GLC Determination of Fluorouracil

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Abstract □ A GLC assay method for fluorouracil, based on the flash-methylation technique using trimethylanilinium hydroxide, was developed. It is a convenient, rapid, and sensitive method for the determination in urine and other biological fluids. An ion-exchange recovery method was also developed, using a combination of strong acid and weak base resins and elution from the latter with aqueous ethanolic ammonia.

Keyphrases □ Fluorouracil—GLC analysis, flash methylation using trimethylanilinium hydroxide □ Ion-exchange chromatography—separation of fluorouracil from urine □ GLC—analysis, fluorouracil, flash methylation using trimethylanilinium hydroxide

The importance of fluorouracil (I) in the treatment of certain forms of cancer was reviewed (1). However, biopharmaceutical studies of this drug have been hampered by the lack of a suitable analytical procedure. Part of the problem is due to the lack of distinctive features which can facilitate the development of a convenient method of assay. The spectrophotometric method (2) lacks sensitivity as well as specificity, and the microbiological assay, although selective, can become unsuitable when the patient is on simultaneous antibiotic therapy. The combination of dialysis and silylation techniques (3) requires overnight operation, so it is not convenient for a routine assay.

Recently, Cohen and Brennan (4) developed a GLC procedure for fluorouracil coupled with extraction using 16% *n*-propanol in ether. The fluorouracil in the extract was silylated with 50% *N,O*-bis(trimethylsilyl)trifluoroacetamide to give a reasonable retention time on the gas chromatograph. In their method, the temperature of the column has to be raised after fluorouracil comes out of the column to permit the emergence of the internal standard, anthracene, in a reasonable time (12 min). Also, use of a silylating agent requires reconditioning of the column every 2–3 days and injection of a mixture of trimethylsilyl donors. The detector also has to be cleaned every 2–3 days.

Brochmann-Hanssen and Oke (5) developed a GLC assay method for barbiturates, phenolic alkaloids, and xanthine bases using flash methylation with 0.1 *M* trimethylanilinium hydroxide. The possibility of the application of this method for the determination of fluorouracil prompted the present study. Also, since the microbiological assay has certain advantages such as simplicity and sensitivity, an improved method for the recovery of fluorouracil from biological fluids would extend its utility. This paper describes an ion-exchange method for recovery and a GLC method for the determination of fluorouracil.

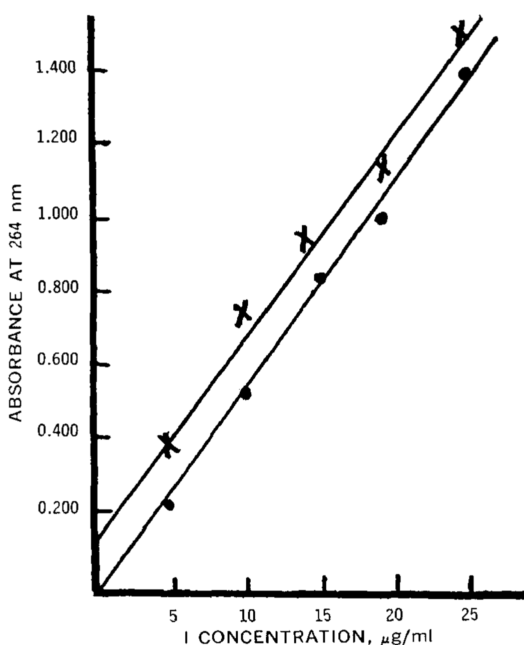

Figure 1—*Calibration curve for ion-exchange method of recovering fluorouracil. Key:* ●, *before the sulfonic acid-type column; and* ×, *after the primary amine-type column.*

EXPERIMENTAL

Microbiological Assays—These assays were performed according to the method of Hunt and Pitillo (6) using *Escherichia coli* (ATCC 9637) as the test organism. Depending on the amount of inoculum, the range of sensitivity was 0.3–3 μg/ml. Although somewhat less sensitive (1–10 μg/ml), the *Bacillus subtilis* (ATCC 6633) plate assay has some advantages; *e.g.*, the culture can be stored as a relatively heat-stable spore suspension and the amount of inoculum can be more easily duplicated. The assay medium for both organisms had the following composition (in grams per liter): K_2HPO_4, 7; KH_2PO_4, 3; sodium citrate, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; glucose, 2; and agar, 15.

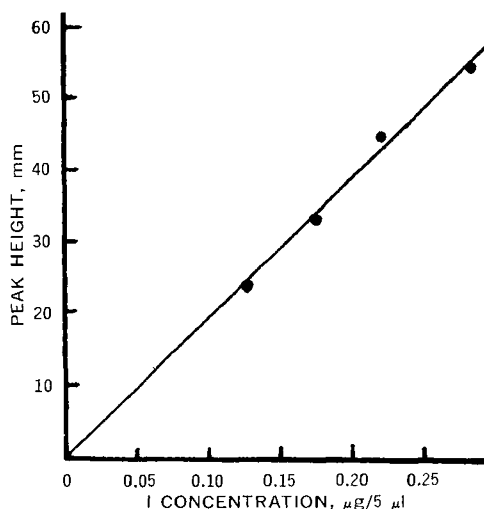

Figure 2—*Calibration curve for fluorouracil extracted from aqueous solution.*

Table I—Recovery of Fluorouracil (I) Added to Urine

I Added, µg/ml	I Recovered, µg/ml (Microbiological)	I Recovered, µg/ml (GLC)
1.24	1.06	1.14
2.48	2.10	2.23
3.72	3.12	4.01
4.96	4.16	5.10

Filter paper disks[1] were dipped in standard solutions of fluorouracil in the appropriate concentration range, placed on the seeded agar plates, and incubated at 37° overnight. A plot of the diameter of the zone of inhibition against the log of the concentration of fluorouracil gave the standard curve.

Ion-Exchange Methods—Analytical grade ion-exchange resins of the sulfonic acid[2] type (100–200 mesh) with 4% cross-linking agent and the primary amine[3] type (50–100 mesh) were used in this study. The acid resin was washed with 2 *N* HCl followed by 50% aqueous ethanol and water. The basic resin was washed with 10% aqueous potassium carbonate followed by 80% aqueous ethanol and water. Both resins must be freshly washed before use.

For the recovery of fluorouracil from urine, a filtered sample (100 ml) was passed through a column (18 × 150 mm) of the acid resin, previously washed as described, at a rate of 5–8 ml/min. The column was then washed with two bed volumes of distilled water. The effluent and wash, as they emerged, were passed through a second column containing the basic resin (18 × 150 mm). In this sequence, fluorouracil passed unabsorbed through the acid resin but was completely retained on the basic resin. After the latter column was washed with water, fluorouracil was eluted by two bed volumes of aqueous ethanolic ammonia [ethanol-water-concentrated ammonium hydroxide (3:1:0.2)].

A gas chromatograph[4] equipped with a flame-ionization detector was used for the GLC analyses. A glass U-shaped column, 0.63 cm o.d. and 183 cm long, was packed with 2.8% OV-210 + 3.2% OV-1 on 80-100-mesh Chromosorb W (MP)[5] and conditioned at 250° for 18 hr. Helium, the carrier gas, was maintained at 55 ml/min, the flow rate for hydrogen was 45 ml/min, and that for air was 300 ml/min. The injector temperature was 250°, the detector temperature was 300°, and the column oven temperature was maintained at 125°. Under these conditions, fluorouracil emerged as a sharp peak with a retention time of 7.7 ± 0.2 min.

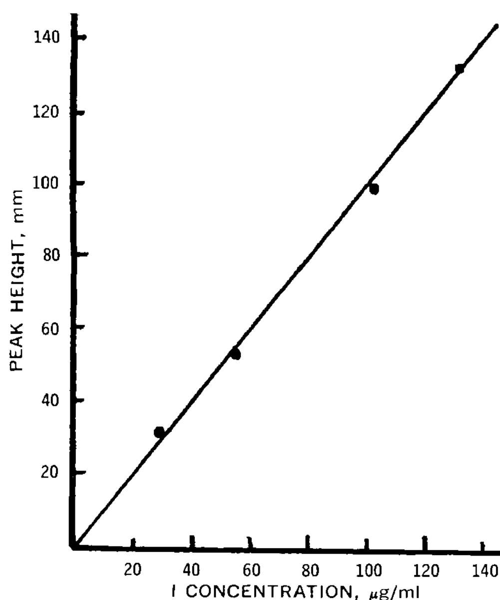

Figure 3—*Calibration curve of fluorouracil extracted from urine.*

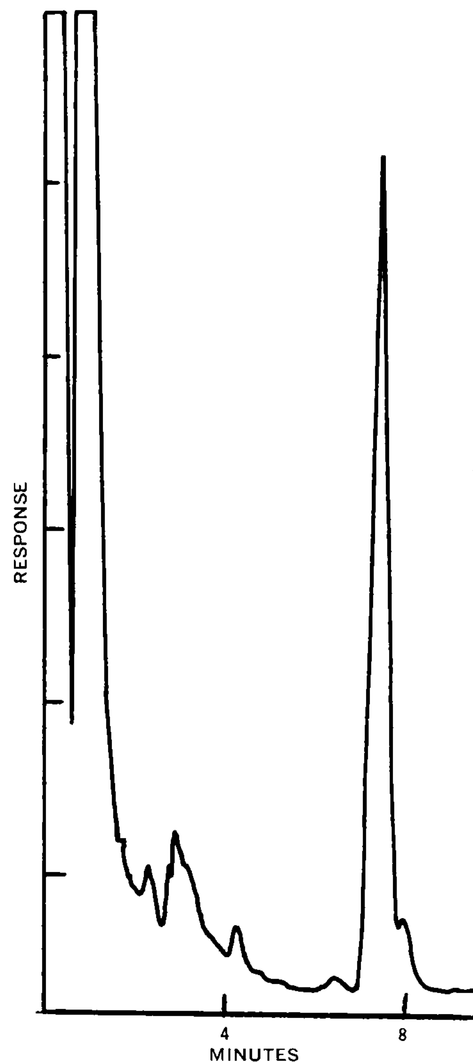

Figure 4—*Gas chromatogram of a urine sample of fluorouracil with the addition of trimethylanilinium hydroxide (fluorouracil concentration = 1.0 mg/ml).*

The extraction method of Cohen and Brennan (4) was followed. If necessary, the pH of the urine sample was adjusted to 6.0 using 1 *M* acetate buffer. After the extraction, a 10-ml aliquot of the ether–*n*-propanol solution was pipeted into a centrifuge tube and evaporated to dryness in a hot water bath (50°) under a gentle stream of air filtered through glass wool.

The evaporated sample was dissolved in 0.2 ml of 0.1 *M* trimethylanilinium hydroxide (5). The reaction mixture was kept cold until injected. A 5-µl sample of the solution was injected into the gas chromatograph. Each analysis was carried out in duplicate.

RESULTS AND DISCUSSION

The recovery of fluorouracil from the ion-exchange procedure based on absorbance at 264 nm is shown in Fig. 1. Because of some leaching from the resin of impurities that absorb in the 260–265-nm region, a blank must be run using the same samples of washed resins. Nevertheless, a method based on absorbance at 264 nm was found to be suitable for biological fluids only for concentrations of 100 µg or higher. For lower concentrations (up to 1 µg/ml), a microbiological assay was used and it gave the expected recovery within the limits of accuracy characteristic of this meth-

[1] Schleicher and Schuell No. 740-E.
[2] Dowex-50, Biorad Laboratories, Richmond, Calif.
[3] Amberlite IR-45, Biorad Laboratories, Richmond, Calif.
[4] Varian model 2100 (Varian Aerograph, Walnut Creek, Calif.) equipped with a Varian model A-25 strip-chart recorder.
[5] Column packing prepared by M. R. J. Perchalski, Medical Research, VA Hospital, Gainesville, Fla.

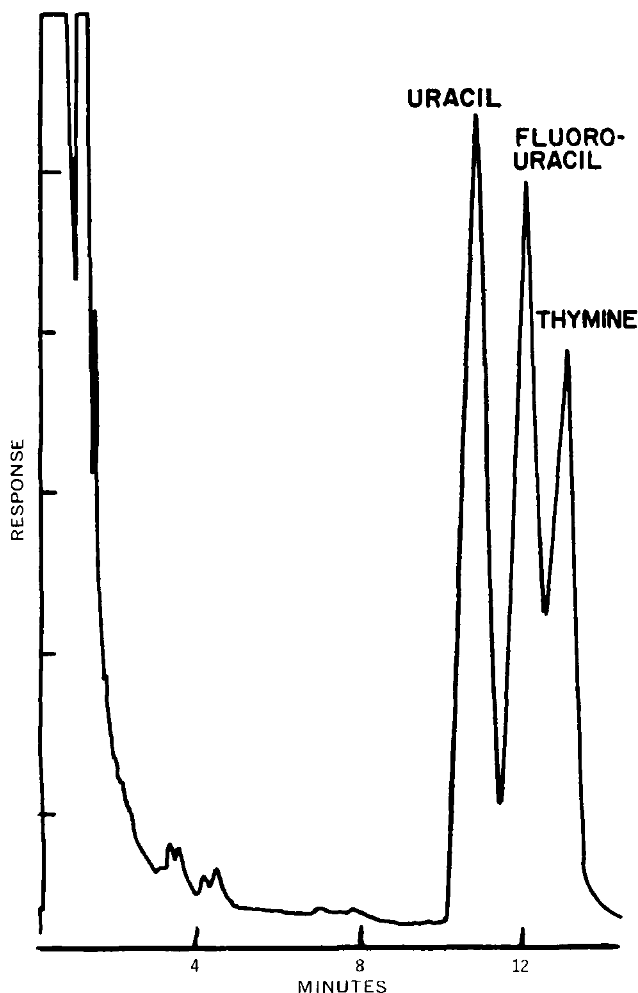

Figure 5—*Gas chromatogram of uracil, fluorouracil, and thymine with the addition of trimethylanilinium hydroxide. Flow rate for helium in this experiment was 27 ml/min.*

od (±20%) (Table I). The applicability of the ion-exchange procedure in the lower concentration range was also tested using GLC (Table I).

Figure 2 shows the standard curve for the GLC determination of fluorouracil after extraction from aqueous solutions. Recovery of added fluorouracil to a urine sample is shown in Fig. 3. The efficiency of extraction was approximately 85%, as indicated by Cohen and Brennan (4).

To establish the identity of the peak corresponding to flash-methylated fluorouracil, it was compared with the peak obtained from fluorouracil methylated with diazomethane. The two showed the same retention time of 7.5 min at the column oven temperature of 125°. Figure 4 shows a typical chromatogram of fluorouracil after extraction from urine sample and flash methylation.

Since biological fluids are likely to contain related pyrimidines such as uracil or thymine, their separation from fluorouracil was studied using these procedures. The three could be separated when the column temperature was maintained at 120°. A typical chromatogram is shown in Fig. 5. The separation of these three components could be effected even more readily when the mixture was subjected to flash ethylation with triethylanilinium hydroxide followed by GLC at a column oven temperature of 100°.

Although the extraction method is more convenient for the GLC assay, the ion-exchange method of recovery has certain advantages. It is capable of providing purer samples of fluorouracil as, for example, when recovery of labeled samples is desired. It is also capable of greater selectivity, especially when separation from certain basic or neutral constituents (such as antibiotics) is necessary.

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