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Studies on the mechanism of metabolic activation of ftorafur (R, S-1-(tetrahydro-2-furanyl)-5-fluorouracil) to 5-fluourouracil

Author
Au, Jessie Lai-Sim

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Studies on the mechanism of metabolic activation of ftorafur

(R,S-1-(tetrahydro-2-furanyl)-5-fluorouracil) to 5-fluorouracil

by

Jessie Lai-Sim Au

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Throughout the many years of association with the University of California, San Francisco, first as a pharmacy student and then a graduate student, I have passaged through many stages of my life. I am indebted to the Faculty of the Pharmacy School and my fellow graduate students for their continuous input on my intellectual and personal growth. I am most grateful to my graduate adviser, Dr. Wolfgang Sadée, for a training process which allowed me to develop in independent and creative thinking; to Dr. Rich B. Meyer, Jr. and Dr. Thomas N. Tozer for serving on my dissertation committee and their many helpful suggestions; and to Dr. Roger Ketchum for his inspirational friendship in the last decade.

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To my family

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ABSTRACT

Studies on the mechanism of metabolic activation of ftorafur (R,S-1-(tetrahydro-2-furanyl)-5-fluorouracil) to 5-fluorouracil

Jessie Lai-Sim Au

The overall objective of this investigation is to establish the mechanism of metabolic activation of ftorafur (FT) to 5-fluorouracil (FU) in order to better the understanding of the observed difference in tissue selectivity of these two antitumor agents. We have studied the metabolism, pharmacokinetics, enzyme systems responsible for the activation, and the extent of conversion of FT.

We have developed high pressure liquid chromatographic (HPLC) assays and gas chromatographic (GC) assays to study the pharmacokinetics and metabolism of FT in animals and patients. The disposition of FT in rats, rabbits, and humans followed two compartment kinetics with mean β-phase half-lives of 5, 2, and 9 hours, respectively. Plasma concentrations of FU in FT-treated animals and patients were exceedingly low ($\leq$ 0.1% of FT levels).

Seven FT metabolites have been identified in plasma and urine samples of experimental animals and humans by physicochemical analyses which include proton nuclear magnetic resonance, chromatographic properties on HPLC, GC, thin layer chromatography, mass spectrometry following GC separation, as well as direct comparison with authentic samples. These metabolites were identified as FU, trans-3'-OH-FT, cis-4'-OH-FT, two enantiomers of trans-4'-OH-FT, dehydro-FT with a double bond in the tetrahydrofuran moiety, and

γ-butyrolactone (in equilibrium with γ-hydroxybutyric acid). Trans-3'-OH-FT was found only in humans and not in animals, while the other metabolites are common to all species studied.

The stereochemistry of the hydroxylated FT metabolites was evaluated by in vivo metabolism studies of the R-FT and S-FT enantiomers, stereospecific enzymatic phosphorolysis, circular dichroism and optical rotatory dispersion analyses. Significantly more 4'-OH-FT was recovered from rat urine following administration of S-FT than R-FT, indicating that either the S-FT was preferentially hydroxylated or the hydroxylated products of R-FT would have the natural stereochemical configurations as the endogenous substrates and would therefore be further metabolized prior to urinary excretion. The latter is supported by the findings that an authentic sample of β, D-4'-OH-FT was quantitatively converted to FU by a horse liver thymidine phosphorylase in vitro. The trans-3'-OH-FT isolated from human urine consists of β,D and α,L anomers in 1:2 ratio. The urinary trans-4'-OH-FT consists of β,D and α,L anomers in different ratios in rabbits (1:4) and humans (5:95). And the cis-4'-OH-FT consists mainly of the α,D anomer.

A few metabolic activation pathways of FT to FU have been established. (1) FT can be hydroxylated at the C-4' position to β, D-4'-OH-FT followed by stereospecific enzymatic phosphorolysis to FU. (2) FT is metabolized to dehydro-FT which is chemically more labile than FT and may spontaneously cleave to FU. However, these two pathways are relatively minor in the overall FT activation. (3) FU was generated from FT upon incubation with the 100,000 x g microsomal fractions of mouse, rat and rabbit liver homogenates, consistent with previous findings of other investigators. The exact mech-

anism of this NADPH-dependent microsomal oxidation of FT to FU and its contribution to the overall FT activation are still unclear. (4) FT is cleaved at the C-2' position to FU and γ-butyrolactone. This metabolic pathway represents one of the major activation pathways of FT in rabbits and humans. It is mediated by the soluble enzymes in the mouse and rabbit liver homogenates, and is not dependent on the hepatic microsomal cytochrome P-450 enzymes.

Pharmacokinetic analysis of the plasma concentrations of FT and FU indicate that the conversion of FT to FU may also occur in target tissues, and the FU generated would be further metabolized without redistributing into the general circulation due to its rapid intracellular metabolism. This is consistent with the observed antitumor activity of FT in spite of the exceedingly low plasma concentrations of FU, and is further supported by the above finding that the major metabolic activation pathway of FT to FU is mediated by soluble enzymes.

Thus, results in this study demonstrate that the mechanism and the location of enzymes responsible for the metabolic activation may contribute to the tissue selectivity of a metabolic prodrug such as FT.

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Chapter I

Introduction

HISTORY

Ftorafur (FT) was synthesized by Hiller et al. (Lavian Academy of Sciences, USSR) in 1966, as part of a program searching for fluorinated pyrimidines with an improved therapeutic index over that of 5-fluorouracil (FU) (43). It bears structural resemblance to FU and 5-fluorodeoxyuridine (FUdR), the two fluorinated pyrimidine antimetabolites in clinical use. It has been proposed that FT is metabolically activated in vivo and represents a chemical depot form of FU (21,30,31,36,51,68,69,81,85). Clinically, FT has shown activity similar to that of FU, but it is less toxic to the bone marrow (40,53,89). The reduced myelosuppression of FT has prompted clinical trials, first in the Soviet Union in 1967 and Japan, and more recently in the United States (41). However, the high neurotoxicity of FT has limited its widespread clinical use in the future (52,88).

CHEMICAL CLASSIFICATION AND SYNTHESIS

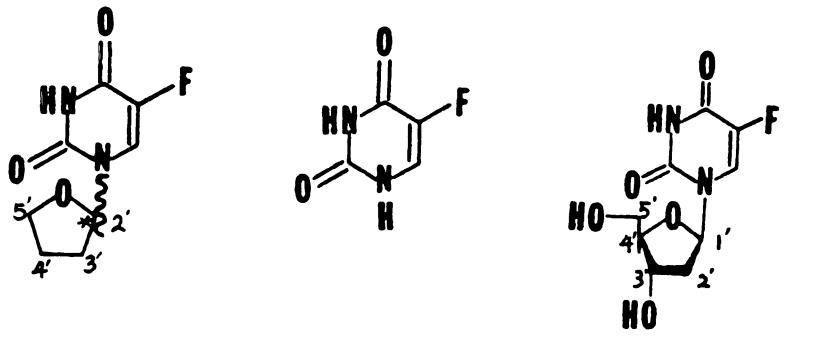
FT represents a new class of heterocyclic compounds named furanidyl pyrimidines. The structure of FT is shown in Figure 1 and compared to those of FU and FUdR. The nomenclature used in FT is in accord with the furan numbering system, whereas the one used in FUdR is in accord with sugar numbering system (Figure 1). Similar to the FU nucleosides, the pyrimidine ring in FT is attached to an asymmetric carbon. Thus, there are two enanotiomers of FT, i.e. R-FT and S-FT.

Numerous chemical schemes have been employed to synthesize FT. The commonly used methods include direct fluorination of tetrahydrofuranyluracil (25), and the condensation of substituted 5-fluorouracil with tetrahydrofuranyl analogs (43,44,45). Both of these methods yield the racemic mixture of R,S-FT, whereas the decarboxylation

of the corresponding 2'-deoxynucleoside uronic acid anomers allows a stereospecific synthesis of R-FT and S-FT (48). Resolution of the racemic FT into R-(+)- and S-(-)-FT has been achieved by fractional crystallization (97). FT is available for clinical use as a racemic mixture.

FT is chemically unstable, and rapidly converts to FU at pH 1 or at temperatures above 80°C (41). The physicochemical properties of FT are listed in Table I.

Figure 1. Structures of FT, FU, FUdR.



* asymetric carbon

Table I - Chemical and physical properties of FT.

Chemical Formula	1-(Tetrahydro-2-furanyl)-5-fluorouracil				
Empirical Formula	$C_8H_9O_3N_2F$				
Mol. Wt.	200.17				
M.P. (41)	164-169° (within 2°)				
Appearance (41)	Odorless white powder, not hygroscopic.				
		at 20°		at B.P.	
Solubility (41) g/l	Water	20		600	
	96% ethanol	21		214	
	acetone	66		150	
	chloroform	50		133	
pK_a (41)	8.04 (FU 7.08, FUdR 8.04)				
UV Absorption Spectra (41)	pH	2	7	12	in EtOH (48)
	max	270 nm	270 nm	270 nm	270 nm (λ_{min} 235 nm)
	ε	8460	8050	6700	7300 (1500)
NMR Spectra (48) (in $CDCl_3$)	δ 7.46 (d, 1, H-6), δ 6.00 (m, 1, H-1'), δ 4.12 (m, 2, H-4'), δ 2.10 (m, H-2',3').				

MECHANISM OF ACTION

FT is neither a substrate nor an inhibitor of the pyrimidine nucleoside phosphorylases (68). It has very little *in vitro* cytotoxicity in microbiological systems and is a weak inhibitor of DNA and RNA synthesis in several mouse tumor cell lines (29,48,68). FU at a concentration of 1×10^{-5} M inhibited DNA synthesis by 90-100 %, measured by ^{14}C-formate incorporation in HeLa cells, whereas the inhibition of FT at 1×10^{-4}M was only 15 % (29,68,69). In tumor-bearing animals, both FU and FT markedly inhibited the incorporation of labelled precursors into nucleic acids and proteins in Ehrlich Ascites cells; however, such effect was absent when the tumor cells were incubated with FT *in vitro* (31). The growth inhibition of FT of cultured chinese hamster cells (65) and E. Coli B3 (81) was accounted for by its slow conversion to FU. The growth inhibition of FU and FT on E. Coli B3, a thymidine-requiring strain, was reversed by uracil, suggesting a similar mode of action for these two agents, which is independent of thymidylate synthetase inhibition (81). The enhancement of FT activity by 18-hour preincubation was probably due to its spontaneous hydrolysis to FU in the culture medium (81). Likewise, Fujita *et al.* (33) reported increased antibacterial activity of FT upon incubation with various organ homogenates; the highest activity was seen after incubation with liver homogenates. In addition, sublines of L1210 leukemic cells (51) and strains of E. Coli B3 (81) were cross-resistant to FU and FT.

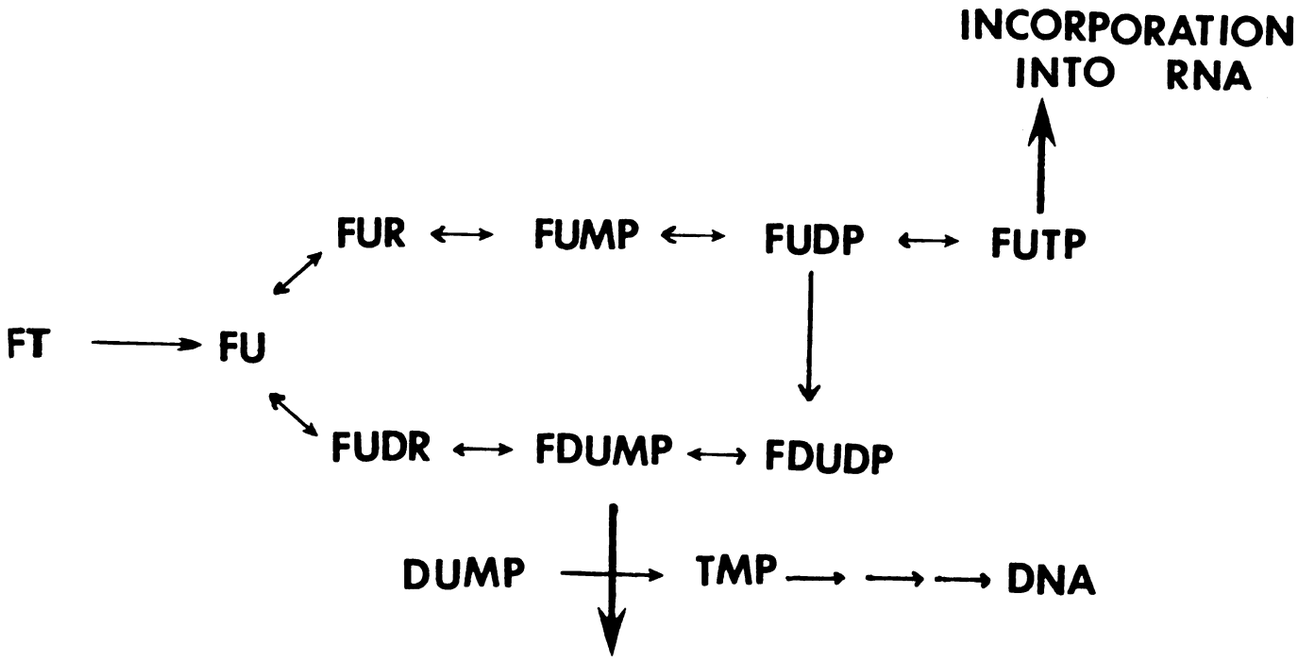
These results lead to the conclusion that FT is a chemical depot form of FU. It has insignificant cytotoxicity *in vitro* and exerts its *in vivo* activity by metabolic activation to FU. The liver has

been proposed as the primary site of FT activation to FU, and the hepatic microsomal cytochrome P-450 enzyme system may be responsible for such activation (33,75). However, nonenzymatic hydrolysis may also occur in the acidic conditions of the gastric juice, particularly when FT is administered orally (72).

The mechanism of pharmacological activity of FT is summarized as follows (Figure 2). FT is metabolized to FU, which is subsequently anabolized to 5- fluorodeoxyuridylate (FdUMP) and 5-fluorouridine triphosphate (FUTP), along with other anabolic products. FdUMP is a potent inhibitor of thymidylate synthetase which catalyzes the methylation of deoxyuridylate to thymidylate, and thus inhibits the de novo thymidylate and consequently DNA synthesis (72,79). Alterations of RNA metabolism by FU may also mediate cell toxicity, which involves inhibition of RNA formation or incorporation of FUTP into RNA (79). The DNA effect of FU is usually considered as its major mode of action (72), although recent data indicate that antitumor effects of FU may be mediated by RNA related mechanism (e.g. 82).

It has been suggested that the liver is the primary site of metabolism and the hepatic microsomal cytochrome P-450 may be the responsible enzymes (21,33,68). This is supported by the following observations during in vitro metabolism studies and in vivo studies in animals. Anti-bacterial activity of FT increased when incubated with human tissue homogenates, and the highest activity was seen with liver homogenates (33). Ohira et al. (75) reported that FT produced a type II binding spectrum with rat hepatic microsomal cytochrome P-450, whereas a type I binding spectrum was seen by Meiren et al. (68). In vitro activation of FT by liver microsomes requires NADPH (30,68); higher rates of FT decomposition were ob-

Figure 2. Schematic representation of metabolic activation of FT.



FT : ftorafur
FU : 5-fluorouracil
FUR : 5-fluorouridine
FUdR : 5-fluorodeoxyuridine
FUMP : 5-fluorouridine monophosphate
FUDP : 5-fluorouridine diphosphate
FUTP : 5-fluorouridine triphosphate
FdUMP : 5-fluorodeoxyuridine monophosphate
FdUDP : 5-fluorodeoxyuridine diphosphate
dUMP : deoxyuridine monophohphate
TMP : thymidylic acid

served with liver microsomes harvested from mice pretreated with phenobarbital and glutathione (33). Pretreatment of tumor-bearing mice with phenobarbital increased the survival time of the animals when treated with FT (75). On the contrary, the elimination of FT and formation of FU were retarded in mice with hepatic lesions induced by chloroform (33). The significance of the liver as the major organ activating FT to FU has not been established in humans. Patients with severe liver malfunction eliminated FT at the same rate as patients without liver damage, as observed by us and others (4,65). Majima and Taguchi (66) pretreated patients with phenobarbital prior to FT administration, and found no difference in response rate when compared to the control group. A recent report of a Phase I-II study of FT and methyl-CCNU in colorectal carcinomas indicated that 4 out of the 5 patients that responded to treatment had hepatic metastasis suggesting that hepatic dysfunction might not significantly alter the conversion of FT to FU (12).

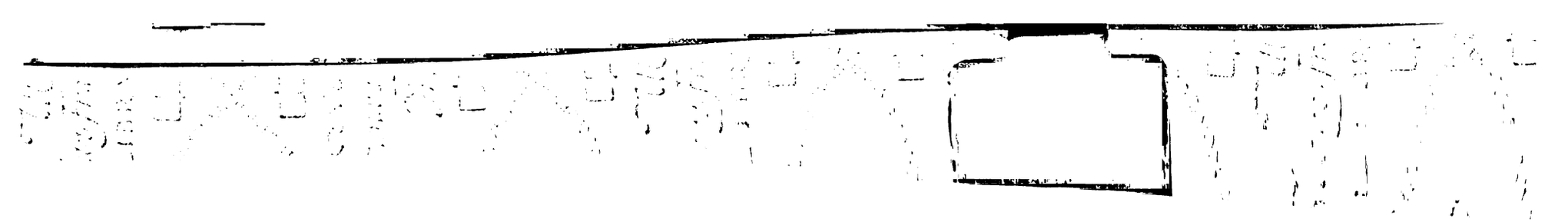
ABSORPTION

Pharmacokinetic studies in patients receiving an oral dose of 1 g FT revealed nearly complete absorption with a peak plasma concentration of 25 μg/ml at 2 hours following administration (22). Similar results were found in rabbits (41). In recent clinical trials in Japan with rectal administration of 1 g FT/d, peak plasma concentrations of 20 μg/ml occurred at 1-4 hours post-administration (41). In contrast to the erratic absorption of FU (26), FT is well absorbed through the gastrointestinal tract (41) with minimal degradation in the gastric juice (22).

DISTRIBUTION

Following i.v., oral, or rectal administration of ^{14}C- and

^{3}H-labelled FT to rats and mice, the administered radioactivity was recovered in the liver, small intestine, stomach, lung, spleen, kidney, tumors, and brain (21,32,33). Highest concentration of FU and FT were found in the liver, implicating the liver as the primary site of metabolic activation of FT to FU (21). FT is more lipid soluble than FU and, therefore, may cross the blood-brain barrier more rapidly (72). Cohen (21) found that within one hour after i.v. administration, the levels of FT in rat brain tissue were similar to those in liver, spleen, kidney and small intestine. Fujita et al. (33) reported that rat brain levels of FT following an i.v. dose of 90 mg FT/kg were 200-300 times higher than those of FU following a similar dose of 30 mg FU/kg. However, the brain levels of FU at 1 hour resulting from FT administration were slightly lower than those found after FU administration (33).



ANTITUMOR ACTIVITIES

Cell Culture Studies. FT has very little in vitro cytotoxicity in microbiological systems and is a weak inhibitor of nucleic acid synthesis in several mouse tumor cell lines (68). The growth inhibition of cultured cells by FT were accounted for by its spontaneous degradation to FU in culture medium (65,81).

Animal Studies. The antitumor activities of FT and FU on several transplanted tumors in rats and mice were compared. Both drugs produced comparable growth inhibition of L1210 leukemia, Sarcoma 180, Sarcoma AK, Walker's carcinosarcoma, Carcinoma HK, and Harding-Passey's melanoma, but neither drug had any activity against Sarcoma 45. FU was more effective against B16 melanoma, Gardener 6C3HED lymphosarcoma, and P388 leukemia (41). In combination chemotherapy of L1210 leukemia, FU plus FT was no more effective than FU alone, and neither of the congeners was synergistic with either adriamycin or actinomycin D (36). When used in combination with methotrexate, synergism was observed with FU but not with FT (36). Sublines of L1210 leukemia were cross-resistant to FT and FU (36).

The similarity of their antineoplastic activities in animals and cross-resistance of cell lines to both drugs support the contention that FT exerts its antitumor effects primarily by activation to FU.

Horwitz et al. (48) compared the in vitro growth inhibitory effect on cultured human fibroblasts by the R-(+), S-(-) isomers, and the racemic mixture of FT, and found no significant difference among these isomers. Similarly, no difference was observed in the inhibitory effects of these isomers on tumor growth in rats bearing AH-130 carcinoma and Yoshida Sarcoma (97).

Clinical Studies. Initial clinical trials with FT were performed in

the Soviet Union in 1967 for treatment of various adenocarcinomas in humans. Daily iv dose of 30 mg FT/kg to a total of 30-40 g produced 50% reduction in tumor size in patients with stomach, colon, and breast cancer (52). The activity of FT against rectal and brain tumors was considered to be superior to that of FU (15). During the clinical trials in Japan, FT was given either orally, rectally, or intravenously to patients. The oral and rectal routes of administration were as effective as the iv administration with minimal gastrointestinal toxicity (22,57,88). The only comprehensive clinical evaluation of FT used as a single agent in the United States was completed in 1976. Valdivieso et al. (89) reported that daily doses of 2 g FT/sq m/d for 5 days, repeated every 3 weeks, produced therapeutic activities comparable to those obtained with 5-day continuous iv infusion of FU against colon, stomach, and lung tumors in patients. Phase I, II, and III clinical trials of FT in combination with methyl-CCNU, adriamycin, and mitomycin have been completed and the results indicate no significant therapeutic advantages of FT over FU (12,94). Phase I clinical studies of FT given orally by multiple daily dosing regimen at 1-2 g/d are now on-going in the United States. Preliminary results indicate that this dosage is well-tolerated with minimal gastrointestinal toxicity (2,22,73,93).

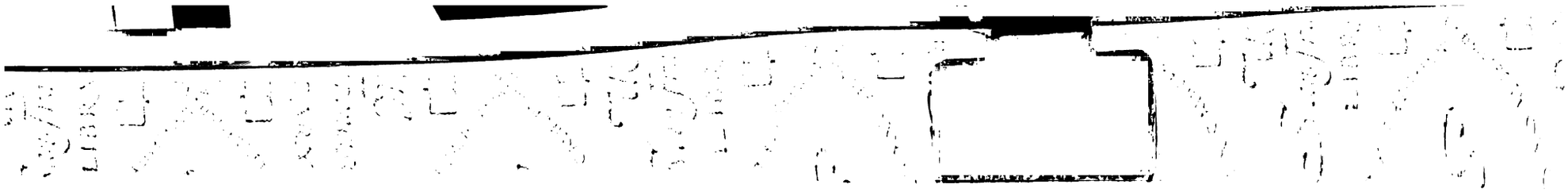
CLINICAL TOXICITIES

FT is toxic to the epithelium of the gastrointestinal tract, the central nervous system, and the bone marrow. These side effects appear to be dose-related and occur at doses greater than 2 g/sq m/d. The gastrointestinal and neurological disturbances are the most common and dose-limiting toxicities of FT (88,93). Incidence of hematological toxicity is less frequent and results of a number

of clinical trials indicate that FT is as effective against solid tumors, but less myelosuppressive than FU when the latter is given by bolus injection (52). The pattern of clinical toxicities of FT, i.e. mild myelosuppression and significant gastrointestinal toxicity, is very similar to that of prolonged infusion of FU and FUdR (72).

Fujita et al. (73) compared the FU concentrations in rat brain after a dose of 90 mg FT/kg or 30 mg FU/kg. They found higher levels of FU, fluoroureido proprionic acid (FUPA), and fluoro-β-alanine (FBAL) one hour after the administration of the FU dose, during which time the neurological toxicity of FT becomes significantly higher than that of FU. This indicates that the active component of FT contributing to its neurotoxicity is unrelated to FU, FBAL or FUPA. The neurological toxicities of FT, including ataxia, dizziness, and less frequently lethargy and headache, are shared by its structural analog, N-1,3-bis-(tetrahydrofuran-2-yl)-5-fluorouracil (FD-1) (66). FD-1 was synthesized in Japan in 1977 and presumably also acts as a FU prodrug (54). The dose-limiting toxicity of FD-1 observed during its clinical trials in Japan is its neurotoxicity, which represents the major factor prohibiting its further use (19,66). One of the major metabolites of FD-1 in rat plasma and brain tissue is γ-butyrolactone (in equilibrium with γ-hydroxybutyrate) derived from the tetrahydrofuran portion of the molecule (54). γ-Butyrolactone is a CNS depressant and is used clinically as a general anesthetic agent (91). γ-Butyrolactone has also been identified as a major FT metabolite in animals and cancer patients in our laboratory (see Chapter 2).

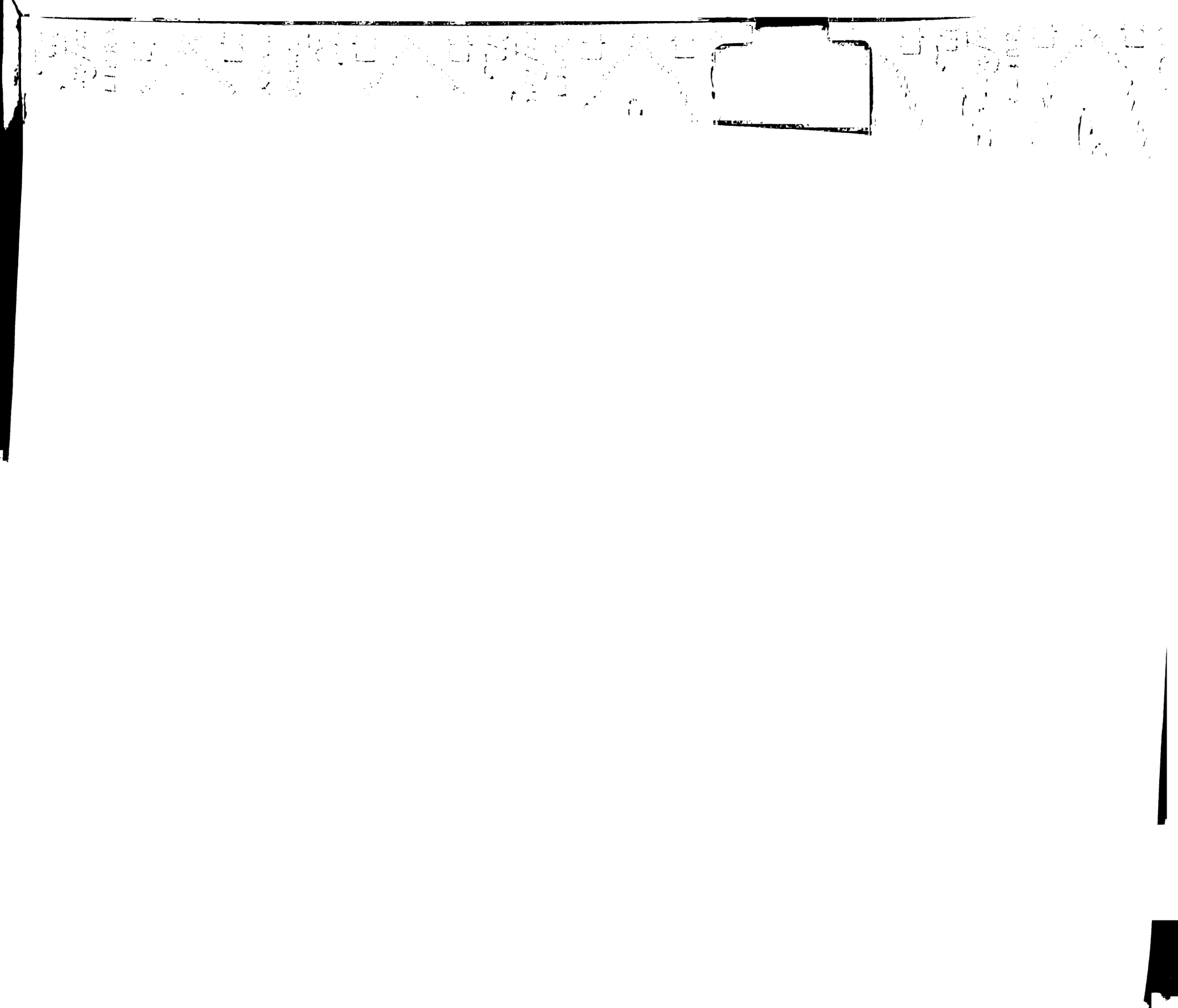
We have studied the metabolism (Chapter 2, References 3,7,8,96),



stereochemistry of FT metabolism (Chapter 3, References 6,8), mechanism of metabolic activation of FT to FU (Chapter 4, References 6,7,8,80,96), pharmacokinetics (Chapter 5, References 6,7,8,96) of FT in animals and in cancer patients, and the kinetics of metabolite formation (Chapter 6, References 5,7). Details of these studies will be presented in the respective chapters.

Chapter II

Characterization of FT metabolites in animals and cancer patients



SUMMARY

Two hydroxylated metabolites (M_1 and M_2) with hydroxyl function on the furan moiety have been isolated from the urine samples of rabbits following the administration of FT. Comparative physicochemical analyses of these metabolites with the authentic 3'-OH-FT and 4'-OH-FT including gas chromatograph-mass spectrometry (GC-MS), proton nuclear magnetic resonance (^{1}H-NMR), UV spectrometry, acid hydrolysis, and circular dichroism confirm their structures to be cis-4'-OH-FT (M_1) and trans-4'-OH-FT (M_2). Following the administration of 2 g/sq m/d of FT to patients with various types of carcinomas, five FT metabolites were identified in the plasma and were isolated from the urine samples. These metabolites were identified as FU, trans-4'-OH-FT (MH-1), cis-4'- OH-FT (MH-2), trans-3'-OH-FT (MH-3), and dehydro-FT with a double bond in the furan moiety (MH-4). The metabolic cleavage of FT at the N_1-C_2 position to form FU and γ-butyrolactone (GBL) as one of the possible products of the tetrahydafuran moiety was studied in patients and rabbits, and in vitro using different subcellular enzyme fractions of mouse and rabbit liver homogenates. GBL was identified by chemical ionization-GC-MS as an FT metabolite generated both in vivo and in vitro. Plasma concentrations of GBL were significantly higher than all other previously isolated FU metabolites in rabbits and patients. In vitro, FT was metabolized to FU by both the 9000xg supernatant fraction and the microsomal pellet of mouse liver homogenate, however, GBL was generated only by the 9000 and 100,000xg supernatant fractions and not by the microsomal pellets of mouse and rabbit homogenate. The other FT metabolites, including trans-3'-OH-FT, cis- and trans-4'-OH-FT and dehydro-FT, were not detectable under in vitro incubation (i.e. < 0.1 % of conversion).

INTRODUCTION

FT undergoes extensive metabolism *in vivo* (13,21,28). Eighty percent of a 100 mg/kg dose of 2-^{14}C-FT administered to rats was eliminated in 24 hours, of which 55 % was expired CO_2, 15 % excreted in the urine as unchanged FT, and 10 % as FU and FU-containing nucleosides and nucleotides (21). The major routes of excretion of ^{14}C-FT in beagle dogs (30 mg/kg) and rhesus monkey (60 mg/kg) were found to be pulmonary (35 % in 24 hours) and renal (30 % in 24 hours). Fluoroproprionic acid was the major urinary metabolite in monkeys, but only a minor one in dogs (28). Tumor-bearing mice eliminated FT at a faster rate. One hundred percent of the administered radioactive FT dose (150 mg/kg) was recovered within 48 hours *via* the renal (51 %), pulmonary (38 %), and fecal (1 %) routes of excretion (60). These studies show that, in spite of some species variation, FT is mainly eliminated by metabolism to FU and FU-related metabolites. This is consistent with the hypothesized mechanism of the antitumor activity of FT that FT acts as a metabolic depot form of FU.

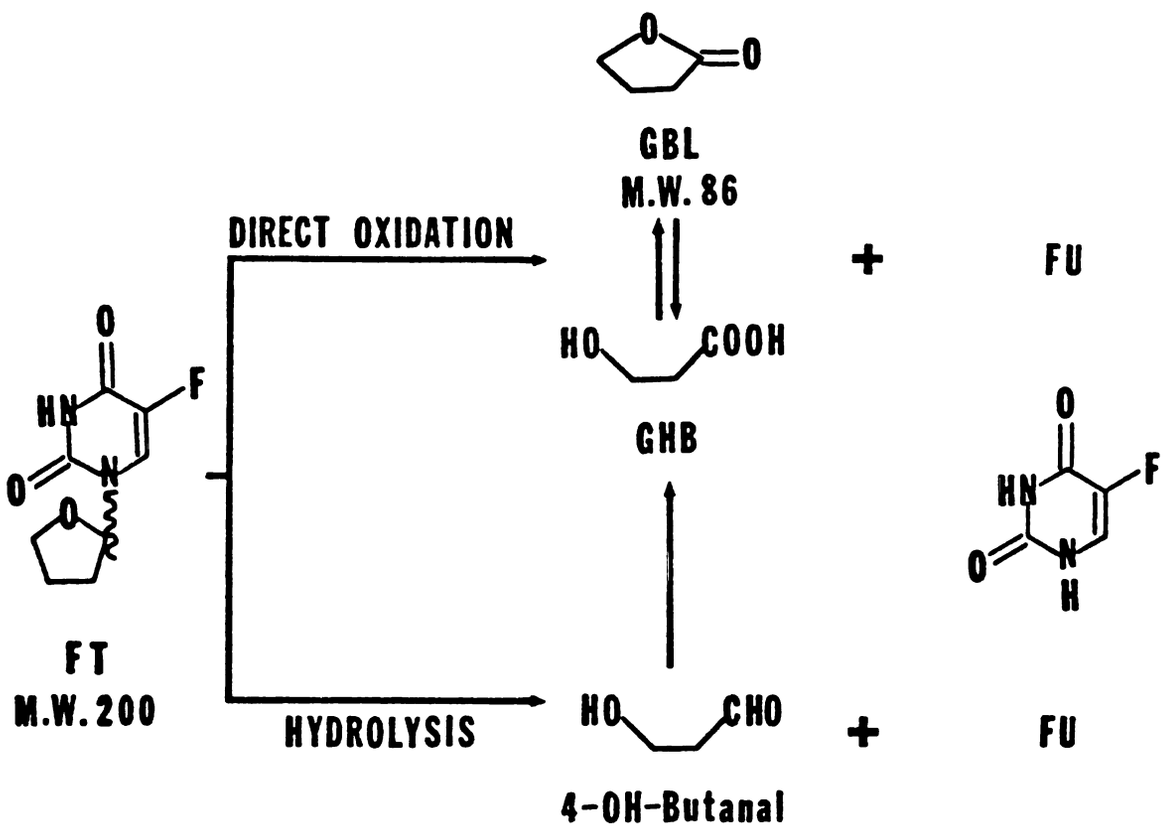
However, several reports support the hypothesis that FU formation may not be the only mechanism of FT activation. Smolyanskaya and Tugarinov (85) have demonstrated the *in vivo* presence of a microbiologically active metabolite fraction of FT in addition to FU. Differences in FT and FU toxicities to mice also support an activation mechanism of FT other than, or in addition to, formation of FU (51), while therapeutic cross-resistance between FT and FU suggests that the major part of the therapeutic mechanism of action is common to FT and FU (36). Pharmacokinetic analysis of FT and FU plasma levels following administration of FT to rabbits and rats

(95) further indicates the presence of metabolites other than FU that might be independently active or serve as intermediate(s) in the formation of FU.

In this study we have used FT specifically labeled either in the tetrahydrofuranyl ring or in the FU moiety in order to investigate metabolic pathways of FT other than formation and subsequent metabolism of FU. With this technique, 2 hydroxylated metabolites of FT, i.e. cis- and trans-4'-OH-FT, were isolated and characterized, and their presence in circulating peripheral blood of rats and rabbits was established. The metabolism of FT was also investigated in cancer patients. In addition to cis-and trans-4'-OH-FT, trans-3'-OH-FT (MH-3) and a dehydrogenated metabolite (MH-4) were identified.

Other possible routes of FT metabolism are the oxidation at the C-2' and C-5' positions by oxidases or dehydrogenases resulting in the formation of chemically labile intermediates that spontaneously cleave to FU and, in the case of C-2' oxidation, to GBL. The latter exists *in vivo* predominantly as γ-hydroxybutyric acid (GHB) (78). An alternative pathway is the hydrolytic cleavage of the N_1-C_2' bond which gives rise to FU and 4-hydroxybutanal which could be further oxidized to GHB, and hence GBL (Figure 3). The extraordinarily high plasma clearance of FU has made it virtually impossible to clearly define the kinetics and extent of FU formation from FT (8,17). Since GBL has a plasma clearance much slower than FU in man (90), the conversion of FT to GBL may serve as an alternative measure of the formation of FU *via* the cleavage of FT at the C-2' position. In this study we have identified GBL as a major FT metabolite generated both *in vivo* and *in vitro*.

Figure 3. Possible pathways of FT activation to GBL and FU.



MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and reagents were of analytical reagent or spectroquality grade. FT was supplied by the chemical and Drug Procurement Section, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. [2-^{14}C]FT [1-(tetrahydro-2-furanyl)-5-fluoro-2-^{14}C-uracil; specific activity, 46 μCi/mg] and [2',5'-^{14}C]FT [1-(tetrahydro-2-furanyl-2'-5'- ^{14}C)-5-fluorouracil, specific activity, 33 μCi/mg] were obtained through the National Cancer Institute from Isotope Synthesis Laboratory, Stanford Research Institute, Menlo Park, CA. Bis-^{15}N-5-fluorouracil ($^{15}N_2$-FU) was synthesized from bis-^{15}N-thiourea (99% ^{15}N enrichment; Koch Isotpoe, Cambridge, MA) (83). 5-Fluorouridine (FUR) and 5-fluorodeoxyuridine (FUdR) were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. 2',3'-Dehydro-5-fluorodeoxyuridine (DHFUdR) was recieved from Dr. T. Khwaja, University of Southern California, Los Angeles, CA. β-D-3'-OH-FT and β,D-4'-OH-FT were generous gifts of Drs. R. Meyer and C. Levinson, University of California, San Francisco, CA.

APPARATUS

GC of FT, dehydro-FT, and hydroxylated FT's were performed on a Varian Aerograph 2700 equipped with a glass column (6 ft x 0.25 inch outside diameter; 2 mm inside diameter) packed with 3 %OV-1 on Gas-Chrom Q (100 to 120 mesh; Applied Science Laboratories, State College, PA). The injector, column, and detector temperatures were 245, 180, and 275°, respectively. Flow rates for helium, oxygen, and hydrogen were 40, 300, and 30 ml/min, respectively. Mass spectra of these compounds were obtained by direct insertion on an AEI MS902 mass spectrometer with isobutane chemical ionization and by use of a gas chromatographic inlet system on a Varian MAT CH-7 electron impact mass spectrometer connected with a Varian Aerograph 2700 gas chromatograph and controlled by a Nova 2-10 computer (GC-MS). ^{14}C activity was measured in a Searle Analytic Mark III liquid scintillation counter.

Analysis of FT and its UV-absorbing metabolites was performed on a high-pressure liquid chromatograph (Waters Associates, Milford, Mass.) equipped with a Model 6000A solvent delivery system, a Model 440 dual wavelength UV absorbance detector at 280 and 254 nm, a Model U6-K injector, and a μBondapak C_{18} column (4 mm x 30 cm; Waters Associates). The column was run at ambient temperature with a flow rate of 2 ml/min at 2300 psi. UV absorption was measured on a Beckman Model 25 spectrophotometer with methanol as solvent. Proton magnetic resonance spectra were obtained on a Varian XL-100 NMR spectrometer equipped with a Nicolet Fourier Transform accessory. Deuterated chloroform, acetone and dimethyl sulfoxide (DMSO) were used as solvents and tetramethylsilane was used as internal standard.

The chemical ionization-GC-MS identification of GBL was obtained

on a Finnigan 3200 quadrupole mass spectrometer equipped with a Finnigan 9500 GC inlet system, and a glass single-jet separator interface. GC separation of samples was carried out using a 24 ft x 2 mm i.d. glass column packed with 3%OV-1 on gas Chrom Q (80-100 mesh, Applied Science Laboratory, State College, PA) at 75° using methane as the reagent gas. Mass chromatograms were obtained using selected ion monitoring at m/e 87 (MH^+ for GBL) and 101 (MH^+ for DVL).

The GC-FID assay of GBL was performed on a Varian Aerograph 2700 equipped with a 6 ft x 2 mm i.d. glass column packed with 5% FFAP (Free Fatty Acid Phase) on gas chrom Q (80-100 mesh, Applied Science Laboratory, State College, PA). The column, injector, and detector temperatures were 155°, 200° and 225°, respectively. Flow rates for the helium, air, and hydrogen gases were 30, 300, and 30 ml/min, respectively.

ANIMAL PROTOCOLS

Pharmacokinetic and metabolism studies of FT were investigated in rabbits and rats. Male and female New Zealand White rabbits weighing 3 to 5 kg received by rapid infusion (2 to 4 min) through an ear vein aqueous solutions (pH 9 to 10) of [2-^{14}C]FT [125 mg/kg (25 μCi/animal)] or [2',5'-^{14}C]FT [30 mg/kg (15 μCi/animal)]. Heparinized blood samples were taken from an opposite ear vein at appropriate times for up to 12 hr. Male Sprague-Dawley rats weighing 250 g were given injections of [2-^{14}C]FT [125 mg/kg (4 to 8 μCi/animal)] into a femoral vein, and blood samples were withdrawn from a femoral artery cannula into heparinized syringes at appropriate time intervals up to 8 hr. Urine samples of both species were collected during the same time period. Blood samples were spun down, and plasma samples and urine samples were frozen for subsequ-

ent HPLC analysis. Storage did not affect drug and metabolite concentrations.

Metabolic conversion of FT to GBL was further investigated in rabbits. Two male New Zealand white rabbits of about 3 kg body weight were given saline solutions of GBL and FT by rapid i.v. infusions over 2-5 minutes through an indwelling catheter in the ear vein. Blood samples were withdrawn through the catheter in the opposite ear. Each rabbit was given a first dose of GBL (25 mg/kg) followed by an equimolar FT dose (60 mg/kg), and then a second dose of GBL (25 mg/kg). The dosing interval was such that the GBL or FT dose was largely (> 95%) eliminated from the circulation at the time when the next dose was administered. In rabbit 1, each dose was given in one day intervals, whereas in rabbit 2, the FT dose was given at 2 hours after the first GBL dose, and the second GBL dose was given at 10 hours after the FT dose. Samples were stored frozen for subsequent GC analysis.

PATIENT PROTOCOLS

The pharmacokinetics and metabolism of FT were studied in five patients with various carcinomas. The clinical data of these patients are detailed in Table II. The patients were randomly recruited from an ongoing phase II clinical trial with FT conducted by the Cancer Research Institute, University of California (San Francisco, CA) and the Northern California Oncology Group. FT dissolved in 5% dextrose in water was infused iv over 30 minutes at a dose of 2 g/sq m as a single chemotherapeutic agent during the first day of therapy and again together with either methyl-CCNU, adriamycin, or mitomycin C on the second day. Urine was collected from patient 5 over 24 hours after the first dose. Serial blood samples were drawn

Table II. Patient data.

patient no.	Age yrs	Sex	Diagnosis	FT g/day	2nd chemotherapeutic agent	Levels of total bilirubin (mg %)[a]	Levels of alkaline phosphatase (IU/liter)[b]	Levels of serum creatinine (mg %)[c]	Levels of BUN (mg%)[d]
1	67	M	Primary hepatoma	3.4	methyl-CCNU	5.9	262	0.9	11
2	66	M	Primary hepatoma	3.0	adriamycin	10.0	220	1.4	33
3	55	M	Primary hepatoma and pleural effusion	3.9	mitomycin C	0.5	68	1.0	13
4	64	M	liver metastasis, pleural effusion, and dirrhosis	3.0	adriamycin	0.6	61	0.7	7
5	68	F	Liver metastasis	3.0	methyl-	0.6	71	0.9	22

[a]Normal range = 0.2-1.5 mg%

[b]Normal range = 23-71 IU/liter

[c]Normal range = 0.5-1.2 mg%

[d]Normal range = 10-20 mg%

through an indwelling i.v. catheter into heparinized tubes and centrifuged, and plasma fractions were immediately extracted with ethyl acetate. The organic layer was dried under nitrogen and reconstituted with methanol for subsequent HPLC analysis.

The metabolic activation of FT to GBL was studied in two additional patients treated under a different protocol. Blood samples were taken from these two patients receiving FT treatment at the Cancer Research Institute at the University of California, San Francisco under the Northern California Oncology Group protocol. The plasma fractions were stored frozen for subsequent analysis. The two patients, KM and CD, were diagnosed to have gastric and pancreatic carcinoma, respectively. Both patients had normal renal and hepatic functions as indicated by their serum blood urea nitrogen, creatinine, bilirubin and alkaline phosphatase levels. Both patients were given a dose of 2 g FT/sq m/d for 2 days by i.v. infusion over 30 minutes, and adriamycin plus BCNU were given in conjunction with FT on the first day.

Isolation of FT metabolites

FT metabolites isolated from rabbit urine are represented by M_1 and M_2, whereas those of human origin are represented by MH-1,2,3 and 4.

Rabbit urine samples were buffered with one-tenth their volume of 0.5 M NaH_2PO_4 and exhaustively extracted with ethyl acetate. The extraction yields of total radioactivity in urine were 34 and 70% following doses of [2-^{14}C]FT and [2',5'-^{14}C]FT, respectively. The organic layer was evaporated under N_2 at 50°. The residue was dissolved in a small volume of methanol:CH_2Cl_2 (2:1, v/v) and placed on a silica gel column (25 x 100 mm, 60 to 200 mesh;

J.T. Baker Chemical Co., Philipsburg, NJ). We collected 30-ml fractions by using gradient elution with 60 ml CH_2Cl_2, 30 ml CH_2Cl_2:methanol (90:10, v/v), and 60 ml CH_2Cl_2:methanol (80:20, v/v). These fractions were analyzed for ^{14}C activity. Fractions 2 to 5 containing 93% of total extractable radioactivity were pooled and evaporated under N_2 at 50°, and the residue was subjected to TLC (Silica Gel F-254; Merck, Darmstadt, Germany) with $CHCl_3$: methanol (70:30, v/v). Four fluorescence-quenching bands with ^{14}C activity at R_f 0.77 (FT), 0.62 (M_1), 0.58 (M_2) and 0.51 (FU) were scraped off and extracted with methanol. In a 10-hr urine collection, 16 mg, 4.5 mg, 500 µg, and 300 µg of FT, M_1, M_2, and FU, respectively were recovered following [2-^{14}C]FT (125 mg/kg) in a 3-kg rabbit.

Both metabolite fractions M_1 and M_2 isolated from TLC plates were then further purified by Sephadex LH-20 gel column chromatography (0.8 g Sephadex LH-20, 25 to 100 µm; Pharmacia Fine Chemicals, Piscataway, NJ) in a column (0.5-mm inside diameter) with 5 ml CH_2Cl_2 and 20 ml CH_2Cl_2:methanol (97:3, v/v) as eluents and on a silica gel column (1 x 27 cm, 60 to 200 mesh; Baker) with 40 ml CH_2Cl_2:methanol (90:10, v/v) as eluent to give chromatographically pure fractions (TLC, HPLC, and GC). GC as well as GC-MS was performed by use of flash methylation with trimethylphenylammonium hydroxide (Pierce Chemical Co., Rockford, IL) under GC conditions described above. Retention times were 0.57, 1.06, and 1.06 min for FT, M_1 and M_2, respectively.

A 24-hour human urine sample (1700 ml) was concentrated in vacuo to about 100 ml. The pH of the urine sample was adjusted to 5 with phosphate buffer and the sample was exhaustively extracted with

ethyl acetate. MH-1-4 were isolated and obtained in chromatographically pure form using column chromatography and thin-layer chromatography as described above. Metabolite MH-4 was recycled several times through reverse-phase HPLC to achieve complete separation from FT. The quantities obtained were 200, 750, 750, and 50 μg for MH-1-4, respectively. Metabolites were characterized by ultraviolet (UV) absorbance in methanol, ^{1}H-NMR in methanol-d_4 or acetone-d_6 (except for MH-4), GC, and GC-MS following flash methylation with trimethylphenylammonium hydroxide (Pierce Chemical Co., Rockford, IL).

In Vitro metabolism studies

5-6 weeks old male CDF-1 mice (obtained through NCI, Bethesda, MD) and 6 months old male dutch rabbits were decapitated and their livers were removed and homogenized in aqueous solution of 1.15 potassium chloride in 0.01 M sodium/potassium phosphate buffer of pH 7.4. Liver homogenates were centrifuged at 9000 X g and 0° for 2 x 15 minutes in a Sorvall Superspeed RC2-B centrifuge, and subsequently at 100,000 X g and 0° for 2 x 1 hour in a Beckman L2- 65B ultracentrifuge. The 9000 X g, 100,000 X g supernatants, and the 100,000 X g microsomal pellets resuspended in buffer were used as enzyme sources. Buffer solutions of 2 mM FT, 0.015 mM $MgSO_4$, and 4 mg NADPH (type I, tetrasodium salt, Sigma Chemicals, St. Louis, MO) added every 20 minutes, were incubated with enzyme solution (final concentration of all preparations standarized to 1 g wet weight of tissue per 4 ml incubation mixture) at 37° for one hour in a Dubnoff metabolic shaker.

Acid hydrolysis of fluorinated pyrimidine nucleosides

Authentic samples of FUR and FUdR were hydrolyzed in 100 μl 2 N

HCl in sealed ampoules under various conditions. The extent of hydrolysis of these compounds to FU was determined by GC-MS using bis-^{15}N-FU as internal standard as described elsewhere (95). These florinated pyrimidine nucleosides were found to quantitatively convert to FU by 2N HCl at 190°C for 2 hours. UV-absorbing FT metabolites isolated from rabbit and human urine were hydrolyzed under the same condition, and the presence of FU as the acid hydrolysis product was confirmed by GC-MS and/or HPLC analyses. This confirms that the FT metabolites contain the unaltered FU nucleus.

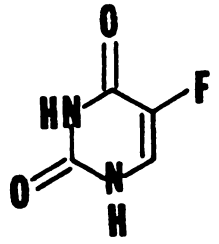
RESULTS

Structural elucidation of FT metabolites

FU and two other hydroxylated FT metabolites were isolated from rabbit urine and will be referred to as M_1 and M_2. FU and four additional FT metabolites were isolated from patient urine and will be referred to as MH-1,2,3 and 4. Table III summarizes the physicochemical properties of these metabolites and the authentic samples of FU, FUR, FUdR, β-D-3'-OH-FT and β-D-4'-OH-FT. The structure assignments of these metabolites will be discussed in this chapter and the structures are summarized in Figure 4, whereas the assignment of their stereochemical configuration will be disscussed in Chapter 3.

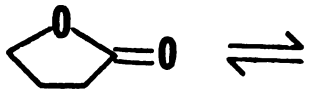
Metabolites M_1 and M_2. Metabolites M_1 and M_2 contained the ^{14}C label following administration of both [2-^{14}C]FT and [2',5'-^{14}C]FT, which indicates that both the FU and tetrahydrofuran ring carbons are present. The UV absorption maximum at 270 nm demonstrates preservation of the FU ring chromophore. This is further supported by the acid hydrolysis of these metabolites to FU (see Figure 8B in Chapter 4). Chemical ionization direct-insertion mass spectrometry gave a quasi-molecular ion for M_1 at m/e 217 ($M^+ + 1$), which suggests insertion of 1 oxygen atom into FT (M.W. 200). GC-MS following permethylation of FT, M_1 and M_2 confirmed the presence of a hydroxyl function and established its location in the tetrahydrofuran moiety through ion fragments observed in the mass spectra of M_1 and M_2 (Figures 5 and 6). Suggested mass fragmentation patterns of the hydroxylated FT metabolites are shown in Figure 5 and 6, structures of mass spectral ions of FT, M_1 and M_2 are given in Figures 5 and 6.

Figure 4. Structures of FT metabolites.



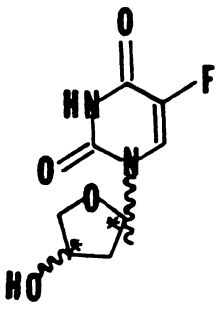
FU

5-fluorouracil



GBL γ-butyrolactone

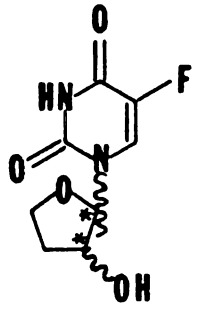
GHB γ-hydroxybutyric acid



4'-OH-FT

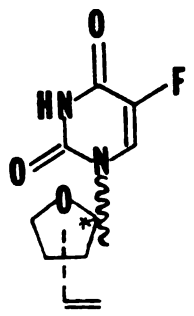
cis-configuration :
rabbit M_1, human MH-2

trans-configuration :
rabbit M_2, human MH-1
(α,L and β,D)



3'-OH-FT

trans-configuration :
MH-3, human only



DEHYDRO-FT

location of the double bond undefined

* Asymetric carbon

Figure 5. Proposed mass fragmentation pattern of MH-1 (trans-4'-OH-FT) and MH-2 (cis-4'-OH-FT).

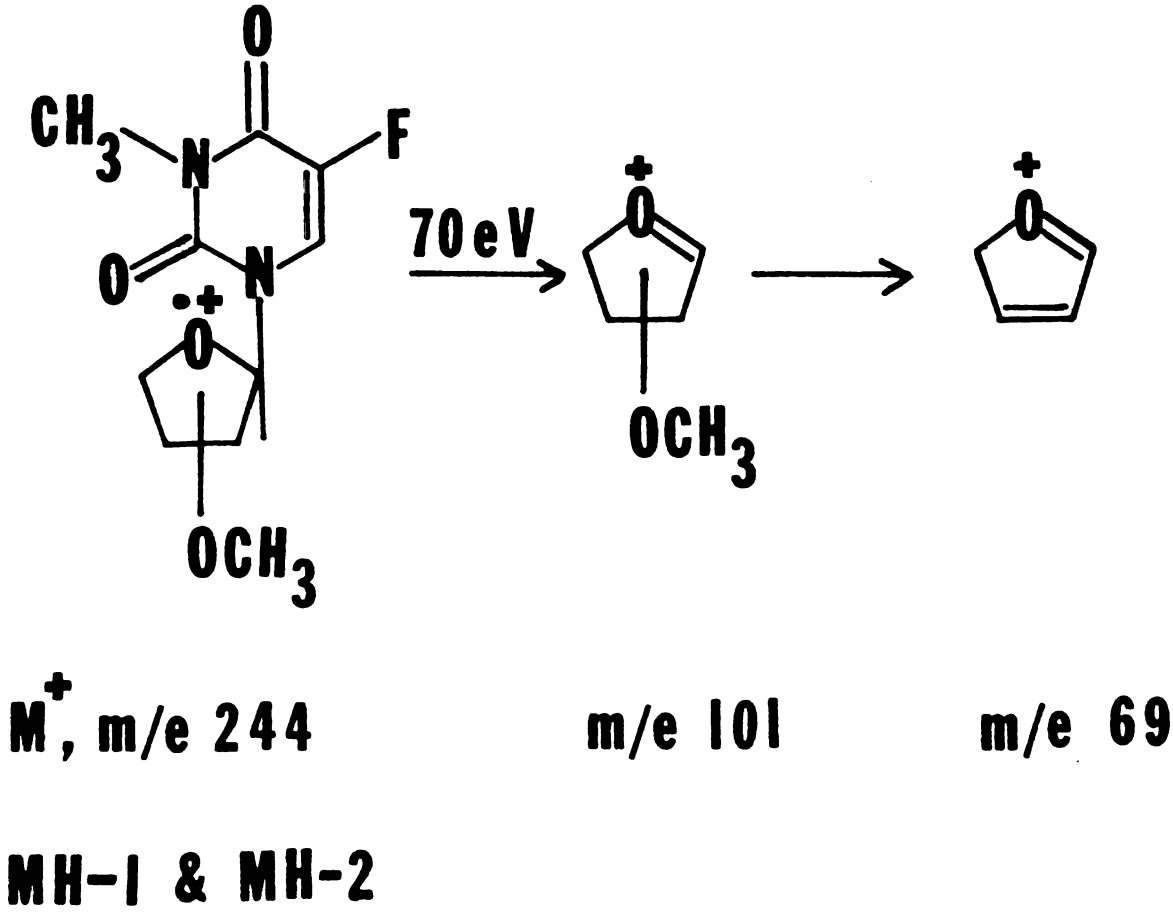
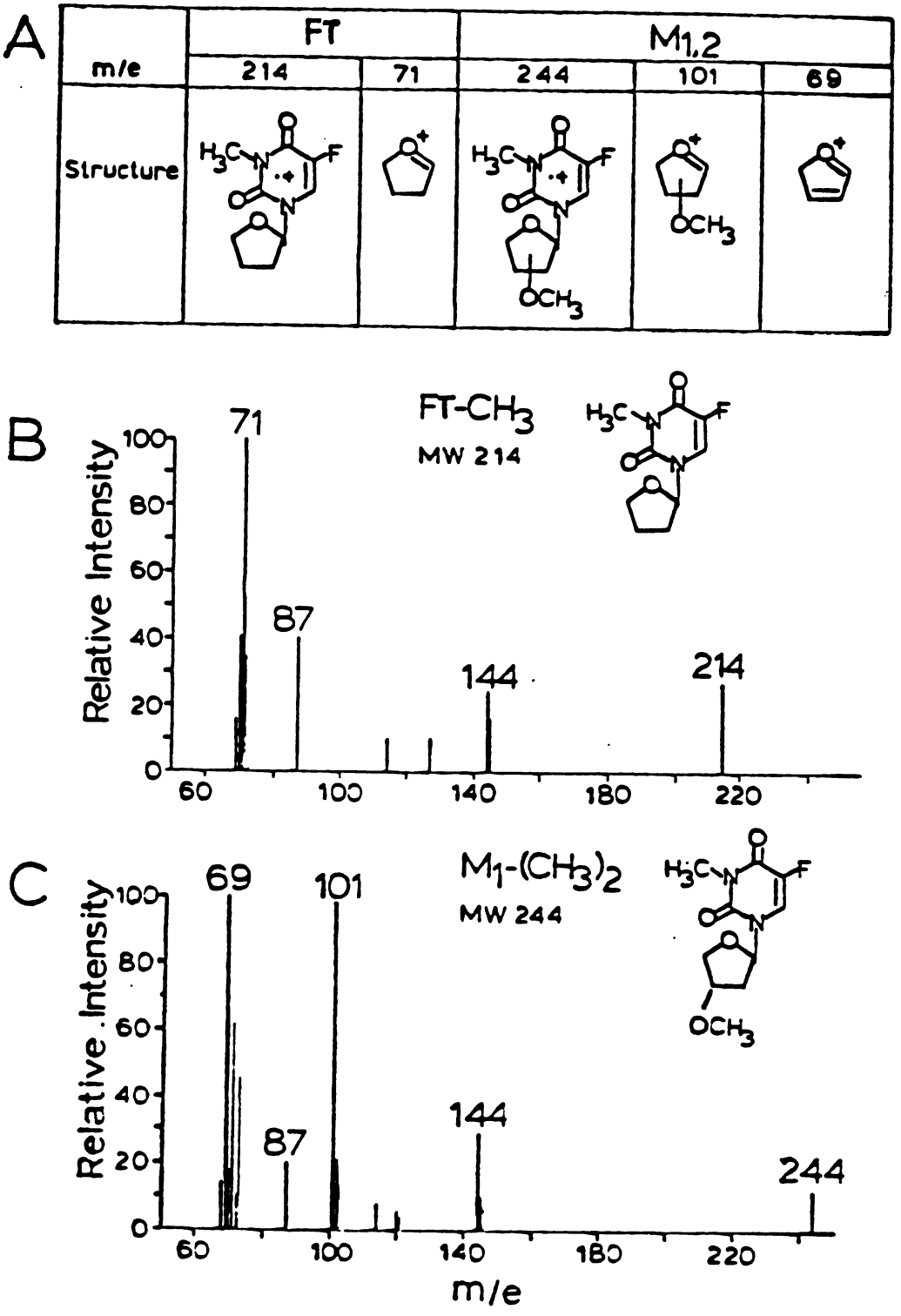


Figure 6. (A). Suggested structures of parent ions and fragments obtained from FT, M_1 (cis-4'-OH-FT), and M_2 (trans-4'-OH-FT) by GC-MS after permethylation.

(B). The mass spectrum of methylated FT.

(C). The mass spectrum of permethylated M_1.



The location of this OH function was studied by proton magnetic resonance spectroscopy. It can be assumed that hydroxylation in C-2' and C-5' of FT results in chemically labile derivatives that are not isolable per se. Hydroxylation at the C-3' and C-4' as the remaining potential sites is readily differentiated by the NMR coupling constants of the anomeric proton in C-2' with FT, FUR, FUdR, β-D-3'-OH-FT, and β-D-4'-OH-FT as references (Table III).

The C-2' anomeric proton in the 'H-NMR spectrum appeared as a doublet-psuedotriplet signal. Lin et al. (64) and Meyer and Levinson (71) recently synthesized isomers of hydroxylated-FT and showed that the splitting pattern and coupling constants of the anomeric proton in these compounds are largely dependent on the conformation of the hydroxyl group in relation to the pyrimidine ring, and that the 'H-NMR spectrum of M_1 is consistent with a structure of cis-4'-OH-FT.

The C-2' anomeric proton of M_2 results in a signal identical to that of the C-1'-H anomeric proton of FUdR, but shifted upfield by 0.09 ppm. This strongly suggests the C-4'-OH position for M_2 with a similar relative configuration as present in FUdR. The triplet configuration of the C-2'-H signal of M_2, which is further split by long-range coupling to C-5-F, is indicative of 2'-deoxyfuranosyl nucleosides in the β configuartion (77). However, it does not preclude existence of an enantiomeric M_2 configuaration.

Metabolites MH-1 and -2. These metabolites are identical to trans-4'-OH-FT (M_2) and cis-4'-OH-FT (M_1) isolated from rabbit urine (96) as confirmed by chromatography, UV absorbance maxima, ^{1}H-NMR and GC-MS (Table III).

Metabolite MH-3. This metabolite has been observed in human but not

Table III. Physicochemical parameters of FT, FU, FUR, FUdR, β,D-3'-OH-FT, β,D-4'-OH-FT, and FT metabolites.

	FT	FUR	FUdR	β,D-3'-OH-FT	β,D-4'-OH-FT	FU	M_1	M_2	MH-1	MH-2	MH-3	MH-4
^{14}C label(2-^{14}C-FT)	+					+	+	+				
(2',5'-^{14}C-FT)	+					-	+	+				
UV_{max} in MeOH,nm	270			270 (pH 1,13)	270 (pH 1,13)	266	270	270	270	270	270	268
ε	9127					8151	9114	8823				
TLC R_f [a]	0.77					0.51	0.62	0.58				
HPLC retention volume, ml	12.8[b]			14.6[c]	10.6[c]	4.0[b]	7.0[b]	5.4[b]	10.6[c]	14.4[c]	14.6[c]	32.7[c]
254:280 nm UV absorbance ratio[c]	0.8	1.0	1.0	0.8	0.9	1.5	0.9	0.9	0.9	0.9	0.9	0.9
GC-MS(permethylated)[d]												
M^+, m/e	214 (27%)						244 (12%)	244 (2%)	244 (2%)	244 (12%)	244 (17%)	see Figure 7
M^+ - FU, m/e	71 (100%)						101 (98%)	101 (98%)	101 (98%)	101 (98%)	101 (100%)	"
M^+ - FU-CH_3OH m/e							69 (100%)	69 (100%)	69 (100%)	69 (100%)		"
^1H-NMR[e]												
C-2'-H	m	d-d	t-d	t@	t-d@		d-t	t-d	t-d*	d-t*	s	
δ, ppm	5.98	5.92	6.30	5.53	6.12		6.15	6.21	6.23	6.00	5.55	
J,Hz		Jd-d=2	Jt=7,Jd=2	Jt=1.7	Jt=6.0,Jd=1.9		Jd=8 Jt=2	Jt=7 Jd=1.5	Jt=6.5 Jd=1.5	Jd=8 Jt=1.5	t,5.55@ Jt=2	
C-6-H	d	d	d	d@			d	d	d*	d*	d	
δ,ppm	7.70	8.34	8.29	7.75			8.14	7.75	7.73	7.97	7.46	
J,Hz	Jd=7	Jd=7	Jd=7	Jd=7			Jd=7	Jd=7	Jd=7	Jd=7	Jd=6.4	
δ,ppm											d,7.70@	
J,Hz											Jd=7	

[a] TLC solvent : $CHCL_3$; MeOH, 70 : 30

[b] HPLC eluent : 15% methanol in 10mM sodium acetate, pH 4.2

[c] HPLC eluent : 5% methanol in 10mM sodium acetate, pH 4.2

[d] Numbers in parentheses = percent abundance

[e] Acetone-d_6, unless specified otherwise

* CD_3OD; @ DMSO-d_6

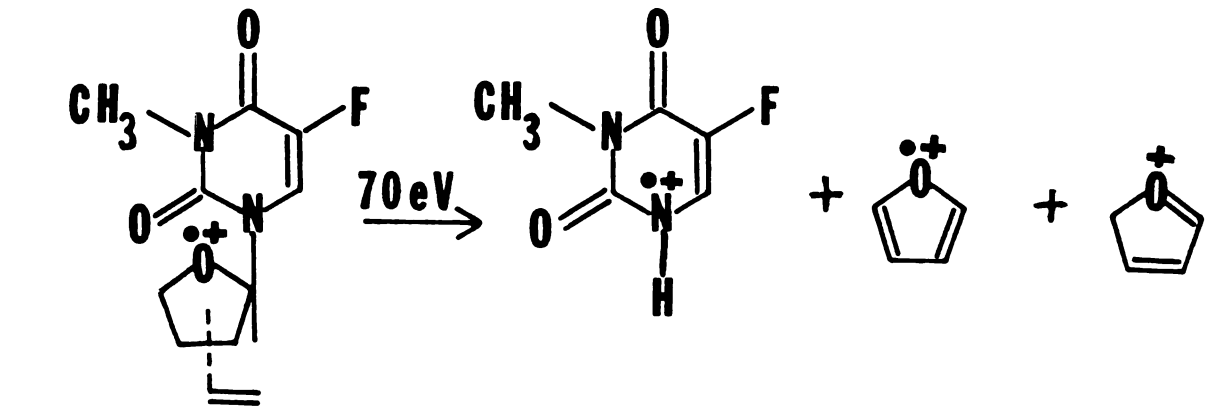
in rat or rabbit plasma and urine. It can be hydrolyzed to FU under strongly acidic conditions and has an UV absorbance maximum at 270 nm which suggests presence of the intact FU nucleus. Mass spectral and ^{1}H-NMR data indicate that the tetrahydrofuranyl moiety is altered in MH-3. The GC-MS spectrum of permethylated MH-3 gave two ion peaks at m/e 244 (M^+, 17%) and m/e 101 (100%), which also occur in the spectra of MH-1 and -2. These ions indicate metabolic insertion of one oxygen atom and addition of two methyl groups by flash methylation. However, the major ion at m/e 69 in the spectra of MH-1 and -2 associated with the tetrahydrofuran moiety is absent in the spectrum of MH-3. The C-6-H signal in the ^{1}H-NMR spectrum of MH-3 in acetone-d_6 is shifted upfield (d,δ7.46 ppm, J_d = 6.4 Hz) when compared to FU (d,δ7.63 ppm, J_d = 6 Hz) while the C-6-protons of FU derivatives with tetrahydrofuran substituent in N-1 are shifted downfield (Table III). The anomeric C-2' proton appear as a singlet at δ5.55 ppm.

The ^{1}H-NMR spectrum of MH-3 in duerterated-DMSO is identical to that of β-D-3'-OH-FT. The C-2' singlet signal observed when acetone-d_6 was used as the solvent now appears as a closely spaced psuedotriplet signal using DMSOd_6 was used as the solvent. This unusual psuedotriplet signal is identical to that of the authentic β-D-3'-OH-FT, but different from the one reported for cis-3'-OH-FT by Lin *et al* (64). Furthermore, the UV spectra of β-D-3'-OH-FT and MH-3 are identical with UV max at 270 nm. These two compounds also coeluted in GC using 3% OV-1 column at 2 minutes following on column flash methylation with trimethylphenylammonium hydroxide and in HPLC with retention volumes of 14.8 and 13.0 ml using two different eluents, i.e. 5% methanol and 3% acetonitrile in 0.01 M sodium acetate

buffer of pH 4.2, respectively. Both compounds have the same 254:280 nm UV absorbance ratio of 0.8. These identical physiochemical properties of MH-3 and β-D-3'-OH-FT indicate that the structure of MH-3 is 3'-OH-FT in a trans-configuration.

Metabolite MH-4. This metabolite was also observed in rabbit plasma and urine (M_3). It is more lipophilic than FT based on their relative chromatographic properties. An UV absorbance maixmum of 268 nm, as well as acid hydrolysis to give FU prove the presence of the intact FU moiety (See Figure 8B in Chapter 4). MH-4 was quantitatively converted to FU within 30 minutes under acid conditions, ie, pH 1 and room temperature, whereas only 20% of FT was hydrolyzed to FU under identical conditions. MH-4 also slowly decomposed to FU when stored in methanol or acetone at 4°C. The isolated amount of this metabolite was insufficient for ^{1}H-NMR studies. However, the GC-MS data of permethylated MH-4 give strong supportive evidence for a dehydrogenated metabolite of FT. While no molecular ion was observed, the fragment ions at m/e 144 (100%), 69 (62%), and 68 (60%) characterize both the FU and the dihydrofuran moieties of MH-4 (Figure 7). The large abundance of the radical cation at m/e 144, which is small in the spectrum of FT, can arise from formation of stable uncharged furan following proton and charge transfer to the FU ring. The furan radical cation at m/e 68 is generated when the charge remains in the furan moiety. This transition cannot occur with MH-1 and -2 due to lack of the preformed double bond, and the ion at m/e 68 is absent in the spectra of MH-1 and -2. The GC-MS spectrum of permethylated MH-4 was compared to an identically treated sample of DHFUdR which also gave no parent ion, but again fragments at m/e 144 (74%), 112 (38%), and 113 (30%) (equivalent

Figure 7. Proposed mass fragmentation pattern of MH-4 (dehydro-FT).



to m/e 68 and 69 of MH-4 plus $-CH_2OCH_3$). An additional peak at m/e 81 (100%) was generated from m/e 113 by loss of CH_3OH. Equivalence of the mass spectra of MH-4 and DHFUdR provides further evidence for the presence of the double bond in the dihydrofuran ring of MH-4. Its exact location, however, cannot be inferred from mass spectral data.

Identification of GBL as an FT Metabolite. GBL, the internal ester of GHB, is formed from GHB under strongly acidic conditions (1). Roth and Giarman (78) reported that GBL is rapidly hydrolyzed to GHB at physiologic pH due to the lactonase activity in plasma. We found that direct extraction of biological samples obtained from FT-treated rabbits and patients as well as from *in vitro* incubation mixtures with chloroform at pH 7.4 yielded little or no GBL measured by GC-FID assay. Addition of sulfuric acid is essential to lactonize the GHB to GBL which is extractable into chloroform. These same biological samples, when extracted under strongly acidic conditions, were found to contain significant quantities of a compound which co-eluted with authentic GBL in the GC-FID and GC-CI-MS analyses under two largely different chromatographic conditions (5% FFAP at 155°C and 3% OV-1 at 75°C). Furthermore, the selected ion monitoring records of the chemical ionization-GC-MS analysis of the plasma extracts of patient CD and of *in vitro* incubation mixtures of FT and mouse hepatic 9000 X g supernatants contained a peak at m/e 87 (MH^+ of GBL) with the identical retention time of authentic GBL that was absent in control samples. Acid treatment of pure FT did not produce detectable amounts of GBL. The observed GC and GC-MS peaks could also have arisen from FT generated from its oxidative metabolites (Figure 4) by the addition of sulfuric acid or by other cleavage products

with the same molecular weight as GBL (e.g. succinaldehyde from C-5' oxidation). However, other cleavage products are chemically rather different from GBL and should, therefore, separate under the GC conditions; moreover, such cleavage products are neutral species and should have been extractable under neutral conditions, where we were unable to observe product formation. The other known FT metabolites were either not detectable during in vitro incubations or present in plasma at approximately 10-fold lower concentrations than GBL; therefore, they cannot serve as a source of the observed GBL. Finally, chemically labile species, e.g., 2'-OH-FT, that may convert to GBL and FU during analysis should have been observable under the mild extraction conditions preceeding HPLC or should have appeared as FU which was not the case. These results provide strong evidence for the presence of GBL as a FT metabolite in biological samples.

In Vitro Metabolism. The results of in vitro metabolism of FT are detailed in Table VII in Chapter 4. It can be summarized as follows. GBL was generated by the soluble enzymes but not the microsomal enzymes of mouse and rabbit liver homogenates, whereas FU was generated by both the soluble and the microsomal enzymes.

DISCUSSION

FU has been identified as an FT metabolite in rats, rabbits and cancer patients. In addition, two new metabolites of FT (M_1 and M_2) have been isolated from rabbit urine and their structures have been determined and confirmed by comparative physicochemical analyses with the authentic 3'-OH-FT and 4'-OH-FT. Metabolites M_1 and M_2 are of potential pharmacological significance since hydroxylation of FT produces chemical species which resemble more closely FU nucleosides. Interaction of hydroxylated metabolites with specific pyrimidine enzymes might result in formation of FU mediated by phosphorylases (e.g., thymidine phosphorylase) or direct pharmacological activity mediated by inhibition of thymidine kinase as an example. Structure-activity relations of substrates for thymidine phosphorylase predict potential affinity of trans-4'- OH-FT if present in the β-D configuration (11).

A few FT metabolites with altered tetrahydrofuran moiety have been isolated from patient urine. MH-1 was found to be identical to M_2 and corresponds to the trans-4'-OH-FT and MH-2 is identical to M_1 and corresponds to the cis-4'-OH-FT. MH-3, corresponding to trans-3'-OH-FT, was observed only in human and not in rat or rabbit plasma and urine, suggesting that the hydroxylation of FT at the C-3' position is species dependent. MH-4 coeluted with M_3 from rabbit urine and corresponds to a structure of dehydrogenated -FT. MH-4 is more acid labile than FT and was found to spontaneously degrade to FU at a faster rate than FT. The lipophilic MH-4 was the second most abundant circulating FT metabolite. The possible role of these FT metabolites serving as metabolic intermediates in FT conversion to FU is investigated and discussed in Chapter 4.

Another FT metabolite common to rabbit and human is GBL. GBL represents the major FT metabolite in plasma, GBL (in equilibrium with GHB) are endogenous compounds found in certain regions of animal and human brain where GHB may play a physiological role in the regulation of nerve activity (23,86); GBL is currently used as a general anesthetic agent with a therapeutic plasma concentration range of 90-100 μg/ml (91), but GHB has been shown to depress both short term memory and critical flicker frequency in man at much lower concentrations (86,87). A structural analog of FT, FD-1 (bis-tetrahydrofuranyl-5-fluorouracil), shares the neurotoxicity of FT, and both compounds are more toxic to the CNS than FU. GBL was also identified as one of the major metabolites of FD-1 in rat plasma and brain tissues (54). Therefore, GBL/GHB may contribute to the effects of FT on the CNS.

Chapter III

Stereochemistry of FT metabolism

SUMMARY

The metabolism of R and S isomers of FT was studied in rats and *in vitro* using different fractions of mouse and rabbit liver homogenates. FT and its metabolites in biological samples were analyzed by gas chromatography and high pressure liquid chromatography. R- and S-FT were metabolized to different extents by individual pathways. Cleavage of R-FT to form γ-butyrolactone by mouse and rabbit liver homogenates was greater than that of S-FT. Cis-and trans-4'-OH-FT are common metabolites in rats, rabbits and humans, however, 3'-OH-FT (trans-configuration) was observed only in man. Both cis- and trans-4'-OH-FT were present in 24-hour rat urine in siginificant amount following S-FT administration, whereas only the trans-4'-OH-FT was detected in small quantity following R-FT administration. Furthermore, the stereochemical configurations of the hydroxylated metabolites isolated from rabbit and patient urine have been established and these metabolite fractions consisted of predominantly the α anomers which can arise only from S-FT. These findings suggest that there is stereoselective hydroxylation of S-FT. However, the hydroxylated metabolites generated from R-FT would have the natural β configuration and might have been preferentially metabolized prior to excretion into urine. An authentic sample of β,D-4'-OH-FT was quantiatively converted to FU by thymidine phosphorylase *in vitro*, and may represent a potential FU prodrug.

INTRODUCTION

A few FT metabolites including FU, 3'-and 4'-OH-FT, dehydro-FT and γ-butyrolactone (GBL) have been identified in rat, rabbit and patient plasma and urine following FT administration (Figure 4). FT was administered as a racemic mixture of R and S isomers, therefore, each of the hydroxylated metabolites would have two chiral centers (Figure 4) and theoretically could be present in two pairs of enantiomers, i.e. the cis-(corresponds to β, L and α, D) and trans-(corresponds to β, D and α,L) isomers. Fractions of the 4'-OH-FT isolated from rabbit and patient urine samples was converted to FU by a horse liver thymidine phosphorylase suggesting that the 4'-OH-FT was partially represented by the β, D configuration (Chapter 2 and 4). However, the absolute configurations of the other hydroxylated FT metabolites have not been defined.

FT may be metabolized to FU via a few pathways (Chapter 4). 1. Cleavage at the C-2' position to FU and GBL represent a major FT activation pathway in rabbits and GBL was a major circulating FT metabolite in patients (7). 2. Hydroxylation of FT at the C-4' position followed by stereospecific enzymatic phosphorolysis to FU (4,96). 3. Dehydrogenation of FT to a chemically more labile dehydro-FT which could undergo nonenzymatic conversion to FU in vivo (4). In vitro metabolism of FT to FU and possibly hydroxylated metabolites was mediated by mouse and rat hepatic microsomal enzymes (13), whereas oxidation at the C-2' position to FU and GBL was mainly mediated by the supernatant fractions of mouse and rabbit liver homogenates (Chapters 2 and 4).

The R and S isomers of FT were equally effective in inhibiting DNA synthesis of the L1210 leukemic cells, growth of human fibro-

blasts in cell culture (48), and tumor growth of AH-130 carcinoma and Yoshida sacroma implanted in rats (97). FT recovered from the combined urine of nine patients receiving racemic R,S-FT treatment showed no optical activity, indicating no enzymatic preference on the degradation on either isomers (48), and both isomers were metabolized by NADPH-dependent hepatic microsomal oxidation and released the same amount of FU determined by its antibacterial activity against Staphylococcus Aureus 209P (97). These observations indicate that there is no significant difference in the overall metabolic activation of the R and S isomers, but do not rule out stereoselectivity in the individual pathways.

In this study, we have compared the previously isolated FT metabolites (MH-1, 2 and 3) with the chemically synthesized authentic compounds and established their structures and stereochemical configurations. We also report here stereoselectivity in the metabolism of FT to 4'-OH-FT in rats and to GBL by mouse and rabbit liver homogenates. Conversion of an authentic sample of β,D-4'-OH-FT to FU by thmidine phosphorylase was studied in vitro.

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and reagents were obtained from commerical sources (Chapter 2). Racemic R,S-FT was supplied by the Chemical Resources Section, Pharmaceutical Resources Branch, Development Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. β,D-3'-OH-FT and β-D-4'-OH-FT were generous gifts of Drs. R. Meyer and C. Levinson, University of California, San Francisco, CA.

Apparatus. Analytical techniques included high pressure liquid chromatography (HPLC), gas chromatography (GC), proton nuclear magnetic resonance (^{1}H-NMR), UV absorbance, circular dichroism (CD), and optical rotation dispersion (ORD). GC analysis of GBL in biological fluids was performed on a 5 %FFAP (Free Fatty Acid Phase) on gas chrom Q column and GC spectra of the permethylated hydroxylated-FT metabolites were obtained on a 3 %OV-1 on a gas chrom Q column (Chapter 2). UV spectra were run on a Cary 118. CD spectra were obtained using a Jasco J-15. ORD of R-and S-FT were measured using a Perkin-Elmer 141 polarimeter. ^{1}H-NMR spectra were run on a Varian FT-80 at 80 MHz. Details of the other apparatus were described previously (Chapter 2).

Separation of the R-and S-Isomers of FT. The racemic FT was separated into R and S isomers by fractional recrystallization (97). The R-(+)-FT and S-(-)-FT thus obtained gave $[\alpha]^{23}$(c 1.0, $CHCL_3$) of +68.5° and -67°, and corresponded to 99% and 98% purity, respectively (cf $[\alpha]^{23}$(c 0.5, $CHCL_3$) of +70° and -70° for R-(+)-FT and S-(-)-FT obtained by Yasumoto et al., 97). R-FT corresponds to the natural β anomers and S-FT corresponds to unnatural α anomers.

Analysis of FT and Its Metabolites in Biological Samples. FT and

its UV absorbing metabolites in the rat urine samples and the in vitro incubation mixtures were analyzed by the previously mentioned HPLC assay (Chapter 5). Concentration of GBL in the in vitro incubation mixtures was analyzed by the GC-flame ionization detection assay described in Chapter 2.

Animal Protocol. Three 200-250 g female Sprague-Dawley rats were given an ip dose of 50-100 mg/kg of R-FT or S-FT in 1 ml of normal saline on day 1. A second FT dose was administered on day 5 when the first FT dose was quantitatively eliminated, animals previously receiving R-FT on day 1 were given S-FT on day 5, and vice versa. Animals were housed in metabolism cages and their urine samples were collected and stored frozen for subsequent analysis.

In Vitro Incubation. R-and S-FT (2mM) were incubated separately with different fractions of mouse and rabbit liver homogenates at 37°C for one hour. Details of the incubation were described previously (Chapter 2).

Incubation of Rat Urine Samples with Glusulase^{R}. Appropriate amount of 20% acetic acid, 2 M sodium acetate buffer, and distilled water were added to 0.5 ml 24-hour rat urine to bring the final volume to 0.9 ml with a pH of 5.2 and a buffer concentration of 0.1 M. 100 µl Glusulase^{R} (each ml contains 176,578 Fishman units of β-glucuronidase and 35,126 units of sulfatase, Endo Laboratory, Garden City, NY) was added to start the incubation at 37°C for 24 hours. Activity of Glusulase^{R} was confirmed by its ability to release free phenophthalein from phenophthalein glucuronic acid.

Thymidine Phosphorylase Incubations. Incubation of β,D-4'-OH-FT with a horse liver thymidine phosphorylase preparation were carried out as described previously (Chapter 4). Similar mixture without

adding the enzyme was used as control to rule out any nonenzymatic degradation. Product (FU) formation and substrate disappearance were monitored by HPLC analysis.

RESULTS

Assignment of Configurations of the Hydroxylated FT Metabolites.

Trans-4'-OH-FT. An authentic sample of β,D-4'-OH-FT was quantitatively converted to FU by thymidine phosphorylase within 3 hours, during which time nonenzymatic degradation was neglible. This enzyme is specific for the β,D anomer of deoxyribonucleosides and was capable of partially converting the MH-1 (5% conversion) and M_2 (20% conversion) isolated from patient and rabbit urine to FU (Table IV). The substrate affinity of the authentic β-D-4'- OH-FT supports our previous contention that the urinary trans-4'-OH-FT partly consists of the β,D anomer and infers that the trans-4'-OH-FT isolated from urine is represented by a mixture of α,L and β,D anomers in ratios of 4:1 and 95:5 for rabbits and patients, respectively.

Trans-3'-OH-FT (MH-3). Trans-3'-OH-FT was identified in human but not animal plasma and urine samples. It is represented by a mixture of β,D and α,L enantiomers. The CD spectra of β,D-3'-OH-FT and MH-3 are depicted in Figure 3, corresponds to $[\theta]_{273}$ + 13600 and $[\theta]_{249}$ 0 for β,D-3'-OH-FT (1.19×10^{-4} M, H_2O) and $[\theta]_{273}$ -4700 and $[\theta]_{252}$ 0 for MH-3 (1.12×10^{-4} M, H_2O). Thus the MH-3 isolated from patient urine represents the trans-3'-OH-FT consisting of the α,L and β,D enantiomers in a 2:1 ratio, calculated as follows.

Assuming a fraction , X, of MH-3 has the β,D configuration, then fraction (1 - X) is represented by the α,L configuration.

$[\theta]_{273}$ (MH-3) = (X) x $[\theta]_{273}$ (β,D-3'-OH-FT) + (1 - X) x $[\theta]_{273}$ (α,L-3'-OH-FT) equation 1

Normalizing $[\theta]_{273}$ to a concentration of 1×10^{-4} M, $[\theta]_{273}$ (β,D-3'-OH-FT) (1×10^{-4} M_1 H_2O) = $\frac{+13600}{1.19}$

Table IV. Enzymatic conversion of FT, β,D-4'-OH-FT, FUdR, and its metabolites to FU by a horse liver thymidine phosphorylase.

				Rabbit urinary		Human urinary		
				cis-4'-OH-FT M_1	trans-4'-OH-FT M_2	cis-4'-OH-FT MH-2	trans-4'-OH-FT MH-1	trans-3'-OH-FT MH-3
		FUdR	FT					
Temperature	β,D-4'-OH-FT	25μM	20μM	20μM	20μM	20μM	20μM	20μM
37° C	---	100% in 4 hours	0	0	20% in 4 hours	---	---	---
25° C								
A. E and S, simultaneously	100% in 4 hours	100% in 4 hours	---	---	20% in 4 hours	0	5% in 4 hours	0
B. S added at 4 hours	---	100% in 4 hours	---	---	20% in 4 hours	---	---	---
C. Additional E at 4 hours	---	---	---	---	20% in 24hours	---	---	---

thus $[\theta]_{273}$ (α,L-3'-OH-FT) (1 x 10^{-4} M, H_2O) = $\frac{-13600}{1.19}$

$[\theta]_{273}$ (MH-3) (1 x 10^{-4} M, H_2O) = $\frac{-4700}{1.12}$ equation 2

Rearranging equation 1 and 2

$$\frac{-4700}{1.12} = \frac{(13600)(X)}{1.19} + \frac{(1 - X)(-13600)}{1.19}$$

X = 0.32

1 - X = 0.68

Therefore,

$$\frac{\alpha,L\text{-}3'\text{-}OH\text{-}FT}{\beta,D\text{-}3'\text{-}OH\text{-}FT} = \frac{1 - X}{X} = \frac{0.68}{0.32} = 2.1:1$$

Urinary Excretion of Hydroxylated Metabolites in Rats Receiving R-and S-FT.

4'-OH-FT. Both cis-and trans-4'-OH-FT were recovered in rat urine following administration of S-FT at 50 and 100 mg/kg doses (Table V). The cumulative amounts excreted in 24 hours were 0.64 ± 0.13 (mean ± standard deviation, n = 3) and 0.54 ± 0.16 (n = 3) of the administered S-FT dose for cis-and trans-4'-OH-FT, respectively. However, only a small amount of trans-4'-OH-FT (0.15% of the dose, n = 2) was recovered in 24-hour rat urine following the 100 mg/kg R-FT dose, and no cis-4'-OH-FT was detected. The amount of 4'-OH-FT excreted in 24-hour urine was not affected by the sequence of administering the FT isomers. Furthermore, the levels of the urinary trans-and cis-isomers of 4'-OH-FT did not alter by incubation with β-glucuronidase and sulfatase suggesting that there are no glucuronide or sulfate conjugates of these compounds in urine. The cumulative amount of unchanged FT excreted in 24-hour ranged from 20 to

Table V. Urinary excretion of 4'-OH-FT in rats following administration of R- and S-FT.

Amout excreted in urine over 24 hours (as % of FT dose)

Rat no.	R-FT (100 mg/kg ip)	S-FT (100mg/kg ip)	R,S-FT (50mg/kg ip)
I. Trans-4'-OH-FT			
1	<0.1 %	0.72%	0.42%
2	0.15%	0.5%	--
3	0.15%	0.4%	--
II. Cis-4'-OH-FT			
1	<0.1%	0.75%	0.41%
2	<0.1%	0.68%	--
3	<0.1%	0.5%	--

40 % of the administered dose.

3'-OH-FT. No 3'-OH-FT was detected in rat urine following either the R-or S-FT doses, consistent with the previous findings that 3'-OH-FT was absent from the plasma and uring samples of rats and rabbits given racemic R,S-FT doses (Chapter 2).

In Vitro Metabolism of R- and S-FT

About 95-98% of the R-and S-FT remained intact at the end of one hour incubation. GBL and FU were the only FT metabolites observed. Other FT metabolites isolated *in vivo* were not detected by the present HPLC assay (i.e. < 0.5 μM). Results of the *in vitro* metabolism of R-and S-FT to GBL and FU by mouse and rabbit liver homogenates are summarized in Table VI. The mouse microsomal pellets were capable of converting both R and S isomers of FT to FU, but not to GBL. Metabolism of R-and S-FT to GBL was mediated by 9000 X g and 100,000 X g supernatant fractions of mouse and rabbit liver homogenates. More GBL was generated from R-FT than S-FT suggesting stereoselective metabolism of R-FT to GBL. However, as noted previously (Chapter 4), oxidation of FT at the C-5' position would yield an unstable 5'-OH-FT structure which may be further converted to FU and succinaldehyde. The latter may undergo redox reaction leading to the formation of γ-hydroxybutyric acid and therefore, GBL. Hence it is uncertain which metabolic pathway, i.e. either attack at the C-5' and/or at the C-2', is stereoselective for R-FT.

Table VI. *In Vitro* metabolism of R- and S-FT by 9,000 X g (9S), 100,000 X g (100S) supernatant fractions, and 100,000 X g microsomal pellets of hepatic homogenates.

	Enzyme Source				
	Rabbit 100S	Mouse 100S	Mouse 9S	Mouse microsomal pellets	Controls[1]
A. GBL (μM)					
R-FT	18.4 ± 2.6[2]	26.3 ± 2.3[2]	52.4[3]	0	0
S-FT	8.3 ± 0.3[2]	16.0 ± 3.4[2]	11.2[3]	0	0
B. FU (μM)					
R-FT	----	3.0[3]	8.8[3]	20.7 ± 8.1[2]	1.9[3]
S-FT	----	2.2[3]	5.5[3]	17.5 ± 4.3[2]	2.0[3]

[1]Incubations with boiled enzymes and without enzymes were used as controls.

[2]Range of two observations.

[3]Single observation.

DISCUSSION

In summary, there are five known hydroxylated FT metabolites including the two enantiomers of trans-4'-OH-FT (β,D and α,L), cis-4'-OH-FT, and the two enantiomers of trans-3'-OH-FT (β,D and α,L). The 4'-OH-FT metabolites are common to rats, rabbits and humans, but the ratios of α,L and β,D anomers are different for rabbits and cancer patients. Trans-4'-OH-FT isolated from urine consists of α,L and β,D anomers in ratios of 4:1 and 95:5 for rabbits and patients, respectively. The low percentage of β,D-4'-OH-FT in patient urine may have been caused by its rapid further metabolism, eg. by thymidine phosphorylase. Cis-4'-OH-FT was recovered in rat urine following S-FT but not R-FT dose, suggesting that cis-4'-OH-FT previously isolated from rabbit and patient urine consists either predominantly or solely of the α,D anomer. Trans-3'-OH-FT was identified in human but not rat or rabbit plasma and urine, thus C-3' hydroxylation probably occurs only in man, and the trans-3'-OH-FT isolated from patient urine is a mixture of α,L and β,D anomers in a ratio of 2:1.

Thus, hydroxylated FT metabolites isolated from animal and patient urine exist predominantly in an α configuration. Moreover, larger amount of hydroxylated metabolites were recovered from rat urine after S-FT than R-FT. These observations indicate that either S-FT is stereoselectively hydroxylated or the hydroxylated metabolites of R-FT are preferentially further metabolized prior to urinary excretion. In contrast, in vitro metabolism of R-FT to GBL and FU is preferred to that of S-FT. This latter pathway represents a major activation pathway of FT (Chapter 4). However, the preferential metabolism of R-FT by this pathway produced little or no difference on the biological activities of the two isomers as observed

by Yasumoto (97). It is conceivable that there are other activation pathways which are stereoselective for S-FT. Smolyanskaya and Tugarinov isolated an active metabolite fraction in addition to FU (85). Jonhson et al. (51) and more recently Harrison et al. (42) reported FT toxicities independent of FU, while the major component of the antitumor activity of FT is presumably due to its conversion to FU (36). GI toxicity of FT has been attributed to the unchanged drug or its active metabolite(s) other than FU (42). Thus, although the R and S isomers of FT have similar antitumor activity in cell cultures and animals, the stereoselective metabolism of these isomers by individual pathways may introduce differences in toxic metabolites formation and their host toxicity.

Chapter IV

Mechanism of metabolic activation of FT to FU

SUMMARY

The possibility of the isolated cis- and trans-4'-OH-FT, trans-4'-OH-FT, dehydro-FT as the potential metabolic intermediates in the FT conversion to FU was studied in vitro. Trans-4'-OH-FT isolated from rabbit and patient urine was converted to FU by a horse liver thymidine phosphorylase to the extent of 20 and 5% , respectively. The stereospecificity of this enzyme suggests that the trans-4'-OH-FT is partially represented by the β-D anomer. Thus, hydroxylation of FT at the C-4' position followed by stereospecific enzymatic phosphorolysis to FU represents one possible activation mechanism of FT to FU. Dehydro-FT is chemically more labile than FT and rapidly converts to FU nonenzymatically under various conditions, and may also serve as a metabolic intermediate. However, lack of correlation between plasma levels of trans-4'-OH-FT, dehydro-FT and FU indicates that these modes of metabolic activation may account for only part of the overall activation of FT in vivo.

It has been established that the cleavage of FT to FU and GBL represents a major pathway of FT activation (Chapter 2, 3 and 5). In vitro, FT was metabolized to FU by both the 9000 x g supernatant fraction and the microsomal pellet of mouse liver homogenate; however, GBL was generated only by 9,000 and 100,000 x g supernatant fractions and not the microsomal pellets of the mouse and rabbit liver homogenates. These results indicate that there are at least two different metabolic pathways of FT activation to FU.

INTRODUCTION

It is assumed that metabolism of FT to active species proceeds solely *via* FU. This assumption is based on the following observations. 1. The antitumor activity and tissue (gastrointestinal and bone marrow) toxicity of FT are similar to those of FU (89). 2. FU is the major product of the *in vitro* metabolism of FT by mouse or rat liver microsomal enzyme preparations (13,97). 3. FT lacks the structural requirements for the pyrimidine phosphokinases (11,68) and therefore cannot undergo direct activation.

FU undergoes a series of metabolic conversions to its active nucleotides. Due to the complex scheme of activation, the tissue selectivity of FU may depend on a number of biochemical determinants (reviewed by Myers *et al.* (72) and Sadee and Wong (79)) as well as its delivery to the target tissues. For example, when given as a prolonged infusion instead of a bolus injection, FU is less myelosuppressive (72,79). The plasma clearance of FU ranges from 0.4-2 l/kg-hr after a bolus injection to 3-60 l/kg-hr after an eight-hour infusion (17,26,27,55). The reason for this drastic difference in FU pharmacokinetics is still obscure. When FU is administered in a form of metabolic prodrug, the kinetics of its release may be related to its bone marrow toxicity; moreover, the tissue selectivity is further complicated by the distribution characteristics, rate, and mechanism of activation of the FU prodrug, such as FT. It is, therefore, important to elucidate the mechanism of metabolic activation of FT to FU and such understanding may aid to the design of potential FU prodrug with greater tissue selectivity and thus improved therapeutic index.

Literature data indicate that microsomal drug-metabolizing en-

zymes such as cytochrome P-450 may participate in the mechanism of FT activation, and the liver has been proposed to be the primary site of activation (21,33,70,75, 97). There are, however, opposing evidence that liver may not be the major site of FT metabolism in humans. This has been discussed in the introduction section in Chapter 1.

In this study we have examined the potential role of the isolated urinary metabolites as the metabolic intermediates of FT activation to FU. The subcellular location of enzymes responsible for individual activation pathways and the relative contribution of individual pathways to the overall activation of FT were evaluated.

MATERIALS AND METHODS

Chemicals and reagents. All chemicals and reagents were obtained as described previously in Chapter 2. SKF 525A was received from the Smith, French and Kline laboratory.

Apparatus. The instruments used include HPLC and GC, and are described in Chapter 2.

Assays of FT and its metabolites in biological samples. GBL was assayed by the GC assay. FT and the other metabolites were analyzed by several HPLC assays. These assays are described in detail in Chapter 2.

In vitro incubations. In vitro metabolism studies of FT by different subcellular fractions of rodent and rabbit hepatic homogenates are described in Chapter 2.

Enzymatic phosphorolysis of FT and its metabolites. Horse liver thymidine phosphorylase (0.1 unit/mg, Pipep Co, Paris, France) and rat liver uridine phosphorylase, purified according to Kraut and Yamada (62), were kindly provided by Dr. D. Santi (University of California, San Francisco). Incubation of FT and its metabolites (10-25 μM) in 0.1 M pH 6.0 potassium phosphate buffer was performed with both enzymes separately at 37° or 25°C at conditions which afforded complete conversion of 25 μM FUdR to FU within 2-4 hours. Similar incubations were carried out without enzymes to rule out any nonenzymatic degradation which might have occurred during the incubation period. Disappearance of substrate and FU formation were monitored by HPLC.

RESULTS

Nucleoside phosphorylase incubations. The rabbit urinary metabolites as well as FT and FUdR were incubated with horse liver thymidine phosphorylases preparation in order to obtain further information on their stereochemistry and metabolic function (Results are listed in Table IV in Chapter III). FT and M_1 (cis-4'-OH-FT) were completely resistant to enzyme-catalyzed phosphorolysis, while FUdR rapidly and quantitatively converted to FU within 2 hr. Trans-4'OH-FT (M_2) was converted to FU to the extent of 20% within 4 hr of incubation; no further FU formation was observed for up to 24 hr. Addition of fresh enzyme preparation did not enhance FU yield, thus excluding enzyme degradation as cause of the low yield of rabbit trans-4'OH-FT conversion to FU. Several independent incubations of this metabolite fraction gave the same low FU yield. Since thymidine phosphorylase is specific for the natural β-deoxyribosides (11), it can be inferred that M_2 exists as a mixture of enantiomers with 20% present in the natural β,D configuration. This is further supported by the latter finding that a sample of authentic β,D-4'OH-FT was quantitatively converted to FU by the thymidine phosphorylase (Chapter 3).

The human urinary metabolites MH-1-4 were incubated with horse liver thymidine phosphorylase and rat liver uridine phosphorylase. Complete phosphorolysis FUdR to FU was observed with both uridine and thymidine phosphorylases. There was no detectable nonenzymatic decomposition of FT and MH-1-4 to FU during the incubation. The trans-4'OH-FT isolated from human urine was converted to FU only to the extent of 5% by thymidine phosphorylase within 24 hours, indicating that this metabolite was also partially represented by the

β,D anomer. FT and MH-2-4 were not substrates for thymidine phosphorylase. Uridine phosphorylase was inactive against FT and MH-1-4.

Dehydro-FT. Dehydro-FT was quantitatively converted to FU within 30 minutes under acid conditions, i.e. pH 1 and room temperature, whereas only 20% of FT was hydrolyzed to FU under identical conditions. Dehydro-FT also slowly decomposed to FU when stored in methanol or acetone at 4°C.

In Vitro Metabolism. Incubations of FT were carried out with different fractions of mouse and rabbit liver homogenates in order to study the nature and origin of the enzyme system responsible for the formation of GBL. The results are represented in Table VII. The 9000 X g supernatant, and to a lesser extent the 100,00 X g supernatant, fractions of liver homogenates from both animal species were capable of metabolizing FT to GBL; however, the 100,000 X g microsomal enzymes did not produce GBL. These data indicate that the cleavage of FT to GBL is mediated by soluble enzymes and not by the microsomal enzymes. FU was generated from FT by the microsomal pellets of mouse liver homogenates, consistent wit the previous findings of Yasumoto et al. using rat hepatic microsomal pellets (97) and by the supernatant fractions. Less FU was recovered in the incubation with supernatant fractions than the microsomal pellets, because of the further metabolism of FU by the soluble enzymes in the supernatant fractions (20). Furthermore, the molar concentrations of GBL after incubation of FT with 9,000 and 100,000 X g supernatant were similar to that of FU in the 100,000 X g microsomal pellet incubations; therefore, the activation of FT to FU and GBL by

Table VII. *In Vitro* metabolism of FT (2mM) to GBL and FU by 9,000 X g (9S), 100,000 X g (100S) supernatant fractions, and 100,000 X g microsomal pellets of hepatic homogenates.

	Enzyme Source			
	9S	100S	100,000 X g microsomal pellets	Controls[1]
A. GBL (μM)				
Rabbit	32.1 ± 5.2 (n = 7)	13.4 ± 6.2 (n = 4)	<2.0 (n = 8)	<2.0
Mouse	31.9 ± 20.5 (n = 2)	21.2 ± 6.7 (n = 4)	<2.0 (n = 4)	<2.0
B. FU (μM)				
Mouse	7.1 ± 1.6[2] (n = 2)	2.6 ± 0.4[2] (n = 2)	19.1 ± 7.6 (n = 4)	2.0 ± 0.1[2]

[1]Incubations without hepatic enzymes and with the boiled hepatic homogenate fractions were used as controls.

[2]Range of results of two observations.

soluble enzymes may be as important as the microsomal FT activation. Other FT metabolites that have been isolated *in vivo* (Figure 4) were not measurable by HPLC in the *in vitro* incubations (i.e. < 0.5 μM or < 0.02% conversion).

DISCUSSION

The rather low conversion (5%) of trans-4'-OH-FT from human urine may have been caused by a more rapid metabolism of the β,D enantiomer prior to excretion in cancer patients when compared to rabbits (4,80). Benvenuto et al. subsequently found that a mixture of these two hydroxylated FT metabolites was partially converted to FU when incubated with plasma and to a lesser extent with aqueous buffer (13). In order to study the activity of the hydroxylated FT metabolites, Lin et al. (63) synthesized enantiomers of 3'-OH-FT and 4'-OH-FT. However, the major products were the α,D and β,L isomers of 4'-OH-FT and the β,D and α,L isomers of 3'-OH-FT. These compounds showed no activity against L1210 at 100 mg/kg doses (63,64), possibly because (1) thymidine phosphorylase is specific for pyrimidine deoxyribosides in the β,D configuration (11), and (2) the phosphorylase activity in L1210 is extremely low (9).

The low extent of enzymatic FU formation together with the presence of rather low plasma concentrations of trans-4'-OH-FT (< 100 ng/ml) suggest that hydroxylation followed by phosphorolysis may represent only a minor activation pathway of FT in man. The pharmacologic significance of cis-4'-OH-FT and trans'-3'-OH-FT remains unknown.

Dehydrogenation of FT to MH-4 results in a chemically mor labile metabolite which could undergo nonenzymatic activation to FU in vivo. Kent and Heidelberger (56) have recently studied the mechanism of activation of a dehydrogenated FU analog, i.e. DHFUdR, and concluded that nonenzymatic conversion to FU was responsible for its in vitro and in vivo cytotoxicity.. Plasma concentrations of MH-4 were the second highest among all FT metabolites and were maintained for up to 24 hours after the FT dose. Contribution of this lipo-

philic metabolite to FU formation and the neurotoxicity associated with FT remain to be determined.

Benvenuto et al. (13) recovered a component with the same chromatographic behavior as the hydroxylated FT metabolites upon incubating 2-^{14}C-FT with rat and mouse liver microsomal cytochrome P-450 enzymes. The enzymes responsible for the formation of dehydro-FT are unknown at present.

Our results demonstrate the existence of two separate metabolic pathways of FT activation to FU, one that is mediated by hepatic microsomal enzymes and one that occurs in the soluble enzyme fraction of the 100,000 x g supernatant. The metabolite GBL/GHB can only be generated in the 9,000 x g and 100,000 x g supernatants but not in the microsomal pellet preparation. The formation of GBL/GHB through the action of soluble enzymes can occur by the two pathways, depicted in Figure 3, that involve oxidative or hydrolytic cleavage of the N-1-C-2' bond of FT. However, a third possibility cannot be excluded, namely the enzymatic oxidation at the C-5' position of FT; the presumably labile product would cleave to FU and succinaldehyde (CHO-CH_2-CH_2- CHO) which can undergo enzymatic reduction at one end and enzymatic oxidation at the other end to yield GHB/GBL. Such metabolic redox reactions between 1,4-butanediol and succinate are known to occur in the cytosol, with GHB as one of the isolable products (24).

The formation of FU from FT in the microsomal pellet is thought to be mediated by cytochrome P-450, requiring NADPH and O_2 (e.g., 21,75,97). Our results rule out the microsomal oxidation of FT at the C-2' position, since the expected oxidation product, GBL/GHB, was not found in the microsomal pellet incubations. Furthermore,

oxidation at the C-3' and C-4' position of FT yield stable metabolic products (Figure 4) and therefore cannot account for the observed FU formation by the microsomal enzymes. The remaining potential molecular site of microsomal oxidation is the C-5' position of FT with resultant formation of succinaldehyde (as previously suggested by Lin et al. (63,64)). The microsomal NADPH/O_2 dependent oxidation at the C-5 position of the C-2 substituted tetrahydrofuran derivative has been previously observed (35); the microsomal oxidation product, a 5-OH-tetrahydrofuran, was further oxidized to the open chain carboxylic acid by soluble enzymes. This observation suggests that GBL/GHB formation in the 9,000 x g supernatant may in part derive from the combined action of the microsomal enzymes (C-5' oxidation) and subsequent redox reactions of the generated succinaldehyde to GBL/GHB by the soluble enzymes. The greater extent of GBL/GHB formation in the 9,000 x g supernatant than that in the 100,000 x g supernatant suggests that such a pathway may exist. Further studies are necessary to clarify the enzymatic mechanism of FT activation.

Our results are important to the understanding of the mechanism of action of FT, because they demonstrate that FT activation may occur in target tissues without the action of cytochrome P-450 which is absent in many tumor cells. Previous findings of exceedingly low FU plasma levels (4,5,22,47), although contested by other workers (13), emphasize the potential role of target tissue activation of FT in its antitumor effects and selective organ toxicity.

The presence of GBL/GHB as a major FT metabolite in plasma of rabbits and patients and the kinetic disposition of GBL/GHB in rabbits indicate that a significant portion of FT is converted to FU and GBL/GHB (Chapter 5,6); the latter may therefore serve as an indicator of FT activation.

Chapter V

Pharmacokinetics of FT in animals and cancer patients

SUMMARY

Plasma concentrations of FT and its UV-absorbing metabolites in biological samples were measured by high-pressure liquid chromatographic assays with a sensitivity range between 20 ng of FU/ml and 100 ng of FT/ml. FU and the cis- and trans-4'-OH-FT were present in rabbit and rat plasma in greatly varying concentrations after FT administration. Pharmacokinetic studies suggest that FU formation proceeds via metabolic intermediates and that the extent of FT activation is variable . The disposition of FT in man followed two-compartment kinetics. The β-elimination half-life of FT ranged from 6 to 16 hours with a plasma clearance of 22-95 ml kg^{-1} hr^{-1}. Plasma concentrations of FU generated *in vivo* were <0.2% of FT concentration indicating that FU, if formed intracellularly, may be further metabolized without extensive redistribution.

Plasma concentrations of GBL following administration of either GBL or FT to rabbits were quantitated by a GC-FID assay using δ-valerolactone as the internal standard. Pharmacokinetic analysis by comparing the area under the plasma concentration-time curves of GBL following equimolar doses of GBL and FT indicates that at least 20 to 40% of the administered FT dose was metabolized to GBL in rabbits. GBL was also present in plasma samples of FT-treated patients in molar concentrations exceeding all other FT metabolites and represents a major circulating FT metabolite in patients.

INTRODUCTION

The assays used to analyze FT in biological fluids include ^{14}C radioactivity analysis after metabolite separation with thin layer chromatography (28), gas-liquid chromatography (GLC) following chemical derivatization (46,93), and high pressure liquid chromatography (HPLC) (4,7,13,96). FT is heat-labile and decomposes to FU when exposed to the high temperature used in GLC (46,95) which complicates the application of GLC to the simultaneous assay of FT and FU. We have measured plasma concentrations of FT and FU and four additional FT metabolites by an HPLC assay using a reverse phase C_{18} column. With some modification, the same assay could be applied to the analysis of urine samples (6). More recently an HPLC awsay using a normal phase column was also described (22). The pharmacokinetics and metabolism of FT in experimental animals and cancer patients have been investigated by us and others using above-mentioned assays.

Following i.v. administration of 2-5 g FT/sq m/d, the disposition of FT in man displayed two-compartment kinetics with a β-phase half-life ranging from 6 to 17 hours and an average plasma clearance of 0.05 l/kg-hr, as observed by us and others (4,13,46). FT has an average volume of distribution (Vd) during the β-phase of 0.5 l/kg (range 0.4-0.8 l/kg) (4,13,32,46), which approximates the volume of total body water and is similar to that of FU. FU does not bind to plasma proteins, whereas FT is bound to the extent of 30-50% at a concentration range of 6-100 μg/ml (13, 28).

There is some species variation in the disposition of FT; half-lives of 40 minutes, 2 hours, 5 hours, and 10 hours were reported for mice (59), rabbits (95,96), rats (21,95,96), and both beagle dogs

and rhesus monkey (28), respectively. Wu et al. (95) reported capacity-limited metabolism in some rabbits given a high dose of 125 mg FT/kg intravenously. This non-linearity of FT disposition was not observed in patients receiving doses of up to 5 g/sq m/d (13).

MATERIALS AND METHODS

Chemicals and Reagents. All chemicals and reagents were obtained as previously described (Chapter 2). 1-Methyl Xanthine was purchased from Aldrich Chemicals.

Animal and Patient Protocols. These have been described in Chapter 2.

HPLC Assays of FT and its Metabolites

Three HPLC assays were used to analyze the biological samples. Plasma samples of rats and rabbits (0.1 to 1.0 ml) were adjusted with distilled water to a total volume of 1 ml, and 0.1 ml of 0.5 M NaH_2PO_4 buffer and 8 ml ethyl acetate were added. After extraction and centrifugation, the organic layer was removed and evaporated under N_2 at 50°. The residue was redissolved in 50 µl methanol, and 5 to 25 µl were injected on the HPLC column with 0.01 M sodium acetate buffer (pH 4):methanol (85:15, v/v) as eluent. The retention times were 6.4, 3.5, 2.7, and 2.0 min for FT, M_1, M_2, and FU, respectively (Figure 8). Overall extraction recoveries were 90, 70, 51, and 58% for FT, M_1, M_2, and FU, respectively. Sensitivity limit of quantitative analysis in plasma was 0.1 µg/ml for all 4 compounds. Standard curves for FT, M_1, M_2 and FU obtained from peak height measurements were linear over a wide concentration range (0.1 to 200 µg/ml).

Plasma samples from patients were similarily extracted with ethyl acetate. The extraction yield was 85% for FT and MH-4, 60% for FU, 50% for MH-1, 70% for MH-2 and -3, and 30% for FUR and FUdR.

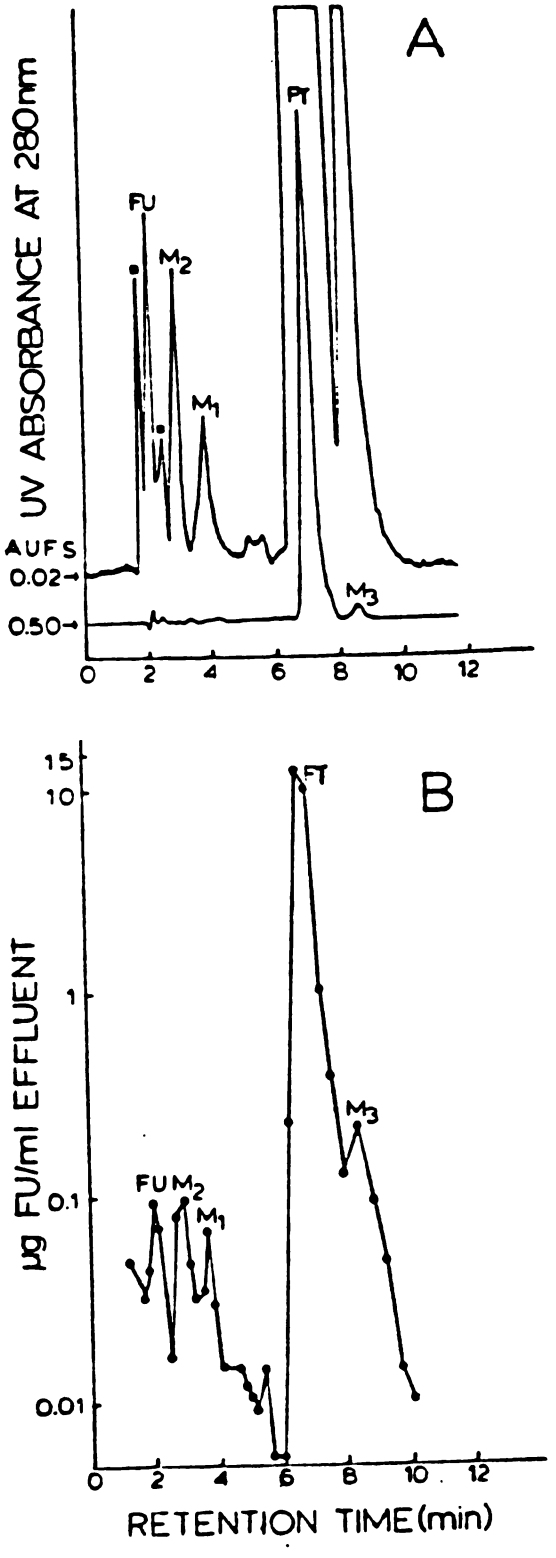
The above HPLC assay was modified by using 0.01 M pH 4 sodium acetate aqueous buffer:methanol (95:5) at a flow rate of 2 ml/min and dual UV absorbance detection at 254 and 280 nm. Under these

Figure 8.

(A). The HPLC tracing of a rabbit plasma extract 8-hour after i.v. administration of FT (125 mg/kg).

■ peak present in control plasma.

(B). FU-specific HPLC tracing of the same chromatogram as shown in (A), measured by GC-MS after acid hydrolysis.



HPLC conditions:

- μBondapak C_{18}-reverse phase column, 4 mm X 30 cm;
- -mobile phase: 15% methanol in 10 mM sodium acetate buffer of pH 4.0, at a flow rate of 2 ml/min

conditions, FU, the sum of MH-2 and -3, FT. and MH-4 as well as FUdR were measurable. Retention volumes of FU, MH-1, MH-2, MH-3, MH-4, FT, and FUdR were 4.7, 10.6, 14.4, 14.6, 39.0, 32.7, and 10.2 ml, respectively. The characteristic UV abosrbance ratios at 254:280 nm of FU, MH-1, MH-2, MH-3, MH-4, FT, and FUdR were 1.45, 0.90, 0.90, 0.80, 0.94, 0.77, and 0.95, respectively. An HPLC record of human plasma extract is shown in Figure 9, which demonstrates the relative concentrations of FT and its metabolites. Separation of MH-1 and FUdR from interfering endogenous substrates and of MH-2 from MH-3 was achieved using 0.01 M pH 4 sodium acetate aqueous buffer:acetonitrile (95:5) at a flow rate of 2 ml/minute. Retention volumes of MH-1, MH-2, MH-3, and FUdR were 7.6, 9.4, 10.0, and 5.2 ml, respectively. However, FU and FUdR were not separated from interfering endogenous substrates using the latter eluent. Therefore, both eluents were used for complete analysis. Sensitivity of this assay was 20 ng/ml for FU and MH-1-3 and 100 ng/ml for FT, MH-4, FUR, and FUdR. The standard curves of FT and FU were linear over a wide range of concentrations. MH-1 was quantitated using standard curves constructed with [2-^{14}C] trans-4'-OH-FT and MH-2 and -3 were quantitated using standard curves constructed with [2-^{14}C] cis-4'-OH-FT. MH-4 was quantitated using standard curves of FT assuming identical chromatographic properties and extinction coefficients at 280 nm.

These HPLC assays were subsequently modified to include 1-methyl xanthine (5 µg in 50 µl methanol) as the internal standard. The eluent used was 15% methanol in 0.01 M sodium acetate buffer of pH 4.2. The retention volumes of FU, trans- and cis-4'-OH-FT, 1-methyl xanthine, FT and dehydro-FT were 3.7, 4.8, 6.6, 8.3, 11.4, and 13.8ml,res-

Figure 9. HPLC tracing of a plasma extract obtained from patient no. 5 at 2-hour following administration of 2 g FT/sq m by i.v. infusion.

* peak corresponds to MH-1 plus an interfering endogenous substance.

MH-1 : trans-4'-OH-FT

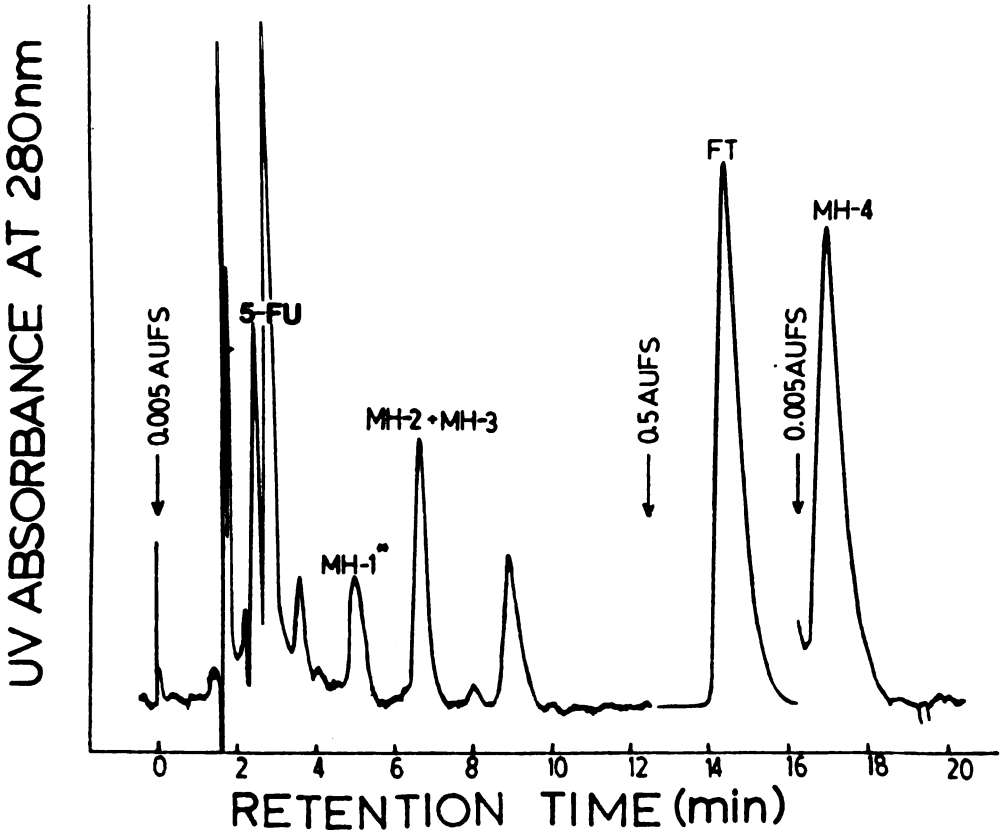
MH-2 : cis-4'-OH-FT

MH-3 : trans-3'-OH-FT

MH-4 : dehydro-FT

HPLC conditions :

- μBondapak C_{18}-reverse phase column, 4 mm X 30 cm
- mobile phase : 5% methanol in 10 mM sodium acetate buffer of pH 4.0 at a flow rate of 2ml/min



pectively (Figure 10). Samples from the *in vitro* incubation mixtures were analyzed using an eluent of 3 % acetonitrile in acetate buffer. Separation of FU from the endogenous interferences present in the liver homogenates was obtained under these conditions. The retention volumes of FU, trans-and cis-4'OH-FT, 1-methyl xanthine, and FT were 5, 10, 12.4, 15.6 and 27.2 ml, respectively. FT concentrations in biological samples were quantitated by FT:1-methyl xanthine UV absorbance ratios at 280 nm. Standard curves of FT concentrations constructed with FT:1-methyl xanthine absorbance ratios obtained from spiked plasma samples were linear from 1 to 400 µg/ml plasma.

Pharmacokinetic Analysis

Pharmacokinetic parameters of FT in humans were obtained by fitting the experimental data with the FITFUN program (Prophet, First Data Computer, Cambridge, Mass.) utilizing a two-compartment first-order kinetic model.

Pharmacokinetic parameter of GBL and FT in rabbits were established by weighted linear regression ($w = 1/y^2$) of the plasma concentration-time curves of GBL and FT using a two-compartment model.

Plasma Assay of GBL

This assay represents a modification of the GC method of Vree and van der Kleijn (89) with a considerably improved sensitivity limit of 200 ng/ml plasma. Plasma samples from patients and rabbits (0.1 to 1.0 ml) were adjusted with distilled water to a total volume of 1 ml and spiked with 5 µg of the internal standard, δ-valerolactone (DVL), in 50 µl chloroform. Concentrated sulfuric acid (0.1 ml) was added to lactonize the GHB to GBL. The plasma samples were then twice extracted with 2 ml chloroform, the pooled organic layers were evaporated to about 50 µl under nitrogen in 3 ml reacti-vials. Up

Figure 10. HPLC tracing of a plasma extract obtained from rabbit 1 at 92 minutes following administration of 60 mg FT/kg.

1 : FU

2 : cis-4'-OH-FT

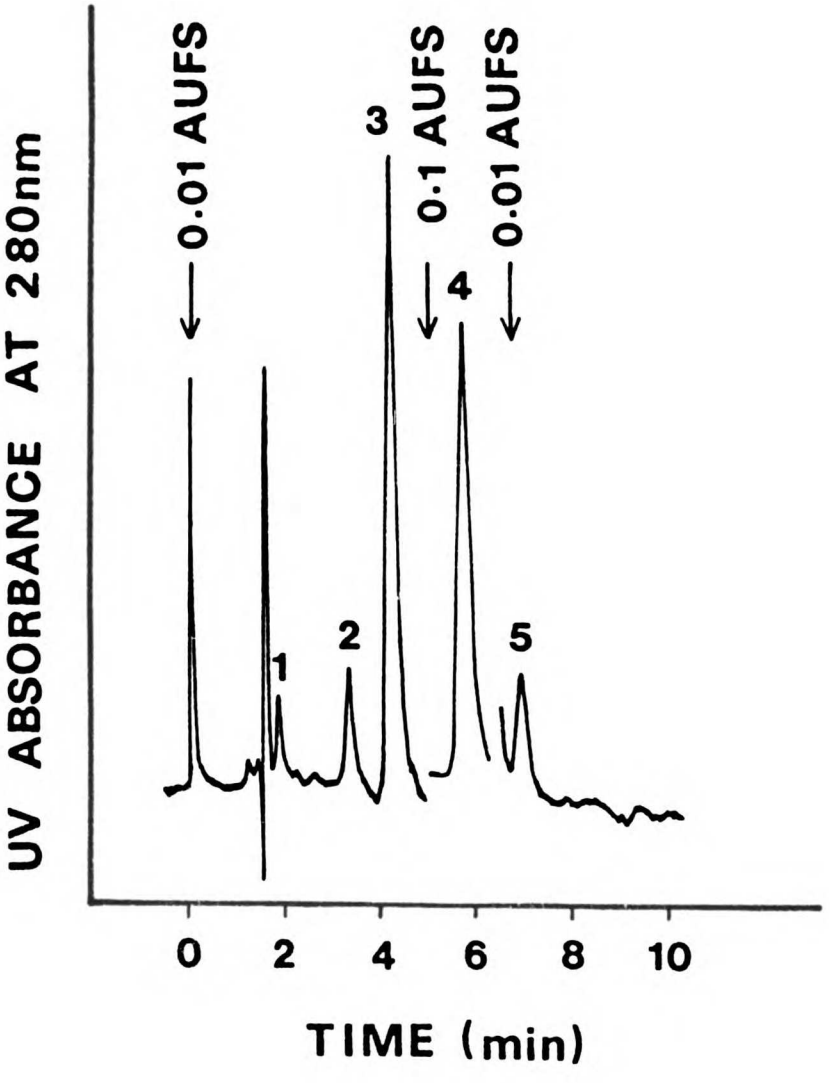
3 : 1-methyl xanthine, the internal standard

4 : FT

5 : dehydro-FT

HPLC conditions :

- μ Bondapak C_{18}-reverse phase column, 4 mm X 30 cm
- mobile phase: 15% methanol in 10 mM sodium acetate buffer of pH 4.2 at a flow rate of 2 ml/min



to 1 μl of the concentrated extracts was injected into the GC. The retention times of GBL and DVL were 2 and 3.5 minutes. Plasma GBL concentrations were quantitated by the GBL:DVL peak height ratios. Standard curves of GBL constructed with the peak height ratios obtained from spiked plasma samples were linear over a concentration range of 0.2 to 200 μg/ml. There is no measurable decomposition of FT to GBL at a concentration up to 400 μg/ml during the assay procedures.

RESULTS

Quantitation of FT, FU, and Hydroxylated Metabolites in Rabbit Plasma after FT Administration. An HPLC record obtained from rabbit plasma following FT administration is shown in Figure 8. This rabbit had relatively high levels of FU as well as M_1 and M_2 and was used for urinary metabolite isolation. One additional metabolite peak (M_3), corresponding to the dehydro-FT (MH-4), can be observed in the HPLC record with a longer retention time than FT.

Plasma concentration-time curves resulting from an iv injection of [2-^{14}C]FT (125 mg/kg) in a rabbit are shown in Figure 11. Total ^{14}C activity was slightly higher than FT specifically measured by HPLC, which declined with a plasma elimination half-life of 90 min. M_1, M_2, and FU plasma concentrations peaked at 2 to 4 hr following FT administration, which was consistently found in several rabbits as well as rat studies.

TableVIII summarizes plasma levels of FT and its metabolites 5 hr following an i.v. dose of 125 mg FT per kg in rabbits and rats. Elimination half-lives of FT were calculated from data collected over a period of 5 to 12 hr after the dose. Initial slow elimination of FT from plasma in some animals indicates capacity-limited metabolism at high doses with an apparent long $t_{1/2}$. The relatively short $t_{1/2}$ in Rabbit 4 (1.5 hr)is similar to the $t_{1/2}$ observed in a previous study at a lower FT dose (30 mg/kg) (95). Plasma levels of M_1, M_2, and FU greatly vary from one animal to another with inconsistent relative concentrations. Plasma levels of FU were found to be relatively high in Rabbits 4 and 5 in contrast to the very low FU levels in rats and those previously measured in rabbits (95). Two hr after FT (30 mg/kg iv) in a rabbit, the FT

Figure 11. Plasma concentration of ^{14}C radioactivity, FT, M_1, M_2, and FU following i.v. administration of ^{14}C-FT (125 mg/kg) in a male New Zealand rabbit (#4).
M_1 : cis-4'-OH-FT
M_2 : trans-4'-OH-FT

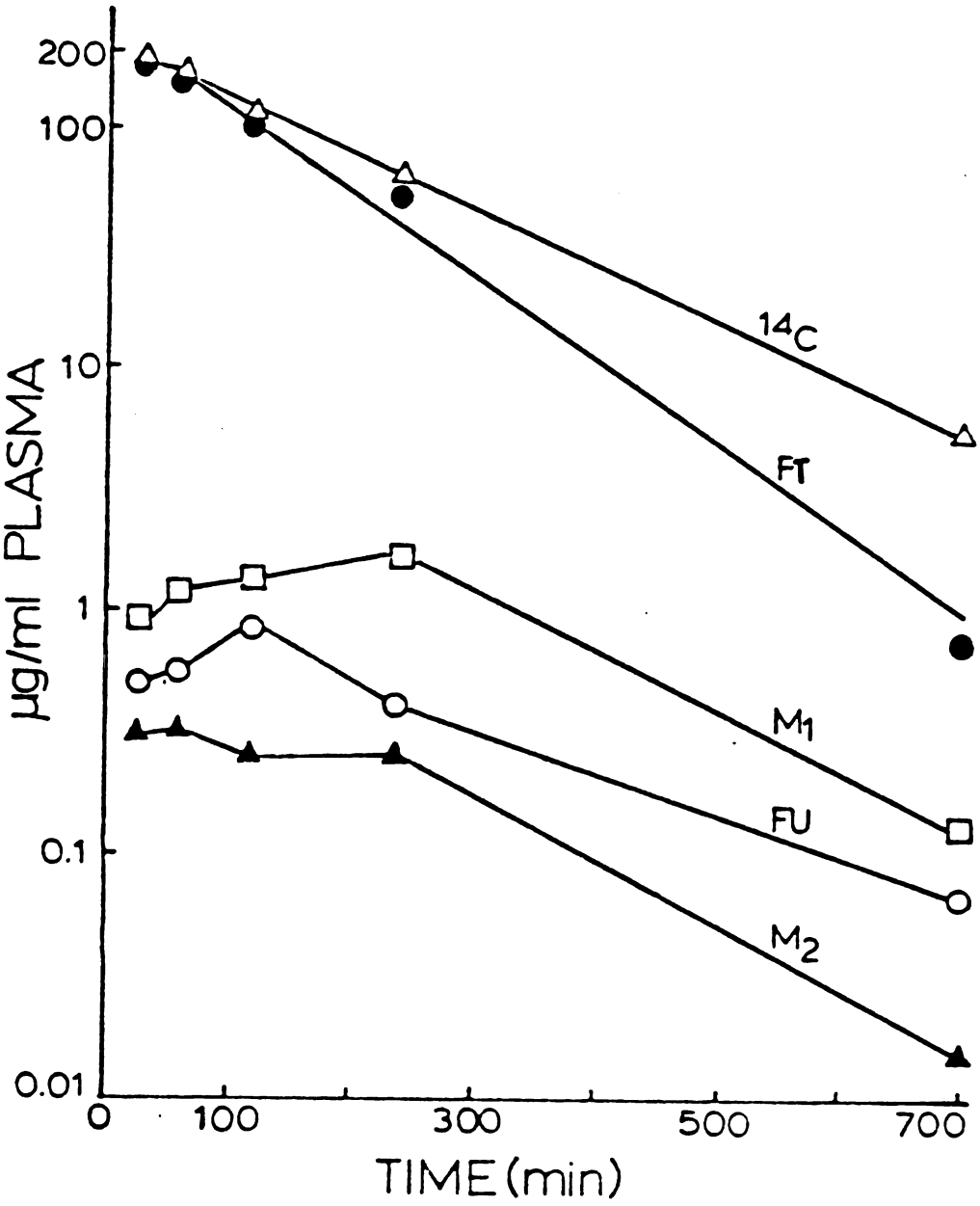


Table VIII. Plasma concentrations of FT, M_1(cis-4'-OH-FT), M_2 (trans-4'-OH-FT), and FU in rats and rabbits 5-hour after administration of 125 mg FT/kg.

	FT μg/ml	M_1 μg/ml	M_2 μg/ml	FU μg/ml	$T_{1/2}$[a] hr
Rabbit 4	23.0	1.2	0.18	0.32	1.5
Rabbit 5	130.0	0.22	0.43	0.24	5.1
Rat 1	72.0	0.1	0.1	<0.1	6.8
Rat 2	116.0	0.21	0.92	<0.1	7.0

[a]Observed over 5 to 12 hours following administration.

level was reported to be 38 μg/ml as measured by GC, and the FU level was reported to be 0.01 μg/ml as measured by GC-MS (95). The same plasma sample analyzed by the HPLC method reported here gave 40, < 0.1, 0.1, and < 0.1 μg/ml for FT, FU, M_1, M_2, respectively. FU levels in Rats 1 and 2 measured by GC-MS (95) were 0.02 and 0.04 μg/ml, respectively. These measurements established validity of the analytical procedures and demonstrated lack of significant detectable metabolite formation in several individual animals of 2 species.

Ethyl acetate-extractable ^{14}C radioactivity from rabbit urine was considerably greater (70% of total ^{14}C) following [2'-5'-^{14}C]FT than after [2-^{14}C]FT (34% of total ^{14}C). The majority of urinary FU metabolites are polar and nonextractable, while the thus-far-identified FT metabolites and FT itself are ethyl acetate extractable. Therefore, separation of the ^{14}C labels in the FU moiety from the ^{14}C label in the tetrahydrofuranyl moiety by extraction indicates extensive cleavage of the FU tetrahydrofuranyl bond in vivo.

The pharmacokinetic parameters of GBL and FT were obtained from their plasma concentrations after administration of GBL and FT separately to the two rabbits. The results are summarized in Table IX. Doses of GBL were given both prior to and after FT administration to determine if FT and/or GBL treatment alters the pharmacokinetics of GBL. Plasma concentration-time profiles of GBL following the administration of the first and second GBL doses to the two rabbits are shown in Figure 12. The GBL plasma concentrations (sum of GBL and GHB) in both rabbits declined biexponentially indicating a two compartment kinetic disposition. The plasma clearance of GBL

Table IX. Pharmacokinetic parameters of GBL and FT in rabbits.

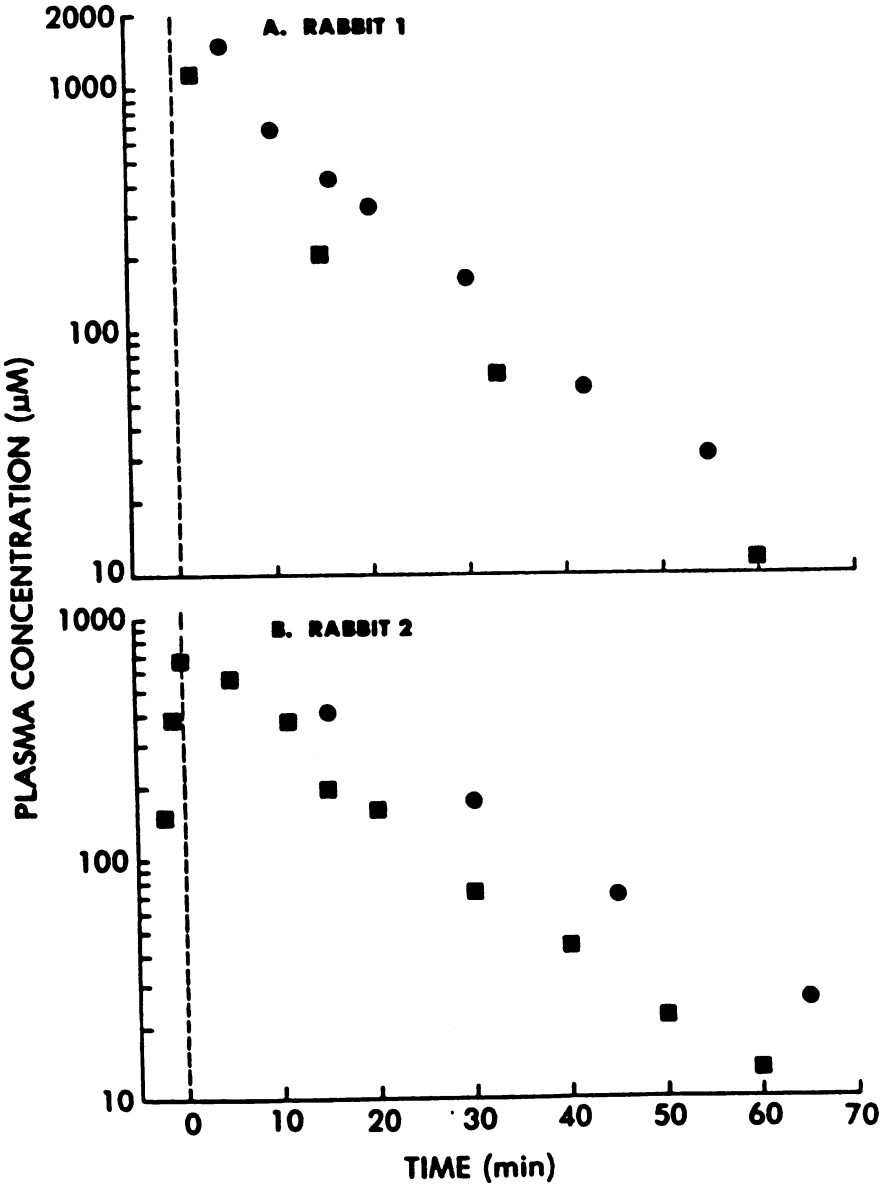
		$T_{1/2}^{a}$ (min)	V_{CL}^{b} (liter/kg-hr)	Vd_{β}^{c} (liter/kg)
A. GBL				
Rabbit 1	1st dose	10.8	0.85	0.22
	2nd dose	9.9	0.46	0.11
Rabbit 2	1st dose	11.2	1.37	0.37
	2nd dose	12.4	0.65	0.20
B. FT				
Rabbit 1		83	0.20	0.40
Rabbit 2		126	0.18	0.54

[a] $T_{1/2}$ is the elimination half-life and is calculated by 0.693 ÷ (the terminal slope of the linearly regressed line (weight = $1/y^2$) of the plasma concentration-time curve).

[b] V_{CL} is the total body clearance and is calculated by (dose of GBL) ÷ (area under the plasma concentration-time curve of GBL, estimated by the trapezoidal rule).

[c] Vd_{β} is the volume of distribution during the elimination phase, and is calculated as $V_{CL} \div \beta$.

Figure 12. Plasma concentration-time profiles of GBL in rabbits after the first (■) and second (●) dose of GBL. Zero time refers to the end of infusion.



in rabbits ranges from 0.5 to 1.4 l/kg-hr, as established in this study, and is greater than that in man (0.13 liter/kg-hr) (90). The plasma concentrations of GBL following the second GBL dose are approximately twice the concentrations following the first dose, while there is little or no change in the elimination half-lives between the two doses of GBL given to the two rabbits. This suggests that FT and/or GBL treatment alters the pharmacokinetic parameters of a subsequent dose of GBL, the cause of which remains unknown.

An HPLC tracing of a plasma extract obtained from rabbit 1 at 92 minutes following FT administration is shown in Figure 10. Other FT metabolites in this rabbit detected by the HPLC assay include FU, cis-4'-OH-FT, and dehydro-FT, whereas only FU and dehydro-FT were observed in the plasma extract of rabbit 2. The disposition of FT in these two rabbits followed two compartment kinetics as reported previously (95). The fractions of FT dose excreted unchanged in 10-hour urine were 3 and 6% for rabbit 1 and 2, respectively.

Plasma concentration-time profiles of GBL and FT following FT administration to the two rabbits are shown in Figure 13. The GBL plasma concentrations peaked in less than one hour in both rabbits, and declined with terminal half-lives of 104 and 260 minutes for rabbit 1 and 2, respectively.

Kinetics of FT and its Metabolites in Patients. An HPLC record of a 2-hour plasma sample obtained from a patient (no. 5) is shown in Figure 9. The plasma concentration-time curves of FT and its metabolites following the first dose of FT to representative patients are shown in Figure 14 and 15. Plasma FT concentrations declined biexponentially, indicating a two-compartment disposition. Pharmacokinetic parameters of FT in these patients are summarized

Figure 13. Plasma concentration-time profiles of GBL (solid symbols) and FT (open symbols) in rabbit 1 (■ , □) and 2 (● , ○), following administration of 60 mg FT/kg.

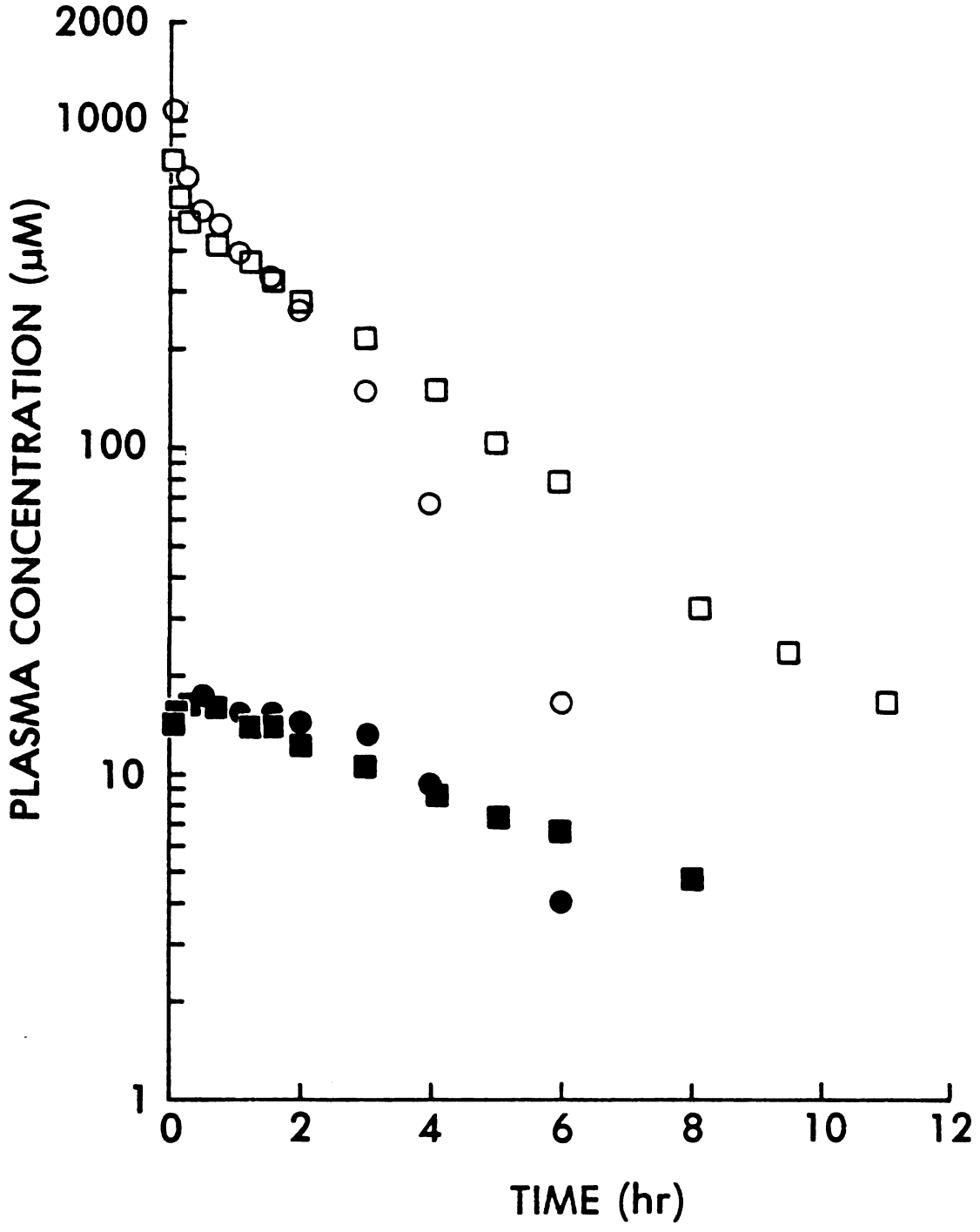


Figure 14. Plasma concentration-time curves of FT and its metabolites, i.e. FU, MH-1 (trans-4'-OH-FT), MH-2 (cis-4'-OH-FT), MH-4 (dehydro-FT), in one patient (no.3) given 2 g FT/sq m by i.v. infusion over 30 minutes. Zero time refers to the end of FT infusion. FU concentrations were corrected for non-metabolically generated FU as described in the text.

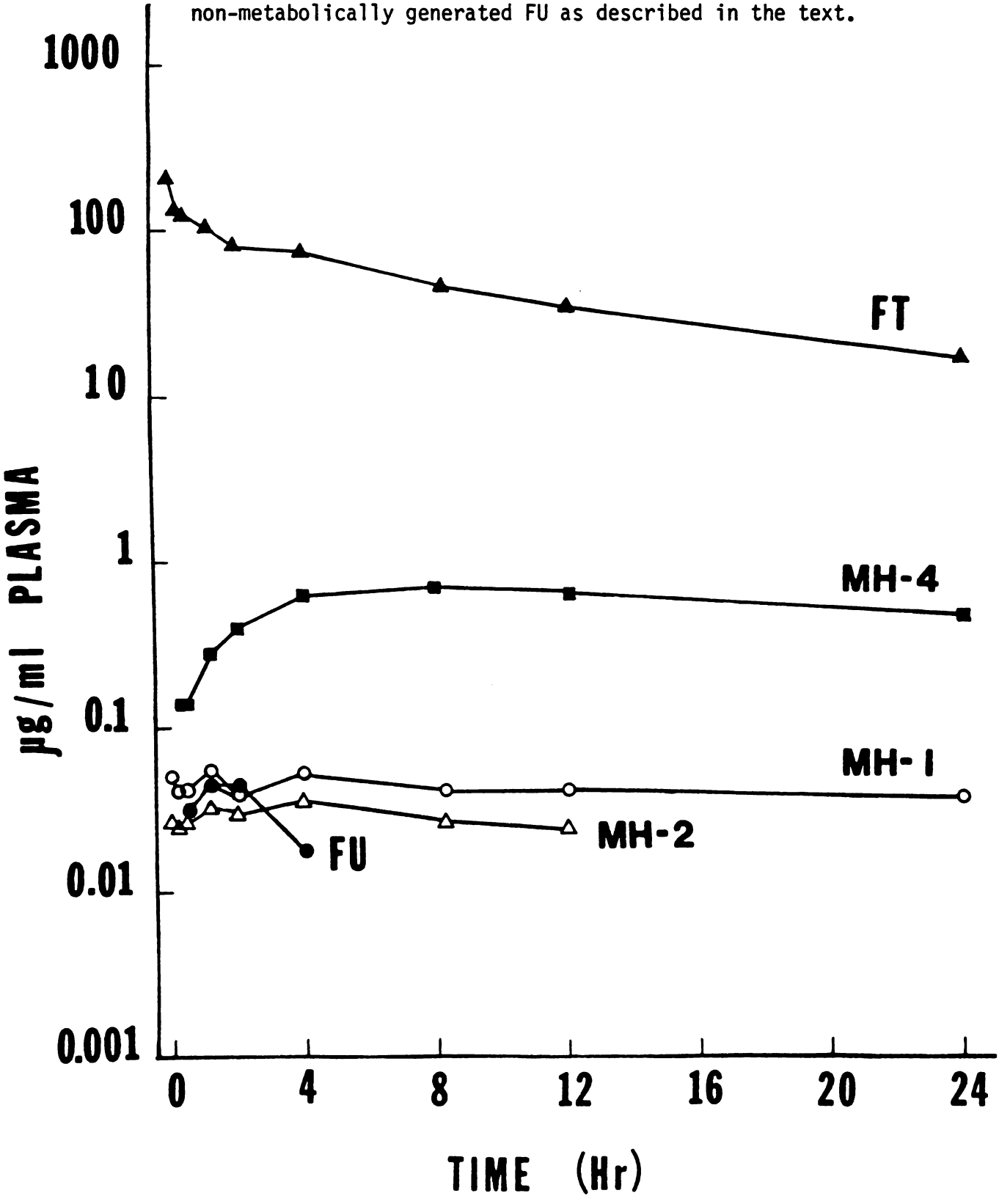
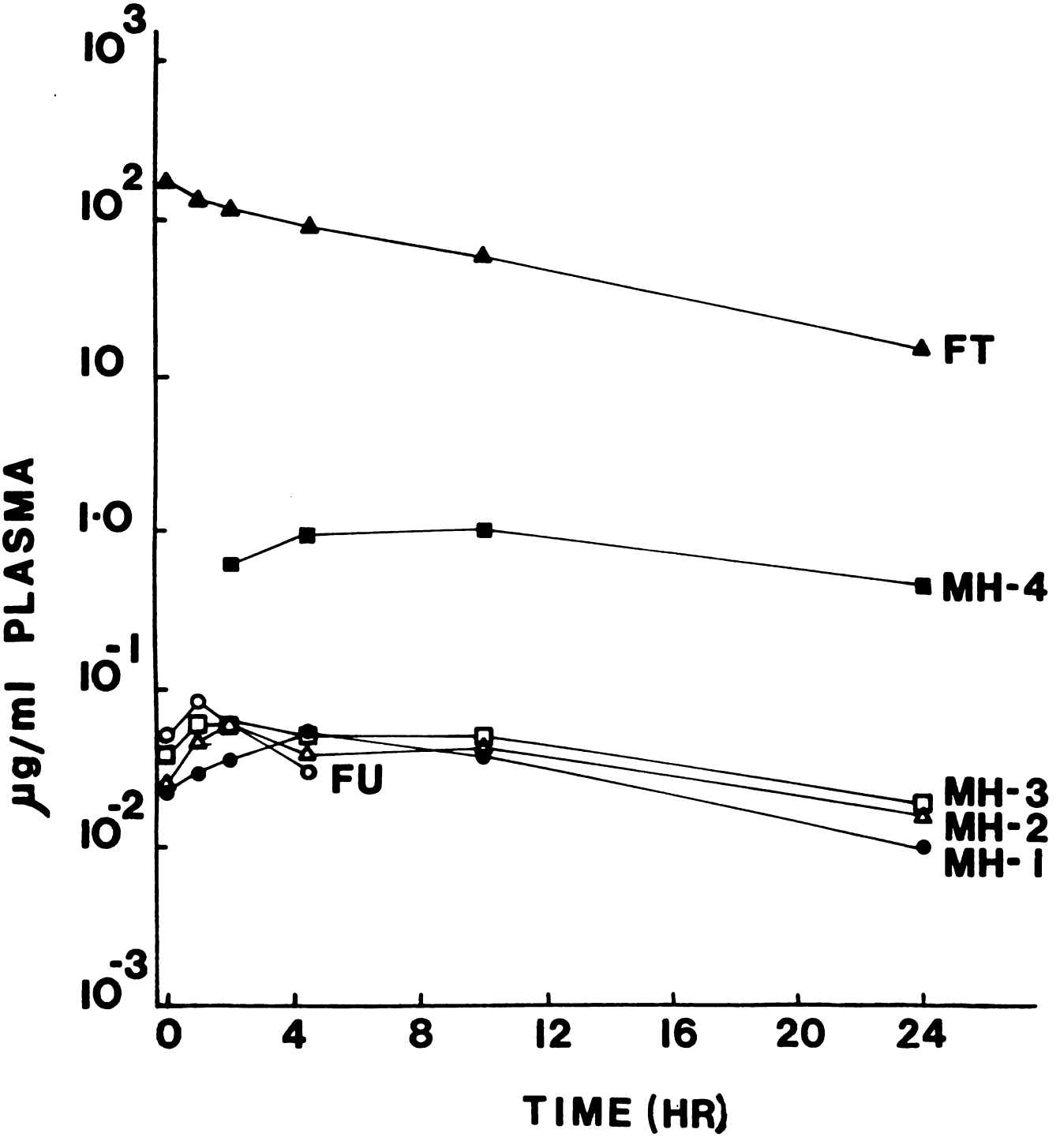


Figure 15. Plasma concentration-time curves of FT and its metabolites, i.e. FU, MH-1 (trans-4'-OH-FT), MH-2 (cis-4'-OH-FT), MH-3 (trans-3'-OH-FT), and MH-4 (dehydro-FT), in one patient (no.5) given 2 g FT/sq m by i.v. infusion over 30 minutes. Zero time refers to the end of FT infusion. FU concentrations were corrected for non-metabolically generated FU as described in the text.



and compared to literature values in Table X. Of the two patients (nos. 1 and 2) with severe liver damage, only patient 1 displayed a prolonged half-life of 16 hours. Plasma samples from patients 1 through 4 obtained after the second dose of FT on the following day showed FT concentrations similar to those of the first day of treatment. This rules out major changes of FT disposition due to repeated dosing or co-administration of other antineoplastic agents, i.e. methyl-CCNU, adriamycin, and mitomycin C. The relative ratios of metabolite concentrations at 4 hours after the FT dose were variable from one patient to another (Table XI). MH-4 was one of the two major detectable circulating metabolites in all patients, reaching plateau concentrations at approximately 4 hours after the dose which were maintained with little (patient 3, Figure 14, and patient 15, Figure 15) or no apparent decline over the 24-hour observation period. The other major circulating metabolite was GBL. The relative molar plasma concentrations of all detectable FT metabolites in patients KM and CD, and rabbits 1 and 2 are listed in Table XII.

Plasma concentrations of FT measured by HPLC in five patients are shown in Figure 16. The observed FU concentrations could have arisen from FT metabolism, chemical decomposition of FT during analysis (0.03%) (95), or contamination of the FT dosage form with FU (determined to be 0.15%). The corrected[1] FU plasma concentrations are shown in Figure 16 and the uncorrected one is shown in Figure 17. The ob-

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[1] Corrected FU concentrations (FU concentrations resulting from FT metabolism) = (observed values at time t) - (Cp^{o}_{FU} e^{-kdt}) - (0.03% x FT concentrations at time t); Cp^{o}_{FU} = plasma FU concentrations resulting from a 30-minute infusion at a dose of 0.15% x FT dose (the plasma clearance value used in the calsulations was taken from the literature : 2.1 l/kg-hr, 26); kd = first order elimination rate constant, literature value = 4.2 hr^{-1}, 26.

Table X. Pharmacokinetic parameters of FT in cancer patients[1]

Patient no.	Elimination[2] half-life hr	Plasma[3] clearance V_{CL} ml/kg-hr	Vd_{β}[4] liter/kg
1	16.0	22	0.51
2	7.5	53	0.58
3	9.4	37	0.49
4	6.0	95	0.78
5	7.6	35	0.39
Range	6-16	22-95	0.4-0.8
Literature values[5]	17.0 (40)	54 (46)	0.4 (46)
	6.7 (46)	50 (13)	0.7 (13)
	8.8 (13)		

[1]Plasma concentration-time curves of FT were coumputer-fitted to a biexponential function $Cp = Ae^{-\alpha t} + Be^{-\beta t}$ using weighted least-squares method (weight = $1/Cp^2$); β phase = elimination of FT from the body and α = first order elimination rate constant.

[2]Half-life = $0.693 \div \beta$.

[3]V_{CL} = dose/AUC where AUC is the area under the plasma concentration-time curve from time when infusion was started to time infinity and was calculated by the trapezoidal rule. AUC during the infusion phase = Cp at the end of infusion X infusion time X 1/2.

[4]Vd_{β} = volume of distribution of FT during the terminal phase = $V_{CL} \div \beta$.

[5]Literature values are obtained either directly from the references or calculated from the data given. Nos in parentheses = reference nos.

Table XI. Plasma concentrations of FT and its metabolites in cancer patients 4-hour afteri.v. administration of 2 g FT/sq m.

Patient no.	Plasma concentration of					
	FT µg/ml	FU[1] ng/ml	MH-1 trans-4'-OH-FT ng/ml	MH-2 cis-4'-OH-FT ng/ml	MH-3 trans-3'-OH-FT ng/ml	MH-4 dehydro-FT ng/ml
1	85	20	20	24	22	not measured
2	65	94	61	70	42	259
3	77	20	not measured	53	45	647
4	43	20	52	63	86	376
5[2]	91	31	53	38	51	950

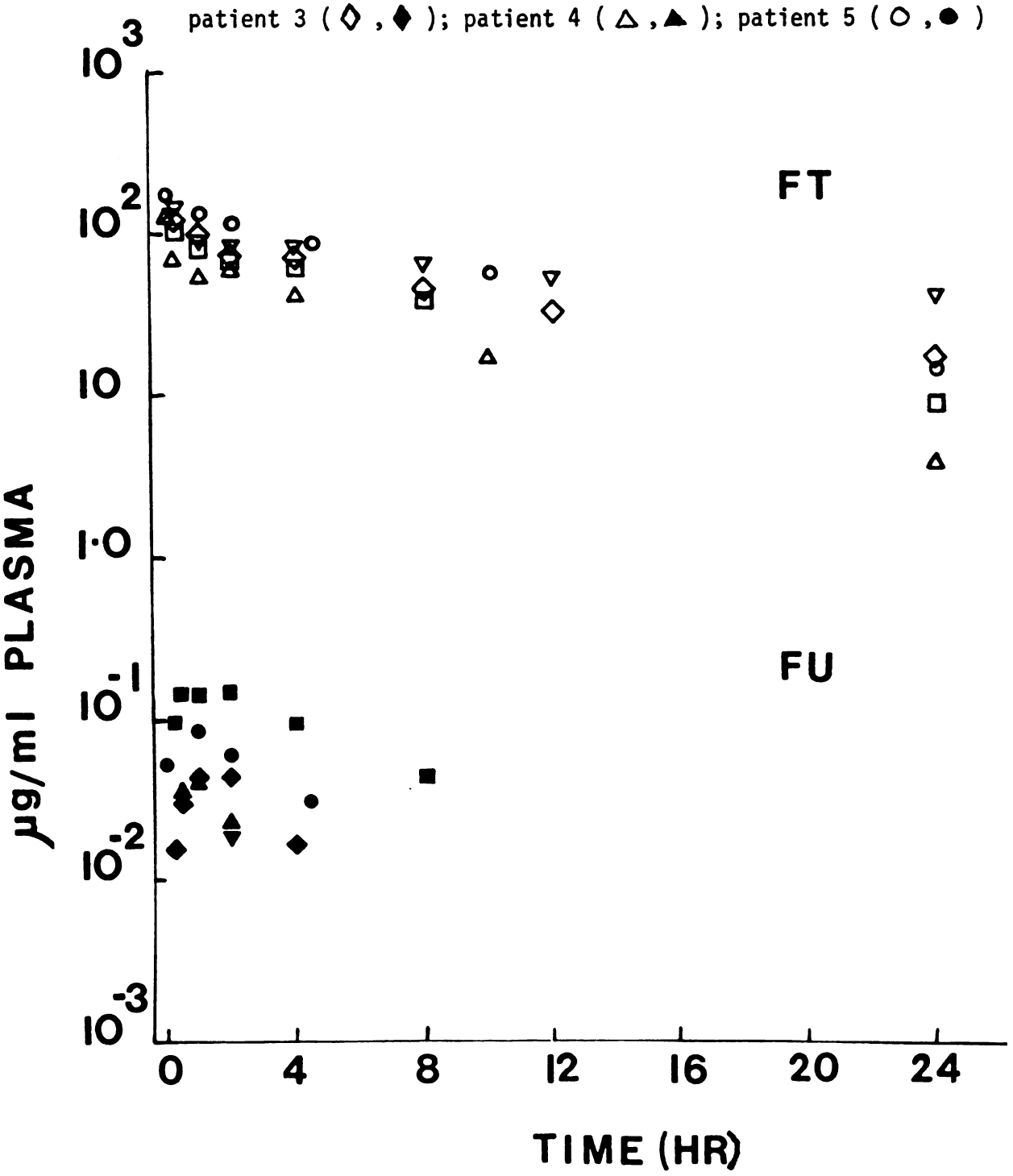
[1]FU concentrations were corrected for non-metabolically generated FU as described in the text.

[2]Concentrations at 4.5 hours after FT administration.

Table XII. Plasma concentrations of FT and its metabolites in patients and rabbits following FT administration. Concentrations are converted to molar concentrations in order to facilitate comparison of the drug and metabolite levels. The molecular weights are : FU, 130; 4'-OH-FT, 216; dehydro-FT, 198; GBL, 86; and FT, 200. FU levels are uncorrected for non-metabolically generated FU.

	Time after dose	FU, μM	cis-4'-OH-FT, μM	dehydro-FT, μM	GBL, μM	FT, μM
Patient CD	2 hr	0.88	0.93	< 0.5	23.8	332
Patient KM	2.5 hr	1.30	<0.5	1.5	18.5	703
Rabbit #1	92 min	1.80	1.8	4.1	15.7	334
Rabbit #2	182 min	0.66	<0.5	2.0	10.5	219

Figure 16. Plasma concetration-time profiles of FT and FU in 5 patients given 2 g FT/sq m by i.v. infusion over 30 minutes. Zero time refers to the end of infusion. FU concentrations were corrected for non-metabolically generated FU as described in the text. patient 1 (▽ , ▼); patient 2 (□ , ■); patient 3 (◇ , ◆); patient 4 (△ , ▲); patient 5 (○ , ●)



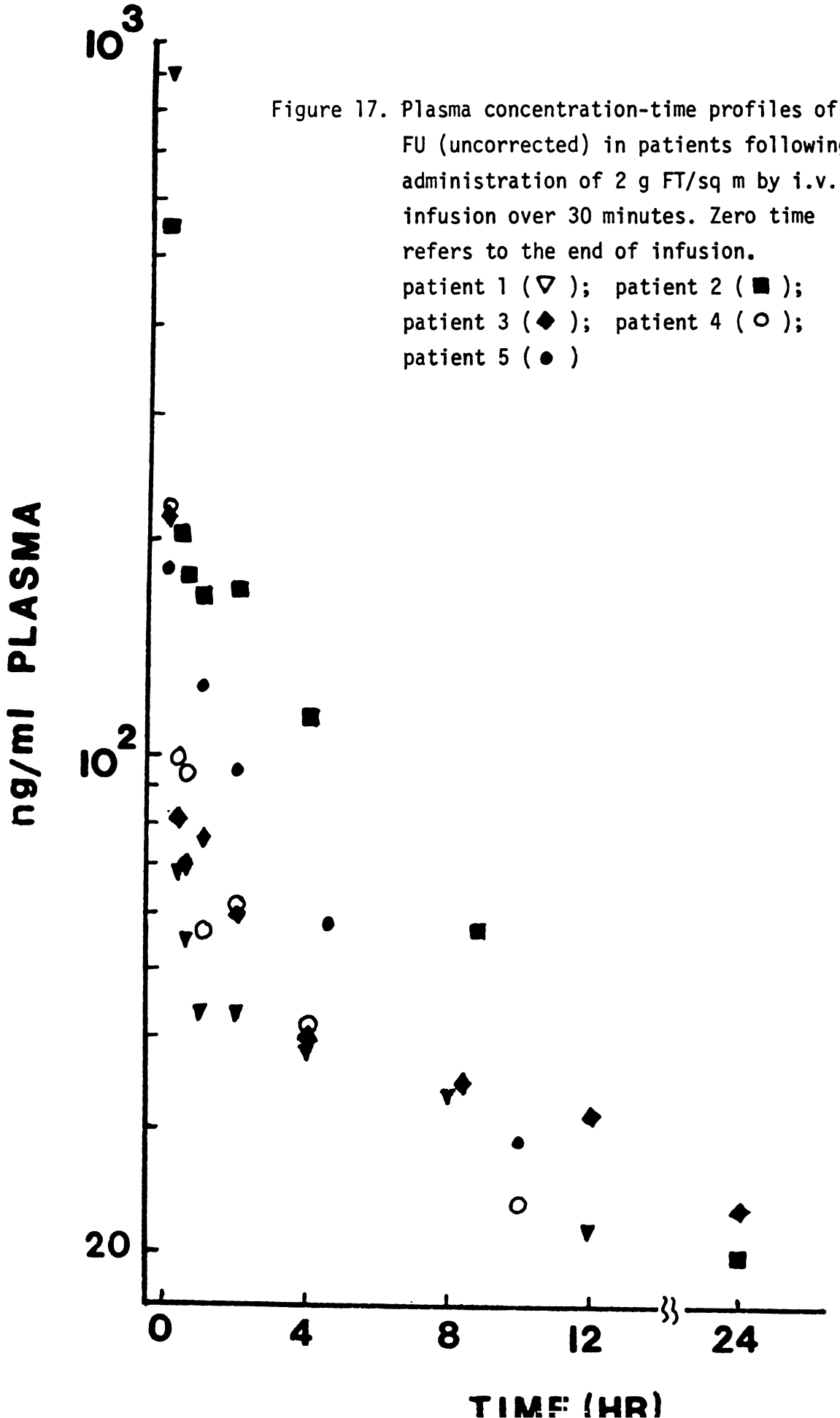
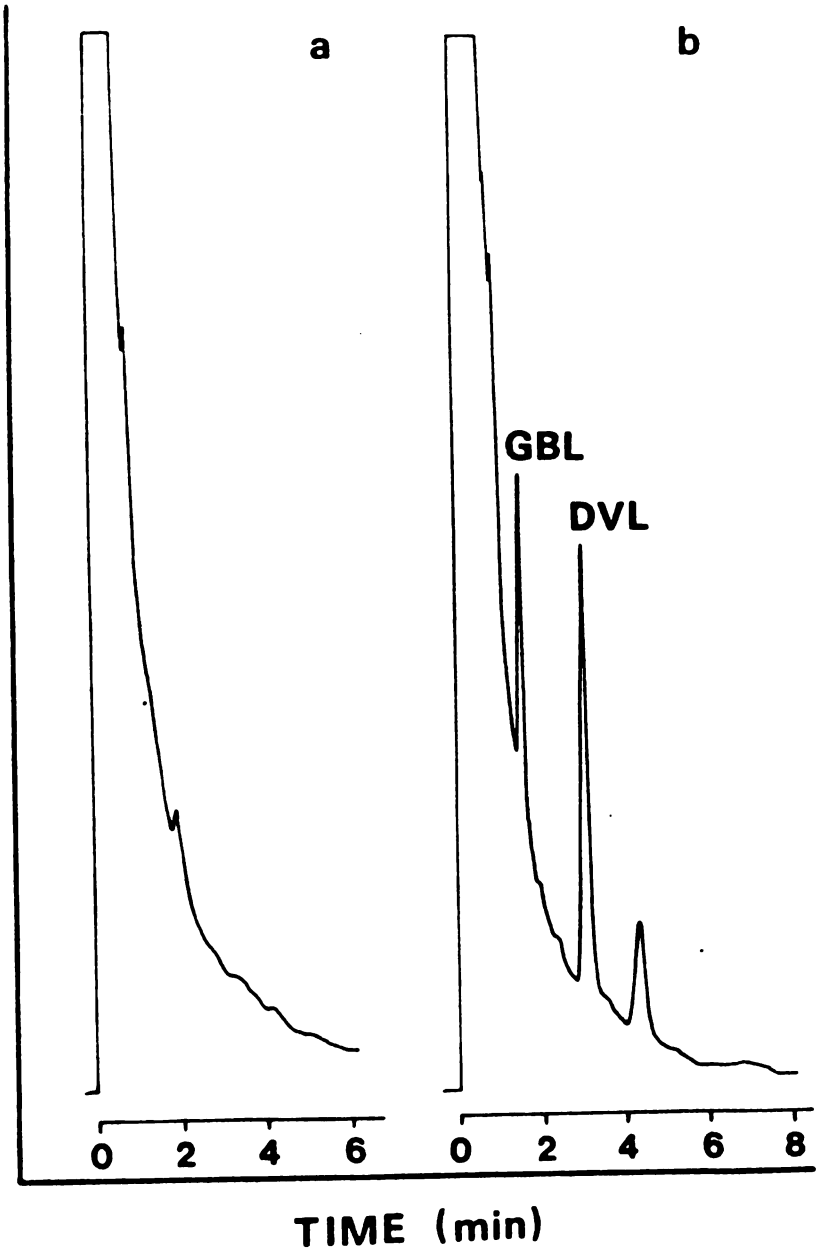


Figure 17. Plasma concentration-time profiles of FU (uncorrected) in patients following administration of 2 g FT/sq m by i.v. infusion over 30 minutes. Zero time refers to the end of infusion.
patient 1 (▽); patient 2 (■);
patient 3 (◆); patient 4 (○);
patient 5 (●)

served initial rapid decline of 5-FU concentrations partially resulted from the FU contamination of the FT dose (Figure 17). After correcting for dosage contaminated plasma FU concentrations peaked at 1-2 hours after FT administration (Figure 16). In patient 1, the corrected FU concentrations were below assay limits (< 20 ng/ml), while in vivo FU formation was evident in other patients; however, FU concentrations dropped to < 20 ng/ml after 8 hours in all patients. Generally, low FU plasma concentrations are consistent with our previous observations in rats and rabbits (95,96). Neither FUR nor FUdR was detectable (< 100 ng/ml in the plasma samples from all five patients.

GC tracings of plasma extract of patient CD obtained before and 2 hours after the administration of FT are shown in Figure 18. The corresponding plasma concentrations of GBL in this patient are 2.05 and 1.12 μg/ml at 2 and 4 hours after FT administration. Similar results were observed in patient KM, with plasma GBL concentrations of 1.59 and 1.00 μg/ml at 2.5 and 4 hours post FT infusion.

Figure 18. GC tracings of extracts of plasma samples of patient CD obtained prior to (a) and 2-hour after FT infusion (b). The 2-hour sample was spiked with 5 μg of DVL, the internal standard. Each chromatogram represents 1/50 of 1 ml plasma extract.



DISCUSSION

It can be inferred from urinary ^{14}C excretion data in rabbits and rats that extensive cleavage of FT to FU does occur *in vivo*. All measured metabolites of FT reached peak plasma levels at 2 to 4 hours following the FT dose. If FU was formed directly from FT without metabolic intermediate, FU plasma levels should have peaked at about 30 min following the dose due to its very short half-life. Therefore, the delayed plasma peak levels of FU and 2 hydroxylated metabolites support the hypothesis of metabolic intermediates in the activation of FT to FU and point to the hydroxylated metabolites as potential intermediates. This hypothesis was tested and the results are discussed in Chapter 4.

There are large discrepancies between the reported FU plasma concentrations resulting from FT administration. Earlier studies in animals reported significant plasma concentrations of FU detected by biological assays (33). Wu *et al.* (95) analyzed FT by GLC method with on-column flash methylation and FU by a column chromatographic GC-MS method, and found that unchanged FT accounted for most of the ^{14}C radioactivity in plasma, while FU concentrations were below 0.15% and 0.4% relative to FT concentrations in rabbits and rats, respectively.

Hills *et al.* (46) studied by a GLC assay the pharmacokinetics of FT and FU formation in patients given a dose of 2.25 g FT/sq m over 30 minutes. In one patient, FU plasma concentrations peaked at above 1 μg/ml at 0.5 to 1 hour after dose and slowly declined over 24 hours; FU concentrations were reported to be about 1 of FT concentrations. Hall *et al.* (40) and Benvenuto *et al.*(13) reported that FU plasma concentration peaked at 5 μg/ml which were maintained

at 1.7 μg/ml for up to 96 hours in patients given rapid i.v. infusion of FT at a dose of 4 to 5 g/sq m, or a total dose of 7-8 g. FU plasma concentrations at above 2-300 ng/ml have been associated with high incidence of toxicity (10,84). Furthermore, the theoretical dose of an FU infusion needed to achieve such concentrations in a 70-kg man is 24 g (or the equimolar amount of 37 g FT) over 96 hours, assuming a plasma clearance of 2.1 l/kg-hr for FU (26)[2]. The rapid exponential fall of initial FU levels (Figure 17, and Figure 1 in reference 13) excludes inhibition of FU metabolism in the presence of FT as a possible cause of the observed high FU plasma concentrations.

In the present study, the plasma concentration ratios of FU to FT in all patients treated with 2 g/sq m/d are below 0.2%, which is lower than those observed by Hills et al. (46) (1%), but in marked contrast to the data of Benvenuto et al. (13) and Hall et al. (40) (initially 5%, steadily increasing with time). Comparable results of low FU concentrations were observed by Diasio et al. (22) and Hornbeck et al. (47). In 9 patients given an oral dose of 1 g FT/d, FU plasma concentration assayed by a normal phase HPLC method reached a maximum of 50 ng/ml (22).

The high FU plasma concentrations reported by other groups can be caused by several factors. FT is chemically labile and slowly decomposes to FU upon storage and possibly during extraction using high salt concentration (46). While such decomposition is quite small, it may be significant due to the very large FT:FU concentration ratio (1000:1) in the biological samples. The hydroxylated metabolites also rapidly converted to FU in plasma at 37° C (13). In

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[2] Dose infused = steady state plasma concentrations X plasma clearance (37).

addition, the dehydrogenated FT metabolite is chemically more labile than FT itself and decomposes to FU during storage. Extraction under acidic conditions employed by Fujita and Kimura (34), and Freudenthal and Emmerling (28), would cause hydrolysis of dehydro-FT to FU, and therefore, introduce an error in FU quantitation. Our study showed that the plasma concentrations of dehydro-FT is the second highest among all FT metabolites, reaching a plateau at 4 hours after the FT dose and remaining essentially constant for up to 24 hours. This kinetic behavior of dehydro-FT is similar to that of the FU concentrations observed by Benvenuto et al. (13) and Hall et al. (40). Our data suggest that previously reported FU plasma concentrations after FT administration are caused by partial or complete in vitro decomposition of the labile dehydro-FT and possibly also the hydroxylated FT metabolites in the plasma.

Chapter VI

Kinetics of formation of FT metabolites

EXTENT OF FT CONVERSION TO GBL IN RABBITS

Equation 1 defines the mathematical relationship between the single dose in the body, the total body clearance (V_{CL}), and the area under the plasma concentration-time curve (AUC) for drugs with linear pharmacokinetic disposition (37). Therefore, for FT

$$Dose_{FT} = V_{CL\ (FT)} \times AUC_{(FT)} \qquad \text{equation 1}$$

Similarily,

$$\text{Amount of GBL formed} = V_{CL\ (GBL)} \times AUC_{(GBL)} \qquad \text{equation 2}$$

Assuming a fraction,F, of the FT dose converts to GBL *in vivo*, then

$$\text{Amount of GBL formed} = F \times Dose_{(FT)} \qquad \text{equation 3}$$

By rearranging equation 1,2, and 3, we obtain

$$F = \frac{V_{CL\ (GBL)}}{V_{CL\ (FT)}} \times \frac{AUC_{(GBL)}}{AUC_{(FT)}} \times \frac{M.W._{(FT)}}{M.W._{(GBL)}} \times 100\% \qquad \text{equation 4}$$

where $AUC_{(GBL)}$ and $AUC_{(FT)}$ are the AUC of GBL and FT resulting from FT administration. Equation 4 was employed to calculate the percentage of FT dose converting to GBL *in vivo* using the V_{CL} of GBL and FT in the individual rabbit (from Table IX in Chapter 5). There are considerable differences in the percentages calculated using the $V_{CL\ (GBL)}$ derived from the first and the second GBL dose due to the changes in pharmacokinetic parameters (Table IX). Although the basic assumption of linear kinetics of GBL appears to be equivocal, an estimate of the range of F can be obtained with

equation 4. The results in Table XIII indicate that the extent of FT conversion to GBL is approximately 20% in rabbit 1 and 40% in rabbit 2.

By injecting tracer amounts of ^{14}C-phenacetin and ^{3}H-acetoaminophen intraportally to rats, Pang and Gillette demonstrated that F established the equation 4 is underestimated by 1/(extent of sequential metabolism of the metabolite) in the case of metabolites with high extraction ratios relative to the parent drugs (74). GBL is eliminated primarily by metabolism via a dehydrogenase, a soluble enzyme (87). The total body clearance of GBL in rabbits ranges from 0.5 to 1.4 l/kg-hr (Chapter 3, Table IX)and is significantly higher than that of FT (0.2 l/kg-hr, Chapter 3, Table IX). Thus it is conceivable that F, estimated by equation 4, is an underestimate of the fraction of FT converted to GBL, and that at least 20 to 40% of the administered FT dose was metabolized to GBL and therefore also FU.

Table XIII. Percentage of the FT dose converting to GBL *in vivo*.

	Using the pharmacokinetic parameters derived from the first dose	Using the pharmacokinetic parameters derived from the second dose
Rabbit 1	24%	13%
Rabbit 2	48%	31%

EXTENT OF FU FORMATION

There is no good estimate of the amount of FU formed following an FT dose. If one assumes the metabolism of FT is solely via FU, then a large fraction of the FT dose is converted to FU since 60-80% of 2-^{14}C-FT dose administered to animals was recovered as FU metabolites (21). One pharmacokinetic approach (37) to determining the extent of prodrug conversion is to compare the plasma clearance and the area under the plasma concentration-time curves of the parent drug and its active metabolite, as shown in equation 5.

$$F = \frac{CL_{FU}}{CL_{FT}} \quad X \quad \frac{AUC_{FU}}{AUC_{FT}} \qquad \text{Equation 5}$$

where F is the fraction of the FT dose converting to FU, CL_{FU} and CL_{FT} represent the plasma clearance of FU and FT respectively, and AUC_{FU} and AUC_{FT} are the areas under the plasma molar concentration-time curves of FU and FT following an FT dose. We have used population estimates of CL_{FU} taken from the literature and, therefore, assume little intersubject or intrasubject variability. The intersubject variability of FU kinetics is indeed small provided that the rate of administration is the same among individuals (17,26,67). However, there is a drastic difference in the CL_{FU} in humans when FU is administered at different rates. Up to 60 fold higher plasma clearance was observed in the same subject receiving FU by slow i.v. infusion rather than by bolus injection (17,18). Nonlinear disposition kinetics has been suggested as a cause of this drastic difference in the CL_{FU} (18). When FU is given in a form of metabolic prodrug, the input of FU follows a first order rate process which is different from a pulse (bolus) injection or a zero

order infusion, the corresponding CL_{FU} would be expected to differ from those following either i.v. bolus administration or i.v. infusion. Another assumption underlying this conventional pharmacokinetic approach is that the distribution of the drug to and from its metabolizing sites is faster than its metabolism. It is of interest to note that the plasma clearance of FU ranges from 0.4 to 60 l/kg-hr depending on the rate of administration (calculated from references 17,26,67),and often exceeds the blood volume of cardiac output (6 l/kg-hr). Jaquez (50) reported a diffusion constant of 1.1×10^{-5} cm/min for FU in Ehrlich Ascites cells at 25 C, and an equilibrium between intracellular and extracellular concentrations was obtained in 1 min. It is, therefore, conceivable that the absolute rate of metabolism of FU may surpass its diffusion rate across the cell, and than FU generated from FT is localized at metabolic sites where it is further metabolized without redistributing back into the systemic circulation. A similar "sequential first-pass" effect of phenactin and its metabolite, acetophenacetin, in an isolated perfused-liver model was noted by Pang and Gillette (74).

We have demonstrated the metabolism of FT to FU can be mediated by al least two distinct mechanisms. One of which may be mediated by hepatic microsomal cytochrome P-450 system, and the other is independent of the cytochrome P-450 system and is mediated by soluble enzymes, and thys may take place in tissues other liver. Kinetics of the formation of GBL, a metabolic product in addition to FU formed <u>via</u> the pathway mediated by soluble enzymes, in rabbits and

patients, and in metabolism studies in vitro indicate that this pathway is equally important as the microsomal degradation. The participation of the soluble enzymes in the activation of FT to FU indicates that FT may be metabolized in tissues other than the liver. Similarily, extrahepatic clearance accounts for 75 %of the total body clearance of FU in dogs (39). Thus, a significant fraction of the FU generated from in FT extrahepatic tissues may be further metabolized without reaching the sampling sites. In this case, FU plasma concentrations may not correctly reflect the extent of exposure of the various target tissues to intracellular FU. In conclusion, it is presently not possible to calculate the conversion rate and extent of FT to FU based on the plasma concentrations of FU generated following FT administration.

Chapter VII

Conclusions and Perspectives

Conclusions and Perspectives

The clinical usefulness of FU in the treatment of solid tumors has been limited by its lack of tissue selectivity. The reduced myelotoxicity of FT has been attributed to its property of releasing FU slowly *in vivo*, and represents a significant advantage over FU. Unfortunately, the clinical use of FT is asociated with a high dose-limiting neurotoxicity. Results of the clinical trials of FT in the United States demonstrated that FT is not superior over FU when given intravenously. At present, the oral applicability of FT is the remaining advantage and Phase I-II clinical trials of oral FT have been initiated.

It is still debatable if an appreciable fraction of the FT dose is indeed metabolized to FU. Pharmacokinetic studies of FT disposition and FU formation have been inconclusive, largely due to the rapid metabolism of FU. Although an alternative hypothesis that FT may be activated to and metabolized *via* other metabolites independent of FU cannot be ruled out, our findings in this study support the following hypothesis. When administered in a form of a metabolic prodrug which is activated in extrahepatic tissues such as FT, the FU generated intracellularly may be localized by further metabolism within the tissues and do not redistribute into the circulation. Thus, a metabolic prodrug may serve as a specific-tissue delivery system of FU, and its tissue selectivity would depend on its distribution into tissues, the mechanism of activation to FU, and the tissue distribution of the enzymes responsible for its activation. In addition, compounds with relatively slow body clearance would be desired to allow sufficient penetration into the generally poorly perfused solid tumors.

One of the FT metabolites isolated during this investigation, i.e. β,D-4'-OH-FT, may be pharmacologically active. Susceptability of this compound to phosphorolysis to FU by thymidine phosphorylase, and the decrease in lipophilicity caused by the addition of the hydroxyl function in the tetrahydrofuran moiety suggest that β,D-4'-OH-FT may serve as a potential FU prodrug that may be devoid of the CNS toxicity of FT. Studies have been initiated to evaluate the biological activity of β,D-4'-OH-FT (not within the scope of this investigation). Results of these initial studies demonstrate that this compound is selectively cytotoxic against cultured human hematopoietic cells with thymidine phosphorylase activity, and thus may act as an FU delivery system specific for tissues with this enzyme (9). One may even speculate on improved therapeutic index with this compound since hepatoma cells have been demonstrated to have elevated ratios of the activities of thymidine and uridine phosphorylases to that of the enzyme responsible for the breakdown of FU (92).

Among the few other FU prodrugs, which have been tested in the last decade, i.e. FD-1 (54), 1-hexylcarbamyl -5-fluorouracil (59) and 5'-deoxy-5-fluorouridine (5'-dFUR), 5'-dFUR have been shown to possess a higher therapeutic index over FU in some animal tumors (3,16,49,61). This compound was subsequently found to be an uridine phosphorylase substrate, a property which may contribute to its improved tissue selectivity (49).

In perspective, development of further FU prodrugs targeted at activation within tumor tissues is a feasible alternative to improve the clinical efficacy of FU.

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Appendix I. TLC separation of GBL and FT.

Conditions: Instantaneous TLC plates, 40 X 80 mm of 0.25 mm thickness, Polygram$^{(R)}$ Sil G/ UV 254.

Detection : FT by UV absorbance

GBL by spraying the plate with the following solutions:

1. 6.25% NaOH in MeOH and 2.5% hydroxylamine HCL
2. Acetic acid, glacial
3. 10% $FeCL_3$ aqueous solution

Lactones give brown-colored spots (58).

Solvents	R_f FT	R_f GBL
$CHCL_3$, 100%	0	0.44
$CHCL_3$: MeOH, 100 : 2	0.13	0.40
$CHCL_3$: Isopropanol : Acetic acid, 100 : 2 : 1	0.10	0.23