Determination of 5-Fluorouracil in Plasma and Liver after Oral Administration of 5'-Deoxy-5-Fluorouridine Using Gas Chromatography-Mass Spectrometry

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A method for determination of 5-fluorouracil (5-FU) has been established for pharmacokinetic study of 5'-deoxy-5-fluorouridine (5'-DFUR), a newly developed prodrug of 5-FU. 5-FU was extracted with diethyl-ether containing 1-propanol and methylated with CH₃I in the presence of (CH₃)₄NOH. The methylated 5-FU was analyzed by gas chromatography-mass spectrometry using ¹⁵N₂-5-FU as an internal standard.

Quantitation was carried out by selected-ion monitoring of molecular ions of N,N-dimethyl-5-FU (m/z 158) and the internal standard (m/z 160). The sensitivity (>2 ng/g sample), specificity and precision of the method were satisfactory for application to clinical studies of this drug. After an oral administration of 5'-DFUR (800 mg/body) to patients with cancer, 5-FU in plasma peaked within 1 h and eliminated with an apparent half life of about 1 h.

Keywords — 5-fluorouracil; 5'-deoxy-5-fluorouridine; GC-MS; selected ion monitoring; pharmacokinetic study

Introduction

5'-Deoxy-5-fluorouridine (5'-DFUR), a pyrimidine nucleoside analog, is a potent anti-cancer agent. A part of its activity is attributed to 5-fluorouracil (5-FU), which is derived from 5'-DFUR in tumour tissues by the action of uridine and thymidine phosphorylases.

Because of this anti-cancer activity of 5'-DFUR, the establishment of a specific assay method for 5-FU is of importance. Several procedures have been reported for the quantitative analysis of 5-FU in biological samples and have been widely utilized clinically. These procedures were, however, found not to be applicable to samples containing both 5-FU and 5'-DFUR concomitantly, because 5'-DFUR interfered with the determination of 5-FU. In the present study we utilized a specific and sensitive analytical method by using a gas chromatographic-mass spectrometric-selected-ion monitoring (GC-MS-SIM) for 5-FU in biological samples.

Materials and Methods

Chemicals and Reagents — 5-FU, ¹⁵N₂-5-FU, ⁵⁻DFUR, ¹⁴C-5-FU and ⁵,⁶-di-hydro-5-FU were supplied by F. Hoffmann-La Roche (Basle, Switzerland). Other reagents used in the present study were commercially obtained and of analytical grade. Stock solutions, 10000 and 100 ng/ml of 5-FU and ¹⁵N₂-5-FU, respectively, were prepared in distilled water.

Equipment — GC-MS-SIM measurements were carried out with a JEOL DX-300 high resolution mass spectrometer (JEOL, Tokyo, Japan) equipped with JMA-2000H mass data analysis system (JEOL).

A glass column, 2 m × 2 mm i.d., was packed with 10% GE SF-96 on 80-100 mesh Gas Chrom Q (Shimadzu, Kyoto, Japan) and conditioned overnight at 240 °C with a 30 ml/min helium gas flow prior to use. The operating temperature for the column was 210 °C, and for the injection port 230 °C. Helium gas flow rate was 40 ml/min. Under these conditions, the retention time of 5-FU was 90 ± 10 s. Ionization voltage and current were 70 eV and 300 μA, respectively. The ions monitored were m/z 158 (molecular ion peak of dimethyl-5-FU) and m/z 160 (molecular ion peak of N,N-dimethyl ¹⁵N₂-5-FU).

Determination of 5-FU — To samples of
plasma (0.2−0.5 ml) or tissues (0.2−1.0 g) was added 500 ng of $^{15}\text{N}_2$-5-FU (an internal standard) dissolved in 5 ml of 0.2 N perchloric acid (PCA). The mixture was homogenized with a glass homogenizer at 0−4 °C, followed by centrifugation at 9000 × g for 10 min. The supernatant fluid was transferred into a 10-ml conical centrifuge tube. The precipitates were washed with 5 ml of 0.2 N PCA and centrifuged as described above. These supernatant fluids were combined and adjusted with 6 N KOH to pH 11 in an ice bath. The precipitates of potassium perchlorate were removed by centrifugation (500 × g for 10 min) and the supernatant fluid was applied to a Dowex 1 × 8 column (formate form, 200−400 mesh, 5 cm × 9 mm i.d.). After washing the column with 30 ml of distilled water, both 5-FU and $^{15}\text{N}_2$-5-FU were eluted with 35 ml of formic acid (100 mM). The eluate was lyophilized and the residue treated three times with 1.0 ml of MeOH. The MeOH solution was centrifuged at 500 × g for 10 min and the supernatant fluid evaporated under a stream of N₂ gas. To the residue were added 0.5 ml of distilled water, 0.5 ml of saturated aqueous solution of (NH₄)₂SO₄, and 9.0 ml of benzene. The mixture was vigorously shaken for 15 min followed by centrifugation at 500 × g for 10 min. The benzene layer was discarded and both 5-FU and $^{15}\text{N}_2$-5-FU were extracted from the aqueous layer with 10 ml of ether containing 20% n-propanol. After separation of the aqueous and organic layers by centrifugation, the organic layer was transferred into a conical centrifuge tube and evaporated under a stream of N₂ gas. The residue was stored overnight in a vacuum desiccator and subsequently dissolved in 100 μl of Me₂NCOCH₃−MeOH (1:3, v/v). An excess amount of CH₃I (100 μl) and 20 μl of 25% (CH₃)₄NOH in MeOH (Eastman Kodak, Rochester, N.Y., U.S.A.) were added to the solution. After 10 min, additional 20 μl of the (CH₃)₄NOH was added to the solution and the mixture was allowed to stand at room temperature for 20 min followed by heating at 60 °C for 2 min. The methylated products in the solution were extracted with 10 ml of cyclohexane−CH₂Cl₂ (17:3, v/v). After evaporation the residue was dissolved in 50−100 μl of EtOAc. An aliquot (2 μl) of this solution was injected into the gas chromatograph−mass spectrometer.

The peak areas of $m/z$ 158 to $m/z$ 160 were measured and the ratio of $m/z$ 158 to $m/z$ 160 was determined with the JMA-2000H Mass Data Analysis System. The ion ratio was converted to concentration by using standard curves.

**Determination of 5'-DFUR** — The concentration of 5'-DFUR in plasma was determined by high performance liquid chromatography (HPLC) according to the procedure reported previously.¹⁰

**Clinical Study** — Cancer patients ($n = 5$; age, 40.8 ± 8.5 years old; body weight, 49.8 ± 2.7 kg) received an oral dose of 800 mg/body of 5'-DFUR in the form of 200 mg capsules 30 min after a meal.

Two ml of blood specimens were collected from the cubital vein with a heparinized syringe at scheduled time periods and the plasma obtained by centrifugation was stocked at −20 °C until analysis.

**Results and Discussion**

**Derivatization of 5-FU**

Several analytical procedures have been used for the determination of tissue levels of 5-FU by a GC-MS method.²⁻⁹ In these procedures, 5-FU is converted to volatile derivatives such as ditrimethyl silyl-5-FU and dimethyl-5-FU prior to GC-MS-SIM analysis.

In the method of trimethylsilylation,⁸ however, unexpectedly high values of the 5-FU concentration were obtained, especially for samples which contained low amounts of 5-FU (5−20 ng/tube) when the samples were analyzed after analysis of a sample with higher amounts of 5-FU. The most likely explanation for this finding is incomplete silylation of 5-FU in the first sample; a low but definite amount of non-derivatized 5-FU was retained in the column or injection port or both and subjected to an on-column silylation with the derivatization reagent present in the second sample. Hence, this procedure was not suitable for determination of 5-FU.

Although dimethylation of 5-FU with diazomethane was successfully used for an assay of
plasma 5-FU concentration by Min and Garland,\textsuperscript{9)} this procedure was also not applicable for determination of 5-FU in the presence of 5'-DFUR because the latter, in tissue samples, was easily converted to dimethyl 5-FU with diazomethane (data not shown). In addition, we found non-selective methylation of 5-FU with this procedure resulting in two major and three minor dimethylated derivatives (I and III) and (II, IV, and V) of 5-FU, respectively (Table I).

Based on these findings, we examined mild methylation of 5-FU with CH\textsubscript{3}I in the presence

<table>
<thead>
<tr>
<th>Derivative</th>
<th>Rf\textsuperscript{a)} value</th>
<th>Rt times (min)</th>
<th>Main fragment in mass spectrum</th>
<th>\textsuperscript{13}C and \textsuperscript{1}H-NMR \textsuperscript{b)} (ppm)</th>
<th>Chemical structure</th>
</tr>
</thead>
</table>
| I | 0.90 | 0.7 | M* 158 (100%)  
\textit{m/z} 157 (70%), 143 (20%), 128 (88%), 113 (34%), 100 (16%), 56 (25%) | \textsuperscript{13}C  
182.6 (C-4), 161.5 (C-2), 144.3 (C-6), 144.2 (C-5), 55.8 (2-OCH\textsubscript{3}), 55.1 (4-OCH\textsubscript{3}) |  
\n \nCH\textsubscript{3}O  
(Fig. 2) |
| II | 0.77 | 1.1 | M* 158 (100%)  
\textit{m/z} 143 (20%), 129 (24%), 128 (22%), 114 (30%), 100 (17%), 73 (32%), 56 (28%) | \textsuperscript{13}C  
158.3 (C-4), 154.7 (C-2), 147.3 (C-5), 135.7 (C-6), 56.8 (2-OCH\textsubscript{3}), 29.1 (1-NCH\textsubscript{3}) |  
\n \nCH\textsubscript{3}O  
(Fig. 2) |
| III | 0.60 | 1.5 | M* 158 (100%)  
\textit{m/z} 129 (4%), 101 (40%), 73 (37%) | \textsuperscript{13}C  
159.5 (C-4), 152.0 (C-2), 141.0 (C-5), 130.3 (C-6), 37.3 (3-NCH\textsubscript{3}), 28.7 (1-NCH\textsubscript{3}) |  
\n \nCH\textsubscript{3}O  
(Fig. 2) |
| IV | 0.19 | 1.7 | M* 158 (100%)  
\textit{m/z} 143 (20%), 129 (18%), 100 (40%), 70 (15%) | \textsuperscript{13}C  
163.9 (C-4), 157.1 (C-2), 137.9 (C-5), 135.3 (C-6), 55.4 (4-OCH\textsubscript{3}), 38.2 (3-NCH\textsubscript{3}) |  
\n \nCH\textsubscript{3}O  
(Fig. 2) |
| V | 0.05 | 4.0 | M* 158 (100%)  
\textit{m/z} 143 (22%), 129 (20%), 101 (38%), 73 (42%) | \textsuperscript{13}C  
167.0 (C-4), 156.5 (C-2), 146.0 (C-5), 131.1 (C-6), 57.2 (2-OCH\textsubscript{3}), 38.2 (3-NCH\textsubscript{3}) |  
\n \nCH\textsubscript{3}O  
(Fig. 2) |

\textsuperscript{a)} Silica thin-layer chromatography, solvent: EtOAc. \textsuperscript{b)} \textsuperscript{13}C and \textsuperscript{1}H-NMR: carbon-13 and proton nuclear magnetic resonance.
of (CH₃)₄NOH. By this method the two nitrogen atoms at positions 1 and 3 were selectively methylated to give N,N-dimethyl-5-FU (III).

In a preliminary study using ¹⁴C-5-FU, 98% of the labeled compound was found to be methylated to N,N-dimethyl-5-FU according to the present procedure. Under these reaction conditions dimethyl 5-FU was not derived from 5'-DFUR to any extent.

GC-MS-SIM

The electron impact mass spectra of both N,N-dimethylated 5-FU and ¹⁵N₂-5-FU are simple (Fig. 1); the base peaks corresponded to respective molecular ions, m/z 158 and 160.

Control samples (1.0 ml of human plasma or 1.0 g of liver) subjected to the assay showed no response in the m/z 158 and m/z 160 ion chromatograms at the retention time corresponding to N,N-dimethyl-5-FU. Analyses of the control samples spiked with 500 ng of ¹⁵N₂-5-FU showed a response at m/z 158 which was 0.2% or less of the value at m/z 160. Taking a value of twice the response of the blank as sensitivity

Fig. 1. Electron-Impact Mass Spectra of N,N-Dimethyl ¹⁵N₂-5-FU (A) and N,N-Dimethyl-5-FU (B)

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**Fig. 2. Metabolic Pathways of 5'-DFUR**
criteria, the assay has a sensitivity of 2 ng/g (or ml).

As described above, interference due to endogenous compounds was not observed in the assay of 5-FU by the present GC-MS-SIM method. However, there is a possibility that a metabolite(s) of 5′-DFUR (Fig. 2) interferes with the assay of 5-FU in the present procedure. 5′-DFUR has been reported to be converted to 5-FU and 5-deoxyribose-1-phosphate (DRP)\textsuperscript{11} which is further metabolized to give deoxy-D-ribitol\textsuperscript{12} (pathway a, Fig. 2). 5-FU was converted to 5-fluorodihydouracil (FUH\textsubscript{2}), 2-fluorouracil (FUPA), 2-fluoro-β-alanine (FBAL), N-carboxy-α-fluoro-β-alanine (CFBAL), and fluoride anion (pathway b).\textsuperscript{13} Anabolic conversion of 5-FU would yield 5-fluorouridine (FUrD), 5-fluorodeoxyuridine (FdUrd) and its phosphorylated derivative (pathway c). Another possible metabolic pathway (pathway e), which has not yet been confirmed, would be that the pyrimidine moiety of 5′-DFUR is metabolized in the same manner as 5-FU.

Among these metabolites, a metabolite having all of the following chemical and physical properties would interfere with the assay of 5-FU: 1. a metabolite that can be eluted with 0.1 M formic acid from the Dowex 1 × 8 ion-exchange column; 2. a metabolite extractable with ether containing 20% PrOH from the aqueous solution, and 3. a metabolite or the methyl derivative that gives peaks at m/z 158 or m/z 160 at a retention time similar to that of the dimethylated 5-FU.

Because 5′-DFUR was not converted to 5-FU under the assay conditions to any extent (see Experimental), metabolites having ribosylated 5-FU structure like those formed via pathway c are unlikely to give interference peaks at m/z 158 and m/z 160.

On the other hand, one of the main catabolic metabolites, FUH\textsubscript{2}, could theoretically interfere with the 5-FU analysis because both FUH\textsubscript{2} and 15\textsuperscript{N}\textsubscript{2}-5-FU (the internal standard) have the same molecular weight. It was confirmed in our preliminary experiment that FUH\textsubscript{2} did not interfere with the 5-FU assay, probably because the metabolite could not undergo the methylation reaction utilized in the present method.

Regarding the catabolic metabolites of 5-FU (pathway b), the four metabolites (i.e., FUPA, CFBAL, FBAL, and fluorine ion) are not efficiently extracted with 20% PrOH in ether due to their high polarity. Even if these metabolites could be extracted by the solvent, the methyl derivatives would not theoretically give any fragment peaks at m/z 158 and m/z 160. The struc-

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure3.png}
\caption{Calibration Curve for 5-FU (Mean ± S. E., n = 3; r = 0.9968)}
\end{figure}

Inset figure shows calibration curve for 5-FU over a low concentration range (mean ± S.E., n = 3; r = 0.9992).
tures of the metabolites formed via a pathway are constituted from 5′-deoxyribose and one of the anabolic metabolites (FUH₂, FUPA, and FBAL). Since the anabolic metabolites and 5-deoxyribose would not give any interferences as discussed above, it is most unlikely that any of these metabolites gives a peak at m/z 158 or m/z 160 at the same retention time as 5-FU.

**Calibration Curve**

A typical standard curve using 500 ng of ¹⁵N₂-5-FU is shown in Fig. 3. Because the labeled compound is only two daltons greater than the compound being determined, the curve slightly deviated from a straight line. However, for the range of the ratios of 5-FU to ¹⁵N₂-5-FU used in the present assay, the deviation was slight with linearity between 5 ng and 1 µg (per tube). This range covered most of the concentrations found in tissue or plasma samples obtained in the clinical field.

**Recovery and Reproducibility**

To evaluate the sample recovery three 1 g human liver samples were spiked with 500 ng of ¹⁴C-5-FU (specific activity, 9.0 µCi/mg) and analyzed. Assay recovery in these samples was 80.8 ± 1.6%. Assay precision was determined for the sample (n = 6) spiked with either 100 or 500 ng of 5-FU. The mean concentrations (± S.E.) were found to be 100.2 ± 8.1 and 475.4 ± 5.7 ng/g liver, respectively.

**Pharmacokinetic Study**

After an oral administration of 5′-DFUR to patients (at a dose of 800 mg/body, n = 5) with cancer, 5-FU in plasma or cancerous tissues (i.e., stomach, colon, breast etc.) was successfully determined by this method without any interference in the chromatogram.

Plasma concentrations of 5′-DFUR were 1.72 ± 0.76, 0.74 ± 0.21, 0.30 ± 0.06, and 0.13 ± 0.03 µg/ml at 1, 2, 3, and 4 h, respectively, while those of 5-FU were 58 ± 19 ng/ml at 1 h, 33 ± 10 ng/ml at 2 h and 14 ± 7 ng/ml at 3 h (Fig. 4). Apparent half-life of 5-FU in the plasma (from 1 to 3 h) was approximately 1 h (Fig. 4).

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**References**

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