5-Fluorouracil and Derivatives in Cancer Chemotherapy: Determination of 5-Fluorouracil in Blood

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Abstract □ The current work describes a dialysis technique which separates 5-fluorouracil from interfering blood components and allows for subsequent GC determinations. The parameters affecting the dialysis and GC are presented. The results indicate that 5-fluorouracil is present in the free and bound states in blood, and the method is capable of distinguishing between them.

Keyphrases □ 5-Fluorouracil—dialysis separation, GC determination from blood □ Dialysis—separation of 5-fluorouracil from blood components, GC determination □ GC—determination of 5-fluorouracil in blood

The use of 5-fluorouracil in the treatment of cancer was summarized previously by Heidelberger (1). A review of the history of this drug shows that biopharmaceutic studies have been hampered by the lack of analytical procedures suitable for the large number of assays such work requires. Clarkson *et al.* (2) reported both microbiological and spectrophotometric methods of analysis. The former suffers from the problems associated with these methods in general, and the latter does not accurately determine the drug at the low level found in blood and tissues. We wish to report a GC method suitable for use in clinical studies.

RESULTS AND DISCUSSION

General Approach—Initial tests in this laboratory indicated that 5-fluorouracil could be detected and accurately determined as the trimethylsilyl derivative by GC. The problem, as with most analytical methods, was the separation of the drug from aqueous solutions and from blood and human plasma.

The solubilities noted in Table I show 5-fluorouracil to be relatively insoluble in nonpolar water-immiscible solvents and relatively soluble in polar organic solvents but, unfortunately, these are also miscible with water. To utilize these solvents, recovery attempts were made by freeze drying samples of plasma and aqueous solutions containing 5-fluorouracil and placing them on diatomaceous earth[1] columns packed in pyridine. In the case of the "clean" aqueous samples, recovery was quantitative as measured by GC but only traces were recovered from the plasma samples. Modifications utilizing different solvents such as methanol, ethanol, and acetone, in which 5-fluorouracil solubility is also appreciable, failed to improve the recovery.

This data suggested two possibilities: (*a*) the 5-fluorouracil was being physically occluded by the proteins during the drying process, and (*b*) the 5-fluorouracil was interacting with the proteins to form nonextractable complexes. After further trials it was decided to attempt to separate the 5-fluorouracil from the proteins by dialysis.

Separation of 5-Fluorouracil by Dialysis—Trial dialyses were conducted using 50 mcg./ml. 5-fluorouracil in an isotonic solution at pH 7.4 to establish that 5-fluorouracil itself dialyzed. The experiments were carried out in cells as shown in Fig. 1.

The results showed that the drug reached a 50:50 equilibrium in less than 6 hr. at 25°. To establish a safety factor to account for the viscosity increase in blood and plasma, a 22-hr. equilibration period was chosen for subsequent work.

[1] Celite 545.

Table I—Solubility of 5-Fluorouracil in Various Solvents at 25°

Solvent	Solubility, g./l.
Water	11.78
Methanol	3.76
Acetone	3.48
Dioxane	2.67
Ethanol	3.5×10^{-1}
Diethyl ether	1.1×10^{-1}
Isopropyl ether	2.6×10^{-2}
Chloroform	1.4×10^{-2}
Carbon tetrachloride	1.2×10^{-3}
Cyclohexane	4.4×10^{-4}

Identical experiments using plasma at pH 7.4 plus 30 mcg./ml. of 5-fluorouracil on one side of the dialyzing membrane and isotonic sodium chloride solution on the other side gave approximately 80% recovery of the drug. This result suggested that the 5-fluorouracil was being partially bound as a nondialyzable fraction. Similar results were obtained with samples of whole blood "spiked" with 5-fluorouracil.

On the assumption that the drug was bound, various experiments were conducted to determine if the addition of sodium chloride, sodium lauryl sulfate, guanidine hydrochloride, and trichloroacetic acid would liberate the 5-fluorouracil. In all cases the yields were not improved. Previous workers (3) demonstrated that complexation phenomena can be highly species dependent. Since 5-fluorouracil can be considered a weak acid, a number of dialysis experiments were conducted using plasma adjusted to pH values between 7 and 10 with appropriate pH value buffers. The apparent pKa of 5-fluorouracil was estimated to be 8.0 based upon spectrophotometric determinations. If the change in 5-fluorouracil species were important to the binding, it would be manifested as a marked change in recovery in the pH range of 7–9. The results are shown in Fig. 2. It is readily apparent that the change in pH resulted in a marked increase in "free" drug in the system. In fact, at pH values above 9.5, essentially all the drug was recovered by the dialysis method.

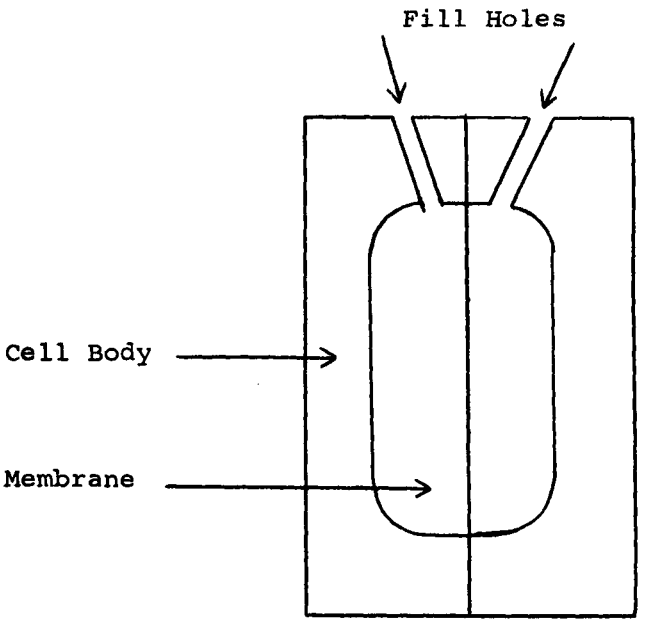

Figure 1—*Dialysis cell for 5-fluorouracil recovery.*

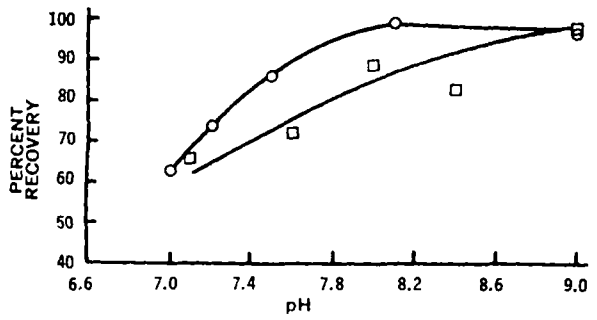

Figure 2—*pH recovery profile of 5-fluorouracil from plasma* (○) *and blood* (□) *at a concentration of 30 mcg./ml. Dialysis and GC procedures are described in the text.*

In parallel experiments on whole blood from volunteers, similar results were obtained with some notable differences. Recoveries at lower pH values were less than those observed with plasma. In examining the two experiments, it was apparent that the plasma and blood samples differed grossly by the absence or presence of cells. Subsequent experiments with whole blood containing 5-fluorouracil, as shown in Fig. 2, indicated that a significant fraction of the drug was bound to the blood cells. This was shown by adding the drug to blood, centrifuging the sample, and assaying the resulting cell-free plasma under conditions that would yield total recovery.

Studies to characterize the overall interactions are currently underway.

GC Determination and Recoveries—As might be expected, the direct GC of 5-fluorouracil was not feasible. Excellent peaks and linearity between peak area and concentration were achieved by formation of the trimethylsilyl derivative. The derivative was formed rapidly at room temperature and was stable if protected from moisture. In the case of dialysates from blood and plasma samples, it was found that an interfering peak developed if the sample was heated or allowed to stand for more than 20 min. Consequently, the derivative was formed simply by adding the silylating reagent and allowing the reaction to proceed at room temperature for 10 min. The completeness of the reaction was verified by following the change in peak area with time at both room temperature and 50°. No further increase was observed after 10 min. at either temperature.

Total recovery of 5-fluorouracil from spiked blood samples was excellent. Typical recoveries are illustrated in Table II.

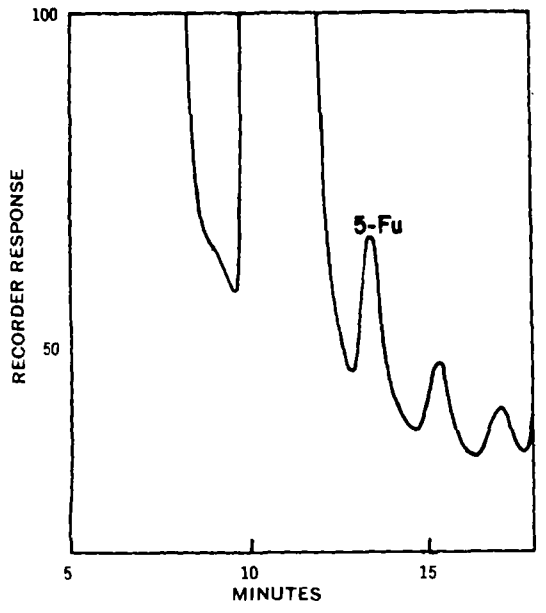

Figure 3—*GC of 5-fluorouracil (5-Fu) recovered from blood at a concentration of 5 mcg./ml. Dialysis and GC procedures are described in the text.*

Table II—Typical Recoveries of 5-Fluorouracil from Blood

Concentration in Blood, mcg./ml.	Percent Recovery[a]
40	98.1
20	99.2
10	100.6
5	97.9

[a] Averages of four determinations at each concentration.

Tests conducted to evaluate the sensitivity of the method showed that drug concentrations down to 1 mcg./ml. could be detected. Figure 3 shows a typical chromatogram of the dialysate of blood samples at 5 mcg./ml. of drug added.

Currently, this procedure is being applied to clinical studies of patients on 5-fluorouracil therapy. These data will be reported subsequently.

EXPERIMENTAL

Method for Total Recovery of 5-Fluorouracil

Collection of Blood Samples—Blood samples were collected for analysis in heparinized Vacutainers[2], having a capacity of 10 ml. The Vacutainers were gently agitated immediately after withdrawing the blood and were used within 30 min.

Dialysis Procedure—*Cells*—Acrylic plastic cells, consisting of two interchangeable compartments (half-cells) with polished surfaces, separated by a semipermeable membrane were used. Solutions were introduced into the assembled cells through screw holes; each compartment was then sealed with a threaded nylon screw plug[3].

Membranes—Membranes were prepared from seamless regenerated cellulose dialysis tubing[4] according to the manufacturer's recommended procedure. The tubing was washed in running tap water for at least 4 hr., heated at 80° in a 0.3% Na_2S solution for 10 min., washed with lukewarm water, and placed in a 0.2% H_2SO_4 solution for 10 min. at room temperature. The acid was then removed by washing with distilled water, and the tubing was cut into strips of single thickness large enough to cover the cell compartments. The membranes were stored in distilled water under refrigeration and were usable for 1 week.

Dialysis—Cells were assembled immediately before filling to prevent the membrane from drying out. By using gas-tight syringes (Hamilton) of 5-ml. capacity, 5.0 ml. of 0.05 *N* sodium hydroxide was added to one compartment of the cell and 4.8 ml. of blood was added to the other compartment. By using a 500-μl. gas-tight syringe (Hamilton), 200 μl. of an aqueous standard solution of 5-fluorouracil was then added to the compartment containing blood, and the compartments were sealed with nylon screw plugs.

The cells were gently agitated on a mechanical shaker for 22 hr., and the dialysates were then removed for GC analysis.

GC—*Evaporation of Sample*—Three milliliters of the dialysate was pipeted into a 50-ml. round-bottom flask with 24/40 ground glass joint, 0.3 ml. of 1 *N* HCl was added, and the sample was evaporated to dryness on a rotary evaporator under reduced pressure with the aid of a water bath at 40°.

Preparation of Silyl Derivative—The residue from evaporation was taken up in 200 μl. of pyridine[5] (used without further purification) delivered by a 500-μl. gas-tight syringe (Hamilton). By using a similar syringe, 200 μl. of *N,O*-bis(trimethylsilyl)acetamide[6] was added. The flask was tightly stoppered, and the mixture was gently swirled for 10 min. at room temperature.

GLC Conditions[7]—The column used was glass, U-shaped, 2.43 m. (8 ft.) × 4 mm. It was packed with 3% methyl silicone gum[8] on

[2] B-D No. 3200 KA V, Becton, Dickinson, and Co., Rutherford, N. J.

[3] Similar cells are available from The Chemical Rubber Co., Cleveland, Ohio, Catalog No. 9375/407 C.

[4] Union Carbide Corp., Films and Packaging Div., Chicago, Ill.

[5] Baker Analyzed Reagent, J. T. Baker Chemical Co., Phillipsburg, N. J.

[6] BSA Specially Purified Grade, Pierce Chemical Co., Rockford, Ill.

[7] A Barber-Coleman Series 5000 Selecta-System was used with a flame-ionization detector.

[8] SE-30, Anspec Co., Inc., Ann Arbor, Mich.

acid-base-washed, silane-treated, flux-calcined diatomaceous earth[9], 80/100 mesh. The column was conditioned 48 hr. at 275°. The operating conditions were: column temperature, 105°; injector temperature, 190°; detector temperature, 250°; and carrier gas, N_2, 50 ml./min.

Sample Injection—Four to eight microliters was injected at 105°, and the chromatogram was run until the 5-fluorouracil peak appeared (approximately 12 min.). *Before injecting the next sample*, it was necessary to increase the column temperature to 175° for 5 min. to drive off other components present in the dialysate having much longer retention times than 5-fluorouracil, which would otherwise interfere with subsequent determinations.

Procedure for Blood and Plasma pH Profiles

Blood Samples—The blood samples were collected in heparinized Vacutainers of 10-ml. capacity, pooled, and mixed prior to pH adjustment.

Plasma Samples—Standard plastic transfer packs containing plasma with anticoagulant citrate dextrose solution USP were used. Subsequent pH adjustment, dialysis, and GC were exactly as described for blood.

Adjustment of pH—Approximately 15 ml. of blood or plasma in a beaker was adjusted to the desired pH by dropwise addition of 1 *N* HCl or 1 *N* NaOH with the aid of a pH meter[10] equipped with a glass–calomel combination electrode. The sample was immediately transferred to a dialysis cell.

Dialysis—Dialysis cells were prepared as described previously and filled immediately after assembly. To one compartment, 5.0 ml. of blood or plasma was added with a 5.0-ml. gas-tight syringe (Hamilton). To the other compartment, 4.80 ml. of 0.9% sodium chloride solution was added, along with 200 μl. of a standard 5-fluorouracil solution containing 0.75 mcg./μl. The cells were sealed with nylon screw plugs and dialyzed 22 hr. as described previously.

Evaporation of Samples, Preparation of Silyl Derivatives, and GLC Conditions—Procedures were the same as described in the description of method for total recovery of 5-fluorouracil.

5-Fluorouracil Standard for GLC—The volume of standard 5-fluorouracil solution equivalent to the 5-fluorouracil present in the dialysate taken for assay was calculated as follows:

$$\text{volume of standard solution} = \text{ml. dialysate evaporated} \times \frac{\text{milliliters standard solution added to cell}}{10} = V_{sl} \quad \text{(Eq. 1)}$$

For example, with 3.0 ml. of dialysate taken for assay:

$$V_{sl} = 3.0 \times \frac{0.200}{10} = 0.060 \text{ ml.} \equiv 60\ \mu\text{l.} \quad \text{(Eq. 2)}$$

This volume, V_{sl}, of 5-fluorouracil standard solution was transferred to a 50-ml. round-bottom flask with a 100-μl. syringe (Hamilton); the 0.3 ml. of 1 *N* HCl was added, and the solution was evaporated to dryness on a rotary evaporator. Subsequent preparation of the silyl derivative and GC was carried out exactly as described for the samples.

Calculation of Percent Recovery—Peak areas for standards and samples were determined with a planimeter. The percent recovery of 5-fluorouracil is given by:

$$\frac{\text{area of sample peak}}{\text{area of standard peak}} \times 100 = \text{percent recovery} \quad \text{(Eq. 3)}$$

[9] Gas Chrom Q, Anspec Co., Inc., Ann Arbor, Mich.
[10] Beckman Expandomatic.

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Mechanism of Action of Tablet Disintegrants: Correlation of Tablet Mean Pore Diameter and Porosity

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Abstract □ Data from a previous study were used to obtain correlations between log mean pore diameter and porosity of tablets. Cornstarch appears to modify this relationship of mean pore diameter and porosity in magnesium oxide and magnesium trisilicate tablets. Dilution of salicylamide with cornstarch and aspirin by three different disintegrants (cornstarch, cation-exchange resin, and waxy maise starch) had little effect on this relationship. The one exception was 10% cationic-exchange resin–90% aspirin mixture. The equation, $\log y = mX + b$, where y is the mean pore diameter and X the porosity, can be used to calculate the mean pore diameter from the more easily obtained porosity.

Keyphrases □ Tablet disintegrants—mechanism of action, correlation of tablet mean pore diameter and porosity □ Disintegrants, tablets—mechanism of action, correlation of tablet mean pore diameter and porosity □ Porosity, tablets—correlated with tablet mean pore diameter □ Pore diameter (mean), tablets—correlated with porosity

Previously the effect of different variables on tablet porosities and tablet mean pore diameters was reported (1). The effect of compression pressure (three levels) and cornstarch concentration (four levels) on porosity and mean pore diameter using four different drugs was studied. A second experiment was used to determine