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Bioanalysis of captopril: two sensitive high-performance liquid chromatographic methods with pre- or postcolumn fluorescent labeling

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Abstract

This study describes the development and comparison of two HPLC methods for the analysis of the antihypertensive drug captopril. The first method is based on a precolumn derivatization of captopril with the fluorescent label monobromobimane (MBB). The second method is based on a postcolumn reaction with the fluorescent reagent *o*-phthaldialdehyde (OPA). Since the disulfide metabolites of captopril can be reconverted to the active drug in vivo, the bioanalysis of captopril should involve both the determination of its free thiol form (free captopril) and the total amount of free thiol and reducible disulfides (total captopril). For total captopril analysis, disulfides were reduced with tributylphosphine (TBP) prior to protein precipitation. Since the reducing agent interfered with the MBB derivatization reaction, this method was not suitable for total captopril analysis. Both methods were validated for the bioanalysis of free captopril in human plasma. After removal of plasma proteins, samples were analyzed without an additional extraction procedure. The limit of quantitation in plasma was 12.5 ng/ml for the MBB method (limit of detection 30 pg) and 25 ng/ml for the OPA method (limit of detection 50 pg). The OPA method was also validated for total captopril analysis in human plasma and urine. The limit of quantitation was 25 ng/ml in plasma and 250 ng/ml in urine (limit of detection 50 pg). We conclude that for the analysis of free captopril the precolumn MBB method is superior to the OPA method since only the derivatization reaction has to be carried out immediately. The postcolumn OPA method is especially suitable for the analysis of total captopril since reducing reagents and high concentrations of endogenous thiols do not interfere with the derivatization reaction.

Keywords: Captopril; Monobromobimane; *o*-Phthaldialdehyde

1. Introduction

The angiotensin converting enzyme (ACE) inhibitor captopril (1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline, Fig. 1), is a widely prescribed antihypertensive drug. This article describes the development and comparison of two HPLC methods with fluorescent derivatization of captopril. The first method is based on precolumn derivatization with monobromobimane (MBB) (Fig. 2). MBB has been used as fluorescent label in the analysis of several important biological thiols and thiol drugs [1–5].

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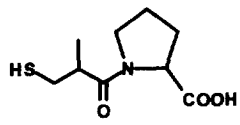

Fig. 1. Structure of captopril.

The other method is based on postcolumn derivatization of captopril with *o*-phthaldialdehyde (OPA). The fluorescent label OPA is commonly applied for the derivatization of primary amines and amino acids. However, when an amine is added as a reagent, OPA can also be used for the selective derivatization of thiols [5–8]. The reaction of captopril with OPA is depicted in Fig. 3.

Like other thiols, captopril undergoes rapid oxidation to disulfide metabolites both in vitro and in vivo [9]. Intracellularly, disulfide metabolites are reduced to the free thiol and as such they can act as a reservoir for free captopril [10]. The kinetics of captopril can be interpreted correctly only when both the free thiol (free captopril) and the total amount of reducible disulfide metabolites (total captopril) are determined.

Several methods have already been reported for the quantitative determination of free and total captopril in biological fluids, including gas chromatography–mass spectrometry (GC–MS) [11,12], radioimmunoassay (RIA) [13] and high-performance liquid chromatography (HPLC) [14–19]. The effective reduction of captopril disulfides with tributylphosphine (TBP) has been reported by several authors [13–16]. Some of the reported methods require very sophisticated and expensive equipment and other methods are very laborious. A major drawback of the existing methods is that relatively large sample volumes are required. Small sample volumes can be of importance when analyzing for captopril in small children [17,20] and in laboratory-animal studies. Both of the methods presented in this paper are sensitive, easy to perform and require only 100 μl of biological sample per assay. The methods were validated for the analysis of either free or total captopril in human plasma and for the analysis of total captopril in urine. In order to test their applicability in clinical and pharmacokinetic studies, the methods were used in a pharmacokinetic experiment in a human volunteer and in an in vivo rat experiment.

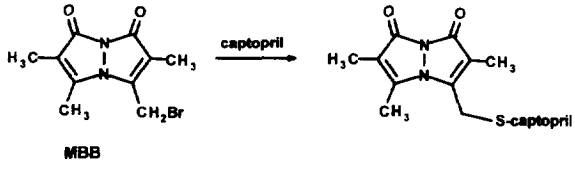

Fig. 2. Derivatization reaction of captopril with monobromobimane (MBB).

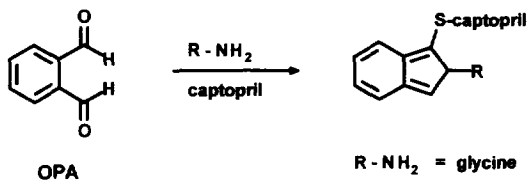

Fig. 3. Derivatization reaction of captopril with *o*-phthaldialdehyde (OPA).

2. Experimental

2.1. Materials

Captopril and MBB were purchased from Sigma (St. Louis, MO, USA). TBP (purum) and OPA (purum) were obtained from Fluka (Buchs, Switzerland). Glycine (analytical grade) was obtained from Merck (Darmstadt, Germany). All solvents were of HPLC quality (Labscan, Dublin, Ireland). All other chemicals were of analytical-reagent grade. Water was purified with a Milli-Q water system (Millipore, Bedford, MA, USA). Captopril tablets (Capoten) were obtained from Bristol-Myers Squibb (Woerden, Netherlands).

2.2. Instrumental

Reaction kinetics were performed on an AMINCO spectrofluorometer SPF-500 (SLM Instruments, Urbana, IL, USA) Bandpasses were 5 nm, high voltage was 1000.

The HPLC analyses were performed on a Waters liquid chromatograph (Waters, Milford, MA, USA) consisting of a 510 pump, an autoinjector 715, a column oven set at 50°C and a fluorescence detector

474. The chromatograph was equipped with a Nova-Pak C_{18} column (60 Å, 4 μm, 15 cm×3.9 mm I.D.), in combination with a μBondapak C_{18} Guard-Pak precolumn (Waters). While using the OPA method, postcolumn reagent was added by a 510 pump via a mixing-T and a mixing coil (1 m×0.5 mm I.D.).

2.3. Methods

2.3.1. MBB method

The fluorescence detector was operated at 400 nm and 480 nm for excitation and emission wavelengths, respectively. The mobile phase consisted of acetonitrile–water–trifluoroacetic acid (20:80:0.1) at a flow-rate of 1.0 ml/min.

2.3.2. Sample preparation for free captopril determination in plasma with MBB method

After the addition of 100 μl 0.1 *M* borate buffer pH 7.5 and 10 μl of 10% MBB in acetonitrile to 100 μl of plasma, the mixture was vortex-mixed for 15 s and allowed to stand at room temperature for 5 min. Proteins were precipitated by addition of 200 μl of acetonitrile and vortexing. After centrifugation at 3000 *g* for 5 min, the supernatant was kept frozen at −20°C prior to analysis. A 10-μl aliquot was injected into the HPLC system.

2.3.3. OPA method

The fluorescence detector was operated at 345 nm and 455 nm for excitation and emission wavelengths, respectively. The mobile phase, consisting of acetonitrile–water–trifluoroacetic acid (15:85:0.1) with 100 mg glycine per liter, was added at a flow-rate of 0.8 ml/min. A 0.1 *M* borate buffer (pH 8.5) containing 100 mg OPA per liter was added as post-column reagent at a flow-rate of 1.2 ml/min.

2.3.4. Sample preparation for free captopril determination in plasma with OPA method

After addition of 100 μl 0.1 *M* borate buffer (pH 7.5) and 300 μl of methanol to 100 μl of plasma, the mixture was vortex-mixed and centrifuged at 3000 *g* for 5 min. A 25-μl aliquot of the supernatant was injected directly into the HPLC system.

2.3.5. Sample preparation for total captopril determination in plasma with OPA method

After addition of 100 μl 0.1 *M* borate buffer (pH 7.5) and 100 μl of TBP (1% in methanol) to 100 μl of plasma, the mixture was vortex-mixed for 15 s and allowed to stand at room temperature for 15 min. Proteins were precipitated by addition of 200 μl of methanol and vortexing. After centrifugation at 3000 *g* for 5 min, the supernatant was allowed to stand at room temperature for at least 1 h. A 25-μl aliquot was injected into the HPLC system.

2.3.6. Sample preparation for total captopril determination in urine with OPA method

A 100 μl sample of diluted urine (1:10) was analyzed as described in Section 2.3.5 for determination of total captopril in plasma.

2.4. Validation of the HPLC methods

The MBB method and OPA method were validated for the analysis of free captopril in human plasma. The OPA method was also validated for the analysis of total captopril in human plasma and urine. The day-to-day reproducibility of the HPLC methods was determined by analyzing plasma and urine samples which were spiked with known amounts of captopril. The samples were assayed as described above. Calibration curves in plasma were made in the concentration range 12.5–1000 ng/ml for the MBB method and 25–1000 ng/ml for the OPA method. The calibration range for total captopril analysis in urine was 250–10 000 ng/ml.

2.5. Human pharmacokinetic experiment

Captopril (25 mg) was taken orally by a male volunteer. Blood samples were taken at 0, 0.5, 1, 2, 6 and 24 h into Venoject tubes containing 0.1 ml EDTA. The samples were centrifuged immediately for 5 min. A 100-μl aliquot plasma was derivatized immediately with MBB and kept frozen at −20°C until analysis. The plasma was analyzed immediately for free captopril and total captopril according to the OPA method. Urine was collected for 24 h and analyzed for total captopril (OPA method).

2.6. Animal pharmacokinetic experiment

The pharmacokinetic experiments were performed in conscious freely moving rats. A bolus dose of captopril of 0.5 mg/kg was administered via a heart-cannula [21]. Blood samples were drawn at 0, 15, 30, 60, 120 and 240 min via the same cannula and collected in heparinized polyethylene tubes. The samples were immediately centrifuged and plasma samples were store at −20°C. The urine was collected in preweighted polyethylene tubes. The urine samples were weighed and an aliquot was stored at −20°C. Both plasma and urine were analyzed for total captopril using the OPA method.

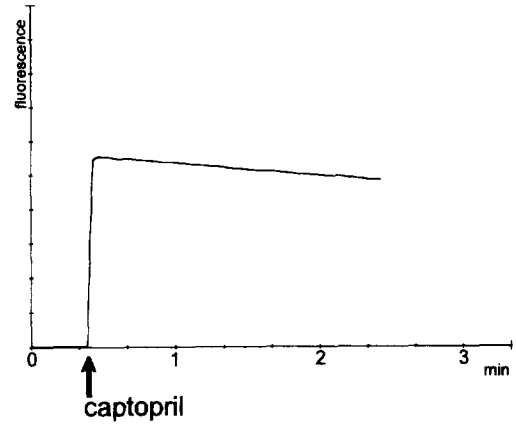

Fig. 4. Reaction course of captopril with OPA in a 2:3 (v/v) mixture of eluent and post column reagent.

3. Results and discussion

3.1. Derivatization procedures

The quantitative analysis of thiols is complicated by the reactive nature of this functional group. Most of the existing analytical methods for thiols are based on a precolumn derivatization reaction, protecting the thiol group from further oxidation [9]. We used MBB as precolumn derivatization reagent because adduct formation of thiols with MBB at room temperature is usually complete within a few minutes [1,2] and MBB adducts are stable at −20°C [2,4,5,9]. The addition of 10 μl of 10% MBB in our experiments was sufficient for complete adduct formation within 5 min.

Fig. 4 shows the formation of the captopril–OPA adduct in a 2:3 (v/v) mixture of mobile phase and postcolumn reagent. The pH of this mixture was 8.5. As can be seen the reaction is complete within seconds, but the formed reaction product is not stable. We concluded that OPA is a suitable fluorescent label for postcolumn labeling of captopril, but not for precolumn labeling. Since free captopril is readily lost from plasma samples in vitro [9,14], the postcolumn OPA method can only be used for free captopril analysis if the samples are analyzed immediately upon collection. With the MBB method only the derivatization reaction has to be carried out immediately.

3.2. Total captopril analysis

The reduction of disulfides can be achieved with different reducing agents, of which TBP and dithiothreitol (DTT) are the most common reducing reagents [2,14–16,22]. Unfortunately the MBB adducts of DTT, a thiol itself, interfered with the chromatography of captopril–MBB.

When TBP was used as reducing agent, optimal MBB derivatization of captopril could only be achieved if the excess of TBP was removed by extraction with *n*-hexane prior to derivatization.

The postcolumn OPA system proved to be especially suitable for total captopril analysis. Reduction of captopril disulfides with TBP was superior to DTT because DTT peaks interfered with the captopril peak in the chromatogram. Fig. 5 shows the results of the optimization of the TBP reduction. Previously spiked plasma samples were analyzed for total captopril. Free captopril was not detectable in these samples (assayed with OPA method). We concluded that 30 min incubation at room temperature with 1% TBP was sufficient for 100% reduction of the captopril disulfides.

3.3. Chromatography

Fig. 6 shows a typical chromatogram of captopril–MBB in a human plasma sample. Figs. 7 and 8 show typical chromatograms of total captopril in

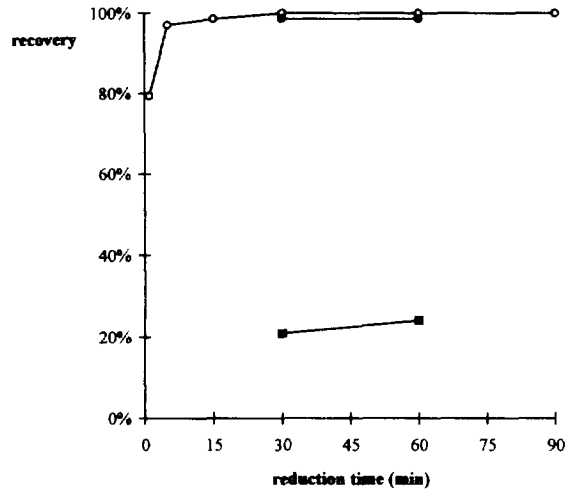

Fig. 5. Effect of incubation time, TBP concentration and temperature on the reduction of oxidized captopril with TBP. (■): 0.1% TBP, room temperature; (○): 1.0% TBP, room temperature; (●): 1.0% TBP, 56°C.

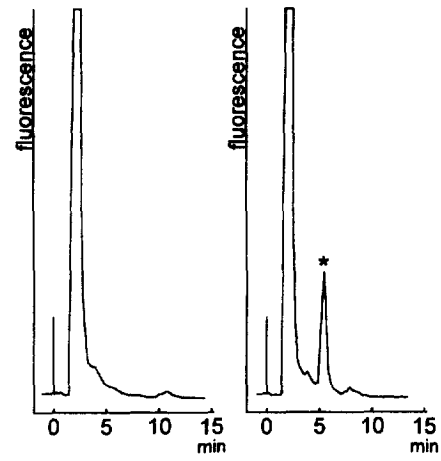

Fig. 7. OPA method: typical chromatogram of total captopril in human plasma. (A) blank; (B) spiked with 250 ng/ml captopril. Samples were treated as described in Section 2.3.5. The fluorescence detector was operated at 8×100 (att.×gain).

plasma and urine with the OPA method. The retention times of captopril–MBB and captopril are 4.8 min and 5.4 min, respectively. An extensive study was required to optimize the chromatographic conditions for both analytical methods. The unusual behavior of captopril and captopril derivatives is thought to be due to hindered rotation around the peptide bond of the proline peptide, which causes the molecule to have stable *cis* and *trans* conformations [23]. Eluting at different retention times, the rotamers of captopril are responsible for peak broadening and unpredictable column interactions. Raising the column temperature to 50°C increased the interconversion of the rotamers and improved peak shapes dramatically.

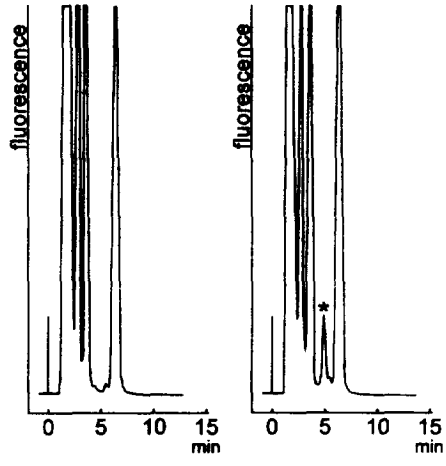

Fig. 6. MBB method: typical chromatogram of captopril–MBB in human plasma. (A) blank; (B) spiked with 250 ng/ml captopril. Samples were treated as described in Section 2.3.2. The fluorescence detector was operated at 8×100 (att.×gain).

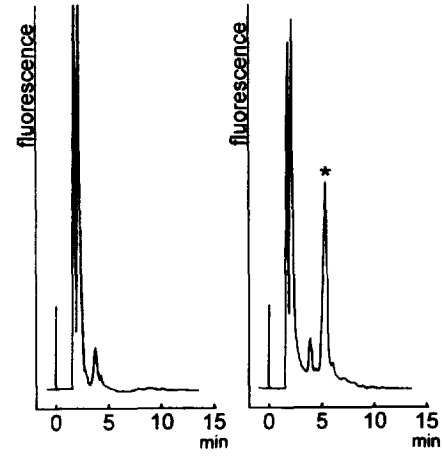

Fig. 8. OPA method: typical chromatogram of total captopril in human urine. (A) blank; (B) spiked with 2500 ng/ml captopril. Samples were treated as described in Section 2.3.6. The fluorescence detector was operated at 4×100 (att.×gain).

Table 1
Retention times of different thiols with the OPA method in various elution systems

Compound	Retention time (min)		
	MeOH–H_2O–TFA 5:95:0.1	ACN–H_2O–TFA 5:95:0.1	ACN–H_2O–TFA 15:85:0.1
Cysteine	1.8	1.7	–
Gluthathion	2.2	2.2	–
N-Acetyl cysteine	3.0	2.8	–
DTT	4.6	4.0	–
Captopril	40.4	24.8	4.4

The column temperature is 50°C; the eluent flow is 1.0 ml/min. All elution systems contain 100 mg/l glycine.

With a small variation of the chromatographic conditions the OPA method can also be applied for the analysis of other thiol-containing compounds. Table 1 gives a list of retention times of these compounds in various elution systems.

3.4. Validation of the HPLC methods

Table 2 shows the results of the day-to-day reproducibility of the free captopril determination with the MBB method and the OPA method in human plasma. The limit of quantitation (LOQ) of free captopril in plasma is 12.5 ng/ml with the MBB method and 25 ng/ml with the OPA method. The limit of detection (LOD) is 30 pg for the MBB method and 50 pg for the OPA method. Both methods showed good linearity and reproducibility in the low nanogram range. Linear calibration curves could be obtained at the low nanogram range [the correlation coefficient was 0.998 for both methods; the slope was 0.85 (MBB) or 2.34 (OPA)].

The OPA method was validated for total captopril analysis in human plasma and urine. Table 3 shows the results of the day-to-day reproducibility. The LOQ in plasma is 25 ng/ml, the LOQ in urine is 250 ng/ml in urine. The LOD is 50 pg total captopril. Good linearity was observed for both plasma and urine calibration curves [the correlation coefficient was 0.999 for both plasma and urine; the slope was 1.68 (plasma) or 28.56 (urine)].

Both methods also showed good reproducibility and accuracy when free or total captopril was analyzed in rat plasma or urine (data not shown).

3.5. Human pharmacokinetic experiment

Fig. 9 shows the plasma levels of free captopril and total captopril following an oral administration of 25 mg captopril to a human volunteer. Maximal total captopril concentrations were reached at 2 h after dosing (396.8 ng/ml). Free captopril concentrations were maximal at 1 h after dosing [146.4 ng/ml (MBB method) or 150.4 ng/ml (OPA method)]. The free captopril levels in plasma when determined with either the MBB method or the OPA method showed a good correlation (the correlation coefficient between the two methods was 0.994).

The urinary recovery of total captopril for 24 h was 78.6% of the dose. Free captopril was not determined in the urine since oxidation of free captopril continues in the bladder, resulting in an underestimation of the actual free captopril concentration after urination.

Although we only performed a single experiment, the pharmacokinetic data are in good agreement with the literature [16,19,24].

3.6. Animal pharmacokinetic experiment

Fig. 10 shows the plasma disappearance of total captopril after administration of an intravenous bolus of 0.5 mg/kg captopril. The curve could be fitted adequately with a two-compartment pharmacokinetic model, resulting in a terminal half-life of 108 min and a clearance of 11.3 ml/kg/min. The urinary recovery of total captopril measured in a period of 10 h after administration was 82% of the dose.

Table 2
Validation of free captopril analysis in human plasma

Spiked amount (ng/ml)	MBB method accuracy		Precision, R.S.D. (%)		OPA method accuracy		Precision R.S.D. (%)	
	Calculated amount (ng/ml)	% of theoretical amount	Within-day	Between-day	Calculated amount (ng/ml)	% of theoretical amount	Within-day	Between-day
50	48.48	97.0	9.27	8.35	48.61	97.2	5.24	4.00
250	254.21	101.7	7.10	2.63	261.63	104.7	3.63	5.39
1000	990.02	99.0	8.95	8.26	985.44	98.5	3.93	5.53

Calibration curves were made in duplo on three consecutive days.

Table 3
Validation of total captopril analysis with the OPA method in human plasma and urine

Spiked amount (ng/ml)	Accuracy		Precision, R.S.D. (%)	
	Calculated amount (ng/ml)	% of theoretical amount	Within-day	Between-day
Plasma				
50	52.77	105.5	3.57	1.44
250	247.57	99.0	2.41	2.34
1 000	991.85	99.2	1.10	1.07
Urine				
500	541.06	108.2	2.48	2.15
2 500	2 572.56	102.9	3.87	3.85
10 000	10 595.68	106.0	1.21	3.11

Calibration curves were made in duplo on three consecutive days.

4. Conclusion

Both HPLC methods are sensitive, easy to perform and require only a small volume of biological sample. The MBB method and the OPA method can be applied for the analysis of free captopril, although the MBB method is superior to the OPA method for this type of analysis with regard to sensitivity and sample stability. The OPA method proves especially suitable for total captopril analysis.

Since both methods can be operated on the same chromatograph with only a small modification of the chromatographic conditions, we prefer a combination of both methods for captopril bioanalysis, using the MBB method for free captopril analysis and the OPA method for total captopril analysis.

Acknowledgments

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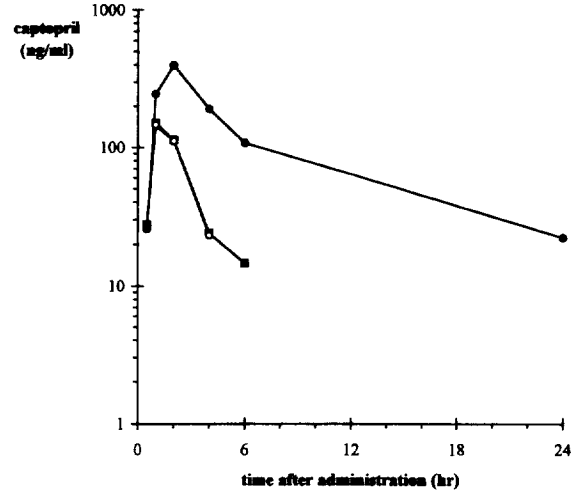

Fig. 9. Plasma concentrations in a healthy human subject after 25 mg oral intake of captopril. (■): free captopril with MBB method; (○): free captopril with OPA method; (●): total captopril with OPA method.

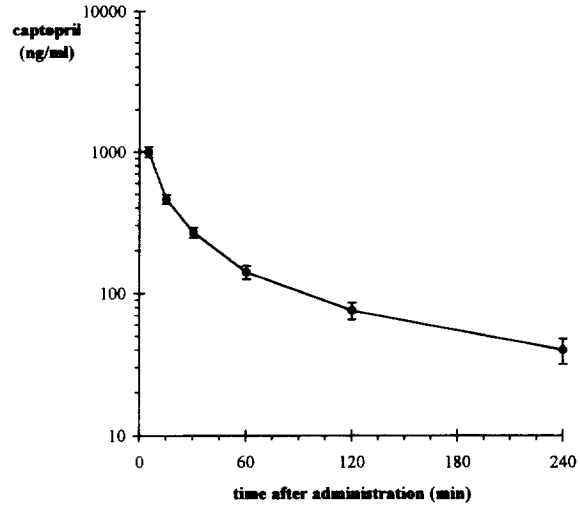

Fig. 10. Plasma concentrations of total captopril in male Wistar rats following an i.v. bolus of captopril of 0.5 mg/kg. Values are mean±S.E.M. ($n=7$).

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