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Note

Determination of a novel 5-fluorouracil derivative in rat and human plasma by high-performance liquid chromatography

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Although 5-fluorouracil (5-FU) remains an important antitumor agent for the treatment of solid tumors in clinical medicine [1, 2] it has severe adverse reactions such as gastrointestinal disorders [3, 4]. Therefore, it is necessary to minimize or improve its side-effects and develop a new derivative from the standpoint of biopharmaceutics and drug design. A novel 5-FU derivative (Fig. 1), 1,3-didecanoyl-2-[6-(5-fluorouracil-1-yl)carbonylamino]glyceride

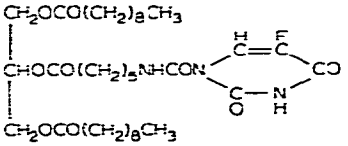

Fig. 1. Chemical structure of DFUG.

(DFUG), was designed in our laboratory. As suggested from the chemical structure, DFUG is a conjugate of 5-FU with triglyceride. The potent antitumor activity of orally administered DFUG was confirmed by basic studies using mice transplanted with tumors in our laboratory. Then, to measure not only the absorbability from the gastrointestinal tract after oral administration of DFUG but also the conversion efficiency of DFUG to 5-FU, it is necessary to develop an assay system for DFUG separate from 5-FU in plasma of both experimental animals and human.

MATERIALS AND METHODS

Reagents

The purified DFUG was provided by Dr. H. Nakao (Sankyo Pharmaceutical

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Co. Ltd., Tokyo, Japan). Methanol, ethyl acetate, and tetrahydrofuran were obtained from Nakarai Chemicals, Kyoto, Japan. Miglyol® 812 was obtained from Dynamit Nobel, Troisdorf-Oberlar, G.F.R. All other chemicals were of reagent grade.

Determination of DFUG in plasma

To 15-ml centrifuge tubes, 100 μl of rat plasma, 1 ml of 0.1 *N* hydrochloric acid, and 4 ml of ethyl acetate were added. The tubes were placed on a reciprocating shaker for 15 min. The aqueous and organic phases were then separated by centrifugation (1500 *g*, 10 min). Then 3 ml of the organic phase were removed with a Pasteur pipette and placed in a 15-ml glass tube. This ethyl acetate extraction of the plasma was repeated one more time and the organic phases were pooled in the conical glass tube. The combined ethyl acetate extracts were then evaporated at room temperature under vacuum. The residue was dissolved in 200 μl methanol of which 50 μl were injected into the chromatograph.

Analysis was performed using a Shimadzu (Kyoto, Japan) Model LC-3A pump and Model SPD-2A UV absorbance detector. The column (25 cm × 4 mm I.D., stainless steel) was packed with nominal 10-μm ODS-silica gel (LiChrosorb ODS, manufactured by E. Merck, Darmstadt, G.F.R.) using a balanced density slurry packing procedure similar to that described by Majors [5]. The mobile phase, methanol—water—tetrahydrofuran (400:10:4), was prepared fresh daily. The flow-rate was 1 ml/min and the pressure approximately 20 kg/cm^2. Detection was UV spectrophotometry at 260 nm. The detector was usually at a sensitivity of 0.01 a.u.f.s. The detector signal was processed and recorded using a Model C-R1A reporting integrator (Shimadzu). Levels were estimated by the chromatographic technique of comparing peaks obtained from rat or human plasma with curves obtained from the plasma to which were added known amounts of DFUG.

RESULTS AND DISCUSSION

The UV absorption spectrum of DFUG is shown in Fig. 2, where the peak of DFUG is at 255 nm. However, determination of DFUG was facilitated at 260 nm, since there is minimal interference by other components eluting near the retention time of DFUG, about 5.8 min in Fig. 3. Fig. 3A shows a typical chromatogram for DFUG from human plasma. For comparison, the chromatogram obtained from DFUG-free human plasma at the same sensitivity is shown in Fig. 3B. There are no peaks in this chromatogram that would interfere with DFUG. In addition, this assay method was applied to a rat plasma sample spiked with DFUG. Though the resulting chromatograms are not shown, it may be mentioned that this assay method is also applicable to rat plasma samples.

The recovery of DFUG added to rat plasma was determined by comparing the peak area from a plasma sample containing 500 ng of DFUG per ml with the results obtained from an aqueous standard of the same concentration. The results (Table I) indicate that recovery is greater than 98%. Plasma samples containing 833.3, 625.0, 416.7, 208.3, 138.9, and 83.3 ng of DFUG per ml

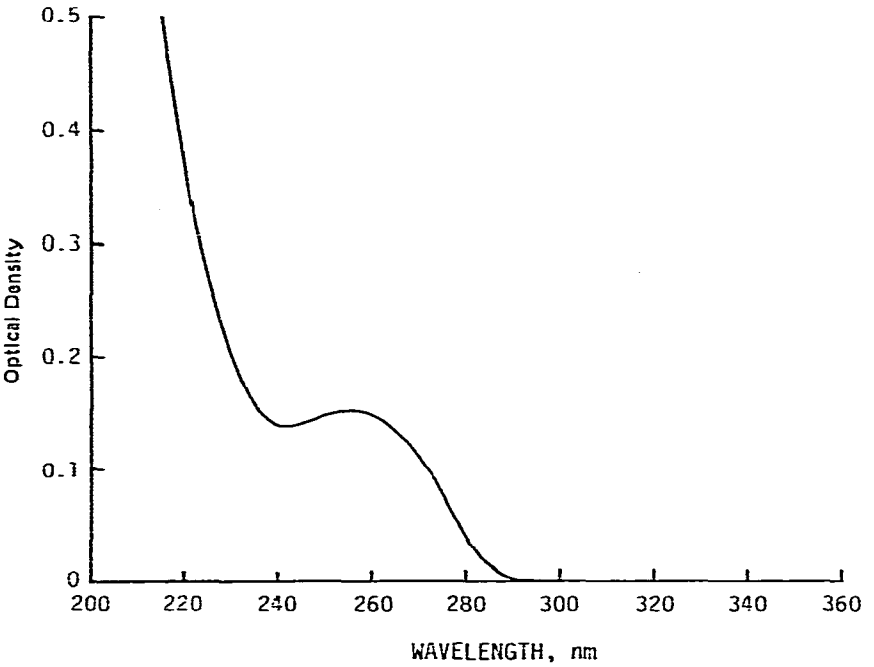


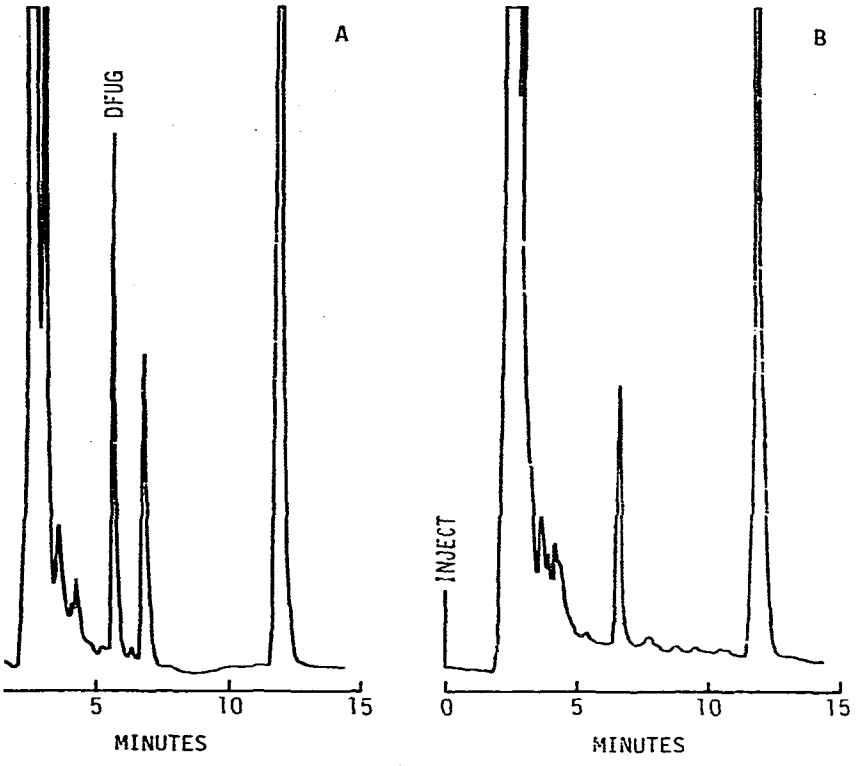
Fig. 2. The UV absorption spectrum of DFUG at a concentration of 10 μg/ml in methanol.

TABLE I

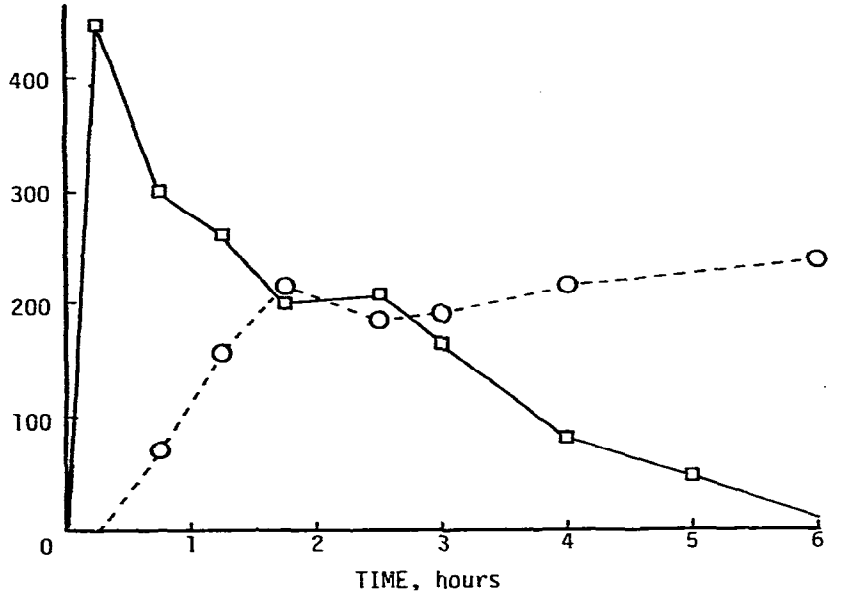
COMPARISON OF PEAK AREAS FOR FOUR ANALYSES OF A STANDARD MIXTURE OF DFUG

Injection	Peak area ($\mu\bar{V}$ × sec)			Peak area ratio
	Mean	Standard deviation	Coefficient of variation (%)	
Serum samples	100,562	3331	3.31	98.4
Aqueous standards	102,191	3151	3.08	100

were prepared and analyzed to determine the standard curve. For each concentration, duplicate injections were made and the sums of the DFUG peak heights were averaged. A linear least-squares regression analysis gave a coefficient of correlation (r) of 0.997. The UV detector was set at its maximum sensitivity (0.005 a.u.f.s.), and 50 μl of ethyl acetate extract dissolved in methanol were injected onto the column. However, up to 100 μl can be injected before resolution of DFUG is lost. Therefore, the limit of detection of this method is about 40 ng/ml. The standard curves were made at least once a day as no internal standard was used in this assay. The standard curves were well reproducible as the standard deviation was 3.5% (n=6 at 208.3 ng/ml). This can probably be attributed to the stability of the column and the accuracy of the selection of the mobile phase. At the early stage of this assay method, methanol—water (100:5) was used as a mobile phase. However, slight inter-



3. A, Representative chromatogram of the HPLC of DFUG in human plasma. B, Chroogram of a blank human plasma.



4. Concentrations of DFUG (□) and 5-FU (○) in plasma of a rat after administration of ng of DFUG per kg body weight, orally.

ference was detected by another component eluting near the retention time of DFUG. This problem was solved by adding tetrahydrofuran to the mobile phase, and the interference was eliminated, as shown in Fig. 3.

A male Wistar rat was administered DFUG, 22 mg/kg, dissolved in 0.1 ml of Miglyol 812 as an oral dose. Venous blood samples were taken for 6 h. Plasma levels of DFUG and 5-FU were both measured by the high-performance liquid chromatographic (HPLC) method (Fig. 4). With respect to the plasma level of 5-FU, several investigators [6, 7] have developed assay methods using reversed-phase HPLC. In this experiment, the same column was used for this purpose. However, as a mobile phase, 0.1% acetic acid was used. As shown in Fig. 4, the peak plasma DFUG level appeared at about 20 min after oral dose. On the other hand, a considerably high plasma 5-FU level was maintained for a long period.

These investigations will be continued with patients suffering from cancer.

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