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Measurement of 5-fluorouracil and its active metabolites in tissue

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SUMMARY

The 5-fluorouracil content of serum, bile, pancreatic juice, liver, pancreas and muscle was measured by reversed-phase high-performance liquid chromatography using a mobile phase of 5 m*M* 1-heptanesulfonic acid in 5 m*M* acetic acid. Free or unmetabolized 5-fluorouracil was extracted from samples with a mixture of light petroleum–*n*-propanol (40:60). The active metabolites of 5-fluorouracil were hydrolyzed with hot perchloric acid to free 5-fluorouracil and the combined 5-fluorouracil content was extracted. The active metabolite fraction was calculated from the difference between the combined and the free fractions. A straight line plot of the peak areas against concentration was achieved and the detection limit was 50 ng/ml. Five minutes after stopping an intravenous infusion of 15 mg/kg of 5-fluorouracil in a dog, the serum contained only the free form, but other body fluids and tissues contained both free and metabolite fractions. The method may be useful to determine the amount of total drug in patient samples.

INTRODUCTION

5-Fluorouracil (5FU) has been used for the chemotherapeutic treatment of various solid tumors for more than twenty years[1,2]. The antineoplastic effects of 5FU are caused by its metabolites, 5-fluoro-2′-deoxyuridine monophosphate (5FdUMP) which inhibits thymidylate synthetase activity and the synthesis of DNA, and 5-fluorouridine triphosphate (5FUTP) which forms fraudulent RNA[3]. Adequate recoveries of these and other metabolites of 5FU are difficult from small amounts of tissue. 5FdUMP forms a tightly binding covalent bond with thymidylate synthetase and 5,10-methylenetetrahydrofolate, and is a competitive inhibitor of deoxyuridine monophosphate (dUMP) binding[4]. 5FUTP incorporates primarily into nuclear RNA, and smaller quantities also incorporate into other RNA[3]. Methods which determine these active metabolites in cell culture use radioactive 5FU, and are not suitable for application to patients[5,6]. Other methods which determine only the free or unmetabolized fractions of 5FU in plasma or serum may be a poor measure of the active metabolites, since 5FdUMP forms an irreversible bond and the elimination kinetics of

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5FU are non-linear which is consistent with a two-compartment metabolic model[7,8]. Steady state plasma levels of 5FU may or may not be achieved depending on the dose administered and the metabolic status of the patient[7,9]. The purpose of this investigation was to devise a means by which 5FU and its active metabolites can be measured in tissue and body fluids.

EXPERIMENTAL

Materials

All chemicals and reagents were either analytical or spectroquality grade. Hydrochloric acid, perchloric acid and light petroleum (b.p. 37.2–57.8°C) were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). Potassium hydroxide, sodium sulfate, sodium acetate, 5FU, 5-fluorocytosine (5FC), 5-fluorodeoxyuridine (5FdU) and 5-fluorodeoxyuridine monophosphate were purchased from Sigma (St. Louis, MO, U.S.A.). Water and *n*-propanol were of HPLC-grade and were obtained from Burdick & Jackson Labs. (Muskegan, MI, U.S.A.). Zetopar® membrane filters, 13-mm diameter, 0.45-μm pore size were purchased from Rainin (Woburn, MA, U.S.A.), high-purity dry nitrogen from T. W. Smith (Brooklyn, NY, U.S.A.) and polypropylene test tubes from Sarstedt (Princeton, NJ, U.S.A.).

Apparatus

The high-performance liquid chromatography (HPLC) system was purchased from Waters-Millipore (Milford, MA, U.S.A.) and consisted of an automatic injector (Model 712), an automatic gradient controller (Model 680), a pump (Model 510), a 300 × 2 mm I.D. μBondapak C_{18} column, a LC spectrophotometer (Model 481) and the data integrator (Model 730). The mobile phase, 1-heptanesulfonic acid (PIC B7) was also obtained from Waters-Millipore. One vial was dissolved in 1 l of water resulting in a solution of 5 m*M* 1-heptanesulfonic acid in 5 m*M* acetic acid.

Chromatographic conditions

The column was equilibrated at room temperature with the mobile phase at a flow-rate of 0.5 ml/min. The injection volume of the samples and standards was 20 μl and the elution of 5FU and 5FC, the internal standard, were detected at 254 nm. Samples and standards were analyzed in triplicate.

Standards

Two sets of standards of 5FU were prepared. 5FU and 5FC concentrations in serum were compared to similar levels in water. Aqueous standards were prepared at a concentration of 1 mg/ml and stored at −70°C. Dilutions with water were made daily for assay and determination of the standard curve which had a range from 0.01 to 1000 μg/ml. Serum concentrations varied from 0.05 to 200 μg/ml of 5FU. In all dilutions the concentration of 5FC was brought to 5 μg/ml. The volume of the aqueous standards or the spiked serum used for assay was 0.2 ml. After extraction and evaporation of the solvent, the recovered 5FU and 5FC was redissolved in 0.2 ml of water.

Sample extraction

The optimal method for extracting 5FU and 5FC from the body fluids and tissues was determined. Since preliminary studies indicated that ethyl acetate extracted substances from the samples that coeluted with 5FU, a light petroleum–*n*-propanol mixture was used to minimize the extraction of the contaminant. In order to determine the appropriate solvent mixture, the recoveries of 1 μg of 5FU and 50 μg of 5FC in 0.2 ml of serum were determined after extraction with 2 ml of light petroleum–*n*-propanol mixtures at ratios of 100:0, 80:20, 60:40, 40:60, 20:80 or 0:100 (v/v), and compared to the unextracted aqueous control. The pH of samples was adjusted to 4.8 with 0.5 ml of 0.8 *M* sodium sulfate and 0.05 ml of 1.5 *M* sodium acetate buffer. The solution was extracted three times with 2 ml of the same petroleum ether–*n*-propanol mixture. The upper organic layers were pooled and evaporated with nitrogen. The residue was dissolved in 2 ml of water. The amount of 5FU and 5FC recovered with each solvent mixture were compared to the unextracted standard.

In order to determine the time required to completely hydrolyze the 5FU active metabolites to free 5FU, two experiments were performed. Amounts of 10 μg of 5FU and 5FdU were brought into separate tubes, and were then incubated in 1 ml of 70% perchloric acid at 85°C for 0, 1, 2, 3 and 4 h. The amount of 5FU obtained from 5FdU hydrolysis was determined for each time. After cooling to room temperature 2.8 ml of 4 *M* potassium hydroxide was added and the tubes were centrifuged at 3000 *g* for 10 min. The supernatant was acidified with 2 *M* hydrochloric acid to pH 3, and then extracted using 30 ml of light petroleum–*n*-propanol (40:60). After two additional extractions the pooled organic layers were evaporated with nitrogen. The residue was dissolved in 2 ml of water. The amount of 5FU produced was compared to the appropriate control tube. In a second experiment 10 μg of 5FU, 5FdU, 5FdUMP and 5FC were dissolved in separate tubes containing 0.2 ml of serum. Then 1 ml of 70% perchloric acid was added to each tube and samples were incubated at 85°C for 2 h. Samples were extracted as before. The amount of 5FU recovered after hydrolysis of each metabolite was compared to control tubes containing unhydrolyzed but extracted metabolites.

Animals

A healthy mongrel dog weighing 20 kg was obtained from Biomedical Assoc. (Friedenburg, PA, U.S.A.). In order to condition the dog to the laboratory environment, a standard laboratory chow was fed (Ralston Purina, St. Louis, MO, U.S.A.) and the animal was housed in a U.S. Department of Agriculture approved facility for at least one week before the experiment.

Following an overnight fast, the dog was anesthetized with an intravenous injection of sodium phenobarbital at a dose of 35 mg/kg and intubated with an endotracheal tube, allowing spontaneous air breathing. A laparotomy was performed. The common bile duct, pancreatic duct and a foreleg vein were cannulated for sample collection. Approximately 1 ml of bile and pancreatic juice, and 3 ml of blood were obtained. Then 1 to 2 g of liver, pancreas and *rectus abdominus* muscle were resected. Samples were placed on ice.

Using another intravenous cannula placed in a second leg, 15 mg/kg of 5FU was administered over 10 min. Five minutes after stopping the infusion, samples of fluids and tissues were again collected. Sera were obtained after centrifugation.

Samples of liver, pancreas and muscle weighing from 150–300 mg and 0.2 ml of serum, bile and pancreatic juice were placed in separate tubes and 5FC was added to concentrations of 5 μg/ml or μg/g. In order to determine the content of free 5FU, tissues were homogenized in 2 ml of the buffered light petroleum–*n*-propanol mixture (40:60, v/v), at pH 4.8 with a Polytron homogenator set at position 7 for 20 s; body fluids were extracted in a similar solution. The aqueous layer was extracted two additional times, and the organic layers were pooled. After evaporation with nitrogen the residue was dissolved in 0.2 ml of water.

Similar quantities of samples were hydrolyzed for 2 h by incubating in 1 ml of 70% perchloric acid at 85°C. After cooling to room temperature 2.8 ml of 4 *M* potassium hydroxide were added, and mixed to precipitate potassium perchlorate. Following centrifugation at 3000 *g* for 10 min the supernatant was acidified with 2 *M* hydrochloric acid to pH 3. 5FU was extracted three times with 30 ml of the buffered petroleum ether–propanol extraction solution. The organic solvent was evaporated with nitrogen and the residue was dissolved in 0.2 ml of water and assayed. The amount of the active 5FU metabolites was calculated from the combined 5FU content minus the free 5FU.

RESULTS AND DISCUSSION

Standard curve

A plot of the 5FU to 5FC area ratio against serum concentration was linear from 0.05–200 μg/ml. The coefficients of variation (C.V.) of the data points were 1 to 3%. A standard curve of 5FU diluted in water was also linear but the C.V. was 0.5 to 1%. Since little 5FU is bound to plasma protein[7], the slightly greater variation seen in the plasma standards can be attributed to the small variation in the recovery of 5FU by the extraction method. The sensitivity of the method is similar to previous reports[10,11]. Other investigators using radioactive 5FU as an internal standard have reported more sensitive methods[12,13].

Extraction and hydrolysis

While light petroleum did not extract any detectable 5FU or 5FC, *n*-propanol extracted 95–97% of the 5FU and 5FC present in serum (Table I). *n*-Propanol,

TABLE I

EXTRACTION OF 5-FLUOROURACIL AND 5-FLUOROCYTOSINE FROM SERUM

Concentrations of 5-fluorouracil and 5-fluorocytosine were 5 μg/ml and 250 μg/ml, respectively. Values are from two experiments.

Light petroleum–n-propanol	*5-Fluorouracil (%)*	*5-Fluorocytosine (%)*
Unextracted aqueous control	100	100
100:0	0; 0	0, 0
80:20	0; 0	2.5; 3.1
60:40	54.7; 58.7	20.5; 25.3
40:60	62.6; 65.6	63.8; 65.2
20:80	83.1; 87.9	88.9; 91.7
0:100	94.4; 99.2	91.4; 98.0

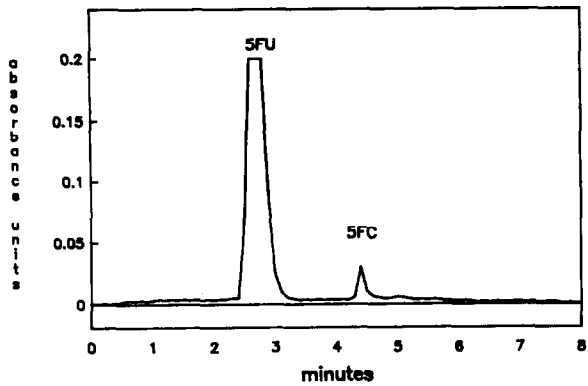

Fig. 1. Chromatogram of a 0.2-ml serum sample which contained 100 μg/ml of 5-fluorouracil (5FU) and 5 μg/ml of 5-fluorocytosine (5FC), and extracted with a mixture of light petroleum–*n*-propanol (40:60, v/v). Retention times for 5FU and 5FC were 2.70 and 4.40 min, respectively. The elution solvent was 5 m*M* 1-heptanesulfonic acid in 5 m*M* acetic acid.

however, also extracts contaminating substances which coelute with 5FU. With a light petroleum–*n*-propanol mixture (40:60) the contaminant was not extracted from serum which contained no 5FU or 5FC. Using this extraction mixture, 64–75% of the 5FU and 5FC was recovered. In Fig. 1 a serum sample containing 100 μg/ml of 5FU and 5 μg/ml of 5FC had retention times of 2.70 and 4.40 min, respectively.

The time required to completely hydrolyze the active metabolites of 5FU was determined. When 5FdU was hydrolyzed, only 1 h was required to achieve near complete conversion to 5FU (Table II). Under the conditions of hydrolysis, the amount of 5FU was stable for at least 4 h. At zero time only 5FdU was present and eluted with a retention time of 3.60 min, whereas only 5FU was detected with a retention time of 2.70 min after hydrolysis.

Hydrolysis of 5FU, 5FdU, 5FdUMP and 5FC with 70% perchloric acid at 85°C for 2 h and extracted as before resulted in the complete conversion of 5FdUMP to 5FU and 93.1% of the 5FdU to 5FU. The amount of 5FU recovered in each tube was

TABLE II

PERCENTAGE OF 5-FLUOROURACIL PRESENT AFTER HYDROLYSIS OF 5-FLUORODEOXYURIDINE

Amounts of 10 μg of 5FU and 5FdU were hydrolyzed in 1 ml of 70% perchloric acid at 85°C from 0 to 4 h. After stopping the reaction with potassium hydroxide and extraction, the amount of 5FU was determined. The percent of 5FU converted from 5FdU was calculated by adjusting for the amount of 5FU lost after extraction of the time-control tubes which contain only 5FU.

Time (h)	*5-Fluorodeoxyuridine (%)*	*5-Fluorouracil (%)*
0	100	0
1	2	98
2	4	96
3	6	94
4	4	96

adjusted by correcting for the proportion of 5FU lost from hydrolysis and extraction in the control tube. Treatment of 5FC with perchloric acid did not result in any 5FU generation. Since 5FdUMP rapidly dissociates from the thymidylate synthetase–5,10-methylenetetrahydrofolate–5FdUMP complex at temperatures greater than 60°C[14], and free bases are liberated from RNA by hydrolysis with hot perchloric acid[15], the method for hydrolysis and extraction is suitable for converting the chemotherapeutic active metabolites in tissue to free 5FU.

Animal experiments

With our extraction methodology no contaminating substances eluted from zero time serum, bile or pancreatic juice with the retention times of 5FU and 5FC. Liver, pancreas and muscle, however, contain substances that elute very near to the 5FU peak. At a light petroleum–*n*-propanol ratio of 40:60, the extraction of the interfering substances was reduced, and as little as 0.1 μg of 5FU in 1 g of tissue can be measured. The chromatogram of the elution of the combined 5FU present in the liver 5 min after stopping the 5FU infusion is shown in Fig. 2. The 5FU content of the liver was 16 μg/g. Although 5FdU was detected in extracted tissues, it was not present after hydrolysis and not in serum.

The content of 5FU in the combined fraction containing free 5FU plus the 5FU obtained after hydrolysis of the active metabolites, the free 5FU fraction and the metabolite fraction in serum, bile, pancreatic juice, liver, pancreas and muscle obtained 5 min after stopping 5FU infusion is listed in Table III. In the serum 5FU was present only in the free form, and the level, 21.0 μg/ml, was similar in magnitude as found by others[16,17]. The concentration of the combined 5FU in bile and pancreatic juice was 70% and 30% respectively of that in the serum. Although 20% of the combined 5FUin the bile was from active metabolites, it comprised only 4% in the pancreatic juice. The concentration of the combined 5FU in bile was 91% of the liver content, suggesting that 5FU was easily excreted by the liver. 5FU secretion in the pancreatic juice was less. The pancreas contains a large proportion of the combined 5FU in the active metabolite fraction, compared to the liver and the muscle.

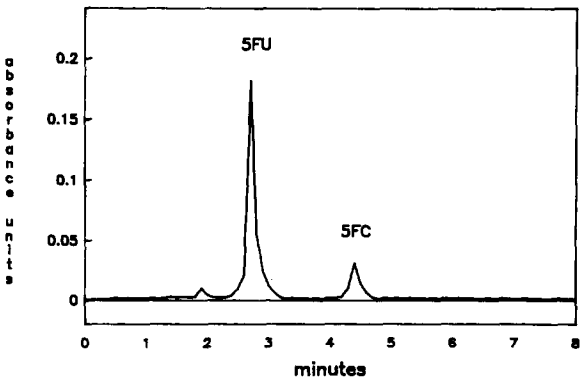

Fig. 2. Chromatogram of a 0.2-g liver sample which was obtained 5 min after cessation of the intravenous infusion of 15 mg/kg of 5-fluorouracil (5FU). The tissue was hydrolyzed with 70% perchloric acid at 85°C for 2 h and extracted with a mixture of light petroleum–*n*-propanol (40:60, v/v). The specimen contained 16 μg/g of 5FU; 5 μg/g of 5-fluorocytosine (5FU), the internal standard, was added.

TABLE III

5-FLUOROURACIL CONTENT IN FLUIDS AND TISSUES

The combined fraction contained free 5FU plus the 5FU obtained after hydrolysis of the active metabolites. The free fraction contained only the unmetabolized 5FU. The active metabolite fraction was calculated by substracting the free from the combined fraction.

Sample	*Combined*	*Free*	*Active metabolites*
Serum	21.0 μg/ml	21.0 μg/ml	0.0 μg/ml
Bile	14.6 μg/ml	11.6 μg/ml	3.0 μg/ml
Pancreatic juice	6.4 μg/ml	6.0 μg/ml	0.4 μg/ml
Liver	16.0 μg/g	14.8 μg/g	1.2 μg/g
Pancreas	23.6 μg/g	13.2 μg/g	10.4 μg/g
Muscle	10.5 μg/g	9.0 μg/g	1.5 μg/g

Reducing 5FU active metabolites, which may be covalently bound to enzymes and RNA, to free 5FU provides a means by which normally unattainable 5FU can be easily measured. Since the chemotherapeutic forms of 5FU can be measured as the free form after hydrolysis, determining the proportion of 5FU in the free and metabolite fractions in samples from biopsies or catheters could be a means by which the penetration of the drug in tissues and body fluids can be evaluated.

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