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Evidence for Conversion of 5-Fluorocytosine to 5-Fluorouracil in Humans: Possible Factor in 5-Fluorocytosine Clinical Toxicity

ROBERT B. DIASIO,[1]* DUANE E. LAKINGS,[2] AND JOHN E. BENNETT[3]

Department of Medicine, Medical College of Virginia-Virginia Commonwealth University, Richmond, Virginia 23298;[1] Laboratory of Chemical Pharmacology, National Cancer Institute;[2] and Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20014[3]

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A gas chromatographic-mass spectrometric method for detecting 5-fluorouracil (5-FU) in serum at concentrations as low as 10 ng/ml was used to determine to what extent 5-FU was present in the serum of patients taking oral 5-fluorocytosine (5-FC). Preliminary studies in two patients and two healthy volunteers given an initial 2-g oral dose of 5-FC demonstrated sustained serum 5-FU levels (>100 ng/ml) during the 5 h after ingestion of drug. Pharmaceutical preparations of 5-FC used in these studies were shown to be insignificantly contaminated with 5-FU (<0.03%), suggesting in vivo conversion of 5-FC to 5-FU had occurred. Serum samples from seven patients with cryptococcal meningitis treated with amphotericin B and 5-FC were examined for 5-FU. Five of these patients had experienced hematological or other toxicity attributed to 5-FC at some time during the course of therapy. Of 41 serum samples, 20 were observed to have 5-FU levels greater than 1,000 ng/ml in the range observed with cancer chemotherapeutic doses of 5-FU known to be associated with hematological toxicity. It is concluded that conversion of 5-FC to 5-FU occurs in humans and furthermore that 5-FU may account for some of the toxicity observed with 5-FC.

5-Fluorocytosine ([5-FC] Flucytosine, Ancobon) is a relatively well-tolerated antifungal agent used in the treatment of systemic candidiasis, cryptococcal meningitis, chromomycosis, and torulopsosis (1). Earlier studies suggested that 5-FC was an ideal drug because susceptible fungi could convert 5-FC to cytotoxic 5-fluorouracil (5-FU) resulting in a disturbance of fungal nucleic acid function, whereas human cells could not convert 5-FC to 5-FU, thus sparing these cells from toxicity (5, 8).

Despite the generally observed absence of clinical toxicity with 5-FC, toxic manifestations, including leukopenia and thrombocytopenia, have been described, particularly when the serum 5-FC level rises above 125 μg/ml, as often occurs with diminished renal function (1, 7). Although such toxicity is known to occur with 5-FU (5), no evidence exists that 5-FU is responsible for these findings in 5-FC-treated patients.

The present study utilized a sensitive gas chromatographic-mass spectrometric (GC-MS) method to determine serum 5-FU levels in patients taking oral 5-FC.

MATERIALS AND METHODS

Chemicals. 5-FU and 5-FC were kindly provided by Edward Miller, Hoffmann-La Roche, Inc., Nutley, N.J. Phenanthrene, acetylnitrile, and methylene chloride were purchased (Fisher Chemical, Fair Lawn, N.J.), as was the silylating reagent, *N,O*-[(bis)-trimethylsilyl]-trifluoroacetamide (Regis Chemical Co., Chicago, Ill.).

Sample preparation. A 1.0-ml portion of serum was first ultrafiltrated through a 25,000-molecular-weight exclusion filter cone (CF 25; Amicon, Boston, Mass.). The ultrafiltrate was alkalinized with 1.0 N NaOH and was then quantitatively transferred to a column (50 by 9 mm) containing AG 1 × 2 anion-exchange resin in the acetate form (BioRad, Richmond, Calif.). The column was washed with 50 ml of deionized water, after which 5-FU was eluted with 25 ml of 0.1 N acetic acid. The eluate was concentrated to about 2.0 ml on a hot plate (60°C) under a stream of nitrogen, and then quantitatively transferred to a Teflon-lined screw-capped silylation vial, after which the contents were evaporated to dryness. Methylene chloride was added to the sample and evaporated to azeotropically remove any remaining trace of water. A 200-μl portion of a 1:1 (vol/vol) *N,O*-[(bis)-trimethylsilyl]-trifluoroacetamide-CH_3CN mixture was added, and the vial was tightly sealed and heated at 150°C for 3 min. The internal standard, phenanthrene, was dissolved in the CH_3CN and was added with the silylating reagent.

GC-MS conditions for 5-FU assay. A Hewlett-Packard 5981A GC-MS with a 5933A data system (Hewlett-Packard Co., Palo Alto, Calif.) was used. The chromatographic column was a glass column (1.0 m by

2.0-mm ID) packed with 3% Dexsil 300 on 100/120 mesh Supelcoport (Supleco, Bellefonte, Pa.). The GC conditions consisted of an initial temperature of 100°C and a temperature program to 250°C at 16°C/min. The helium carrier-gas flow rate was maintained at 22 ml/min, with the injection port temperature at 250°C. The eluent from the chromatographic column was passed through an all-glass jet separator at 300°C and into the ion source. The MS was operated at 70 eV with a source temperature of 110°C and the dodecapol at 240°C.

Under these conditions, the mass spectra of the trimethylsilyl derivative of 5-FU had a molecular ion at electron mass (*m/e*) 274.1, with a base peak at 259.1, with an additional peak at 273.1. The GC retention time of 5-FU was 2.0 min. 5-FC also had a molecular ion at *m/e* 273.1 but was detected at a retention time of 2.8 min. The internal standard, phenanthrene, had a strong molecular ion at *m/e* of 178.1 and was detected at a retention time of 6.0 min (9).

The other major and minor nucleic acid bases which could be present in the serum samples do not have these ions in their mass spectra (D.B. Lakings, unpublished data) and therefore could not interfere with the detection and quantitation of 5-FU.

The level of 5-FU in each serum sample was determined by monitoring *m/e* 259.1. The ratios of *m/e* 274.1/259.1 and 273.1/259.1 were used to locate 5-FU on the chromatogram. Calculations were made from the relative weight response of standard 5-FU solutions which were run with the serum samples. Recovery of 5-FU from serum with this technique was greater than 90% for concentrations from 100 to 500 ng/ml, with sensitivity extending to 10 ng/ml (9).

Pharmaceutical preparations of 5-FC. 5-FC capsules (Ancobon; Hoffmann-La Roche) were sampled for 5-FU analysis from six different pharmacy lots from the hospital pharmacies of the Medical College of Virginia Hospitals and the National Institutes of Health Clinical Center. The age of these preparations varied from 12 to 38 months. Capsules from these pharmacy lots were utilized in the initial pharmacokinetic studies in the two patients and two volunteers given 5-FC.

Clinical studies. Two adult male patients with cryptococcal meningitis having normal renal function and no underlying disease and two healthy adult male volunteers were studied with their initial dose of 5-FC. Each was fasted for at least 1 h before being given a 2-g oral dose of 5-FC. Serum samples were collected before drug administration and at various times over 6 h.

The samples from the two volunteers were analyzed immediately after collection. The samples from the two patients were collected under sterile conditions and stored at −70°C before analysis for 5-FU. Preliminary studies measured 5-FU levels in stock solutions of 5-FC (in water and in pooled human serum) initially and at various intervals over 6 months, demonstrating no evidence for conversion of 5-FC to 5-FU when these solutions were stored at −70°C.

Forty-one serum samples collected during the previous 24 months from seven patients with cryptococcal meningitis were available for analysis. The seven patients had been initially treated with amphotericin B (intravenous, 0.3 mg/kg per day) and 5-FC (oral, 150 mg/kg per day) with modification of the inital dose in the presence of renal dysfunction. At least once weekly, laboratory studies were performed to monitor hematological toxicity, renal dysfunction, and hepatic dysfunction, with further adjustment of drug dosages. Serum samples from both peak and valley time points were collected approximately every 2 weeks and analyzed for 5-FC by bioassay (2), after which the sample was stored at −70°C for later 5-FU analysis.

RESULTS

5-FU in pharmaceutical preparations of 5-FC. The contents of a 500-mg capsule of 5-FC from each of the six pharmacy lots were dissolved in deionized water (500 ml) and analyzed for 5-FU by the GC-MS method. Table 1 lists the concentration of 5-FU (in nanograms per milliliter) for each of these 1.0-mg/ml 5-FC solutions. The present contamination of 5-FC by 5-FU is shown to be insignificant, ranging from 0.0010 to 0.0274%.

Serum 5-FU level after initial dose of 5-FC. Figure 1 shows the serum concentration-time curves for serum 5-FU after oral administration of 2 g of 5-FC with two volunteers and two patients. Peak serum levels of 5-FU were noted at the 2 h time point corresponding to the peak serum level of 5-FC (5-FC data not shown). The serum 5-FU levels were then observed to

TABLE 1. *Extent of 5-FU contamination of 5-FC pharmacy preparation*

Lot no.	5-FC Concn (mg/ml)	5-FU Concn (ng/ml)	5-FU%[a]
1	1.0	83	0.0083
2	1.0	22	0.0022
3	1.0	10	0.0010
4	1.0	26	0.0026
5	1.0	169	0.0169
6	1.0	274	0.0274

[a] 5-FU% = (5-FU/5-FC) × 100.

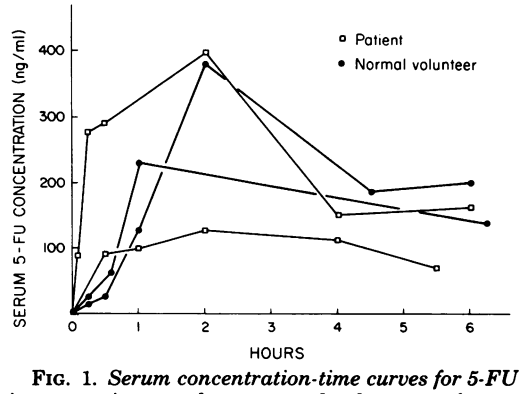

FIG. 1. *Serum concentration-time curves for 5-FU in two patients and two normal volunteers after an initial oral dose of 2 g of 5-FC.*

decrease, remaining in the range of 80 to 200 ng/ml between 4 and 6 h.

Serum 5-FU level in patients during 5-FC therapy. Table 2 summarizes the clinical characteristics of the seven patients with cryptococcal meningitis treated with combined amphotericin B and 5-FC. Five of the seven patients had toxic manifestations, either gastrointestinal or hematological, attributed to 5-FC at some time during the treatment period. The serum 5-FU level obtained by GC-MS and the serum 5-FC level previously obtained by bioassay are shown

TABLE 2. *Clinical findings in cryptococcal meningitis patients on combined amphotericin B and 5-FC*

Patient no.	Age (sex)	Underlying disease	Renal insufficiency[a]	5-FC serum level (μg/ml)	5-FU serum level (ng/ml)	5-FU%[b]	Leukocyte count[c]	Other toxicity
1	40 (M)	None	Yes	29	73	0.25	7,900	Diarrhea
				33	36	0.11		
				50	1,590	3.18		
				80	2,050	2.56		
				155	1,490	0.96		
				80	620	0.78		
				65	687	1.06		
2	40 (F)	Systemic lupus erythematosus	No	128	1,930	1.51	2,000	No
				145	2,080	1.43		
				125	1,430	1.14		
3	65 (M)	Chronic glomerulonephritis	Yes	60	724	1,21	3,700	No
				53	1,245	2.35		
				150	1,050	0.70		
				200	3,060	1.53		
				25	2	0.01		
				29	121	0.42		
4	26 (M)	None	Yes	70	689	0.98	2,400	No
				120	1,350	0.13		
				34	575	1.69		
				53	247	0.47		
				58	620	1.07		
				44	660	1.50		
5	56 (M)	Cirrhosis	Yes	115	1,320	1.15	5,900	No
				113	1,040	0.92		
				113	1,360	1.20		
				110	995	0.90		
				200	1,160	0.58		
6	47 (M)	Lymphoma (non-Hodgkin's)	No	54	216	0.40	7,300	No
				67	142	0.21		
				90	619	0.69		
				38	193	0.51		
				75	986	1.31		
				54	354	0.66		
				54	1,060	1.96		
				47	315	0.67		
7	70 (M)	Chronic lymphocytic leukemia	Yes	159	2,080	1.31	8,200[d]	Platelet[e]
				120	1,710	1.43		
				106	1,880	1.77		
				2	69	3.45		
				68	1,070	1.57		
				100	1,210	1.21		

[a] Blood urea nitrogen > 20 mg/100 ml or creatinine > 2.0 mg/100 ml.
[b] 5-FU% = (5-FU/5-FC) × 100.
[c] Lowest leukocyte count recorded.
[d] Decrease from 47,000 to 8,200.
[e] Decrease from 248,000 to 71,000.

for each of the 41 serum samples examined. The ratio of 5-FU concentration to 5-FC concentration is shown as 5-FU percent. Figure 2 depicts the treatment course of patient 6, a 47-year-old male with a non-Hodgkin's lymphoma (previously treated with chemotherapy) who had a relatively benign course with normal renal function, no clinical manifestations of 5-FC toxicity, and serum 5-FC levels that had been consistently within the therapeutic range. The patient received a full course of combined amphotericin B and 5-FC, requiring no adjustment in the daily dose of either drug. The serum 5-FU levels were observed to be approximately 1,000 ng/ml or lower throughout this period. Figure 3 illustrates the treatment course of patient 7, a 70-year-old male with chronic lymphocytic leukemia (not on concomitant antileukemic chemotherapy) whose course was complicated by renal dysfunction, leukopenia (relative) and thrombocytopenia, and serum 5-FC levels in the toxic range, with four of six serums having 5-FC levels above 100 μg/ml. Because of these toxic manifestations, the daily dose of 5-FC was reduced four times during the course of treatment. Serum 5-FU levels were greater than 1,000 ng/ml on three separate occasions during the 28 days of treatment, with 5-FU detected in the serum even 5 days after discontinuation of 5-FC. Figure 4 illustrates that a somewhat linear relationship exists between the serum 5-FC level and the serum 5-FU level in the 41 serum samples, with a Spearman rank correlation coefficient of 0.789 ($p < 0.001$).

DISCUSSION

Earlier metabolic studies in humans used radiolabeled 5-FC as a tracer, with separation and quantitation of drug and suspected metabolites by thin-layer chromatography (8, 12, 13). These studies demonstrated that 90% or more of the administered 5-FC (oral or parenteral) was excreted into the urine unchanged, with no 5-FU being detected in the patients studied. It was concluded that 5-FC was not metabolized in humans, providing an explanation for the generally observed minimal clinical toxicity of this fluoropyrimidine drug (5). Recent reviews of the clinical pharmacology of 5-FC (1), however, have noted that toxicity does indeed occur with 5-FC with features similar to those described with 5-FU (5, 10), raising the question again as to whether 5-FU could be formed from 5-FC. Regarding this question, it should be noted that in

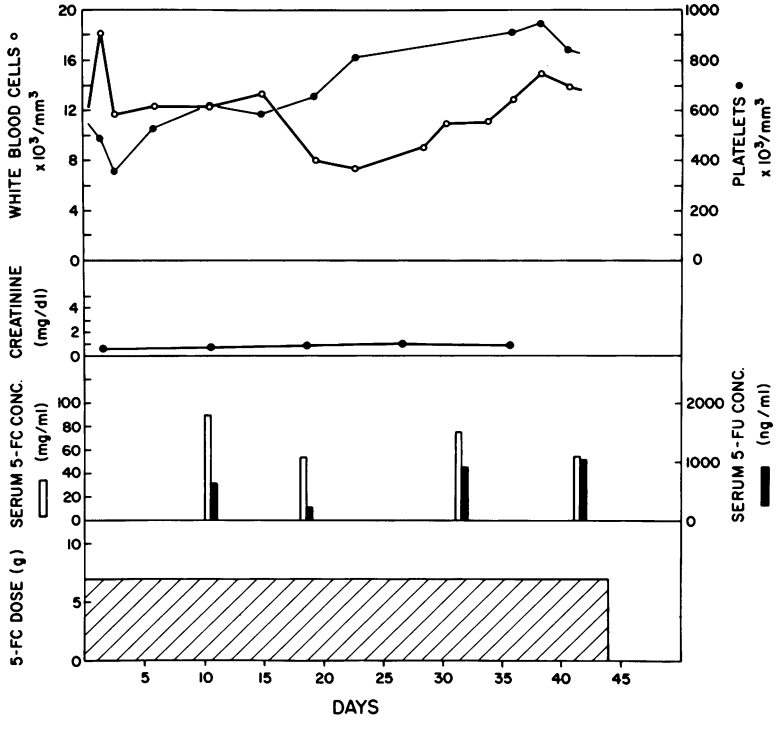

FIG. 2. *Graphic representation of clinical course of patient 6, who tolerated amphotericin B and 5-FC well, with no evidence of renal dysfunction and no hematological toxicity. Serum levels of 5-FU and 5-FC are shown. (Note: where more than one blood sample was drawn on a particular date, only the highest 5-FU level is shown with the corresponding 5-FC level from the same sample.)*

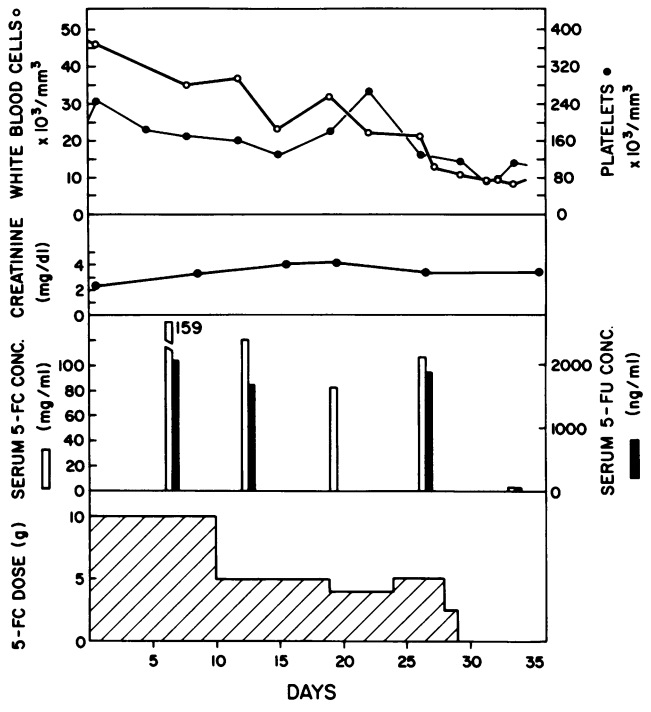

FIG. 3. *Graphic representation of clinical course of patient 7, whose course with amphotericin B and 5-FC was complicated by renal dysfunction with evidence of hematological toxicity, and very elevated serum 5-FC levels, necessitating adjustment of the daily 5-FC dose on five occasions (see note for Fig. 2).*

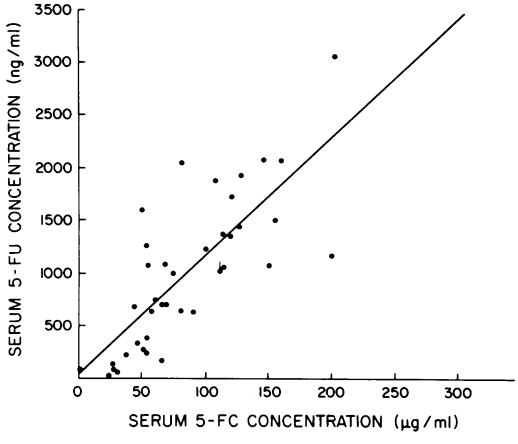

FIG. 4. *Relation of serum 5-FC level and serum 5-FU level for each 41 serum samples. The line is generated by least-squares regression of the 41 points—Spearman rank correlation coefficient of 0.789 ($p < 0.001$).*

the earlier metabolic studies: (i) α-fluoro-β-ureido-propionic acid, a known metabolite of 5-FU, was detected in the urine of several 5-FC-treated patients (8, 12) and (ii) the method used to detect 5-FU was limited to a sensitivity threshold of 1 μg/ml or greater, a rather insensitive method for studying 5-FU, even in patients being given 5-FU (3, 10). It was with this background that we chose the more sensitive analytic technique of GC-MS for determining 5-FU levels in patients given 5-FC.

Initial examination of 5-FC pharmaceutical preparations (Table 1) revealed insignificant contamination by 5-FU, with the 5-FU% less than 0.03. Drug from the same pharmacy lots listed in Table 1 was used to dose the two patients and two volunteers. In each of these individuals, serum 5-FU levels were observed to attain levels that could not have been explained by contamination of the 5-FC by 5-FU. Figure 1 shows that the serum 5-FU level appeared to peak at approximately 2 h after 5-FC administration, with the level then decreasing to a lower but still detectable (100 to 200 ng/ml) range by 5 h. These serum concentration-time curves for 5-FU are similar to those observed for 5-FC itself after 5-FC administration (2). This finding, together with the fact that 5-FU in the blood has a half-life of 10 min (3), suggests that 5-FU was continuously formed from available 5-FC. The relation between the serum 5-FU level and serum 5-FC level is further clarified by the results shown in Table 2. In addition to providing fur-

ther support for in vivo formation of 5-FU from 5-FC, serum 5-FU levels can be seen to vary somewhat linearly with serum 5-FC levels (Fig. 4), with more elevated 5-FU levels found at higher 5-FC levels.

Although the present study did not address the question as to where 5-FC might be converted to 5-FU, the data nevertheless provide a basis for future studies. In contrast to many microorganisms known to have the enzyme cytosine deaminase permitting conversion of 5-FC to 5-FU (11), no evidence exists in the literature for the existence of this enzyme in humans. Studies by one of us have also failed to detect cytosine deaminase activity in human liver, gastointestinal mucosa, or bone marrow (R.B. Diasio, unpublished data). The data from the present study do not suggest that 5-FC is extensively deaminated to 5-FU, with the 5-FU% being less than 4. These data are compatible with the findings of the earlier studies (8, 12, 13), with greater than 90% of the drug not being metabolized. The possible role of intestinal microflora in deaminating 5-FC has previously been suggested (8). Studies are currently underway to examine the role of intestinal microflora in the formation of 5-FU from 5-FC.

The 5-FU levels measured in the 41 serum samples obtained from seven patients on combined amphotericin B and 5-FC are of particular interest because of the hematological toxicity noted in several of these patients. Although it is impossible retrospectively to state that the 5-FU was the cause of the toxicity, it is of interest to compare these levels with levels obtained in cancer chemotherapeutic regimens of 5-FU. Thus, it is known that serum 5-FU levels of 1,000 ng/ml or greater are found after rapid intravenous infusion of 12 to 15 mg of 5-FU per kg (3), a regimen known to be associated with bone marrow and gastrointestinal toxicity (5, 10). Serum 5-FU levels below 1,000 ng/ml are typically found after a 24 h infusion of 30 mg of 5-FU per kg (4, 6), a regimen associated with only rare toxicity in spite of administration of twice as much drug (9). Thus, a possible mechanism for toxicity in patients treated with amphotericin B and 5-FC could be the following: amphotericin B causes renal dysfunction resulting in higher and prolonged serum levels of 5-FC. The 5-FC is then deaminated, resulting in elevated serum levels of 5-FU, which in turn can cause toxicity in human bone marrow and gastrointestinal mucosa.

In summary, the present study has demonstrated that conversion of 5-FC to 5-FU occurs in humans with the implication that elevated serum 5-FU levels (1,000 ng/ml or greater) may account for the toxicity that has been associated with 5-FC. Further studies are planned to determine the site of 5-FU formation as well as prospective clinical studies to assess the value of monitoring serum 5-FU levels as a predictor of toxicity.

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