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Gas Chromatographic-Mass Fragmentographic Determination of 5-Fluorouracil as a Metabolite of New 5-Fluorouracil Derivatives

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5-Fluoro-5′-deoxyuridine and ethyl (±)-6-*n*-butoxy-5-fluoro-5,6-dihydrouracil-γ-5-carboxylate are new pro-drug forms of 5-fluorouracil that have been developed as antitumor agents. A gas chromatographic–mass fragmentographic determination of the active substance 5-fluorouracil in biological materials following administration of each compound was established. The detection limit for 5-fluorouracil was 1 ng/ml of plasma and 1—5 ng/g of wet weight of visceral tissues.

Keywords——5-fluoro-5′-deoxyuridine; ethyl (±)-6-*n*-butoxy-5-fluoro-5,6-dihydrouracil-γ-5-carboxylate; 5-fluorouracil; active metabolite; gas chromatography–mass fragmentography

5-Fluorouracil (5-FU) and the derivative 1-(tetrahydro-2-furanyl)-5-fluorouracil (ftorafur) have been widely used in cancer chemotherapy, and a combined therapy of uracil plus ftorafur (UFT), which has a greater antitumor effect than ftorafur alone, has recently been investigated.[1,2] Furthermore, 5-fluoro-5′-deoxyuridine (I) and ethyl (±)-6-*n*-butoxy-5-fluoro-5,6-dihydrouracil-γ-5-carboxylate (II) (Chart 1) have recently been developed as antitumor agents.[3–5]

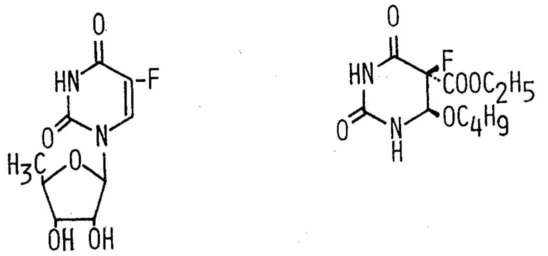
I II

Chart 1. The Structures of New 5-Fluorouracil Derivatives

For basic and clinico-pharmacological studies and also for comparative investigation of these 5-FU derivatives, it seems important to establish a quantitative method for the determination of the active metabolite 5-FU in biological materials after administration of each derivative. We have already reported methods employing gas chromatography–mass fragmentography (GC–MF) with an electron impact[6–8] or a chemical ionization[9] method for assay of 5-FU in biological materials following administration of ftorafur or UFT. On the other hand, although combined methods using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC)[10] or TLC and microbiological assay[11] were reported for determining 5-FU after treatment with compound II, there are no reports on the determination of 5-FU after administration of compound I.

In the present study, we examined a sensitive method for the assay of 5-FU as an active metabolite of compounds I and II on the basis of the previous reports.[6–8]

Experimental

Materials——Compounds I and II were synthesized and purified in our laboratory.[12,13] 5-FU and [1,3-$^{15}N_2$]-5-FU (95% enrichment) were obtained from Sigma (St. Louis, MO, U.S.A.) and PCR (Gainesville, FL, U.S.A.), respectively. *N*,*O*-Bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and pyridine for use as silylating agents were purchased from Pierce (Rockford, IL, U.S.A.). The on-column methylating reagent, a solution of 20—25% phenyltrimethylammonium hydroxide in methanol, was obtained from Tokyo Chemical

Industry Co. (Tokyo, Japan) and diluted to 2% with methanol before use. All other chemicals used were liquid chromatographic and analytical grade materials (Wako Pure Chemicals Co., Osaka, Japan).

The samples of blood and visceral tissues were collected from male AH-130 tumor-bearing rats (170—180 g, 6—7 weeks old).

Instrumentation——A JEOL JMS-D 300 mass spectrometer with an electron impact ion source connected to a JEOL JGC-20kP gas chromatograph (Tokyo, Japan) was used.

The conditions of the gas chromatograph were as follows: a coiled glass column (1 m × 2 mm I.D.) packed with 3% OV-17 on Chromosorb W AW (DMCS), 80—100 mesh (Gaschro Kogyo Co., Tokyo, Japan); injector, column and ion source temperatures of 210, 150 and 180°C for the measurement of the trimethylsilyl derivatives and of 280, 200 and 180°C for the measurement of the methyl derivatives; and flow rate of helium as a carrier gas, 30 ml/min.

The mass spectrometer was used under the following conditions: ionization energy, 70 eV; ionization current, 300 μA; accelerating voltage, 3 kV; and ion multiplier voltage, 1.4 kV. The peaks of the molecular ion at m/z 274 (rel. int., 36.1%) and 276 (38.2%) of the bis(trimethylsilyl) derivatives and at m/z 158 (100%) and 160 (100%) of the dimethyl derivatives of 5-FU and [1,3-$^{15}N_2$]-5-FU as an internal standard were selected for mass fragmentographic analysis.

Extraction Procedure——The extraction procedure for GC–MF determination of 5-FU after administration of compounds I and II was carried out in the same way as for the samples following administration of ftorafur or UFT.[6-8] However, the ethyl acetate extract prepared from the samples obtained after treatment with compound II was further dissolved in 100 μl of methanol and subjected to TLC (Kieselgel HF_{254}, Type 60, Merck, Darmstadt, Germany) with chloroform: methanol: aqueous ammonia (75: 25: 1). The fraction containing 5-FU with *Rf* 0.20 was extracted with methanol, and this methanol extract was dried under nitrogen gas after the removal of the silica gel by centrifugation.

Derivatization——The ethyl acetate extract containing 5-FU prepared from the samples obtained after treatment with compound I was dissolved in 100 μl of a freshly prepared solution of 25% BSTFA in pyridine, and the solution was kept at 80°C for 20 min to allow trimethylsilylation to occur. Then 1—3 μl of this solution was injected into the gas chromatograph–mass spectrometer. On the other hand, the methanol extract prepared from the samples obtained after administration of compound II was dissolved into 100 μl of 2% phenyltrimethylammonium hydroxide in methanol, then an aliquot was introduced into the gas chromatograph through the injection port.

Calibration Curves——The calibration curves for the determination of 5-FU by GC–MF were prepared by plotting the ratio of the peak height of 5-FU to that of the internal standard [1,3-$^{15}N_2$]-5-FU (as their trimethylsilyl derivatives for the samples of compound I or as methyl derivatives for the samples of compound II) against concentration. These calibration plots were linear.

Results and Discussion

The methods for assay of 5-FU in plasma and visceral tissues after administration of ftorafur or UFT[6-8] could be adapted to the determination of 5-FU in samples obtained after administration of compound I, but these methods could not be used for the samples of compound II.

Compound I remained in the aqueous layer after chloroform extraction under acidic conditions, under which protein was denatured and enzymes were inactivated,[8] and could be extracted with ethyl acetate together with 5-FU. Compound I had no significant effect on the determination of 5-FU. Known amounts of compound I were added to the plasma and homogenates of various organs of AH-130 tumor-bearing rats and these were then assayed for 5-FU. The results showed that the decomposition of compound I to 5-FU during the entire procedure was 0.01 w/w% or less.

Compound II was extracted quantitatively with chloroform under acidic conditions from the aqueous layer, but its metabolic intermediate, 6-*n*-butoxy-5-fluoro-5,6-dihydrouracil,[10,11] remained partially in the aqueous layer. This metabolite was extracted with ethyl acetate together with 5-FU, and then had an appreciable effect on the measurement of 5-FU because of its decomposition to 5-FU during the derivatization procedure. Therefore, in the present study the TLC procedure was employed for the separation of 6-*n*-butoxy-5-fluoro-5,6-dihydrouracil from 5-FU in the ethyl acetate extract. The *Rf* values of 6-*n*-butoxy-5-fluoro-5,6-dihydrouracil and 5-FU on TLC, as described in the experimental section, were 0.57 and 0.20, respectively, In addition, the *Rf* value of compound II under the same TLC conditions was

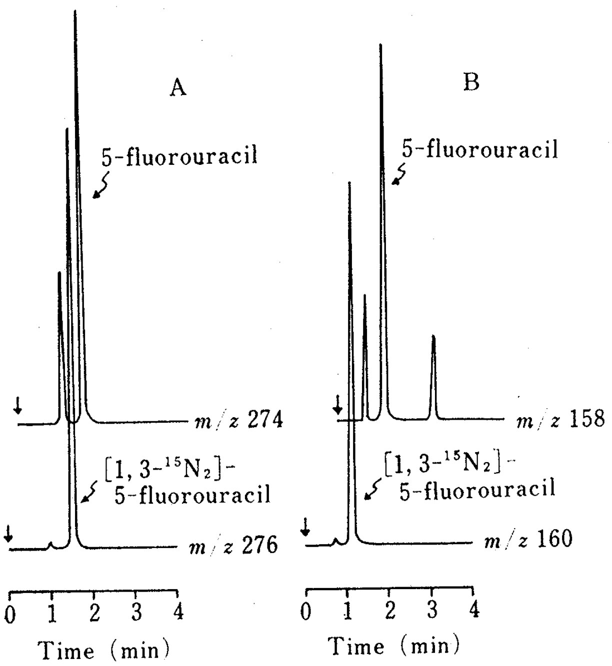

Fig. 1. The GC–MF Separation Pattern based on Trimethylsilylation for 5-FU as a Metabolite of Compound I(A) and Methylation for that of Compound II(B) extracted from Plasma of AH-130 Tumor-bearing Rats with [1,3-$^{15}N_2$]-5-FU as an Internal Standard

0.67. Methanol was used as a solvent for the extraction of the fraction containing 5-FU from the TLC plate, because the recovery was quantitative. To obtain a derivative of 5-FU from the TLC plate suitable for GC–MF, the procedures of trimethylsilylation with BSTFA and methylation with an on-column methylating reagent were compared. The trimethylsilylation procedure was often affected by silica gel, which dissolved slightly in methanol, while the methylation procedure was found to give a better result.

Thus, GC–MF methods based on trimethylsilylation for the metabolite 5-FU of compound I and on methylation for that of compound II were employed in the present study.

The typical mass fragmentograms showing the separation by GC–MF of the trimethylsilyl and the methyl derivatives of 5-FU extracted from plasma of AH-130 tumor-bearing rats after administration of compound I or II are shown in Fig. 1, with [1,3-$^{15}N_2$]-5-FU as an internal standard. These chromatograms show a good separation of 5-FU from plasma components. The chromatographic separations of the visceral tissues extract were in good agreement with those of the plasma extract. The retention times of the trimethylsilyl and methyl derivatives of 5-FU were 1.5 and 1.2 min, respectively. The detection limits for 5-FU in the present methods were 1 ng/ml of plasma and 1—5 ng/g wet weight of visceral tissues. The reproducibility of the present methods was ±3.3%. Furthermore, the absolute recoveries of 5-FU from plasma and visceral tissues during the entire procedure in the present methods were *ca.* 85 and 80%, respectively.

Thus, it is suggested that, in the case of many 5-FU derivatives, the concentration of the active metabolite 5-FU in biological materials after administration of each compound can be determined by utilizing the present methods or previous methods.[6-8]

Methods for the quantitative determination of compound I or compound II and its metabolic intermediate, 6-*n*-butoxy-5-fluoro-5,6-dihydrouracil, are under investigation.

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