HFI esters of S-methyl captopril and captopril chromatographed readily on a packed column eluting at 3.4 min and 9.8 min, respectively. Furthermore, these same conditions also facilitated chromatography of the disulfide dimer of captopril which eluted at 12.6 min (Fig. 3a). Chromatography of a urinary extract of a patient receiving captopril (50 mg) chronically using SIM-MS analysis shows peaks at 3.4 min, 9.8 min and 12.6 min (Fig. 3b), which by comparison of both EI and CI (isobutane) mass spectra of authentic standards confirmed the presence of S-methyl captopril, captopril and the disulfide dimer.

Similarly extracts of rat urine (see Methods) gave an almost identical profile to that obtained from human urine with only the S-methylcaptopril, captopril and the disulfide dimer evident. There were some very small unknown peaks in both rat and human urinary extracts eluting between 4 and 8 min which were also present in control urine extracts devoid of captopril. It was evident, however, that if urine was stored at room temperature for even relatively short periods (1 h) or subjected to more than one thawing an extra peak sometimes appeared in the chromatogram at 5.2 min and levels of S-methyl captopril and captopril fell. This peak was identified as the sulfone (S-dioxide) of S-methyl captopril by its EI and CI mass spectra and by comparison with authentic sulfone prepared from the oxidation of S-methyl captopril (see Methods) which gave identical retention times and mass spectra to the unknown peak (Figs. 1b and 2b). Although this compound was well extracted from salt-saturated urine (recovery approximately 50%) there did not appear to be significant amounts in either fresh rat or human urine obtained over 24 h after captopril administration.

Captopril, the dimer of captopril and the sulfone of S-methyl captopril gave

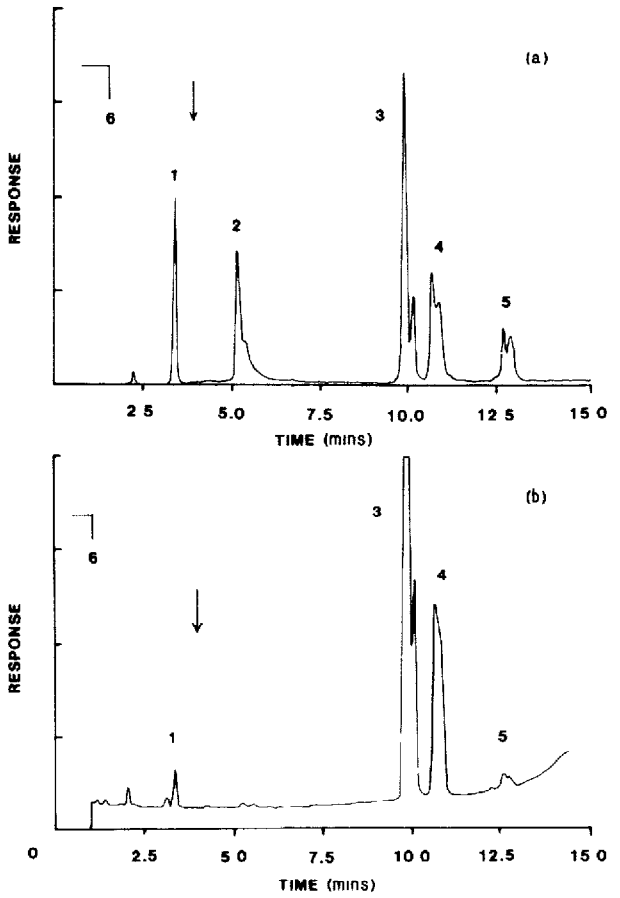


Fig. 3. Traces from EIMS SIM analysis of the HFI esters of (a) aqueous extract of S-methyl captopril (1), S-methyl captopril sulfone (2), captopril-NEM (3), the internal standard YS-980-NEM (4), and captopril disulfide dimer (5): and (b) urinary extract of a patient receiving captopril (50 mg p.o. t.i.d.) showing excretion of S-methyl captopril (1), captopril (3), and the disulfide dimer (5). The arrow denotes possible elution time of a potential sulfoxide metabolite of S-methyl captopril. Event 6 denotes time during which helium effluent was vacuum diverted away from the MS source.

split chromatographic peaks. The presence and amount of these split peaks were not altered by removing peroxide impurities in the ethyl acetate by elution through alumina. Both EI and CI (isobutane) GC—MS gave identical mass spectra of all portions of the split peaks suggesting that the composition of the split peaks was uniform, and probably, one compound. This phenomenon also occurs in HPLC analysis [7, 9, 14] and has been attributed to inter- and intra-molecular bonding of captopril [15]. Epimerization at the C-2 position of the side chain by derivatization is another possibility contributing to split peaks, however, this is unlikely since this phenomenon also occurs with HPLC analysis where derivatization was not used and where chromatography was performed at ambient temperature [14]. The possibility of a mixture of stereoisomers contributing to the dual peaks cannot entirely be ruled out although product literature from Squibb suggests a pure DL stereo-

isomer of captopril is used. This splitting of the peak did not, however, affect reproducibility when peak areas were used for quantitation. Analyses of urinary extracts were greatly simplified and detection limits vastly improved by using SIM analysis of the ions at *m/z* 264, 334 and 366 together with the ion at *m/z* 282 which was the most intense ion of the YS-980 EI mass spectrum (thiazolidine moiety). Columns were conditioned daily with two 1-μl injections of bis-trimethyltrifluoro acetamide (BSTFA) and two 1-μl injections of 1 μg of derivatized captopril. The use of HFI ester of captopril [11, 13] and the use of NEM for derivatizing the thiol of captopril in blood at the time of collection have previously been described [11—13]. We have used a combination of these two treatment procedures similar to that described by Matsuki and co-workers [11, 13] with SIM analysis [13] for measurement of captopril and metabolites in plasma or urine. The use of a close structural analogue of captopril (YS-980) in this method is an improvement over existing GC procedures for measuring free captopril which have either used a captopril derivative which did not undergo S-alkylation with NEM in the method [11, 13] or a deuterated analogue of captopril whose fragment ions interfere with quantitation of captopril at low concentrations [12]. In addition, complicated and time-consuming extraction steps previously reported have been considerably abbreviated allowing shorter sample preparation time, hence shorter analysis time.

All compounds that were quantitated exhibited linear calibration curves from the limit of detection to at least 10 μg for captopril and disulfide dimer and 3 μg for S-methyl captopril. All curves passed through the origin. Coordinates for these curves were; captopril ($y = 0.265x + 0.0009$), S-methyl captopril ($y = 0.045x - 0.0003$) and disulfide dimer ($y = 0.041x + 0.002$).

Reproducibility studies for both compounds were evaluated by performing replicate assays of control urine samples containing different amounts of both compounds (Table I). Within-assay variability for captopril was 4.6% at 270 ng/ml and 2.7% at 3.7 μg/ml (Table I). Corresponding values for S-methyl captopril were 2.1% at 250 ng/ml and 2.5% at 1.1 μg/ml and for the dimer 12.6% at 2 μg/ml.

Inter-assay variability of these same samples was evaluated over a period of four weeks ($n = 7$). Coefficients of variation for captopril were 9.1% at 270 ng/ml and 8.7% at 2.7 μg/ml (Table I). Similarly, S-methyl captopril gave variabilities of 8.3% at 250 ng/ml and 6.5% at 1.1 μg/ml and 15.1% for the dimer at 2 μg/ml. Detection limits for captopril, S-methyl captopril and the dimer using the method described were 1 ng/ml, 10 ng/ml and 25 ng/ml re-

TABLE I

ASSAY PARAMETERS FOR MEASUREMENT OF CAPTOPRIL, S-METHYL CAPTOPRIL AND DISULFIDE DIMER IN URINE

| Parameter | Captopril | S-methyl captopril | Disulfide dimer |
|---|---|---|---|
| Detection limit (ng/ml) | 1 | 10 | 25 |
| Reproducibility within-day (%) | 4.6 (270 ng/ml)<br>2.7 (3.7 μg/ml) | 2.1 (250 ng/ml)<br>2.5 (1.1 μg/ml) | 12.6 (2 μg/ml) |
| Reproducibility day-to-day (%) | 9.1 (270 ng/ml)<br>8.7 (2.7 μg/ml) | 8.3 (250 ng/ml)<br>6.5 (1.1 μg/ml) | 15.1 (2 μg/ml) |

TABLE II

EXCRETION OF CAPTOPRIL, S-METHYL CAPTOPRIL AND THE DISULFIDE DIMER OF CAPTOPRIL IN URINE OF MAN AND RATS

Dose administered by gavage of an aqueous solution.

| Metabolite | Rat (10 mg/kg)* | | | | Man** | |
|---|---|---|---|---|---|---|
| | 0—6 h | *n* | 6—24 h | *n* | 0—6 h | *n* |
| Captopril | 18 3 ± 9 5 | 5 | 2.8 ± 1.6 | 5 | 18 3 ± 4.2 | 6 |
| Captopril disulfide dimer | 1.80 ± 0.29 | 4 | 0.76 ± 0.70 | 5 | 3.06 ± 1 26 | 8 |
| S-Methyl captopril | 2.69 ± 0.88 | 5 | 1 71 ± 1.10 | 5 | 0.97 ± 0.14 | 5 |

*All values expressed as a percentage of dose, mean ± standard error.
**Patients on captopril chronically, 50- or 100-mg dose.

spectively (Table I). However, the detection limits described were more than adequate for urine and plasma samples encountered. Recoveries of S-methyl captopril and captopril were greater than 80% and 50% for the dimer and sulfone of S-methyl captopril. Similar recoveries and variability estimates were found for plasma samples.

The levels of captopril and the metabolites in urinary extracts of both man and rat are shown in Table II. The 24-h urinary excretion of free captopril in rat was a sizeable proportion (21.1%) of the dose administered and it was the same (18.3%) in the 0—6 h period in both rat and man.

Previous studies in both man [4] and rat [16] have shown that only about 50—60% of a captopril dose is absorbed hence the excretion of unchanged captopril in this study would increase by approximately a factor of two. Excretion of free captopril in man over 6 h assuming the bioavailability was 50% would therefore approach 36% of the dose. However, the values reported here are lower than previously reported using radiolabelled captopril and TLC separation procedures which have shown 37% excretion of unchanged captopril in man following a 100-mg dose after 8 h [4] and 47% in the rat [6]. These differences in excretion of free captopril may reflect the increased specificity of our GC—MS assay methodology over TLC—radiometric procedures and this is supported by a recent GC—MS study which showed that 20.9% of a 10 mg/kg dose of captopril is excreted as unchanged captopril in the rat over 24 h [13]. The disulfide dimer of captopril was present in both human and rat urine, albeit only a small proportion of the captopril administered. In two of the eight human urine samples no dimer was detected whilst one subject excreted almost 10% of this metabolite suggesting a large individual variability in dimerization of captopril. Urinary excretion of the dimer in the rat was significantly less than in man ($p < 0.005$) (Table II).

The presence of the disulfide dimer of captopril in urine was not unexpected since this material forms spontaneously from aqueous solutions of captopril and may in addition also form enzymatically, probably through the mixed function oxidase system [17]. Incubation of rat serum or human plasma with captopril has been shown to result in about 10% formation of dimer over 30

min [5]. In view of the ease of formation of the dimer the levels encountered in this study were surprisingly low, in agreement with a previous study in man [4] using TLC procedures. The excretion in the rat however was three-fold lower than in a previous study [6]. The small excretion of dimer may reflect the action of reductases similar to those present for oxidized glutathione [18, 19]. Moreover, the lower but consistent values found in the present study are more likely to reflect real excretion data rather than problems in assay methodologies. The suggestion that only a small amount of captopril oxidation to the dimer occurs is supported by the lack of S-methyl captopril oxidation products. Although the sulfone is extractable into ethyl acetate from salt-saturated urine neither rat nor human urine contained measureable amounts. Large variations in excretion of dimer may occur if urine is not properly stored or captopril is not completely alkylated to prevent spontaneous dimer formation and this may in part explain the previously reported higher level in the rat [6].

Spontaneous formation of the oxidation products of S-methyl captopril was observed when old thawed urines were reanalysed again suggesting that storage is critical. This may be an explanation for the formation of large amounts of the apparent sulfoxide of S-methyl captopril (3%) in the rat using a previously described radiolabelled procedure [6].

In the present studies, the urinary excretion of S-methyl captopril was evident in both man and rat and was about three-fold higher ($p < 0.005$) in rats when urine was collected over a 6-h period. It is not clear at this stage whether this reflects differences in the doses given to the two species, or to different clearances, or a lower degree of S-methylation in humans.

## CONCLUSIONS

In summary, a simple, sensitive and specific GC—MS method for quantitation and identification of S-methyl captopril, S-methyl captopril sulfone, captopril and the disulfide dimer in biological fluids is presented. Data are presented to show excretion values for those metabolites in human and rat urine after pharmacological doses of captopril. In the present study S-oxide formation of S-methyl captopril, as the sulfone was not observed to be a significant pathway of captopril elimination in both man and rat. The formation of other oxidized polar disulfide conjugates of captopril could account for other routes of captopril elimination and at present we are examining these.

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