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Determination of total captopril in dog plasma by HPLC after prelabelling with ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F)

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Abstract: A HPLC method has been developed for the determination of captopril, an angiotensin-converting enzyme (ACE) inhibitor, in dog plasma. The plasma was deproteinized with trichloroacetic acid (TCA) containing N-(3-mercapto-1-oxopropyl)-piperidine-2-carboxylic acid (SQ 25,233) as an internal standard, adjusted to pH 9.5 with borate buffer containing tri-*n*-butyl phosphine (TBP) to reduce the oxidized captopril, and treated with a thiol specific reagent, SBD-F. The fluorescent derivatives of captopril and the internal standard were separated from other interfering peaks on a reversed-phase column and determined by HPLC. The proposed method was simple, selective and sensitive. The total plasma concentration of captopril reached a maximum of 3.04 ± 0.54 μg/ml (mean ± S.E.) 60 min after administration of the drug.

Keywords: *SBD-F, a thiol specific fluorogenic reagent; captopril, an angiotensin-converting enzyme inhibitor; HPLC–fluorescence detection; plasma level of total captopril.*

Introduction

Captopril (1-(D-3-mercapto-2-methyl-1-oxopropyl)-L-proline) is a potent angiotensin-converting enzyme (ACE) inhibitor [1–4]. Its pharmacokinetics have been recently reported [5–8]. Methods for the assay of captopril, such as gas chromatography [9], gas chromatography–mass spectrometry (GC–MS) [10–11], a radiochemical method [8], and high-performance liquid chromatography (HPLC) [12–14], in blood samples have some disadvantages in respect of simplicity, selectivity and sensitivity.

A new fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (SBD-F), has recently been developed [15] for thiol compounds. Its favourable properties include: high reactivity to thiol compounds so that these compounds can be

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detected at low concentrations; lack of fluorescence itself; solubility in biological buffers; stability for more than 1 week at room temperature in water; and stability of its derivatives for more than 1 week at pH 9.5 and 4°C. These properties prompted its use to determine captopril.

The present work reports the HPLC determination of SBD–captopril in tri-*n*-butyl phosphine (TBP) treated plasma after oral administration of 50 mg captopril to dogs.

Experimental

Materials

Captopril(1-(D-3-mercapto-2-methyl-1-oxopropyl)-L-proline), captopril disulphide and captopril–glutathione disulphide were obtained from Sankyo (Tokyo, Japan). *N*-(3-Mercapto-1-oxopropyl)-piperidine-2-carboxylic acid (SQ 25,233), used as an internal standard, was obtained from E. R. Squibb and Sons (Princeton, NJ, USA) (Fig. 1). All other chemicals used in this work were of analytical reagent grade. SBD-F [15] was synthesized in the laboratory (the compound decomposed at above 280°C).

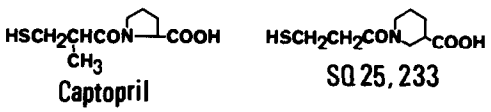
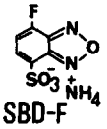


Figure 1
Structures of captopril, SQ 25,233 and SBD-F.



Apparatus

· A Waters high-performance liquid chromatograph equipped with a U6K Universal Injector, a 6000A Pump and an Auto Sample Processor Model 710B was used. A fluorescence spectrophotometer 650-10S (Hitachi, Tokyo, Japan) equipped with an 18-μl flow cell and a 150-W xenon lamp was used with excitation and emission wavelengths set at 385 nm and 515 nm, respectively. A 300 × 3.9 mm i.d. column packed with 8–10-μm Bondapak C_{18} (Waters Assoc.) was used at ambient temperature. Methanol–1% phosphoric acid (35:65 v/v) was employed as a mobile phase at a flow rate of 1.5 ml/min. The elution solvent was filtered through a type HA filter (0.45 μm, Millipore) and degassed just before use. Infrared (IR) spectra were recorded using potassium bromide (KBr) discs with a JASCO Model IRA-2 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL Model FX-100 spectrometer at 100 MHz using tetramethylsilane (TMS) as an internal standard.

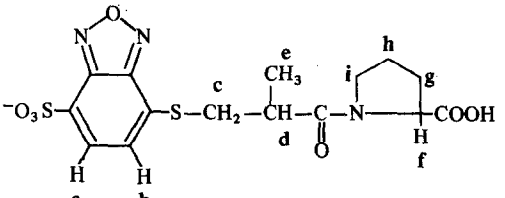
Dogs

Six adult, male, purebred beagles (body weight, 10–12 kg) were fasted 16 h before oral administration of single doses of captopril (two 25-mg tablets with 10 ml water).

Preparation of SBD–captopril

Captopril (650 mg) and SBD-F (62 mg) dissolved in 5 ml 0.1 M borate buffer (pH 9.5) was heated at 60°C for 30 min; the reaction mixture was then evaporated *in vacuo*. The

residue was dissolved in benzene–acetic acid (7:1) and applied to a 120 × 30 mm i.d. column packed with 100–200 mesh silica gel (Kanto Chemical Co.). The column was eluted with benzene–methanol (6:4 v/v) and the fractions corresponding to a yellow band were collected. The fractions were evaporated to dryness *in vacuo*. The residue was dissolved in 3 ml of 1% acetic acid and subjected to gel permeation chromatography (Sephadex G-10, Pharmacia Fine Chemicals, Sweden) using 1% acetic acid as an eluent. The fluorescent fractions were collected and lyophilized to obtain the yellow powder (yield 60%). Analysis calculated for $C_{15}H_{16}N_3O_7S_2Na.H_2O$: C, 39.56; H, 3.98; N, 9.23; S, 14.08. Found: C, 39.33; H, 3.79; N, 8.99; S, 13.88. NMR* (in DMSO-d_6) δ: 7.76 (1H, d, J_{ab} = 7.2 Hz, *a*), 7.41 (1H, d, J_{ab} = 7.2 Hz, *b*), 4.40 (1H, q, *f*), 3.36–3.60 (2H, m, *i*), 2.95–3.36 (3H, m, *c* + *d*), 1.68–2.28 (4H, m, *g* + *h*), 1.17 (3H, d, *J* = 6.6 Hz, *e*). IR maxima (KBr discs), cm^{-1}: 3430 (COOH), 1730 (COO), 1630 (CON). Excitation and emission maxima of the adduct in 0.1 M borate buffer (pH 9.5) were 388 nm and 517 nm, respectively. The structure of the adduct was unequivocally established on the basis of the instrumental data.



SBD-captopril

Assay for total (free and oxidized) captopril in dog plasma

A 2-ml portion of fresh blood drawn with a heparinized syringe, just before administration of the drug and thereafter at frequent intervals for 6 h, was poured into a glass tube cooled with a mixture of crushed ice and water. The heparinized whole blood was immediately centrifuged at 1200***g*** and 0°C for 5 min to separate plasma. To 0.5 ml plasma was added an equal volume of 10% trichloroacetic acid (TCA) containing SQ 25,233 (5 μg) as an internal standard; the solution was immediately mixed in a vortex mixer for about 10 s and centrifuged at 1200***g*** and 0°C for 5 min. To 50 μl supernatant was added a mixture of 100 μl borate buffer (2.5 M; pH 9.5, prepared with 2.5 M boric acid and potassium hydroxide) containing 4 mM disodium edetate, with 50 μl SBD-F (0.4 mg/ml) in 2.5 M borate buffer (pH 9.5) and 10 μl TBP (0.2 g/ml) in N,N-dimethylacetamide) (DMA); DMA was selected because of the high solubility of TBP in that solvent. The solution was vigorously mixed and allowed to stand at 60°C for 1 h. A 30-μl aliquot of the cooled reaction mixture was analysed by HPLC.

Reduction of captopril disulphide and captopril–glutathione disulphide with TBP

Dog plasma (0.5 ml) spiked with known amounts of captopril (0.125–2.0 μg), captopril disulphide (0.125–2.0 μg) or captopril–glutathione disulphide (0.29–4.65 μg) was treated as described above except that the internal standard was not added. After separation by HPLC, the peak heights corresponding to SBD–captopril were measured and the reduction ratios of captopril disulphide and captopril–glutathione disulphide with TBP were determined, based on the peak heights of free captopril.

* Abbreviations used for ^{1}H-NMR: d = doublet, q = quartet, m = multiplet.

Calibration graph for total captopril in dog plasma

Dog plasma (0.5 ml), spiked with known amounts of captopril (0.125–2.0 μg), was analysed according to the proposed analytical procedure. The peak height ratios of SBD–captopril to the SBD–internal standard (5 μg) were plotted against the plasma concentration of the added captopril.

Derivatization of captopril in dog plasma with SBD-F

To 0.5 ml freshly prepared dog plasma was added 0.5 ml TCA (10%) containing captopril (2 μg). The solution was immediately mixed for about 10 s and centrifuged at 1200*g* and 0°C for 5 min. To 50 μl supernatant was added the mixture of 100 μl borate buffer (2.5 M; pH 9.5, prepared with 2.5 M boric acid and potassium hydroxide) containing 4 mM disodium edetate, 50 μl SBD-F (0.4 mg/ml) in 2.5 M borate buffer (pH 9.5) and 10 μl TBP (0.2 g/ml) in DMA. The solution was vigorously mixed and heated at 60°C. At certain time intervals, 30-μl aliquots of the reaction mixture were analysed by HPLC. The reaction yield was calculated from the peak height of derivatized captopril and that of the authentic SBD–captopril.

Derivatization of captopril with SBD-F in borate buffer (pH 9.5)

The mixture of 50 μl captopril (2 μg/ml water), 100 μl borate buffer (2.5 M; pH 9.5) containing 4 mM disodium edetate, 50 μl SBD-F (0.4 mg/ml) in 2.5 M borate buffer (pH 9.5) and 10 μl TBP (0.2 g/ml) in DMA was treated as described above. Aliquots (30 μl) of the reaction mixture were analysed by HPLC and the reaction yield was calculated as described above.

Results and Discussion

Reaction conditions for derivatization of total captopril in plasma

SBD-F specifically reacts with thiol-containing compounds in alkaline medium to yield fluorescent compounds [15]. In the borate buffer solution (pH 9.5), captopril was derivatized with the reagent at 60°C for 30 min (Fig. 2).

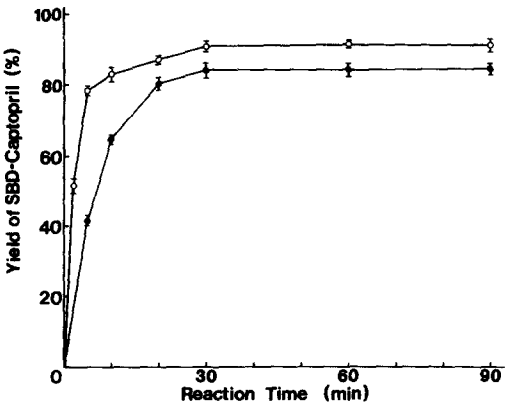


Figure 2
Time courses for derivatization of captopril with SBD-F at 60°C and pH 9.5 in solutions containing 10 μl TBP (0.2 g/ml) in DMA. —○—: in pH 9.5 buffer; —●—: in dog plasma adjusted to pH 9.5. Concentrations: captopril, 2.2 μM; SBD-F, 40.5 μM. Each point and bar indicate the mean and S.D., respectively (n = 10).

Captopril circulates in the reduced form, the oxidized form and the mixed disulphides with glutathione or cysteine [6, 16]. Shindo *et al.* reported that a significant amount of captopril (reduced form) was recovered in the urine after administration of ^{14}C/labelled captopril disulphide to the rat or dog [16]. In an experiment *in vitro* with rat liver, captopril disulphide was similarly converted to the reduced form of captopril [16]. Therefore, captopril (reduced form) seems to be generated from the oxidized form or the mixed disulphides of captopril by glutathione reductase in the circulation or at the reactive site of captopril. Therefore, in this experiment, the total captopril in the forms of reduced, oxidized and mixed disulphides was determined.

Of several known reducing reagents, it was found that TBP was best for the reduction of disulphide because of its good reduction ratio [13]. Thus in the present work, TBP was selected for reduction of the oxidized captopril. Indeed, captopril disulphide and captopril–glutathione disulphide were reduced quantitatively (more than 95%) at pH 9.5 and 60°C for 1 h and the SBD-adduct formed from free captopril was stable for 3 h under the conditions of reduction.

Captopril in dog plasma adjusted to pH 9.5 was also derivatized at 60°C in the presence of TBP. As shown in Fig. 2, the yield of the derivative rose initially as the reaction time was increased; the yields in plasma and in the buffer solution were similar after 30–90 min. From these results, conditions of derivatization of total captopril in plasma with SBD-F were selected to be 60°C for 1 h in the borate buffer (pH 9.5).

HPLC separation of SBD–captopril and internal standard from interfering peaks

The next set of experiments were devised for the chromatographic separation of SBD–captopril and the selection of an internal standard. Several analogues of captopril were examined for suitability as an internal standard for separation on 8–10-μm Bondapak C_{18}. Among those analogues tested, SQ 25,233 was selected because of its reactivity with SBD-F and because its peak was well separated from interfering peaks that originated from plasma.

The fluorophores of cysteine and glutathione, the main thiol components in plasma, are eluted earlier and separated from the SBD–captopril on 8–10-μm Bondapak C_{18}. The amine components that exist in great amounts in plasma do not generate fluorescence under the experimental conditions. Thus, no interfering peaks close to those of SBD–captopril and SQ 25,233 were recognized on the chromatogram obtained from plasma (Fig. 3). Furthermore, the short time for analysis was advantageous for routine use.

Recovery of captopril after addition to plasma

A known amount of captopril was added to plasma containing 10 μl of TBP (0.2 g/ml) in DMA and the overall recovery was determined with reference to authentic SBD–captopril. At 60°C and pH 9.5 for 1 h, the recovery of captopril added to the plasma was 84.3 ± 2.4% (mean ± S.D.). In contrast, the recovery at pH 9.5 without plasma was 91.3 ± 1.8% (mean ± S.D.). The recoveries were not enhanced by an increase in SBD-F (from 0.4 to 3.2 mg/ml) and a rise in pH (from pH 9.5 to pH 11.5) of plasma. The reason for the lower recovery in the plasma is not known. The recovery of SQ 25,233 (internal standard) was almost the same as that of captopril. In the experiments, the plasma captopril levels were determined by the peak height ratios of captopril to the internal standard, which was added to the plasma.

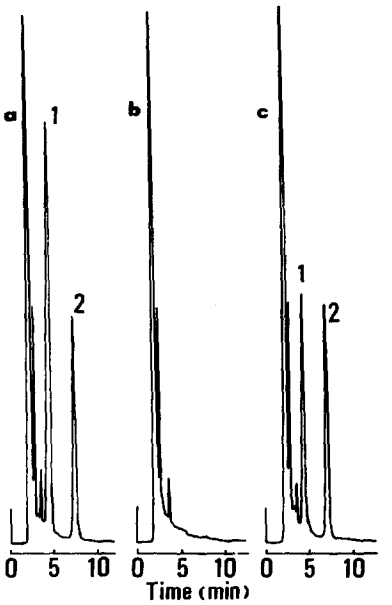


Figure 3
Chromatograms: a, standard SBD-adducts (15 ng captopril and 37.5 ng SQ 25,233); b, blank plasma reacted with SBD-F; c, plasma sample of the drug treated dog (30 min after oral administration with 50 mg captopril). 1, SBD–captopril; 2, SBD–SQ 25,233.

Linearity and sensitivity of determination of plasma captopril

When the peak height ratio of the SBD–captopril to the SBD–internal standard was plotted against the concentration of captopril added to the fresh pooled plasma of six dogs, good linearity was observed. The relative standard deviations (RSD) (n = 10) for 421, 183, 99, 54 and 26 ng/ml (mean values) of captopril were 3.0, 2.5, 2.3, 2.0 and 4.6%, respectively. The slope, intercept and correlation coefficient of the calibration graph were 0.784 ± 0.024, −0.037 ± 0.024 (mean ± S.D.) and 0.996 (n = 25), respectively. Therefore, the proposed method was thought to be suitable for the present purpose.

Detection limits (signal-to-noise ratio of 3, n = 5) were 270 pg (for 3.75 ng injected) and 240 pg (for 0.93 ng injected), respectively. The sensitivity by this procedure was comparable to that obtained by GC–MS [10], a radiochemical [17] method or an electrochemical method [14].

Total plasma captopril

As shown in Fig. 4, the total plasma captopril after administration of 50 mg in dogs reached its maximum at 60 min at a level of 3.04 ± 0.54 μg/ml (mean ± S.E.).

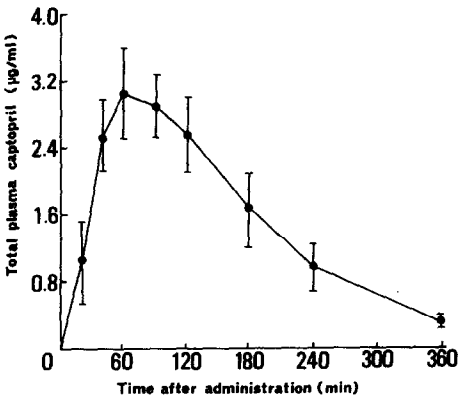


Figure 4
Time courses of total plasma captopril concentration in dogs. Each point and bar indicate the mean and S.E. of results obtained from six dogs. Each dog was given orally 50 mg of captopril in a single dose.

Matsuki *et al.* reported that the plasma free captopril level in the dog reached its maximum after 60 min and its disulphide level reached its maximum after 90 min, determined by a GC–MS method [11]. In the present work, the plasma free captopril level determined at the same time as the total level (data not shown) also reached its maximum at 60 min, and the plasma captopril disulphide level (calculated from the total captopril (Fig. 4) minus free captopril) also reached its maximum at 90 min; thus the present result was the same as that in the work of Matsuki *et al.* [11].

In conclusion, the proposed method is simple, selective and sensitive for the assay of plasma levels of total (free plus oxidized) captopril.

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