[Chem. Pharm. Bull. 30(11)4107—4113(1982)]

Quantitation of 6-Amino-2-phenylamino-9-β-D-ribofuranosyl-9*H*-purine (CV-1808) and Its Metabolite, 2-(4-Hydroxyphenyl)aminoadenosine, in Human Serum and Urine by High Performance Liquid Chromatography using a Fluorimetric Detector[1)]

Yoshitatsu Hayashi,* Sohachiro Miyake, Motoaki Kuwayama, Masatoshi Hattori and Yoshiro Usui

Central Research Division, Takeda Chemical Industries, Ltd., Jusohonmachi, Yodogawa-ku, Osaka, 532, Japan

(Received April 28, 1982)

A high performance liquid chromatographic method using a fluorimetric detector for determination of the quantities of 6-amino-2-phenylamino-9-β-D-ribofuranosyl-9*H*-purine (CV-1808, **1**) and its metabolite, 2-(4-hydroxyphenyl)aminoadenosine (**2**), in human serum and urine is presented.

Compounds **1** and **2**, after chromatographic extraction from urine or serum with a Sep-Pak C_{18} cartridge, are allowed to react with propionic anhydride in the presence of triethylamine and the quantities of the resulting propionyl derivatives of **1** and **2** (**1-P** and **2-P**) are determined by high performance liquid chromatography on a μPorasil column. The detection limits of **1** and **2** are 5.0 and 10.0 ng/ml in urine and 1.0 and 2.0 ng/ml in serum, respectively. For a more sensitive determination of the amount of **1** in serum, a concentrated eluate of **1-P** from the μPorasil column is rechromatographed on a mini-column (10 cm × 2 mm I.D.) packed with Lichrosorb SI-60 (5 μm). With this method, a detection limit of 0.1 ng/ml for **1** in serum is obtained.

Keywords——6-Amino-2-phenylamino-9-β-D-ribofuranosyl-9*H*-purine; 2-(4-hydroxyphenyl)aminoadenosine; high performance liquid chromatography (HPLC); fluorimetric determination; blood levels and urinary excretion

6-Amino-2-phenylamino-9-β-D-ribofuranosyl-9*H*-purine (CV-1808, **1**), synthesized by Marumoto *et al.*,[2)] shows distinct coronary vasodilating activity[3)] and has been tested for clinical use. As an aid to the pharmacokinetic study of **1**, we developed a quantitation method for **1** and its metabolite,[4)] 2-(4-hydroxyphenyl)aminoadenosine (**2**), in serum and urine using high performance liquid chromatography (HPLC).

HPLC is being used more often for the analysis of drugs in biological fluids because of its simplicity and specificity. However, its sensitivity depends on the detection device used and the properties of the compounds to be analyzed. Other factors can influence the detection limit of the compounds, *e.g.*, the solvents or the properties of coexisting impurities. An ultraviolet (UV) detection method initially applied for the quantitation of **1** and **2** was unsatisfactory at the nanograms per milliliter level in the biological fluids under study. A fluorimetric detection method, which was expected to have higher sensitivity because of the strong fluorescence of **1** at 366 nm (emission maximum) on excitation at 285 nm, also had insufficient sensitivity owing to quenching and interference by biological contaminants. In order to avoid these effects and to improve the sensitivity and selectivity of the analytical method, the formation of a more favorable fluorigenic derivative was attempted. Yoshioka *et al.*[5)] reported a very sensitive fluorimetric determination of adenosine content after conversion of adenosine into 1,N^6-ethenoadenosine with chloroacetaldehyde, and Yuki *et al.*[6)] determined the amount of adenine fluorimetrically after treatment with glyoxal hydrate trimer. In the present study, we found that **1** and **2** extracted from biological fluids can be successfully converted into propionyl derivatives by reaction with propionic anhydride. Propionylated **1** and **2** exhibit intense blue fluorescence at the emission maximum of 410 nm with an excitation maximum

of 320 nm, and can be determined by normal phase HPLC with fluorimetric detection.

The method allows very sensitive and specific determination of the amounts of **1** and **2** in urine and serum with no quenching or interference from biological substances.

Experimental

Chemicals and Materials——All chemicals used in this work were of reagent grade and no further purification was conducted, except for PrOAc, which was redistilled at 101—102°C. Sulfatase (type H-1, 24600 units/g, solid) was purchased from Sigma Chemical Co., Ltd. Compounds **1** and **2** were supplied by Drs. Marumoto and Furukawa of this Division.

Preparation of the Tetrapropionyl Derivative of 1 (1-P)——A mixture of **1** (150 mg), triethylamine (0.5 ml) and propionic anhydride (1 ml) was heated at 80°C for 60 min and the resulting solution was evaporated to dryness in a stream of nitrogen. The residue was dissolved in $CHCl_3$ (0.5 ml) and purified by column chromatography [silica gel (Kieselgel 60), 80 g, $CHCl_3$] to obtain a pale yellow solid (140 mg). NMR ($CDCl_3$) δ: 1.0—1.4 [12H, m, $(CH_3)_4$], 2.1—2.6 [6H, m, $(CH_2)_3$], 2.9 (2H, q, CH_2), 4.4 (3H, $H_{4'}$, $2H_{5'}$), 5.65 (1H, t, $H_{3'}$), 5.95—6.15 (2H, m, $H_{1'}$, $H_{2'}$), 7.0—7.7 (6H, phenyl, NH), 7.9 (1H, s, H_8), 8.95 (1H, s, NH). MS *m/z*: 582 (M^+). UV λ_{max}^{MeOH} nm: 250, 275, 323. *Anal.* Calcd for $C_{28}H_{34}N_6O_8$: C, 57.75; H, 5.88; N, 14.42. Found: C, 57.24; H, 6.02; N, 13.71.

Preparation of the Pentapropionyl Derivative of 2 (2-P)——A mixture of **2** (40 mg), triethylamine (0.1 ml) and propionic anhydride (0.5 ml) was heated at 80°C for 60 min and the resulting solution was evaporated to dryness in a stream of nitrogen. The residue was dissolved in $CHCl_3$ (0.5 ml) and subjected to thin-layer chromatography (TLC) (plate; Kieselgel 60 F_{254}, solvent; EtOAc). The main spot (*Rf* 0.58) was extracted with MeOH. Removal of the solvent by evaporation gave a pale brown solid (54 mg) as **2-P**. NMR ($CDCl_3$) δ: 1.0—1.4 [15H, $(CH_3)_5$], 2.15—2.95 [10H, $(CH_2)_5$], 4.34 (3H, $H_{4'}$, $2H_{5'}$), 5.60 (1H, t, $H_{3'}$), 5.95—6.10 (2H, $H_{1'}$, $H_{2'}$), 7.05—7.57 (5H, phenyl, NH), 7.85 (1H, s, H_8), 8.84 (1H, s, NH). MS *m/z*: 654 (M^+). UV λ_{max}^{MeOH} nm; 249, 272, 320. *Anal.* Calcd for $C_{31}H_{38}N_6O_{10}$: C, 56.88; H, 5.85; N, 12.84. Found: C, 56.32; H, 5.77; N, 12.45.

Apparatus——The fluorescence spectra and intensities were measured with a Shimadzu RF-502 spectrofluorophotometer. The fluorescence spectra and excitation and emission maxima are uncorrected.

Chromatographic Conditions——The HPLC system consisted of a Waters 6000 A pump, a Rheodyne 7120 loop injector (100 μl) and a Jasco FP-110C fluorescence detector. The detector was equipped with a mercury lamp and was set at 313 nm for excitation and 410 nm for emission. The mobile phase consisted of

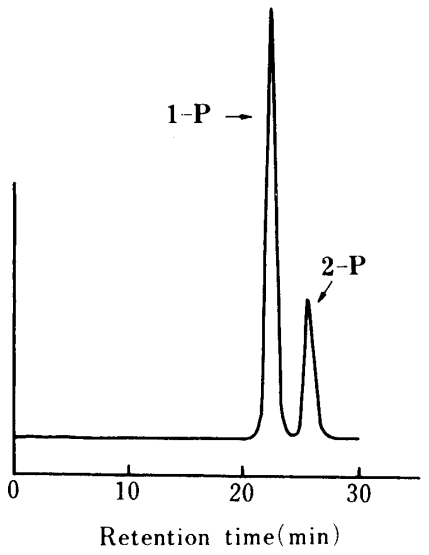


Fig. 1-a. Chromatogram of **1-P** and **2-P**

Condition A
Column: μPorasil, two 30 cm × 3.9 mm I.D.
Mobile phase: *n*-hexane–PrOAc–methyl cellosolve (50: 50: 3, v/v).
Flow rate: 1 ml/min.
Amount injected: 140 ng of **1-P** and **2-P**.

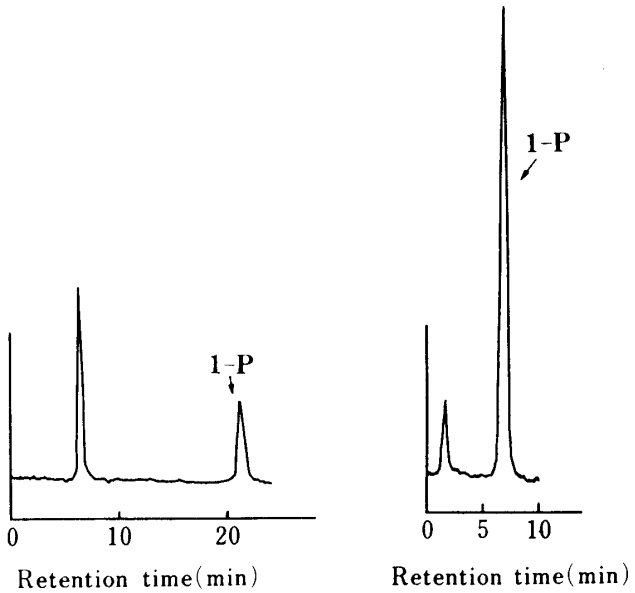


Fig. 1-b. Chromatograms of **1-P** under Different Chromatographic Conditions

Left: condition A (see Fig. 1-a).
Right: condition B.
Column: Lichrosorb SI-60 (5 μm), 10 cm × 2 mm I.D.
Mobile phase: same as condition A.
Flow rate: 0.2 ml/min.
Amount injected: 1.4 ng of **1-P**.

n-hexane–PrOAc–methyl cellosolve (50: 50 : 3, v/v). For the chromatographic separation, two 30 cm × 3.9 mm I.D. μPorasil columns connected in series were used at a flow rate of 1 ml/min (condition A). For more sensitive determination of the amount of **1**, the effluent of **1-P** was reinjected on a mini-column (10 cm × 2 mm I.D.) packed with Lichrosorb SI-60 (5 μm) and eluted at a flow rate of 0.2 ml/min (condition B).

An example of the chromatograms of a mixture of **1-P** and **2-P** under condition A is shown in Fig. 1-a. The chromatograms for 1.4 ng of **1-P** under conditions A (left) and B (right) are shown in Fig. 1-b.

Assay Procedure for 1 and 2 in Urine——For the quantitative determination of unconjugated **1** and **2**, 1 ml of urine sample was directly passed through a Sep-pak C_{18} cartridge which had been pre-washed with 5 ml of EtOH and 5 ml of H_2O successively. For determination of the total amount of unconjugated and conjugated **1** or **2** in urine, 5 ml of 0.1 M acetate buffer (pH 5) and 4 mg of sulfatase were added to 1 ml of urine sample and the solution was incubated at 37°C for 16 h. The resulting solution was passed through a Sep-pak C_{18} cartridge pre-washed as described above. The cartridge was successively washed with 10 ml of H_2O and 6 ml of 5% EtOH, then the **1** and **2** adsorbed on the cartridge were eluted with 2 ml of EtOH. The eluate was evaporated to dryness in a stream of nitrogen and 4 ml of EtOH was added to the residue. A 0.5-ml portion of the solution was transferred to a glass tube and evaporated to dryness in a stream of nitrogen. Next, 95 μl of propionic anhydride and 5 μl of triethylamine were added to the residue, and the mixture was left standing at 80°C for 60 min. The solution was evaporated to dryness and the residue was dissolved in 0.2 ml of the mobile phase. A 100-μl portion of the solution was subjected to HPLC (condition A).

Assay Procedure for 1 and 2 in Serum——To 2 ml of serum sample was added 5 ml of 5% trichloroacetic acid solution. The mixture was vigorously shaken for 2 min, and the supernatant was transferred to another tube. This extraction procedure was repeated three times in all. The combined supernatant was worked up as described in the assay procedure for urine except that all of the eluate from the Sep-pak C_{18} cartridge was used for the propionylation. When the concentration of **1** in serum was lower than 1 ng/ml, the fractionated effluent of **1-P** from the column under condition A was evaporated to dryness and the residue was redissolved in 0.2 ml of the mobile phase followed by rechromatography of a 100-μl portion of the solution under condition B.

Calculations——Linear calibration curves were constructed by plotting the peak heights of **1-P** and **2-P** *versus* the amounts of **1** and **2** applied in the range of 0—100 ng for condition A and the amount of **1** applied in the range of 0—2.0 ng for condition A→B.

Results and Discussion

Chemical Structures and Fluorescence Characteristics of Acyl Derivatives

Compound **1** is a strongly fluorescent substance with an excitation maximum at 285 nm and an emission maximum at 366 nm in EtOAc (Fig. 2). However, the fluorimetric determination (by HPLC) of **1** in biological fluids was unsuccessful because of marked quenching and interference from biological substances. In trying to improve the detectability of **1**, we found that the reaction product of **1** with propionic anhydride exhibits a more intense blue fluorescence, with excitation and emission maxima shifted to longer wavelengths; the fluorescence intensity was five times stronger than that of **1** (Fig. 2). When the reaction product was subjected to TLC on a silica gel plate, developed with BuOAc, two blue fluorescent spots (*Rf* values: main, 0.2; minor, 0.5) were observed. To elucidate their structures, the products were isolated by means of preparative TLC. The main product was assumed to be a tetrapropionate (**1-P**) (Chart 1) and the minor one to be a pentapropionyl deriv-

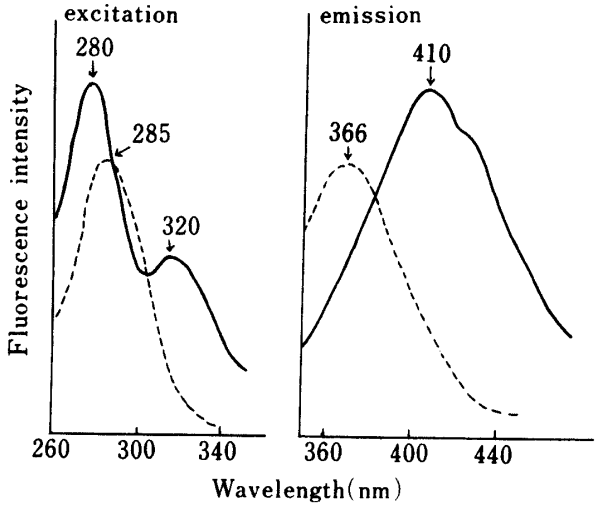
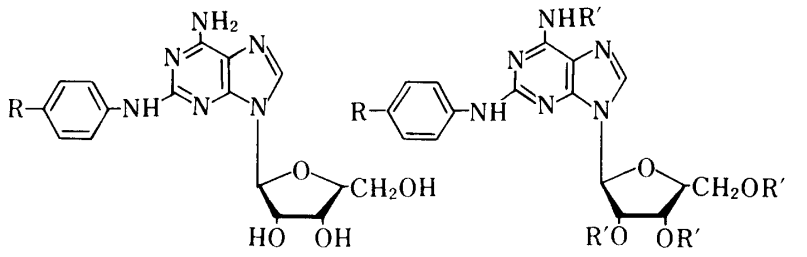


Fig. 2. Excitation and Emission Spectra of **1** and Reaction Products of **1** after Propionylation

Reaction products: 10 μg of **1** was heated with propionic anhydride (95 μl) and triethylamine (5 μl) at 80°C for 60 min, followed by evaporation and dissolution of the residue in EtOAc.
Solvent: EtOAc.
Concentration: 1 μg/ml.
---- : **1**.
—— : reaction products of **1**.



1 : R=H
2 : R=OH

1-P : R=H, R'=$COCH_2CH_3$
2-P : R=$OCOCH_2CH_3$, R'=$COCH_2CH_3$

Chart 1

TABLE I. Fluorescence Intensities of **1-P** and **2-P**

Solvent	Concentration, 1 μg/ml					
	n-Hexane	PrOAc	EtOAc	Methyl cellosolve	MeOH	H_2O
1-P	100	99	98	4	0.6	0
2-P	57	47	47	1.5	0.2	0

The fluorescence intensities were measured with excitation at 313 nm and emission at 410 nm. The intensity of **1-P** in *n*-hexane was taken as 100.

ative of **1** from the results of UV, nuclear magnetic resonance (NMR) and mass spectra (MS), as described in the experimental section. The longer wavelength shift of the excitation and emission maxima of acyl derivatives in EtOAc may be the consequence of the so-called "red shift" due to the formation of the 6-amide bond.

The fluorescence characteristics of acetyl and butyryl derivatives of **1** were also investigated and were very similar to those of **1-P**. The yields of tetraacylated compounds were measured by HPLC. Propionylation and butyration gave tetraacylated compounds in yields of 82.4 and 82.2%, respectively, *i.e.*, about twice the yield (48.3%) of acetylation of **1**. Propionylation was selected for the quantitation of **1** and **2**, because of the high reaction yield and the ease of evaporation of the remaining acylating agent from the reaction mixture. The main propionylation product of **2** (80% yield) was similarly confirmed to be **2-P** by UV, NMR and MS. The fluorescence intensities of **1-P** and **2-P** are strong in EtOAc, PrOAc and *n*-hexane, but very weak in methyl cellosolve and MeOH (Table I). Thus, the mixture of *n*-hexane–PrOAc–methyl cellosolve (50 : 50 : 3, v/v) was selected as the mobile phase of HPLC with a μPorasil column for good separation of **1-P** and **2-P** (Fig. 1).

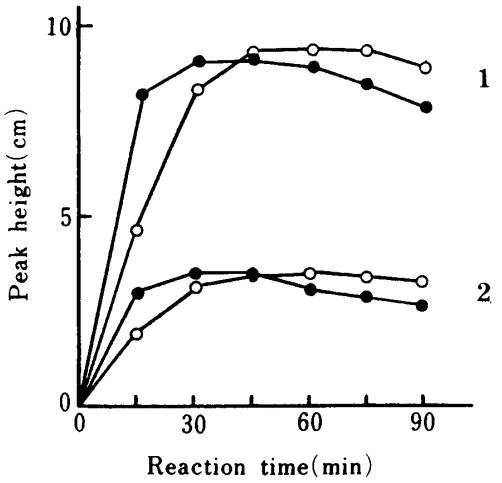


Fig. 3. Effect of Reaction Time at 80 and 100°C

1 and **2** (100 ng each) were heated with propionic anhydride (95 μl) and triethylamine (5 μl) for various reaction times and subjected to HPLC (condition A) to measure the peak heights of **1-P** and **2-P**.
○—○ : 80°C.
●—● : 100°C.

Optimal Reaction Conditions for Propionylation of 1 and 2

Excess propionic anhydride (95 μl) and tri-

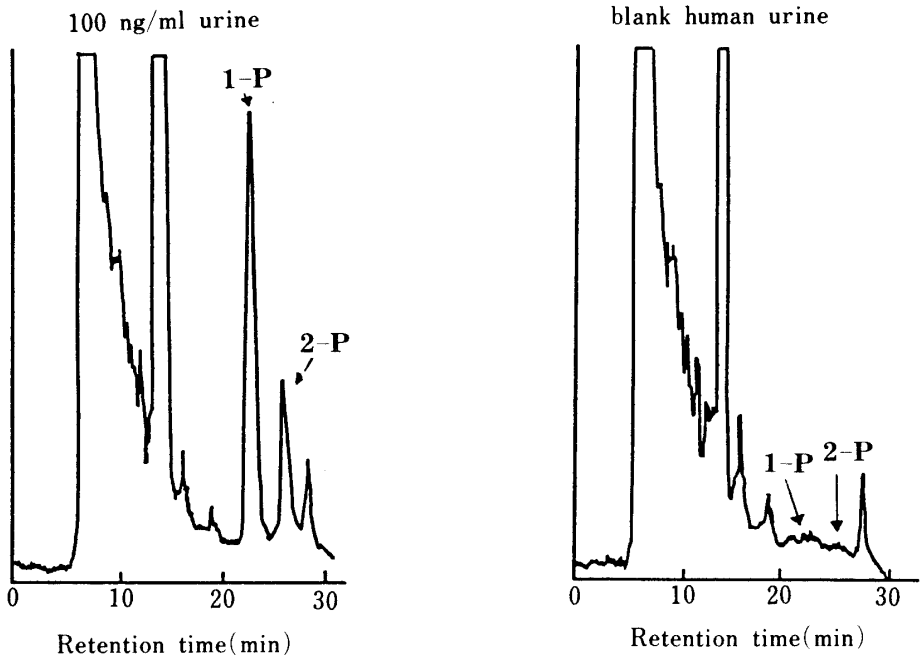


Fig. 4. Chromatograms of Extracts from Human Urine with or without **1** and **2**

ethylamine (5 μl) were added to 100 ng of **1** and **2**, and the optimal conditions for the propionylation were examined by varying the reaction time from 15 to 90 min at 80 and 100°C. At selected times, the reaction mixture was subjected to HPLC to measure the peak heights of **1-P** and **2-P**; maximal heights were obtained from 45 to 75 min at 80°C and from 30 to 45 min at 100°C (Fig. 3), respectively. As prolongation of the reaction time resulted in a decrease in peak heights of both **1-P** and **2-P**, acylation conditions of 60 min at 80°C were selected. The calibration curves obtained by this method were linear in the range of 2 to 100 ng of **1** and **2**. The calibration curve obtained for the determination of low concentrations of **1** was also linear in the range of 0.2 to 2 ng of **1**.

HPLC Separation and Recovery of 1 and 2 in Urine and Serum

Examples of chromatograms of extracts from a blank human urine sample and the same urine containing 100 ng each of **1** and **2** per ml in urine, using condition A, are shown in Fig. 4, in which clear separation of **1-P** and **2-P** from other biological substances can be seen. A chromatogram of the extract from human serum containing 1 ng/ml of **1** is also shown in Fig. 5-a. The mean recoveries from human urine were 92% for **1** and 90% for **2** at the level of 100 ng/ml and the coefficients of variation were below 5% (Table II). In the case of serum, the mean recoveries were 87% for **1** and 82% for **2** at a concentration of 10 ng/ml (Table III). The detection limits of **1** and **2** were estimated to be 5.0 and 10.0 ng/ml in urine and 1.0 and 2.0 ng/ml in serum, respectively. The method could be used to determine the

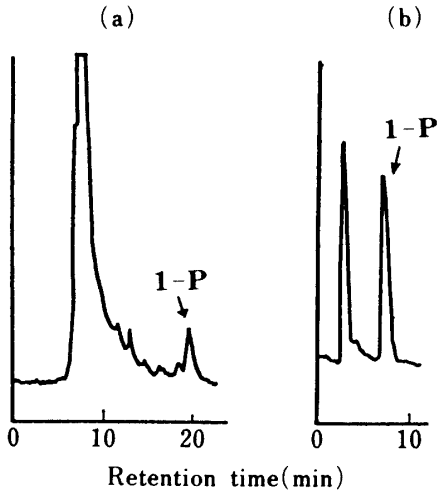


Fig. 5. Chromatograms of Extracts from Human Serum containing **1**

(a) 1 ng/ml serum, condition A.
(b) 1 ng/ml serum, condition B.

Table II. Recoveries of 1 and 2 added to Urine

Added (ng/ml, urine)	n	Recovery			
		1 (%)	c.v. (%)	**2** (%)	c.v. (%)
50	5	98	3.3	97	3.8
100	5	92	2.4	90	4.0
200	5	96	2.9	97	4.0

Table III. Recoveries of 1 and 2 added to Serum

Added (ng/ml, serum)	n	Recovery				HPLC condition
		1 (%)	c.v. (%)	**2** (%)	c.v. (%)	
0.4	5	84	5.5	—		A→B
1.0	10	76	2.2	—		A→B
10.0	5	87	4.6	82	4.2	A
50.0	4	91	5.0	87	3.7	A

concentrations of **1** and **2** in biological fluids of humans given a large oral dose of the drug, but in the case of administration of a small dose such as 0.35 mg/man, a more sensitive assay for concentrations below 1 ng/ml in serum was required. Ishii[7] has reported on micro-HPLC using a micro-column as a system for increasing analytical sensitivity, and Yoshioka *et al.*[8] determined the levels of adenine and adenosine and their nucleotides at femtomole levels by HPLC on a mini-column. In our study, for more sensitive detection of **1-P**, the effluents of **1-P** from the μPorasil column were concentrated and rechromatographed on a mini-column (10 cm×2 mm I.D.) packed with Lichrosorb SI-60 (5 μm) using the same mobile phase as in condition A. Five times higher sensitivity than that under condition A was obtained by this method. Fig. 5-b shows a chromatogram obtained from a serum sample containing 1 ng/ml of **1**. Using this method, **1** could be determined at a minimum concentration of 0.1 ng/ml in serum.

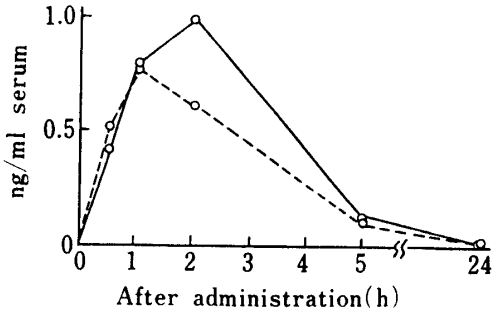


Fig. 6. Serum Levels of **1** after Oral Administration to Men

Dose: **1**, 0.35 mg/man, *p.o.*
—— : volunteer A.
---- : volunteer B.

Blood and Urinary Levels after Oral Administration of 1 to Man

After oral administration of **1** to healthy volunteers at a dose of 0.35 mg/man, the blood and urinary levels of **1** and **2** were followed for 24 h. Fig. 6 shows the serum levels of **1**. The metabolite **2** was not present in a detectable amount in any serum sample. Maximum concentration of **1** in serum was observed at 1—2 h after drug administration and the concentration and the concentration decreased below the detection limit after 5 h. The total urinary excretion of **1** and **2** within 24 h after the administration was about 40% of the dose. In urine, **1** and **2** were mainly present as the free forms, with a small amount of conjugated **2**.

Our proposed method is specific, sensitive and reproducible for the assay of **1** and **2** in biological fluids and is applicable to pharmacokinetic studies of the drug.

Acknowledgement We are grateful to Drs. E. Ohmura and M. Nishikawa of this division for their encouragement throughout this work.

References and Notes

1) This work was presented at the 30th Meeting of the Kinki Branch, Pharmaceutical Society of Japan, 1980.
2) R. Marumoto, Y. Yoshioka, O. Miyashita, S. Shima, K. Imai, K. Kawazoe and M. Honjo, *Chem. Pharm. Bull.*, **23**, 759 (1975).
3) K. Kawazoe, N. Matsumoto, M. Tanabe, S. Fujiwara, M. Yanagimoto, M. Hirata and K. Kikuchi, *Arzneim.-Forsch.*, **30**, 1083 (1980).
4) K. Yoshida, T. Kondo, T. Kobayashi and S. Tanayama, *J. Takeda Res. Lab.*, **40**, 153 (1981).
5) M. Yoshioka and Z. Tamura, *J. Chromatogr.*, **123**, 220 (1976).
6) H. Yuki, C. Sempuku, M. Park and K. Takiura, *Anal. Biochem.*, **46**, 123 (1972).
7) D. Ishii, *Bunseki*, **1980**, 92.
8) M. Yoshioka, A. Nakamura, H. Iizuka, K. Nishidate, Z. Tamura and T. Miyazaki, *Nucleic Acids Res.*, **8**, s61 (1980).