BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **15**: 427–432 (2001)
DOI: 10.1002/bmc.95

ORIGINAL RESEARCH

Determination of captopril in biological samples by high-performance liquid chromatography with ThioGlo™3 derivatization

Nukhet Aykin, Rachel Neal, Mozow Yusof and Nuran Ercal*

Department of Chemistry, University of Missouri-Rolla 142 Schrenk Hall, Rolla, MO 65409, USA

Received 2 May 2000; revised 25 January 2001; accepted 30 January 2001

ABSTRACT: Captopril, a well-known angiotensin converting enzyme (ACE) inhibitor, is widely used for treatment of arterial hypertension. Recent studies suggest that it may also act as a scavenger of free radicals because of its thiol group. Therefore, the present study describes a rapid, sensitive and relatively simple method for the detection of captopril in biological tissues with reverse-phase HPLC. Captopril was first derivatized with ThioGlo™3 [3H-Naphto[2,1-b]pyran,9-acetoxy-2-(4-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)phenyl-3-oxo-)]. It was then detected by fluorescence-HPLC using an Astec C_{18} column as the stationary phase and a water:acetonitrile:acetic acid:phosphoric acid mixture (50:50; 1 mL/L acids) as the mobile phase (excitation wavelength, 365 nm; emission wavelength, 445 nm). The calibration curve for captopril was linear over a range of 10–2500 nM and the coefficient of variation acquired for the within- and between-run precision for captopril was 0.5 and 3.8%, respectively. The detection limit of captopril with this method was found to be 200 fmol/20 μL injection volume. Its relative recovery from biological samples was determined to the range from 93.3 to 105.3%. Based on these results, we believe that our method is advantageous for captopril determination. Copyright © 2001 John Wiley & Sons, Ltd.

INTRODUCTION

Captopril (Fig. 1), an inhibitor angiotensin converting enzyme (ACE), has also been postulated as a free radical scavenger because of its terminal sulfhydryl group (Bagchi *et al.*, 1989; Andreoli, 1993). Some *in vitro* studies indicate that captopril functions as an antioxidant both by scavenging reactive oxygen species (ROS) and by increasing the activities of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (De Cavanagh *et al.*, 1995). Captopril has been shown to decrease serum lipid peroxide concentrations in diabetic patients and also to enhance the antioxidant capacity in hypertensive patients (Schnider *et al.*, 1990).

There have been several methods for measuring captopril levels, although quantitation of its plasma concentration has been problematic due to its relative instability. Captopril is easily converted to its disulfide dimer and forms conjugates with other thiols (Wieling *et al.*, 1996). Previous techniques that were based on liquid–liquid extraction (Wieling *et al.*, 1996), electroanalytical (Nunez-Vergara *et al.*, 1996), and radioimmunoassay (Wong *et al.*, 1981) were time-consuming and required considerable care.

In this paper, we suggest a more sensitive, practical and rapid method for captopril quantitation with reverse-phase HPLC. The derivatizing agent, ThioGlo™3, is a naphtopyranone based fluorescent thiol probe synthesized by Covalent Associates Inc. (Yang and Langmuir 1991). In our method, ThioGlo™3 reacts with the free sulfhydryl group of captopril to form a fluorescent adduct. The reaction between ThioGlo™3 and captopril can be seen in Fig. 2. Moreover, some preliminary results have been obtained from Sprague–Dawley rats by administering 50 mg/kg of captopril in order to show the applicability of this method to biological studies.

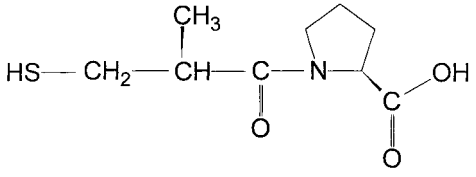

Figure 1. Chemical structure of captopril.

*Correspondence to: N. Ercal, Department of Chemistry, University of Missouri-Rolla, 142 Schrenk Hall, Rolla, MO 65409, USA.
Email: nercal@umr.edu

Abbreviations used: ACE, angiotensin converting enzyme; PBPB, *P*-bromophenylbromide; ROS, reactive oxygen species.

EXPERIMENTAL

Reagents and chemicals

Acetonitrile, acetic acid, water and phosphoric acid (all HPLC grade) were purchased from Fisher Scientific (Houston, TX, USA). Captopril was obtained from Sigma (St Louis, MO, USA) and

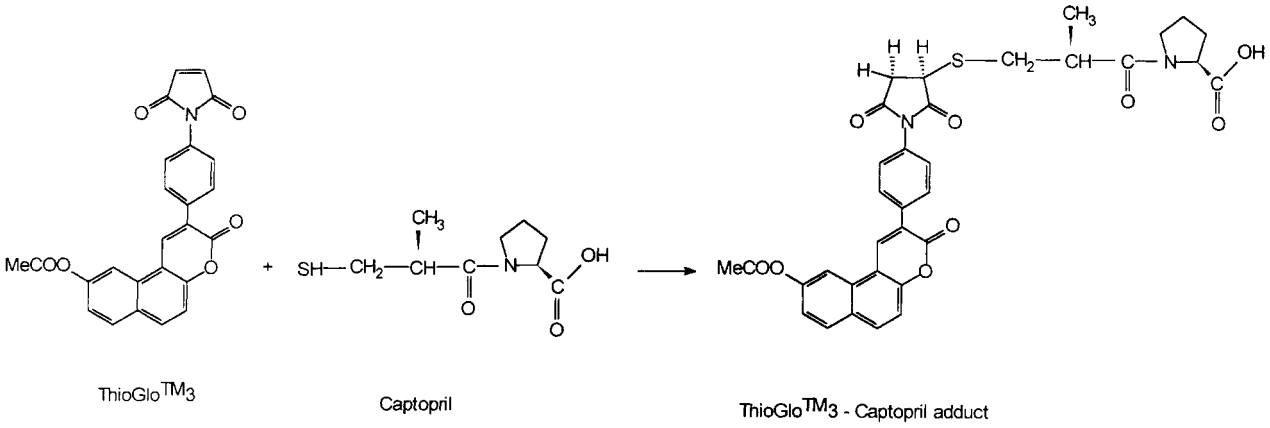

Figure 2. Formation of fluorescent ThioGlo™3-captopril adduct.

Table 1. Captopril in sample matrices ($n = 7$) and standards ($n = 7$) for within-run and between-run precision. Relative recovery is reported as the average relative recovery of three samples spiked with 250 nM captopril in each sample matrix; N/A = not applicable

Sample matrix	Liver	Lung	Kidney	Plasma	Standard
Between run precision ($n = 7$)	1.48%	4.12%	1.67%	1.99%	3.80%
Within run precision ($n = 7$)	0.70%	1.02%	2.80%	1.00%	0.50%
Percentage relative recovery	99.3 ± 2.5%	99.4 ± 6.3%	94.0 ± 3.3%	107.5 ± 11.6%	N/A

ThioGlo™3 was purchased from Covalent Associates Inc. (Woburn, MA, USA).

Animals

Adult male Sprague–Dawley rats, which were used in the experiments, were obtained from the UMR Animal Colony. Their weights were in the range of 400–600 g. The rats were housed in stainless steel cages in a temperature-controlled (25°C) room with a 12 h light–dark cycle and allowed standard rat chow (Purina Rat Chow) and water *ad libitum*. After overnight fasting, six rats were anesthetized according to the University of Missouri animal care regulations and were administered 50 mg/kg body weight of captopril (i.g.) or saline (i.g.). After 1 h, blood samples were collected via intracardiac puncture. The animals were then killed and liver, lung and kidney samples were obtained. Plasma was separated from blood immediately and kept at −70°C with tissue samples until derivatization with ThioGlo™3.

HPLC system

The HPLC system (Shimadzu) consisted of a model LC-10A pump, a Rheodyne injection valve with a 20 μL filling loop and a Model RF 535 fluorometer operating at an excitation wavelength of 365 nm and an emission wavelength of 445 nm. The HPLC column (Astec, Whippany, NJ, USA) is 100 × 4.6 mm i.d. and was packed with 3 μm particles of C_{18} packing material. Quantitation of the peaks from the HPLC system was performed with a Chromatopac Model CR601 integrator (Shimadzu). The mobile phase was water–acetonitrile (50:50, v/v) and was adjusted to a pH of 2.5 through addition of 1 mL/L of acetic and phosphoric acids. The ThioGlo™3 derivatives were eluted from the column isocratically at a flow rate of 1 mL/min.

Assay procedures

Calibration and captopril recovery. A calibration curve was constructed by injecting ThioGlo™3-derivatized standards in a volume of 20 μL. Within-run precision was determined by successively injecting the same sample seven times and comparing the peak areas for the captopril derivatives for the seven injections. The between-run precision was obtained by derivatizing the same sample at seven different times, and running the derivatized samples at seven different times (Table 1). Moreover, relative recovery was determined by spiking the liver, kidney, lung, and plasma samples with 250 nM of

Table 2. Captopril in tissues 1 h after intragastric administration of 50 mg/kg captopril. After derivatization with ThioGlo™3, the ThioGlo™3-captopril adducts formed were measured by HPLC and sample captopril concentration were compared to known standards; N/D = not detectable

	Captopril concentration	
Sample	Control ($n = 3$)	Mean ± SD ($n = 3$)
Plasma	N/D	84894 ± 7725 nM
Liver	N/D	0.22 ± 0.06 nmol/mg protein
Lung	N/D	1.98 ± 0.95 nmol/mg protein
Kidney	N/D	9.84 ± 2.39 nmol/mg protein

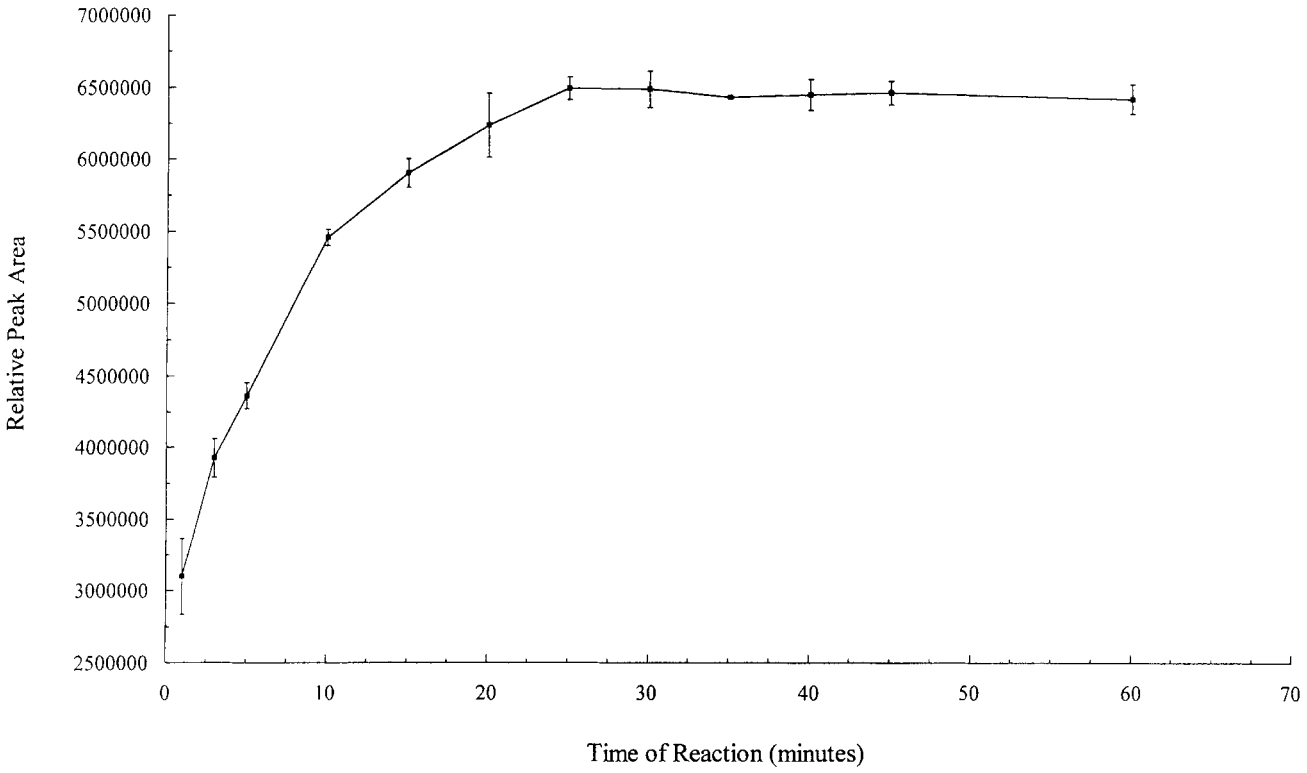

Figure 3. Reaction time of ThioGlo™3-captopril adduct ($n = 5$). Peak areas are reported as mean values ± standard deviation (SD).

captopril and comparing the results to those obtained from the standards with the same concentration (Table 2).

Sample derivatization. Plasma was obtained by centrifugation of blood samples for 10 min at 3000 rpm. Tissues (0.140 g/mL) were homogenized in 100 mM Tris buffer containing 10 mM borate and 5 mM serine with 1 mM diethylenetriaminepentaacetic acid as described (Neal *et al.*, 1991). Plasma, as well as liver, kidney and lung tissue homogenates were derivatized with ThioGlo™3, which reacts with the free sulfhydryl group of captopril to form fluorescent derivatives. A 0.5 mM ThioGlo™3 solution in acetonitrile (750 μL) was added to 150 μL of water (HPLC grade) and 100 μL of the diluted samples. The resulting solution was mixed and then incubated at room temperature for 25 min. Figure 3 shows that the reaction is complete at 25 min. At the end of the incubation period, 10 μL of 2 N HCl solution were added to stop the reaction. The final pH of the solution should be around 1.0, which is important for stabilizing the derivatives. The samples were filtered through a 0.2 μm acrodisc and injected onto a 3 μm C_{18} column in a reverse-phase HPLC system. The Bradford Method was used to determine the protein content of the tissue samples (Bradford *et al.*, 1976).

RESULTS

In this study, the derivatization of captopril with ThioGlo™3 and separation of ThioGlo™3-captopril fluorescence adduct by the reverse phase HPLC system were examined. Figure 4 shows the chromatogram of the ThioGlo™3-captoril adduct which is separated from the hydrolysis peaks of ThioGlo™3. Kidney tissue chromatograms before and after being spiked with 250 mM of captopril, are illustrated in Fig. 5(A) and (B).

Calibration curve

A calibration curve was constructed by plotting integrated peak areas vs captopril concentrations (10, 25, 125, 250, 500, 1250 and 2500 nM) at regular intervals. Another calibration curve was obtained by injecting 20 μL of ThioGlo™3-derivatized standards containing 250 mg of liver tissue. Figure 6 shows the 'captopril standard' and 'captopril standard in liver tissue matrix' which were obtained from results of seven different standard curves for each. Linearity was obtained over a full range of 10–2500 nM ($r = 0.999$). Calibration graphs were calculated by weighted linear regression ($W = X^{-1}$) on the responses of calibration samples vs the corresponding nominal concentrations.

Sensitivity, stability, reproducibility and relative recovery

The lower detection limit obtained for captopril was 10 nM (S/N = 3). The coefficient of variation (CV) for

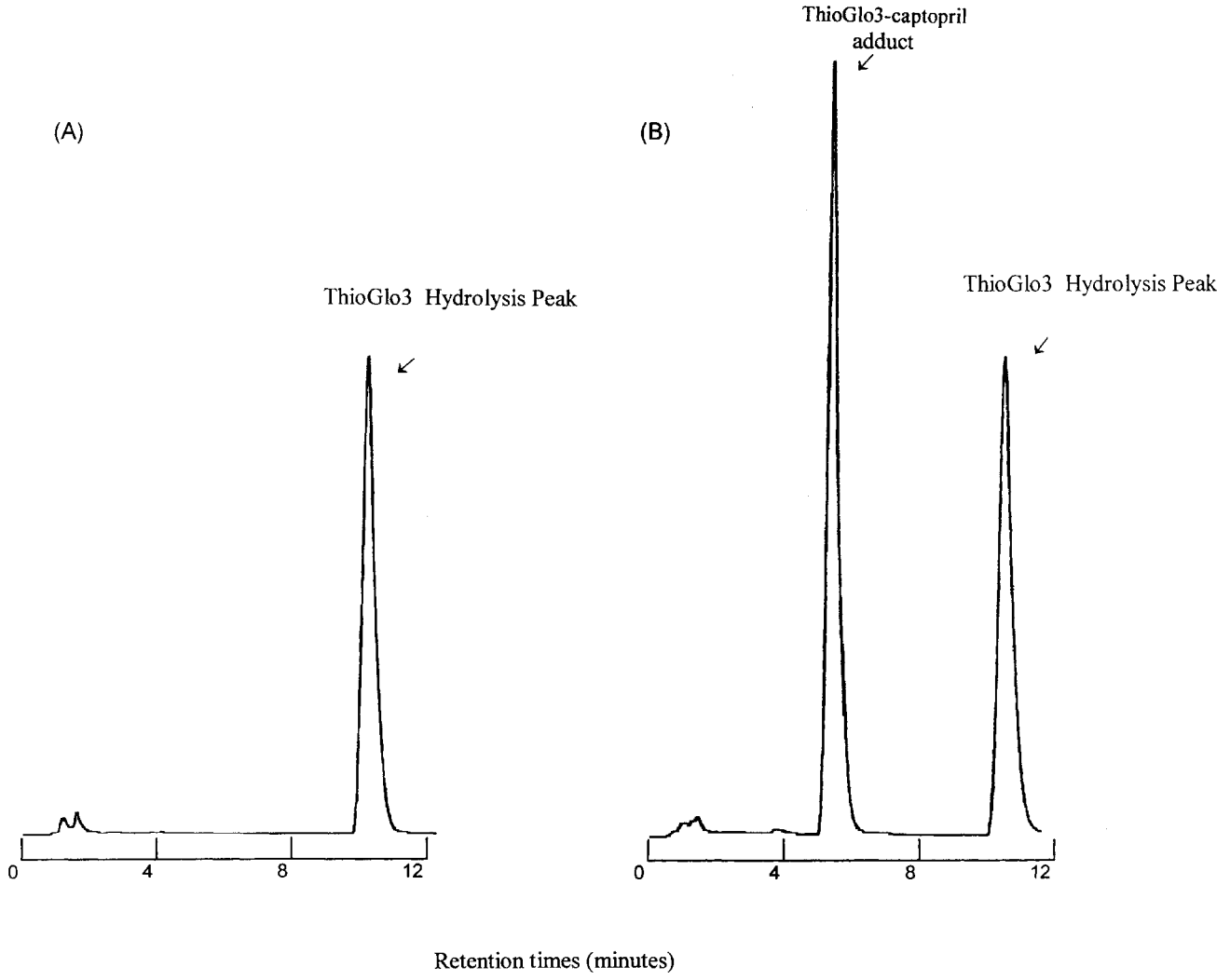

Figure 4. (A) Standard chromatogram containing ThioGlo™3 hydrolysis peak without captopril peak ($n = 7$). (B) Standard chromatogram containing peaks from the ThioGlo™3-captopril adduct ($n = 7$). Peak areas are reported as mean values ± standard deviation (SD).

within-run and between-run precision was 2.2 and 3.6%, respectively, at this level. Plasma and tissue samples were derivatized with ThioGlo™3 and in order to determine the stability of ThioGlo™3 derivatives, the standards and samples were injected onto the HPLC system periodically during the course of 2 weeks storage at 4°C. The coefficient of variation (CV) for within-run precision and between-run precision were found to be 0.5 and 3.8%, respectively. For relative recovery, spiking of liver, lung and kidney tissue homogenates and plasma was performed with 250 nM of captopril. Results were compared with the standard curve for the same concentration. Mean relative recoveries of captopril in the different tissues are shown in Table 2.

Results of biological sample analysis

The captopril concentrations in samples of plasma, liver, lung and kidney tissues taken from Sprague–Dawley rats are shown in Table 2. One hour after the rats received intragastric administration of 50 mg/kg captopril, they were sacrificed and the samples were collected. The concentration of captopril in plasma was higher than that in the tissues. Following a 1 h exposure to 50 mg/kg of captopril, accumulation was highest in the kidney tissue and lowest in the liver tissue.

DISCUSSION

Captopril [(2S)-1-[3-mercapto-2-methylpropionyl]-L-proline] has been widely used in medicine as an angiotensin-converting enzyme inhibitor since 1981, for the treatment of hypertensive and congestive heart failure patients (Florey, 1982; Heel *et al.*, 1980; Romankiewicz *et al.*, 1983). Moreover, it has been suggested that

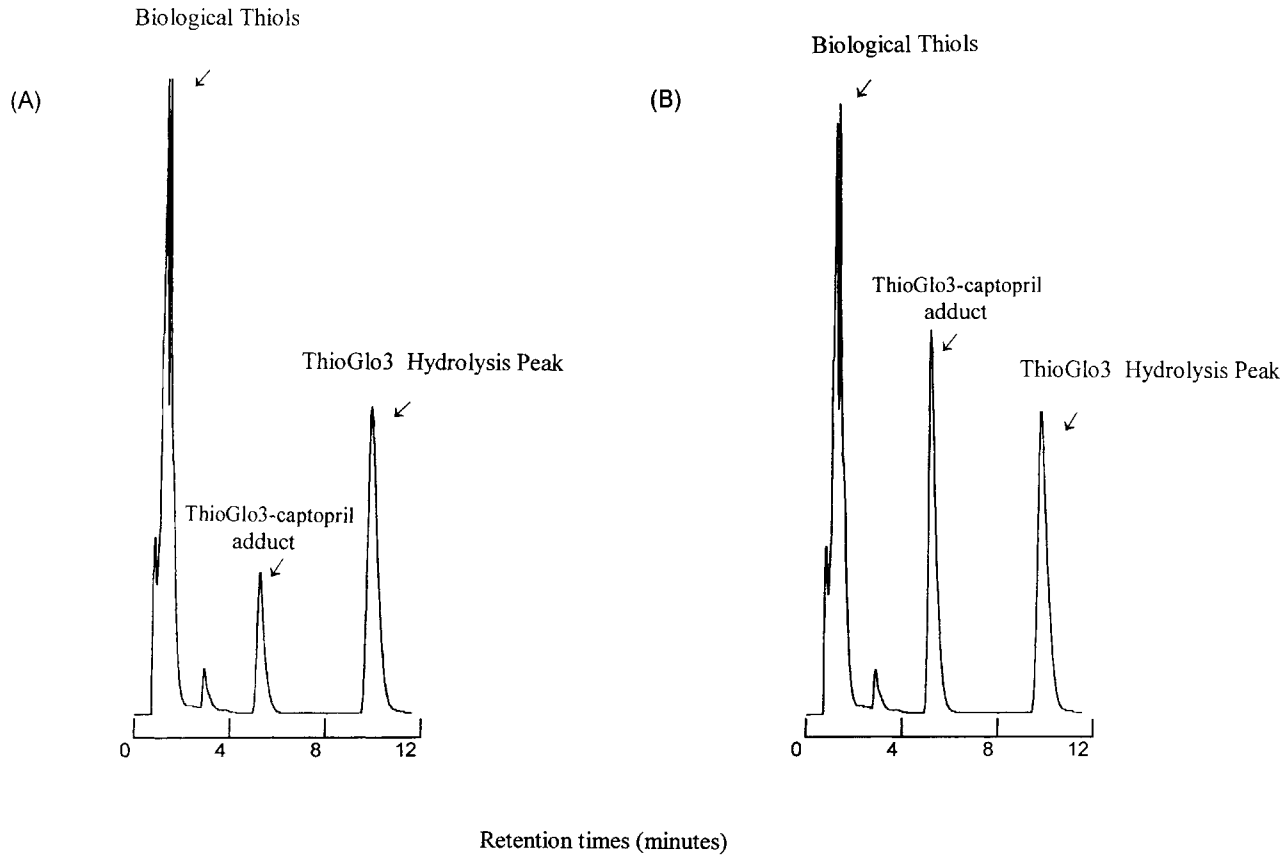

Figure 5. (A) Chromatogram of kidney before spiking with captopril. (B) Chromatogram of kidney spiked with 125 nM captopril.

captopril may act as a free radical scavenger because of its sulfhydryl group. Captopril was shown to decrease lipid peroxide levels caused by diabetic protenuria (Ha and Kim, 1992). It also reduces oxidative stress-induced cataract formation (Bhuyan *et al.*, 1992). As a result of its extensive use, many different methods have been reported for captopril quantitation, including gas chromatography (Matsuki *et al.*, 1982), gas chromatography–mass spectrometry (Ito *et al.*, 1987), and radioimmunoassay (Wong *et al.*, 1981).

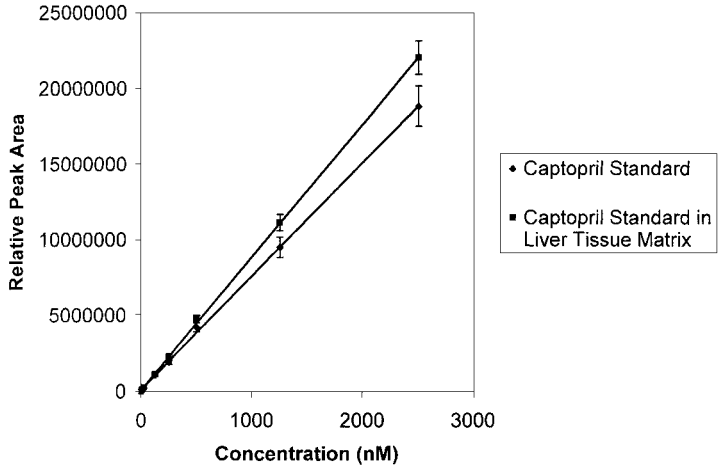

Figure 6. Standard curves obtained from captopril and captopril in liver tissue matrix.

HPLC has been used lately to monitor captopril levels in biological samples. The chromatographic method described by Gao *et al.* (1992) needs 1 h reflux of captopril-pBPB (*p*-bromophenylbromide) adduct and then to be vacuum dried. In addition, this method required column switching during HPLC operation (Gao *et al.*, 1992). Another method for captopril determination with HPLC has been reported by Bahmaei *et al.* (1997). Although the method has good relative recovery, again it requires preparation of captopril-pBPB and thiosalicyclic acid-pBPB adduct and a solid phase extraction step. Li *et al.* (1996) published a method for captopril determination in human plasma, which is quite similar to the methods above. A low detection limit and a high relative recovery are the plusses of this method. However, the overall, procedure is time-consuming with several refluxing, washing, and vacuum evaporation steps that are not practical for clinical use (Li *et al.*, 1996). The method provided by Bald *et al.* (1996) is, again, a long process for clinical application. Captopril and its derivatizing agent, 1-benzyl-2-chloro-4-methyl-pyridinium bromide, need to be stirred in a Tris buffer for 1 h and then a washing step must be applied (Bald *et al.*, 1996).

The procedure that we suggest is relatively simple and gives the lowest detection limit, as compared to HPLC methods described above. Our method has a 10 nM detection limit and linearity over a range of 10–2500 nM, whereas the detection limits are reported as 70 nM in Bahmaei *et al.* and 50 nM both in Gao *et al.* and in Bald *et al.* The only method which has the same limit of detection with our method is described by Li *et al.*. However, the authors stated that their lower limit of quantitation (LLOQ) is 24 nM, which is higher than our LLOQ (10 nM). In this study, we also show that our results are reproducible with low coefficients of variations. The samples can be subjected to long-term injections without degradation. Each sample requires 11 min to run and captopril's retention time is 5.2 min. Application of the present assay to biological material, including plasma, liver, lung and kidney samples has also been presented. This method can be applied for determination in human tissue and plasma in clinical settings.

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