Table II—Plasma Levels of Acetaminophen (μg/0.5 mL) Following Administration of 325.0 mg of Acetaminophen at 0 and 6 h

Subject	Hours After Treatment													
	0	1	2	3	4	5	6	7	8	9	10	12	16	24
1	N.D.[a]	1.421	0.905	0.936	0.671	0.496	0.449	0.711	1.196	1.368	0.911	0.626	0.237	0.078
2	N.D.	1.058	0.923	0.615	0.464	0.294	0.238	0.343	1.688	1.178	0.814	0.454	0.187	0.065
3	N.D.	1.512	0.989	0.702	0.517	0.436	0.333	1.074	1.506	1.010	0.701	0.381	0.119	N.D.
4	N.D.	1.357	1.049	0.744	0.530	0.406	0.292	1.169	1.494	0.982	0.777	0.384	0.109	N.D.
5	N.D.	1.110	0.758	0.584	0.399	0.271	0.210	0.189	0.671	0.711	0.811	0.376	0.143	0.073
6	N.D.	1.239	1.542	0.798	0.617	0.361	0.249	0.651	1.954	1.233	0.861	0.400	0.165	0.078
7	N.D.	2.249	1.557	1.181	0.939	0.801	0.523	0.696	2.703	2.055	1.609	0.752	0.278	0.094
8	N.D.	1.265	1.360	1.110	0.702	0.554	0.441	0.885	2.212	1.354	0.878	0.494	0.172	0.056
9	N.D.	1.696	1.200	0.811	0.551	0.439	0.378	0.279	2.199	1.488	1.117	0.551	0.230	0.089

[a] N.D.—not detectable.

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Determination of a New Antihypertensive Agent (2*R*,4*R*)-2-(2-Hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic Acid in Blood by High-Performance Liquid Chromatography with Electrochemical Detection

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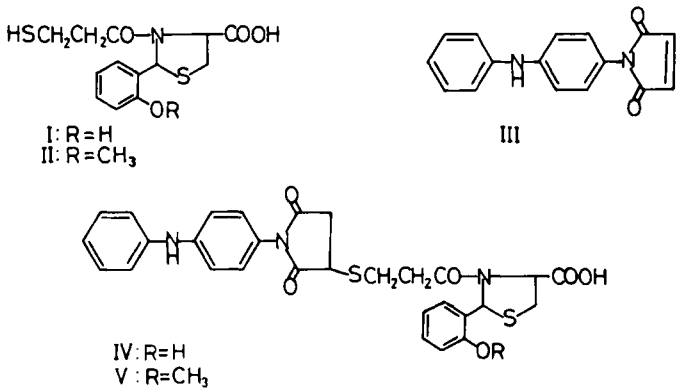
Abstract □ A sensitive method has been developed for the determination of a new antihypertensive agent, (2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid (I), in human blood. Because the drug is unstable in biological fluids, it was immediately derivatized by treating the freshly drawn blood specimens with *N*-(4-anilinophenyl)maleimide. The adduct was separated and determined by high-performance liquid chromatography with electrochemical detection on a reverse-phase column. The assay method was satisfactory with respect to the sensitivity and precision, providing a quantitation limit of 2 ng/ml and coefficient of variation of 3%. Preliminary pharmacokinetic data were obtained by orally administering I to two patients with essential hypertension.

Keyphrases □ Antihypertensive agents—(2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid, determination in human blood, high-performance liquid chromatography with electrochemical detection □ High-performance liquid chromatography—determination of (2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid in human blood, electrochemical detection □ (2*R*,4*R*)-2-(2-Hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid—determination in human blood, high-performance liquid chromatography with electrochemical detection

(2*R*,4*R*) - 2 - (2-Hydroxyphenyl) - 3 - (3-mercaptopropionyl)-4-thiazolidinecarboxylic acid (SA 446, I), a newly developed potent and specific inhibitor of angiotensin-converting enzyme, appears to be a promising antihypertensive agent (1, 2). This drug is several times more potent *in vitro* than captopril, a specific and orally active inhibitor of angiotensin-converting enzyme proven useful in patients with hypertension and congestive heart failure (3, 4). The lower therapeutic dose of I requires a more sensitive method for the determination of the drug in biological fluids for pharmacokinetic studies. A sensitive GC method using an electron capture detector has been reported, but this procedure requires a two-step derivatization (5). High-performance liquid chromatography (HPLC) with



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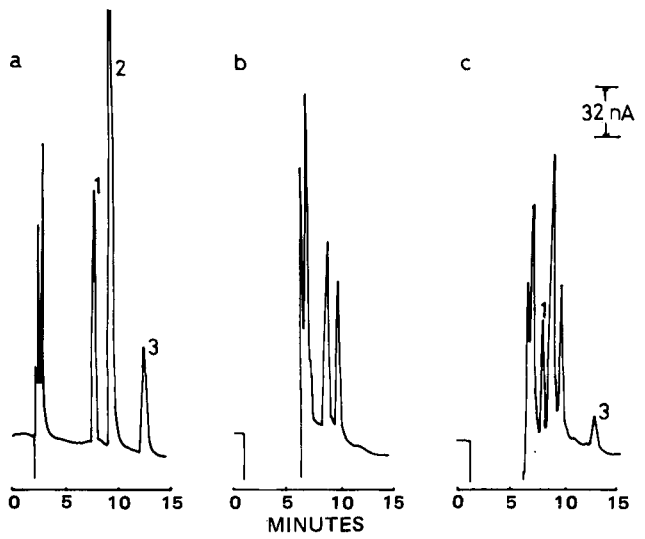

Figure 1—*Chromatograms obtained with (a) a standard 5-ng sample, (b) a blank sample, and (c) a blood sample of a patient orally administered 50 mg of I (325 ng/mL). Key: (1) I–*N*-(4-anilinophenyl)maleimide adduct; (2) N-(4-anilinophenyl)maleimide, (3) internal standard–N-(4-anilinophenyl)maleimide adduct.*

electrochemical detection (ED) continues to gain popularity for the sensitive and selective determination of trace components in complex biological samples (6, 7).

In our previous work we developed a sensitive method for the quantitation of captopril at therapeutic doses in human blood. Captopril was derivatized with *N*-(4-dimethylaminophenyl)maleimide into the electrochemically active adduct, which was then separated and determined by HPLC–ED (8). It was also found that *N*-(4-anilinophenyl)maleimide (III) was a better derivatization reagent for thiol compounds than *N*-(4-dimethylaminophenyl)-maleimide because of its higher sensitivity to ED (9). This paper describes a new method for the determination of I in whole blood, which involves derivatization with *N*-(4-anilinophenyl)maleimide into the electrochemically active form followed by HPLC–ED.

EXPERIMENTAL

Instruments—The apparatus used for this work was a high-performance liquid chromatograph[1] equipped with an electrochemical detector[2]. The applied potential was set at +1.0 V *versus* a silver–silver chloride reference electrode. A sample was introduced by a universal injector[3] with an effective volume of 2 mL. A reverse-phase column[4] (30.5 × 0.4-cm i.d.) was used under ambient conditions. A 1:1 mixture of acetonitrile and 0.8% $NH_4H_2PO_4$ (pH 3.0, adjusted with phosphoric acid) was used as a mobile phase at a flow rate of 1 mL/min.

Materials—(2*R*,4*R*)-2- (Hydroxyphenyl) -3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid[5] and the internal standard[5], (2*R*,4*R*)-2-(2-methoxyphenyl)-3- (3-mercaptopropionyl) -4-thiazolidinecarboxylic acid (SA 427, II) were used as received. *N*-(4-Anilinophenyl)maleimide was prepared in-house as described in a previous paper (9). All other chemicals were of analytical reagent grade.

Standard Solutions—Standard solutions of I and the internal standard were each prepared by dissolving 2.0 mg of pure sample in 5.0 mL of methanol. These solutions were stable at least for 1 week when stored at −20°. A solution of 10 mg of *N*-(4-anilinophenyl)maleimide dissolved in 1.0 mL of acetone could be stored unchanged in a refrigerator for several months.

[1] Model ALC/GPC 202; Waters Associates, Milford, Mass.
[2] Model VMD 101; Yanagimoto Co., Kyoto, Japan.
[3] Model U6K; Waters Associates, Milford, Mass.
[4] μBondapak C_{18}; Waters Associates, Milford, Mass.
[5] Santen Pharmaceutical Co., Osaka, Japan.

Table I—Recovery of I Added to Human Blood

I Added, ng/mL	I Found, ng/mL	Recovery, %[a]
40	29.5	73.7±1.94
100	75.1	75.1±1.59
200	151	75.6±1.74
600	449	74.9±1.93
1000	740	74.0±1.77

[a] Mean ± *SD*; $n = 6$.

Subjects and Medication—Two female patients with essential hypertension were orally given 50 or 30 mg of I in capsule form after an overnight fast. The subjects were kept supine during the study period. Blood samples were withdrawn prior to administration of the drug and at intervals up to 2 h thereafter.

Assay Procedure—Duplicate 0.5-mL aliquots of each whole blood sample were poured immediately into tubes containing 20 μL of a 1% *N*-(4-anilinophenyl)maleimide solution and 0.3 mL of 0.033 M sodium-potassium phosphate buffer (pH 6.85). After addition of 100 ng of the internal standard, the tube was vortexed and then allowed to stand at 0°C for 30 min. The samples were frozen in a dry ice–acetone bath until analyzed.

The thawed sample was extracted twice with 2 mL of ether–hexane (1:1). After addition of ~500 μg of glutathione to consume the excess reagent, the aqueous layer was kept at 0°C for 20 min and then deproteinized with 3 mL of acetone. After centrifuging at 1500×*g* for 5 min, the precipitate was washed with 3 mL of acetone. The supernatant and washings were combined and evaporated to ~1 mL *in vacuo* at room temperature. The concentrate was diluted with 6 mL of water and applied to a reverse-phase cartridge[6]. After washing with 2 mL of water, the adducts of I and the internal standard with *N*-(4-anilinophenyl)maleimide were eluted with 8 mL of acetonitrile. The effluent was evaporated to dryness *in vacuo* below 40°C. The residue was dissolved in 0.2 mL of methanol, an aliquot of which was applied to the HPLC.

A calibration curve was constructed by assaying blood samples spiked with known amounts of I in the manner described above. The peak height ratio of I to the internal standard (II) was plotted against the blood concentration of I.

Derivatization of I—To a solution of 10 μg of *N*-(4-anilinophenyl)-maleimide in 1 ml of 0.033 M sodium-potassium phosphate buffer (pH 6.85) kept at 0°C in an ice-bath was added 4.0 μg of I. The reaction was terminated by extracting with 2 mL of ether–hexane (1:1) to eliminate the excess reagent. A 10-μL aliquot of the remaining aqueous layer was applied to the HPLC.

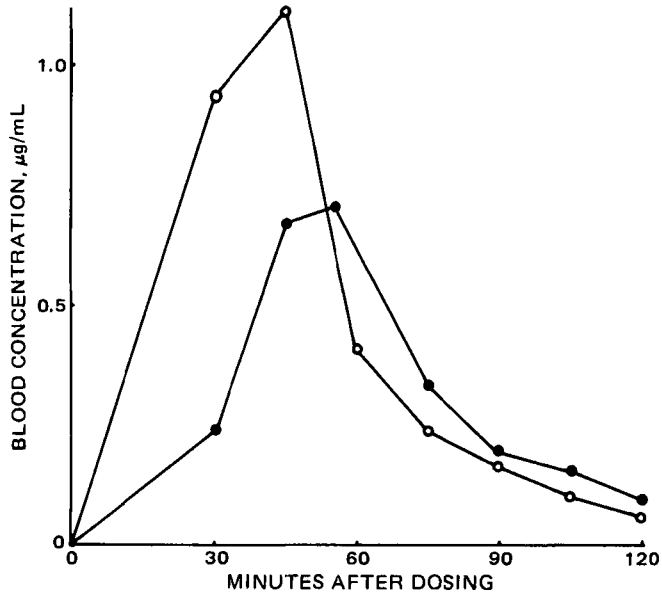

Figure 2—*Change in blood level after oral administration of I in two patients with essential hypertension. Key: (○) subject A, dose 50 mg; (●) subject B, dose 30 mg.*

[6] Sep-pak C_{18}; Waters Associates, Milford, Mass.

Table II—Accuracy and Precision of the Present Method for Determination of I in Human Blood

I Added, ng/mL	I Found, ng/mL[a]	CV, %
40	41± 1.2	3.0
200	200± 4.2	2.1
1000	1007±21	2.1

[a] Mean ± *SD*; $n = 6$.

RESULTS AND DISCUSSION

It is well known that thiol compounds are unstable, especially in biological fluids because of their high reactivity. In addition, the thiol compound I exhibits no prominent UV absorption responsive to UV detection. Therefore, appropriate precolumn labeling is needed for sensitive detection of I. The use of a derivatization reagent both reactive towards the thiol group and responsive to ED seemed to be promising. Accordingly, *N*-(4-anilinophenyl)maleimide, readily obtainable by a known method (9, 10), was chosen for investigation as a derivatization reagent.

First, the effect of reaction time on the formation of the adduct was investigated. After treatment with *N*-(4-anilinophenyl)maleimide in phosphate buffer at 0°C, I was quantitatively derivatized into the adduct (IV) in 10 min. The internal standard–*N*-(4-anilinophenyl)maleimide adduct (V) was similarly formed from II which was used as the internal standard. Among the columns tested, an octadecylsilane reverse-phase column[4] was the most favorable for efficient separation of the compounds. A hydrodynamic voltammogram of the I–*N*-(4-anilinophenyl)maleimide adduct indicated that the maximum sensitivity was obtainable at the potential of ~ +1.0 V, which was therefore used for the electrochemical detection. The electrochemical behavior of the internal standard–*N*-(4-anilinophenyl)maleimide adduct was similar to that of the I–*N*-(4-anilinophenyl)maleimide adduct. Typical chromatograms of the adducts formed from I and the internal standard (II) are illustrated in Fig. 1a. The detection limit (S/N = 2 at 4 nA full scale) was estimated to be 40 pg for I.

The next effort was focused on establishing a clean-up procedure for I in biological fluids. Compound I in human blood was immediately derivatized with *N*-(4-anilinophenyl)maleimide to prevent oxidative decomposition. Excess reagent was eliminated by extraction with ether–hexane and treatment with glutathione; the glutathione–*N*-(4-anilinophenyl)maleimide adduct was eluted at the solvent front on the chromatogram. More polar solvents such as ether or ethyl acetate were effective for removal of excess reagent, but reduced the recovery rate because the adducts of I and internal standard were also partially lost. The deproteinized sample was applied to a reverse-phase cartridge[6], and the adducts of I and the internal standard were eluted with acetonitrile. No interfering peaks were present with the blank specimen, as shown in Fig. 1b and c. Interferences of endogenous polar substances due to saturation of the output of the detector were overcome by disconnecting the detector for ~5 min after injection (11).

A known amount of I was added to the blood, and the overall recovery was estimated. Compound I spiked at five levels was recovered at the rate of >70% (Table I). In addition, the mean recovery rate for 100 ng of the internal standard (II) was 73.9±1.1%. When the peak height ratio of I/internal standard was plotted against the concentration of I (20–1000 ng/mL) spiked in a human blood specimen, a linear correlation was observed with the regression line defined by a slope of 11.96, a *y*-intercept of 0.018, and a correlation coefficient of 0.9998. The proposed method was also satisfactory with respect to both accuracy and precision (Table II).

Table III—Pharmacokinetic Parameters of I in Patients with Essential Hypertension

Subject	Age, yr	Body wt., kg	Dose, mg	t_{max}, min	C_{max}, μg/mL	$AUC_{0\to\infty}$, μg·min/mL	$t_{1/2}$, min
A	45	65.6	50	45	1.12	54.6	22
B	29	49.8	30	60	0.712	40.7	24

The present method was then applied to the determination of I in blood samples taken from two patients (subjects A and B) orally administered 50 and 30 mg of I, respectively. The blood concentration–time profiles observed are illustrated in Fig. 2; the individual pharmacokinetic data of the two patients are listed in Table III. The I levels reached maximum values, 1.12 and 0.721 μg/mL, at 45 and 60 min after administration, respectively. The blood concentrations decreased to 0.063 and 0.095 μg/ml at 120 min, with half-lives of 22 and 24 min, respectively.

The proposed method for the determination of I in human blood is satisfactory with respect to sensitivity and reliability and, hence, would be useful for clinical pharmacokinetic studies. The newly developed method may be widely applicable to the determination of biologically and pharmacologically important thiol compounds.

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