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A MATHEMATICAL MODEL OF THE KINETICS OF 5-FLUOROURACIL AND ITS CATABOLITES IN FRESHLY ISOLATED RAT HEPATOCYTES

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Abstract—A mathematical model for the kinetics of 5-fluorouracil (FUra) catabolism in liver cells is proposed. It is based on published data for the metabolism of FUra by isolated rat hepatocytes. The model relies on biochemical knowledge of the catabolic pathway. The key-steps are: (i) the cellular uptake and the conversion of the unchanged drug to dihydrofluorouracil (FUH_2) and subsequently to α-fluoro-β-alanine (FBAL); (ii) the cellular fluxes of the 2 catabolites, FUH_2 and FBAL. Water is partitioned between the extracellular and intracellular spaces. The first step is described by Michaelis–Menten kinetics and the other processes by first-order kinetics. Satisfactory fitting of the model validates these simplifications and provides values for the parameters describing the process. The model indicates that (i) the kinetics of FUra disappearance are non linear, the V_{max} of the first step being between 3.1 and 5.0 μM/min and the K_m between 12 and 37 μM; (ii) the rate limiting step is the degradation of FUH_2 (the major intracellular catabolite) with a rate constant of 0.1 to 0.02 min^{-1}; (iii) the FUH_2 transmembrane exchange is active; (iv) the exchange of the final catabolite FBAL is by diffusion.

5-Fluorouracil is one of the most widely-used antineoplastic agents in the treatment of solid tumors. Rapid degradation of this drug has been observed both *in vitro* and *in vivo* [1–5], principally in the liver [6]. In a recent study, the metabolism of FUra† by isolated rat hepatocytes has been investigated using highly-specific high performance liquid chromatography [7]. This technique, because of its great specificity and speed compared with previous methodologies [1, 2, 8], allowed the measurement in cellular as well as in extracellular fluids of unmetabolized FUra, its presumptive catabolites and anabolites. Exposure of hepatocytes in suspension in 30 μM FUra resulted in a rapid and complete conversion of FUra to several catabolites, namely FUH_2, FUPA and FBAL. By describing the concentration–time curve of each compound, a number of key steps in the catabolism of FUra were identified. However, such analysis cannot provide precise quantitative information about the metabolism of FUra.

In this paper, a mathematical model was developed to describe qualitatively and quantitatively the kinetics of the enzymic transformations and transmembrane exchanges involved in the catabolism of FUra. The model was fitted to the experimental data to test its validity and to estimate the parameters characterizing FUra's catabolism.

MATERIALS AND METHODS

Experimental

The experimental procedures have been described in detail [7]. Freshly-isolated rat hepatocytes in suspension were exposed over 2 hours to 30 μM of [^{3}H] FUra. The time course of the unchanged FUra and its catabolites was obtained through analysis of the extracellular and the intracellular ^{3}H using high performance liquid chromatography. The published data represented the mean values of 3 experiments, whereas in the present study data from each experiment were treated separately. Twelve time points were analyzed in experiment 1 and 2, and 10 in experiment 3.

Modeling

General assumptions. The model is based on what is known of FUra catabolism in liver cells [7]. The degradative pathway of FUra undergoes the following sequence: (i) transport of FUra from the extracellular to the intracellular compartment followed by a rapid reduction to FUH_2; (ii) conversion of FUH_2 to the transient intermediate FUPA; (iii) rapid conversion of FUPA to FBAL, the final catabolite observed under these conditions. The detection of the various catabolites in the extracellular medium indicates they exchange between

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† Abbreviations: FUra, 5-fluorouracil; FUH_2, dihydrofluorouracil; FUPA, α-fluoro-β-ureidopropionic acid; FBAL, α-fluoro-β-alanine.

the inner and the outer fluids. Furthermore, since no transformation was detected in the extracellular medium, the decrease by 15 min of the concentration of extracellular catabolites suggests reversible exchanges.

In the present model, some simplifications have been made: (i) unmetabolized FUra was not detected within the hepatocytes at any time after they were exposed to 30 μM FUra so that the transmembrane transport for unmetabolized FUra and the conversion of FUra to FUH_2 could not be differentiated. (ii) In these experiments the levels of FUPA in the intracellular and in the extracellular compartments were very low and FUPA was not detected within cells after 25 min. As a further simplification FUPA was not included in the model.

Mathematical formulation. Five state variables were used to describe the extracellular and the intracellular kinetics of FUra and its two metabolites FUH_2 and FBAL: the extracellular concentrations of FUra, FUH_2 and FBAL and the intracellular concentrations of FUH_2 and FBAL. The mathematical model (Fig. 1) takes into account catabolic and transport processes, and was written as a set of mass–balance differential equations expressing the various rates of exchange and transformation related to the state variables. The extracellular and the intracellular compounds were assumed to be in homogeneous solution, evenly dispersed either in the extracellular medium or throughout the total available water space of the cell without binding to any cellular macromolecules. The transport of FUra and its reduction to FUH_2 were described by a single irreversible and saturable Michaelis–Menten process. In contrast, first order kinetics for the conversion of FUH_2 to FBAL was assumed. All fluxes of FUra catabolites between the extracellular and the intracellular compartment were treated as reversible first order processes. The equations of the model are:

$$dC_1/dt = -V_m \times C_1/(K_m + C_1)$$
$$dC_2/dt = (V_{in}/V_{ex} \times K \times C_4) - (K_{-1} \times C_2)$$
$$dC_3/dt = (V_{in}/V_{ex} \times K' \times C_5) - (K'_{-1} \times C_3)$$

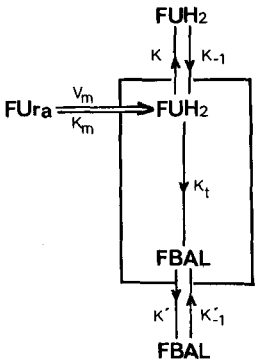

Fig. 1. Proposed model of the catabolism of FUra in rat hepatocytes. The FUra transport and the intracellular conversion of FUra to FUH_2 are represented by Michaelis–Menten kinetics. The intracellular conversion of FUH_2 to FBAL and the exchanges of these two metabolites are represented by first-order kinetics.

$$dC_4/dt = (V_{ex}/V_{in} \times V_m \times C_1/(K_m + C_1) - ((K_t + K) \times C_4) + (V_{ex}/V_{in} \times K_{-1} \times C_2)$$
$$dC_5/dt = (Kt \times C_4) + (V_{ex}/V_{in} \times K'_{-1} \times C_3) - (K' \times C_5)$$

where:

The variables ($C_i(t)$; $i = 1, \ldots, 5$) are the concentrations (μM) of extracellular FUra ($i = 1$), FUH_2 ($i = 2$) and FBAL ($i = 3$) and intracellular FUH_2 ($i = 4$) and FBAL ($i = 5$).

The parameters K, K_{-1}, K', K'_{-1} are the rate constants (min^{-1}) for the transmembrane exchanges of FUH_2 and FBAL, respectively.

V_m (μM/min) is the maximum rate and K_m (μM) the Michaelis–Menten constant for both the transport of FUra and its intracellular conversion to FUH_2;

K_t is the rate constant (min^{-1}) of the transformation of FUH_2 to FBAL.

V_{ex} and V_{in} (ml) are the volumes of the extracellular and the intracellular fluids, respectively. It should be noted that the time–course of the state variables depends only on the volume ratio.

At the beginning of the incubation period, the initial values of the various concentrations were: $C_1(O) = Co$, $C_i(O) = 0$, $i = 2,3,4,5$, where Co is the initial FUra concentration (μM).

In order to calculate the time–course of the variables for a given set of parameter values, the set of differential equations was integrated numerically with the Kutta–Merson variable step method [9].

Statistical methods

The unknown parameters were estimated by maximum likelihood. The model was fitted to the full set of concentration data for each experiment. The measurement error of a data point $C_i(t)$ was assumed to arise from a zero mean gaussian distribution with a standard deviation $s_i(t)$, depending on the variable (i) and the time (t). The errors of separate variable and successive data points were supposed to be uncorrelated. Accordingly, maximizing the likelihood of the data related to the parameters is equivalent to minimizing the following weighed least-squares function:

$$CRI(P) = \sum_{i=1}^{5} \sum_{j=1}^{N} \frac{[C_i(T_j)\ M_i(P,T_j)]^2}{[s_i(T_j)]^2}$$

where:

P is the vector of unknown parameters,

N is the number of measurement times (T_j),

$C_i(T_j)$, $M_i(P, T_j)$, $s_i(T_j)$ represent for variable i at time T_j, the values of the experimental concentration, the concentration predicted by the model and the standard deviation of the experimental error, respectively.

The following formula for the standard deviation of the experimental error was used to provide the weights in the least-squares function:

$$s_i(t) = 0.04 \times \left[C_i(t) + \max_J [C_i(T_j)] \right]$$

where

$\max_J C_i(T_j)$ is for variable i the maximal value of the observed concentrations.

This equation reflects the conditions where the absolute error on an experimental concentration increases with the measured amounts. It can be noted that this noise model provided a satisfactory weighting of the data. Minimization of the objective function related to the parameters was performed with Marquardt's second order algorithm [10].

The variability of the parameter estimates caused by experimental error, was obtained from the asymptotic variance–covariance matrix [11, 12]. The square-root of a diagonal element of the covariance matric was used as the S.D. of the corresponding parameter estimate [13].

The goodness-of-fit of the model to the experimental data was ascertained using the non parametric number-of-runs test for each variable of a given experiment [14]. This goodness-of-fit test relies on the signs of the residuals, the difference between the observed and the predicted concentrations. Each run is composed of one sequence of residuals with identical signs. The critical minimum number of runs, for a 5% risk of rejecting the true model, is 4 and 3 for sequences of 12 and 10 residuals, respectively.

Numerical methods

Routines for numerical integration and parameter estimation were written in FORTRAN 77 and executed on a D.E.C. VAX 11/780 computer.

RESULTS AND DISCUSSION

Relevant quantitative and qualitative results of the various transformation and exchange processes were obtained with this mathematical model. Figure 2 shows the concentrations of extracellular and intracellular FUra, FUH_2 and FBAL in each experiment with the corresponding curves generated by the two-compartment model. The number of runs

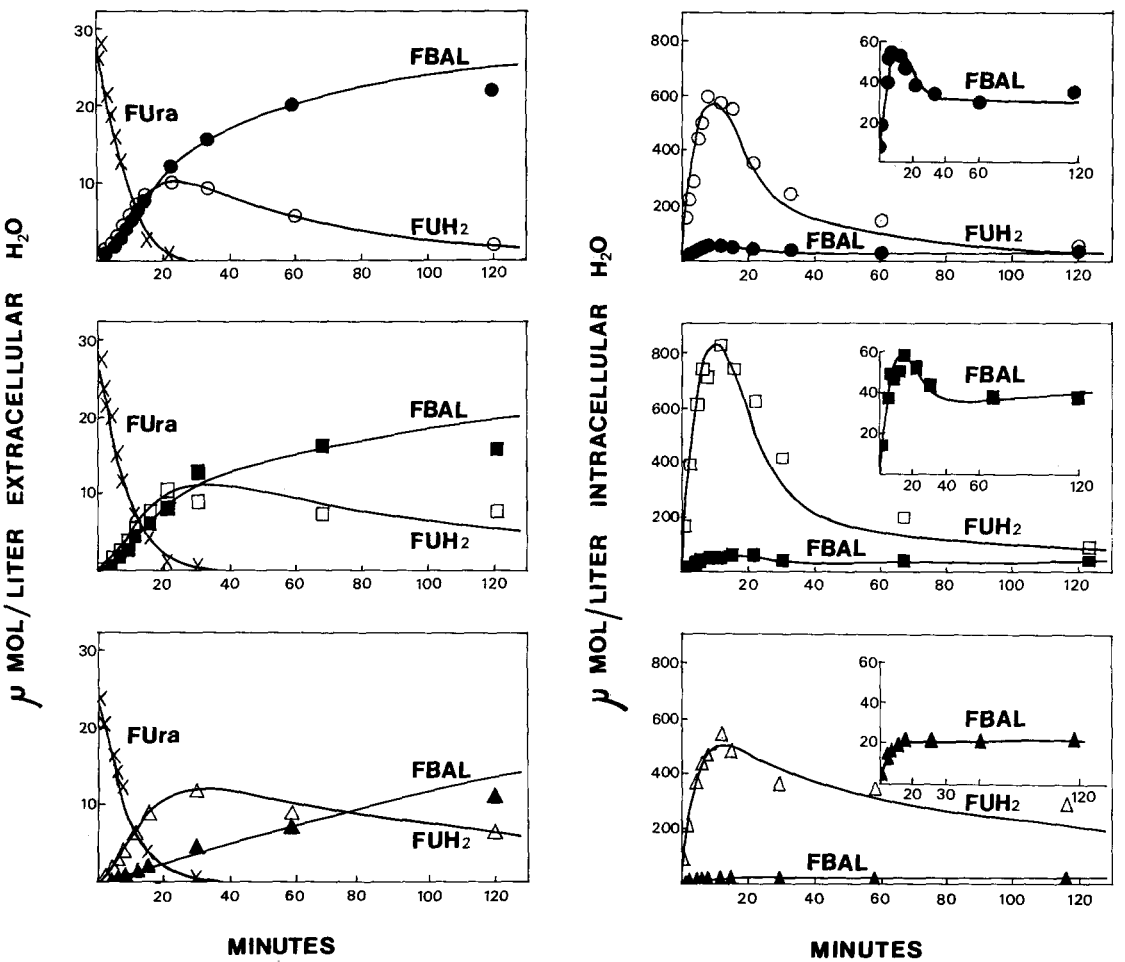

Fig. 2. Time course of extracellular and intracellular concentrations of FUra and its catabolites after exposure of hepatocytes to 30 μM [^{3}H] FUra in three experiments. Experimental concentrations are represented by symbols (○, ●: exp. 1; □, ■: exp. 2; △, ▲: exp. 3). Solid lines are the concentrations predicted by the model using the optimal parameter values for each experiment (Table 1). The inset represents the expanded time course for the appearance of the intracellular FBAL.

Table 1. Estimated parameter values of the catabolism of FUra*

Experiment	1	2	3
Co (μM)	26.7 ± 0.6	25.8 ± 0.6	22.4 ± 0.6
$100 \times V_{in}/V_{ex}$	1.23 ± 0.13	1.01 ± 0.11	1.44 ± 0.15
V_m (μM/min)	3.1 ± 0.5	4.1 ± 1.0	5.0 ± 2.0
K_m (μM)	12.0 ± 4.0	25.0 ± 10.0	37.5 ± 20.0
Kt (min^{-1})	0.102 ± 0.01	0.064 ± 0.006	0.023 ± 0.002
K (min^{-1})	0.13 ± 0.02	0.09 ± 0.01	0.15 ± 0.02
K_{-1} (min^{-1})	0.047 ± 0.006	0.022 ± 0.003	0.073 ± 0.01
K' (min^{-1})	1.16 ± 0.1	0.97 ± 0.1	0.56 ± 0.08
K'_{-1} (min^{-1})	0.016 ± 0.002	0.018 ± 0.002	0.009 ± 0.002

* The estimates ±S.D. were obtained for the 3 separate experiments by fitting the model to the concentrations of FUra and its catabolites.

for the extracellular and intracellular concentrations of FUH_2 and FBAL suggest that the model is consistent. In contrast, the predicted unmetabolized FUra levels underestimated the measured levels at the beginning of the extracellular time course and overestimated them in the late part of the kinetic, with a value of only 2 runs. The optimal parameter values with standard deviations are given in Table 1. The exchange characteristics were derived from the estimates of the associated parameters. For a given compound, the intracellular to extracellular equilibrium "partition coefficient" was the ratio: $(K_{-1}/K)/(V_{in}/V_{ex})$ (Table 2).

Our mathematical model adequately represents the time-course of FUra and its catabolites under the experimental conditions described previously [7]. The agreement between the curves predicted by the model and the experimental data was satisfactory. In addition, the small size of standard deviations indicates the meaningfulness of the parameter estimates. The difference in the parameter values between experiments reflects the variations observed in the three experiments. However, a minimal difference between the predicted and the measured extracellular FUra levels can be detected with a runs test. As this bias does not influence the fitting of the two metabolites FUH_2 and FBAL, it may be considered only as a minor phenomenon; nevertheless, these data suggest that another process which was omitted in the initial simplifications, because of the lack of available information, may slightly affect the unmetabolized FUra kinetics. It is intended that this model will be updated when further studies on the catabolism of FUra will be reported.

Comparison of parameter values

Transformation processes. The rate-limiting step in the degradative pathway of FUra appears to be the conversion of FUH_2 to FBAL via FUPA (K_t). The apparent first order rate constant V_m/K_m, representing the transport of FUra and its conversion to FUH_2, was much higher than the rate constants for the degradation of FUH_2 and its exit from cells. This may explain why the maximal intracellular FUH_2 level is very high.

Exchange processes. The rate constant for diffusional release of FBAL was much more rapid than for FUH_2 ($K' \gg K$). In these experimental conditions, the cellular uptake of FUra was much more rapid than that of the other compounds ($V_m/K_m \geqslant K_{-1}$ and K'_{-1}). In addition, as the first enzymic step is saturable [6], it may become rate-limiting at higher extracellular FUra concentrations.

FBAL diffusion and FUH_2 active transport

The closeness of the FBAL "partition coefficient" to 1 strongly suggests that exchange of FBAL occurs through a diffusion process. The FUH_2 partition coefficient was much higher than 1. As the measured concentration was representative of unbound catabolite [7], this partition coefficient suggests that the net transmembrane flow can be opposite to the concentration gradient of the free compound and that transport for FUH_2 into the cell is likely active.

FUPA: a transient intermediate

The kinetics of the intracellular catabolites of FUra, at this 30 μM extracellular FUra, did not allow the transformation of FUH_2 to FUPA and FUPA to FBAL to be resolved. The assumption that the conversion and exchange of FUPA were not rate-limiting, under these conditions, was ascertained by fitting the model to the experimental data. In addition, a model including FUPA was also

Table 2. "Partition coefficient" of the FUra catabolites*

Experiment	1	2	3
FUH_2	29.4	24.2	33.7
FBAL	1.12	1.84	1.17

* The values have been calculated using the equation $(K_{-1}/K)/(V_{in}/V_{ex})$. This ratio indicates the nature of the intracellular–extracellular exchange (a ratio of 1 can be described as a diffusional process).

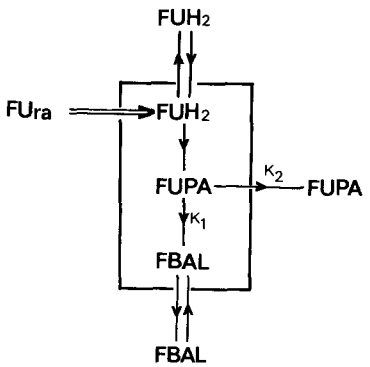

Fig. 3. Model used for the simulation of the catabolism of FUra including FUPA. The model of Fig. 1 was expanded in order to take into account the FUPA as an intermediate catabolite in the conversion of FUH_2 to FBAL. Two first-order processes were added to describe the conversion of FUPA to FBAL and the exit of FUPA of the cell.

investigated using computer simulation (Fig. 3). First order kinetics were assumed for the transformation of FUPA to FBAL (K_1) and for its exit from the cell (K_2). Very rapid kinetics for the first process ($K_1 = 20\,min^{-1}$) and rapid kinetics for the second ($K_2 = 1.5\,min^{-1}$) were included in the model with other parameters at their optimal values (Table 1). The prediction of the model for the extracellular and intracellular concentrations of FUra, FUH_2 and FBAL were not significantly changed. Extracellular FUPA values calculated using the model were in agreement with the experimental data. In contrast, the intracellular FUPA levels, predicted with this model, underestimated the measured levels over the time of exposure of the hepatocyte suspension to the drug. This may result from the difficulties of measuring precisely the intracellular levels of FUPA, which were close to the sensitivity limit on the HPLC technique. So more sophisticated modelling based on this fragmentary data set was not carried out.

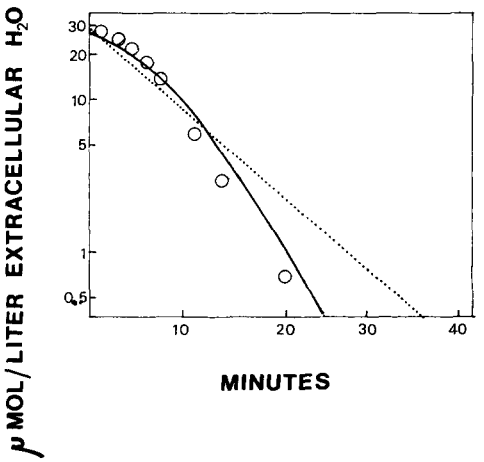

Fig. 4. Representative extracellular FUra time-course in a semi-logarithmic scale. The curves were calculated using parameters estimated by global fitting of the data with Michaelis–Menten kinetics (———) and with first-order kinetics (– – – – –).

Michaelis–Menten kinetics

No improvement could be observed when Michaelis–Menten kinetics instead of first order kinetics were assumed in the conversion of FUH_2 to FBAL, so that no saturation of this step was detected at 30 μM extracellular FUra. The nonlinearity of the overall process of FUra transport and conversion to FUH_2 was apparent on modelling the experimental data. Figure 4 shows the improvement in the fit to the extracellular FUra time-course when Michaelis–Menten instead of first-order kinetics were used. The unmetabolized drug showed a convex log concentration–time curve consistent with a nonlinear process.

The mathematical model allowed the maximum velocity (V_m) and the Michaelis–Menten constant (K_m) to be estimated (Table 1). The V_m were in a narrow range and the K_m agreed with the values obtained by Shiotani and Wever using purified dihydrouracil dehydrogenase [15]. However, Metzler and Tong [16] demonstrated, concerning the mathematical properties of the Michaelis–Menten kinetics, that the more measurement error is important, the more it appears difficult to estimate the V_m and K_m, which in consequence can be upwards biased and less accurate. This aspect was slightly apparent when the data of the three experiments were compared (Table 1).

In summary, a nonlinear model is proposed for the transport, transformation and exchange of FUra and its catabolites in liver cells. The quality of the global curve-fitting to the extracellular and intracellular concentration of FUra and catabolites suggests that the structure of the model is consistent. Relevant quantitative results of the kinetics of the various transformation and of the exchange processes were obtained, and comparisons between the respective rates of the different steps were assessed. This mathematical model may be helpful, as a predictive tool, in exploring whether and to what extent, changes in an elementary conversion or exchange process could modulate the metabolism of FUra.

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