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# Determination of SA-446 in Human Whole Blood and Urine by Electron Capture-Gas Liquid Chromatography

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A highly sensitive and specific gas chromatographic method for the determination of SA-446 [(2*R*,4*R*)-2-(2-hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid] in human whole blood and urine was established. The mercapto group of SA-446 was alkylated completely with pentafluorobenzyl bromide (PFBB) and then the remaining hydroxyl and carboxyl groups were methylated with diazomethane. As little as 10 picograms of the derivatized SA-446 is detectable by the electron capture technique. The mean recoveries of SA-446 from whole blood and urine by the present method were 95.5% and 93.5%, respectively. The calibration curves for SA-446 were linear in the concentration ranges of 10 ng/ml to 10000 ng/ml of whole blood and 0.1 μg/ml to 100 μg/ml of urine. The standard deviation with this method was below 3.0%. This method is highly sensitive, and a small sample size of 250 μl is enough to determine SA-446 in human whole blood and urine.

**Keywords**——SA-446; pentafluorobenzyl bromide; diazomethane; electron capture–gas liquid chromatography; derivatization; whole blood; urine

(2*R*, 4*R*)-2-(2-Hydroxyphenyl)-3-(3-mercaptopropionyl)-4-thiazolidinecarboxylic acid (SA-446) is one of the potent angiotensin converting enzyme inhibitors synthesized in our laboratory[1] and may prove to be a useful antihypertensive agent.[2] This paper describes a micro-determination method for SA-446 in human whole blood and urine by electron capture–gas liquid chromatography. The present method has a 2-step derivatization procedure prior to gas liquid chromatography. First, the mercapto group of SA-446 was alkylated with penta fluorobenzyl bromide (PFBB) and then the remaining hydroxyl and carboxyl groups were methylated with diazomethane. This method is suitable for the determination of SA-446 in human whole blood and urine.

## Experimental

**Apparatus**——A Shimadzu GC-5A gas chromatograph equipped with a $^{63}$Ni electron capture detector was used. The glass column (2.0 m × 3 mm i.d.) was filled with 0.5% OV-17 on 80—100 mesh Gas-Chrom Q. The flow rate of nitrogen carrier gas was 75 ml/min. Samples were applied by on-column injection. The other operating conditions for chromatography are indicated in the figure legends. Results were quantified by the use of a Shimadzu integrator, Chromatopac C-R1A. Melting points were determined in open capillary tubes with a Yamato melting point apparatus and are uncorrected. Specific rotations were measured with a JASCO DIP-4 polarimeter. Infrared (IR) spectra were recorded on a JASCO A-302 spectrometer. $^1$H-Nuclear magnetic resonance (NMR) spectra were taken on a JEOL TMN-PMX 60 spectrometer with $SiMe_4$ as an internal standard. Mass spectra were measured with a Hitachi M-52 machine equipped with a direct inlet system under the following conditions: ionization voltage, 70 eV; ion accelerating voltage, 3.0 kV; ion source temperature, 90°C.

**Materials**——SA-446 was synthesized by Santen Pharmaceutical Co., Ltd. (Osaka Japan). PFBB was purchased from Tokyo Kasei Co., Ltd. (Tokyo, Japan). *p*-Toluenesulfonyl-*N*-methyl-*N*-nitrosoamide for the preparation of diazomethane was from Nakarai Chemicals Ltd. (Kyoto, Japan). Deionized water, with a specific resistance of more than 10 MΩcm, was used after being degassed and bubbled through with nitrogen. All other reagents were of analytical reagent grade.

**Preparation of Standard Compounds**

**(1) (2*R*,4*R*)-2-(2-Hydroxyphenyl)-3-(*S*-2,3,4,5,6-pentafluorobenzyl-3-mercaptopropionyl)-4-thiazolidine-**

**carboxylic Acid (2)**——SA-446 (**1**, 2.4 g, 7.7 mmol) and potassium carbonate (2.1 g, 15.2 mmol) were dissolved in water (50 ml). PFBB (2.0 g, 7.7 mmol) was added to the above solution with stirring at room temperature. The reaction mixture was stirred for 4 h, then adjusted to pH 3 with glacial acetic acid, and extracted with ethyl acetate. The extract was washed with water, dried over $Na_2SO_4$, and concentrated *in vacuo*. Recrystallization from chloroform gave 2.2 g (58%) of **2** as fine needles: mp 108—110°C. $[\alpha]_D^{25}$ +108.7° ($c$=0.9, MeOH). IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1725 (COOH), 1630 (CON). $^1$H-NMR (acetone-$d_6$) $\delta$: 2.17—3.10 (4H, m, $COC\underline{H}_2$-$C\underline{H}_2S$), 3.10—3.59 (2H, m, $C_5$–H), 3.73 (2H, s, benzylic H), 4.90 (1H, t, $J$=7.5 Hz, $C_4$–H), 6.49 (1H, s, $C_2$–H), 6.62—8.13 (4H, m, aromatic H), 7.33—9.10 (2H, br, O$\underline{H}$ and COO$\underline{H}$). MS $m/z$: 493 ($M^+$).

**(2) Butyl (2*R*,4*R*)-2-(2-Hydroxyphenyl)-3-(*S*-2,3,4,5,6-pentafluorobenzyl-3-mercaptopropionyl)-4-thiazolidinecarboxylate (3)**——*N*,*N*-Dimethylformamide dibutyl acetal (2.6 g, 12.8 mmol) was added to compound **2** (1.3 g, 2.6 mmol) in absolute benzene and the mixture was stirred at 80°C for 1 h, then washed with water, dried over $Na_2SO_4$, and concentrated *in vacuo*. The residual oil was purified by silica gel column chromatography and yielded a crystalline product. Recrystallization from carbon tetrachloride gave 0.50 g (36%) of **3** as colorless prisms: mp 103—103.5°C. $[\alpha]_D^{25}$ +104.5° ($c$=1.0, MeOH). IR $\nu_{max}^{KBr}$ cm$^{-1}$: 1742 (COO), 1638 (CON). $^1$H-NMR ($CDCl_3$) $\delta$: 0.69—1.15 (3H, m, $OCH_2CH_2CH_2C\underline{H}_3$), 1.15—2.20 (4H, m, $OCH_2C\underline{H}_2C\underline{H}_2CH_3$), 2.24—3.05 (4H, m, $COC\underline{H}_2C\underline{H}_2S$), 3.09—3.45 (2H, m, $C_5$–H), 3.61 (2H, s, benzylic H), 4.22 (2H, t, $J$=6.0 Hz, $OC\underline{H}_2CH_2CH_2CH_3$), 4.94 (1H, t, $J$=7.0 Hz, $C_4$–H), 6.37 (1H, s, $C_2$–H), 6.66—8.06 (4H, m, aromatic H), 7.00—9.30 (1H, br, OH). MS $m/z$: 549 ($M^+$).

**(3) Methyl (2*R*,4*R*)-2-(2-Methoxyphenyl)-3-(*S*-2,3,4,5,6-pentafluorobenzyl-3-mercaptopropionyl)-4-thiazolidinecarboxylate (4)**——Diazomethane ethereal solution[3] (20 ml, 12 mmol) was added to compound **2** (0.80 g, 1.6 mmol) in ether (10 ml). The solution was allowed to stand overnight, then concentrated *in vacuo*. The residual oil was purified by silica gel column chromatography to give 0.56 g (66%) of **4**. $[\alpha]_D^{25}$ +113.0° ($c$=1.2, MeOH). IR $\nu_{max}^{film}$ cm$^{-1}$: 1750 (COO), 1659 (CON). $^1$H-NMR ($CDCl_3$) $\delta$: 2.07—2.96 (4H, m, $COC\underline{H}_2$-$C\underline{H}_2S$), 3.06—3.33 (2H, m, $C_5$–H), 3.56 (2H, s, benzylic H), 3.81 (3H, s, $COOC\underline{H}_3$), 3.87 (3H, s, $OC\underline{H}_3$), 4.82 (1H, dd, $J$=8.0 and 7.5 Hz, $C_4$–H), 6.28 (1H, s, $C_2$–H), 6.74—8.17 (4H, m, aromatic H). MS $m/z$: 521 ($M^+$).

**(4) Butyl (2*R*,4*R*)-2-(2-Methoxyphenyl)-3-(*S*-2,3,4,5,6-pentafluorobenzyl-3-mercaptopropionyl)-4-thiazolidinecarboxylate (5)**——Diazomethane ethereal solution (20 ml, 12 mmol) was added to compound **3** (0.90 g, 1.6 mmol) in ether (10 ml). The solution was allowed to stand overnight and then concentrated *in vacuo*. The residual oil was purified by silica gel column chromatography to give 0.59 g (65%) of **5**. $[\alpha]_D^{25}$ +108.6° ($c$=1.2, MeOH). IR $\nu_{max}^{film}$ cm$^{-1}$: 1742 (COO), 1658 (CON). $^1$H-NMR ($CDCl_3$) $\delta$: 0.73—1.11 (3H, m, $OCH_2CH_2CH_2C\underline{H}_3$), 1.11—2.00 (4H, m, $OCH_2C\underline{H}_2C\underline{H}_2CH_3$), 2.18—2.95 (4H, m, $COC\underline{H}_2C\underline{H}_2S$), 3.03—3.31 (2H, m, $C_5$–H), 3.55 (2H, s, benzylic H), 3.86 (3H, s, $OC\underline{H}_3$), 4.22 (2H, t, $J$=6.0 Hz, $OC\underline{H}_2CH_2CH_2CH_3$), 4.80 (1H, dd, $J$=9.0 and 7.0 Hz, $C_4$–H), 6.29 (1H, s, $C_2$–H), 6.72—8.15 (1H, m, aromatic H). MS $m/z$: 563 ($M^+$).

**Assay Procedure for SA-446 in Whole Blood and Urine**——A 250 μl aliquot of fresh whole blood or urine was added to a glass-stoppered test tube containing 2 ml of 0.05 M tris-HCl buffer (pH 9) and 250 μl of 0.2% (v/v) PFBB–acetonitrile solution. The mixture was immediately stirred with a vortex-mixer and allowed to stand at room temperature for 30 min. Next 200 μl of 1 N acetic acid was added, the whole was stirred with the vortex-mixer, and 4 ml of ethyl acetate containing 440 ng (or 4400 ng) of compound **3** (internal standard) was added in the case of whole blood (or urine). The tube was shaken for 10 min and centrifuged at 3000 rpm for 10 min. The organic layer was transferred to another glass-stoppered test tube and the remaining aqueous layer was extracted with 4 ml of ethyl acetate. The combined organic layer was concentrated to about 2 ml under a stream of nitrogen, and then diazomethane ethereal solution (2 ml, *ca.* 1.5 mmol) was added. The mixture was stirred, then the tube was stoppered tightly and allowed to stand overnight at room temperature. The resulting solution was evaporated to dryness at 30°C under a stream of nitrogen. The residue was dissolved in 2 ml of acetone and a 5 μl aliquot of this solution was injected into the gas chromatograph.

**Calculations**——The peak areas for compounds **4** and **5** were measured. A calibration curve was drawn by plotting the peak area ratio of compounds **4** to **5**. The contents of compound **1** in blood and urine were calculated from the calibration curve.

## Results and Discussion

### Derivatization of SA-446

For GC–ECD, compound **1** was derivatized to compound **4** (Fig. 1). PFBB, which has commonly been used for derivatization of organic acids, phenols and mercaptans,[4–6] was used for the conversion of compound **1** into a highly electron-capture sensitive compound. Under the present experiment conditions, the mercapto group of compound **1** reacted nearly quantitatively with PFBB.

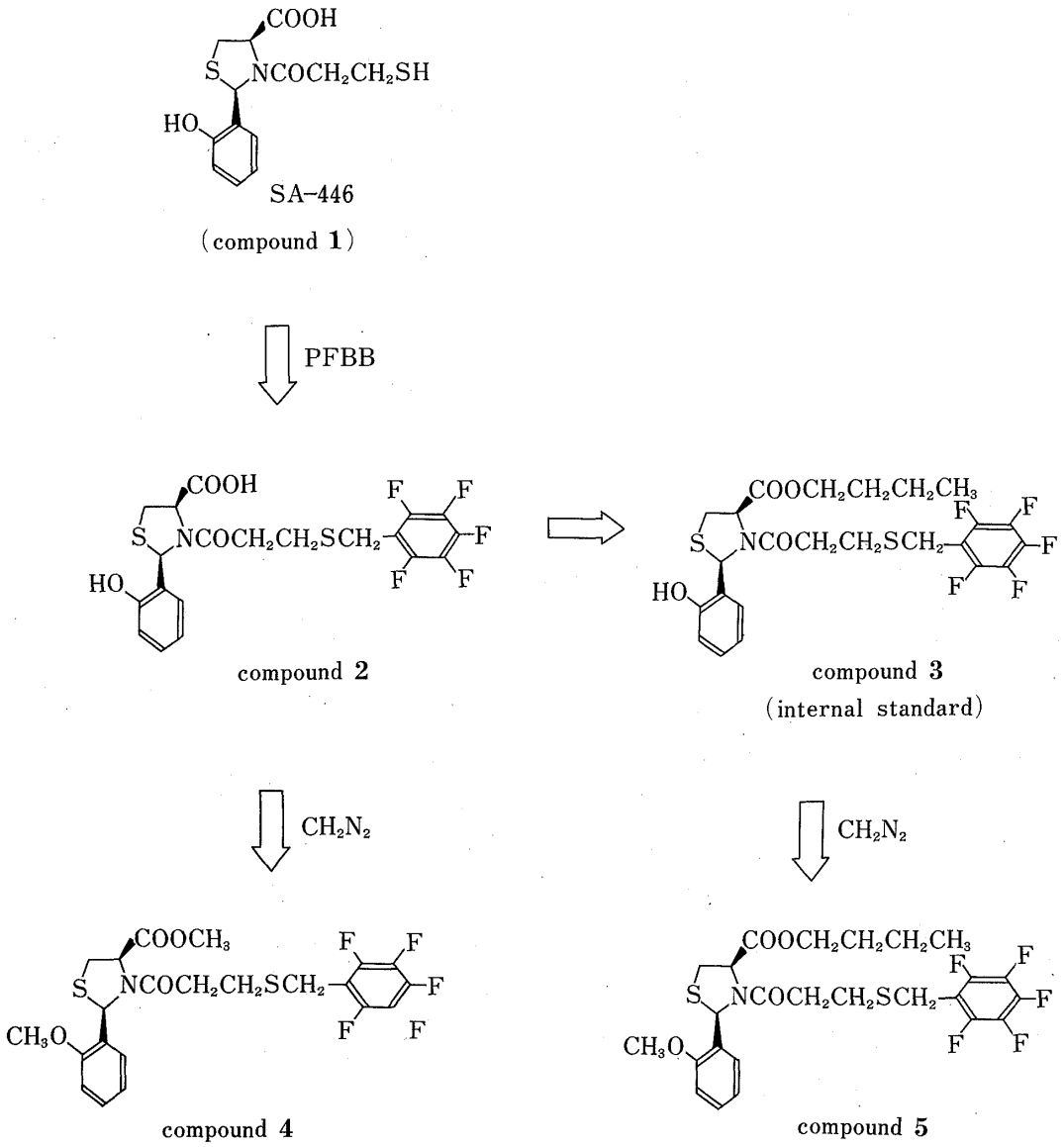


Fig. 1. Derivatization of SA-446 with Pentafluorobenzyl Bromide and Diazomethane

## Optimum Conditions for Derivatization

**Pentafluorobenzylation**——Pentafluorobenzylation of compound **1** in aqueous solution at a concentration of 1 μg/ml was examined at pH 9 at 2, 25 and 45°C. The reaction mixture after pentafluorobenzylation was acidified with 100 μl of 1 N acetic acid and extracted with ethyl acetate. The extract was methylated under the conditions shown below. The degree of pentafluorobenzylation was calculated from the total recovery. This reaction was complete at 25°C in 15 min or at 45°C in 5 min (Fig. 2). Under the standard conditions, the optimum pH range for this reaction was from 8 to 11, and high recovery (96%) was obtained (Fig. 3).

**Methylation with Diazomethane**——Diazomethane ethereal solution (2 ml, *ca.* 1.5 mmol) was added to 2 ml of ethyl acetate containing 400 ng of compound **2** and 440 ng of compound **3** and the reaction was carried out at 25°C. As shown in Fig. 4, the recoveries of both compounds at each reaction time were similar, and methylation was completed in about 6 h. Yields of compounds **4** and **5** after completion of the reaction were 95.7 and 95.6%, respectively. Thus, for practical applications, the reaction was performed overnight at room temperature.

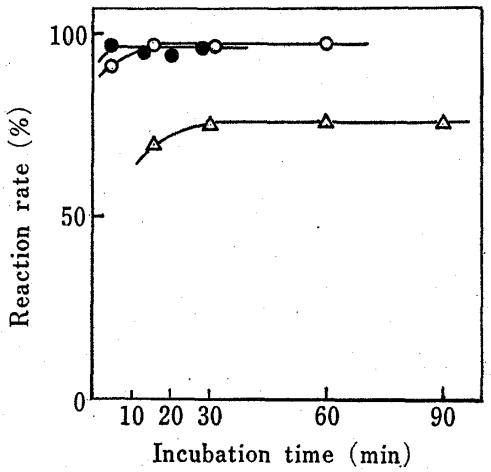


Fig. 2. Time Course of Adduct Formation from SA-446 with PFBB

—△—: incubation at 2°C.
—○—: incubation at 25°C.
—●—: incubation at 45°C.

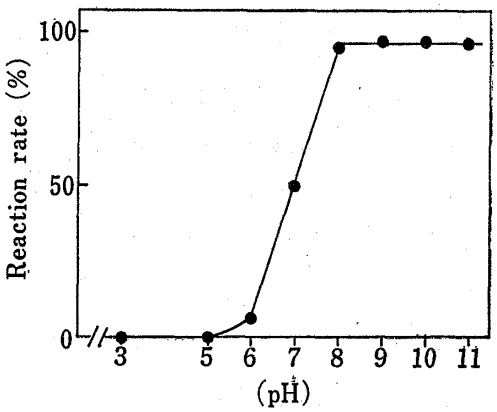


Fig. 3. Effect of pH on the Pentafluorobenzylation of SA-446 with PFBB at 25°C for 30 min

## SA-446 in Whole Blood and Urine

**Extraction**——Whole blood and urine were pentafluorobenzylated without compound **1** according to the procedure described in "Experimental," and 400 ng (or 4000 ng) of compound **2** and 440 ng (or 4400 ng) of compound **3** were added in the case of whole blood (or urine). The mixture was acidified with various volumes of 1 N acetic acid and extracted with ethyl acetate. Extraction efficiency was determined on the basis of the methylation yield as described below. As shown in Fig. 5, compounds **2** and **3** were completely extracted in the volume range of 150 μl to 400 μl of 1 N acetic acid.

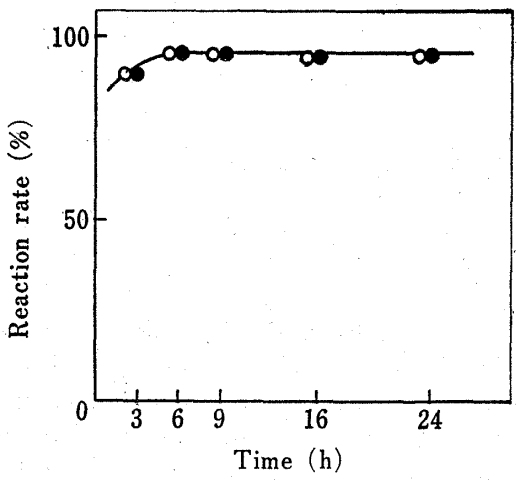


Fig. 4. Time Course of Methylation of Compounds **2** and **3** with Diazomethane

—○—: compound **2**.
—●—: compound **3**.

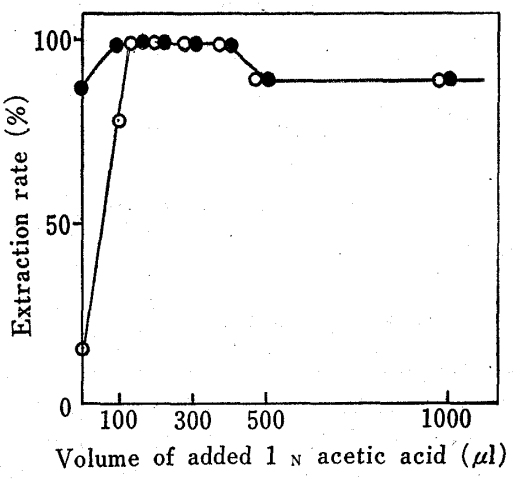


Fig. 5. Effect of Acidification on the Extraction of Compounds **2** and **3** from Whole Blood and Urine with Ethyl Acetate

—○—: compound **2**.
—●—: compound **3**.

**Methylation**——400 ng (or 4000 ng) of compound **2** and 440 ng (or 4400 ng) of compound **3** were added to extracts of whole blood (or urine) without compound **1** which had been treated according to the procedure described in "Experimental." The mixture was methylated with

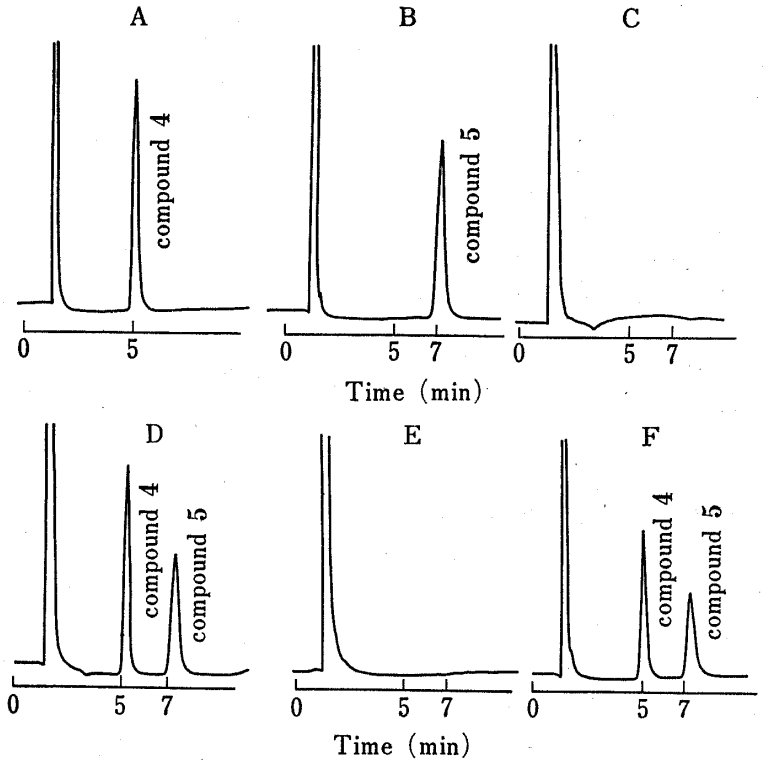


Fig. 6. Chromatograms of Compounds **4**, **5** and Extracts from Human Whole Blood and Urine with or without Addition of Compounds **1** and **3**

A: 300 pg of compound **4**, B: 700 pg of compound **5**, C: treated whole blood, D: treated whole blood with compound **1** (1 $\mu$g/ml of blood) and compound **3** (1.8 $\mu$g/ml of blood), E: treated urine, F: treated urine with compound **1** (10 $\mu$g/ml of urine) and compound **3** (18 $\mu$g/ml of urine).
Chromatography conditions: column, 2.0 m×3 mm i.d., 0.5% OV-17 (80/100); injector temperature 300°C, column oven 270°C, detector 310°C.

diazomethane at room temperature for 16 h and the recoveries of compounds **4** and **5** in whole blood and urine were 91.3 and 90.8%, 90.6 and 92.4%, respectively.

**Gas Chromatography**——Typical gas chromatograms of compound **1** derivatized in human whole blood and urine are shown in Fig. 6. No peak interfering with the determination of compound **4** or **5** was observed on the chromatogram.

**Calibration Curves and Recovery**——Compound **1** was added to 250 $\mu$l of fresh human whole blood and urine, and the mixture was immediately treated according to the assay procedure described in "Experimental." As shown in Fig. 7, linear calibration curves for compound **1** in whole blood and urine were drawn by plotting ratios of peak areas (compound **4**/compound **5**) against concentrations of compound **1**. The total recoveries of compound **1** after derivatization and extraction were 95.5% in whole blood and 93.5% in urine.

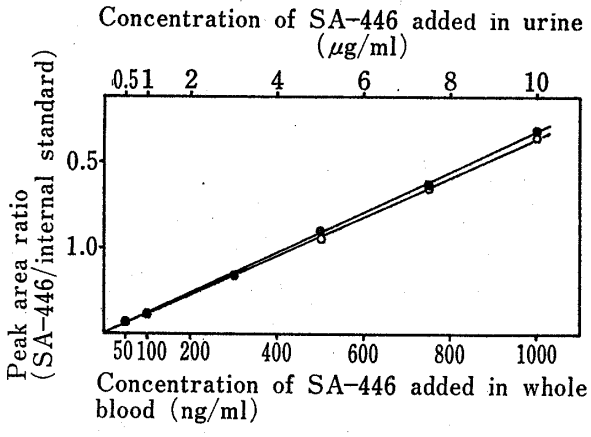


Fig. 7. Calibration Curves for SA-446 added to Whole Blood and Urine

—○—: in whole blood.
—●—: in urine.

Table I. Recovery of SA-446 added to Human Whole Blood and Urine

| Biological fluids | Added | Mean of measured values | Recovery (%) ±S.D.[a] |
|---|---|---|---|
| Whole blood | 50 (ng/ml) | 46.5 | 93.0±2.2 |
| | 500 | 487 | 97.4±1.3 |
| | 5000 | 4790 | 95.8±1.7 |
| | | Mean recovery | 95.5 |
| Urine | 0.5 (μg/ml) | 0.452 | 90.4±0.7 |
| | 5.0 | 4.65 | 93.0±0.7 |
| | 50 | 48.6 | 97.2±0.8 |
| | | Mean recovery | 93.5 |

Number of determinations: 5.
a) Standard deviation (%).

## Stability of SA-446 in Biological Fluids

Since thiol compounds are easily oxidized to form –S–S– bonds,[7-9] the stability of compound **1** in whole blood and urine was examined. A 10 μl aliquot of compound **1** aqueous solution at a concentration of 125 μg/ml was added to 250 μl of blood or urine, and the mixtures were stored at room temperature (22°C) and at 2°C. As shown in Table II, compound **1** in whole blood was unstable; the residual amount decreased with increase of temperature and with the passage of time. A similar tendency was observed in urine, but the compound was more stable than in blood. Consequently, it is considered that biological samples containing thiol compounds should be treated immediately.

Table II. Stability of SA-446 in Biological Fluids (Residual Amount, Percent)

| Biological fluids | Incubation temp. (°C) | Incubation time (min) 0 | 5 | 15 | 30 | 60 | 120 | 180 | 240 | Rate constant $k$ ($min^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| Whole blood | 22 | 100 | 92.7 | 83.2 | 66.4 | 45.7 | 21.7 | 12.8 | 11.1 | $1.27\times10^{-2}$ |
| | 2 | 100 | 95.8 | 93.3 | 89.1 | 79.0 | 60.8 | 49.5 | 36.7 | $4.28\times10^{-3}$ |
| Urine (pH 6.5) | 22 | 100 | 95.5 | 94.2 | 91.1 | 83.9 | 65.5 | 53.6 | 43.4 | $3.58\times10^{-3}$ |
| | 2 | 100 | 100.1 | 100.0 | 99.7 | 98.4 | 94.9 | 90.5 | 85.4 | $8.83\times10^{-4}$ |

## Reproducibility and Sensitivity

The intra-assay reproducibility for SA-446 (**1**) was determined by assaying five replicate whole blood and urine samples containing the drug added at concentrations ranging from 50 ng/ml to 5000 ng/ml and 0.5 μg/ml to 50 μg/ml, respectively. The standard deviation was below 3.0% and good reproducibility was obtained at each concentration (Table I). Compound **4** is highly electron-capture sensitive. As little as 10 pg of this compound when injected into the gas chromatograph gave a peak with a signal-to-noise ratio of 10. Quantitative results for SA-446 (**1**) were obtained in the concentration ranges of 10 ng/ml to 10000 ng/ml of whole blood and 0.1 μg/ml to 100 μg/ml of urine. The detection limit of SA-446 (**1**) was 1 ng/ml of biological samples.

The present study suggests that the gas chromatographic method established here is applicable to the practical determination of SA-446 (**1**) in human whole blood and urine.

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## References and Notes

1) M. Oya, T. Baba, E. Kato, Y. Kawashima, and T. Watanabe, *Chem. Pharm. Bull.*, **30**, 440 (1982).
2) T. Iso, H. Yamauchi, H. Suda, K. Nakata, K. Nishimura, and J. Iwao, *Jpn. J. Pharmacol.*, **31**, 875 (1981).
3) T.J. De Boer and H.J. Backer, *Rec. Trav. Chim.*, **73**, 229 (1954).
4) F.K. Kawahara, *Anal. Chem.*, **40**, 1009 (1968).
5) F.K. Kawahara, *Anal. Chem.*, **40**, 2073 (1968).
6) Kung-Tat Ng, *J. Chromatogr.*, **166**. 527 (1978).
7) R.F. Bergström, D.R. Kay, and J.G. Wagner, *Life Sci.*, **27**, 189 (1980).
8) R. Saetre and D.L. Rabenstein, *Anal. Chem.*, **50**, 276 (1978).
9) K.K. Wong and J. Dreyfuss., *Pharmacologist*, **20**, 213 (1978).