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Pharmacokinetic Modelling of 5-FU Production from Capecitabine—A Population Study in 40 Adult Patients with Metastatic Cancer

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Aims: *To model the biotransformation steps of 5-FU production from capecitabine and identify patient characteristics that may influence the drug disposition.* Methods: *Blood samples and demographic data were collected from two phase I studies in which adult patients received oral capecitabine for various malignancies. Capecitabine, 5′-deoxy-5-fluorocytidine (5′-DFCR), 5′-deoxy-5-fluorouridine (5′-DFUR) and 5-fluorouracile (5-FU) concentration-time data were analysed via a population approach using NONMEM.* Results: *Forty patients and 75 pharmacokinetic time-courses were available for analysis. Capecitabine pharmacokinetics was ascribed to a one compartment model from which 5′-DFCR, 5′-DFUR and 5-FU were sequentially produced. Capecitabine oral absorption was characterized by a rapid first order input ($K_a = 2.1 \pm 0.3\,hr^{-1}$) with a lag time ($0.28 \pm 0.11\,hr$), but related inter-occasion (IOV) and inter-subject (ISV) variabilities for these parameters, 167% and 110%, indicated that this oral absorption was highly variable. The capecitabine CL ($CL_{10} = 218 \pm 18$ L/hr, ISV = 18%) and 5′-DFUR elimination rate constant ($K_{34} = 5.3 \pm 2.0\,hr^{-1}$, ISV = 25%) were influenced by total bilirubin (BILT). The elimination rate constant of plasma 5-FU (K_{40}) was $66 \pm 24\,hr^{-1}$ (ISV = 34%). The final pharmacokinetic model was validated using 2000 bootstrap runs and provided non-parametric statistics of the parameters (median, 2.5th and 97.5th percentiles).* Conclusions: *This study supported the possibility of modelling a complex sequential metabolic pathway which produces pharmacologicaly active compounds from a prodrug. Only BILT significantly influenced the pharmacokinetics but this effect was not considered as relevant for dosing adjustment.*

KEY WORDS: capecitabine; 5-FU; population pharmacokinetics; drug metabolism; anticancer drugs.

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INTRODUCTION

Capecitabine is an oral prodrug that is converted to the cytotoxic agent 5-fluorouracil (5-FU). After oral administration, capecitabine is converted to 5′-DFCR (5′-deoxy-5-fluorocytidine) mainly in the liver, then 5′-DFCR is converted to 5′-DFUR (5′-deoxy-5-fluorouridine) both in liver and tumor tissues, then 5-FU is finally produced from 5′-DFUR by thymidine phosphorylase, preferentially in tumor tissues (Fig. 1). Mean pharmacokinetic parameters of capecitabine and its metabolites have been mainly determined from non-compartmental methods (1). Also, the integrated pharmacokinetics of the sequence 5′-DFUR > 5-FU > FBAL has been analyzed *via* population approach methods (2,3). However, the pharmacokinetic modelling of the full capecitabine > 5′-DFCR > 5′-DFUR > 5-FU sequence has not been studied yet.

The objectives of this study were then to develop a full integrated population pharmacokinetic model for the 5-FU production from capecitabine, including the research of covariate effects on the pharmacokinetic

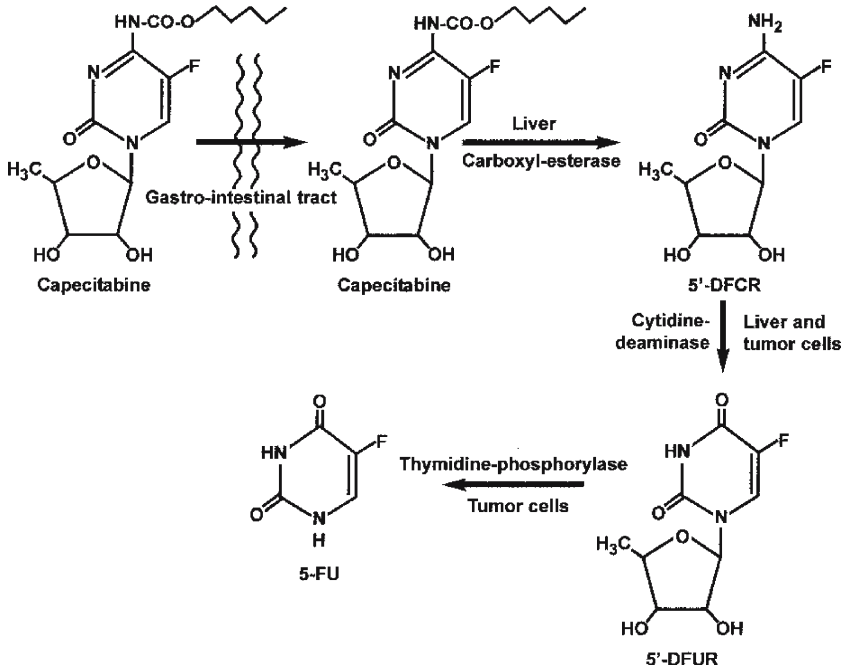

Fig. 1. Capecitabine metabolism.

parameters of interest. Because there were only 40 patients in this study, the stability and predictive performance of the population pharmacokinetic model were assessed using a bootstrap procedure.

METHODS

Patients

Patients were receiving capecitabine according to two phase I studies, for which capecitabine pharmacokinetic evaluation took place in the Pharmacology Department of the "Centre René Huguenin, Saint-Cloud, France". Capecitabine was combined to either irinotecan 200–250 mg/m^2 (90 min-infusion on day1) or to irofulven 0.4 mg/kg (30 min-infusion on day1), depending on the study. Patients were diagnosed with metastatic cancer and were receiving second or third line chemotherapy. The protocols were approved by the local Ethics Committee in France (CCPPRB) and informed consent was approved from each patient. Morphological and physiological characteristics of the patients are summarized in Table I.

Capecitabine Administration and Sampling Design

Capecitabine was administered orally every 12 hr within 30 min of a breakfast or diner. Patients received 1400, 1700, 2000 or 2300 mg/m^2/day. For most patients, two pharmacokinetic evaluations took place on days 1 and 15. Plasma concentration-time data were obtained prior to dosing then at times centered around 0.25, 0.5, 1, 1.5, 2, 2.5 3, 3.5, 4, 6, 8 and 12 hr after drug intake. Blood samples were immediately centrifuged to yield plasma. Plasma samples were then assayed by HPLC to quantify capecitabine, 5′-DFCR, 5′-DFUR and 5-FU as previously described

Table I. Characteristics of the 40 Patients (25 males/15 females) Studied

Item	Mean	Median	Range
Age, year	54	54.5	30–73
Body weight, kg	65	68	41.5–95
Height, cm	168	169	150–178
Body surface area, m^2	1.70	1.80	1.40–2.10
Serum albumin, g/l	37	37	30–46
Serum creatinine, μmol/l	85	85	58–113
Total bilirubin, μmol/l	10.1	8.8	3–22
Number of samples per patient	9.3	10	4–14
Dose/m^2, mg/m^2	1900	2000	1400–2300

(4). The limits of quantification for capecitabine, 5′-DFCR, 5′-DFUR and 5-FU were respectively 0.03, 0.05, 0.05 and 0.05 μM.

Population Pharmacokinetic Modelling of Capecitabine and Metabolites

Data were analysed using the nonlinear mixed effect modelling software program NONMEM (version V, level 1.1, double precision) with the DIGITAL FORTRAN compiler (5). The first-order method is based on first-order Taylor series linearizations of the prediction, with respect to the dependence on parameters. The derivative approximation of the function can be based on population (FO method) or individual (FOCE method) parameter estimate. The theoretically best choice is the FOCE method (with INTERACTION if the residual error is heteroscedastic). However this method is complex and computer-intensive, and can be problematic in complex situations. So the FO method was finally used.

The pharmacokinetics of capecitabine and metabolites were studied simultaneously. Capecitabine and metabolite concentrations were converted to molar concentrations for the analysis. Capecitabine data was analysed first, including covariate modelling. The pharmacokinetic parameters of the parent were then used to produce the input function into the metabolite compartments (see Fig. 2). Then parameters were no more restricted and estimated by simultaneous fitting. Since capecitabine and metabolite concentrations were observed at the same times, a correlation is likely to exist between these observations. So, a L2 item was added to the database to allow, if necessary, the estimation of covariance terms between residual variabilities of the 4 compounds (5, see NONMEM user's guide, Guide V section 12.4.2). The metabolite distribution volumes are not identifiable and fixed to 1, so only output constant rates could be estimated for the metabolites (K_{ij}). All clearance and volume terms are apparent parameters, i.e., V/F, CL/F etc..., where F is the bioavailability fraction.

Several error models were investigated (i.e., proportional, exponential and additive error models) to describe inter-subject (ISV) and residual variabilities. Inter-occasion variability (IOV) was also considered.

The influence of continuous covariates was modelled according to the following equation, using CL for example,

$$\mathrm{CL} = \mathrm{TV(CL)} * \{\mathrm{BW}/\mathrm{median(BW)}\}^{\theta \mathrm{BW}}$$

where TV(CL) is the typical value of clearance for a patient with the median covariate value and θ_{BW} is the estimated influential factor for BW (it can be a positive or a negative, effect, value).

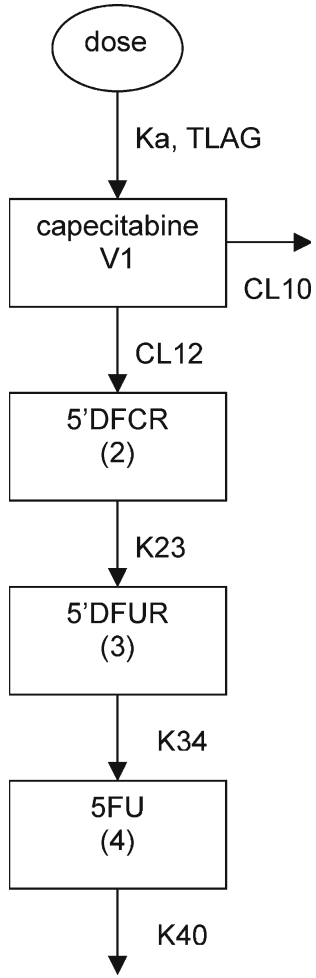

Fig. 2. Compartmental model describing the 5-FU plasma production from oral capecitabine administration.

Categorical covariates including gender and combined anticancer drugs were tested as, considering a CL induction of one combined drug for example,

$$\mathrm{CL} = \mathrm{TV(CL)} * (1 + \theta_{\mathrm{DRUG}} * (\mathrm{DRUG} = 0 \text{ or } 1)),$$

where 0 or 1 denote absence or presence of the drug.

Or in the case of an inhibitory drug effect,

$$CL = TV(CL)/(1 + \theta_{DRUG} * (DRUG = 0 \text{ or } 1))$$

Covariates were selected in the final population model if (i) their effect was biologically plausible, (ii) they produced a minimum reduction of 11 units of the objective function value (OFV), (iii) they produced a reduction in the variability of the pharmacokinetic parameter, assessed by the associated ISV and (iv) the relative standard error (SE) of covariate parameter estimate was lower than 50%. Plausible covariates were size descriptors (BW, BSA, heigth) for CL or K terms and distribution volume, age for CL or K terms. BILT and serum albumin were considered as rough markers of hepatic function. Gender and combined drugs were also likely to influence CL or K terms.

For evaluation of the goodness-of-fit, the following graphs were compared: observed concentrations vs. predictions (PRED-DV), weighted residuals (WRES) vs. time and weighted residuals vs. PRED (WRES-PRED) as well as the corresponding graphs issued from the POSTHOC estimation step (IPRED-DV...). Diagnostic graphics and distribution statistics were obtained using the R program (6).

From the final population model, possible relationships between AUC of capecitabine and metabolites were investigated according to the individual pharmacokinetic parameters provided by the POSTHOC option. The relationships are derived in the Appendix.

The accuracy and robustness of candidate and final population models were assessed using a bootstrap method. Briefly, this includes the following steps,

(i) from the original data set of n individuals, B bootstrap sets of n individuals are drawn with replacement (resampling),

(ii) for each of the B bootstrap sets, the population pharmacokinetic parameters are estimated,

(iii) with the B estimates of each population pharmacokinetic parameter, descriptive statistics (mean, median, confidence interval between the 2.5th and 97.5th percentiles etc...) of the population parameters can be estimated,

(iv) to validate the model, the parameters estimated from the bootstrap must be close to estimates obtained from the original population set.

The entire procedure was performed in an automated fashion using Wings for NONMEM (7).

RESULTS

Patients

For the 40 patients, 75 pharmacokinetic courses were available. A total of 1426 time-plasma concentrations were observed including 373 capecitabine, 354 5′-DFCR, 363 5′-DFUR and 336 5-FU concentrations. Latest sampling times with quantifiable total capecitabine or metabolite concentrations were 8–10 hr after dosing (after 10 hr, only 10 samples were quantifiable per 1426 samples). A major part of the observed concentrations took place between 0.25 and 4 hr. Patients characteristics are summarized in Table I.

Capecitabine-Metabolites Pharmacokinetic Model Building

A one-compartment model adequately described the isolated capecitabine data (SUBROUTINES ADVAN2 TRANS2). At this step, capecitabine clearance, CL, was 257 ± 21 l/hr. The absorption was rapid, characterized by a first-order input, $K_a 3.1 \pm 0.7\,\mathrm{hr}^{-1}$ with a mean TLAG 0.33 ± 0.06 hr.

Connecting the sequence of metabolites to the capecitabine compartment resulted in a system including at least 8 pharmacokinetic parameters plus 8 ISV parameters plus 4 residual variability parameters and 1 or more IOV parameters. For such a complex model, the use of the ADVANs 5 or 6–8 subroutines were too much time-consuming. So the explicit solutions for each compound concentration were finally coded in the control streams in the $PRED section (see Appendix A). These mathematical equations were verified by simple ordinary least square fittings of few individual datasets. Figure 3 illustrates the curve-fittings of capecitabine and its metabolites obtained in two subjects.

In a first step, the capecitabine pharmacokinetic parameters were fixed to approximate the metabolite parameters, then all parameters were freely estimated with no restrictions. Attempts to estimate elimination of intermediate metabolites from the body (K_{20} or K_{30}) always provided values near zero. The TLAG parameter was tested again and was definitively included in the model. At this step, the use of FO or FOCE methods was reconsidered. The use of FOCE method always led to an abnormal termination of the program, whereas the runs performed with the FO method were successful (including achievement of the covariance step). The FO method using logarithmic transformation of the data was also used, since this is an attractive alternative with less parameter bias. However this resulted in more biased results in terms of both DV-PRED and DV-IPRED data. The FO method was then used for every run.

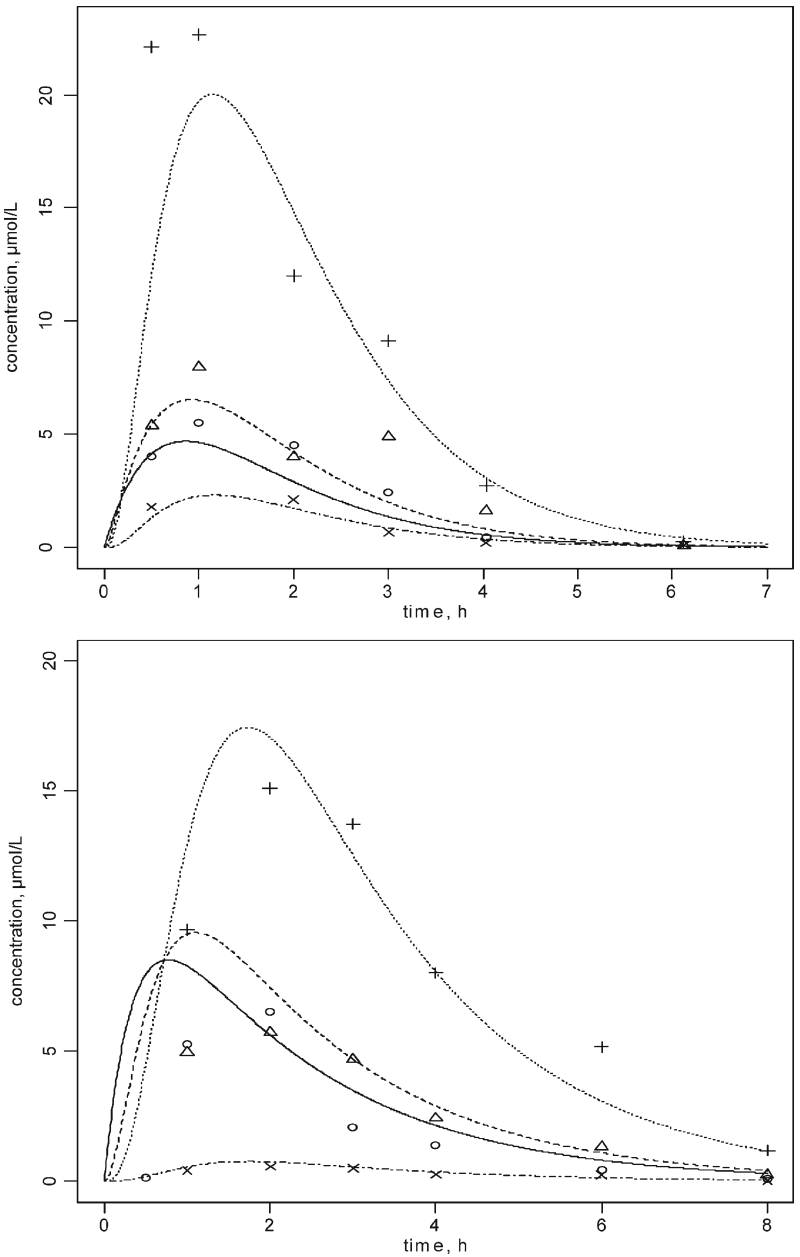

Fig. 3. Ordinary least squares curve-fittings for two subject using the Fig.1 model model and the equations derived in the Appendix. Key: —- , o capecitabine; --- Δ 5′-DFCR; …, + 5′-DFUR; -.-.-, × 5-FU. Concentrations are μmol/l.

All of the ISVs were estimated, then deletion of very small ISVs with large SEs (>100% coefficient of variation) was considered to keep only significant terms. The ISV on CL_{12} was definively fixed to zero with no detriment to the fit, since estimates were always in the range 10^{-3}–10^{-2} with much larger SE (10^{-1}). Covariance terms between ISVs were also considered, especially between V_1 and CL_{10} and between the successive rate constants K_{23}, K_{34} and K_{40}, but did not result in significant decrease in OFV. Addition of covariance terms between the residual error terms led to $a > 150$ units decrease in OFV. IOV could be estimated for K_a or TLAG or V_1. Different combinations of parameters, including IOV estimates on K_a, V_1 or TLAG indicated that IOV on K_a led always to the lowest OFV. However, this resulted in a very large SE (CV $\gg$ 100%) on the corresponding ISV estimate. Finally, ISV on K_a was deleted, but ISV on TLAG was kept for its deletion resulted in a 34 units increase in OFV. The basic candidate model included an IOV for K_a and covariance terms between residual errors for capecitabine and 5′-DFCR, and for 5′-DFUR and 5-FU. Also, ISVs on K_a and CL_{12} were not significant and fixed to zero. This basic model was subjected to a bootstrap analysis, which provided 312 successful runs with achieved covariance step per 400 programmed runs. The median parameters were similar to the NONMEM estimates of the original database and all 2.5th percentile estimates were above zero.

Covariate Modelling

POSTHOC parameters derived from the basic model indicated significant correlations between BSA and $CL_{10}(r = +0.353)$ and between BILT and $K_{34}(r = -0.335)$ or $K_{40}(r = +0.420)$, suggesting an increase in capecitabine and 5-FU elimination related to BSA and a decrease in metabolite production (mainly 5-FU) related to BILT. An intermediate model including BSA and BILT effects on CL_{10} and BILT effects on K_{34} and K_{40} terms was tested. Then, successively, the covariate effect parameter with the largest coefficient of variation was removed from the model. This was repeated until all covariate effect parameters had acceptable accuracies (coefficient of variation <50%) and a further deletion induced $a > 11$ units increase in OFV. Finally, BILT had a positive effect on CL_{10} and a negative effect on K_{34}. Increased BILT levels suggest liver dysfunction and some degree of altered metabolism. The positive effect of BILT on CL_{10} suggested a relative decrease in capecitabine transformation to 5-FU by an increased elimination *via* the non transformation pathway. The negative effect on K_{34} also indicated a decreased 5-FU formation. The following equations described the final covariate model

$$CL_{10}\ (l/hr) = 218*(BILT/8.8)^{+0.32}$$

$$K_{34}\ (hr^{-1}) = 5.70*(BILT/8.8)^{-0.36}$$

Table II summarizes the final population pharmacokinetic estimates including the bootstrap verification. Figure 4 depicts the observed and predicted capecitabine and metabolite times courses and Fig. 5 depicts goodness-of-fit plots.

Given the final population model, individual AUCs of plasma capecitabine and metabolites were estimated (Fig. 6). As shown, the relationship between capecitabine AUC and metabolites was significant but would not allow accurate prediction of 5-FU AUC from capecitabine AUC.

Table II. Population Pharmacokinetic Parameters of Capecitabine and Bootstrap Results

Parameter	Mean	SE	Median[a]	2.5th–97.7th percentiles[b]
K_a, hr^{-1}	2.07	0.27	2.29	0.91–2.66
TLAG, hr	0.28	0.11	0.16	0.01–0.36
V_1, l	338	31	290	206–391
TV.CL_{10}, l/hr	218	18	207	172–252
BILT effect on CL10	+0.32	0.08	+0.24	+0.10 to +0.44
CL_{12}, l/hr	12.9	5.10	10.4	7.25–20
K_{23}, hr^{-1}	10.7	3.10	7.9	5.8–14.3
K_{34}, hr^{-1}	5.30	2.0	4.30	3.0–7.60
BILT effect on K_34	−0.36	0.09	−0.42	−0.68 to −0.20
K_{40}, hr^{-1}	66	24	59	38–112
Res. variability[c], CAP μM	3.83	0.88	4.10	3.1–4.9
Res. variability, 5′-DFCR μM	3.72	1.04	3.7	2.7–4.8
Res. variability, 5′-DFUR μM	5.81	0.92	6.6	5.2–7.8
Res. variability, 5-FU μM	0.64	0.25	0.67	0.40–0.92
IOV K_a %	167	69	187	1–256
ISV TLAG %	110	95	143	31–1200
ISV.V_1 %	136	31	125	8–170
ISV.CL_{10} %	18	12	27	4–43
ISV K_{23} %	50	24	43	25–88
ISV K_{34} %	25	10	20	3–36.5
ISV K_{40} %	34	14	30	4–42

Key: SE, Standard error of estimate; TV, typical value; ISV, intersubject variability; BILT, total bilirubin.

[a] Statistics on 1922 bootstrap runs (2000 programmed, 1922 runs were successful).

[b] non parametric 95% confidence interval based on the 2.5th–97.5th percentiles.

[c] Covariance between residual variability terms, $\text{cov}(\varepsilon^2_{cap}, \varepsilon^2_{5'\text{-DFCR}}) = 3.1 \pm 1.8$ and $\text{cov}(\varepsilon^2_{5'\text{-DFUR}}, \varepsilon^2_{5\text{-FU}}) = 2.6 \pm 0.8$.

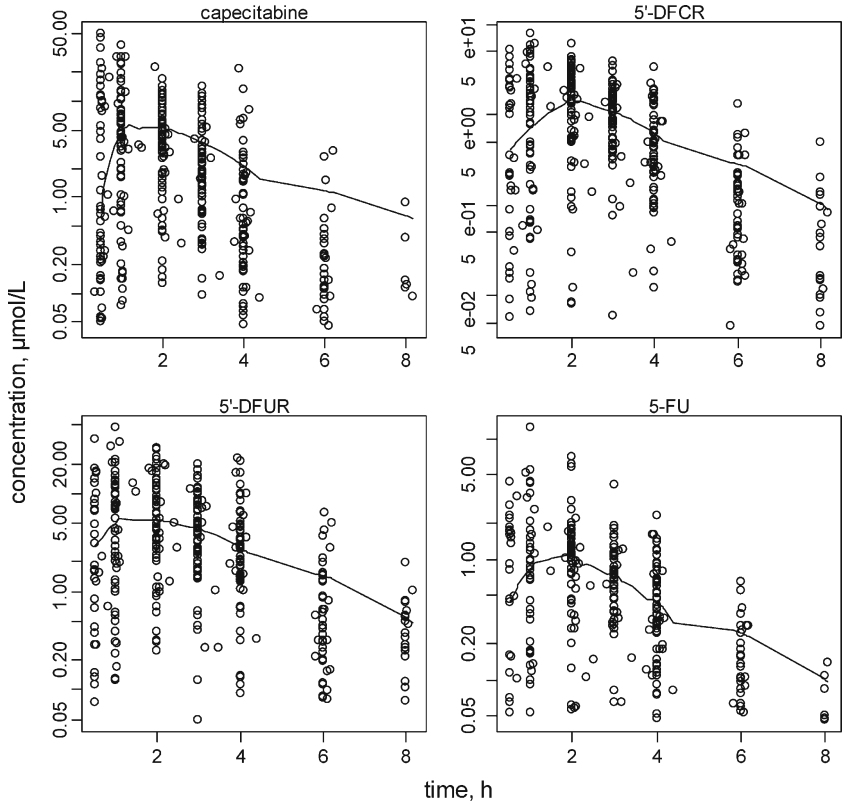

Fig. 4. Log-scale observed (o) and predicted (solid line) plasma concentrations in μmol/l vs. time profiles (solid line, mean prediction) normalised for a 4596 μm oral dose of capecitabine. Observed and predicted concentrations are normalized for this mean dosing.

DISCUSSION

The pharmacokinetics of plasma capecitabine and its metabolites were satisfactorily described by the multi-compartmental model depicted in Fig.2. The capecitabine CL estimate, 231 l/hr, was lower but in the order of previously reported estimates from non-compartmental analyses, 347 l/hr/1.8 m^2 (8), 311 to 378 l/hr/1.8 m^2 (1) and 315 l/hr/1.8 m^2 (9). The capecitabine $T_{1/2}$, 1 hr, was also in the range of previous estimates (1). The absorption process was rapid ($K_a 2\,hr^{-1}$) and variable with a TLAG.

The basic parameter estimates of K_{12}, K_{23}, K_{34} and K_{40} suggested flip-flop pharmacokinetics. However, previous reports on capecitabine pharmacokinetics support the fact that the elimination of metabolites is clearly rate-limited, i.e., apparent half-lives of the three metabolites were

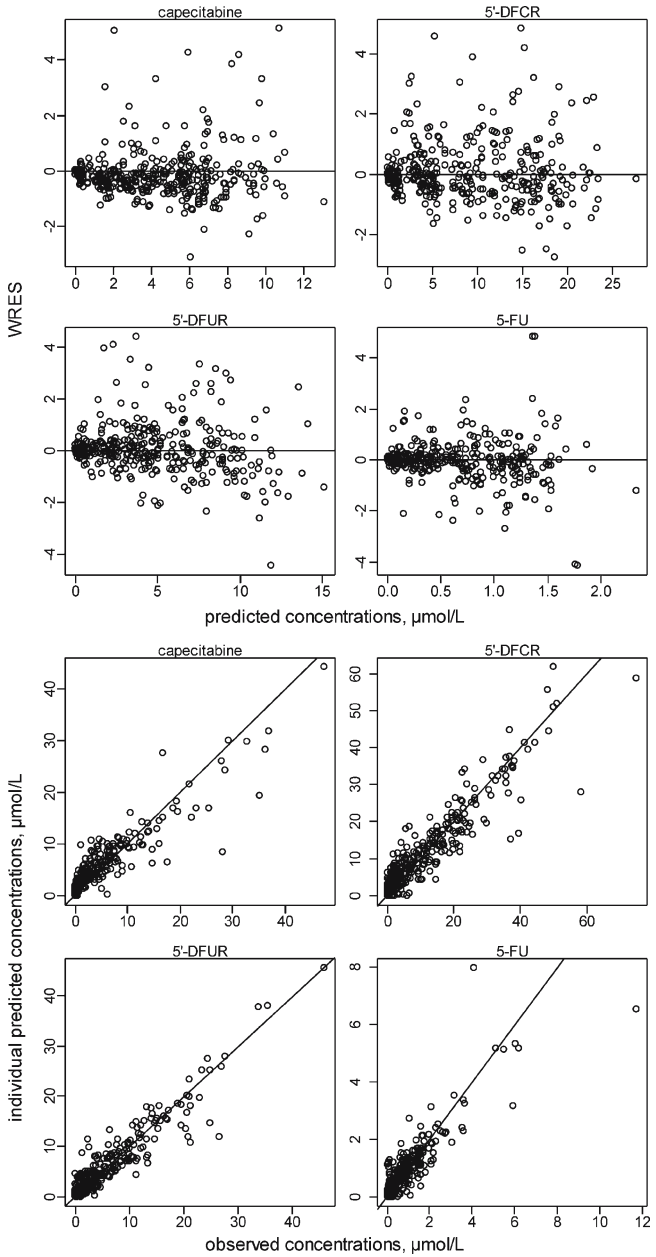

Fig. 5. Model goodness-of-fit plots : WRES vs. predicted concentrations (solid line = zero level) and individual predicted vs. observed plasma concentrations in μmol/l (solid line = line of identity).

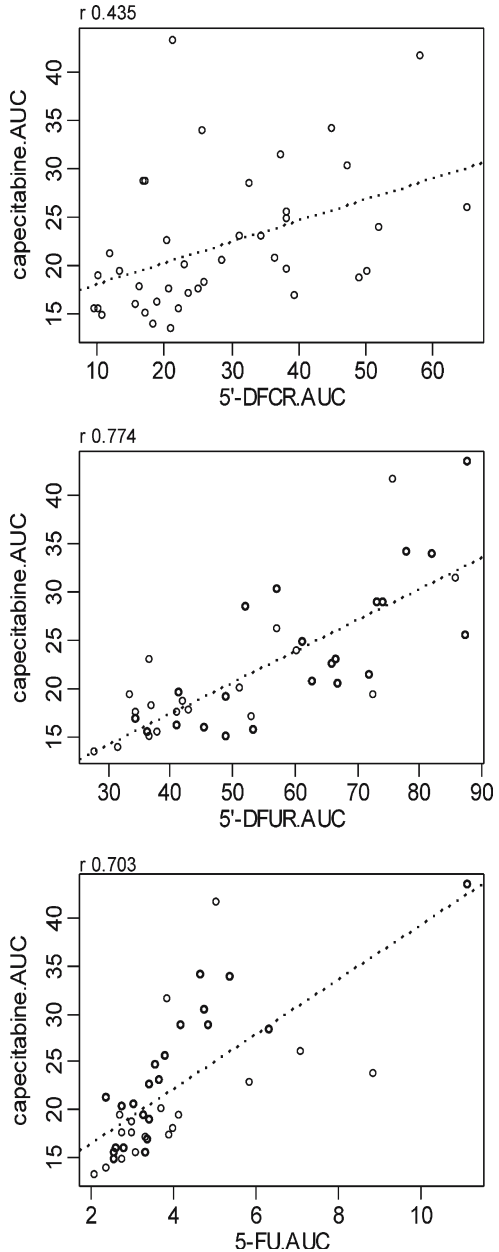

Fig. 6. Individual predictions of capecitabine and metabolite AUCs and correlations between the prodrug AUC and the metabolites AUC (expressed in μmol*hr/l). Median dotted line = regression line.

close to the capecitabine half-life (1). Also, previous population pharmacokinetic studies clearly showed that 5-FU elimination rate, K_{40}, was much greater than 5′-DFUR elimination rate, K_{34} (2, 3). To further verify this point, the model was simplified assuming the fast exponential process was not apparent in the data. The corresponding control stream was then rerun and population parameter estimates were similar to the original values.

The 5-FU elimination rate constant, 66 l/hr, was similar to those previously reported in two population studies, 65 l/hr ($CL/V = 1150/17.8$) and 67 l/hr ($CL/V = 1190/17.8$) with comparable ISV, 30% and 33% (2, 3). Other comparisons are less straightforward. When the modelling includes 5′-DFUR as directly produced from oral capecitabine, the K_a estimate is an hybrid parameter that could hardly be approximated by the product $K_a * K_{12} * K_{23} (=0.84\,hr^{-1})$ in our model. The reported values were 1.48 and $1.09\,hr^{-1}$ (2, 3). Also, our error model parameters are not fully comparable to those reported in the two previous population studies, since IOV or ISVs could be separately estimated in this study for K_a, CL_{10} and K_{23}.

The covariate submodeling identified only BILT as a significant covariate. The BILT effect on K_{34} (5′-DFUR elimination rate constant), −0.36, was greater but comparable to that previously reported, −0.20 (2). This BILT effect was evaluated given variations of +30% or −30% from the BILT median value. This resulted in capecitabine, 5′-DFCR and 5-FU variations from +12% to −8%. The 5′-DFUR AUC variation was between −2% and +2%. These effects were negligible on a clinical viewpoint. Unexpectedly, although a trend was observed between BSA and CL_{10} using the POSTHOC estimates, there was finally no significant influence of BSA in the final model. A BSA effect on 5′-DFUR CL was first reported with no therapeutic consequences (2), then in a second study, this effect was no more observed (3).

Problems related to the population modelling of such a complex metabolic pathway have been fully dicussed elsewhere (2). The greatest complexity of our system, modelling of 4 successive compounds, was resolved, in terms of efficiency and possibility to perform numerous runs, by the development of the analytical solution for the differential system connected to the model. The elimination of 5-FU was also assumed to be linear, as in previous studies (2,3), but this did not resulted in poor predictive performance for 5-FU compared to the other compounds as shown by the diagnostic plots (Fig. 5).

In conclusion, this study supports the possibility to estimate population parameters from a multiple-response model describing the pharmacokinetics of four sequentially related compounds. The significant BILT effect on pharmacokinetics was not related to significant variations in

capecitabine and metabolite exposures, including 5′-DFUR and 5-FU. Finally, although it could be reasonably expected, it was not possible to accurately relate 5′-DFUR and 5-FU exposures from capecitabine plasma data.

ACKNOWLEDGMENTS

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APPENDIX A

The differential system connected with the model depicted in Fig. 2 is

$$dX_1/dt = K_a D - (k_{10} + k_{12})X_1 \quad X_1 = 0, t = 0 \tag{A.1}$$
$$dX_2/dt = k_{12}X_1 - k_{23}X_2 \quad X_2 = 0, t = 0 \tag{A.2}$$
$$dX_3/dt = k_{23}X_2 - k_{34}X_3 \quad X_3 = 0, t = 0 \tag{A.3}$$
$$dX_4/dt = k_{34}X_3 - k_{40}X_4 \quad X_4 = 0, t = 0 \tag{A.4}$$

where K_a is the absorption rate and $CL_{10} = k_{10}V_1$, $CL_{12} = k_{12}V_1$

The Laplace transforms $Z_i(s)$ of X_i are

$$Z_1(s) = \frac{DK_a}{(s + K_a)(s + \lambda_1)} \tag{A.5}$$
$$Z_2(s) = \frac{DK_a k_{12}}{s(s + K_a)(s + \lambda_1)(s + \lambda_2)} \tag{A.6}$$
$$Z_3(s) = \frac{DK_a k_{12} k_{23}}{s(s + K_a)(s + \lambda_1)(s + \lambda_2)(s + \lambda_3)} \tag{A.7}$$
$$Z_4(s) = \frac{DK_a k_{12} k_{23} k_{34}}{s(s + K_a)(s + \lambda_1)(s + \lambda_2)(s + \lambda_3)(s + \lambda_4)} \tag{A.8}$$

where $\lambda_1 = k_{10} + k_{12}$, $\lambda_2 = k_{23}$, $\lambda_3 = k_{34}$ and $\lambda_4 = k_{40}$. The AUCs are then readily obtained,

$$AUC_1 = Z_1(0) = D/(\lambda_1 V_1)$$
$$AUC_2 = Z_2(0) = Dk_{12}/(\lambda_1 k_{23})$$
$$AUC_3 = Z_3(0) = Dk_{12}/(\lambda_1 k_{34})$$
$$AUC_4 = Z_4(0) = Dk_{12}/(\lambda_1 k_{40})$$

Using the Heaviside's formula, the solutions giving the profiles of the metabolites 2,3 and 4 following oral absorption are

$$C_2(t) = \frac{K_a k_{12}}{V_2}\left\{\frac{e^{-K_a t}}{(K_a-\lambda_1)(K_a-\lambda_2)} + \frac{e^{-\lambda_1 .t}}{(\lambda_1-K_a)(\lambda_1-\lambda_2)} + \frac{e^{-\lambda_2 .t}}{(\lambda_2-K_a)(\lambda_2-\lambda_1)}\right\}$$

$$C_3(t) = \frac{K_a k_{12} k_{23}}{V_3}\left\{\frac{e^{-K_a t}}{(K_a-\lambda_1)(K_a-\lambda_2)(K_a-\lambda_3)} + \frac{e^{-\lambda_1 .t}}{(\lambda_1-K_a)(\lambda_1-\lambda_2)(\lambda_1-\lambda_3)} + \frac{e^{-\lambda_2 .t}}{(\lambda_2-K_a)(\lambda_2-\lambda_1)(\lambda_2-\lambda_3)} + \frac{e^{-\lambda_3 .t}}{(\lambda_3-K_a)(\lambda_3-\lambda_1)(\lambda_3-\lambda_2)}\right\}$$

$$C_4(t) = \frac{K_a k_{12} k_{23} k_{34}}{V_4}\left\{\frac{e^{-K_a t}}{(K_a-\lambda_1)(K_a-\lambda_2)(K_a-\lambda_3)(K_a-\lambda_4)} + \frac{e^{-\lambda_1 .t}}{(\lambda_1-K_a)(\lambda_1-\lambda_2)(\lambda_1-\lambda_3)(\lambda_1-\lambda_4)} + \frac{e^{-\lambda_2 .t}}{(\lambda_2-K_a)(\lambda_2-\lambda_1)(\lambda_2-\lambda_3)(\lambda_2-\lambda_4)} + \frac{e^{-\lambda_3 .t}}{(\lambda_3-K_a)(\lambda_3-\lambda_1)(\lambda_3-\lambda_2)(\lambda_3-\lambda_4)} + \frac{e^{-\lambda_4 .t}}{(\lambda_4-K_a)(\lambda_4-\lambda_1)(\lambda_4-\lambda_2)(\lambda_4-\lambda_3)}\right\}$$

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