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Highly sensitive method for the determination of 5-fluorouracil in biological samples in the presence of 2′-deoxy-5-fluorouridine by gas chromatography–mass spectrometry

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ABSTRACT

A highly sensitive and convenient gas chromatographic–mass spectrometric (GC–MS) method is described for the determination of 5-fluorouracil in the presence of 2′-deoxy-5-fluorouridine (which breaks down into 5-fluorouracil during ordinary GC derivatization) in biological samples such as plasma and urine. After extraction with ethyl acetate, 5-fluorouracil and 5-chlorouracil, the latter being used as an internal standard, were converted into their *tert.*-butyldimethylsilyl derivatives by allowing the mixture to stand for 30 min at room temperature and were assayed by electron-impact ionization GC–MS. Under these conditions, 2′-deoxy-5-fluorouridine did not decompose or interfere with the determination of 5-fluorouracil. The assay method, including the extraction and *tert.*-butyldimethylsilyl derivatization of 5-fluorouracil, showed good linearity in the range 0–100 ng/ml for 5-fluorouracil in plasma (detection limit 0.5 ng/ml) and urine (detection limit 1 ng/ml). The usefulness of this method was demonstrated by determining plasma concentrations of 5-fluorouracil in rats treated intravenously with 5-fluorouracil and 2′-deoxy-5-fluorouridine.

INTRODUCTION

The biochemical and pharmacological importance of 5-fluorouracil (5-FU) and its nucleosides and nucleotides has been demonstrated [1–3] and 2′-deoxy-5-fluorouridine (FdUrd), an anabolite of 5-FU, is also used in clinical oncology by infusion in the treatment of malignant neoplasms [4–7].

It is well known that the clearance rate of 5-FU is very high [8,9], and that this compound is metabolized to fluorouridine (FUrd), FdUrd and 5,6-dihydrouracil [10]. Therefore, a specific, sensitive and simple analytical method is required to quantitate the 5-FU in biological samples which circumvents possible interferences from the metabolites of 5-FU and some endogenous substances such as uracil.

Several analytical procedures utilizing gas chromatography–mass spectrometry (GC–MS) [11–15] and gas chromatography (GC) [16–18] were used for the

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determination of 5-FU in biological samples. In these procedures, 5-FU was converted into trimethylsilyl [12] or methyl [13–18] derivatives prior to GC–MS or GC analysis. However, these procedures were found not to be applicable to samples containing 5-FU and concomitant FdUrd, because it is assumed that FdUrd decomposes to 5-FU during the derivatization process and thus that it interferes with the determination of 5-FU. Williams *et al.* [18] described a GC method to quantitate fluoropyrimidine nucleosides and 5-FU, and pointed out that FdUrd was partially decomposed to 5-FU on the GC column. Thus a correction was required in the quantitation of 5-FU in the presence of FdUrd. Odagiri *et al.* [15] and Min and Garland [11] described that 5′-deoxy-5-fluorouridine (5′-DFUR) was easily converted to dimethyl 5-FU during methylation with diazomethane of 5-FU in the presence of 5′-DFUR.

In the trimethylsilylation of 5-FU, unexpectedly high values of the 5-FU concentration were obtained, because the non-derivatized 5-FU on the injection port was being silylated by the derivatizing reagent present in the following sample [12].

Although plasma concentrations of 5-FU were monitored using high-performance liquid chromatography (HPLC) [19–28], these assay methods were limited to pharmacokinetic studies, where 5-FU concentrations are maintained at rather high levels by continuous intravenous (i.v.) infusion.

N-Methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) as a *tert.*-butyldimethylsilyl (TBDMS) derivatizing agent was used in GC analysis of amino acids [29] and in GC–MS analysis of monohydroxyeicosatetraenoic acids [30] and 2-acetylaminofluorene [31], and it was shown that TBDMS derivatives were thermally stable and had favourable fragmentations upon electron-impact (EI) ionization.

There have been few *in vivo* studies on the kinetics of FdUrd decompisition to 5-FU, probably due, in part, to the lack of suitable analytical methods.

In the present study, we developed a specific, sensitive and simple analytical method by GC–MS to determine 5-FU in plasma and urine samples in the presence of FdUrd. The selected ion of TBDMS-derivatized 5-FU was monitored, and it was found that FdUrd does not decompose to 5-FU during TBDMS derivatization. The usefulness of the method was demonstrated by determining plasma concentrations of 5-FU in rats treated intravenously with 5-FU or FdUrd.

EXPERIMENTAL

Apparatus and GC–MS conditions

GC–MS measurements were made using a Shimadzu Auto GC–MS 9020-DF (Shimadzu, Kyoto, Japan) system. A wide-bore open tubular G-250 column (50% phenyl methyl silicone; 20 m × 1.2 mm I.D.; 0.5 μm film thickness; Chemicals Inspection & Testing Institute, Tokyo, Japan) was used for the GC sep-

aration in order to obtain high resolution and sensitivity. The columns were conditioned at 250°C for at least 24 h.

The analytical conditions for the GC–MS method were as follows: the column oven temperature was 180°C, and the injection port and ion source temperatures were 250°C. The flow-rate of the carrier gas, helium, was 30 ml/min. The ionization voltage and acceleration voltage were 20 eV and 3 kV, respectively.

The ions selected were *m/z* 301 (fragment ion peak of TBDMS-5-FU) and *m/z* 317 (fragment ion peak of TBDMS-5-chlorouracil).

Chemicals and reagents

All the chemicals and reagents used were of analytical reagent grade. Ethyl acetate, chloroform and cyclohexane were used without further purification. 5-FU and 5-chlorouracil (5-ClU) were purchased from Sigma (St. Louis, MO, U.S.A.). FdUrd was provided by Wako (Osaka, Japan). MTBSTFA was obtained from Tokyo Kasei (Tokyo, Japan). The stock solutions of 5-FU (100 μg/ml of acetonitrile) and 5-ClU (100 μg/ml of acetonitrile) were stored at 4°C. FdUrd was dissolved in acetonitrile and diluted with the same solvent before use. The 5-FU and FdUrd solutions used for the i.v. dosing of the rats were prepared at a concentration of 15 mg/ml by dissolving each solid in 7% (w/v) $NaHCO_3$ aqueous solution before use.

Sample preparation

Plasma and urine from rats were mainly used to find the optimal analytical conditions of 5-FU. We added 50 ng of 5-ClU (internal standard) and 0.5 ml of a saturated aqueous solution of $(NH_4)_2SO_4$ to 0.5 ml plasma or 0.2 ml urine.

Each sample was extracted by vigorously shaking with 5 ml of ethyl acetate for 10 min. After centrifugation, the organic layer was collected in a glass tube and evaporated to dryness under either a gentle nitrogen or air stream at room temperature. The residue was dissolved in 50 μl of dry acetonitrile. A 50-μl volume of MTBSTFA reagent was added and the mixture was allowed to stand for 30 min at room temperature. After the TBDMS derivatizing reaction had been completed, 100 μl of cyclohexane and 200 μl of 0.05 *M* NaOH were added in this order, and vortex-mixed to extract the derivatives into the organic layer as well as to remove some interfering substances and concomitant FdUrd. After centrifugation, another 5 μl of MTBSTFA reagent were again added to the cyclohexane layer to prevent the decomposition of TBDMS-5-FU to 5-FU. An aliquot (2 μl) was injected into the GC–MS system.

Standard curves

Standard samples were prepared by adding various concentrations of 5-FU [from 1 to 300 ng/ml and 50 ng of the internal standard (5-ClU)] to the untreated plasma or urine samples. The ratio of the GC–MS chromatographic ion peak height corresponding to 5-FU and that corresponding to the internal standard

was plotted against the spiked amount of 5-FU in the sample. The linear regression line was determined by the least-squares method.

Drug administration and sampling in the pharmacokinetic study in rats

The GC–MS method was applied in the pharmacokinetic investigation of 5-FU after single bolus i.v. injection of 5-FU or FdUrd. Sprague–Dawley strain male rats (eight weeks of age, three animals for each group) received the 5-FU and/or FdUrd intravenously at a dose of 15 mg/kg through the tail vein. Food and water were given *ad libitum*. Blood specimens were collected from the inferior *vena cava* into a heparinized syringe at scheduled time periods. Plasma obtained by centrifugation was stored at −20°C until analysed.

Determination of FdUrd

The concentration of FdUrd in plasma was determined by HPLC according to the procedure reported by Lacreta and Williams [28].

RESULTS AND DISCUSSION

Derivatization of 5-FU

In order to determine suitable TBDMS derivatization conditions using the MTBSTFA reagent, 5 μg/ml 5-FU and 5-ClU or 50 μg/ml FdUrd solutions in acetonitrile were added to an equal volume of MTBSTFA reagent. After the mixture had been left to stand at room temperature for varying reaction times or at 70°C for 1 h, the aliquot was injected into the GC–MS system to monitor the ion intensity of TBDMS-derivatized 5-FU and 5ClU. Both 5-FU and 5-ClU were almost completely converted into TBDMS derivatives within 30 min at room temperature. The ion peak intensities that correspond to the derivatives of 5-FU and 5-ClU did not change for the 2 h of the reaction period and after heating at 70°C for 1 h. These TBDMS derivatives of 5-FU and 5-ClU were stable for at least one week when stored in a dark cool (4°C) place.

Under the same reaction conditions used here, FdUrd did not decompose to 5-FU during a 1-h period at room temperature and thus FdUrd did not interfere with the 5-FU analysis by GC–MS. A trace amount (less than 0.3%) of TBDMS-5-FU found at early reaction times (ranging from 5 min to 1 h) was estimated to be contaminated as trace 5-FU was found as an impurity in FdUrd. When the reaction was carried out at room temperature for 2 or 6 h, 0.5% or about 10% of the FdUrd, respectively, was decomposed and derivatized to the TBDMS-5-FU. In the reaction at 70°C for 1 h, 7% of the FdUrd was transformed to TBDMS-5-FU (Fig. 1).

Gas chromatography–mass spectrometry

The EI mass spectra of both di-TBDMS derivatives of 5-FU and 5-ClU are shown in Fig. 2. These spectra were simple and gave intense fragment ion peaks at

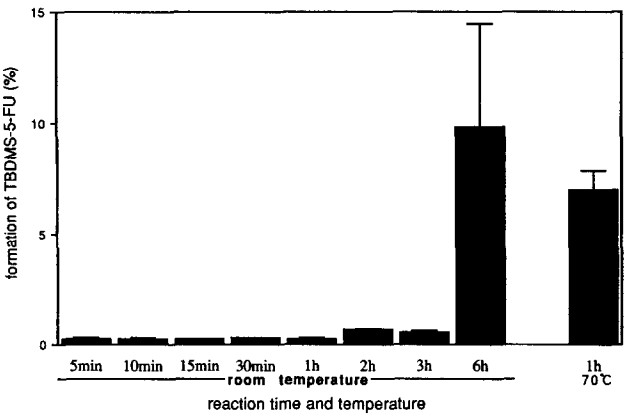

Fig. 1. Decomposition of FdUrd and subsequent formation of TBDMS-5-FU under the TBDMS-derivatizing conditions. (Each column and bar represents the mean ± S.D. of three experiments).

m/z 301 and 317, corresponding to the [M − 57] ions of TBDMS-5-FU and TBDMS-5-ClU, respectively. The intensity due to the base peak of TBDMS-5-FU was about ten times higher than that of TMS-5-FU.

Under the operating conditions, selected-ion monitoring (SIM) chromato-

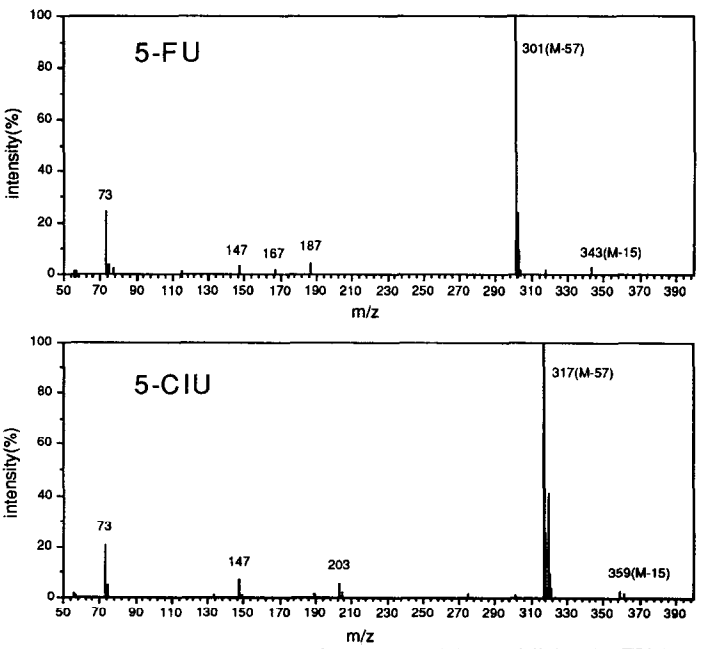

Fig. 2. EI ionization mass spectra of di-*tert.*-butyldimethylsilylated 5-FU (upper) and the internal standard 5-ClU (lower).

grams of standard, control and spiked rat plasma and urine samples are shown in Fig. 3.

The peaks due to the TBDMS-5-FU and TBDMS-5-ClU appeared at 2.7 and 4.0 min, respectively, after injection. The minimum detectable amount was estimated to be approximately 1 pg per injection at a signal-to-noise ratio of 3 for 5-FU, as judged from the peak intensity of the standard. The samples from the control plasma (0.5 ml) or control urine (0.2 ml) of rat showed no responses at the retention times corresponding to TBDMS-5-FU and TBDMS-5-ClU by monitoring the *m/z* 301 and 317.

Furthermore, the control samples from beagle dog, monkey and human plasma showed no interfering peaks due to endogenous substances. The metabolic products of 5-FU such as 5-FUrd, phosphorylated compounds of FUrd or those

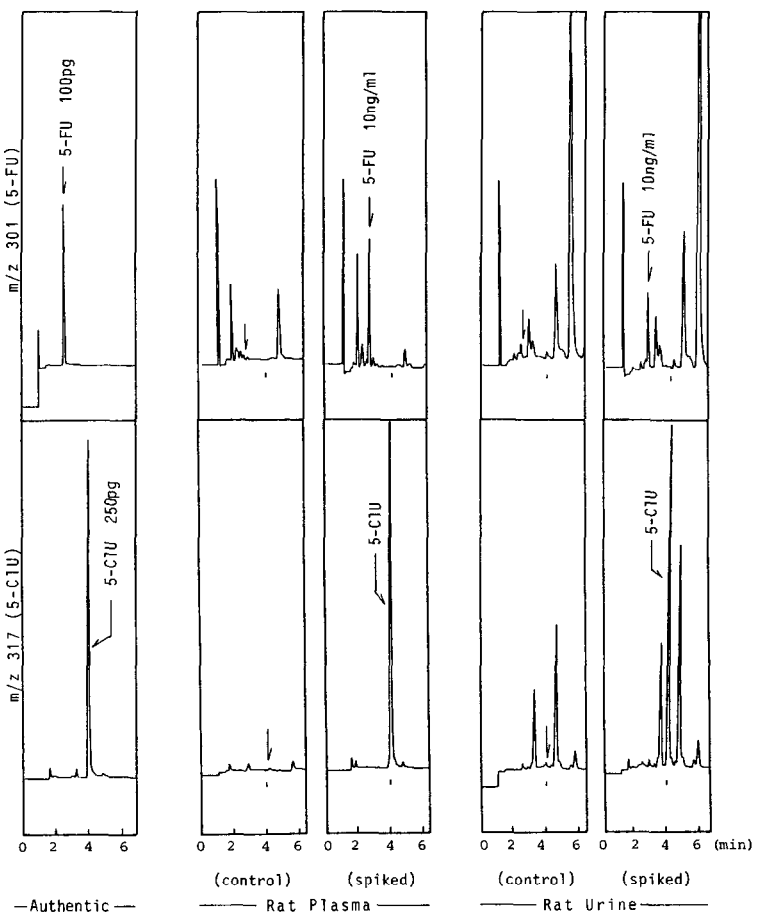

Fig. 3. SIM chromatograms (*m/z* 301 for 5-FU and *m/z* 317 for 5-ClU as the internal standard) of di-*tert.*-butyldimethylsilylated samples.

of 5-fluorodihydrouacil did not interfere with the 5-FU analysis in either plasma or urine under the present assay conditions.

Calibration curve, reproducibility and recovery

To evaluate the linearity and reproducibility of the present method, 5-FU was added in the range of 1–100 ng/ml to 0.5-ml plasma and 3–300 ng/ml to 0.2 ml urine samples, respectively. Reproducibility was determined by carrying out seven determinations at each concentration.

Standard curves were expressed at the peak-height ratio of 5-FU to the internal standard (y) against the concentration of 5-FU (x). The data presented in Table I indicate that the typical standard curves for plasma and urine were found to be linear over the concentration ranges of 0–100 ng/ml in plasma and 0–300 ng/ml in urine. The correlation coefficients of linear regression were 0.9983 and 0.9962 for a total of five concentration points in seven samples of plasma and urine, respectively. The reproducibility (coefficient of variation at each concentration point) was 4–20% for plasma and 7–15% for urine. The limit of detection (defined as twice the minimum standard deviation) was 0.5 ng/ml for plasma and 1.0 ng/ml for urine.

The total analytical recovery was studied at concentrations of 1–100 ng/ml for 5-FU in plasma and 10–100 ng/ml in urine, respectively, and by analysing three samples at each concentration. The peak heights were compared with those obtained for the standard. Over the concentration ranges tested in the analysis, recoveries from plasma were 47–57% and those from urine were about 64%.

Thus, our proposed method enables the determination of 5-FU concentrations in plasma and urine at minimum levels of 0.5 and 1.0 ng/ml, respectively. This

TABLE I

PRECISION OF A GC–MS ASSAY METHOD OF 5-FU IN RAT PLASMA AND URINE

Values represent the mean ± S.D. of seven samples.

Spiked concentration (ng/ml)	Plasma		Urine	
	Peak-height ratio (5-FU/5-ClU)	Percentage of theory[a]	Peak-height ratio (5-FU/5-ClU)	Percentage of theory[a]
1	0.008 ± 0.002	83.2 ± 18.1	–	–
3	0.025 ± 0.005	92.8 ± 19.6	0.017 ± 0.002	113.7 ± 15.7
10	0.104 ± 0.008	114.4 ± 9.2	0.058 ± 0.006	114.0 ± 11.5
30	0.274 ± 0.010	100.1 ± 3.7	0.153 ± 0.010	100.2 ± 6.9
100	0.909 ± 0.039	99.9 ± 4.2	0.505 ± 0.026	99.4 ± 5.2
300	–	–	1.526 ± 0.107	100.1 ± 7.0

[a] Calculated from the slope of the respective calibration plots based on the equation $y = 0.00911x$ ($r = 0.9983$) in plasma and $y = 0.00508x$ ($r = 0.9962$) in urine, where x is the spiked concentration of 5-FU (ng/ml) and y is the peak-height ratio and is expressed as (found concentration/spiked concentration) × 100.

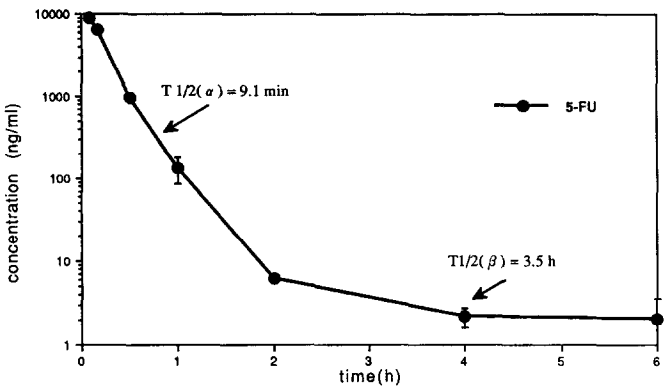

Fig. 4. Plasma profile of 5-FU after an intravenous bolus injection of 5-FU at 15 mg/kg to rats. (Each point is expressed as the mean ± S.D. of three animals.)

method may be useful to quantitate 5-FU in samples from patients treated with FdUrd, 5-FU and/or prodrugs of 5-FU. As an application, we performed a pharmacokinetic study, in which plasma concentrations of 5-FU in rats treated with 5-FU or FdUrd were determined by using the proposed GC–MS method.

Pharmacokinetic study

The plasma concentration–time profiles of 5-FU and FdUrd were studied in male Sprague–Dawley strain rats after i.v. bolus doses of 15 mg/kg 5-FU or 15 mg/kg FdUrd, respectively. Plasma levels of 5-FU (after i.v. injection of 5-FU) decreased biphasic exponentially with the first elimination half-life of about 9 min and with the second elimination half-life of about 3.5 h (Fig. 4).

This result on first elimination half-life is similar to those reported by Au *et al.* [25]. After an i.v. administration of FdUrd, plasma levels of FdUrd rapidly declined with a half-life of about 4 min, and the levels of 5-FU as a metabolite of FdUrd decreased with a half-life of about 11 min (Fig. 5).

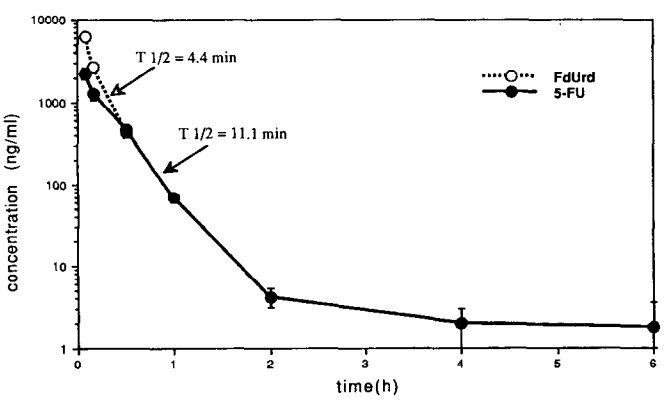

Fig. 5. Plasma profiles of FdUrd and 5-FU after an intravenous bolus injection of FdUrd at 15 mg/kg to rats. (Each point is expressed as the mean ± S.D. of three animals.)

CONCLUSION

A higly sensitive, simple and specific GC–MS assay method has been developed for the determination of 5-FU in plasma and urine in the presence or absence of FdUrd. It can be routinely used for monitoring sub ng/ml amounts of 5-FU in plasma and urine samples from patients treated with FdUrd, 5-FU and/or prodrugs of 5-FU.

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