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ACKNOWLEDGMENTS AND ADDRESSES

Received August 2, 1976, from the *Instituut voor Hygiëne en Epidemiologie, Department of Farmatoxicologie, Afdeling Farmacopee en Standaarden, Juliette Wytsmanstraat 14, 1050 Brussels, Belgium.*

Accepted for publication December 23, 1976.

The author thanks Apr. A. van den Bulcke (I.H.E.-Brussel) and Dr. W. Van Gampelaere (Sidmar Gent) for valuable advice and Dr. J. W. Lightbown, Director, National Institute for Biological Standards and Control, Hampstead, London, England, for careful editing of this paper. The author also thanks Mr. Legrand (I.H.E.-Brussel) for the statistical analysis and Gist Brokades, Holland, for gifts of oxytetracyclines.

Kinetics and Mechanisms of Drug Action on Microorganisms XXIII: Microbial Kinetic Assay for Fluorouracil in Biological Fluids and Its Application to Human Pharmacokinetics

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Abstract □ The apparent first-order generation rate constant, k_{app}, of *Escherichia coli* is a function of fluorouracil concentration, C, *i.e.*, $1/(k_0 - k_{app}) = k_1(1/C) + k_2$, where k_0 is the constant in the absence of drug. A routine procedure, using only six counts to establish a daily calibration curve (at 125 and 225 min after medium inoculation and with 0, 15, and 30 ng of fluorouracil/ml), assayed unknown drug concentrations in plasma or urine diluted to 15–30 ng/ml with standard deviations of 12%. Uracil completely antagonized the inhibitory action of fluorouracil but not of 5-fluorouridine, 5-fluorodeoxyuridine, chloramphenicol, or tetracycline, so fluorouracil could be assayed in the presence of the latter compounds by kinetic studies with and without uracil. Fluorouracil degraded in generating cultures of *E. coli*, and the drug effects on filtered organisms persisted on their inoculation into drug-free medium. Potential products of fluorouracil solvolysis, barbituric and isobarbituric acids, had no significant effect on *E. coli* generation. Human plasma protein binding of fluorouracil averaged 10%. The pharmacokinetics of intravenously administered fluorouracil to cancer patients showed a dose-dependent two-compartment body model with a faster terminal phase of elimination at the lower dose. Total and metabolic plasma clearances increased with a decreasing intravenous dose and exceeded hepatic plasma flow to indicate extrahepatic metabolism. The initial distribution phase and the renal clearance did not appear dose dependent, and the terminal semilogarithmic plots of plasma levels against time were linear for both 0.5- and 1.0-g doses. A possible explanation is product- or metabolite-inhibited metabolism. Infusion studies of 1.0 g of fluorouracil showed increased metabolic clearances to confirm dose dependency and to be consistent with this postulate. The oral absorption of unchanged fluorouracil was highly variable between 1 and 15% of the administered dose, showing a large first-pass effect.

Keyphrases □ Fluorouracil—microbial kinetic analysis in biological fluids, pharmacokinetics in humans □ Microbial kinetics—analysis, fluorouracil in biological fluids □ Pharmacokinetics—fluorouracil in humans, studied using microbial kinetic analysis □ Antineoplastic agents—fluorouracil, microbial kinetic analysis in biological fluids, pharmacokinetics in humans

Facile, sensitive, and nontedious assays for fluorouracil in biological fluids are needed for the proper and routine evaluation of its pharmacokinetics and for the evaluation of proper dosage regimens. The bioavailability on oral administration also needs to be assessed. This paper reports the development of a microbial kinetic assay of fluorouracil in biological fluids and its application to human pharmacokinetics.

The GLC analysis of fluorouracil in biological fluids requires its separation and derivatization. Separation by 22-hr dialysis with subsequent trimethylsilylation permitted GLC analysis sensitive to drug concentrations down to 1 μg/ml (1). Separation for a similar GLC analysis was also effected by an 80% extraction from biological fluids buffered at pH 6 into 16% 1-propanol in ether (2). A sensitivity to drug concentrations down to 0.5 μg/ml was shown (2), and a reproducibility of ±5% was claimed. An ion-exchange recovery of the drug, with a subsequent GLC flash-methylation technique using trimethylanilinium hydroxide, also was reported; the apparent sensitivity was 1.0 μg/ml of urine (3). A recently published isotope dilution mass fragmentographic assay (4) claimed a standard deviation of ±6% for 10 ng/ml extracted from plasma.

A sensitive classical disk–agar plate microbiological assay based on the correlation of zones of inhibition of *Streptococcus faecalis* with fluorouracil was developed and applied to the time course of the drug in plasma and urine; the estimated error was ±25%/zonal measurement (5).

Fluorouracil lessens the generation rate of logarithmic phase microorganisms, and the degree of decrease can be related to the drug concentration (6). This ability provided the basis for a sensitive assay of fluorouracil in biological fluids below the drug levels necessary for complete inhibition of generation. The tedious and time-consuming separation procedures vital for GLC assays and the time lag necessary for organism incubation in the disk–agar plate studies were avoided. Also, since the inhibitory effect of fluorouracil on microbial generation can be reversed by the addition of excess uracil, fluorouracil can be specifically assayed in the presence of other antibacterial agents that do not show this reversibility. The assay was applied to the study of fluorouracil pharmacokinetics in humans.

EXPERIMENTAL

Organism—Replicate slants of *Escherichia coli* (ATCC 12407) were prepared from a single colony and stored at 4°.

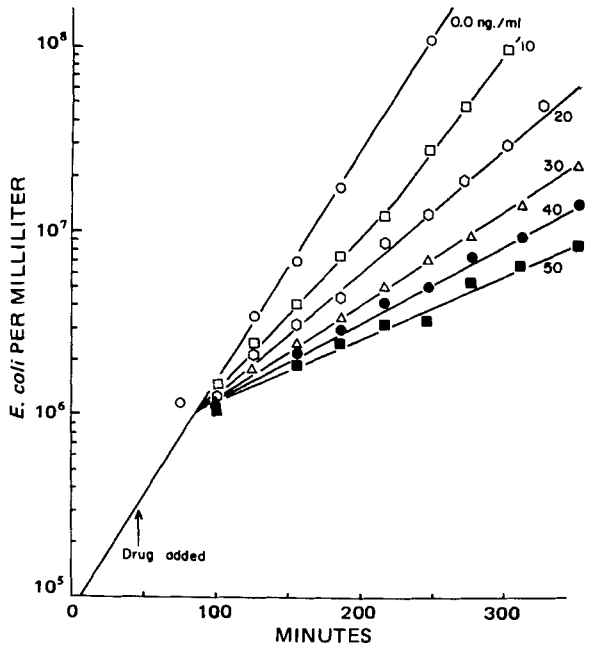

Figure 1—*Semilogarithmic plots of* E. coli *per milliliter against time in Anton's medium as affected by the stated fluorouracil concentrations at 37.5° and pH 7.*

Culture Medium and Materials—Anton's medium (7) was used with 3% casamino acids[1]. The media were passed through a 0.45-μm membrane filter[2] and autoclaved at 120° for 15 min. The final pH of the buffered medium was 7.0.

The stable (8) neutral aqueous solutions of fluorouracil[3], floxuridine[4], 5-fluorouridine[4], and uracil[5] were used in *in vitro* studies and could be stored indefinitely at room temperature after being autoclaved for 15 min. Chloramphenicol[6] was dissolved in sterile water and used without autoclaving.

Monitoring Generation Rates—The kinetics of bacterial generation in the logarithmic growth phase were monitored by counting[7] the numbers of cells with time (6, 9, 10). Predetermined volumes of a 37.5° balanced growth culture of *E. coli*, typically 9.9 ml of 3.5×10^5 cells/ml, were pipetted into 25-ml loosely capped conical flasks in a 37.5° constant-temperature bath equipped with a shaker. The biological fluids to be assayed were diluted with sterile water, medium, ultrafiltered plasma, or urine without drug; then 0.100 ml of fluorouracil was added by a micropipet to give final concentrations of 10–50 ng/ml in the cultures after 15 min of culture growth. The numbers of organisms at a specific time were obtained by micropipetting 0.50 or 1.0 ml into known volumes of a previously filtered[2] sterile solution of 0.85% sodium chloride and 1.0% formaldehyde to obtain counts in the range of 10,000–20,000/50 μl[7].

Construction of Calibration Curves—The apparent generation rate constants, k_{app}, of fluorouracil-affected cultures were obtained from the slopes of the linear plots (Fig. 1) of the natural logarithm of the number, N, of organisms per milliliter against time, t, in the 100–250-min interval after culture inoculation in accordance with (6, 9, 10):

$$\ln N = k_{app}t + \text{intercept} \qquad \text{(Eq. 1)}$$

Plots (Fig. 2) of these apparent generation rate constants, k_{app}, against fluorouracil concentration, FU, were nonlinear. Linear plots (Fig. 3) could be obtained (8) in accordance with:

$$y = 1/(k_0 - k_{app}) - 1/k_0 = k_1(1/[\text{FU}]) + k_2 \qquad \text{(Eq. 2)}$$

where k_0 is the first-order generation rate constant in the absence of drug, and $k_2 = 0$ for a simple saturable receptor site model (11).

Calibration curves were prepared for fluorouracil in plasma ultrafiltrate at various dilutions with sterile water, medium, or fluorouracil-free plasma ultrafiltrate. The plasma ultrafiltrate was prepared by ultrafiltration of plasma through a filter cone[8] in a centrifuge at 1600 rpm. Al-

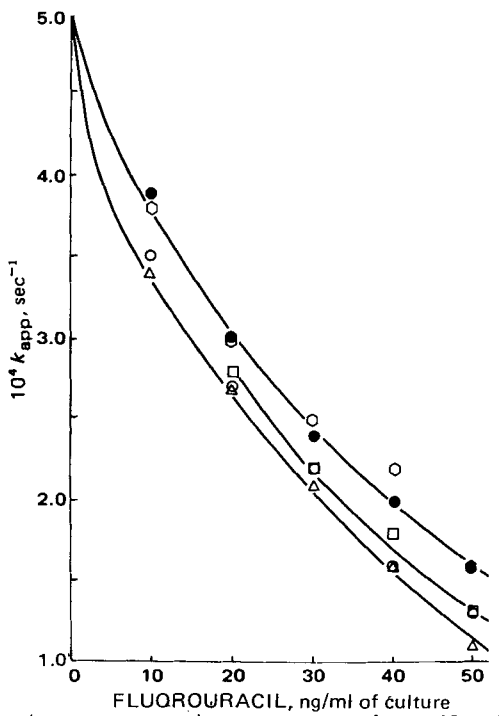

Figure 2—*Apparent generation rate constant,* k_{app}, *of* E. coli *in seconds*$^{-1}$ *at 37.5° and pH 7 as a function of fluorouracil concentration in Anton's medium and in the presence of diluted plasma ultrafiltrate. A 0.100-ml aliquot of plasma ultrafiltrate containing fluorouracil, diluted 1:2000 (○), 1:400 (△), 1:100 (□), 1:20 (●), and 1:5 (○) with sterile water or medium, was added to 9.9 ml of the generating culture.*

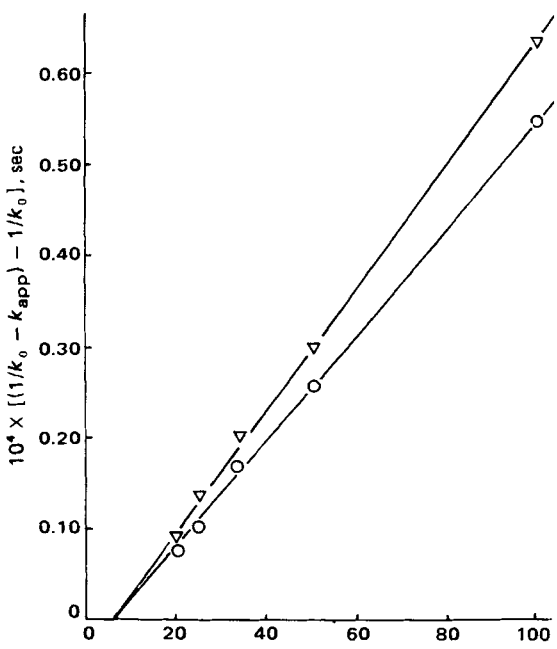

Figure 3—*Typical linear plots of a function,* $1/(k_{app} - 1/k_0) - 1/k_0$, *of the apparent generation rate constants at 37.5° and pH 7 in the presence,* k_{app}, *and absence,* k_0, *of fluorouracil against the reciprocal of the fluorouracil concentration in Anton's medium in the presence of diluted plasma ultrafiltrate. An aliquot (0.100 ml) of the plasma ultrafiltrates containing fluorouracil, diluted 1:2000 (○) and 1:5 (▽) with sterile water or medium, was added to 9.9 ml of the generating culture.*

[1] Difco Laboratories, Detroit, Mich.
[2] Millipore 0.45-μm HA filters.
[3] Hoffmann-La Roche Inc., Nutley, N.J.
[4] Courtesy of J. Fox, Sloan Kettering, Institute for Cancer Research, New York, N.Y.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] Boehringer Mannheim, West Germany.
[7] Coulter counter models B and ZBI, Coulter Electronics Co., Hialeah, Fla.
[8] Ultrafiltration membrane cones 2100 CF 50, Amicon Corp., Lexington, Mass.

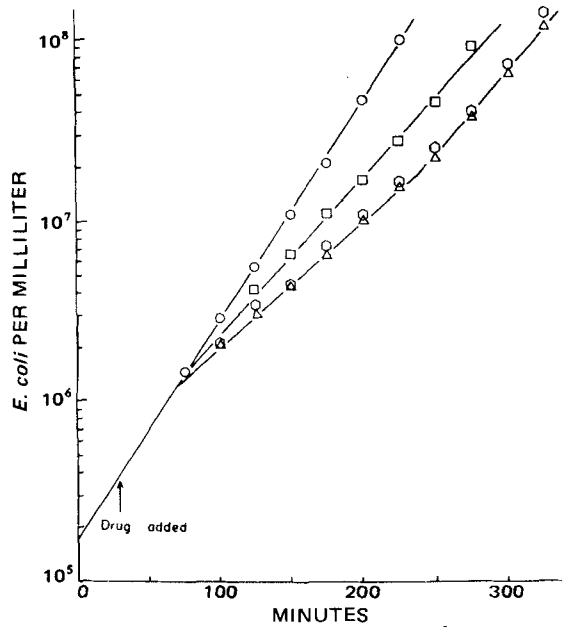

Figure 4—*Semilogarithmic plots of* E. coli *per milliliter against time in Anton's medium at 37.5° and pH 7 for 20 ng of fluorouracil/ml in the presence of diluted urines. An aliquot (0.100 ml) of the diluted urines containing fluorouracil, diluted 1:2000 (△), 1:400 (○), and 1:100 (□) with sterile water, was added at the time designated by the arrow to 9.9 ml of the generating culture. The other curve (○) is for the generation of a drug-free culture.*

though the generation rates of *E. coli* in the absence of fluorouracil were not significantly different in ultrafiltered plasma diluted 100- and 20-fold, the generation rates of fluorouracil-affected organisms were significantly modified by the content of plasma ultrafiltrate that had an antagonistic effect on fluorouracil inhibition of microbial generation rates (Figs. 2 and 3).

Therefore, all plasma samples to be assayed for fluorouracil were ultrafiltered and diluted to the proper range for assay with previously prepared ultrafiltered plasma without fluorouracil, preferably from the same individual. However, there were no significant differences among the generation rates of fluorouracil-affected cultures for the same amount of fluorouracil-free plasma ultrafiltrate from different individuals. The calibration curve constructed for aqueous solutions of fluorouracil could be employed when plasma samples were diluted 200-fold or more with sterile water.

The addition of urine to fluorouracil-affected generating cultures antagonized the generation rate inhibition of fluorouracil. If a urine sample could be diluted more than 1:400 (Fig. 4) with water to obtain 15–30 ng/ml, a calibration curve constructed from aqueous solutions of fluorouracil could be used. Dilution of urine containing fluorouracil with blank urine not containing any fluorouracil minimized this error, but there were significant differences in the antagonistic action of different blank urines from the same individual. A modified procedure was applicable to the assay of urine samples that could not be diluted more than 1:400. Fluorouracil itself was used as an internal standard.

A reasonable first approximation of k_2 in Eq. 2 is zero (11) (see also the intercepts in Fig. 3); if the total concentration of fluorouracil, $[FU]_T$, consists of the sum of the unknown concentration in the urine, $[FU]_U$, and that due to the added concentration of standard, $[FU]_S$, then, from Eq. 2:

$$y = \frac{1}{k_0 - k_{app}} - \frac{1}{k_0} \simeq \frac{k_{app}}{k_0(k_0 - k_{app})} = \frac{k_1}{[FU]_S + [FU]_U} \quad \text{(Eq. 3)}$$

which can be rearranged to:

$$\frac{1}{y} = k_0 \frac{(k_0 - k_{app})}{k_{app}} = \frac{1}{k_1}([FU]_S + [FU]_U) \quad \text{(Eq. 4)}$$

so that:

$$[FU]_S = k_1\left(\frac{1}{y}\right) - [FU]_U \quad \text{(Eq. 5)}$$

or:

$$[FU]_S = k_1 k_0\left(\frac{k_0 - k_{app}}{k_{app}}\right) - [FU]_U \quad \text{(Eq. 6)}$$

The value for the generation rate constant, k_0, in the absence of fluorouracil could be estimated from the slope of the logarithm of a generating culture against time when urine without drug was added to the culture. The values of y (Eq. 5) or $(k_0 - k_{app})/k_{app}$ (Eq. 6) were calculated from this k_0 and the values of the apparent generation rate constants, k_{app}, obtained at three different concentrations of $[FU]_S$ added to the sample urine and when $[FU]_S = 0$. Plots of the added concentrations of fluorouracil, $[FU]_S$, against $1/y$ (Eq. 5) or $(k_0 - k_{app})/k_{app}$ (Eq. 6) provided a straight line where the negative of the intercept was an estimate of the fluorouracil in the urine to be assayed, $[FU]_U$. This approximate procedure for dilute drug concentrations in urine had an average error of ±18% in four separate studies.

Procedures for Routine Assay of Fluorouracil in Biological Fluids—The consistent linearity of the plots of the natural logarithm of organisms against time from 100 to 250 min after drug addition to the generating culture (Fig. 1) permitted the routine estimation of slopes (or generation rate constants, k_{app}) from the counts of two samples obtained at 125 and 225 min after inoculation of 3×10^5 organisms.

The linearity of calibration curves in accordance with Eq. 2 (Fig. 3) permitted the daily estimation of k_1 and k_2 parameters from k_{app} values obtained at only two fluorouracil concentrations and from the k_0 value obtained from a drug-free generating culture where equivalent amounts of drug-free plasma ultrafiltrate or drug-free urine had been added. The two concentrations of drug, [FU], chosen were 15 and 30 ng/ml, straddling a concentration of 20 ng/ml that experimentally gave the minimum standard deviation of the calculated y parameter of Eq. 2. Three replicate assays were made at each concentration.

The slope, k_1, and intercept, k_2, of the line between these two points were obtained from the plot of the averaged y values against 1/[FU] in accordance with Eq. 2. The amount of fluorouracil in the diluted biological sample could be calculated from its observed k_{app} values (average of three replicates) and the derived y parameter from a rearranged Eq. 2:

$$[FU] = k_1/(y - k_2) \quad \text{(Eq. 7)}$$

All assayed samples were diluted into the response range given by the 15 and 30 ng/ml. The unknown original concentration was calculated from the necessary dilution.

This method of routine assay was challenged on each of 5 separate days, with a new calibration curve on each day. An aqueous solution of fluorouracil was diluted so that the drug concentration in the medium was 20 ng/ml. Three separate assays were performed on each day, and the averages ± *SD* for each day were: 20.3 ± 0.5, 22.1 ± 0.4, 20.8 ± 2.5, 19.5 ± 1.6, and 24.3 ± 0.9. The overall average was 21.4 ± 1.9, $n = 15$, with a standard deviation (in percent of the mean) of 7%. A similar study for plasma solutions of fluorouracil diluted 10-fold on addition to the culture had an average error for 20 ng/ml of 12%.

An analysis of variance of the calibration curves showed a significant variation among days. Thus, it was necessary to construct a daily calibration curve for the routine assay.

Antibacterial Effects of Derived Nucleosides and Potential Metabolites of Fluorouracil and Their Possible Inhibition by Uracil—The possible inhibition of the antibacterial effect of fluorouracil, 5-fluorouridine, and floxuridine on microbial generation by uracil was studied in similar systems, Anton's medium and 2.5×10^5/ml inocula. Similar studies were conducted with mixtures of fluorouracil, tetracycline, and/or chloramphenicol with and without uracil. The antimicrobial effects of potential products of fluorouracil solvolysis, barbituric and isobarbituric acids, were studied in the same system. The stability of fluorouracil in refrigerated whole blood, plasma, ultrafiltered plasma, and urine was monitored over 6 days.

Effect of Filtrates from Fluorouracil-Inhibited Microbial Cultures on Fresh Organisms and of Transference of Affected Organisms to Fresh Medium—A culture in logarithmic growth was divided into five portions. One served as the control, and the other four had 30 ng of fluorouracil/ml. Each of the four cultures was vacuum filtered through a sterile membrane filter (0.45 μm) at 123, 153, 254, and 279 min, respectively, after the parent culture was prepared. The filtrates were stored at 4° overnight.

The organisms were washed with sterile medium at 37.5°. The membrane filter was submerged in 50 ml of fresh medium, and the flask was shaken vigorously. Aliquots of the cultures prepared from the organisms filtered at the various times were monitored for generation rates. Aliquots (9.7 ml) of cultures also were added to 0.30 ml of uracil solution to give a uracil concentration of 32 μg/ml, and the generation rates were monitored. In some cases 1 ml of these cultures was diluted 10-fold into fresh medium at 37.5° and the generation rates were monitored.

Aliquots (8.0 ml) of the stored filtrates were warmed to 37.5° and added to 2.0 ml of culture for a population of 4×10^5 organisms/ml. In some cases, fluorouracil was added 15 min after the addition of filtrate to the culture to increase the drug concentration by 20 ng/ml. The generation rates of these cultures were monitored.

Human Plasma Protein Binding of Fluorouracil—Human plasma (3–4 ml), adjusted to pH 5.8, 7.2, and 9.0 with dropwise addition of 0.5 *N* HCl or 0.5 *N* NaOH and containing 2-^{14}C-fluorouracil[3] at 100, 1.0, and 0.1 μg/ml, was ultrafiltered at 1000 rpm through cone filters[8] in a centrifuge for 15 min to ultrafilter 20% of the volume. The cones, with a 50,000 molecular weight cutoff, were previously soaked in distilled water for 1 hr and centrifuged for 10 min at 1500 rpm. Aliquots (200 μl) of the ultrafiltrate and original plasma were assayed with 10 ml of a scintillation fluid[9] by liquid scintillation spectroscopy[10]. The possible plasma quenching of the counts was challenged by adding 25 μl of 0.520 μCi/ml of 2-^{14}C-fluorouracil to 200 μl of plasma and plasma ultrafiltrate and counting. The plasma counts were within 1% of the ultrafiltrate, indicating no significant quenching by plasma proteins.

Fluorouracil binding to the filter cones was determined by again filtering plasma ultrafiltrates, adjusted to various pH values and with added 2-^{14}C-fluorouracil at 1000 rpm for 3 min to effect 50% ultrafiltration. Aliquots (200 μl) of the original ultrafiltrate and the filtered ultrafiltrates were added to 10 ml of scintillation fluid and counted in a liquid scintillation spectrophotometer[10].

Pharmacokinetics of Fluorouracil Administered to Human Patients—Three patients who were receiving fluorouracil[11] for diagnosed metastatic carcinomas gave their informed consent for these studies[12].

Tests including alkaline phosphatase, serum glutamic-oxaloacetic transaminase, serum glutamic-pyruvic transaminase, prothrombin time, total proteins, and albumins were normal except for elevated levels of serum glutamic-oxaloacetic transaminase and serum glutamic-pyruvic transaminase in Patient RC. Urinalyses were all normal. Electrocardiograms, chest X-rays, and blood pressures were normal, as were blood tests that included hematocrit, hemoglobin, white blood cells, leucocytes, differential, erythrocytes, and platelets. In some studies, the patients were given morphine or thorazine concurrently, but other anticancer drugs were not administered.

The patients were supine during all experiments. An arm vein was catheterized 2 hr prior to drug administration for blood sampling during the intravenous bolus and oral studies. Veins in both arms were catheterized for the infusion studies. Isotonic saline was infused through the indwelling catheter at the rate of 50 ml/hr for 2 hr before drug administration until the end of each study. The patient was water loaded orally with 10 ml/kg in the hour before drug administration.

Intravenous bolus administration was effected over 1 min (20 ml × 50 mg/ml) or 10 sec (10 ml × 50 mg/ml) in the opposite arm from the catheter, except for Patient JC where venipuncture was difficult. The infusion of the dose (1000 mg) in isotonic saline was effected in the arm opposite that used for sampling. The drug was normally administered orally in 100 ml of orange juice, except for the 1-g study in Patient JC where distilled water was used. Patients were fasted for 12 hr before and 2 hr after oral administration. Patient RC had nausea and vomiting on oral administration and could not be studied by this route.

Blood samples were withdrawn from the catheter used for saline drip after a 2-ml blood withdrawal for flushing purposes. They were added to iced heparinized tubes until centrifugation and separation of plasma. The plasma ultrafiltrates were stored at 4°. The pH and volume of a urine collection were measured, and a portion was ultrafiltered and refrigerated. Urine pH values were ~7.5, except for Patient RC whose urine pH ranged between 8.0 and 8.7.

RESULTS AND DISCUSSION

Fluorouracil Assay by Microbial Generation—The described method is rapid and sensitive. It does not necessitate the tedious separative techniques from biological fluids required prior to GLC analysis (1–4), nor is it as time consuming as the classical disk-plate assay (5). Its accuracy is of the same order (±5%) as GLC for a complete calibration curve and is ±12% for the routine method proposed. Its sensitivity, however, is four to 20 times greater than that claimed for GLC. Twenty samples can be analyzed by one operator within an 8-hr working day.

[9] Scinti-Verse, Fisher Scientific Co., Fair Lawn, N.J.
[10] Beckman liquid scintillation, Beckman Instruments, Fullerton, CA 92634.
[11] Injectable solution, Hoffmann-La Roche Inc., Nutley, N.J.
[12] The protocol was approved by the Committee for the Protection of Human Subjects and the Clinical Research Center Advisory Board of the J. Hillis Miller Health Center, University of Florida.

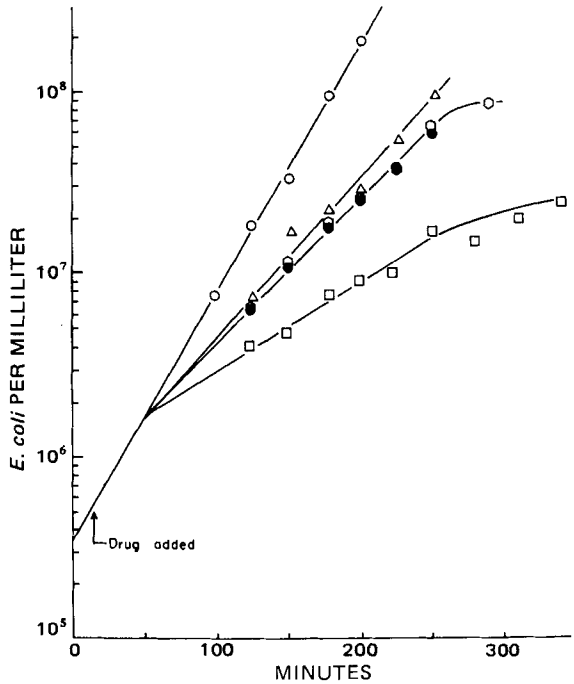

Figure 5—*Semilogarithmic generation curves of* E. coli *in Anton's medium at 37.5° and pH 7 without drug (○), with 20 ng of fluorouracil/ml (△), with 500 ng of chloramphenicol/ml (○), with the combination (□), and with the combination plus 10 μg of uracil/ml (●). These results are typical of assays in plasma and urine.*

Reversal of Fluorouracil Effect by Uracil in Presence of Tetracycline and Chloramphenicol—The inhibition of microbial generation effected by fluorouracil is reversed by uracil (6, 11). In Anton's medium, concentrations of uracil of 0.200 μg/ml completely reversed fluorouracil action at 0.020 and 0.030 μg/ml, where the generation rates in the 160 min after drug addition were 0.54 and 0.36, respectively, of the generation rate without fluorouracil.

Studies of microbial generation in the presence of no drug, 20 ng of fluorouracil/ml, 0.50 μg of chloramphenicol/ml, and the combination alone or with 10 μg of uracil/ml (Fig. 5) showed that the fluorouracil effect

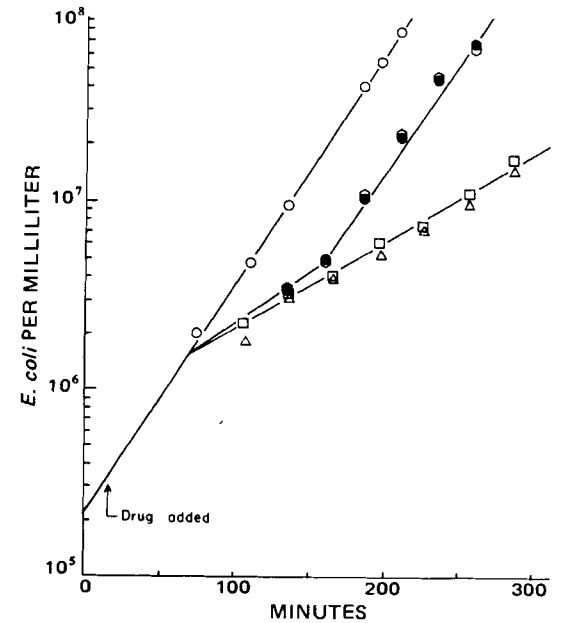

Figure 6—*Semilogarithmic generation curves of* E. coli *in Anton's medium at 37.5° and pH 7 without drug (○), with 9.5 ng of floxuridine/ml alone (○) and combined with 0.4 μg of uracil/ml (●), and with 60 ng of 5-fluorouridine/ml alone (□) and combined with 2.15 μg of uracil/ml (△).*

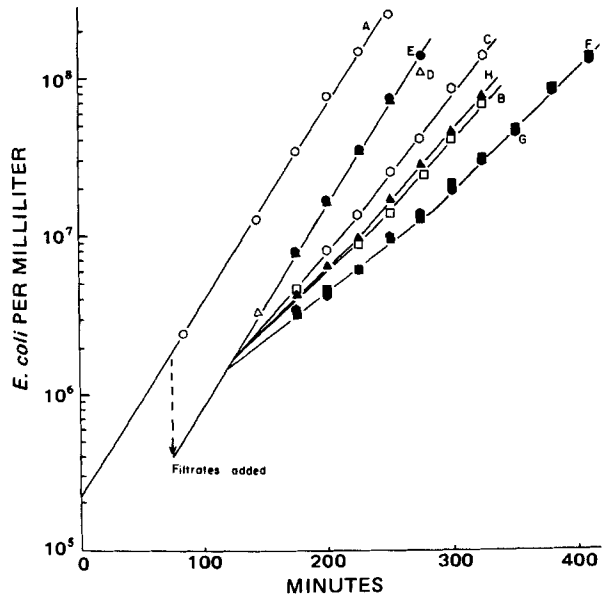

Figure 7—*Semilogarithmic plots of* E. coli *per milliliter against time at 37.5° and pH 7 when a fresh inoculum was introduced into the filtrates taken at various times from a fluorouracil-affected generating culture in Anton's medium. The curves and times after inoculation for the various filtrations were: B, 123 min (□); C, 153 min (○); D, 254 min (△); and E, 279 min (●). The generating culture of curve A (○) was diluted with the filtrates at the time indicated by the arrow to give the other generating curves. Curves F (■) and G (●) represent generating cultures with an additional 20 ng of fluorouracil/ml in media prepared from dilution of A with filtrates at 254 and 279 min, respectively. The drug was added at 90 min. Curve H (▲) represents a generating culture with 15 ng of fluorouracil/ml prepared from dilution of A with fresh Anton's medium.*

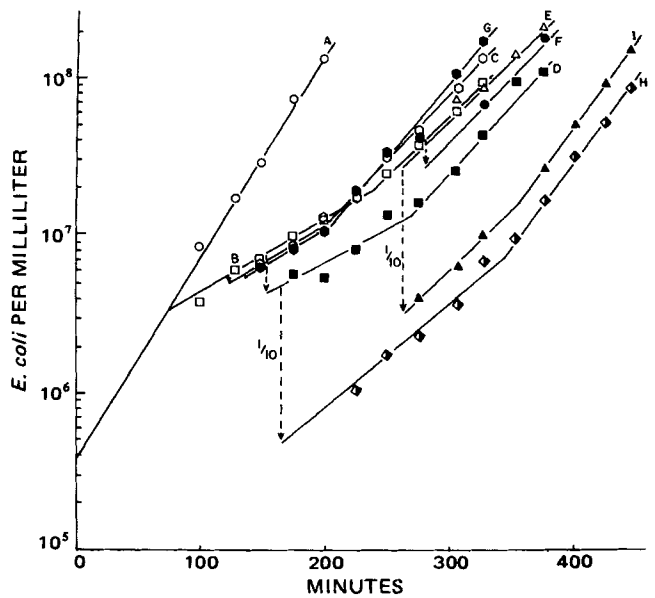

Figure 8—*Semilogarithmic plots of* E. coli *per milliliter against time at 37.5° and pH 7 in Anton's medium when inoculated with organisms filtered at various times from a previously fluorouracil-affected (30 ng/ml) generation curve B (□). The generation curves for the resuspended organisms in fresh media taken at the various times of filtration of curve B were: C, 123 min (○); D, 153 min (■); E, 254 min (△); and F, 279 min (●). Curve G (●) is for the 123-min filtered organisms generating in the presence of 3 μg of uracil/ml. Curves H (◆) and I (▲) are the generation curves after 1:10 dilution with fresh medium of the cultures of curves D and E, respectively, at the time indicated in the graph. Curve A (○) is for the generation of fresh inoculum in fresh medium without added drug.*

Table I—Apparent Total Binding of Fluorouracil to Human Plasma Protein and the Cones Used for Ultrafiltration

pH	Fluorouracil, μg/ml 100[a]	1.00[a]	0.10[b]	Average ± *SD*
5.7	3.1	6.0	7.4	5.5 ± 2.2
7.2	11.7	12.5	13.5	12.6 ± 0.9
9.0	7.8	8.4	6.6	7.6 ± 0.9
			Overall average	8.6

[a] Counts per minute were 8000. [b] Counts per minute were 1500.

in the presence of chloramphenicol was completely reversed by uracil. The generation rate of the triple mixture was the same as that of chloramphenicol alone. Similar results were obtained with a mixture of 0.49 μg of chloramphenicol/ml, 0.049 μg of tetracycline/ml, and 0.020 μg of fluorouracil/ml with and without added uracil, 2 μg/ml. The generation rate constant of 1.6×10^{-4} sec^{-1} of the combination was changed to 3.0×10^{-4} sec^{-1} on the addition of uracil, a rate constant that was the same for the mixture of chloramphenicol and tetracycline alone.

This phenomenon permits the assay of the fluorouracil content of biological fluids in the presence of other antibacterial agents. The microbial generation rates need to be studied in the presence and absence of added uracil, and the difference in the inhibitory rate constant can be assigned to fluorouracil action.

Action of Potential Nucleoside Derivatives and Solvolysis Products of Fluorouracil with and without Uracil Addition on Microbial Generation—Potential products of fluorouracil solvolysis (8), barbituric acid and isobarbituric acid at 1 μg/ml, had no significant effect on *E. coli* generation under the stated conditions. Solutions (2 μg/ml) of fluorouracil in whole blood, plasma, ultrafiltered plasma, and urine stored at 4° showed no significant loss in fluorouracil potency by this assay over 6 days and thus implied that no significant degradation occurred with these storage conditions.

Floxuridine and 5-fluorouridine have significant inhibitory effects on the generation of *E. coli* (6). Uracil did not reverse the action of 5-fluorouridine and had a negligible effect on floxuridine (Fig. 6), in contrast to the complete reversibility of the action of the precursor, fluorouracil (Fig. 5). Studies of floxuridine effects at 30, 400, and 500 ng/ml showed an initial phase of inhibition for 100 min and a subsequent increase in generation equivalent to a control without added drug. This result was in contrast to fluorouracil and 5-fluorouridine action, where the second phase of the higher generation rate was always significantly less than that of the control.

The accepted mechanism of fluorouracil antimicrobial or anticancer action is by its incorporation into 5-fluorouridine or floxuridine and their monophosphates, compounds that block DNA synthesis (6, 12). The lack of uracil reversal of the antibacterial action of these nucleosides implies that uracil interferes with the incorporation of fluorouracil into such nucleosides instead of antagonizing the nucleosidic or nucleic acid action *per se*.

Effect of Filtrates from Fluorouracil-Inhibited Microbial Cultures on Fresh Organisms and of Generation of Transferred Affected Organisms in Fresh Medium—Semilogarithmic plots of organisms against time over longer periods showed biphasic generation curves (6, 12). The initially inhibited generation rate in phase I (the portion used in the described assay) increased to a new steady state (phase II) after reasonable linearity for approximately 2 hr. The effect of dilution of the fluorouracil-affected organisms with fresh medium was studied (6) in peptone broth; the addition of the drug was not readily reversed by dilution. It took about 150 min for the generation rate on dilution in phase I to increase significantly. Diluted cultures were inhibited further to the expected extent by additional drug. These studies were repeated with 1:10 and 1:100 dilution with Anton's medium, and the results were the same.

It was suggested (6) that the slower phase II of fluorouracil-affected microbial generation, particularly observed in the nonbinding Anton's medium, was due to drug degradation in the presence of generating *E. coli*. Aliquots of a generating culture of *E. coli* in Anton's medium with 30 ng of fluorouracil/ml were filtered at various times and added to a fresh culture of organisms, 4:1 (Fig. 7). The fluorouracil contents of the diluted filtrates, presumably 24 ng/ml, were 17 ng/ml for the 123-min filtrate, 6 ng/ml for the 153-min filtrate, and 0 ng/ml for the 254- and 279-min filtrates as determined from a calibration curve constructed in fresh medium.

These results prove that fluorouracil is inactivated by the generating

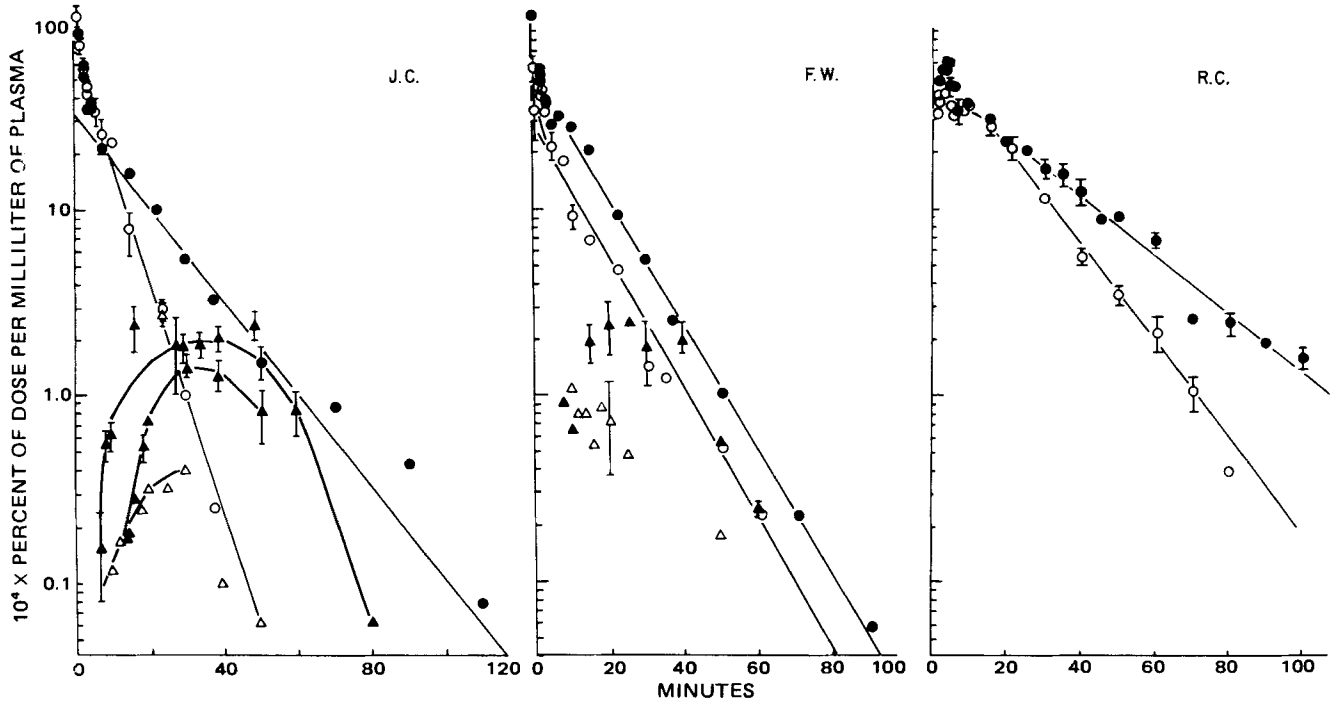

Figure 9—*Semilogarithmic plots for plasma fluorouracil levels in 10^4 μg/ml ÷ dose in micrograms against time on intravenous administration of 1000 (●) and 500 (○) mg and on oral administration of 1000 (▲) and 500 (△) mg. The vertical lines at several points designate the range of the average ± SD when three or more replicate assays were made.*

organism and/or an antagonist to this drug is produced by the generating organisms. However, when an additional 20 ng of drug/ml was added to the fresh culture-diluted filtrates, the resultant generation rate was equivalent to that expected from completely fresh medium. This finding denies the postulate of a produced antagonist and demonstrates the microbial inactivation of fluorouracil (Fig. 7). Studies of fluorouracil effects on microbial generation of *E. coli* at various inoculum sizes showed, after the initial inhibition, greater acceleration of generation rates with increased inocula, confirming the microbial inactivation of fluorouracil.

The filtered cells associated with these filtrates were resuspended in fresh medium without fluorouracil. Generation rates of these cultures and cultures with an added 1000-fold excess of uracil were monitored. The generation rates of the filtered organisms in fresh medium were similar to those observed prior to the removal of fluorouracil by filtration (Fig. 8). This result demonstrates the irreversibility of fluorouracil action. The drug is irreversibly incorporated into the organism, and the effects of these metabolites persist until the homeostatic processes compensate for the insult, which is consistent with the established mechanism where phosphorylated derived nucleosides would not readily be removed from the cell. Dilution of the generating drug-pretreated organism had no significant effect on this irreversibly affected generation rate (Fig. 8).

The addition of uracil to the resuspended organisms had no effect on the previously modified generation rate. This result further demonstrates that uracil antagonizes fluorouracil incorporation into active anabolites or catabolites and has no direct effect on deblocking DNA synthesis.

Human Plasma Protein Binding of Fluorouracil—The cone bindings for various concentrations of fluorouracil in percent of concentration ± *SD* were: 2.5 ± 0.4 at 0.1 μg/ml and 1850 cpm, 4.4 ± 1.8% at 0.4 μg/ml, 2.7 ± 2.8% at 1.0 μg/ml and 18,500 cpm, 3.6 ± 0.9% at 4.0 μg/ml and 1100 cpm, and 1.5 ± 0.5% at 100 μg/ml and 1100 cpm. The overall average was 3% with an averaged standard deviation of 1.5%. There was no significant difference between cone bindings at pH 7.9 and 7.1.

The total binding to plasma protein and the filter cones was not sig-

Table II—Pharmacokinetic Parameters for Intravenous Bolus Administration of Fluorouracil

Parameter	Patient RC[a] (F) Weight, kg 44.7	45.0	Patient FW[a] (M) Weight, kg 69.0	67.4	Patient JC (M) Weight, kg 98.6	—
Dose, D_0, mg	1000	500	1000	500	1000	500
α^b, min^{-1}	—	—	—	0.5	0.5	1.2
β^b, min^{-1}	0.036	0.059	0.081	0.082	0.058	0.137
$(t_{1/2})_\alpha$, min	—	—	(~10)	1.5	1.4	0.6
$(t_{1/2})_\beta$, min	19	12	8.6	8.4	12	5.1
A^b, μg/ml	—	—	—	27	94	—
B^b, μg/ml	50	35	60	15	33	32
$V_{extrap}{}^c$, liters	20	15	17	33	31	16
$V_C{}^d$, liters	—	—	—	12.1	10.6	—
AUC^e, μg/ml-min	1411	496	782	203	723	278
$V_{pss}{}^f$, liters	20	17	16	30	24	13
Total clearance, $Cl_{tot}{}^g$, ml/min	709	1008	1278	2469	1383	1801
Renal clearance, $Cl_{ren}{}^h$, ml/min	101	147	91	86	140[k]	157
Metabolic clearance[i], ml/min	608	861	1187	2383	1243	1644
Percent of dose excreted in urine [j]	14.2	14.6	7.1	3.5	10.1	8.7

[a] The creatinine clearances for Patients RC and FW were 83 and 74 and 65, 60, 70, and 50, respectively. [b] Parameters for time dependence, t in minutes, for plasma levels, b in micrograms of fluorouracil per milliliter of plasma: $b = (Ae^{-\alpha t} + Be^{-\beta t})$. [c] D_0/B. [d] Estimates of apparent volume of distribution of central compartment, $V_C = D_0/(A + B)$. [e] Area under plasma level–time curve by trapezoidal rule. Terminal area estimated from last plasma level, b_n, and observed β, *i.e.*, b_n/β. [f] Pseudo-steady-state overall apparent volume of distribution, $V_{pss} = Cl_{tot}/\beta$. [g] Total clearance, $Cl_{tot} = D_0/AUC$. [h] Renal clearance, $Cl_{ren} = (U_\infty/D_0)\ Cl_{tot}$, where U_∞ is total amount of drug excreted unchanged in urine. [i] $Cl_{tot} - Cl_{ren}$. [j] $10^2\ U_\infty/D_0$. [k] One infusion study gave a $Cl_{ren} = (\Delta U/\Delta t)/b_{avg}$ = (450 μg/min)/3.0 μg/ml = 150 ml/min at steady state.

nificantly concentration dependent (Table I), although the total binding at pH 7.2 did significantly exceed that at pH values of 5.7 and 9.0. Correction for cone binding implies a protein binding of fluorouracil of 10% at pH 7.2 and ~3% at the other pH values.

Pharmacokinetics of Fluorouracil on Bolus Intravenous Administration—The time courses of the plasma levels as percent of dose per milliliter of plasma on intravenous bolus injection are shown in Fig. 9, and the derived pharmacokinetic parameters are listed in Table II. A two-compartment body model is indicated by the biphasic semilogarithmic plots. The slow mixing for Patient RC did not permit observation of an α-phase when the drug was injected and taken from opposite arms. The half-lives for the preliminary distribution (α) phases for the other two subjects ranged from 0.6 to 1.5 min. The percent of dose–time plots of Fig. 9 for the initial data of both 1000- and 500-mg doses were reasonably superimposable when plotted in such a manner and do not deny the premise that the apparent 10–12-liter volume of distribution of the central compartment and the initial distribution rates are dose independent.

Various other pharmacokinetic parameters were calculated for the two studies where the data were adequate for estimation of α and A values. The first-order two-compartment body model where the intravenously administered drug distributes between the central compartment, C, and the peripheral compartment, P, and is eliminated or metabolized to E is shown in Scheme I.

$$E \xleftarrow{k_{CE}} C \underset{k_{PC}}{\overset{k_{CP}}{\rightleftarrows}} P$$

Scheme I

The estimated microscopic rate constants (13) in minutes^{-1} for Patients FW (500-mg dose) and JC (1000-mg dose) were, respectively: k_{PC}, 0.23, 0.17; k_{CP}, 0.17, 0.22; and k_{CE}, 0.18, 0.17. The apparent volumes of distribution of the central compartment, V_c = dose/(B + A), were 11.9 and 7.9 liters; of the peripheral compartment, $V_p = V_c k_{CP}/k_{PC}$, they were 8.9 and 9.9 liters. The total steady-state volumes of distribution, $V_{ss} = V_c + V_P$, were 21 and 18 liters, respectively. The areas (AUC) under the plasma level–time curves, calculated from the parameters given in Table II for the sum of exponentials fit of the plasma level, $b = Ae^{-\alpha t} + Be^{-\beta t}$, were 237 and 757 μg/ml-min from $AUC = A/\alpha + B/\beta$. Thus, the respective total clearances (dose/AUC) for these studies of Patients FW and JC were 2110 and 1321 ml/min, respectively, which were similar to those clearances determined from areas calculated by the trapezoidal rule (Table II).

There were lower plasma levels as percent of dose for the 500-mg bolus as opposed to the 1000-mg bolus in all three subjects (Fig. 9), thereby demonstrating a significant pharmacokinetic dose dependency. In two (JC and RC) out of the three studied subjects, the apparent first-order terminal rate constant of drug elimination was significantly higher at the lower dose, which could imply a saturable metabolism of fluorouracil. However, the continued linearity of the terminal semilogarithmic plots implies that it cannot be assigned to substrate-dependent metabolism alone since an increase in the terminal slope with time would be anticipated at the higher doses. A possible explanation could be product-inhibited metabolism, which would significantly decrease the slope of the terminal phase (Fig. 9) at the higher dose.

A possible alternative explanation may be based on the premise that fluorouracil is initially transformed reversibly to 5,6-dihydrofluorouracil. The subsequent irreversible process of ring opening of the latter may be a saturable process. Thus, higher plasma levels would promote the accumulation of the fluorouracil in equilibrium with the dihydrofluorouracil.

The dose-dependent metabolic clearances, Cl_{met} (Table II), exceeded the normal hepatic plasma flow of 812 ml/min (14). This result indicates that metabolism occurs at sites other than in the liver, and a high first-pass metabolism would be predicted on oral administration.

The renal clearances (Table II) in the three subjects did not appear to be dose dependent and averaged 124 (RC), 89 (FW), and 149 (JC) ml/min, in contrast to the two subjects studied by Clarkson *et al.* (5) on drug infusion with renal clearances of 159 and 180 ml/min. However, these subjects may have had normal renal clearances (one had an inulin clearance of 109 ml/min) whereas Patients RC and FW had significantly low creatinine clearances of 79 and 61 ml/min, respectively. The percent of drug previously reported (5) to be excreted unchanged in the urine on single intravenous injection was 16% (7.8% with impaired renal function) and was consistent with the present values (Table II).

Occasional microbial generation studies of plasma samples containing fluorouracil were made with added uracil. In all cases, the inhibitory action of the drug was completely reversed. This finding indicated no significant concentration of derived nucleoside in the plasma.

Table III—Pharmacokinetic Parameters on the Oral Administration of Fluorouracil Solutions

Parameter	Patient JC (M), 107.5 kg			Patient FW (M), 65.3 kg	
Dose, D_0, mg	1000	1000	500	1000	500
AUC_{po}[a], μg/ml-min	47	110	5.5	89	16
Percent of dose excreted in urine[b]	1.24	0.33	0.14	0.67	0.16
Percent apparent bioavailability					
From $10^2\ AUC_{po}/AUC_{iv}$[c]	12.4	3.3	1.6	9.4	4.6
From $10^2\ (U_\infty)_{po}/(U_\infty)_{iv}$[d]	15.1	6.4	0.8	11.3	8.0

[a] Area under plasma level–time curve by trapezoidal rule. Terminal area estimated from last plasma level, b_n, and observed β at 500-mg iv dose, *i.e.*, b_n/β. [b] $10^2(U_\infty)_{po}/D_0$, where U_∞ is total amount of drug excreted unchanged in urine. [c] From ratios of AUC for same doses, oral/intravenous. [d] From ratios of total amounts of drug excreted unchanged, U_∞, for same doses, oral/intravenous.

Pharmacokinetics of Fluorouracil on Constant-Rate Intravenous Infusion—Two studies, with Patients RC and FW, of constant-rate (k_0) intravenous infusion (6.67 mg/min) of 1000 mg (D_0) of fluorouracil were conducted with total areas under the plasma level–time curves of 336 and 197 μg/ml-min, respectively. Thus, the respective clearances, $Cl_{tot} = D_0/AUC$, were 2977 and 5085 ml/min. The average plasma levels on attainment of steady state (b_{ss}) were 2.65 μg/ml ± 0.46 (SD) for RC and 1.42 μg/ml ± 0.28 (SD) for FW. Thus, the respective estimated total clearances calculated from these values ($Cl_{tot} = k_0/b_{ss}$) were 2517 and 4697 ml/min, values consistent with the total clearances calculated from the AUC values. However, the averages of these total clearances, 2747 (RC) and 4891 (FW) ml/min, greatly exceeded, by more than twofold, the total clearances obtained on bolus intravenous injection of either 500 or 1000 mg of fluorouracil (Table II). When the total clearance was divided by the respective average pseudo-steady-state volume of distribution for each subject (Table II), the estimated elimination rate constants ($\beta = Cl_{tot}/V_{P_{ss}}$) were 0.15 (RC) and 0.20 (FW) min^{-1}. This finding confirms the indicated dose-dependent pharmacokinetics of fluorouracil. Slow infusion of the drug that increased the metabolic rates would give a lessened steady-state concentration of metabolites and would support the hypothesis of product-inhibited metabolism.

Patient RC excreted unchanged 7.1% of the dose in the urine, a value significantly less than the 14.4% observed on intravenous bolus administration. This decrease in the urinary excretion is normally expected with an increase in the rate of a parallel metabolism of a drug.

Pharmacokinetics of Fluorouracil on Oral Administration of Solutions—As previously reported (5, 15), the absorption of fluorouracil on oral administration was highly variable among and within subjects (Fig. 9). First approximations of the fraction of drug absorbed unchanged can be made from the ratios of drug excreted unchanged or from the ratios of the area under the curve, oral to intravenous for equivalent doses. These ratios ranged from 0.01 to 0.15 in the five studies conducted (Table III), where the ratios of the AUC values reasonably correlated with the ratios of the renal excretion of unchanged drug. These are not accurate quantitative values for bioavailability but are only coincident with true bioavailabilities if, and only if, elimination rates are dose independent.

These apparent bioavailabilities were much less than the 0.50 and 0.80 values reported for two subjects by Cohen *et al.* (15). However, their subjects had carcinomas of the colon metastatic to the liver. Other investigators (5, 16) indicated much lower amounts of drug available to the plasma on oral absorption.

The plasma fluorouracil levels of solutions (Fig. 9) peaked 10–15 min after oral administration; at the higher 1000-mg dose, they were prolonged, indicating a sustained zero-order absorption. The apparent bioavailabilities (Table III) at the 1000-mg oral dose always exceeded those at the lower 500-mg doses. This finding is consistent with a dose-dependent first-pass effect. Since hepatic clearances on intravenous administration (Table II) always exceeded hepatic plasma flow by more than twofold, there should have been negligible drug in the plasma by the oral route. The fact that significant drug was absorbed orally is indicative of highly saturable first-pass phenomena when high concentrations of drug enter the liver on oral administration and/or additional extrahepatic metabolic and elimination pathways that are not mediated by the absorption process.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 18, 1976, from *The Beehive, College of Pharmacy, University of Florida, Gainesville, FL 32610.*

Accepted for publication December 16, 1976.

Supported in part by Grant NIH-RR-82 from the National Institutes of Health, Bethesda, MD 20014.

The technical assistance of George Perry and Kathleen L. Eberst is gratefully acknowledged. The authors thank the nursing staff of the Clinical Research Center, Shands Hospital, University of Florida, for assistance in performing the clinical studies.

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Factors Influencing Comparative Bioavailability of Spironolactone Tablets

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Abstract □ The bioavailability of spironolactone from 10 tablet formulations, selected to provide a wide range of specifications and *in vitro* dissolution rates, was assessed from the plasma and urinary levels of its major unconjugated metabolite, canrenone, in a study of balanced incomplete block design using 11 healthy subjects. Significant but weak correlations existed between the amount of spironolactone in solution at 40 min *in vitro* and the area under the plasma concentration–time curve for canrenone and urinary canrenone excretion. The correlations between *in vitro* dissolution and bioavailability parameters appeared to be weakened by two tablet formulations, one with dibasic calcium phosphate as the principal excipient and the other formulated from micronized spironolactone bulk drug. Measurement of *in vitro* dissolution of spironolactone tablets is of value for quality control purposes, provided no major alteration is made in the formulation.

Keyphrases □ Spironolactone—bioavailability in humans related to *in vitro* dissolution, various tablet formulations compared □ Bioavailability—spironolactone in humans, related to *in vitro* dissolution, various tablet formulations compared □ Dissolution, *in vitro*—spironolactone, related to human bioavailability, various tablet formulations compared □ Diuretics—spironolactone, bioavailability in humans related to *in vitro* dissolution, various tablet formulations compared

Spironolactone[1], a synthetic steroid lactone having properties compatible with specific competitive antagonism of aldosterone and other mineralocorticoids (1–3), is of clinical value in the treatment of congestive heart failure, hepatic ascites, primary aldosteronism, and essential hypertension (4). The original tablet formulation of spironolactone had incomplete bioavailability, and reformulation yielded a tablet that proved to be fourfold superior with regard to plasma levels of the principal unconjugated metabolite (canrenone) (5–8), pharmacological activity (9, 10), and therapeutic efficacy (8).

It was suggested that the spironolactone absorption was limited by its dissolution rate, and some evidence indicated that the improved bioavailability of the new formulation could be related to more rapid *in vitro* dissolution (11). The new formulation of spironolactone (25-mg tablets) is still used, and little has been published on spironolactone bioavailability in recent years.

A new 100-mg spironolactone tablet was shown to be bioequivalent to four 25-mg tablets (12). Spironolactone was included in the long list of drugs for which more information on bioavailability is considered desirable (13, 14).

This paper presents the comparative bioavailability of 10 tablet formulations of spironolactone. The objective of the study was to determine whether dissolution *in vitro* is of value in predicting *in vivo* bioavailability and which factors in tablet specification may be important to bioavailability.

[1] Aldactone, G. D. Searle & Co.

EXPERIMENTAL

Formulations—Three experimental and seven production batch tablets were studied. All tablets were chemically equivalent inasmuch as they all complied with the BP monograph requirements for spironolactone. The tablets were selected specifically to provide the wide range