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GLC Assay for 5-Fluorouracil in Biological Fluids

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Abstract □ A rapid, sensitive GLC method of analysis for 5-fluorouracil was developed to follow the disposition of the drug in patients on 5-fluorouracil therapy. The free drug is removed from aqueous biological samples in a single extraction step, derivatized by silylation, and chromatographed using flame-ionization detection. An internal standard is utilized to quantitate the results. The procedure is rapid, sensitive (0.2 mcg./ml. plasma), specific for the intact 5-fluorouracil molecule, and suitable to support pharmacokinetic studies of 5-fluorouracil in animals and humans.

Keyphrases □ 5-Fluorouracil—GLC analysis in biological fluids □ GLC—analysis, 5-fluorouracil in biological fluids

Detailed pharmacokinetic studies that would provide information useful to the clinician would be desirable to optimize cancer chemotherapy with 5-fluorouracil. The drug is still given empirically by many different dosage schedules, with some recent evidence suggesting that the oral route of administration differs in activity and toxicity from the intravenous route (1). Disposition studies performed previously were limited by the absence of a rapid, sensitive method of analysis capable of determining free 5-fluorouracil in large numbers of biological samples. Studies using radioactively labeled drugs showed wide variability due to extensive metab-

Table I—Recovery of 5-Fluorouracil Added to Water, Human Plasma, and Human Whole Blood

5-Fluorouracil Added, mcg./ml.	Water		Plasma		Blood	
	Peak Height Ratio[a]	Percent Recovery[b]	Peak Height Ratio[a]	Percent Recovery[b]	Peak Height Ratio[a]	Percent Recovery[c]
4.83	1.42	78.9 ± 2.50	1.45	80.6 ± 3.24	1.47	81.7
1.94	0.60	82.2 ± 3.10	0.58	79.4 ± 3.47	0.56	76.7
0.96	0.28	80.0 ± 2.74	0.27	77.1 ± 3.05	0.28	80.0
0.48	0.14	82.3 ± 3.85	0.14	82.3 ± 3.75	0.13	76.5

[a] Anthracene concentration was 3.2 ng./μl. injected. [b] Average of three determinations ± standard deviation. [c] Represents a single determination. Human blood was extracted according to the described procedures to determine binding to red blood cells.

olism (2) and illustrated the nonspecificity of this method of analysis for compounds of this type.

Clarkson *et al.* (3) performed some comparative disposition studies on a limited number of patients; they utilized a spectrophotometric assay procedure when the blood levels were greater than 100 mcg./ml. and a microbiological assay for lower blood levels. In most instances the spectrophotometric procedure was not sensitive enough for clinical studies, while the microbiological procedure lacked accuracy and was not readily adaptable to large numbers of samples. Windheuser *et al.* (4) developed a GC method which requires extended (22-hr.) dialysis to remove the drug from blood samples and which would be difficult to perform on a routine basis.

The present work describes an analytical method based upon a single extraction of the drug into a nonaqueous solution, with subsequent formation of a trimethylsilyl derivative for GLC detection. The concentrations of 5-fluorouracil were quantitated using an internal standard. The method is applicable to biological samples including blood and urine and can be performed on a routine basis for clinical studies of patients on 5-fluorouracil therapy.

EXPERIMENTAL

Reagents—All chemicals and solvents used were of analytical reagent grade, and water was glass double distilled.

Procedure for Biological Samples—Blood samples were collected in citrated Vacutainers[1], mixed gently, and centrifuged to allow separation of the plasma. Then the plasma was frozen until the sample was to be analyzed. Urine samples were either collected and directly frozen or lyophilized and stored in a similar fashion.

A plasma or urine sample was adjusted to pH 6 by the addition of a known volume of a pH 4.7, 0.5 *M* acetate buffer (*e.g.*, 125 μl. of acetate buffer was required per milliliter of human plasma). Then 1.0 ml. of this sample was added to a flask already containing 0.4 g. of anhydrous sodium sulfate and 15.0 ml. of a solution of 16% *n*-propanol in ether. This mixture was shaken vigorously for 5 min. and then allowed to stand for 15 min. or until the ether layer cleared. Depending upon the concentration of 5-fluorouracil, a suitable volume (1.0–10.0 ml.) of the ether solution was pipeted into either a: (*a*) 15.0-ml. centrifuge tube if more than 5.0 ml. was used, or (*b*) 5.0-ml. vial[2] if a smaller volume of ether was used. Then 25 μl. of an anthracene solution in pyridine containing 16 mcg./ml. was added as an internal standard, and the solvent was removed under a gentle stream of nitrogen with the reaction vial immersed in a 55° water bath.

Derivatization—The evaporated sample was dissolved in 100 μl. of a freshly prepared solution of 50% *N,O*-bis(trimethylsilyl)-trifluoroacetamide (I), containing 1% trimethylchlorosilane[3] as a catalyst in silation grade pyridine[3]. This reaction mixture was allowed to stand for 5 min. at room temperature, and then 1–10 μl. was injected into the gas chromatograph.

Gas Chromatography—The assay was performed on a gas chromatograph[4] equipped with a flame-ionization detector. A glass coiled column, 1.83 m. (6 ft.) × 0.63 cm. (0.25 in.) o.d., was packed with 3% OV-1 on 100–120-mesh Gas Chrom Q[5], conditioned at 250° for 72 hr. and injected periodically with trimethylsilyl donors[6]. Nitrogen as a carrier gas was maintained at 40 ml./min., while the flow rates for air and hydrogen were 250 and 30 ml./min., respectively. The injector and detector temperatures were both maintained at 200° throughout the procedure.

The sample was injected at a column-oven temperature of 110° and the 5-fluorouracil eluted as a sharp peak at ~5.5 min. The temperature was then increased to 160° (heating rate 50°/min.) and the anthracene (internal standard) peak appeared after 12 min. (from time of injection). After 2–3 days of use, the column was reconditioned at 250° overnight and a mixture of trimethylsilyl donors[6] was injected. The detector was cleaned every 3–4 days with repeated injections of trichlorotrifluoroethane[7] and was removed periodically and further cleaned by sonication in acetone.

RESULTS AND DISCUSSION

5-Fluorouracil is representative of the analytical challenges now faced by workers in biopharmaceutics and clinical pharmacology. Its water solubility, even as the free acid, and nonfluorescent properties make specific, sensitive, routine methods of analysis difficult to develop. The described extraction procedure gives the highest constant percentage extracted (80%) in a single extraction of many explored and was the only method that appeared quantitatively useful. Any increase in the polarity of the "nonaqueous phase" destroys the immiscibility with aqueous samples. Saturation with sodium sulfate also appeared to optimize extraction of 5-fluorouracil by salting it out of the aqueous medium. Since no improvement in extractibility was seen at more acidic pH's, pH 6 was selected, on the basis of the reported pKa of 8.1 (5), to minimize the extraction of other weakly acidic compounds which could be present in blood samples and possibly silate and interfere with the chromatographic assay procedure. This is a critical consideration in this case since the partition coefficient of 5-fluorouracil does not allow a "cleanup" extraction. Plasma blanks were always carried through the assay procedure to verify the absence of any interfering material.

The extraction procedure gave identical results for aqueous, plasma, and blood samples to which 5-fluorouracil had been added[8]. These results (Table I) indicate no interference with the assay in the presence of plasma proteins or red blood cells which would affect the percentage extracted. The percent recovery is based upon the addition of 5-fluorouracil to ether (Table II). In view of this and since the extraction was not complete, a standard curve was prepared by using aqueous samples to which various concentrations of 5-fluorouracil had been added and carrying them through

[1] Becton, Dickinson and Co., Rutherford, N. J.
[2] Reacti-vial, Pierce Chemical Co., Rockford, Ill.
[3] Pierce Chemical Co., Rockford, Ill.
[4] Varian model 1440 (Varian Aerograph, Walnut Creek, Calif.) equipped with a Perkin-Elmer Hitachi recorder, model 156.
[5] Applied Science Laboratories, State College, Pa.
[6] Silyl 8, Pierce Chemical Co., Rockford, Ill.
[7] Freon 113, Pierce Chemical Co., Rockford, Ill.
[8] Obtained from either International Chemical and Nuclear, Irvine, Calif., as a solid, or Hoffmann-La Roche, Nutley, N. J., as the dosage form.

Table II—GLC Data for 5-Fluorouracil Added to Ether

5-Fluorouracil Added, mcg./ml.	Peak Height Ratio[a,b]
4.83	1.80
1.94	0.73
0.96	0.35
0.48	0.17

[a] Anthracene concentration was 3.2 ng./μl. injected. [b] Average of three determinations.

the entire assay procedure. Figure 1 shows a typical chromatographic separation from a human plasma sample. The sharpness of the peaks enabled a quantitation to be made using peak height ratios rather than the more cumbersome peak area ratios. A linear standard curve (Figs. 2 and 3) corresponding to original concentrations of 5-fluorouracil of from 0.2 to 80 mcg./ml. was obtained and used to determine directly the drug levels of samples in this concentration range after accounting for any dilution changes. Higher concentrations of 5-fluorouracil required different concentrations of internal standard and the curve was linear up to 300 mcg./ml.

Working from observations of Windheuser *et al.* (4) that 5-fluorouracil could be silylated quantitatively and chromatographed led to development of the final GLC assay procedure. Compound I had the advantage of much more volatile fluorinated by-products after silylation, which appear under the solvent peak and do not interfere with the quantitation of 5-fluorouracil as did *N,O*-bis-(trimethylsilyl)acetamide under our assay conditions. To be certain of the specificity of the method for the free drug molecule, the analysis was performed on a GLC interfaced with a mass spectrometer[9]. The peak corresponding to 5-fluorouracil at 5.5 min. was shown by fragmentation and the appearance of a parent peak at

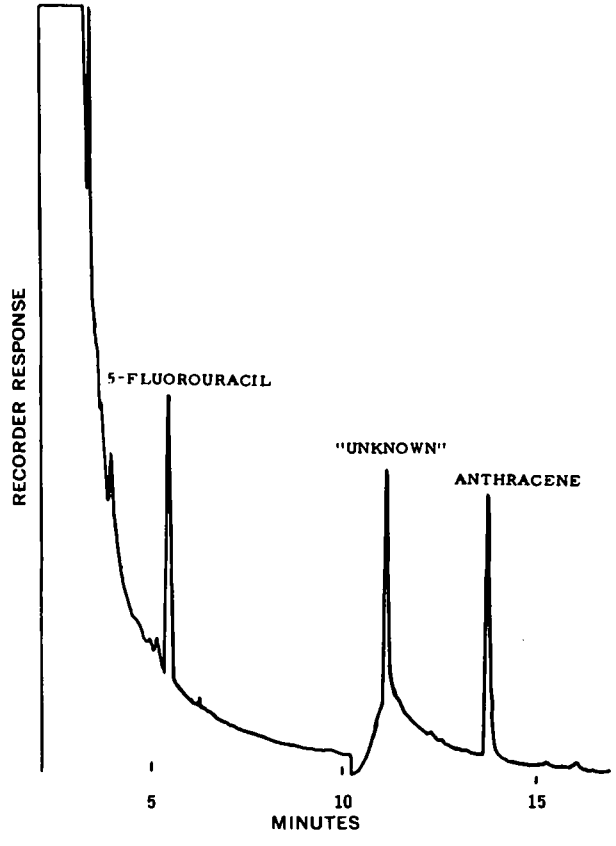

Figure 1—*Gas chromatogram of a plasma sample of 5-fluorouracil with the addition of anthracene. (5-Fluorouracil concentration = 4.83 mcg./ml.; anthracene concentration = 3.2 ng./μl. injected.)*

[9] Varian model CH-7, Varian Mat Divisions, Palo Alto, Calif.

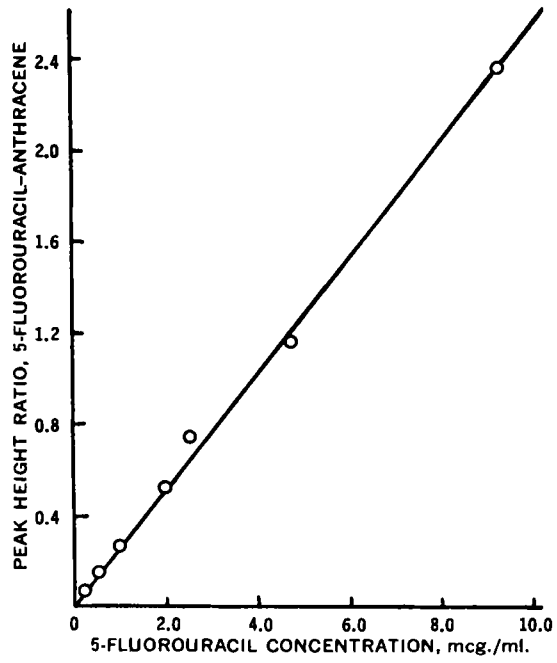

Figure 2—*Calibration curve for 5-fluorouracil extracted from aqueous solution at low 5-fluorouracil concentrations. (Anthracene concentration = 3.2 ng./μl. injected.)*

274 *m/e* to be 5-fluorouracil with the addition of two trimethylsilyl groups.

No suitable internal standard could be found for the column operating temperature at which the assay is performed. Anthracene was selected for its inertness, purity, and good flame-ionization response; however, the column had to be programmed to obtain reproducible results for peak height ratio calculations. This actually turns out to be an advantage, since the higher temperatures elute impurities present in the biological samples before the anthracene peak comes out (the broad peak labeled "unknown" in Fig. 1). Otherwise, to avoid interferences, these impurities would have to be eluted before the next sample could be injected.

The presence of the fluorine atom suggested that electron-capture detection may improve sensitivity and eliminate extraneous plasma peaks and other interferences. Much work has been done correlat-

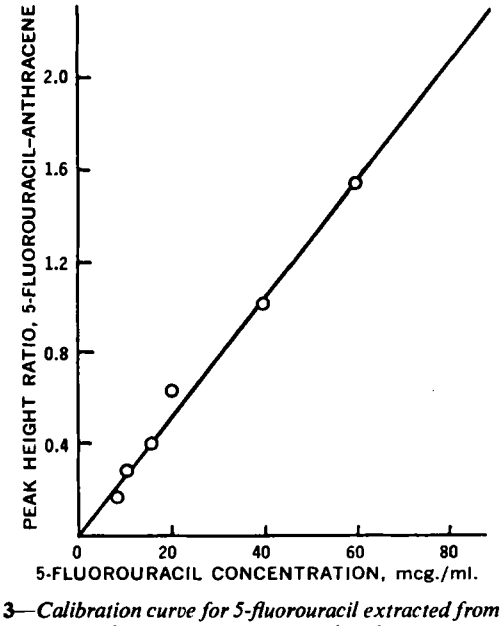

Figure 3—*Calibration curve for 5-fluorouracil extracted from aqueous solution over a wide concentration range. (Anthracene concentration = 3.2 ng./μl. injected.)*

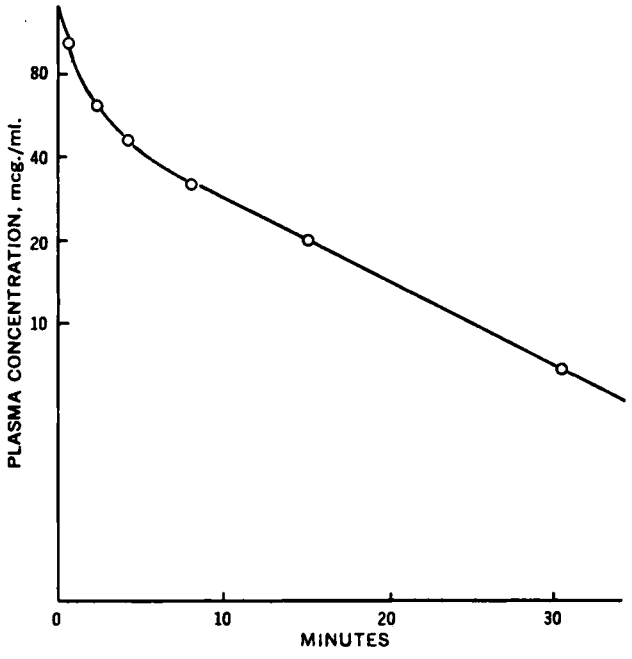

Figure 4—*Plasma concentration of 5-fluorouracil in a patient (E.J.) given a single intravenous dose of 15 mg./kg.*

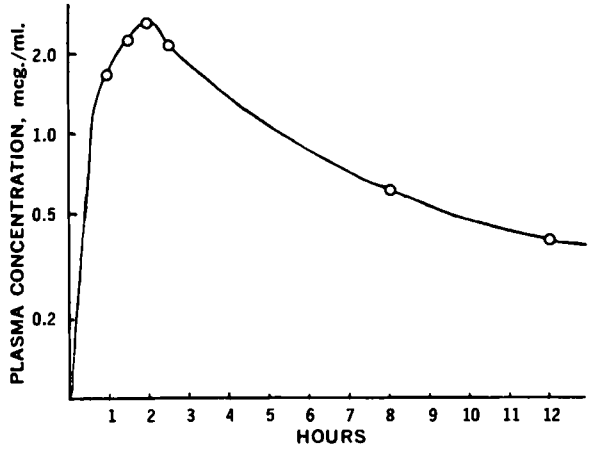

Figure 5—*Plasma concentration of 5-fluorouracil in a patient (I.B.) given a single oral dose (in solution) of 15 mg./kg.*

ing the structures of derivatives with electron-capture sensitivity (6–10), the presence of a single chlorine atom, for example, on chlorpromazine allowed a sensitive electron-capture analysis to be performed (11). Since the analytical conditions were essentially worked out, a ^{63}Ni detector was substituted for the flame-ionization detector; however, sensitivity could not be improved by more than a factor of two using this approach. A modification of the present method is currently being pursued in these laboratories in an effort to increase sensitivity, which may enable the quantitation of 5-fluorouracil in trace concentrations in body tissues and fluids other than plasma and urine.

Since the reason for development of an assay procedure of this type is to support biopharmaceutical and clinical pharmacology studies designed to improve drug therapy, data from two different patients receiving therapeutic doses of 5-fluorouracil have been included. Figure 4 illustrates the plasma time course of the drug in a patient (E.J.) receiving 15 mg./kg. by intravenous push, and Fig. 5 illustrates the same data for a different patient (I.B.) receiving a comparable dose in solution orally. No interfering peaks were encountered either from possible metabolites or other medication taken by these patients. The sensitivity and applicability of the method to these patients indicate that the method is suitable for these types of studies, and several clinical studies are currently being performed.

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