Journal of Chromatography, 146 (1978) 512—517
Biomedical Applications

CHROMBIO. 236

Note

Quantitative analysis of 5-fluorouracil in human serum by selected ion monitoring gas chromatography—mass spectrometry

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(First received December 6th, 1977; revised manuscript received March 30th, 1978)

5-Fluorouracil (5-FU) is a pyrimidine analog currently used in the treatment of cancer of the breast, ovary, and gastrointestinal tract. Since its clinical introduction more than fifteen years ago, a great deal of experience has been gained in using this drug in many different dose schedules by various routes of administration, both as a single agent and as part of combined regimens, Nevertheless, a basic understanding of the clinical pharmacokinetics has been lacking, particularly with regard to the concentration—time relationship which determines adequate therapeutic effect or unacceptable toxicity. Thus, there has been a lack of pharmacological rationale for the many schedules currently used [1].

The approach to the study of 5-FU pharmacokinetics has been hampered by the fact that 5-FU has a relatively short half-life with serum levels dropping below 1μg/ml soon after a standard dose (500 mg/m^2) [2]. Until recently, the analytical methodology capable of measuring 5-FU at levels lower than 1 μg/ml has been unavailable [3]. Reported methods for the analysis of 5-FU in physiological fluids have included gas—liquid chromatography (GLC) [4—6] and more recently gas chromatography—mass spectrometry (GC—MS) [7—9]. These methods, particularly those by GC—MS, provide the sensitivity needed to

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measure 5-FU in the range found clinically. Unfortunately, the sample preparation from physiological fluids has given 5-FU recoveries of 40—75% depending on the method used. A method permitting greater recovery would be desirable.

In this report, a method which provides for quantitative recovery of 5-FU from serum is described.

MATERIALS AND METHODS

Chemicals

5-FU was obtained as a gift from Hoffman-LaRoche (Dr. E. Miller, Nuttey, N.J., U.S.A.). Phenanthrene, acetonitrile, and methylene chloride were purchased from Fischer Chemicals (Fair Lawn, N.J., U.S.A.). The acetic acid was obtained from J.T. Baker (Phillipsburgh, N.J., U.S.A.). The silylating reagent, bistrimethylsilyltrifluoroacetamide (BSTFA) were purchased from Regis (Chicago, Ill., U.S.A.).

Serum samples: standard and patient samples

Serum standards (100, 200, 300, and 400 ng/ml) were prepared in quadruplicate by adding appropriate amounts of 5-FU to pooled human serum, such that the total volume of drug plus serum was 1.0 ml. The pooled serum was obtained from healthy male adults on no medication.

Serum samples were obtained from three patients with colon cancer being treated with 500 mg/m^2 of 5-FU. Two of the patients received the drug intravenously, while the third was given 5-FU by the oral route. Serum samples were obtained prior to receiving drugs and at various time intervals following administration.

Preparation of samples for analysis

Prior to the analysis of 5-FU in serum samples, a clean-up step was required to remove proteins and related compounds which interfere with the chromatographic separation and quantitation of 5-FU. The following procedure was developed to provide a relatively clean sample for GC—MS analysis and quantitative recovery of 5-FU from serum.

Ultrafiltration. A 1.0-ml aliquot of serum was placed in a CF 25 Amicon ultrafiltration cone (Amicon, Boston, Mass., U.S.A.) and centrifuged at 2000 *g*. The contents remaining in the cone were washed twice with 1.0 ml of deionized water and recentrifuged into the same collection tube. The pH of the ultrafiltrate was adjusted to 13 with 1.0 *N* KOH.

Anion exchange resin. The alkalinized ultrafiltrate was then quantitatively transferred to a 50 × 9 mm column containing AG 1-X2 anion-exchange resin in the acetate form (resin regeneration procedure described previously [10]). The collection tube was rinsed twice with 1.0 ml of deionized water, with the wash being placed on the column after the sample was completely on the resin. The column was washed with 10 rinses of 5.0 ml of deionized water. 5-FU was then eluted with 25 ml of 0.1 *N* acetic acid and collected in a 50-ml beaker. This volume was concentrated to approximately 2 ml on a 60° hot-plate under a stream of nitrogen, and then quantitatively transferred to a 4.5 ml silylation vial (made from Corning 9826 culture tubes). The beaker was washed twice

with 1.0 ml of 0.1 *N* acetic acid, with the wash being added to the silylation vial. The entire volume was then evaporated to dryness on a 60° hot-plate under a stream of nitrogen. 1.0 ml of methylene chloride was added to the dry contents of the silylation vial and evaporated to remove azeotropically any remaining traces of water.

Silylation

Patient samples and 5-FU serum standards were silylated with 200 μl of a BSTFA—CH_3CN (1:1, v/v) mixture. The silylation vials were tightly sealed with a PTFE-lined screw-cap and heated at 150° for 3 min. An internal standard (phenanthrene) was dissolved in a CH_3CN and added to the sample with the silylation mixture.

Chromatographic and mass spectrometry conditions

A Hewlett-Packard 5981A gas chromatograph—mass spectrometer with a Hewlett-Packard 5933A data system (Hewlett-Packard, Palo Alto, Calif., U.S.A.) was used to analyze for 5-FU. A 1 m × 2 mm I.D. glass column packed with 3% Dexsil 300 on 100—120 mesh Supelcoport (Supelco, Bellefonte, Pa., U.S.A.) was used for the chromatography.

Volumes (4 μl) of the derivatized solution were injected into the GC—MS system. The initial temperature was 100° and was programmed to 250° at 16° per min. The helium carrier gas flow-rate was maintained at 22 ml/min. and the injection port temperature was 250°. The eluent from the chromatographic column was passed through an all glass jet separator at 300° and into the ion source of the MS. The MS was operated at 70 eV with a source temperature of 240° and the dodecapole at 110°.

Calculations

The level of 5-FU in serum was calculated from the relative weight response (RWR) of a standard 5-FU solution. The equations were:

$$\text{ng 5-FU per aliquot} = \frac{\text{Area 5-FU}}{\text{Area IS}} \times \frac{\text{ng IS}}{\text{RWR std}}$$

where:

$$\text{RWR std} = \frac{\text{Area 5-FU std}}{\text{Area IS}} \times \frac{\text{ng IS}}{\text{ng 5-FU std}}$$

and IS = internal standard; area = area of peak on chromatogram; ng = nanograms of compound; RWR std = relative weight response of standard 5-FU solution to an internal standard.

RESULTS AND DISCUSSION

Mass spectrum of 5-FU

In Fig. 1, the mass spectrum of the trimethylsilyl derivative of 5-FU is presented. The molecular ion is at *m/e* 274.1, and the base peak is at 259.1. From the spectrum, the *m/e* 259.1 ion was selected for quantitation of 5-FU in plasma samples. To determine the location of 5-FU on the selected ion chromato-

grams and to provide specificity to the analysis, *m/e* 273.1 and 274.1 were also monitored and the 273.1:259.1 and 274.1:259.1 ratios calculated.

A selected ion chromatogram of a standard solution containing 5-FU and 5-fluorocytosine (5-FC), a compound with similar GLC and MS characteristics,

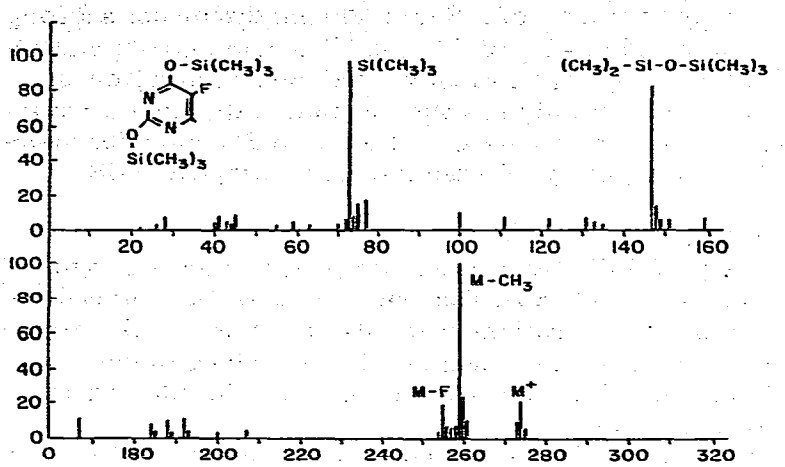

Fig. 1. Mass spectrum of 5-fluorouracil.

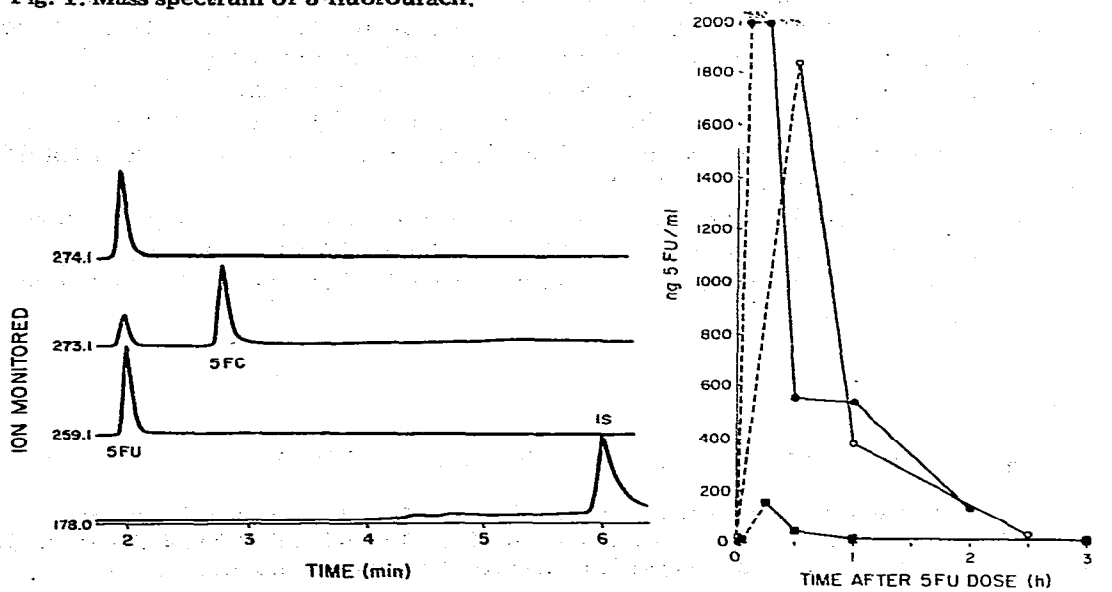

Fig. 2. Selected ion chromatograms of 5-FU, 5-FC, and phenanthrene (IS). Sample: 100 ng each 5-FU, 5-FC, and IS silylated with 200 µl CH_3CN—BSTFA (1:1, v/v) at 150° for 3 min. GLC conditions: column, 3% Dexsil 300 on 100—120 mesh Supelcoport, 1 m × 2 mm I.D., glass; initial temperature, 100°; program rate, 16°/min; final temperature, 250°; helium flow-rate, 22 ml/min; injection, 4 µl. MS conditions: separator temperature, 300°; source temperature, 240°; dodecapole temperature, 110°; ionizing voltage, 70 eV.

Fig. 3. 5-FU serum concentration in three cancer patients after administration of dose of 500 mg/m². Sample preparation as outlined in text. GC—MS conditions as given in Fig. 2. ●, ○, 475 mg 5-FU, i.v. push; ■, 400 mg 5-FU, p.o.

along with the internal standard, phenanthrene, is shown in Fig. 2. The highest detected voltage for each of the four selected ions (*m/e* 178.0, 259.2, 273.1 and 274.1) on the chromatogram is normalized to 100. The peaks at retention time (t_R) 2.0 min are ion fragments of 5-FU. 5-FC has an *m/e* 273.1 in its mass spectrum, and this ion is detected at a t_R of 2.8 min. Phenanthrene has a strong *m/e* 178.0 and this ion is detected at a t_R of 6.0 min. The complete separation of the ions of the two fluorinated pyrimidines shows that interference from similar compounds is unlikely. Other pyrimidines such as uracil, thymine, or cytosine do not have ion fragments corresponding to those of 5-FU and will, therefore, not interfere with 5-FU analysis by selected ion monitoring GC—MS.

Recovery of 5-FU from serum

To determine the recovery of 5-FU from serum samples, a known amount of 5-FU was added to normal serum and carried through the analytical procedure. Standards of 5-FU at various concentrations were also analyzed to determine the RWR standard value, to evaluate the linearity of the analysis method, and to calculate the 273.1:259.1 and 274.1:259.1 ion ratio values for identification of the 5-FU peak in the serum samples.

In Table I, the average RWR standard value and the average ion ratio values are presented. The standard deviation (S.D.) and the relative standard deviation (R.S.D.) are also given. The analysis of 5-FU was found to be linear from 10 ng to 1000 ng and had a minimum detectable limit of less than 1 ng.

Also in Table I, the recovery of 5-FU added to serum samples is presented. Quadruplicate analyses at 100, 200, 300 and 400 ng 5-FU were made, and the average value at each level is given. Quantitative recovery of 5-FU was found for all samples with an overall R.S.D. of 12.

The standard RWR value was found to vary slightly day-to-day; however, in-day variations were less than 5%. A new RWR std value was determined daily, and 5-FU standards were analyzed periodically during the day to evaluate the stability of the system.

TABLE I

STANDARD RWR, ION RATIOS, AND RECOVERY FROM SERUM

RWR std = average of 20 independent analyses of 100, 200, and 300 ng 5-FU standards. %R-100, 200, 300, 400 ng = percent recovery of 100, 200, 300 and 400 ng 5-FU added to 1.0 normal human serum. Four independent analyses at each concentration.

	Value	S.D.	R.S.D.
RWR std	0.705	0.064	9
273.1:259.1 ratio	9.88	0.08	0.8
274.1:259.1 ratio	19.42	0.26	1.3
%R-100 ng	103	11	10
%R-200 ng	108	11	10
%R-300 ng	93	12	13
%R-400 ng	94	12	13

Serum 5-FU levels in patients receiving 5-FU therapy

The concentration of 5-FU in serum for the three cancer patients receiving 5-FU therapy was determined using the analytical procedure. Blood samples for each of the patients were obtained at various times following the administration of the drug. Two of the patients received the drug by intravenous push, while the third received an oral dose.

The 5-FU serum levels for the three patients plotted versus time are presented in Fig. 3. The patients receiving the drug by the intravenous route had much higher serum levels than the patient taking an oral dose. The highest concentration of 5-FU was at 5 min after intravenous administration and was greater than 7000 ng/ml. The serum levels of the free drug decreased quickly, and very little drug was detected at 3 h. This method thus permits detection of 5-FU at sufficiently low levels to enable pharmacokinetic analysis.

NOTE ADDED IN PROOF

In a recent article [11], a method for the determination of 5-FU in plasma by electron-capture GLC was described. The technique has sufficient sensitivity for nanogram detection, and the sample preparation procedure gave over 80% recovery of 5-FU. No pharmacokinetic data were given.

ACKNOWLEDGEMENTS

This investigation was supported in part by an American Cancer Society Grant, No. IN-105B, and in part by an institutional grant of the Medical College of Virginia.

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