mixture was washed with ice-cooled 5% aqueous Na_2CO_3 and water, then dried over $MgSO_4$. The solvent was evaporated off and the residue was chromatographed on a silica gel column with benzene–AcOEt (4: 1). After concentration of the eluate, the addition of ether–petr. ether (1: 2) afforded crystals of (+)-**6**, (1.28 g) mp 107—108°, $[\alpha]_D^{20}$ +26.8 (c=5.00, MeOH). NMR ($CDCl_3$) δ: 2.19 (3H, s, >N–CH_3), 2.33 (6H, s, $C_{2,6}$–CH_3), 2.61 (2H, t, –CH_2CH_2N<), 3.48 (2H, s, –CH_2–C_6H_5), 3.63 (3H, s, –$COOCH_3$), 4.16 (2H, t, –$COOCH_2$–CH_2–), 5.10 (1H, s, C_4–H), 5.82 (1H, s, >NH). *Anal.* Calcd for $C_{26}H_{29}N_3O_6$: C, 65.12; H, 6.10; N, 8.76. Found: C, 65.05; H, 6.12; N, 8.66.

(−)-2-(N-Benzyl-N-methylamino)ethyl Methyl 2,6-Dimethyl-4-(*m*-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate ((−)-(6))——Treatment of (+)-**4** (1.1 g) as described for the preparation of (+)-**6** gave (−)-**6** (0.91 g) as crystals, mp 106—107°, $[\alpha]_D^{20}$ −26.7 (c=5.00, MeOH). The NMR spectrum of this sample was identical with that of (+)-**6**. *Anal.* Calcd for $C_{26}H_{29}N_3O_6$: C, 65.12; H, 6.10; N, 8.76. Found: C, 65.10; H, 6.21; N, 8.76.

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Determination of Fluorescein and Fluorescein Monoglucuronide excreted in Urine

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A simple method for the fluorometric determination of fluorescein and fluorescein monoglucuronide in urine was developed. Free fluorescein was determined by measuring its native fluorescence at 494 nm excitation and 516 nm emission. The conjugate was determined as fluorescein after hydrolysis at 37° for 20 min with β-glucuronidase. This method was applied to investigate the glucuronidation function of Wistar rats with liver injury in comparison with that of normal rats.

Keywords——fluorescein; fluorescin; fluorescein monoglucuronide; β-glucuronidase; fluorometry; glucuronidation; conjugation function; liver

As reported previously,[2)] fluorescein (F) and fluorescein monoglucuronide (FG) are excreted in the urine of animals administered F. For metabolic studies of F, a precise method for the determination of both F and FG is required.

Although F had been determined by measuring its absorbance,[3)] Menaker and Parker[4)] devised a fluorometric method for the determination of urinary and biliary F in order to eliminate the interference of the bile pigments and urochromogens. However, they neglected the interference of fluorescent FG with the determination of F. Later, Webb and his coworkers[5)] confirmed the presence of FG and estimated the total fluorescein (TF) in urine photometrically after hydrolysis with sodium hydroxide, despite the decomposition of F by the alkali.[6,7)] Thus, no precise method has been reported for the simultaneous determination of F, FG and TF in urine.

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The present paper describes a reliable method for the fluorometric determination of urinary F, FG and TF using β-glucuronidase.

Experimental

Apparatus——A Hitachi MPF-2A fluorescence spectrophotometer and a Toa HM-12A digital pH meter were used.

Materials and Reagents——F and FG were prepared as described previously.[2,6] β-Glucuronidase from bovine liver was purchased from Tokyo Zoki Seiyaku (Tokyo, Japan). All other reagents were of analytical grade and were used without further purification. GOT activity determination kit was obtained from Daiichi Pure Chemicals Co. (Tokyo, Japan).

Animals and administration of F——Male Wistar rats (250 g) were used. F in 1% sodium bicarbonate solution was orally administered just after urination for blank determination. The first urine after administration was collected for examination.

Induction of Acute Liver Injuries with Carbon Tetrachloride or D-(+)-Galactosamine HCl——In order to produce cytotoxic type injury, 0.5 ml of a mixture of carbon tetrachloride and olive oil (1: 1, v/v) was orally administered 24 hours before F administration. To produce hepatitic type injury, 10% D-(+)-galactosamine HCl aqueous solution was intraperitoneally injected three times at intervals of four hours. F was then administered at 48 hours after the first injection of the amino sugar. In either case, the injuries were identified in terms of GOT activity.

Procedure for Determination of TF——A 50 μl (130 U) aliquot of β-glucuronidase is added to a mixture of 200 μl of 0.2 M acetate buffer (pH 5.0) and 100 μl of urine and the whole is incubated at 37° for 20 min. After dilution with 5.0 ml of 0.2 M carbonate buffer (pH 9.15) to produce the maximum fluorescence,[6] the fluorescence intensity is determined at Ex. 494 and Em. 516 nm against a similarly treated urine blank. The working curve is prepared by the same procedure using a urine solution of F as a standard.

Procedure for Determining Apparent Fluorescence Intensity of F (fF_a)——The procedure is similar to that for TF determination, but β-glucuronidase is added after the carbonate buffer and incubation is omitted.

Results and Discussion

Selection of Wavelengths and Derivation of Equations for Determination

As shown in Fig. 1, the fluorescence of F was much more intense than that of FG, but the fluorescence of FG and urine was not negligible at any wavelength. To minimize the interference, 494 and 516 nm were selected as the excitation and emission wavelengths for fluorescence determination of F, respectively. At these wavelengths, the fluorescence intensity ratio of F to FG was 53.8 when the fluorescence intensity of FG solution was measured before and after complete hydrolysis.

For the calculation of the amounts of F and FG from the observed values of fTF and fF_a, the working curve for F and the following equations were used. Because fTF is equal to the sum of the true fluorescence intensity of F (fF_t) and the fluorescence intensity of F released from FG, we have

$$fF_t + (fF_a - fF_t) \times 53.8 = f\mathrm{TF} \tag{1}$$

Therefore,

$$fF_t = \frac{53.8 \times fF_a - f\mathrm{TF}}{52.8} \tag{2}$$

$$f\mathrm{F\ from\ FG} = f\mathrm{TF} - fF_t = 1.02\,(f\mathrm{TF} - fF_a) \tag{3}$$

From equation (3), the molar ratio (FG/TF) is directly obtained as

$$\mathrm{FG/TF} = 1.02 \times \frac{f\mathrm{TF} - fF_a}{f\mathrm{TF}} \tag{4}$$

Hydrolysis of FG with β-Glucuronidase

As depicted in Fig. 2, FG in 270 μl of aqueous buffer was hydrolyzed completely within 15 min by 65 U of β-glucuronidase. However, in order to increase the hydrolysis capacity and reaction rate, 130 U of β-glucuronidase in 230—1070 μl of reaction mixture was used for

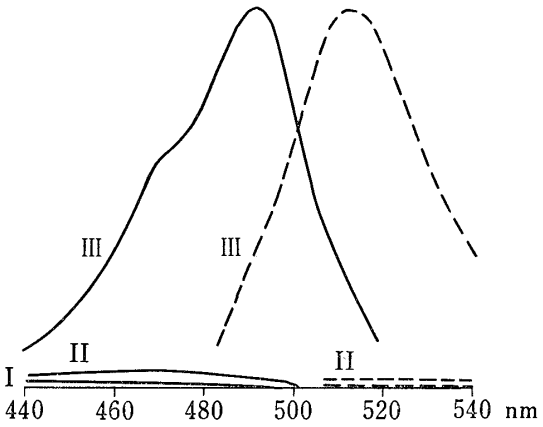

Fig. 1. Fluorescence Spectra of Urine (I) and Fluorescein Monoglucuronide before (II) and after (III) Hydrolysis

Solid lines are the excitation spectra observed at 512 nm and broken lines the emission spectra excited at 490 nm. Hydrolysis was performed with 130 U of β-glucuronidase in a 230 μl reaction mixture at 37° for 20 min.

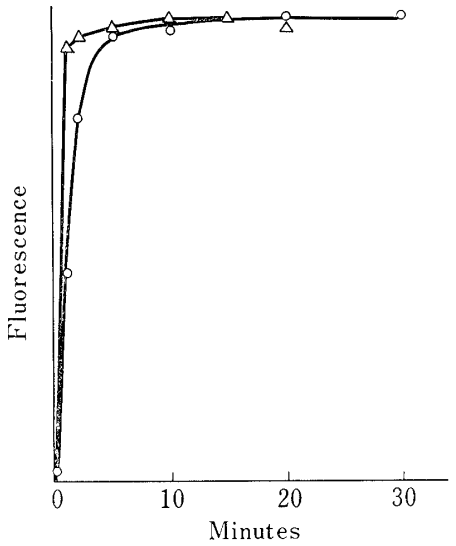

Fig. 2. Hydrolysis of Fluorescein Monoglucuronide with β-Glucuronidase

The system consisted of 20 μl (5×10^{-10} mol) of fluorescein monoglucuronide solution, 200 μl of 0.2M acetate buffer (pH 5.0) and 50 μl of β-glucuronidase solution (Δ, 130 U; o, 65 U). Incubation was done at 37° and stopped by adding 200 μl of ethyl alcohol.

the following investigations. FG at final concentrations up to 11.1 μM was completely hydrolyzed at 37° for 20 min. The presence of β-glucuronidase did not influence the fluorescence intensity of F, at least up to 260 U.

Effect of Urine on the Recoveries of F and FG

As indicated by Rollefson and Stoughton,[8] chloride which is present in the urine may cause quenching of the fluorescence of F. Therefore, the effect of urine on the recovery of F was examined.

Although a relatively large volume of urine (500 μl) did not affect the determination of F at the 0.4 μM level, the recovery of FG fell with increasing volume of urine (Fig. 3).

The pH of urine, in the range from 4.00 to 8.00, had no effect on the enzymatic hydrolysis of FG.

Stability of FG in 0.2M Acetate Buffer (pH 5.0) or Urine

As shown in Tables I and II, FG was stable for about one month both in 0.2 M acetate buffer (pH 5.0) at 6° and in urine at 0°. However, the conjugate was gradually hydrolyzed at room temperature to release F. Therefore, the collected urine should be preserved in an ice-bath or a refrigerator before determination.

Recoveries of the Ratio of FG to TF from Artificial Samples

In order to test the validity of the equations and the established procedures, 5 artificial samples were prepared by mixing different amounts of F and FG, and determined. The recoveries of FG/TF were close to 100%, as shown in Table III.

Effect of Fluorescin on the Determination

As stated previously,[2] fluorescin (Fi) was also present in a smaller amount together with F and FG in urine. Although Fi was easily oxidized to F in alkaline media, this was not the case in urine. Therefore, the effect of Fi on the determination of F and FG can be neglected.

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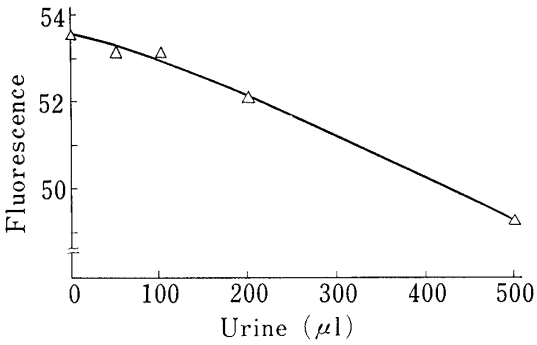

Fig. 3. Effect of Urine on the Recovery of Fluorescein Monoglucuronide

The system consisted of 20 μl (1×10^{-9} mol) of fluorescein monoglucuronide; 500 μl of a mixture of urine and water, 500 μl of 0.2 M acetate buffer (pH 5.0) and 50 μl (130 U) of β-glucuronidase solution. Incubation was performed at 37° for 60 min.

Table I. Stability[a] of 50 μM Fluorescein Monoglucuronide in 0.2M Acetate Buffer (pH 5.0) at 18—20° or 6°

Time (Day)	0	7	26	40
6°	1.001	1.001	1.000	0.9997
18—20	1.001		0.9616	

a) Stability is expressed as the FG/TF molar ratio.

Table II. Stability[a] of Fluorescein Monoglucuronide in Urine

Time (Day)	0	1	6	25
0°	0.8643		0.8594	0.8526
18—20°	0.8643	0.8560	0.7506	

a) Stability is expressed as the FG/TF molar ratio.

Table III. Recovery of the Ratio of FG to TF from Artificial Samples

FG Added (10^{-11} mol)	F Added (10^{-11} mol)	TF Found (10^{-11} mol)[a]	Recovery of TF (%)[a]	Recovery of FG/TF (%)[a]
54.10	10.05	63.4±2.4	98.8±3.8	99.1±0.3
54.10	20.10	73.5±1.8	99.2±2.4	100.2±1.1
54.10	30.15	83.6±1.4	99.3±1.7	100.0±1.0
18.03	10.05	27.8±0.5	98.9±1.8	100.0±1.0
36.07	10.05	45.2±0.6	98.0±1.3	100.4±0.7

a) Mean±S.D.
b) The volume of the reaction mixture was 315 μl, comprising 65 μl of sample solution containing various amounts of fluorescein and fluorescein monoglucuronide aqueous solution, 200 μl of 0.2 M acetate buffer (pH 5.0) and 50 μl of enzyme solution containing 130 U of β-glucuronidase. Incubation and determination were performed as described in "Experimental".

Application of the Method to the Determination of F and FG in Urine of Normal and Liver-damaged Wistar Rats Administered F

Glucuronidation is an important detoxication pathway of the liver for the excretion of exogenous and endogenous compounds. Various methods have been developed for testing this conjugation function, using benzoic acid,[9] sodium benzoate,[10] menthol,[11] salicylamide,[12] chloramphenicol[13] acetaminophen,[14] or isoketopinic acid.[15] However, because the procedures for determination of these glucuronides are tedious and time-consuming, they have not been used as routine tests.

F is a diagnostic drug in ophthalmology, and is usually used at an intravenous dose of 500 mg. Our preliminary results showed that 75.17±5.74% of F was excreted as FG in

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Table IV. Conjugation of Fluorescein in the Urine of Wistar Rats (Male, 250 g) administered 10 mg/kg of Fluorescein

	n	FG/TF±S.D. (%)
Normal rats	6	11.2±3.8
Acute CCl_4 injury	6	4.4±6.3
Acute D-(+)-galactosamine HCl injury	3	5.8±2.5

the first 2 hours in the urine of human subjects orally administered 5 mg of F. Therefore, F may be considered as a possible substrate for testing the liver conjugation function. This possibility was tested by the present analytical method as follows.

Three doses of 0.1, 1.0 and 10 mg per kg body weight of F were separately administered to normal and acutely liver-damaged rats. Although the doses of 0.1 and 1.0 mg/kg did not reflect the conjugation function, the conjugation of F decreased when F was administered at a dose of 10 mg/kg (Table IV). This result shows that a large dose of F may reflect the liver conjugation function.

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Sustained Release of Drugs from Ethylcellulose Microcapsules Containing Drugs dispersed in Matrices

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The use of ethylcellulose microcapsules possessing a reservoir system to achieve sustained release of sulfamethizole or 5-fluorouracil was examined *in vitro*. As a first step, the drugs were dispersed in cellulose acetate matrices, then the matrices were microencapsulated by means of coacervation-phase separation of ethylcellulose from diethyl ether solution by nonsolvent addition.

The surfaces of microcapsules were rounded, whereas the milled matrices were sharp-edged. In microcapsules of both drugs, longer release half-lives were obtained than with the corresponding matrices.

Keywords——microcapsule; matrix; sulfamethizole; 5-fluorouracil; coacervation-phase separation; sustained release; photomicrograph

Many attempts have been made to achieve sustained release of drugs.[2,3)] Drugs microencapsulated by poorly water-soluble polymers may be employed for this purpose. The authors have demonstrated the usefulness of enteric coated microcapsules in producing sustained release of an antibacterial agent, sulfamethizole, from microcapsules coated with carboxymethylethylcellulose.[4)] We have also studied ethylcellulose microcapsules containing chemo-

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