Rapid Catabolism of 5-Fluorouracil in Freshly Isolated Rat Hepatocytes as Analyzed by High Performance Liquid Chromatography

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The catabolism of 5-fluorouracil (FUra) has been investigated in freshly isolated rat hepatocytes in suspension by a new and highly specific high performance liquid chromatographic methodology. This technique permits rapid and simultaneous quantitation of FUra catabolites—dihydrofluorouracil (FUH2), α-fluoro-β-uridinopropionic acid (FUPA), and α-fluoro-β-alanine (FBAL) with FUra nucleosides and nucleotides. Analysis of intracellular and extracellular H2 was evaluated from 1 min to 2 h after exposure of the cells to 30 μM [3H]FUra. FUra is rapidly cleared from the incubation medium with only one-half of the unmetabolized drug remaining after 8 min when the cytocr is as low as 3.5%. Transport of FUra into the hepatocytes is much slower than its catabolism to FUH2 so that unmetabolized FUra is not detected within the cell as early as 1 min after exposure to this agent. Utilizing an initial [3H]FUra concentration of 30 μM, intracellular FUH2 (the major intracellular catabolite) reaches its peak level of 637.4 ± 85.8 μM within 11.5 min and subsequently declines as FUH2 leaves the cells and/or is further catabolized to FUPA and FBAL. The peak transmembrane concentration gradient of FUH2 (642 ± 93/1) is attained within 1 min. Low levels of FUPA appear in the intracellular and extracellular fluids. A 50-fold transmembrane gradient for FBAL is generated within 3 min, which decreases as FBAL approaches equilibrium across the cell membrane over the next hour. FBAL is the main extracellular product of FUra catabolism after 129 min. Incorporation of H from [3H]FUra into RNA and protein is minimal (<1% of the total intracellular H) and no drug was found to be bound to cellular macromolecules by a minicolumn chromatographic exclusion technique. Total H in the cell suspension between 1 min and 2 h is accounted for by the total of intracellular and extracellular FUra and catabolites, indicating that these catabolites are the major products of FUra metabolism by the hepatocyte. These studies indicate that FUra is rapidly transported into and catabolized by hepatocytes and confirm the critical role that the liver plays in the clearance of this fluoropyrimidine.

5-Fluorouracil, synthesized in 1957 (1) and studied extensively by Heidelberger and co-workers (2–4), is still today a major agent utilized in the treatment of several malignancies including carcinomas of the breast, gastrointestinal tract, and ovary. The cytotoxic effects of this antimetabolite are based, upon two distinct biochemical mechanisms: 1) the conversion of FUra1 to 5-fluoro-2'-deoxyuridine 5'-monophosphate which binds to thymidylate synthetase and thereby inhibits de novo synthesis of dTMP and, hence, DNA synthesis (2, 5–7); 2) the inhibition or alteration of RNA maturation and function as a consequence of 5-fluorouridine 5'-triphosphate incorporation into RNA (8, 9). Studies over the past 2 decades have clarified the importance of these anabolic pathways; in contrast, knowledge of the catabolic pathway is limited (10). In previous studies on the mechanism of action of fluoropyrimidines, rapid degradation of FUra was indicated both in vivo and in vitro in normal tissues of mice and humans (11–13) and FUH2 has been observed in plasma of patients given FUra (14, 15). The liver appears to be the major site of FUra catabolism (4) with the initial step postulated to be reductive degradation to FUH2 followed by conversion to FUPA, a compound readily excreted in the urine (12, 13). These two initial reactions utilize the same pathways as uracil and thymine (16–18). In the next proposed catabolic step, FUPA is converted to FBAL, with the release of CO2 and presumably NH3 (13). In addition, previous studies indicate the presence of large amounts of radiolabeled urea in urine of patients exposed to [2,14C]FUra (12, 19). This latter finding led to the proposal that α-fluoro-β-guanidinopropionic acid is formed as well as FUPA with subsequent cleavage to urea and CO2 (4, 12, 19).

Uncertainties regarding the pathways and extent of catabolism of FUra have been based, in part, upon the inadequacies of the analytical techniques that have been available. Previous methods for separating the various catabolites of FUra have included thin layer chromatography (20) and low pressure "ion exchange chromatography" (12, 13). Both methods are limited by poor resolution; furthermore, these techniques would fail to measure FUH2 because of the instability of this compound with the drastic alterations in pH and the length of time required to resolve FUra and its catabolites. The present studies describe a new and highly specific high performance liquid chromatographic method which, by its greater specificity and speed, permits the evaluation of the parent drug and its catabolites, including very unstable compounds such as FUH2 in 25 min.

This new HPLC technique has been utilized to assess liver
catabolism and anabolism of FUra for the first time in the isolated rat hepatocyte system, a model for the study, at the cellular level, of biosynthetic, catabolic, and transport phenomena in the liver. This system permits analysis of drug-cell interactions within seconds, which is particularly important when there are rapid transport and catabolic events. In addition, the isolated hepatocyte system eliminates complexities of studies with the intact liver or liver slices such as alterations in blood flow, uncertainties about drug concentration at the cell membrane site because of large unstirred extracellular spaces, and contributions to drug transport and metabolism by other cell types in the liver (i.e., hepatic reticuloendothelial cells).

This report analyzes aspects of the kinetics of [6-3H]FUra catabolism in hepatocytes within 1 min after exposure of the cells to radiolabeled compound. This paper demonstrates (i) rapid catabolism of FUra by the freshly isolated hepatocyte, (ii) rapid release of FUra catabolites into the extracellular compartment, and (iii) insignificant anabolism of FUra by liver cells over an interval of up to 2 h. These data indicate the crucial role of the liver in the rapid elimination of FUra and, hence, the important contribution of this tissue in determining the interval over which tumor cells are exposed to this drug in vivo.

MATERIALS AND METHODS

Preparation of Hepatocyte Suspension—Studies were performed utilizing rat hepatocytes in suspension isolated from male Sprague-Dawley rats by a modification of the collagenase perfusion technique of Berry and Friend (21) as previously described (22). Cell viability, as determined by trypan blue exclusion, was 90% or greater in these experiments. Hepatocytes were suspended to a final cytocrit of 5.5 to 5% and were incubated at 37°C in Krebs-Henseleit buffer containing 0.25% gelatin and 10 mM glucuron. pH was maintained at 7.4 by passing warmed and humidified 95% O2 and 5% CO2 over the cell suspension.

Incubation Conditions and Extraction of Extracellular 3H—Throughout the incubation, the hepatocyte suspension was stirred by a Teflon paddle in specifically designed flasks as described previously (23). The experiment was initiated with the addition of sufficient [3H]FUra (15 mCi/mM) to achieve a final concentration of 30 μM and portions of the cell suspension (0.5 ml) were layered on 400 μl of inert silicone oil of density 1.2 (24) in 1.5-ml plastic microcentrifuge tubes. The tubes were centrifuged at 15,000 g for 3 min. This procedure, described in detail previously (25), permits separation of the extracellular space that accompanies the cell pellet by sonic oscillation, the sonicate was acidified with 10% trichloroacetic acid. The acid precipitate was then separated into dry ice/acetone. Times of incubation in the text represent the interval over which tumor cells are exposed to this drug in vivo.

The residue was dissolved in 100 μl of 0.15 M sodium chloride, 5 μl of sodium dodecyl sulfate, and 5 μl of formic acid. The samples were then injected into the GC/MS system.

Extracellular and Intracellular Space Determinations—The cell pellet less the [14C]inulin space. This technique has been described in detail previously (23, 25, 26). This report analyzes aspects of the kinetics of [6-3H]FUra catabolism in hepatocytes within 1 min after exposure of the cells to radiolabeled compound. This paper demonstrates (i) rapid catabolism of FUra by the freshly isolated hepatocyte, (ii) rapid release of FUra catabolites into the extracellular compartment, and (iii) insignificant anabolism of FUra by liver cells over an interval of up to 2 h. These data indicate the crucial role of the liver in the rapid elimination of FUra and, hence, the important contribution of this tissue in determining the interval over which tumor cells are exposed to this drug in vivo. In the extracellular fluid presented as the GC carrier (flow 1.55% recovery based upon 48 runs).

GC-MS Analysis—The identity and purity of the FUH1 liquid chromatography peak were confirmed by GC-MS. This analysis was performed using a Hewlett-Packard 5890A GC-MS interfaced to a Hewlett-Packard 5973A data system. This study was carried out in chemical ionization mode using methane as reagent gas (ion source pressure 1 torr, source temperature 200°C). The ionization energy and emission current were 15 eV and 300 μA, respectively. Helium served as the GC carrier (flow 30 ml/min) and GC separations were accomplished using a glass column (2.8 m × 3 mm inner diameter) packed with 3% OV-275 on Chromosorb WHP (80 to 100 mesh). The GC oven was programmed at 210°C. The HPLC fraction with a retention time of 7.76 min was collected, concentrated to dryness at 50°C under stream of nitrogen, and derivatized using iodopentane.

The residue was dissolved in 100 μl of methanol, and portions of 2 μl were then injected into the GC/MS system.

Incorporation of H into RNA and Protein—After disruption of the cell pellet by sonic oscillation, the sonicate was acidified with 10% trichloroacetic acid. The acid precipitate was then separated into RNA and protein fractions by the methodology described previously (27). Radioactivity in each fraction was determined, and the incorporation of radiolabeled drug was expressed in picomoles/μg of RNA and picomoles/mg of protein contained in the trichloroacetic acid precipitate using thecin reaction (28) and the technique of Lowry et al. (29), respectively.

Stability of FUra Catabolites—The pH stability of nonlabeled FUH1 was assessed to determine the optimum conditions for preserving this compound. The analysis of the unmodified standard FUH1 was analyzed by GC/MS (see above) and no degradation was observed in FUH1 stored at −20°C for 1 week when the pH was maintained between 7 and 8. However, rapid and complete
breakdown of the FUH₂ appeared with higher or lower pH values (data not shown).

The stability of [³H]FUra catabolites in both the extracellular medium and in the cell extract was assessed using the HPLC methodology described above in samples stored at pH 7.4 and -20°C over an interval of 6 h to 1 week (Fig. 1). In the cell extract, there was less than 3% breakdown of the FUH₂ in 12 h and a degradation of about 20% in 1 week, while the amount of FBAL increased proportionately. These data indicate the relative instability of FUH₂ in the presence of cellular protein and the requirement that analysis be performed within 12 h after the cell extract is obtained. In the extracellular medium, [³H]FUra and its catabolites FUH₂, FUPA, and FBAL were found to be stable over 1 week at -20°C.

Chemicals—[6-³H]FUra (18 Ci/mmol) was purchased from Moravek Biochemicals Inc. (Brea, CA) and then purified by the HPLC technique described above. [Carboxyl-¹⁴C]inulin (2.5 Ci/g) was obtained from Amersham-Searle (Irvine, CA). Nonlabeled standards FUra, FUH₂, FUPA, and FBAL were generous gifts of Hoffmann-LaRoche Laboratories. 5-Fluorouridine 5'-monophosphate, 5-fluoro-2'-deoxyuridine 5'-monophosphate, 5-fluorouridine 5'-diphosphate, 5-fluoro-2'-deoxyuridine 5'-diphosphate, 5-fluorouridine 5'-triphosphate, and 5-fluoro-2'-deoxyuridine 5'-triphosphate were obtained from Sierra Bioresearch (Tucson, AZ). All other chemicals employed were products of Sigma (St. Louis, MO).

RESULTS

HPLC Analysis of FUra and Its Catabolites FUH₂, FUPA, and FBAL—Fig. 2 represents an HPLC chromatogram demonstrating separation of nonradiolabeled FUra and its presumptive catabolites FBAL, FUH₂, and FUPA. This chromatographic technique unambiguously resolves FUra from these metabolic products with R values (mean of three experiments ± S. D.) of 2.0 ± 0.1 between peaks I and II, 6.98 ± 0.44 between peaks II and III, and 10.83 ± 0.41 between peaks III and IV. The resolution values were >1.25, confirming the specificity of this method (31). As the FUH₂ was demonstrated to be an unstable derivative of FUra (see above), it appeared possible that the peak identified as FUH₂ may actually have been a breakdown product of FUH₂ degradation. Therefore, the identity of the FUH₂ peak was evaluated by GC/MS analysis. The FUH₂ chromatographic peak was derivatized as described under "Materials and Methods" and the analysis of this sample produced the mass spectrum shown in Fig. 3. The dipentylated derivative of FUH₂ was identified from its methane chemical ionization mass spectrum ([M⁺] = m/z 273 (100%); [M + 29]⁺ = m/z 301 (8%); [M + 41]⁺ = m/z 313 (4%)). This methane chemical ionization-MS fragmentation was identical with that obtained from an authentic specimen of FUH₂.

Analysis of Intracellular ³H after Incubation of Hepatocytes with [³H]FUra—Fig. 4 shows the HPLC analysis of intracellular ³H 4.5 min after incubation of the hepatocyte suspension with 30 μM [³H]FUra. The resolution of the radiolabeled catabolites was comparable to that obtained with the nonradiolabeled compounds (Fig. 2). After 4.5 min, the major...
intracellular constituent was FUH₂. In addition, there were significant levels of FUPA and FBAL. In this and other experiments, no unmetabolized FUra was detected within the hepatocytes when the suspension was sampled as early as 1 min and as late as 2 h after exposure of the hepatocytes to [³H]FUra. This indicates that FUra that enters the hepatocyte is rapidly catabolized and suggests that transport may be rate limiting to catabolism in this system.

Evaluation of intracellular ³H up to 2 h after incubation with 30 μM [³H]FUra failed to demonstrate the presence of nucleoside or nucleotide derivatives of FUra.

Time Course of Appearance of Intracellular Catabolites—The profile of the time course of appearance of FUH₂, FUPA, and FBAL in the intracellular water is shown in Fig. 5. The predominant catabolite in the intracellular water is FUH₂ which quickly achieves its peak intracellular concentration of 637.4 ± 85.8 μM in approximately 11.5 min. Subsequently, intracellular FUH₂ declines to a level of 144.66 ± 65.73 μM by 2 h.

Within 1 min after the initial exposure of the hepatocyte suspension to [³H]FUra, intracellular FUPA and FBAL can be detected. By 30 min, FBAL has reached a steady state level of 31.5 ± 4.9 μM within the intracellular water which is maintained for the remaining 1½ h of the experiment. Intracellular FUH₂ appears transiently, reaching a maximum concentration of 37 ± 1.4 μM in 5 min; by 25 min, FUPA is no longer detectable within the cell.

Analysis of the Time Course of Disappearance of Extracellular FUra and Appearance of Extracellular Catabolites—Analysis of extracellular FUra and its catabolites over 1 min to 2 h after exposure of hepatocytes to [³H]FUra is illustrated in Fig. 6. The extracellular level of FUra declines to one-half of the initial level within 8 min and no unchanged drug is detectable after 30 min. As the level of extracellular FUra declines, catabolites of FUra that have been synthesized within the cell appear in the extracellular compartment. Extracellular FUH₂ gradually increases, reaching a peak level of 10.6 ± 0.61 μM at approximately 22 min, and subsequently declines. FBAL also appears rapidly in the medium, reaches levels equivalent to those of FUH₂ by 22 min but remains the major extracellular catabolite as the FUH₂ level declines. Extracellular FBAL finally reaches a steady state level of 18.5 ± 1.4 μM. Extracellular FUPA is detected within 1 min and then declines very slowly over 2 h. Total ³H added as FUra can be accounted for the sum of the catabolites in the extracellular and intracellular water; this indicates that FUra is converted essentially quantitatively to these catabolites.

Assessment of Intracellular Binding of FUra and Its Catabolites—Binding of intracellular ³H to cell macromolecules was assessed by the minicolumn technique of Fry et al. (30) in which free ligand remains in the column while ligand-macromolecular complexes pass through the column during centrifugation. This procedure was carried out using cell extract taken at times ranging from 11 min when FUH₂ has reached its maximum concentration up to 2 h when FUH₂ has declined and FBAL is at steady state. All radioactivity from the cell extract that was applied to the minicolumn was trapped within the column, indicating that intracellular ³H was not bound to cellular constituents.

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**Fig. 4.** HPLC chromatogram of intracellular ³H after incubation of a hepatocyte suspension for 4.5 min with 30 μM [³H]FUra. Timed fractions of 0.2 ml were collected for the first 9 min followed by 0.5-ml fractions over 25 min under the HPLC conditions described under "Materials and Methods." Retention times are indicated on the abscissa.

**Fig. 5.** Time course of appearance of FUH₂, FUPA, and FBAL in the intracellular water after exposure of cells to 30 μM [³H]FUra. The inset represents the expanded time course for appearance of FUPA and FBAL. At the indicative times, portions of cell suspension were separated by centrifugation and extracted with total intracellular ³H analyzed by HPLC as described under "Materials and Methods" and in the legend to Fig. 4. Corrections were made for extracellular FUra and its catabolites that accompany cells through the oil.

**Fig. 6.** Analysis of extracellular FUra and its catabolites after exposure of hepatocytes to 30 μM concentration of [³H]FUra. The inset represents FUPA levels in the same experiment. At the indicated time, portions of cell suspension were separated by centrifugation and the total extracellular ³H assayed by HPLC as described under "Materials and Methods."
Incorporation into RNA and Proteins—Over an interval of 7.5 min to 2 h after exposure to $[^3]H$FUra, incorporation of radiolabeled drug represented 0.09 ± 0.001 pmol/ug of RNA and 3.37 ± 0.25 pmol/mg of protein in the acid-insoluble fraction of the cell extract (mean of 18 time points ± S. D.). This represents less than 1% of the total intracellular radiolabel. This, along with the absence of nucleotide derivatives of FUra, indicates that the catabolites formed from FUra are the main components of intracellular $^3H$ and that anabolism is an insignificant factor following exposure of liver cells to FUra under these conditions.

DISCUSSION

These studies provide the first detailed analysis of the rapid formation of FUra catabolites and their disposition in the freshly isolated rat hepatocyte system. This approach was made possible by the development of a new HPLC methodology that permits rapid and simultaneous identification of all FUra catabolites and anabolites. The study of hepatocytes in suspension further permits quantitation of rapid transport and catabolic processes without complexities encountered in studies with the intact liver or liver slices, i.e. the presence of hepatic reticuloendothelial cells, large unstirred extracellular spaces, and changes in blood flow to the whole tissue.

These results clarify a number of key steps in the catabolic pathway of FUra in the rat hepatocyte (Fig. 7). (i) The initial degradation reaction is thought to be the reduction of FUra to FUH by dihydrouracil dehydrogenase (4). Low levels (0.8% of the total radioactivity) of FUH have been detected previously in high speed supernatant fractions of mouse liver exposed to $[2-^14C]FUra$ (12). In contrast, under the conditions of these experiments, FUH was shown to be the major constituent of intracellular $^3H$ after incubation of the hepatocytes with 30 $\mu$m $[^3]H$FUra. FUH achieves a maximal transmembrane chemical gradient of 642 ± 93/1 with respect to extracellular FUH, within 1 min, and a maximum intracellular level of 637.4 ± 85.8 $\mu$m is achieved in 22 min. Since no catabolites of FUra appeared to be bound to cellular constituents, the data suggest that FUH exits from the cells very slowly relative to the rate at which it is synthesized, and that the rate of FUH synthesis is much faster than its rate of degradation. Furthermore, because intracellular FUH never declined to equilibrium levels across the cell membrane (a transmembrane gradient of 22.3 ± 5.5/1 was still present after 2 h of incubation), active transport for this compound into the cell is possible; however, longer intervals of exposure would be required to determine whether, in fact, a steady state gradient for FUH is actually sustained.

(ii) The second step of FUra catabolism is thought to proceed via degradation of FUH to FUPA (4). The FUPA levels in these experiments were low both in the intracellular (37 ± 1.4 $\mu$m) and extracellular (1.14 ± 0.025 $\mu$m) compartments, suggesting that in the liver, FUPA is a transient intermediate in the transformation of FUH to FBAL. Because FBAL accumulates to a much greater level than FUPA, the data indicate that the rate of FUPA conversion to FBAL is rapid compared to the rate of FUPA formation from FUH.

These findings differ from results published previously which indicated that FUPA was the major catabolite of FUra degradation in liver homogenates (12). A possible explanation for the differences in these studies is that the instability of FUH2 did not permit the measurement of this compound by the techniques employed, and that the measured levels of FUPA actually represented the sum of FUH2 and FUPA.

(iii) Subsequently, FUPA is converted to FBAL (4). In the present studies, FBAL appears within 1 min in the cell water at a level of 8.1 ± 1.8 $\mu$m, indicating the rapidity of FUra catabolism. A 50-fold transmembrane gradient for FBAL is generated within 3 min, which decreases during the 1 h to approach equilibrium across the cell membrane. The quantitative increase in the formation of FBAL as FUra and the catabolites decline indicates that FBAL is probably the final catabolite of $[^6]H$FUra formed by the hepatocytes. Previous studies (32) also suggested that FBAL is the main extracellular product of FUra catabolism.

Since FUra is labeled in the 6-hydrogen, it has not been possible, as yet, to study the fate of the remainder of the molecule such as the formation of CO2 or urea (12, 13) or possible degradation of FBAL (13, 32, 33). However, the total radioactivity applied to the HPLC system was recovered for both intracellular and extracellular $^3H$ as FUra and its catabolites FUH2, FUPA, and FBAL so that formation of other catabolites that might involve the 6(C-H) position of the FUra molecule by the liver must be negligible. Heidelberger et al. (12, 13) suggested the possible formation of another catabolite, $\alpha$-fluoro-$\beta$-guanidinopropionic acid, simultaneously with FUPA. However, our studies indicate that this compound is not formed in hepatocytes under these conditions. Absolute verification of the absence of $\alpha$-fluoro-$\beta$-guanidinopropionic acid would not require an $\alpha$-fluoro-$\beta$-guanidinopropionic acid standard to exclude the unlikely possibility that $\alpha$-fluoro-$\beta$-guanidinopropionic acid co-elutes with FUra or the other catabolites described herein.

These findings indicate the rapid catabolic conversion of FUra to FUH2, FUPA, and FBAL and significant conversion of FUra to nucleosides and nucleotides in liver cells. The data support the critical role of the liver as a major factor in the clearance of FUra from the circulation. As some pharmacokinetic studies indicate that the plasma clearance of FUra exceeds the hepatic blood flow (34, 35), degradation of FUra in other tissues in vivo is possible as well. The isolated hepatocyte system provides an opportunity for a highly quantitative assessment of the transport and metabolic steps involved in FUra degradation by the liver and will be utilized subsequently as a model for exploring how these processes might be modulated to increase the chemotherapeutic effectiveness of this agent.

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