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Studies on the Metabolites of Fluorescein in Rabbit and Human Urine[1]

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After oral administration of fluorescein to rabbits and human subjects, the urinary metabolites were examined. Two new metabolites, fluorescin and fluorescin monoglucuronide, were identified in addition to the main metabolite, fluorescein monoglucuronide. In both conjugates, glucuronic acid was found to be linked to a phenolic OH group *via* an ether bond.

Keywords——fluorescein; fluorescin; fluorescein monoglucuronide; fluorescin monoglucuronide; trimethylfluorescin; structure; thin-layer chromatography; gas chromatography; metabolite

Fluorescein (F) has been used for a long time as a diagnostic drug in medicine,[3-8] especially in ophthalmology.[3] Fluorescein glucuronide (FG) has been reported to be the sole metabolite of F in mammalia.[9,10] However, the previous evidence for FG, that its incubation with β-glucuronidase gave F, is obviously insufficient to determine the structure of the metabolite.

In this investigation, the authors have attempted to characterize the chemical structure of the urinary metabolite, FG. The present study also deals with two new metabolites isolated from human and rabbit urines.

Experimental

Apparatus——A Hitachi MPF-2A fluorescence spectrophotometer, a Shimadzu GC-4APF gas chromatograph equipped with a flame ionization detector, and a Toa HM-12A digital pH meter were used. The wave-lengths given here are uncorrected. All concentration procedures were performed with a rotatory evaporator under reduced pressure below 10°.

Materials and Reagents——Trimethylfluorescin was synthesized from fluorescin (Fi) by the Ullmann method,[11] and purified by column chromatography (silica gel 60, Merck, Art. 7734) using benzene as an eluent to give pale yellow crystals, mp 138—139°. Calcd for $C_{23}H_{20}O_5$: C, 73.39; H, 5.36. Found: C, 73.41; H, 5.35. β-Glucuronidase from bovine liver was purchased from either Tokyo Zoki Seiyaku (Tokyo, Japan) for fluorometry or Worthington Biochemical Corp. (New Jersey, U.S.A.) for gas-liquid chromatography (GLC).

All other reagents, except triethylamine, which was purified by boiling with phthalic anhydride followed by distillation, were of analytical grade and were used without further purification.

Ion-exchangers used were SP-Sephadex C-50 from Pharmacia Fine Chemicals (Uppsala, Sweden), Amberlite XAD-2 from Rohm and Haas Co. (U.S.A.), and Dowex 50W×8 from Muromachi Kagaku Co. (Tokyo, Japan). Microcrystalline cellulose was purchased from Funakoshi Yakuhin Co. (Tokyo, Japan). Precoated thin-layer chromatography (TLC) aluminum sheets (silica gel 60, Merck, Art. 5553) were used.

1) This work was presented at the 99th Annual Meeting of the Pharmaceutical Society of Japan, Sapporo, August, 1979.
2) Location: *7-3-1, Hongo, Bunkyo-ku, Tokyo 113, Japan.*

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4) E. Koch, *Deutsches Arch. Klin. Med.*, **140**, 39 (1922).
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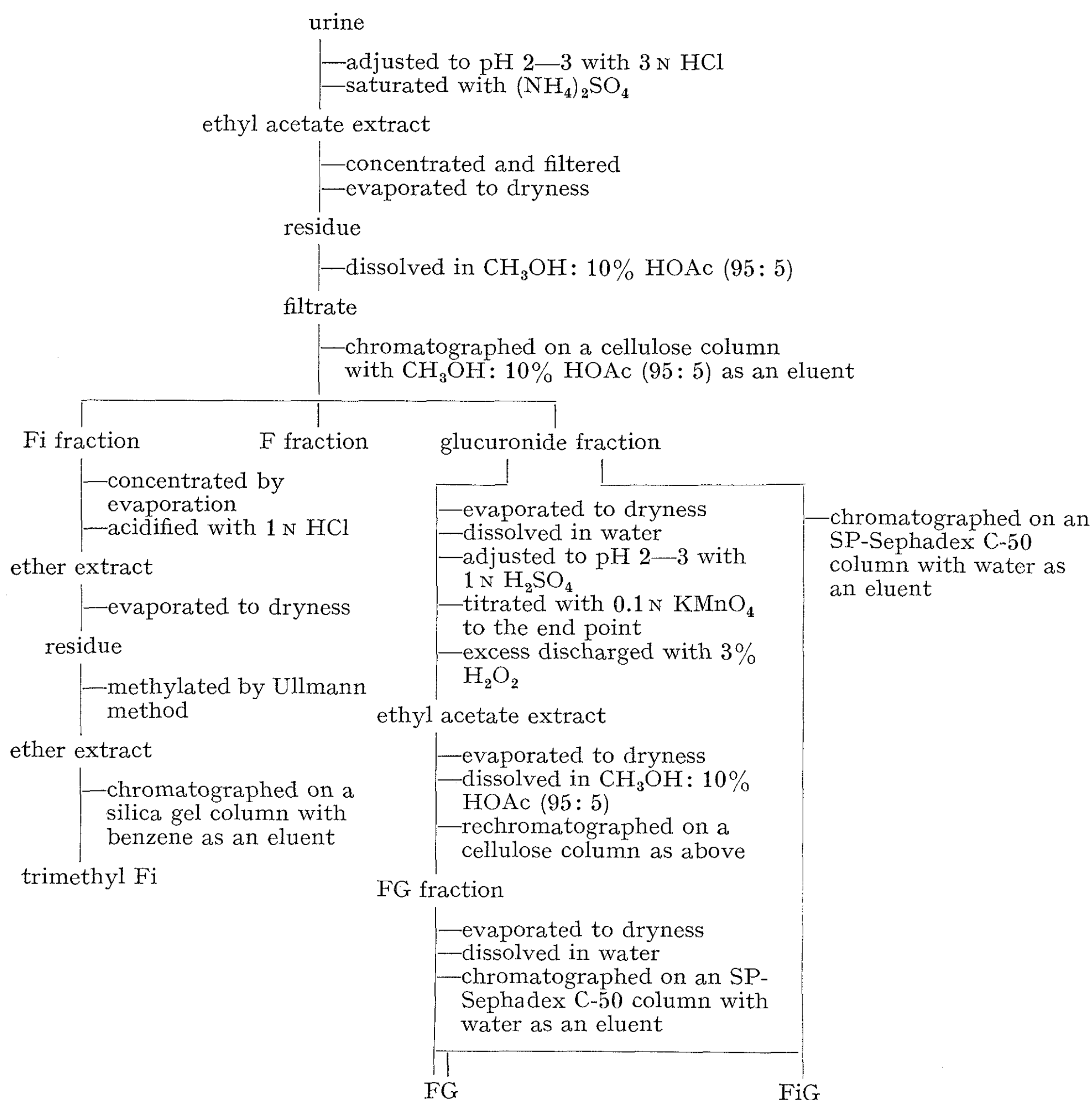

Chart 1. Isolation of Metabolites

Oral Administration of F——F was administered to a rabbit as reported by Webb *et al.*[10] Human subjects were given 50 or 5 mg of F in a colorless gelatin capsule containing no other additives.

Partial Purification of FG, Fi, and FiG——Urine specimens were collected from a rabbit just after urination and treated according to the procedure indicated in Chart 1.

Biliary Cannulation——Anesthesia was induced and maintained in a rabbit with ether. After a mid-line incision (*ca.* 6—8 cm) in the abdominal wall, the duodenum was exposed and the bile duct ligated just before the entrance of the duodenum. A second ligature was placed loosely around the duct between the liver and the first ligature. The duct was opened between these two ligatures, and a polypropylene cannula was introduced 0.5—1 cm into the duct and secured by tightening of the second ligature. The duodenum and intestine were replaced and then the abdominal wall was closed.

Hydrolysis of Glucuronide(s)——One hundred μl of urine was mixed with 200 μl of 0.2 M acetate buffer (pH 5.0) and 130 U (or 0.03 U for GLC) of β-glucuronidase and incubated for 20 min (or 1.5 hr for GLC) at 37°. The resulting aglycones and glucuronic acid were analyzed by TLC and/or GLC[12] using a blank and a control preparation without addition of the enzyme as references. Isolated FG fraction was dissolved in 0.2 M acetate buffer (pH 5.0) and treated similarly.

Gas Chromatographic Analysis of D-Glucuronic Acid——For the analysis of D-glucuronic acid liberated from FG by the enzymatic hydrolysis described above, trimethylsilyl (TMS) derivatization was carried out by the method described by Matsunaga *et al.*[12] In this study, Dowex 50W × 8 was used in place of Amberlite

12) I. Matsunaga, T. Imanari, and Z. Tamura, *Chem. Pharm. Bull.*, **18**, 2535 (1970).

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CG-120. The TMS derivative obtained was then analyzed with a column (1.5 m×4 mm i.d. glass tube) packed with 1.5% Silicone QF-1 on Chromosorb W at 120°, or a column (2 m×4 mm i.d. glass tube) packed with 2% silicone GE XF-1105 on Chromosorb W at 170°.

Gas Chromatographic Analysis of Methylated Fi——Fi in urine was isolated and methylated by the procedure described in Chart 1 and the resulting methylated Fi was analyzed with a column (1.5 m×4 mm i.d. glass tube) packed with 2% Silicone GE XE-60 or 1.5% Silicone QF-1 on Chromosorb W (60—80 mesh) at 245°.

Results and Discussion

Thin-Layer Chromatogram of Urine

A two-dimensional silica gel chromatogram of the ethyl acetate extract of urine revealed that a nonfluorescent compound with a slightly smaller *Rf* value than that of F changed to a fluorescent compound with the same *Rf* value as F after exposure to air for 8—12 hr (Fig. 1a). The chromatographic behavior and the oxidizability properties of this spot coincided with those of Fi, indicating the probable existence of Fi in the urines. Since the spot near the starting point delivered a trace amount of F after standing for 12 hr, it is presumably the glucuronide described by Webb *et al.*[10] In view of the presence of Fi, the presence of FiG in urine was also presumed.

Isolation of Metabolites

The metabolites were isolated by the procedure shown in Chart 1. The eluate from the cellulose column was divided into Fi fraction, F fraction and glucuronide fraction in the order of elution on the basis of TLC examination. It was found that the glucuronide fraction probably contained FG and FiG, since F and Fi were released upon enzymatic hydrolysis (Fig. 1c). In order to purify FG and FiG, potassium permanganate and/or SP-Sephadex C-50 were used (Chart 1). A further attempt to crystallize FG in the free form, or as a salt of cinchonidine[13] or brucine, was unsuccessful. However, the isolated FG was not contaminated with either glucuronic acid or any other fluorescent compound when examined by GLC and TLC.

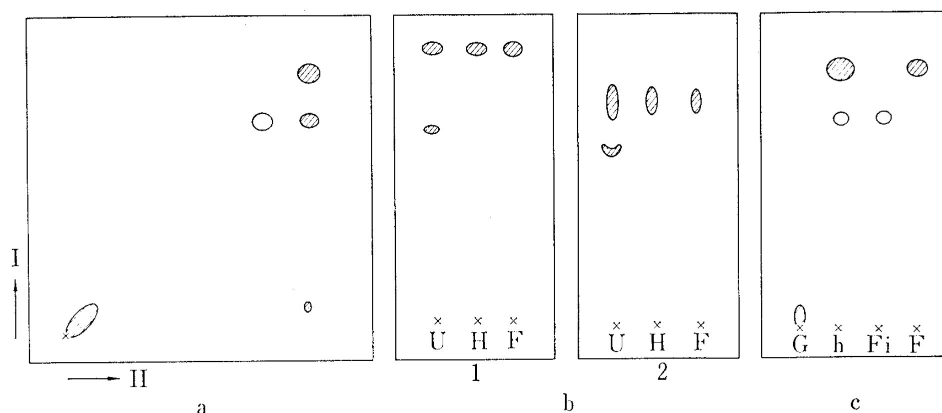

Fig. 1. TLC Analysis of Urinary Metabolites of F

Samples were applied at the "X" marks. Detection of fluorescent spots was performed with a UV lamp. Open circles represent spots that became fluorescent after standing. (a), a two-dimensional chromatogram of the ethyl acetate extract of urine using acetone: benzene (3:1, v/v). Before the second development, the plate was allowed to stand for 8—12 hr; (b_1), solvent=*n*-BuOH: MeOH: H_2O (6.0: 2.1: 2.2, v/v/v), U=urine specimen, H=hydrolysate of urine with β-glucuronidase, F=florescein; (b_2), solvent=MeOH: EtOAc (3:1, v/v); (c), a chromatogram of the glucuronide fraction. The solvent used was the same as in "a", G=glucuronide fraction, h=hydrolysate of glucuronide fraction with β-glucuronidase, F=fluorescein, Fi=fluorescin.

13) A. A Di Somma, *J. Biol. Chem.*, **133**, 277 (1940).

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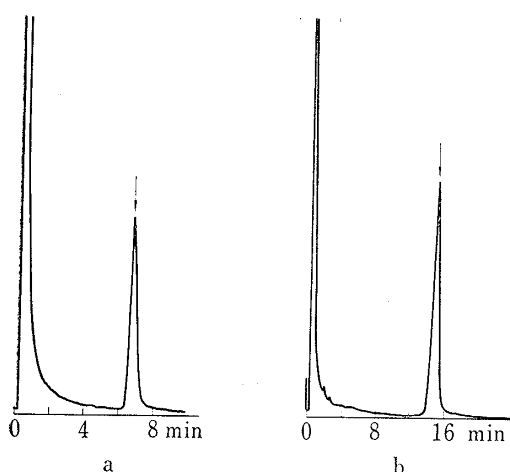

Fig. 2. Gas Chromatograms of Methylated Fi from Urine

Column: 1.5% QF-1 (a) or 2% XE-60 (b) on chromosorb W in a 1.5 m×4 mm i.d. glass column.
Temperature for a and b: column, 245°; injection port, 280°.
Carrier gas: N_2 25 ml/min (a) or 22.5 ml/min (b).
The arrows show the elution position of authentic trimethylfluorescin.

Identification of Fi

The methylated Fi fraction (Chart 1) showed a similar *Rf* value, as well as a yellowish color and fluorescence after standing for 10—15 min, to that of authentic trimethylfluorescin. Further evidence was obtained by GLC using two different liquid phases (Fig. 2); this methyl derivative gave the same retention times as authentic trimethylfluorescin.

Identification of FG and FiG

Isolated FG was positive to the naphthoresorcinol test.[14] After hydrolysis with β-glucuronidase, only F and glucuronic acid were obtained; these were identified by TLC and GLC (Fig. 3), respectively. The molar ratio of F (determined by fluorometry) to glucuronic acid (determined by GLC) was 1.1. The pH dependence of the fluorescence intensity of FG at fixed wavelength (Fig. 4) was similar to that of monomethylfluorescein but not to that of fluorescein methyl ester.[15] Therefore, FG is a monoglucuronide, which should have an ether linkage between glucuronic acid and F (Fig. 5).

When FG was reduced with zinc powder, a new nonfluorescent spot with a slightly smaller *Rf* value than FG appeared on the TLC plates together with a small spot of FG (Fig. 6b). This chromatogram was quite similar to that of isolated FiG (Fig. 6c), which in turn produced a

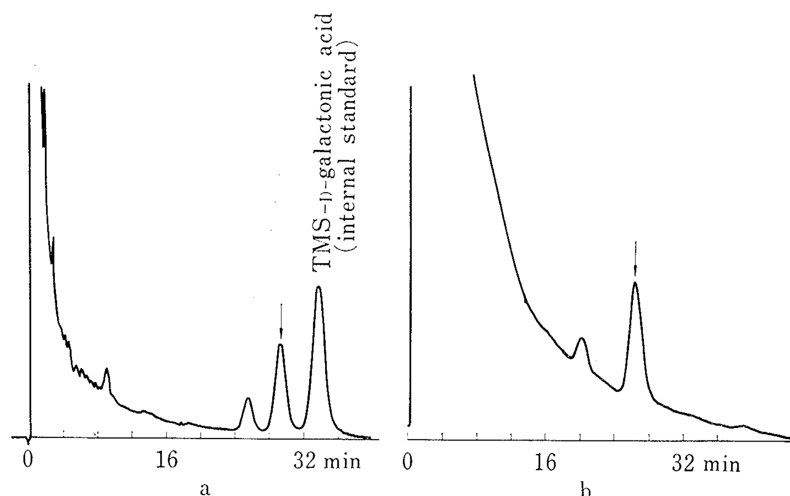

Fig. 3. Gas Chromatographic Identification of Glucuronic Acid in Enzymatic Hydrolysates of FG

Column: 2% XF-1105 (a) or 1.5% QF-1 (b) on Chromosorb W in a 2 m×4 mm (a) or a 1.5 m×4 mm (b) i.d. glass column.
Temperature: Column, 170° (a), 120° (b); injection port, 225° (a), 150° (b).
Carrier gas: N_2: 29 ml/min (a) or 29.4 ml/min (b).
The arrows indicate the elution positions of authentic TMS-gulonic acid.

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14) G.B. Maughan, K.A. Evelyn, and J.S.L. Browne, *J. Biol. Chem.*, **126**, 567 (1938).
15) S.C. Chen, H. Nakamura, and Z. Tamura, *Chem. Pharm. Bull.*, **27**, 475 (1979).

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fluorescent compound with the same Rf value as FG, as shown in Fig. 6d.

Origin of Fi

To examine the origin of Fi, a rabbit was subjected to biliary cannulation to block enterohepatic circulation, and administered 25 mg of F intravenously. Two-dimensional TLC of the urine demonstrated the presence of Fi, indicating the reduction of F to Fi by the animal itself. When 5 mg of F was orally administered to 9 healthy male human subjects, the amount of Fi found in urine obtained during 10-hour after the β-glucuronidase treatment was about 0.1 mg (2%).

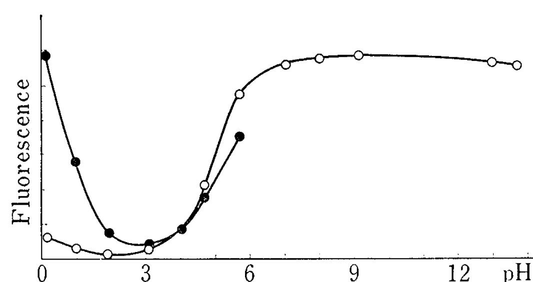

Fig. 4. Effect of pH on the Fluorescence Intensity of Isolated FG at a Concentration of 2×10^{-7} M

The fluorescence intensity was measured with excitation at 430 nm (●) or 470 nm (○) and emission at 516 nm.

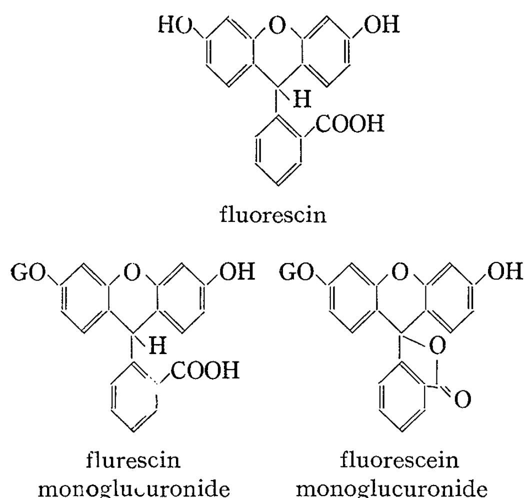

Fig. 5. Structures of Urinary Metabolites of F

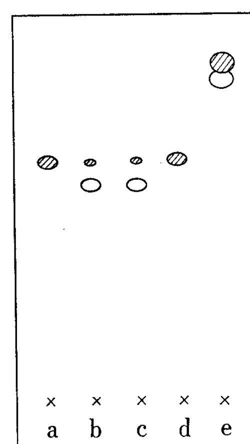

Fig. 6. TLC Analysis of FiG

The symbols used are the same as in Fig. 1. Samples used for a, b, c, d, and e were FG, FG reduced with zinc, FiG, FiG oxidized with air, and a mixture of Fi and F, respectively. The solvent system used was the same as in Fig. $1b_1$.

Other Metabolites That May be Present

Attempts to detect other metabolite in urine were performed by TLC. The spraying of 15 N sulfuric acid, which can make all fluorescein derivatives, including nonfluorescent diglucuronide, fluorescent by conversion to the protonated form,[15] did not show up any additional spots other than F, Fi, FG, and FiG. Complete disappearance of the conjugate spot after the reaction with β-glucuronidase (Fig. 1b) indicated that no other conjugate such as sulfate was present in the rabbit and human urines.

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