A PILOT STUDY TO IDENTIFY SOURCES OF VARIABILITY IN 5-FLUOROURACIL PHARMACOKINETICS AND TOXICITY

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To my parents, my family and my husband
for their encouragement, suggestion and help.

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GLOSSARY

Abbreviations

3′-UTR	3′-untranslated region
5-CU	5-chlorouracil
5-FdUMP	5-fluoro-2´-deoxyuridine-5´-monophosphate
5-FdUDP	5-fluoro-2´-deoxyuridine-5´-diphosphate
5-FdUrd	5-fluoro-2´-deoxyuridine
5-FdUTP	5-fluoro-2´-deoxyuridine-5´-triphosphate
5-FU	5-fluorouracil
5-FUDP	5´-fluouroruidine 5´-diphosphate
5-FUH_2	5,6-dihydrouracil-5-fluorouracil
5-FUMP	5-fluorouridine-5´-monophosphate
5-FUrd	5-fluorouridine
5-FUTP	5´-fluorouridine-5´-triphosphate
AIO	Association of Medical Oncology
ALT	alanine aminotransferase
AF	atrial fibrillation
AP	alkaline phosphatase
AST	aspartate aminotransferase
AUC	area under the plasma concentration-time curve
$AUC_{0-\infty}$	area under the plasma concentration-time curve extrapolated to infinity
BMI	body mass index
bp	base pair
BSA	body surface area
C	cytosine
°C	Degree Celsius
CDC	Center for Disease Control and Prevention
CDDP	cisplatin
CFBAL	N-carboxy-α-fluoro-β-alanine
CH_2-THF	5,10-methylenetetrahydrofolate
CID	collision-induced dissociation

CL	clearance
CL_{Cr}	creatinine clearance
CL_{tot}	total body clearance
CL_m	total clearance of 5-FUH_2
CL_{met}	metabolic clearance of 5-FU converted to 5-FUH_2
CL_{res}	residual clearance of 5-FU
C_{max}	peak plasma concentration
CMF	cyclophosphamide, methotrexate and 5-fluorouracil
CO_2	carbon dioxide
COV	value of the covariate of respective patient from the study population
$C\bar{O}V$	median value of the covariate in the study population
CP	cyclophosphamide
CR	complete response
CRF	case report form
Cr_{serum}	serum creatinine
C_{ss}	plasma concentration at steady state
CV	coefficient of variation
CVI	continuous intravenous infusion
dL	deciliter
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DMSO	dimethyl sulfoxide
DPD	dihydropyrimidine dehydrogenase
DPYD	gene encoding for DPD
ECG	electrocardiogram
EDTA	ethylenediaminetetra acetic acid
EPI	epirubicin
F_m	fraction of the initial dose of 5-FU converted to 5-FUH_2 divided by the volume of distribution of 5-FUH_2
FA	folinic acid
FAC	fluoroacetate
FBAL	α-fluoro-β-alanine

FDA	Food and Drug Administration
FHPA	2-fluoro-3-hydroxypropanoic acid
FUPA	α-fluoro-β-ureidopropionic acid
g	gram
G	gaunine
γGT	gamma-glutamyl transpeptidase
GI	gastrointestinal
GOF	goodness-of-fit
h	hour
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
ICD	incidence
INF	interferon
IRI	irinotecan
i.v.	intravenous
K_{30}	elimination rate constant of 5-FUH_2
K_{el}	overall eliminate rate constant of 5-FU
KH_2PO_4	potassium dihydrogenphosphate
KST	Klinik und Poliklinik für Strahlentherapie
LC-MS-MS	liquid chromatography-tandem mass spectrophotometry
LDH	lactate dehydrogenase
LLOQ	lower limited of quantification
MgCl	magnesium chloride
mRNA	messenger RNA
MTHFR	methylenetetrahydrofolate reductase
MTX	methotrexate
mV	millivolt
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NH_2	ammonia
NONMEM	non-linear mixed effects modeling
OPRT	orotate phosphoribosyl transferase
PALA	N-phosphoroacetyl-L-aspartic acid

PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
pH	negative decadic logarithm of H_30^+ concentration
PR	partial response
PRPP	5´-phosphoribosyl-1-pyrophosphate
PVI	protracted venous infusion
Q	inter-compartmental clearance
QC	quality control sample
RNA	ribonucleic acid
rpm	round per minute
RT	retention time
SD	standard deviation
SOPs	standard operating procedures
SRM	selected reaction monitoring
$t_{1/2}$	plasma elimination half-life
$t_{1/2\lambda z}$	terminal half-life
TDM	therapeutic drug monitoring
TS	Thymidylate synthase
TSER	5′-promoter enhancer region
TYMS	gene encoding for TS
U	uracil
UH_2	dihydrouracil
UV	ultraviolet light
V_c	central volume of distribution
V_d	volume of distribution
V_m	volume of distribution of 5-FUH_2
V_p	peripheral volume distribution

Important definitions of terms

Pre-study phase:

The preparations for the study was performed prior the start of clinical investigations. During this phase:

- information about the components of 5-FU was collected
- the protocol and the patient information and informed consent form were drawn up,
- the blank CRF was drawn up
- the opinion of the relevant ethics committee was requested
- relevant SOPs were put together, and specific SOPs were drawn up
- the relevant state authorities ("Regierungspräsidium") were notified of the study,
- the subjects were insured.

Start of the study, enrolment:

The study started with obtaining the informed consent by signing the informed consent form by the first patients. A patient was enrolled for the study when all results of the clinical screening examination necessary for eligibility were available to the clinical investigator and the patient was considered eligible according to inclusion and exclusion criteria.

End of the study:

The regular termination of the study for a subject was the signing of the case report form by the principal investigator after the final examination, which is approximately 28 days after the last blood sampling or – in the case ongoing adverse events – after restitution of adverse events or when the adverse events could be explained satisfactorily.
The study ends as a project when the final report is signed and a joint publication has been accepted by an international journal.

Drop out:

A subject, who has prematurely been withdrawn from the study within the time of enrolment until last blood sampling, was considered a drop out.

1. Introduction

5-Fluorouracil (5-FU) is an analogue of uracil that was synthesized by Heidelberger and colleagues in 1957 (Grem, 2000), which differs from uracil by virtue of a fluorine atom in place of hydrogen at the carbon-5 position of the pyrimidine ring (fig. 1). Over the years, 5-FU has continued to be useful in the treatment of solid tumors, including breast cancer, gastrointestinal (GI) adenocarcinoma, and squamous cell carcinomas arising in the head and neck (Anonymous, 1997; Decatris et al, 2004; Ilson, 2003; Rich, et al., 2004; Wilke & Custem, 2003).

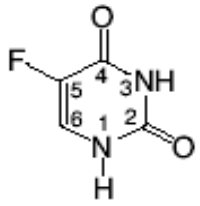


Figure 1: Chemical structure of 5-FU

5-FU therapy is characterized by considerable interpatient variability in pharmacokinetics, toxicity, and responses. The routes of administration currently used are i.v. bolus or continuous intravenous infusion (CVI) for up to 5 days and usually administered along with folinic acid (FA) or other chemotherapeutic agents and/or radiation to increase the anticancer activity of 5-FU and decrease host toxicity.

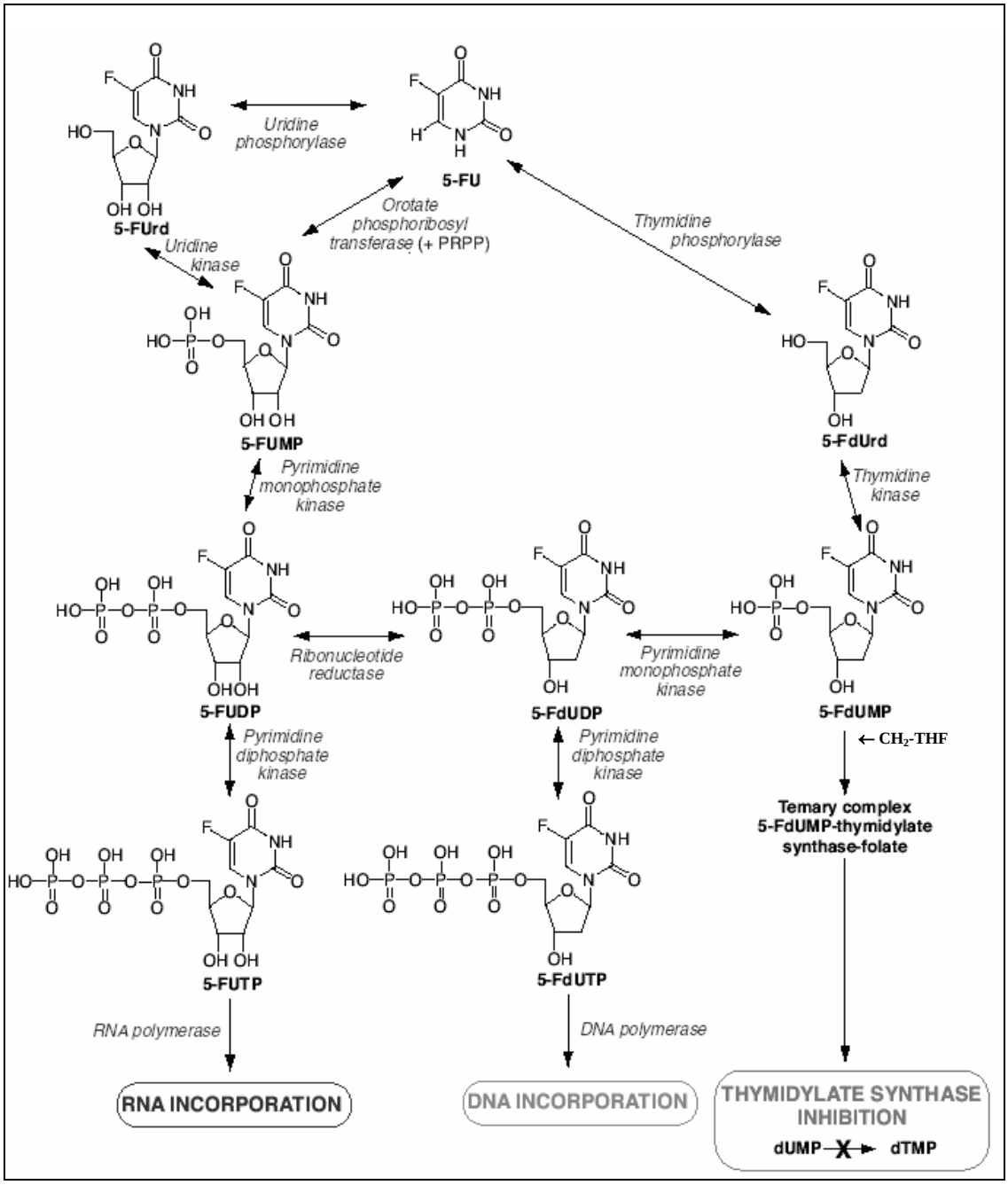
Blood dyscrasias, especially leukopenia, are the most common adverse effects of 5-FU therapy. Cardiac toxicities including chest pain, tightness of the chest, dyspnea, and cardiogenic shock have also occurred (2-5% of cases). Other frequent reactions include stomatitis, GI disturbance (nausea, vomiting, and diarrhea) and hand foot-syndrome (i.e., dermal pain in hands and feet) (Grem, 2000). The toxicity profile of 5-FU is schedule dependent: reducing the rate of injection to a slow infusion over several hours causes less myelosuppression but often lead to more severe stomatitis, diarrhea, and hand-foot syndrome (Meta-Analysis Group in Cancer, 1998).

1.1 Metabolic activation and catabolism of 5-FU

5-FU is a prodrug that requires an anabolic conversion into cytotoxic nucleotides, using several enzymes of the pyrimidine metabolic pathway. These nucleotides can be formed by three routes as illustrated in fig. 2: (1) conversion of 5-FU to 5-fluorouridine-5´-monophosphate (5-FUMP) by orotate phosphoribosyl transferase (OPRT); (2) sequential conversion of 5-FU to FUMP by uridine phosphorylase and uridine kinase; (3) sequential conversion of 5-FU to 5-fluoro-2´-deoxyuridine-5´-monophosphate (5-FdUMP) by thymidine phosphorylase and thymidine kinase (Grem, 2000; Longley et al., 2003). The anti-tumor activity results from inhibition of thymidylate synthase (TS), an enzyme required for *de novo* pyrimidine synthesis, by 5-FdUMP, as well as from incorporation of 5-FU active metabolites into RNA and DNA (fig. 2).

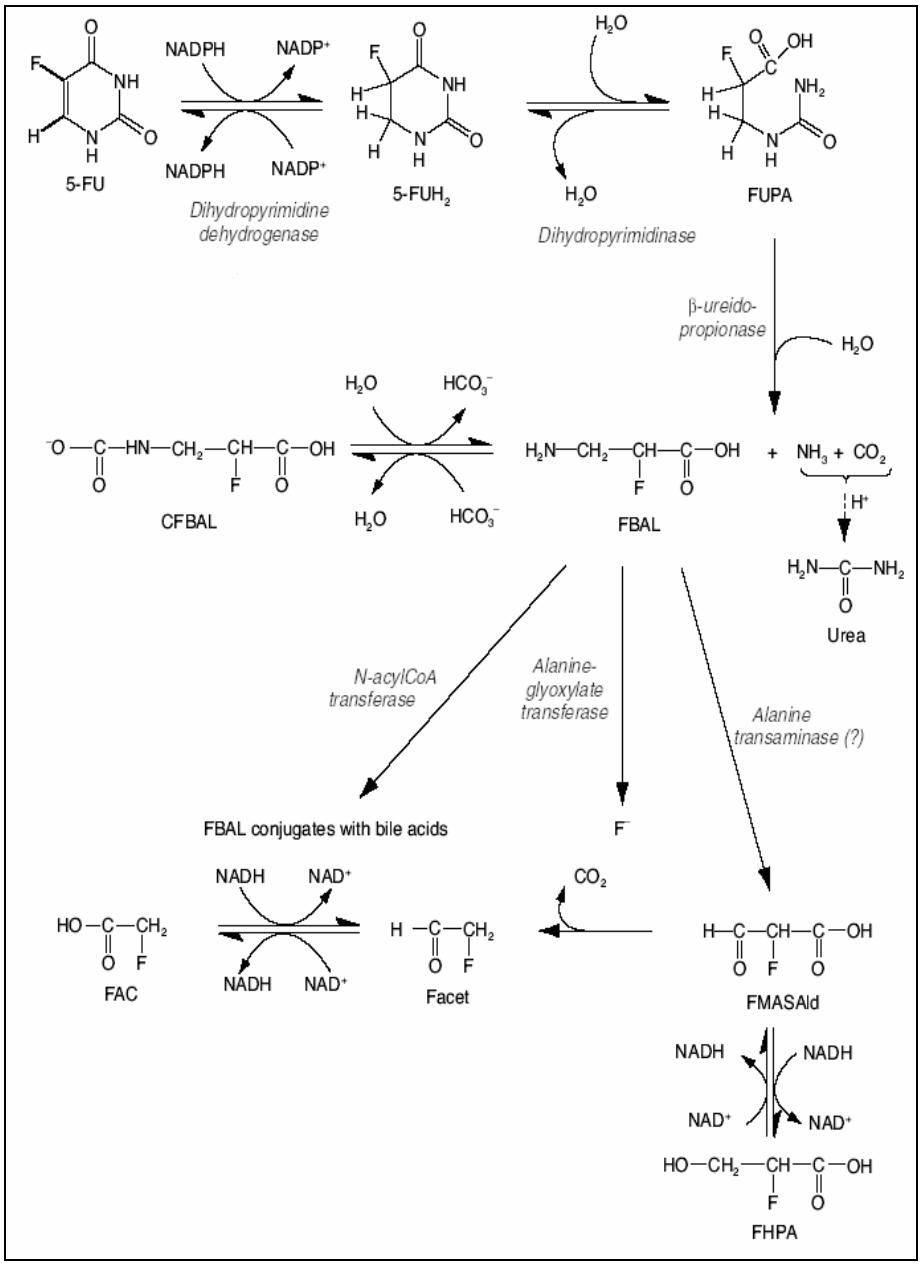
Only a small part of the 5-FU dose is activated via these routes, as in humans 80-90% of the administered dose is degraded mainly in the liver to 5,6-dihydro-5-fluorouracil (5-FUH_2) by dihydropyrimidine dehydrogenase (DPD) (Diasio & Harris, 1989), the rate-limiting step in 5-FU catabolism. 5-FUH_2 is further cleaved to form α-fluoro-β-ureidopropionic acid (FUPA) by dihydropyrimidinase. FUPA appears to be a transient intracellular catabolite and is then converted to α-fluoro-β-alanine (FBAL), carbon dioxide (CO_2), and ammonia (NH_2) by β-ureidopropionate. Other than the release of small amounts of free fluoride anion (F^-) from FBAL, further metabolisms of FBAL has been found, including N-carboxy-α-fluoro-β-alanine (CFBAL), three conjugates of FBAL with bile acids, two metabolites of FBAL by transamination (2-fluoro-3-hydroxypropanoic acid (FHPA) and fluoroacetate (FAC) (fig. 3).

Figure 2 Pathways for the anabolic metabolism of 5-FU



5-FU = 5-fluorouracil; 5-FUrd = 5-fluorouridine; PRPP = 5´-phosphoribosyl-1-pyrophosphate; 5-FUMP = 5´-flourouridine-5´-monophosphate; 5-FUDP = 5´-fluouroruidine 5´-diphosphate; 5-FUTP = 5´-fluorouridine-5´-triphosphate; 5-FdUrd = 5-fluoro-2´-deoxyuridine; 5-FdUMP = 5-fluoro-2´-deoxyuridine-5´-monophosphate; 5-FdUDP = 5-fluoro-2´-deoxyuridine-5´-diphosphate; 5-FdUTP = 5-fluoro-2´-deoxyuridine-5´-triphosphate; CH_2-THF = 5,10-methylenetetrahydrofolate

Figure 3 Pathways for the catabolic metabolism of 5-FU



5-FU = 5-fluorouracil; 5-FUH^2 = 5,6-dihydro-5-fluorouracil; FUPA = α-fluoro-β-ureidopropionic acid; FBAL = α-fluoro-β-alanine, NADPH = nicotinamide adenine dinucleotide phosphate (reduced form); NADP$^+$ = nicotinamide adenine dinucleotide phosphate (oxidized form)

1.2 Clinical pharmacokinetics

1.2.1 Absorption and distribution

Oral administration of 5-FU gives rise to erratic and unpredictable plasma concentrations due to the great variability in bioavailability (0-80%) (Iyer & Ratain, 1999). The unpredictable and significant interpatient variation in 5-FU absorption after

oral administration may be partially due to differences in 5-FU catabolism by DPD enzyme in the intestines and liver. This effectively rules out oral treatment with 5-FU.

Whether given by i.v. bolus or infusion, 5-FU readily distributes in tissue and extracellular fluid, including intestinal mucosa, bone marrow, liver, brain, cerebral spinal fluid, and neoplastic tissue. Reported volumes of distribution (V_d) ranged from 13 to 18 L (Iyer & Ratain, 1999; Diasio & Harris, 1989).

1.2.2 Plasma pharmacokinetics

The pharmacokinetic profile of 5-FU, as shown in table 1 and 2, varies according to dose and schedule of administration. After i.v. bolus of doses of 400-600 mg/m² once a week for 6 weeks, peak plasma concentration (C_{max}) values of up to 500 μM have been reported (table 2). The plasma half-life ($t_{1/2}$) of 5-FU is extremely short, about 6-22 min. The clearance (CL) of 5-FU is much faster with CVI than with i.v. bolus administration and increases as the dose rate decrease (table 1 & 2). Concentration of 5-FU in bone marrow were much lower and maintained for a shorter period after CVI administration than after bolus dosing, which is consistent with the reported decrease of myelosuppression of CVI (Fraile et al., 1980).

In addition to dose- and schedule-dependent variations in 5-FU pharmacokinetics, 5-FU clearance varies considerably between individuals on a given schedule. A number of studies indicated that the elimination of 5-FU are nonlinear (Wagner et al., 1986; Schaaf et al., 1987). The following nonlinear pharmacokinetic characteristics are noted with increasing doses of 5-FU: an increase in bioavailability, area under the plasma concentration-time curve (AUC), and plasma $t_{1/2}$ and a decrease in hepatic extraction ratio and total CL (CL_{tot}) (Iyer & Ratian, 1999). Although the change in 5-FU CL or AUC with increasing 5-FU dosage on a given schedule may be linear over a certain dose range, with higher dosages the decrease in clearance and increase in AUC may change disproportionately. This nonlinear behavior represents saturation of metabolic processes at higher drug concentrations, leading to difficulty in predicting plasma levels or toxicity at higher doses.

Table 1 Pharmacokinetics of 5-FU given by i.v. bolus

Investigator	Dose ($mg/m^2/d$)	*n*	$t_{1/2}$ (min)	CL (L/h)	C_{max} (µM)	V_d (L)
Di Paolo et al., 2001	370	110	21.6 ± 3	87.19 ± 5.05	285.9 ± 31	35.8 ± 3.6
Bocci et al., 2000	370	20	12.6 ± 1.2	25.43 ± 2.3	372.1 ± 59.0	7.2 ± 1.3
Macmillan et al., 1978	400	8	11.4 ± 1.5	75.9 ± 14.8	469 ± 85	18 ± 3.2
Casale et al., 2004	400	18	20	52.09	425.8	32.55
Heggie, et al., 1987	500	10	12.9 ± 7.3	60.6 ± 0.74	420 ± 102	14 ± 6.2
Van Groeningen, et al., 1988	500	15	1.9-18.7	56.92	Not stated	17.4 ± 6.7
Zhu et al., 2004	500	22	10.6 ± 1.3	58.65 ± 23.46	456.6 ± 199.1	13.2 ± 5.3
Coustère et al., 1991	500	10	6.9 ± 3.9	48.6 ± 12.6	Not stated	Not stated
Larsson et al., 1996	500	82	15	122.71	341 ± 34	18.05
Larsson et al., 1996	600	18	9.3-19.5	41.21	Not stated	14.4 ± 3.8
Sandström et al., 1996	600	21	11	79	Not stated	24

$t_{1/2}$ = half-life, CL = clearance, C_{max} = peak plasma concentration, V_d = volume of distribution

Table 2 Pharmacokinetics of 5-FU given by continuous infusion

Investigator	Duration of infusion	Daily dose (mg/m^2)	*n*	C_{ss} (µM)	CL (L/h)	V_d (L)
Fraile et al., 1980	96 h	1000-1100	6	1.3 ± 0.1	-	-
Erlichman et al., 1986	120 h	1250-2250	15	3.4 ± 0.4	182.58-245.82	-
Spicer et al., 1988	120 h	300-500	25	0.005-0.01	-	-
Grem et al., 1993a	120 h	64-200	24	0.30 ± 0.04	311.1 ± 33.66	-
Grem et al., 1993b	120 h	2000	19	6.5 ± 0.9	270.40 ± 33.05	-
Harris et al., 1990	120 h	300	7	0.13 ± 0.01	-	-
Fleming et al., 1992	120 h	1000	57	2.1	257.35 ± 69.77	-
Petit et al., 1988	120 h	450-996	7	2.6 ± 0.2	-	-
Jodrell et al., 2001	Protracted	300	58	0.23-2.0	-	18.0 ± 3.3
Yoshida et al., 1990	Protracted	190-600	19	1.15 ± 0.15	207.37	-
Anderson et al., 1992	Protracted	176-300	3	0.32	-	-

CL = clearance, C_{ss} = steady-state plasma concentration, V_d = volume of distribution

1.3 Special populations

Several studies have investigated the influence of body surface area (BSA), weight, gender, and age on 5-FU CL (Chansky et al., 2005; Etienne et al., 1998; Fleming et al., 1992; Lu & Diasio, 1995; Milano et al., 1992; Port et al., 1991; Sloan et al., 2002). Multiple linear regressions have confirmed a moderate relationship between BSA and 5-FU CL and BSA (r^2 = 0.602, p <0.001; Port et al., 1991). In 26 cancer patients with an equal 5-FU dose/m², the average CL in males was by 0.22 L/min higher than that in females (Port et al., 1991). In another study, women had a 10% lower 5-FU CL than men (p = 0.0005; Milano et al., 1992). Additionally, a pharmacokinetic study in 22 cancer patients showed that the mean $AUC_{0-\infty}$) was 72% greater in female patients than in males (p = 0.002; Zhu et al., 2004). This may in part explain why women experience

toxicity more frequently and with more severity than men (Chansky et al., 2005; Sloan et al., 2002).

Concerning the effect of increased age on 5-FU CL, both a moderate decrease (in one study by 0.072 L/min with every 10-year increase of age) (Port et al., 1991; Etienne et al., 1998) and no change (Milano et al., 1992) have been reported. This discrepancy may also reflect different methods of statistical analysis: age was considered as a continuous variable in some studies, while age classes were examined both alone and in covariance matrices in others. Similarly, DPD activity was not correlated with age (Etienne et al., 1994; Lu et al., 1993) but appears to be influenced by gender. The hepatic *in vitro* DPD activity in women seems to be higher than in men (Lu et al., 1995). In contrast, the DPD activity in PMBC was found to be 15% lower in women than in men (Etienne et al., 1994). The significant interpatient variability in 5-FU CL, tumor response, and toxicity may be explained in part by genetic differences in the activity of DPD (see section 1.7) (Milano & Etienne, 1994; Terret et al, 2000).

In a study in 187 head and neck cancer patients, no association between 5-FU CL and hepatic function (AST, ALT, AP, GGT, LDH, bilirubin) or nutrition status (albumin, prealbumin, transferrin) following 5-day CVI of 5-FU was found (r <0.25; Fleming et al., 1992). The influence of liver metastases on 5-FU pharmacokinetics after an i.v. bolus administration was studied in comparing between metastatic (n = 16) and nonmetastatic GI cancer patients (n = 18). Both groups displayed similar pharmacokinetics. No effect of liver metastases on 5-FU CL was observed. Ten (66%) and 13 (72%) patients with and without liver metastases, respectively, experienced any kind of 5-FU related toxicity (Maring et al., 2003).

Similarly, a trial on 24-h CVI of 5-FU with FA in patients with elevated serum bilirubin or mild renal dysfunction (n = 64) showed that 5-FU toxicities did not appear to be related to (mild) organ dysfunction. There was also no relationship between 5-FU CL and either serum bilirubin (p = 0.517) or serum creatinine (p = 0.396) (Fleming et al., 2003). In a colorectal carcinoma patient with end-stage renal insufficiency on maintenance hemodialysis therapy, pharmacokinetics of 5-FU and its metabolite, 5-FUH_2, were in the ranges reported in literature for patients with normal renal function, implying no need for dose adjustment (Rengelshausen et al., 2002). However, the

terminal inactive metabolite FBAL excreted renally accumulated in this patient. Whether this accumulation may have any clinical consequences is unknown to date. Because renal elimination of unchanged 5-FU accounts for only 10% of dose (Diasio & Harris, 1989), renal dysfunction should have, *a priori*, a minimal effect on 5-FU pharmacokinetics. There does not seem to be a need for 5-FU dose adjustments in patients with liver metastases or mild to moderate hepatic and renal dysfunction.

1.4 Pharmacodynamics

Measurements of systemic exposure towards 5-FU have been correlated with the incidence of toxicity, tumor response, and survival (table 3). Equivocal findings exist regarding the relationship between 5-FU plasma concentrations or exposure and response (table 3). In some studies, no relationship (Findley et al., 1996; Grem et al., 1993b; Thyss et al., 1986) was found, others suggested that improved responses occur with a target 5-FU AUC of approximately 30 mg·h/L during CVI (Hillcoat et al., 1978; Gamelin et al., 1996; Milano et al., 1994; Vokes et al., 1996).

Several investigators have described a significant correlation between 5-FU pharmacokinetics and toxicity (table 3). Despite the differences in 5-FU schedules, serious toxicity (leukopenia, diarrhea, stomatitis, and HFS) tends to increase with AUC values greater than 25-30 mg·h/L during CVI (Au et al., 1982; Milano et al., 1988; Milano et al., 1994; Santini et al., 1989; Thyss et al., 1986). Lower “critical” AUC values around 8-9 mg·h/L have been determined for i.v. bolus administrations (Di Paolo et al., 2001, 2002). These findings suggest that pharmacokinetic monitoring might be used to adjust 5-FU doses in order to avoid or minimize serious toxicity. This approach has been used successfully in patients receiving infusional 5-FU in several clinical trials (Gamelin et al., 1998; Santini et al., 1994; Ychou et al., 2003). However, not all patients with relatively high 5-FU systemic exposure experience serious 5-FU toxicity, and some patients have toxicity despite relatively low 5-FU systemic exposure (Gamelin et al., 1996; Milano et al. 1988; Thyss et al., 1986), suggesting that other factors also contribute to clinical toxicity.

Table 3 Summary of clinical pharmacokinetic studies with 5-FU

5-FU regimen	Co-med.	*n*	PK parameter	Drug effect (s) investigated			Ref.
				Groups compared or correlation studies	PK parameter in the groups (mean) or PD relationship	*p* value	
Weekly 8 h CVI: initial dose 1 g/m^2, individually increased every 3 wk by 250 mg/m^2 steps according to toxicity.	FA	43	C_{ss}	OR vs. NR	1,800-2,000 µg/L vs.< 1,800 µg/L	< 0.01	Gamelin et al., 1996
		152	C_{ss}, $AUC_{0\text{-}8h}$	Toxicity and 5-FU plasma levels	C_{ss} >3,000 µg/L ($AUC_{0\text{-}8h}$ 24 mg·h/L correlated to acute toxicity	= 0.0001	Gamelin et al., 1998
72 h CVI: dose 2 g/m^2/day	PALA & FA	27	C_{ss}	Incidence (ICD) of serious toxicity (GI, granulocytopenia, thrombocytopenia) at $C_{ss} \leq 8.9$ µM vs. $C_{ss} > 9$ µM	ICD of GI toxicity: $C_{ss} \leq 8.9$ µM = 1% vs. $C_{ss} > 9$ µM = 14%	= 0.02	Grem et al., 1993b
					ICD of granulocytopenia: $C_{ss} \leq 8.9$ µM = 14% vs. $C_{ss} > 9$ µM = 41%	= 0.01	
					ICD of thrombocytopenia: $C_{ss} \leq 8.9$ µM = 0% vs. $C_{ss} > 9$ µM = 14%	= 0.07	
72 h CVI 185-3500 mg/m^2/day	Dipyridamole	42	C_{ss}	Risk of leukopenia and mucositis and C_{ss}	$C_{ss} > 2$ µM associated with risk of toxicity	-	Trump et al., 1991
5-day CVI 2-6 cycles: mean daily dose: 960 mg/m^2 (650-1,300)	None	26	Total cycle AUC	High vs. low probability of toxicity	30 vs. < 30 mg·h/L	< 0.01	Milano et al., 1988
5-day CVI: dose 1 g/m^2/day	CDDP	170	$AUC_{0\text{-}5days}$ and $AUC_{0\text{-}3days}$	$AUC_{0\text{-}5days}$ for toxic vs. non-toxic cycles	34 vs. 26 mg·h/L	< 0.001	Santini et al., 1989
				$AUC_{0\text{-}3days}$ for toxic vs. non-toxic cycles	11 vs. 5.5 mg·h/L	< 0.01	
5-day CVI: 1 g/m^2/day, dose adaptation on day 3 based on $AUC_{0\text{-}48h}$. Dose reduction 15-100% for $AUC_{0\text{-}105h}$ <30 mg·h/L	CDDP	186	Average AUC per cycle	CR vs. PR >75% vs. PR <75% vs. NR	29.66 ± 4.9 vs. 28.68 ± 5.9 vs. 27.41 ± 4.7 vs. 27.21 ± 4.7 mg·h/L	0.05	Milano et al., 1994
				Overall survival	longer overall survival at AUC > 29 mg·h/L	0.001	
5-day CVI 500 mg/m^2/day, 3 cycles	CDDP & FA	39	C_{ss}	NR vs. CR or PR	0.67 µM vs. 1.00 µM	= 0.007	Schneider et al., 1995
				Toxicities and 5-FU concentration	5-FU concentration > 1 µM associated with all grade 4 toxicities.	-	
5-day CVI 1.2 g/m^2, not > 2 g	CDDP	27	AUC	NR vs. PR + stable	36.1 mg·h/L vs. 19.2 mg·h/mL (median)	= 0.05	Hillcoat et al., 1978
				Toxic (≥ grade 2) vs. non-toxic group	14.5 mg·h/L vs. 27.6 mg·h/mL (median)	NS	

CVI = continuous intravenous infusion, Co-med = co-medication, FA = folinic acid, CDDP = cisplatin, INF = interferon, PALA= N-phosphoroacetyl-L-aspartic acid, PK = pharmacokinetic, PD = pharmacodynamic, C_{ss} = steady-state plasma concentration, C_{max} = peak plasma concentration, CL = clearance, AUC = area under the plasma concentration-time, OR = objective response, CR = complete response, PR = partial response, NR = no response, ICD = incidence, - = not stated

Table 3 continued

5-FU regimen	Co-med.	*n*	PK parameter	Drug effect (s) investigated			Ref.
				Groups compared or correlation studies	PK parameter in the groups (mean) or PD relationship	*p* value	
5-day CVI 640 mg/m^2/day	IFN, FA & CDDP	89	C_{ss} and AUC	CR vs. all other	2.01 μm (31.2 mg·h/L) vs. 1.54 μM (23.9 mg·h/L)	= 0.02	Vokes et al., 1996
				Toxicity and C_{ss}	C_{ss} associated with leukopenia and mucositis	= 0.01, = 0.04	
5-day CVI: dose 1 g/m^2/day	CDDP	29	AUC	Toxic vs. non-toxic cycles	≥ 30 vs. < 30 mg·h/L	< 0.001	Thyss et al., 1986
5-day CVI: dose 7.5-15 mg/kg/day	Thymidine	24	CL and C_{ss}	Toxic vs. non-toxic group	CL: 72.0 ± 37.3 vs. 32.0 ±16.8 L/kg/day	= 0.001	Au et al., 1982
				Change in WBC count	Leukopenia more frequent with C_{ss} >1.5 μM (25 mg·h/L)		
7-28 days CVI: 190-600 mg/m^2/day	None	19	C_{ss} and AUC_{0-72h}	Toxic vs. non-toxic group	0.198 ± 0.088 (AUC_{0-72h} 12.53 ± 5.55) vs. 0.106 ± 0.059 mg/L (AUC_{0-72h} 6.32 ± 3.84 mg·h/L)	< 0.05	Yoshida et al., 1990
				Effective vs. non-effective	0.163 ± 0.097 (AUC_{0-72h} 10.12 ± 6.05) vs. 0.134 ± 0.074 mg/L (AUC_{0-72h} 8.31 ± 5.21 mg·h/L)	NS	
PVI 200 mg/m^2/day 12 wk followed by a 2-wk rest period	CDDP & FA	22	C_{ss}	Grade 0-1 vs. grade 2 GI toxicity	0.23 μM vs. 0.54 μM	= 0.02	Grem et al., 1993a
				PR vs. stable	0.27 μM vs. 0.23 μM	NS	
PVI 300 mg/m^2/day until the tumor response become refractory to treatment	± INF	30	Plasma concentration defined as C_{5-FU}	Non-toxic vs. toxic group	≥ 5 μm vs. < 5 μM (650 μg/L)	-	Findley et al., 1996
				Tumor response and C_{5-FU}	No correlation		
PVI 300 mg/m^2/day for 26 wk	None	64	C_{ss}	C_{ss} and PD endpoints (response, toxicity) - C_{ss} and diarrhea - C_{ss} and hand-foot syndrome - C_{ss} and stomatitis - C_{ss} and tumor response	No relationship	 = 0.164 = 0.41 = 0.949 = 0.182	Jordrell et al., 2001

PVI = protracted venous infusion, CVI = continuous infusion, Co-med = co-medication, FA = folinic acid, CDDP = cisplatin, INF = interferon, PK = pharmacokinetic, PD = pharmacodynamic, C_{ss} = steady-state plasma concentration, C_{max} = peak plasma concentration, CL = clearance, AUC = area under the plasma concentration-time, PR = partial response

Table 3 continued

5-FU regimen	Co-med.	*n*	PK parameter	Drug effect (s) investigated			Ref.
				Groups compared or correlation studies	PK parameter in the groups (mean) or PD relationship	*p* value	
Weekly i.v. bolus: initial dose 500 or 600 mg/m^2, individually escalated by 20% every wk until dose limiting toxicity developed	None	21	$AUC_{0\text{-}90\ min}$	Risk of toxicity and $AUC_{0\text{-}90\ min}$	Slightly higher risk of toxicity at $AUC_{0\text{-}90\ min}$ > 18 mg·h/L	-	Van Groeningen et al., 1988
i.v. bolus 5 consecutive days every 5 wk: 370 mg/m^2/day	FA	110	CL, C_{max}, AUC of 5-FU	GI toxicity: grade ≥ 2 vs. ≤ grade 1 toxicity	CL: 35.28 ± 3.31 vs. 56.30 ± 3.60 L/h/ m^2	< 0.005	Di Paolo et al., 2001
					C_{max}: 55.97 ± 12.51 vs. 31.48 ± 3.45 mg/L	< 0.01	
					AUC: 13.59 ± 2.05 vs. 7.91 ± 0.44 mg·h/L	< 0.001	
			AUC of $5\text{-}FUH_2$	GI toxicity: grade ≥ 2 vs. ≤ grade 1 toxicity	AUC: 12.91 ± 0.85 vs. 10.51 ± 1.02 mg·h/L	> 0.05	
i.v. bolus for 5 consecutive days every 5 wk: 370 mg/m^2/day.	FA	26	CL, C_{max}, AUC of 5-FU	Grade ≥ 3 vs. ≤ grade ≤ 2 toxicities	CL: 21.73 ± 4.81 vs. 48.86 ± 5.11 L/h/ m^2	< 0.05	Di Paolo et al., 2002
					C_{max}: 34.56 ± 5.48 vs. 18.80 ± 1.98 mg/L	< 0.05	
					AUC: 25.80 ± 10.15 vs. 8.51 ± 0.69 mg·h/L	< 0.05	
			C_{max}, AUC of $5\text{-}FUH_2$	Grade ≥ 3 vs. ≤ grade 2 toxicities	C_{max}: 4.78 ± 0.39 vs. 4.36 ± 0.23 mg/L	> 0.05	
					AUC: 8.74 ± 1.29 vs. 8.09 ± 0.95 mg·h/L	> 0.05	
i.v. bolus 500 mg/m^2/day once a wk for 6 consecutive wk followed by a 2-wk rest period.	FA	22	AUC	Grade 0-2 vs. grade 3 or 4 toxicity	14.5 ± 6.3 mg·h/L vs. 14.8 ± mg·h/L	< 0.899	Zhu et al., 2004
Mayo regimen: 425 mg/m^2 repeated on days 2 to 5, every 4 wk for 6 courses	FA	30	C_{max} and AUC in plasma	Plasma:			Jansman et al., 2002
				- Overall toxicity and AUC	No correlation either between AUC or C_{max} and toxicity	= 0.346	
				- Overall toxicity and C_{max}		= 0.863	
				- Mucositis and AUC		= 0.987	
				- Mucositis and C_{max}		= 0.162	
			C_{max} and AUC in saliva	Saliva:			
				- Overall toxicity and AUC	No correlation either between AUC or C_{max} and toxicity	=0.882	
				- Overall toxicity and C_{max}		= 0.746	
				- Mucositis and AUC		= 0.896	
				- Mucositis and C_{max}		= 0.154	

Co-med = co-medication, FA = folinic acid, CDDP = cisplatin, EPI = epirubicin, CP = cyclophosphamide, PK = pharmacokinetic, PD = pharmacodynamic, C_{ss} = steady-state plasma concentration, C_{max} = peak plasma concentration, CL = clearance, AUC = area under the plasma concentration-time

1.5 Pharmacogenetics

1.5.1 Dihydropyrimidine dehydrogenase (DPD)

Severe 5-FU-related toxicity is seen in DPD-deficient patients, which can be fatal (Diasio et al., 1998; Houyau et al., 1993; Lyss et al., 1993). Pharmacokinetics of 5-FU were modified in a patient with a low DPD activity, with 90% of the administered dose being recovered unchanged in urine, a prolonged $t_{1/2}$ of about 160 min, and a markedly decreased CL_{tot} of 71 mL/min/m^2 already after a 5-FU test dose (25 mg/m^2; Diasio et al., 1988). Corresponding values in patients with normal DPD activity are <20%, 13 ± 7 min, and 594 ± 198 mL/min/m^2, respectively (Diasio & Lu, 1994; Heggie et al., 1987). A comparable AUC threshold (25-30 mg·h/L) was also observed for severe toxicity (section 1.6). Hence, a low DPD activity leads to a marked reduction in 5-FU CL with an increased likelihood of developing severe 5-FU induced toxicity.

The DPD enzyme is encoded by the polymorphic *DPYD* gene which is located on chromosome 1p22 and consists of 23 exons (Johnson et al., 1997; Wei et al., 1998; Meinsma et al., 1995). To date, more than 30 mutations have been identified in cancer patients (table 4: Ploylearmsaeng et al., 2006). *DPYD*2A* is the most common one seen in cancer patients with severe 5-FU associated toxicity (van Kuilenburg et al., 1999, 2001, 2002). This allele carries a G to A point mutation in the 5′-splicing site of intron 14 (exon 14-skipping mutation, IVS14+1G>A), leading to skipping of exon 14 immediately upstream of the mutated splice donor site in the process of DPD pre-mRNA splicing (fig. 4). The mature DPD*2A mRNA lacks a 165-nucleotide segment encoding the amino acids 58-635 (Wei et al., 1996; van Kuilenburg et al., 2001), resulting in a loss of enzyme activity. When this mutation is present in a heterozygote, a 50% reduction of the normal activity leads to a roughly 50% reduction in 5-FU CL (Maring et al., 2002). In patients homozygous for this variant allele, DPD activity is completely lacking, and the 5-FU toxicity may become life-threatening. Other *DPYD* aberrations associated with DPD deficiency have been reported (table 4), but their clinical consequences are less clear and sometimes equivocal (table 5, reviewed in Ploylearmsaeng et al., 2006).

Table 4 The mutations of *DPYD* and their reported relationship to DPD*

*DPYD**	Exon	Genotype	Effect	DPD enzyme activity		
				Normal	Reduced	Unclear
-	2	61C>A	Stop codon			
*12	2	62G>A	AA exchange	√		
-	2	74G>A	AA exchange			√
*9A	2	85T>C	AA exchange	√	√	
-	3	100 del A	Truncated protein		√	
-	4	257C>T	AA exchange		√	
*7	4	Del TCAT 295-298	Truncated protein		√	
-	6	496A>G	AA exchange	√	√	
-	6	545T>A	AA exchange			√
-	6	601A>C	AA exchange		√	
-	6	632A>G	AA exchange		√	
*8	7	703C>T	AA exchange		√	
-	7	731A>C	AA exchange			√
-	8	775A>G	AA exchange	√		
-	8	812 del T	Truncated protein			√
*11	10	1003G>T	AA exchange		√	
-	10	Del TG 1039-1042	Truncated protein		√	
-	10	1097G>C	AA exchange		√	
-	10	1108A>G	AA exchange		√	
-	11	IVS11+1G>T	Exon 11 skipping	√		
*12	11	1156G>T	Stop codon		√	
-	11	1217T>C	AA exchange	√		
-	11	1218G>A	AA exchange	√		
-	11	1236G>A	-	√		
-	12	1475C>T	AA exchange		√	
*4	13	1601G>A	AA exchange	√		√
*5A	13	1627A>G	AA exchange	√	√	
-	13	1651G>A	AA exchange	√		
*13	13	1679T>G	AA exchange			√
-	13	1714C>G	AA exchange			√
*2A	14	IV14+1G>A	Exon 14 skipping	√	√	
-	14	1896T>C	AA exchange			√
*3	14	1897 del C	Truncated protein		√	
*2A	14	IV14+1G>A	Exon 14 skipping	√	√	
-	13	1714C>G	AA exchange			√
-	19	2303C>A	AA exchange	√		
-	19	2329G>A	AA exchange			√
-	21	2657G>A	AA exchange			√
-	22	2846A>T	AA exchange		√	
-	23	2921A>T	AA exchange			√
-	23	2933A>G	AA exchange		√	
*10	23	2983G>T	AA exchange		√	
-	23	3067C>T	AA exchange	√		
*9B	2/21	85T>C + 2657G>A	AA exchange			√
*5B	13/Intron 13	1627G>A+Intron 13 39C>T	AA exchange			√
*2B	13/14	1627A>G + IV14+1G>A	AA exchange + truncated protein			√
-	Intron 10	Intron 10 15T>C	-	√		
-	Intron 13	Intron 13 40G>A	-			√

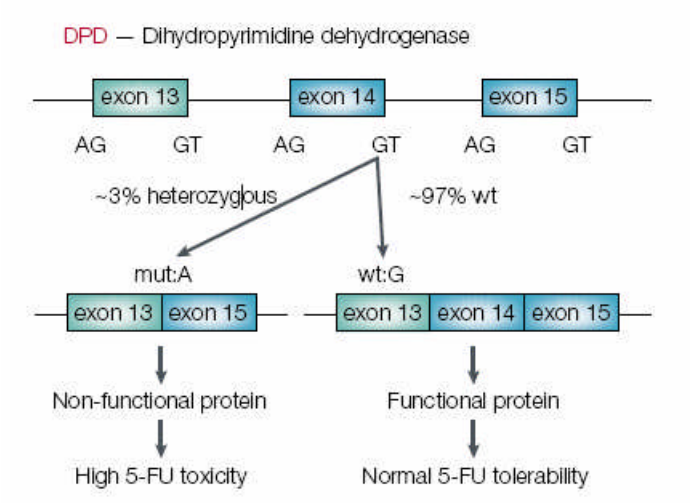
* reviewed in Ploylearmsaeng et al. (2006), AA = amino acid

Table 5 Variant *DPYD* alleles and patients characteristics suffering 5-FU toxicity*

***DPYD* alleles**	**Sex**	**Age***	**Cancer**	**Chemotherapy**	**Toxicity (grade)**
*DYPD*2A*	M	76	Basal cell	5% 5-FU cream	diarrhea (severe), mucositis (severe), skin rash
	F	44	Rectal	5-FU/FA	mucositis (4), leukopenia (4), thrombocytopenia (4), pancytopenia (4)
	M	73	rectal	5-FU	leukopeina (4)
	F	48	Breast	CMF	leukopenia (4)
	M	63	Colon	5-FU/FA	leukopenia(4), diarrhea (3)
	F	50	Rectal	5-FU	leukopenia (4), diarrhea (3)
	M	78	Colon	5-FU/FA	leukopenia (4), thrombocytopenia (4)
	F	72	Colon	5-FU/FA	leupenia (4), mucositis (3)
	F	59	Breast	5-FU/tamoxifen	diarrhea (4), N/V, leukopenia, cardiotoxicity, thrombocytopenia, hyperpigmentation,
	F	60	Colon	5-FU/FA	leukopenia (4), hair loss, thrombocytopenia (4), fever (40°C), diarrhea, mucositis
	M	65	Colon	5-FU	pancytopenia (4), mucositis (4)
	F	66	Breast	5-FU	Severe toxicity
	F	57	Colon	5-FU	Severe toxicity
	M	79	Colon	5-FU	Severe toxicity
	M	54	Colon	5-FU	Severe toxicity
	F	44	Colon	5-FU/FA/IRI	diarrhea (4), neutropenia (4) and death
*DPYD*2A*, *DPYD*5*, R21X & *DPYD*9A*	F	42	Ovarian	5-FU/FA	mucositis (4), anemia (3), thrombocytopenia (3), leukopenia (3), alopecia, erythrodermaia of skin
*DPYD*2A*, *DPYD*5*, M166V & D949V	M	73	Colorectal	5-FU/FA	neutropenia, mucositis, exfoliation of the skin, diarrhea, AF and death
*DPYD*2A*, *DPYD*5*	F	60	Colon	5-FU	Severe toxicity
*DPYD*2A*, A777S, D949V	F	58	Colon	5-FU/FA	neutropenia, N/V, mucositis (4) and death
*DPYD*2A*, *DPYD*9A*	F	40	Colon	5-FU	Severe toxicity
*DPYD*2A*, *DPYD*13A*	F	40	Breast	CMF	neutropenia, ataxia and deteriorate neurologically
*DPYD*4A*	-	-	-	5-FU	Severe hematopoietic toxicity
*DPYD*4*, *DPYD*6*, *DPYD*13*, IVS10+15T>C	-	-	-	5-FU	Severe toxicity
*DPYD*4*, *DPYD*5B*, *DPYD*9A*, IVS13+40G>A	M	35	Colon	5-FU/FA	cardeogenic with severe insufficient left ventricular, reduced renal function, hepatic encephalopathy with somnolence, myclonus & seizure
*DPYD**5	F	49	Breast	5-FU	Severe toxicity
*DPYD*5*, M182K, A77S	F	53	Rectal	5-FU/Rx	N/V (4), disoriented, somnolent, arrhythmia and death
*DPYD*5*,*DPYD*9A*,	F	66	Colon	5-FU	Severe toxicity
*DPYD*6*	M	53	Rectal	5-FU	Severe toxicity
*DPYD*9A*	F F M M	-. - - -	Gastric Gastric Gastric Rectal	MTX/5-FU MTX/5-FU MTX/5-FU 5-FU/FA	nausea (1) nausea (2) nausea (2) no toxicity (0)
*DPYD*9A*, 496A>G, 2846A>T	M	51	Rectal	5-FU	Severe toxicity
*DPYD*9A*, 496A>G	M	63	Colon	5-FU	Severe toxicity
*DPYD*11*, *DPYD*12*	F	57	Breast	5-FU	leukopenia, mucositis (4), thrombocytopenia
812delT	M	-	Colon	MTX/5-FU	nausea (1)
I543V	M	-	Esophageal	5-FU/CDDP/Rx	nausea (2)
L572V	F	-	Gastric	MTX/5-FU	arrhythmia (2)

* reviewed in Ploylearmsaeng et al. (2006)
F = female, M = male, CMF = cyclophosphamide, methotrexate and 5-fluorouracil, FA = folic acid, IRI = irinotecan, MTX = methotrexate, Rx = radiotherapy, AF = atrial fibrillation, *age in year, - = data not show, N/V = nausea and vomiting

Figure 4 The effect of the IVS14+1G>A mutation



The wild-type DPYD gene contains a GT nucleotide sequence at exon 14(wt), required for normal catabolism of 5-FU to 5-FUH_2. A G-to-A transition at the exon 14 splice site leads to skipping of exon 14 of the mutated splice donor site in the process of DPD pre-mRNA splicing (mut). Patients with low hepatic DPD activity cannot efficiently metabolize 5-FU and its accumulation causes toxicity (Relling & Dervieux, 2001)

Considering the common use of 5-FU in cancer patients and the relatively high prevalence of *DPYD* mutations associated with a decrease or lack of DPD activity in the normal population, patients who are to receive 5-FU should theoretically benefit from genetic screening. It has been suggested to analyze the DPD activity (various phenotyping methods) and to screen the DPD mutations (use allele-specific polymerase chain reaction-based methods), at least for carriers of *DPYD*2A*, to identify persons at risk for toxicity prior to 5-FU therapy (Omura 2003; van Kiulenburg et al., 2003), to reduce doses in heterozygotes or to avoid the use of 5-FU in homozygotes.

However, the presence *DPYD*2A* is not the only reason for severe 5-FU-related toxicity. Indeed, studies on populations of individuals phenotypically deficient in DPD activity and of patients with 5-FU toxicity detected *DPYD*2A* in only 14 of 22 (64%: van Kuilenburg et al., 2002) and in 6 of 25 (24%: van Kuilenburg et al., 1988) subjects, respectively. In a prospective study carried out in 351 patients receiving 5-FU (± FA) as a bolus or infusional therapy to assess the impact of DPD mutations on 5-FU toxicity, there was no evidence for a pivotal role of this mutation (Kollmannsberger et al., 2001). In 14 patients with severe 5-FU-related toxicity sequencing of ten *DPYD* exons containing most of the known mutations was carried out, and in 3 patients no mutations have been detected at all (Collie-Duguid et al., 2000). Furthermore, the complexity of the *DPYD* gene with 23 exons, the still increasing number of sequence variations, and

the mostly unclear clinical relevance of the majority of mutations reported to date limit the usefulness of single mutation genotyping tests.

1.5.2 Thymidylate synthase (TS)

In addition to the enzyme catabolizing 5-FU, far more information is available regarding polymorphisms of the target enzyme thymidylate synthase (TS). Genetic polymorphisms in the gene encoding TS (called *TYMS*) have also been shown to influence response (Marsh et al., 2001; Pullarkat et al., 2001) and toxicity (Lecomte et al., 2004) of 5-FU-based therapy, although protein expression levels were not linked to 5-FU pharmacodynamics (Tomiak et al., 2001; Westra et al., 2005).

The *TYMS* is located on chromosome 18p11 (Hori et al., 1990). The polymorphisms have been identified, characterized by variable numbers of a 28-bp tandem repeat sequence in 5′-promoter enhancer region (*TSER*), with double (2R) and triple repeats (3R) being the predominant alleles (Pullarkat et al., 2001; Villafranca et al., 2001). The 3R/3R genotype is associated with higher tumor TS mRNA compared to either 2R/2R (p = 0.004) or 2R/3R (p = 0.05) (Pullarkat et al., 2001). In 24 metastatic colorectal cancer patients, the 2R/2R genotype was nearly twice as common in responders to a 5-FU therapy compared to non-responders (40% vs. 22%, respectively). A decrease in median survival was also linked to increasing numbers of *TSER* repeats (median survival 16, 14 and 12 months for 2R/2R, 2R/3R and 3R/3R, respectively) (Mash et al., 2001). This is confirmed in a study in 50 metastatic colorectal cancer patients receiving 5-FU, a higher response rate was observed in patients with lower numbers of *TSER* repeats (50% for 2R/2R, 15% for 2R/3R, and 9% for 3R/3R: Pullarkat et al., 2001).

The associations between polymorphisms in the *TYMS* promoter and 5-FU toxicity were investigated in 90 patients with colorectal cancer treated with 5-FU (Lecomte et al., 2004). Patients with a 2R/2R, 2R/3R, or 3R/3R genotype had grade 3 or 4 toxicities in 43% (6 of 14), 18% (8 of 44), and 3% (1 of 28), respectively ($p < 0.02$). In this study, response to 5-FU and survival of patients did not differ between the *TYMS* genotype groups.

1.5.3 Methylenetetrahydrofolate reductase (MTHFR)

Directly linked to the 5-FU-mediated inhibition of TS is the presence of intracellular folate. Methylenetetrahydrofolate reductase (MTHFR) also plays an important role in the intracellular action of 5-FU. The substrate of MTHFR is necessary for the formation of the cytotoxic inactive ternary complex between the active metabolite of 5-FU and TS. Folic acid, a precursor of this substrate, is therefore added in various 5-FU containing regimens to increase the intracellular reduced folate pool.

So far, two MTHFR polymorphisms have been recognized but most of them concerned to the response of 5-FU. A common C677T transition in exon 4 of the MTHFR gene results in a thermolabile enzyme variant with lower specific activity (Stern et al., 2000). In 2 studies with 45 and 51 colorectal cancer patients, respectively, the C677T genotype appeared to affect the folate pool (Wisotzkey et al., 2000; Kawakami et al., 2001). In the latter study, no effect of the C677T genotype on overall survival after an oral 5-FU-based chemotherapy was seen (Karakamie et al., 2001). In a recent study of 98 colorectal cancer patients, the C677T mutation was also associated with higher response rates after 5-FU/FA chemotherapy (Etienne et al., 2004). The effect of the single point mutation was small, and results were ambiguous, so that further studies would be useful to elucidate the exact role of the MTHFR polymorphism and to identify patient groups who would benefit from genotyping.

1.6 Conclusion

A major problem in 5-FU therapy is the prediction of the outcome of therapy, both in terms of tumor response and host toxicity. 5-FU forms an integral part of the many chemotherapy regimens. However, its use has been marred by variable pharmacokinetics, unpredictable response rates and seemingly random toxicity. Pharmacogenetic variability in drug metabolizing enzyme systems is a major determinant of variations in these outcomes. Unpredictable disposition of drugs may result in an undertreatment failing to provide therapeutic effects, or an overtreatment leading to excessive toxicity. The current practice in 5-FU therapy is to dose patients based upon BSA, which may be imprecise. Numerous attempts have established relationships between the concentration of 5-FU and its desired or undesired effects.

The majority of studies has focused on the toxic effects of 5-FU. AUC values between 25-30 mg·h/L should be achieved, but not exceeded during 5-day CVI. For other regimens, therapeutic plasma concentration or AUC ranges are not as well defined. They should be established and used in prospective studies to adjust doses *a posteriori*.

Pharmacogenetically derived dosing regimens may offer an alternative to the above-mentioned approaches in 5-FU therapy. Despite the many studies that focused on potential genes interfering with 5-FU therapy, only few factors indeed have been proven to affect 5-FU efficacy and/or toxicity. Most consistent data are available regarding the role of DPD in 5-FU chemotherapy. The impact of DPD deficiency on 5-FU pharmacokinetics and the development of severe toxicity are obvious. Whether or not to screen patients on DPD deficiency before starting chemotherapy is, however, an issue of debate (Behnke et al., 2002; Innocenti & Ratain, 2002; Raida et al., 2001). Since genetic aberrations in the *DPYD* gene explain less than half of all cases of extreme 5-FU-related toxicity, costly screening will only be partially preventive. Therefore, a solid cost-benefit analysis, preferentially embedded in a large prospective clinical trial, will be needed to establish the benefit of *DPYD* genotyping. Screening (by means of genotyping or phenotyping) might become standard clinical practice as soon as a rapid, sensitive, and cheap test becomes available.

Since TS is the important target enzyme of 5-FU, and since genetically controlled expression of this enzyme varies considerably between individuals, genotyping of the promoter of *TYMS* may prove useful to identify patients who are likely to respond to 5-FU, but results from protein level quantification studies challenge the genotype-effect relationship. The combination of a functional DPD activity test and of genotyping of *DPYD*, perhaps also genotyping of *TYMS*, before 5-FU administration may be valuable for the identification of patients who are likely both to tolerate and to respond to 5-FU. However, the validity of these assumptions and the clinical usefulness remain to be established in larger clinical trials.

2. Rationale and objectives

2.1 Rationale

It is now well recognized that the pharmacogenetic syndrome DPD deficiency, associated with severe or lethal toxicity after 5-FU administration, is primarily caused by molecular defects in the *DPYD* gene that result in complete or partial loss of DPD activity (van Kuilenburg, 2004). At least 30 variant alleles have been identified in DPD deficient patients (van Kuilenburg 2004), with *DPYD***2A* being most frequently implicated in patients suffering from severe 5-FU toxicity (van Kuilenburg, 2004). However, the presence of *DPYD***2A* is not the only reason for severe 5-FU toxicity (van Kuilenburg et al., 2002, Kollmannsberger et al. 2001). Besides, the known variant *DPYD* alleles do not entirely explain the polymorphic DPD activity and toxic response to 5-FU (Collie-Duguid et al., 2000). Hence, other factors, including novel mutations, may contribute to DPD deficiency.

Although the relationship between DPD phenotype and *DPYD* genotype is not entirely elucidated, it is well recognised that one of the major risk factors in cancer patients for developing severe and life-threatening toxicity after 5-FU therapy is represented by mutations in *DPYD* (Collie-Duguid et al., 2000; Johnson et al., 2002). Complete and partial DPD deficiency affect around 0.1 and 3-5% of the general population, respectively (Etienne et al., 1994). These individuals have no symptoms in the absence of drug treatment, but are at risk for developing toxicity if exposed to 5-FU (Diasio & Lu, 1994). Thus, the Association of Medical Oncology (AIO) of the German Cancer Society has recommended to pre-screen patients for DPD activity and/or *DPYD* genotype before 5-FU therapy and to modify therapy accordingly (Köhne et al., 2002). However, neither all mutations leading to an altered enzyme function are identified nor are therapeutic drug monitoring (TDM) schemes for 5-FU established which would be suitable for use in clinical routine.

Additionally to the 5-FU-catabolizing enzyme, genetic polymorphisms in the TS gene (*TYMS*) have been shown to influence response (Mash et al., 2001; Pullarkat et al., 2001) and toxicity (Leconte et al., 2004) of 5-FU-based therapies. Another enzyme, MTHFR, also plays an important role in the intracellular action of 5-FU. A polymorphism that may influence the efficacy of 5-FU by influencing folate pools is

that of the *MTHFR* gene (Etienne et al., 2004; Wisotzkey et al., 1999). Otherwise, a large-scale assessment of the role of each *TYMS* and *MTHFR* polymorphism individually is now required to determine whether prospective assessment is warranted in patients prior to 5-FU-based chemotherapy.

However, no clear studies have quantified the extent and variability of influence of DPD genotype/phenotype, of TS genotype and of MTHFR genotype on 5-FU pharmacokinetics. Since 5-FU pharmacokinetics show high individual variability causing difficulties in predicting efficacy and toxicity, it is important to elucidate other factors influencing this variability. To estimate their extent of influence on pharmacokinetics and/or pharmacodynamics would help to optimise cancer therapy. Additionally, since DPD catalyzes the metabolic transformation of 5-FU to 5-FUH_2, investigations have been carried out to correlate pharmacokinetic parameters of these two analytes in plasma to the occurrence and severity of 5-FU toxicity (Di Paolo et al., 2001, 2002). The results of these studies showed that the increased 5-FU/5-FUH_2 AUC ratio was related to the severity of 5-FU toxicity in patients treated with adjuvant bolus 5-FU plus FA. These suggest that the $AUC_{5\text{-}FU}/AUC_{5\text{-}FUH2}$ ratio may be a useful parameter to assess the metabolic activity of patients during 5-FU administration. However, further studies will be required to prove and validate the usefulness of the AUC ratio in various regimens.

Thus, in this study, DPD activity, DPD, TS, and MTHFR genotype and other factors were correlated to 5-FU, and 5-FUH_2 pharmacokinetics and 5-FU toxicity.

2.2 Objectives

1. Identification of the DPD, TS and MTHFR genotype and of the DPD phenotype in 30 patients before start of 5-FU administration
2. Investigation of 5-FU pharmacokinetics and its metabolite, 5-FUH_2, and toxicity of 5-FU in these patients
3. Correlation of individual factors including DPD phenotype, genotyping of DPD, TS, and MTHFR to 5-FU pharmacokinetics and adverse events.
4. Establishing a method based on individual factors and 5-FU pharmacokinetic parameter allowing reliable prediction of clinical 5-FU effects (if applicable).

3. Patients and methods

3.1 Study design

An open, non-randomized, consecutive period phenotyping study design in patients with colorectal, gastric, or locally advanced esophageal cancer treated with 5-FU was used in this pilot study. Phenotyping and genotyping for enzymes related 5-FU metabolism was carried out before the initiation of the chemotherapy. Then, 5-FU plasma concentrations were monitored during and after the first administration of 5-FU during the first chemotherapy cycle (table 6).

3.2 Patients

This study was approved by the Ethics Committee of the University of Cologne, Germany, and was conducted according to the Declaration of Helsinki in its amended version of Edinburgh, Scotland, 2000, and national and international legal stipulations and guidelines. Patients to be treated with 5-FU at the radiologic oncology department (Klinik und Poliklinik für Strahlentherapie: KST), University of Cologne, were eligible to participate in this study.

Patients were informed about the procedures and the aims of the study both verbally and in written form, and they were enrolled after giving written informed consent to participate. Inclusion and exclusion criteria were taken into consideration.

3.2.2 Sample size

The number of patients was calculated by sample size estimation using WinBiAS (version 7.01, epsilon Verlag, Darmstadt, Germany) under the following conditions: interindividual variability in 5-FU pharmacokinetics is determined to at least 30% by DPD activity. Hence, in order to test between various parameters for a linear coefficient of correlation $\rho = 0.5477$ with a power of 90% on a level of significance of $p = 0.05$, $n = 30$ patients were required.

3.2.1 Patient selection

Eligibility requirements for this study included:

- Caucasian

- Histologically and/or cytologically proven colorectal, gastric, or locally advanced esophageal cancer
- age 18-70 years
- Karnofsky performance status ≥ 70%
- life expectancy ≥ 3 months (as assessed by the attending physician)
- adequate hematopoietic reserve (leukocyte count ≥ 3,000 cells/μL, absolute neutrophil count ≥ 1,500 cells/μL, platelet count ≥ 100,000 cells/μL and hemoglobin level ≥ 10 g/dL)
- chemotherapy regimen containing i.v. bolus administration (450 mg/m^2/day within 5 min) or continuous long-term infusion of 5-FU (2 to 2.6 g/m^2/24 h and 650-1000 mg/m^2/24 h for 5 days), besides, other regimens will be considered by the investigator, too
- normal hepatic function (total bilirubin level ≤ 2.0 mg/dL, aspartate aminotransferase and alanine aminotransferase ≤ 2.5 times upper limit of normal range)
- normal renal function (serum creatinine ≤ 1.5 mg/dL)
- willing and capable to confirm written consent to enrolment after thorough information has been provided
- normal finding in the medical history and physical examination unless the investigator considers an abnormality to be related to the neoplastic disease or clinically irrelevant.

Exclusion criteria included the following:

- hypersensitivity against 5-FU and/or related drugs
- hypersensitivity against inactive ingredients of the preparation
- history of drug allergy
- acute hay fever
- present or past serious infection
- malnutrition (BMI < 18.5 kg/m^2)
- females only: pregnancy, lactation
- history of relevant hepatic disorders or diseases
- history of relevant gastrointestinal disorders or diseases

- history of treatment with 5-FU or other chemotherapeutic agents or radiotherapy
- acute or chronic diseases which could affect absorption and/or metabolism
- drug and/or alcohol dependence
- concomitant treatment with drugs known to interfere with 5-FU pharmacokinetics and/or –dynamics, which are not part of the chemotherapeutic regimen, such as cimetidine, levamisol, metronidazole, warfarin, allopurinol and thiazide diurectics.
- history or suspicion of HIV infection, active or chronic viral hepatitis
- subjects who were known or suspected not to be capable of understanding and evaluating the information that is given to them as part of the formal information policy (informed consent), in particular regarding the discomfort to which they will be exposed.
- subjects who known or suspected not to comply with the study directives and/or known or suspected not to be reliable or trustworthy.

3.3 Treatment plan and evaluations

Patients underwent a physical examination, performance status determination, electrocardiogram (ECG), complete blood count with platelet and differential counts, serum chemistry profile, and urinalysis within 1-14 days before initiating therapy. Chemotherapy was delivered on an inpatient basis. Prior to the initiation of 5-FU therapy, all patients were required to obtain a central vascular catheter through the subclavian vein. 5-FU was administered via a device pump in 24-h CVI for 5 days in a mixture of 0.9% saline.

For patients with colorectal cancer, each cycle of treatment consisted of 5-FU 650 or 1,000 mg/m²/day for 5 days and radiotherapy, while the patients with oesophageal cancer were treated with 5-FU 650 or 1,000 mg/m²/day and cisplatin 20 mg/m²/day for 5 days with radiotherapy. Cisplatin was administered in a mixture of 0.9% saline

The administration of anti-emetic agents was permitted at any time as an intervention or a prophylaxis. Concurrent treatment with drugs known to interfere with 5-FU pharmacokinetics and/or –dynamics (see exclusion criteria) was prohibited.

Toxicity was evaluated with particular attention to diarrhea, mucositis, hand-foot syndrome and hematologic effects. After 5-FU administration, blood was taken 2 or 3 times/week for controlling hematologic parameters (hemoglobin, white blood cell count, platelet count). The toxic events were prospectively recorded, evaluated and graded during the period of blood sample collection and thereafter, up to the following cycle of chemotherapy according to Center for Disease Control and Prevention (CDC) toxicity criteria. A patient history update, physical examination (including ECG, blood pressure, heart rate, body temperature and body weight) and evaluation of clinical chemistry tests were also performed at the end of study evaluation and long-term follow up data were also obtained for the patients.

Table 6 Overview: study procedures

Study phase	Pre-study	Phenotyping period	PK period[(1)]	Post study[(2)]
Period		1	2	
Study day relative to day of drug intake	-14 - - 1	- 14 - 0	0 - 5	+28
Informed consent	×			
Medical history	×			
Medical history update and re-check for inclusion and exclusion criteria		×	×	×
Physical examination	×	×*	×*	×*
Check for inclusion/exclusion criteria	×	×**	×**	×
Determination of adverse events	×	×	×	×
Serology (Hepatitis & HIV)***	×			
Hematology	×			×
Clinical chemistry	×			×
12-lead ECG at rest	×			×
Recumbent blood pressure/pulse rate	×	×		×
A spot urine sampling for phenotyping		×		
Administration of chemotherapy			×	Depended on schedule
Blood sampling for genetic analysis		×		
Blood sampling for PK analysis of 5-FU			×	
Hematologic parameters (3 times/week)			×	
Check for CDC toxicity criteria			×	×

[(1), (2)]Chemotherapy cycle 1 and 2, respectively, *short physical check up, **short physical check up, patients were asked about their well-being, *** in case of suspicion or knowledge of infection, PK = pharmacokinetic

3.4 Urine and blood sample collection

3.4.1 Urine samples

Single spontaneous urine samples, ≥ 20 mL, for DPD phenotyping were collected from patients after an overnight fast (at least 10 h) between 09:00 and 10:00 a.m. before the start of the chemotherapeutic period. The urine samples were stirred and 4 aliquots of 5 mL each were drawn and stored at -80°C until analysis.

3.4.2 Blood samples

Blood samples were drawn from a peripheral vein by venipuncture for DPD, TS, and MTHFR genotyping, and for 5-FU plasma pharmacokinetics. For genotyping, 8 mL of blood (2 aliquots of 4 mL each) was drawn from each patient in EDTA containing tubes (Sarstedt, Nümbrecht, Germany). This whole blood was stored at -20°C until assayed. For 5-FU pharmacokinetics, 4.5 mL of blood were drawn and collected in Li^+-heparinized tubes (Sarstedt, Nümbrecht, Germany) before dosing (pre-dose), and at the following times relative to the beginning of 5-FU infusion of the first cycle: 36, 48, 108 h and at the end of infusion, and at the following times relative to the end of infusion: 5, 30, 60 and 90 min. Samples were mixed by inversion, immediately placed on ice, and centrifuged at 3500 rpm for 10 min at +4°C. Two aliquots of the resulting plasma were stored at -80°C until analyzed.

3.5 Phenotyping analysis

DPD phenotyping was evaluated by using the dihydrouracil-uracil concentration ratio (UH_2/U) in urine. U and UH_2 were quantified with liquid chromatography-tandem mass spectrometry (LC-MS-MS).

3.5.1 Chemical and reagents

Standards: Uracil (U) (Acros Organics, Ort, New Jersey, USA) and dihydrouracil (UH_2) (Sigma-Aldrich, Steinheim, Germany) were purchased as crystalline form, pure >95%.

Other reagents: Formic acid (98-100%) and sodium chloride (NaCl) were of analytical grade and were supplied by Merck, Darmstadt, Germany. Methanol (Roth, Karlsruhe,

Germany) was of HPLC grade. All water used was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA, USA).

3.5.2 Stock solutions, calibration standard samples

Standard solutions of U and UH_2 were prepared by dissolving U and UH_2 powder in methanol-water (1:1) at a concentration of 1,905.26 µM and 1,696.91 µM, respectively, and stored at -20°C. The working solutions for quantification in urine were prepared with 0.9% NaCl. Analytical standards in the concentrations of U/UH_2 of 210.56/115.16, 104.80/57.32, 20.90/11.43, 10.49/5.71, 2.08/0.90, and 1.04/0.57 µM, were prepared by diluting the stock solutions with 0.9% NaCl solution. QC samples (QC_{1-3}) were also prepared in the same way containing the following concentrations of U/UH_2: 116.04/258.72, 61.04/136.09, and 12.20/2.05 µM.

3.5.3 Urine sample preparation

Urine samples were centrifuged at 18,600 g for 10 min at 4°C, and 200 µL of the supernatant was transferred into HPLC glass vials (give manufacturer here). Twenty µL of the sample was injected into the LC-MS-MS system for detection.

3.5.4 LC-MS-MS analytical conditions

The LC-MS-MS system consisted of a Surveyor® MS pump (Firmware 1.1), a Surveyor autosampler (Firmware 2.1), fitted with a tempered tray and a column oven, coupled to a TSQ® Quantum triple quadrupole mass spectrometer (Thermo Finnigan, San José, CA, USA).

Separation was achieved by gradient elution with a mobile phase A (0.1% formic acid) and mobile phase B (pure methanol), programmed as follows: 0-0.5 min 97% A : 3% B, 0.5-1 min the conditions were changed gradually to the second mixture of mobiles phase, 1-4 min 90% A : 10% B, and 4-5 min 97% A : 3% B, at a flow rate of 0.3 mL/min through an Aquasil analytic column (100 x 3 mm, 5 µm particle size, Thermo Electron, Runcorn, UK) maintained at 20°C, preceded by a guard column (10 x 3 mm, 5 µm particle size) of the same material. Subsequent to sample injection, the

autosampler syringe and injection needle were repeatedly rinsed with methanol. Total duration of each sample analysis was 5 min.

The flow from the column was directed into a Thermo Finnigan TSQ® Quantum electrospray interface using nitrogen as both the sheath and auxiliary gas. The sheath gas was set at 60 instrument units, and flow-meter reading of auxiliary gas was set at 2 instrument units. A source collision-induced dissociation (CID) energy of 1.0 *e*V was applied in order to break up elution solvent clusters. Analytes were ionized by positive electrospray ionization (3500 V), and detected by tandem mass spectrometry (MS-MS) using the selected reaction monitoring (SRM) mode. The scan rate of the mass spectrometer for each SRM scan was set at 0.5 sec/scan. Integration of the detector output was performed using LC-Quan (rev. 1.3) software (Thermo-Electron, San José, USA) to determine peak areas.

3.5.5 Mass spectra and assay validation

Various procedures were performed to validate the assay according to the FDA guideline (U.S. FDA, 2001). Mass spectra of target compounds are shown in fig. 5. The ions used in the quantification were *m/z* 113 → 70.48 for U (RT: 3:94 min) and *m/z* 115.15 → 74.24 for UH_2 (RT: 3.70 min).

To quantify the compounds of interest in urine samples, calibration curves were obtained from mass chromatograms of calibration standards. The calibration standards and QC samples were processed as described above and analyzed by LC-MS-MS.

The lower limit of quantification (LLOQ) for U was 2.9 *μM*, with an accuracy and precision of 3.1% and 2.9%, respectively (n = 5). The LLOQ of UH_2 was 1.8 *μM*, with an accuracy and precision of 7.3% and 1.8%, respectively.

The r^2 of the calibration curve was 0.999 for U and 0.998 for UH_2. The intra-day variability of LC-MS-MS measurement was obtained by analysis of five QCs of each concentration on the same day. The intra-day precision of U varied between 2.6 and 3.1%, while for UH_2, this value varied between 1.1 and 6.2%. The accuracy at the tested concentrations ranged from 99.08-114.90% for U and 97.2-116.3% for UH_2 (table 7). Further validation, including inter-day assessment, did not carried out because this assay could be done in the one day.

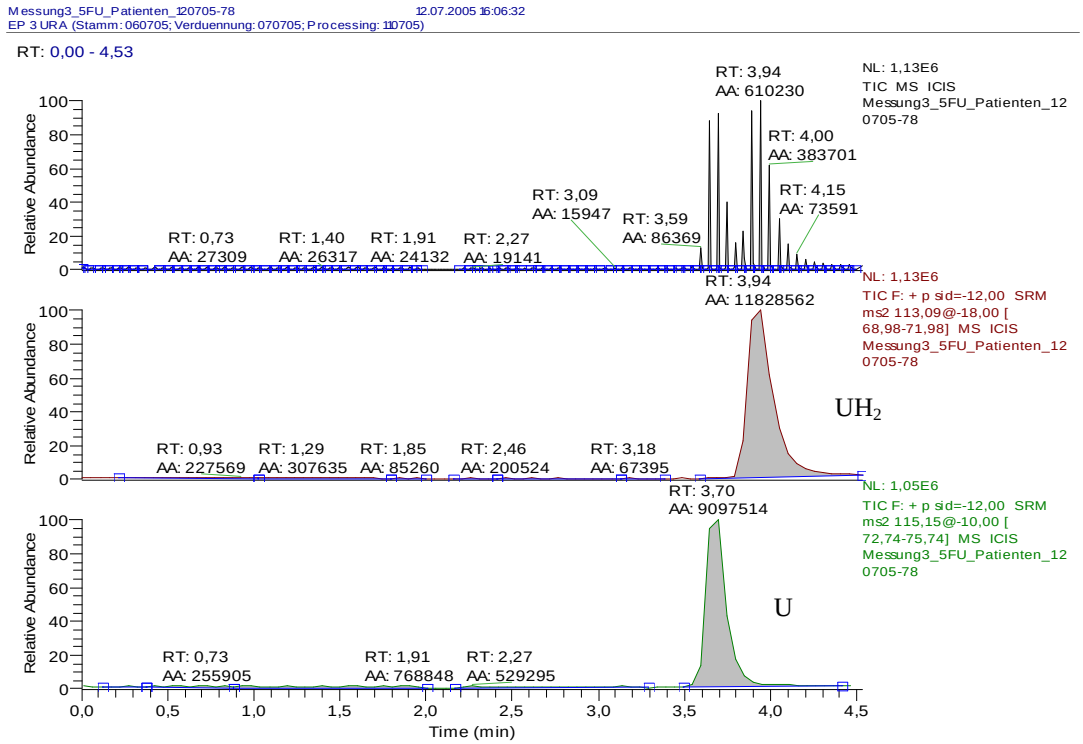


Figure 5 Mass spectra of U and UH_2

Table 7 Intra-day accuracy and precision for U and UH_2

Theoretical concentration (μM)	*n*	Experimental concentration (μM) (mean ± SD)	CV (%)	% deviation (accuracy)
Intra-day variability of U				
116.14	5	$118.91 \pm 3.14 \times 10^{-3}$	6	2.5
61.09	5	$60.93 \pm 2.71 \times 10^{-3}$	4.4	-0.2
12.21	5	$14.02 \pm 4.37 \times 10^{-4}$	3.1	14.9
Intra-day variability of UH_2				
258.72	5	$290.37 \pm 3.33 \times 10^{-3}$	1.1	12.4
136.09	5	$139.92 \pm 0.72 \times 10^{-3}$	6.2	2.8
27.20	5	$31.62 \pm 8.59 \times 10^{-4}$	2.9	16.3

3.6 Genetic analyses

3.6.1 Isolation of genomic DNA

DNA was isolated from EDTA-anticoagulated blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

Briefly, 200 µL of blood and 200 µL of buffer AL were added to 20 µL of proteinase K in a 2 mL microcentrifuge tube. The mixture was mixed by pulse-vortexing for 15 sec,

incubated at 56°C for 10 min, and briefly centrifuged to remove drops from the inside of the lid. Then, 200 µL of ethanol (96%) was added to the sample; the mixture was mixed by pulse-vortexing for 15 sec and briefly centrifuged. Then the samples were applied to the QIAamp column (in a 2 mL collection tube) and centrifuged at 8000 rpm for 1 min.

After centrifugation, the QIAamp column was placed in a clean 2 mL collection tube, and the tube containing the filtrate was discarded. 500 µL of buffer AW1 was given onto the QIAamp column, and the sample was centrifuged at 8000 rpm for 1 min, again the QIAamp column was placed in a new collection tube and the tube containing the filtrate was discarded. Thereafter, 500 mL of buffer AW2 was given onto the QIAamp column, and the sample was centrifuged at 14000 rpm for 5 min. The QIAamp column was placed in a clean 1.5 mL microcentrifuge tube and the collection tube containing the filtrate was discarded. Finally, 200 µL buffer AE was added onto the QIAamp column, the sample was incubated at room temperature for 1 min, and then centrifuged at 8000 rpm for 1 min. After centrifugation, the filtrate contained the DNA, and the QIAamp column was discarded. In average, a 200 µL of a whole blood sample typically yielded 6 µg of DNA in 200 µL in eluting buffer (30 ng/µL), which was in accordance with the value indicated by the manufacturer.

3.6.2 Analysis of the *DPYD* gene

3.6.2.1 Polymerase Chain Reaction amplification of *DPYD* genomic DNA

The polymerase chain reaction (PCR) was used to amplify 23 exonic regions of the *DPYD* gene from genomic DNA. The specific primers used to amplify each exon are listed in table 8.

The PCR amplification of all exons (except exon 1, see below) was performed in a 50-µL reaction mixture consisting of 5 µL PCR-buffer (10x), 4.6 µL $MgCl_2$ (25 mM), 2 µL dNTPs (10 mM), 1 µL of each primer (25 µM), 0.5 µL HotStarTaq DNA-polymerase (5 U/µL) (Qiagen, Hilden, Germany), 100 ng of genomic DNA of each patient used as the template (except for exon 3 and 8, 300 ng of genomic DNA were used in these cases), and purified water for molecular biology analysis (Sigma-Aldrich) was added to make 50 µL reaction volumes.

Amplification of exon 1 was carried out with 5 µL PCR-buffer (10x), 1 µL $MgCl_2$, 2 µL dNTPs, 1 µL of each primer, 2.5 µL DMSO (5%), 0.5 µL HotStarTaq DNA-polymerase, 100 ng of genomic DNA of each patient, and purified water was added to make 50 µL reaction volumes.

The PCR amplifications were run in a MultiCycler programmable thermal cycler-200 (MJ Research Laboratories, Waltham, MA, U.S.A). The PCR amplification program was as follows: denaturation at 95°C for 15 sec, 34 cycles of 30 sec at 94°C, 1 min at 50-60°C (the exact annealing temperature used for each exon is given in table 8) and 1 min at 72°C, and a final elongation step of 10 min at 72°C.

3.6.2.2 Gel documentation and purification of the PCR amplification product

After the PCR amplification, 5 µL of glycerol blue was added to each PCR product. The PCR product was separated by 80 mV electrophoresis on a 1.6% agarose gel containing 5 mM cytidine (Fluka) and visualized with ethidium bromide staining. An appropriate basepair marker was included into each gel electrophoresis run. The PCR-fragments were identified on the agarose gel by comparing the length of the fragment to the marker ladder, and photographically documented under UV transilluminator with UV light at a wavelength of 302 nm. The length of each PCR product that contained the exon investigated is listed in table 8.

Subsequently, the PCR product was purified using the QIAquick gel extraction kit (Qiagen). Briefly, the gel slice containing the DNA band of interest was excised and weighed in a vapor sterilized 2 mL tube. Three volumes of buffer QG were added to 1 volume of gel. The sample was incubated at 50°C for 10 min in a thermal block and mixed by vortexing the tube. One gel volume of isopropranol was added to the sample and the sample was mixed. The mixture was applied onto a QIAquick column (in a 2 mL containing tube) and centrifuged for 1 min at 13,200 rpm. The flow-through was discarded, and the QIAquick column was placed back into the same collection tube. For purification, 500 µL QG buffer was added to the QIAquick column, the sample was centrifuged for 1 min at 13200 rpm, and the flow-through was discarded.

After placing the QIAquick column into the same collection tube, 750 µL of buffer PE was added to QIAquick column for washing. The sample was incubated at room

temperature for 5 min, centrifuged at 13,200 rpm for 1 min, followed by discarding the filtrate. The QIAquick column was centrifuged again at 13,200 rpm for 1 min, the eluent was discarded, and the column was placed on a clean, sterile 1.5 mL tube. Finally, the DNA was eluted with 30 µL of buffer EB (10 mM Tris·Cl, pH 8.5) applying the buffer directly on the silica membrane of the QIAquick column. The QIAquick column was discarded. The eluent contained purified DNA which was either directly used for sequencing or stored at -20°C until analysis.

3.6.2.3 DNA sequencing

The DNA sequencing of each exon was carried out by the dideoxynucleotide chain termination method (enzymatic method) using BigDye Terminator kit V. 1.1 (Applied Biosystems). The sequencing reactions were prepared to analyze the 23 coding exons of the *DPYD* gene using sequencing primer sets as specified in table 9. The sequencing reactions were carried out in 10 µL reaction mixtures in microtubes containing 7 µL of purified PCR products used as the template, 1 µL of specific sequencing primer (3.2 µM), 1.5 µL of BigDye Terminator kit V.1.1, and buffer for BigDye Terminator Kit V.1.1 added to reach a total volume of 10 µL. However, for exon 1 this reaction consisted of 7 µL of purified PCR products, 1 µL of specific sequencing primer (3.2 µM), 4 µL of BigDye Terminator kit V 1.1, and deionized water added to reach a total volume of 20 µL.

Amplification of the sequencing reactions was performed in a MultiCycler PTC 200 (MJ Research) thermocycler, programmed for 25 temperature-step cycles of 96°C (10 s), 50-60°C (30 s; see annealing temperature for each exon in table 9), and 60°C (4 min) at a ramp speed of 1°C/s.

3.6.2.4 Purification of sequencing products

To generate high quality DNA sequence data, the sequencing products were purified under filtration procedure with MultiScreen HV plate (MAHVN45, Millipore).

Briefly, the dry G_{50} Superfine grade Sephadex® (G-50-50, Sigma-Aldrich) was filled into all wells of MultiScreen column loaders (Millipore). Then, 300 µL of distilled

water were added to the Sephadex® powder within each well. The plates were allowed to incubate at room temperature for 3 hours with no agitation, and then centrifuged at 910 rpm for 5 min 2 times for packing the mini-columns, and the filtrates were discarded.

Subsequently, the Sephadex-columns were placed in the MultiScreen HV plate with 15 μL of distilled water and followed by adding the sequencing reaction product to the center of each column. By centrifugation at 910 rpm for 5 min, the filtrate containing the purified sequencing products was collected in 96-well plates which were analyzed directly or stored at -20°C until analysis.

3.6.2.5 Sequence analysis

Ten μL of the purified sequencing product was filled in a MicroAmp Optical 96-well plate (Applied Biosystems) with 10 μL distilled water. The MicroAmp optical 96-well plate was closed with septa strip (Applied Biosystems) and briefly centrifuged at 910 rpm to ensure that each sample was positioned correctly at the bottom of the well. Sequence analysis was carried out using an ABI Prism® 3100 Genetic Analyzer 16 capillary sequencer (Applied Biosystems, Foster City, CA, U.S.A.).

Table 8 Oligonucleotides and annealing temperatures used to amplify genomic DNA for *DPYD* genotyping

Exon	Forward-Primer	5´→3´	Reverse-Primer	5´→3´	Annealing temperature (°C)	Fragment (bp)
1	F3x1DPD	GCGGACTGCTTTTACCTTTG	Rx1DPD	TGCTCTGCGGGTAGGTG	58	493
2	Fx2DPD	GTGACAAAGTGAGAGAGACCGTGTC	Rx2DPD	GCCTTACAATGTGTGGAGTGAGG	60	284
3	F2x3DPD	ATTTTTATTCCTCCAAACTTA	R2x3DPD	CCCAAATAATGAAGAATGACT	55	473
4	Fx4DPD	GGTAGAAAATAGATTATCTC	R2x4DPD	GATTTGCTAAGACAAGCTG	55	245
5	Fx5DPD	GTTTGTCGTAATTTGGCTG	Rx5DPD	ATTTGTGCATGGTGATGG	55	284
6	Fx6DPD	GAGGATGTAAGCTAGTTTC	Rx6DPD	CCATTTGTGTGCGTGAAGTTC	50	357
7	Fx7DPD	GTCCTCATGCATATCTTGTGTG	Rx7DPD	GCTTCTGCCTGATGTAG	55	360
8	Fx8DPD	GCCCCACATCGTGCTATGAAC	Rx8DPD	GTCTGAAGGCAGTCATTCTG	57	461
9	Fx9DPD	CCCTCCTCCTGCTAAT	R2x9DPD	GAACAATGTGCTGCTGAG	55	242
10	Fx10DPD	GATAGTGACACTTCATCCTG	Rx10DPD	CTGTTGGTGTACAACTC	55	340
11	Fx11DPD	ACTGGTAACTGAAACTCAG	Rx11DPD	CAATTCCCTGAAAGCTAG	52	442
12	F2x12DPD	ACGACTCACTATAGGGCA	Rx12DPD	GAAGCACTTATCCATTGG	55	453
13	Fx13DPD	CGGATGACTGTGTTGAAGTG	Rx13DPD	TGTGTAATGATAGGTCGTGTC	57	439
14	F2x14DPD	TCCTCTGCAAAAATGTGAGA	Rx14DPD	CAGCAAAGCAACTGGCAGATTC	60	415
15	F2x15DPD	GTTTTGCTATCTTACCCTGCTA	R2x15DPD	AAAGAGTGTATGGATTCAGAGA	55	504
16	Fx16DPD	AACGGTGAAAGCCTATTGG	Rx16DPD	TAGTAACTATCCATACGGGGG	50	223
17	Fx17DPD	CACGTCTCCAGCTTTGCTGTTG	Rx17DPD	CGGGCAACTGATTCAAGTCAAG	55	269
18	Fx18DPD	TGGGATGTGAGGGGGTGAATG	Rx18DPD	TTCAGCAACCTCCAAGAAAGCCAC	60	247
19	Fx19DPD	TGTCCAGTGACGCTGTCATCAC	Rx19DPD	CATTGCATTTGTGAGATGGAG	60	300
20	Fx20DPD	GAGAAGTGAATTTGTTTGGAG	Rx20DPD	CACAGACCCATCATATGGCTG	60	424
21	F3x21DPD	GCGAAGTACCTTTGCTATT	R3x21DPD	TGCTGGTTGTGTTATCATACTATA	50	1287
22	Fx22DPD	GAGCTTGCTAAGTAATTCAGTGGC	Rx22DPD	AGAGCAATATGTGGCACC	60	291
23	F2x23DPD	CCCCAAAATCCACAGGTAGAAGAC	Rx23DPD	GGTGACATGAAAGTTCACAGCAAC	60	269

Table 9 Sequencing primers and annealing temperatures used to sequence DPYD

Exon	Sequencing Primer	5´→3´	Annealing temperature (°C)
1	F2x1DPD	CTCTACTCCCTCCCTCCCTTCT	60
2	SeqFx2DPD	AAACAAATGCCAACATATTTC	55
3	SeqF2x3DPD	CCATGATCAATATAATC	50
4	SeqRx4DPD	CCCACAGATAATAGAGAA	55
5	SeqRx5DPD	TGCATGGTGATGGTAGTG	55
6	SeqRx6DPD	ATTGTTTTGCTCCATCATTTCT	55
7	SeqFx7DPD	ATTTTTCTACTGATGCCTGTT	55
8	SeqFx8DPD	TAATGACACTGGCTTTTCTTC	55
9	SeqRx9DPD	GCTGAGCTTGATTTTGA	55
10	SeqFx10DPD	TAGTGACACTTCATCCTGGA	55
11	SeqFx11DPD	CTGCATATTGACTTAATATCA	55
12	SeqFx12DPD	CGTTAGCTTTTCATTTTTATAG	55
13	SeqFx13DPD	ATGCTGTGTTGAAGTGAT	55
14	SeqFx14DPD	TCTGCAAAAATGTGAGAAG	55
	SeqRx14DPD	GCAAAGCAACTGGCAGATTCTT	55
15	SeqFx15DPD	ATCTTACCCTGCTATTTTCTA	55
16	SeqFx16DPD	GGTGAAAGCCTATTGGTATAT	55
17	SeqFx17DPD	CCAGCTTTGCTGTTGTTCCAGTAC	55
18	SeqFx18DPD	GAGGGTTTGAATGGGTTTTAAC	55
19	SeqFx19DPD	CAAGTGGTCAGTGTGCTAAC	55
20	SeqRx20DPD	TGGCTGTAATCAAGTCTC	55
21	SeqRx21DPD	GCAGTAAATAAACATTTTAAC	55
22	SeqRx22DPD	GCCATAAAAACAAGAAGAAAC	55
23	SeqFx23DPD	CCTTTGTGGTCAGTGACATC	55

3.6.3 Analysis of thymidylate synthase (*TS)* gene

PCR analysis of thymidylate synthase (*TS)* gene polymorphism was performed as previously described by Iacopetta et al. (2001). PCR amplification of the TS promoter enhancer region containing the double and triple tandem repeats was carried out using the following primers: Forward 5´ AAAAGGCGCGCGGAAGGGGTCCT 3´

Reverse 5´ TCCGAGCCGGCCACAGGCAT 3´

PCR reactions were carried out in 42 µL volumes containing 4 µL of DNA preparation, 0.2 µL of *Rapidozym Polymerase* (5U/µL) (Rapidozym, Berlin, Germany), 0.5 µL of each primer at a final concentration of 10 µM, 3.0 µL $MgCl_2$ at a final concentration of

50 *mM*, 4 µl DMSO, 4.2 µL of 10 x buffer without Mg^{2+}, and 1.0 µL of dNTPs (2 *mM*), finally, purified water for molecular biology analysis (Sigma-Aldrich) was added to make 42 µL reaction volumes.

Following hot-start denaturation at 94°C for 4 min after which time DNA was added, a total of 32 PCR cycles were performed in a MultiCycler programmable thermal cycler-200 (MJ Research) under the following conditions: denaturation at 94°C for 40 s, annealing at 62°C for 40 s and extension at 72°C for 1 min. Final extension was at 72°C for 5 min.

PCR products containing triple repeats (144 bp) were distinguished from those containing double repeats (116 bp) by electrophoretic separation on 3% agarose gels. Patients who were homozygous for the triple repeat (3R/3R) displayed only the 144-bp PCR product; those homozygous for the double repeat (2R/2R) displayed only the 116-bp PCR product, while heterozygous individuals (2R/3R) showed both 144- and 116-bp PCR products.

3.6.4 Analysis of methylenetetrahydrofolate reductase (*MTHFR)* gene

Genotyping for the methylenetetrahydrofolate reductase (*MTHFR)* C677T polymorphism was performed using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method reported by Shrubsole et al. (2004) with minor modifications.

The PCR reactions were run in a MultiCycler programmable thermal cycler-200 (MJ Research). Each 40 µL of PCR mixture contained 2 µL of DNA, 4 µL of 10 x PCR buffer without Mg^{2+}, 1 µL of $MgCl_2$ (50 *mM*), 1 µL of dNTPs (2 *mM*), 0.5 µL of each 10 *mM* primer (forward primer 5´ TGAAGGAGAAGGTGTCTGCGGGA 3´ and reverse primer 5´ AGGACGGTGCGCTGAGAGTG 3´), 1.2 µL of *Rapidozym Polymerase*, and purified water for molecular biology analysis was added to make 40 µL reaction volumes.

The reaction mixture was initially denatured at 94°C for 2 min. The PCR was carried out in 38 cycles of 94°C for 20 s, 64°C for 10 s and 72°C for 10 s. The PCR was completed by a final extension cycle at 72°C for 7 min.

After complete PCR amplification, PCR products were separated electrophoretically in a 3% agarose gel. Each positive PCR product (198 bp fragment) was digested with *Hinf* I (Fermentas) in a 10-µL reaction volume, containing 5 µL PCR product, 3.5 µL purified water for molecular biology, 1 µL 5 x Tango buffer, and 0.5 µL (10 units) of *Hinf I*, at 37°C for 12 hours. The products of the incubations with the restriction enzyme from each patient were loaded with 10 µL bromphenol blue on a 3.2% agarose gel and detected by ethidium bromide staining. The C→T substitution at nucleotide 677 creates a *Hinf* I digestion site. The PCR product (198 bp) with the T allele was digested to two fragments (175 bp and 23 bp), whereas the PCR product with the wild-type C allele was not digested by *Hinf* I.

3.7 Analytical assay for pharmacokinetic studies

3.7.1 Chemical and Reagents

Analytical standards: 5-FU (Sigma, Steinheim, Germany) was purchased as crystalline form, pure >95.0%, and its metabolite, 5-fluoro-5,6-dihydrouracil (5-FUH_2), (26.5% pure) was supplied by Syncom (Groningen, the Netherlands). 5-Chlorouracil (5-CU), the internal standard, was obtained from Acros Organics, New Jersey, USA.

Other reagents: Potassium dihydrogenphosphate (KH_2PO_4) and orthophosphoric acid were of analytical grade. Ethyl acetate, acetonitrile, and isopropranol were of HPLC grade. These reagents were purchased from Merck (Darmstadt, Germany). All water used in the study was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, USA). Pooled drug-free plasma samples from healthy volunteers were used for the validation of the method.

3.7.2 Stock solutions and standards

The drugs were dissolved in Milli Q water to yield a stock solution of 1 mg/mL each. Working solutions of 5-FU, 5-FUH_2 and 5-CU were prepared by dilution of the stock solution in KH_2PO_4 (50 *mM*, adjusted to pH 4 with 85% orthophosphoric acid). All solutions were stored at -20°C.

3.7.3 HPLC instrumentation and conditions

The HPLC system consisted of a Water 2690 Separations Module (Waters, Milford, MA, USA) with a PDA Waters 996 photodiode array detector. Detection of 5-FU, 5-FUH_2 and 5-CU were carried out at 265, 220 and 270 nm, respectively. Separation of compounds was performed on an Ultrasphere ODS C_{18} analytical column (5 µm, 250 x 4.6 mm, Beckman Coulter^{TM}, Fullerton, CA, USA), operating at 25˚C (column heater).

Elution was performed under gradient condition controlled by a gradient proportioning valve. The elution program was: mobile phase A (50 mM K_2HPO_4) for 17 min, mobile phase B (pure acetonitrile) 0-50% over 1 min and maintained at 50% for 5 min. Initial conditions were restored by decreasing mobile phase B to 0% over 1 min, and the column was equilibrated with 100% mobile phase A for 5 min. For 5-FU analytics, the pumps were run at a flow rate of 1.0 mL/min, while for 5-FUH_2, a flow rate of 0.8 mL/min was chosen. Analytical run-time was 25 min. Instrument control, chromatogram recording and peak integration was performed with the Millennium 2.1 software (Waters).

3.7.4 Sample preparation

Plasma samples were allowed to thaw at room temperature and vortexed briefly. The plasma sample (700 µL) was pipetted into a 15-mL glass tube with a PTFE-lined screw cap. After addition of the internal standard (20 µL 5-CU, 100 µg/mL), 7 mL of ethyl acetate-isopropranol (95:5) were added as extraction solvent and then vortexed for 30 s. Following centrifugation (10 min, 3000 rpm) to separate the phases, the entire organic layer was transferred into a conical glass centrifuge tube and evaporated to dryness in a vacuum rotation evaporator (Jouan GmbH, St. Herbleain, France) at room temperature. The dried residue was dissolved in 100 µL of 50 *mM* KH_2PO_4, vortexed for 30 s, and 40 µL were injected into the HPLC system.

3.7.5 Calibration and calculation

Standard plasma calibration samples of 5-FU were prepared by spiking blank plasma with an appropriate volume of working solution to give concentrations in the range

0.005-75.0 μg/mL. Similarly, calibration curves for 5-FUH_2 were prepared by adding 5-FUH_2 in concentrations of 0.01-7.5 μg/mL.

Four quality control samples (QC_{1-4}) of 5-FU were prepared in plasma at concentrations of 0.015, 0.15, 1.0 and 75.0 μg/mL, and for 5-FUH_2 the QC_{1-4} were 0.03, 0.15, 1.0 and 3.5 μg/mL. These samples were subjected to the same sample preparation procedure as described above.

Calibration curves were constructed by plotting peak area ratios of each analyte to that of the internal standard *(y)* versus the nominal plasma drug concentration *(x)*. The line of best fit was determined using weighted (1/*x*) linear least-squares regression analysis. 5-FU and 5-FUH_2 concentrations of controls and unknown samples were calculated from the calibration curves using the software. To evaluate linearity of the calibration curves, 5 calibration curves were prepared and analyzed. The curves were judged linear if the correlation coefficient r was >0.99 as calculated by weighted linear regression.

3.7.6 Analytical method validation

The analytical method validation employed for the quantitative determination of 5-FU and 5-FUH_2 in plasma was performed according to the guidance proposed by Shah et al. (1992) and according to the FDA guideline on analytical method validation (2001). 5-FU and 5-FUH_2 were validated separately.

3.7.6.1 Validation of 5-FU

Specificity and retention time

Several human plasma samples from different healthy subjects were tested for the absence of interfering compounds. The retention times of endogenous compounds in plasma were compared with those of 5-FU and the internal standard (5-CU). Under the conditions of the assay, 5-FU and 5-CU were eluted at about 5.4 min and 10.7 min, respectively. No significant interfering peaks that could affect the compounds of interest were observed (fig. 6).

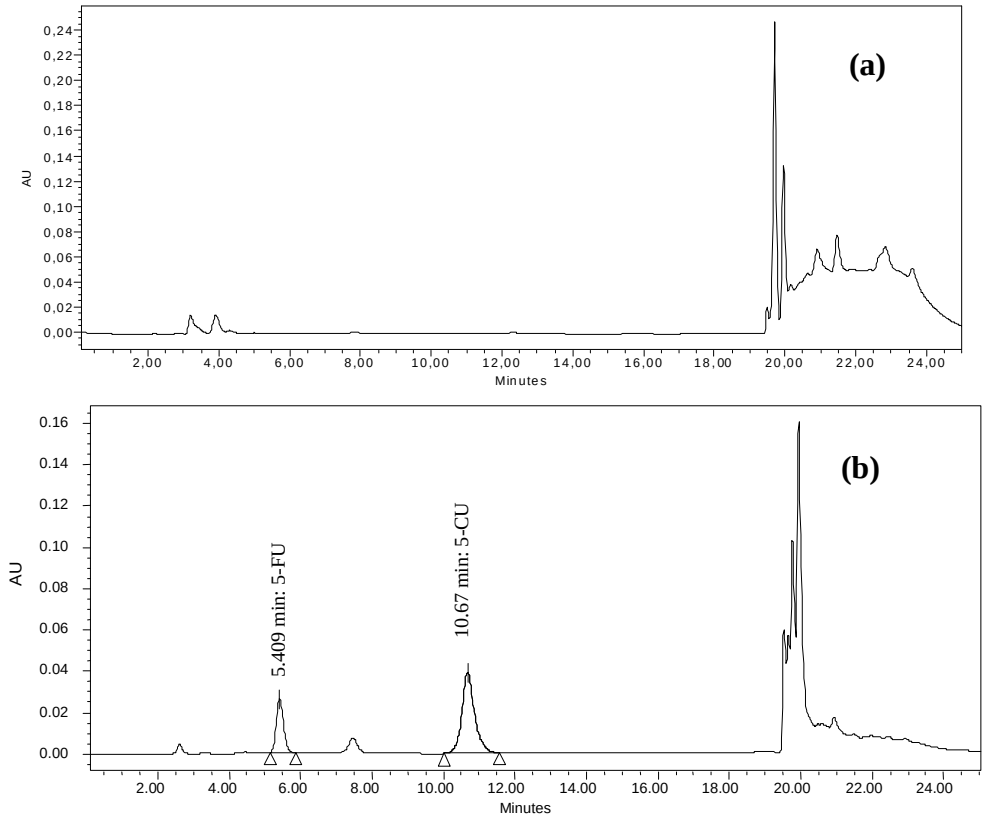


Figure 6 Chromatographic separation of 5-FU and internal standard 5-CU

(a) blank plasma; (b) blank plasma spiked with 5-FU (1.0 µg/mL) and 5-CU eluted at 5.4 and 10.7 min, respectively

Extraction recovery

The overall recoveries of 5-FU, expressed as the ratio peak heights of the validation QC samples low (0.015 µg/mL), middle (1.0 µg/mL) and high (75.0 µg/mL) concentrations to those in corresponding standard solutions, was 65.16 ± 2.67% (n = 9): mean recoveries of 5-FU was 61.72 ± 0.56, 65.60 ± 0.13 and 68.15 ± 0.29% for the low, middle and high concentrations, respectively. The recovery of internal standard (5-CU) measured at the concentration used in the analysis (100 µg/mL) was 72.73% (n = 3).

Determination of the lower limit of quantification (LLOQ)

The LLOQ is the lowest concentration of the standard curve that can be measured with acceptable accuracy and precision (CV ≤20%). The LLOQ of 5-FU was 0.005 µg/mL (n = 5); the signal-to-noise ratio at this concentration was about 5. The precision and the accuracy were 6.32% and 104%, respectively.

Linearity, accuracy and precision

The standard curve for 5-FU over the range 0.005-75 µg/mL exhibited good linearity with average of correlation coefficient (r^2) = 0.999295 (table 10). The deviation of the interpolated concentrations of standards in the daily calibration curves of 5-FU were within the acceptable range of 85-115%. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards (table 11).

Table 10 Calibration standard concentrations of 5-FU (*5 analytical runs*)

Theoretical concentration (µg/mL)	Experimental concentration (µg/mL)					
	Day 1	Day 2	*Day 3*	Day 4	Day 5	Mean ± SD
0.005	0.0044	0.0044	0.0052	0.0045	0.0046	$0.0047 \pm 2.73 \times 10^{-4}$
0.0075	0.0074	0.0074	0.0073	0.0074	0.0074	$0.0074 \pm 4.76 \times 10^{-5}$
0.01	0.0101	0.0097	0.0094	0.0092	0.0104	$0.0098 \pm 4.39 \times 10^{-4}$
0.1	0.107	0.108	0.101	0.110	0.0977	$0.105 \pm 4.62 \times 10^{-4}$
5.0	5.16	5.21	5.17	5.30	5.28	5.22 ± 0.06
25.0	26.03	26.24	26.00	26.13	25.61	26.00 ± 0.21
75.0	73.80	73.55	73.82	73.56	74.11	73.77 ± 0.21
r^2 (a)	0.999360	0.999060	0.999381	0.999066	0.999606	$0.999295 \pm 2.08 \times 10^{-4}$

r^2 = correlation coefficient of the linear regression analysis, (a) = linear weighted regression, formula: y = a + bx

Table 11 Calibration standards of 5-FU *(5 analytical runs)*

Parameter	Calibration standard (µg/mL)						
	0.005	0.075	0.01	0.1	5.0	25.0	75.0
Mean	0.00467	0.0074	0.0098	0.105	5.22	26.00	73.77
%CV	5.89	0.65	4.50	0.44	1.11	0.81	0.28
%deviation	-6.34	-1.81	-2.36	+5.0	+4.5	+4.01	-1.64

CV = coefficient of variation

To assess the accuracy and the precision of the method, five replicates per concentration of QCs were analyzed on the same day and once a day during 5 days to determine the intra- and inter-day reproducibility, respectively.

The precision of the method at each concentration was calculated as the coefficient of variation (CV). A CV of less than 15% was accepted, except for the LLOQ, where it should not be more than 20%. The accuracy of the procedure was evaluated by calculating the relative difference (% deviation) between the measured mean concentrations and the theoretical concentrations. The method was considered accurate when the deviation from the theoretical concentration was less than 20% at the LLOQ

and less than 15% at the remaining levels. The data for the validation of intra- and inter-day accuracy and precision of the method are presented in table 12. The results show CVs which were within the acceptable range.

Table 12 Intra-day and inter-day accuracy and precision of the method for 5-FU

Theoretical concentration (µg/mL)	n	5-FU		
		Experimental concentration (µg/mL) (mean ± SD)	CV (%)	% deviation (accuracy)
Intra-day variability				
0.015	5	$0.015 \pm 1.09 \times 10^{-3}$	7.15	0
0.15	5	$0.1498 \pm 3.12 \times 10^{-3}$	2.07	0.13
1.0	5	1.06 ± 0.016	1.50	5.60
75.0	5	75.01 ± 0.26	0.34	0.011
Inter-day variability				
0.015	5	$0.0144 \pm 4.9 \times 10^{-4}$	3.40	4
0.15	5	$0.151 \pm 1.63 \times 10^{-3}$	1.08	0.4
1.0	5	$1.078 \pm 9.96 \times 10^{-3}$	0.92	7.8
75.0	5	74.99 ± 0.052	0.07	9.33×10^{-3}

Stability

Stability testing of the 5-FU was performed on both in plasma and stock solution. For stability in plasma, QCs of 0.015 and 75 µg/mL were analyzed in replicate (n = 3) for determination of long-term stability, short-term temperature stability, freeze thaw stability and post-preparative stability.

Three aliquots of each QCs were thawed at room temperature (20°C at day-light exposure) and kept at this temperature for 6 hours and analyzed. The long-term stability in frozen human plasma (-80°C) was determined by storing 3 aliquots of each QCs under the same conditions as the study samples (at -80°C) and periodic analysis over 3 months. Prior to their analyzes, samples were brought to room temperature and vortex-mixed well. Samples were analyzed immediately after preparation (reference values or freshly prepared QCs) and after storage.

The freeze-thaw stability was determined after 3 refreeze and thaw cycles. Three aliquots at each of QCs were stored at the -80°C for 24 h and thawed unassisted at room temperature. When completely thawed, the samples were refrozen for 24 h under the

same conditions. The freeze-thaw cycles were repeated two more times, and then analyzed immediately on the third cycles.

The stability of processed samples (extracted plasma samples), including the resident time in the autosampler, was also determined by analyzing three aliquots at each QC over a period of 24 h. The stored samples in autosampler (4°C) were determined concentrations on the basis of original calibration standards (freshly prepared samples).

For the stability of stock solution of 5-FU, three aliquots of were injected into the chromatograph immediately after preparation (time 0) and at 6 h after bench-top storage at room temperature.

The drug was considered stable if more than 90% of the intact drug was retained at the end of the study period. 5-FU is quite stable under the conditions of storage chosen in this study (-80°C). It was found to be stable during three freeze thaw cycles and during incubation after 6 hours at room temperature. The long- term stability of 5-FU in plasma samples stored at -80°C was investigated for 3 months. Compared to the reference values, there was no statistical difference. Processed plasma samples containing 5-FU and 5-CU in mobile phase were also found to be stable at room temperature in the autosampler for 24 h. Stock solution of 5-FU was also stable for at 3 months if stored at -20°C (table 13).

Table 13 Stability of 5-FU *(n = 3)*

Stability condition	Time	Concentration (µg/mL)	% recovery (mean ± SD)
5-FU in plasma			
Storage, -80˚C	3 months	0.015	103.12 ± 2.25
		75.0	101.60 ± 3.08
		Overall % recovery	102.36 ± 2.80
Bench top, 20˚C	6 hours	0.015	100.76 ± 0.05
		75.0	95.87 ± 1.79
		Overall % recovery	98.32 ± 2.75
Freeze and thaw (x3)		0.015	101.26 ± 1.52
		75.0	100.43 ± 2.43
		Overall % recovery	100.71 ± 1.95
5-FU in extracted samples			
On-system (4˚C)	24 hours	0.015	92.49 ± 1.64
		75.0	93.90 ± 2.46
		Overall % recovery	93.20 ± 2.21
5-FU in stock solution			
Bench top, 20°C	6 hours	1.00 mg/mL	99.47 ± 0.28

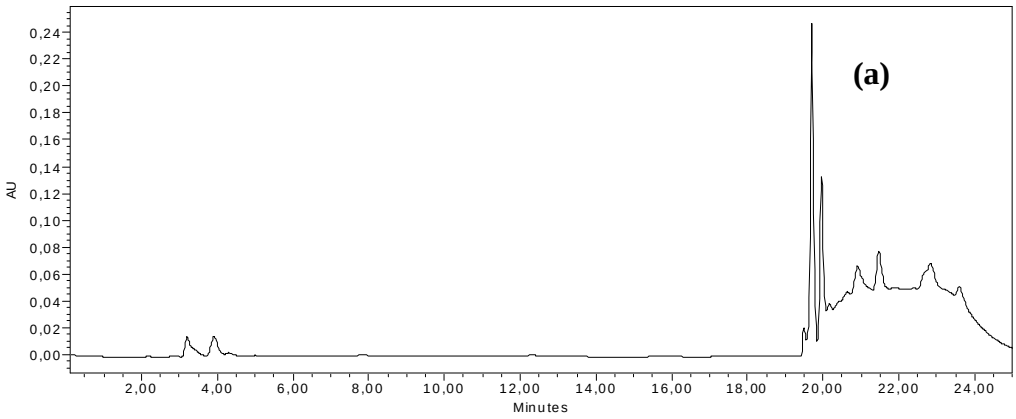
3.7.6.2 Validation of 5-FUH_2

The analytical method validation employed for the quantitative determination of 5-FUH_2 in plasma was performed according to the guidance proposed by Shah et al. (1992) and according to the FDA guideline on analytical method validation (2001). The validation method of 5-FUH_2 were determined in a similar way to the validation of 5-FU, this part presents only the result of validation of 5-FUH_2.

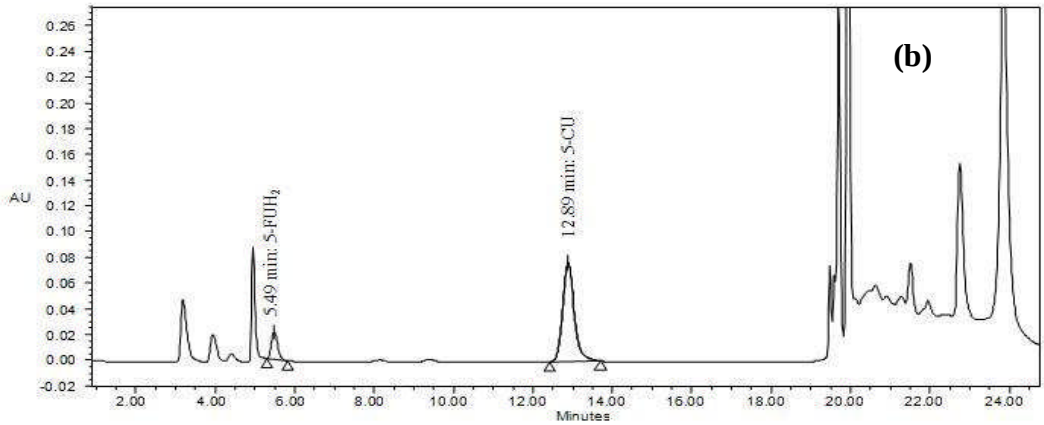
Specificity and retention time

The retention time of 5-FUH_2 and 5-CU (internal standard) was approximately 5.49 min and 12.89 min, respectively (fig. 7).

Figure 7 Chromatographic separation of 5-FUH_2 and internal standard 5-CU

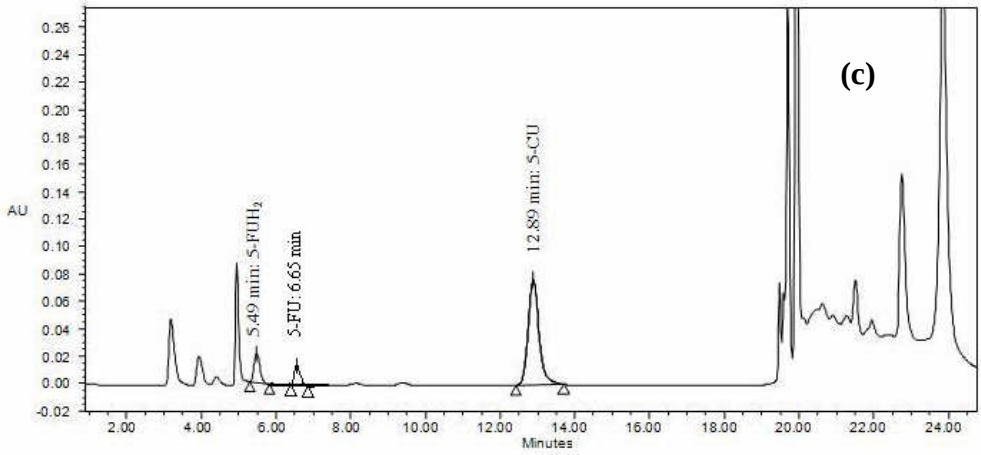


(a) Blank plasma extract



(b) blank plasma sample spiked with 5-FUH_2 (1.0 µg/mL) and 5-CU which eluted at 5.48 and 12.89 min, respectively

Figure 7 Cont.



(c) blank plasma sample spiked with 5-FUH_2 (1.0 µg/mL), 5-FU (1.0 µg/mL) and 5-CU which eluted at 5.49, 6.7 and 12.89 min, respectively, the chromatogram was recorded at 220 nm

Extraction recovery

The overall recovery of 5-FUH_2 averaged 70.93 ± 1.8 (n = 9): mean recoveries at low (0.03 µg/mL), middle (1.0 µg/mL), and high QCs (3.5 µg/mL) was 71.17 ± 2.2%, 70.40 ± 1.63%, and 71.23 ± 1.35, respectively.

Determination of the lower limit of quantification (LLOQ)

The LLOQ of 5-FUH_2 was 0.01 µg/mL (n = 5); the signal-to-noise ratio at this concentration was about 5. The precision and the accuracy were 1.96% and 93.78%, respectively.

Linearity, accuracy and precision

The calibration curves of 5-FUH_2 were linear (average r^2 = 0.99905; table 14). The deviation from 5 validation analytical runs (5 days) was within the acceptable range of 85-115% (table 15).

The intra-day and inter-day precisions at the concentrations of the four QCs were <8% and <4%, respectively, and the average accuracy showed values ranging within the acceptable range of 85-115%. A summary of the analysis is presented in table 16.

Table 14 Calibration standard concentrations of 5-FUH$_2$ (*5 analytical runs*)

Theoretical concentration (µg/mL)	Experimental concentration (µg/mL)					
	Day 1	Day 2	*Day 3*	Day 4	Day 5	Mean ± SD
0.01	0.0094	0.0114	0.0091	0.00814	0.0081	$0.0093 \pm 1.20 \times 10^{-3}$
0.025	0.0275	0.0278	0.0260	0.0260	Excluded	$0.0258 \pm 2.31 \times 10^{-3}$
0.05	0.0490	0.0510	Excluded	0.0520	0.0540	$0.0525 \pm 1.80 \times 10^{-3}$
0.1	0.0870	0.0864	0.1143	0.1021	Excluded	0.0974 ± 0.011
0.5	0.5313	0.4369	0.5281	0.5142	0.5600	0.514 ± 0.041
1.0	1.0334	0.9963	1.0255	1.0249	1.00	1.016 ± 0.015
7.5	7.4462	7.5762	7.4363	7.4570	7.4380	7.47 ± 0.053
r^2 (a)	0.999389	0.99755	0.999410	0.999787	0.9991	$0.99905 \pm 7.8 \times 10^{-4}$

r^2 = correlation coefficient of the linear regression analysis, (a) = linear weighted regression, formula: y = a + bx

Table 15 Calibration standards of 5-FUH$_2$ *(5 analytical runs)*

Parameter	Calibration standard (µg/mL)						
	0.01	0.025	0.05	0.1	0.5	1.0	7.5
Mean	0.0093	0.0258	0.0525	0.0974	0.514	1.016	7.47
%CV	12.93	8.95	3.43	11.29	7.98	1.48	0.71
%deviation	-7.2	+3.2	+5.0	+2.6	+2.8	+1.6	-0.4

CV = coefficient of variation

Table 16 Intra-day and inter-day accuracy and precision of the method of 5-FUH$_2$

Theoretical concentration (µg/mL)	n	5-FUH$_2$		
		Experimental concentration (µg/mL) (mean ± SD)	CV (%)	% deviation (accuracy)
Intra-day variability				
0.03	5	$0.0322 \pm 1.72 \times 10^{-3}$	0.53	7.33
0.15	5	$0.149 \pm 4.12 \times 10^{-3}$	2.76	-0.67
1.0	5	1.009 ± 0.017	1.68	0.9
3.5	5	3.461 ± 0.071	2.05	-1.11
Inter-day variability				
0.03	5	$0.033 \pm 2.50 \times 10^{-3}$	7.58	10
0.15	5	$0.150 \pm 5.84 \times 10^{-3}$	3.89	0
1.0	5	1.03 ± 0.052	5.05	3
3.5	5	3.48 ± 0.052	9.20	0.57

Stability

We assumed that 5-FUH$_2$ might not be stable because instability of 5-FUH$_2$ had been observed previously under various conditions (van den Bosch et al., 1987; Ackland et al., 1997; Casale et al., 2002; Maring et al., 2005). Thus, in this study the stability testing of 5-FUH$_2$ was not performed. However, to avoid the degradation of 5-FUH$_2$ during analysis, the optimum requirements for storage and processing during the analysis was followed as described below according to the previous reports.

- Due to the instability of 5-FUH_2 in plasma at room temperature (Maring et al., 2005; van den Bosch et al., 1987), thus the samples were placed on ice immediately after the collection, separate the plasma as quickly as possible and freeze it (-28°C or -80°C) until analysis.
- 5-FUH_2 appears stable only 2 freeze-thaw cycles (Maring et al., 2005; van den Bosch et al., 1987), once-thawed samples were used and thawing was proceed to 4-5°C.
- At low concentration of 5-FUH_2 in extracted and reconstituted plasma sample appears stable for no longer than 20 h at ambient temperature (Maring et al., 2005; Ackland et al., 1997), therefore each analytical run was not longer than 20 h and the cooling of the autosampler compartment was set at 4°C.
- For all standards, controls, stock solution and standard solution of 5-FUH_2 was diluted with K_2HPO_4 and prepared weekly and stored at -20°C in the dark when not in use as recommended by Casale et al (2002). Besides, the QCs samples and standard calibration samples were prepared freshly for every analytical run.

3.7.7 Application to clinical pharmacokinetic studies

As shown above, the present method fulfilled the requirements for validation of bioanalytical methodologies (Shah et al., 1992; FDA, 2001), making this method suitable for the quantification of plasma concentrations of 5-FU and 5-FUH_2. For routine use, separate calibration samples for 5-FU and 5-FUH_2 were included in each analytical run in duplicate (in the beginning and at the end of each sample queue). An analytical run consisted of 8 QC samples, twice 9 calibration standards (including blank plasma and a zero sample), and all processed unknown samples of two patients of the study (9 samples/patient) to be analyzed as one batch. After each 4^{th} sample with unknown 5-FU or 5-FUH_2 content, 2 QCs were analyzed. Each analytical run took about 20 h.

For accepting the results of an analytical run, at least 2/3 of the QC samples had to be within ±15% of their respective values; 1/3 of the QC sample (not all replicates at one concentration) were allowed to be outside the ±15% range of the nominal value. If the QC sample of the particular run did not meet these criteria, the run was repeated.

In case a single patient sample with unknown content of the analytes deviated from the theoretical level to be expected according to the individual concentration versus time profile, this sample was verified by repeating the analysis. When the initial result was confirmed (difference <15%), the first value was reported. If the difference between two determinations ranged between 15-30%, the mean of these two values was reported. If the difference was >30%, a third determination was performed. If the difference between any two of these three values was <15%, the median of these values was reported.

Analysis of plasma concentrations in the samples of the 30 patients of the study was performed as described above. Figure 8 depicts the chromatograms of a plasma sample at t = 36 h after the beginning of continuous infusion over 5 days of 5-FU.

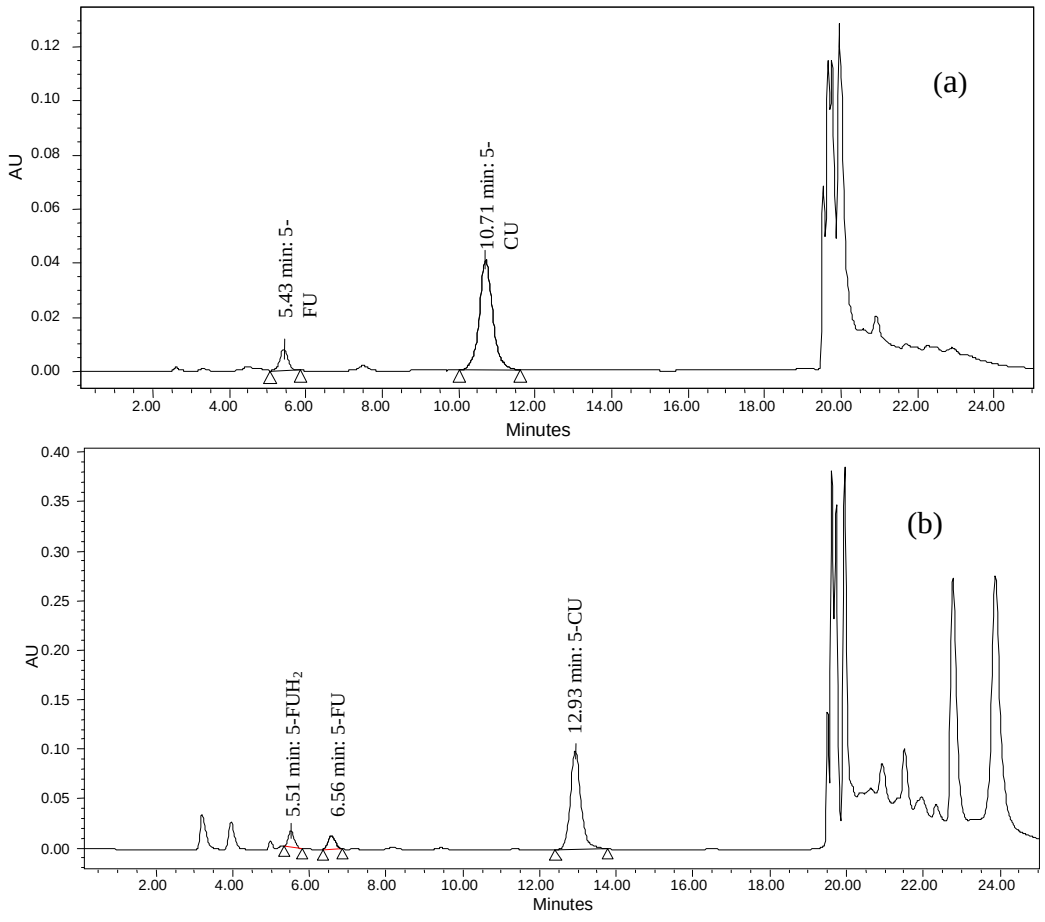


Figure 8 Representative Chromatograms of a patient plasma sample at 36 h after the start of a continuous infusion over 5 days of 5-FU

(a) Chromatogram of a patient plasma sample of 5-FU monitored at 265 nm (flow rate of 1.0 mL/min); (b) Chromatogram of a patient plasma sample of 5-FUH_2 monitored at 220 nm (flow rate of 0.8 mL/min).

3.8 Data analysis

3.8.1 Population pharmacokinetic model development

The NONMEM program version V, level 1.1 (NONMEM Project Group, UCSF, San Francisco, CA, U.S.A.) was used to develop a compartmental population pharmacokinetic model to describe the pharmacokinetics of 5-FU and its main metabolite (5-FUH_2) and their variability (interindividual and residual), as well as the influence of covariates on disposition of 5-FU and 5-FUH_2. Fitting was performed with the "first-order conditional estimates" algorithm, taking interaction between the parameters into account. Additional statistical analysis was done with Statistical Product and Service Solutions (SPSS) for Windows, version 11.0 (SPSS Inc. Chicago, IL, U.S.A.) to confirm the results obtained from the NONMEM analysis.

3.8.1.1 Algorithm of model building:

Step 1: building a basic model without covariates, including

- development of a structural pharmacokinetic model for 5-FU
- development of a combined structural pharmacokinetic model for 5-FU and 5-FUH_2: the dataset included concentrations of both 5-FU and 5-FUH_2; model parameters allowed the simultaneous estimation of 5-FU and 5-FUH_2 pharmacokinetics

Step 2: building the final model including all relevant covariates

Step 3: model assessment

In order to characterize that the present model is representative of the data in hand, the following criterias were taken into account:

a) Difference in minimum value of objective function (ΔOFV)

The goodness-of-fit (GOF) of the model to the data was evaluated based on changing in minimum value of objective function (OFV), which is proportional to minus twice the log-likelihood, provided by NONMEM. The discrimination models were compared by using a log-likelihood ratio test, which is the ΔOFV for the two models and which was referenced to its asymptotic χ^2 distribution (with *n* degrees of freedom equal to the

difference in the number of parameters between different models). The ΔOFV of ≥3.84 was required to indicate that the model with the lowest OFV was associated with the better model ($p < 0.05$). The log likelihood ratio was used to simultaneously estimate population values of fixed-effects parameters (e.g. CL, V_d) and values of random-effects parameters (inter-individual and residual variability).

b) The plausibility of estimated parameters and their 95% confidence intervals

c) Quality of goodness of fit plots (GOF)

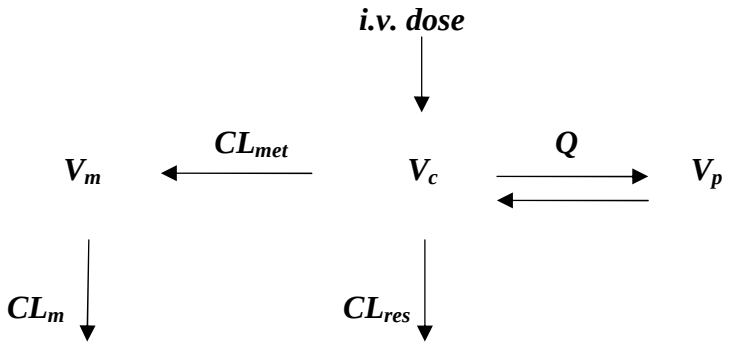
GOF plots included the following (all were presented as X vs. Y): (1) observed vs. predicted concentrations, (2) predicted concentrations vs. weighted residuals (deviations of predictions from observed concentrations) and (3) time vs. weighted residuals. A more randomly distribution of predicted vs. observed concentration across the line of unity and less systemic distribution of weighted residual compared with those in the base model indicate a better fit.

3.8.1.2 Basic model building:

The mixed effects model consists of a structural, a statistical and a covariate model. Building the basic model means choosing the structural and statistical model.

Concerning the structural model, initially the 5-FU concentration-time data were modelled separately from 5-FUH_2 without covariates in order to determine the initial estimates of the pharmacokinetic parameters and to determine whether a one-, two-, or three-compartment model best described the patient data. The model that best fit the concentration-time profiles of 5-FU was selected for further analysis: i.e. to establish a combined model (parent-metabolite structural model). The values of 5-FU pharmacokinetic parameter [total clearance (CL), intercompartmental clearance (Q), central and peripheral volume of distribution (V_c, V_p) obtained from this model were used as inputs values to develop the combined pharmacokinetic model. Then, the values of these parameters were “fixed”, allowing the first estimation for the 5-FUH_2 pharmacokinetic parameters. Finally, the pharmacokinetic parameters of 5-FU and 5-FUH_2 were calculated simultaneously to refine the combined model. 5-FU was assumed to be eliminated only from the central compartment; and 5-FUH_2 was assigned to a separate compartment (fig. 9).

Figure 9 Structural pharmacokinetic model of 5-FU and 5-FUH$_2$



Boxes indicate compartments. Arrows indicate first-order processes.

V_c = central volume of distribution of 5-FU, V_p = peripheral volume of distribution of 5-FU, V_m = volume of distribution of 5-FUH$_2$, CL_{met} = metabolic clearance of 5-FU converted to 5-FUH$_2$, Q = inter-compartmental clearance, CL_{res} = elimination clearance of 5-FU other than intercompartment clearance and conversion to 5-FUH$_2$ (residual elimination clearance), CL_m = total clearance of 5-FUH$_2$

In modelling 5-FU and 5-FUH$_2$ simultaneously, several parameters could not be globally identified under the conditions used in this study, mainly because no urine for 5-FU and 5-FUH$_2$ quantification was collected. The following parameters were not globally identifiable: residual elimination clearance (CL_{res}) and metabolic clearance (CL_{met}) of 5-FU, volume of distribution of 5-FUH$_2$ (V_m), and total clearance of 5-FUH$_2$ (CL_m). However, combinations of these parameters were identifiable: $CL_{met}/(V_c*V_p)$ or $(CL_{res} + CL_{met})/V_c$: in which V_c and V_p is central and peripheral volume of distribution of 5-FU, respectively.

Therefore, the combined model was parameterized in terms of clearance and volume of distribution for 5-FU, while fraction of the metabolite and elimination rate constant (K_{30}) was parameterized for 5-FUH$_2$. To aid the identification of pharmacokinetic parameters of 5-FUH$_2$, V_m was arbitrarily set to 100 L, so the model could be run in a more stable fashion.

The statistical model accounts for interindividual and residual variability. Variability is usually assumed to follow normal distribution with a mean of zero. The interindividual variability (η) is described as the individual's deviation from the population mean (P_{pop}) of a kinetic parameter. As individual pharmacokinetic parameters (P_i) are usually log-normally distributed, the interindividual variability in pharmacokinetic parameters was estimated using an exponential error model: $P_i = P_{pop}*e^{\eta}$

Residual variability (ε), which includes the intra-individual variability, measurement error and any model misspecification error, etc., corresponds to the differences between observed (C_{obs}) and predicted (C_{pred}) concentration by individual parameters (P_i). The residual variability was calculated separately for 5-FU and 5-FUH_2 and was described by a proportional error model used the following equation: $C_{obs} = C_{pred} + C_{pred}*\varepsilon$

3.8.1.3 Final model building (Covariate model):

After the basic model (combined pharmacokinetic model of 5-FU and 5-FUH_2) was constructed, individual estimates of the pharmacokinetic parameters were generated from the basic model to assess whether additional patients' covariates influenced the pharmacokinetic parameters. The OFV obtained from the basic model was considered as a starting value to test the significance of covariates. At first, relationships between individual estimates of pharmacokinetic parameters and patient covariates were explored graphically: scatter plots for individual pharmacokinetic parameter estimates vs. continuous covariates or box and whiskers plots for pharmacokinetic parameters vs. categorical covariates were evaluated. NONMEM used multiple linear regressions to assess and quantify the relationship between the pharmacokinetic parameters and the covariate(s). The selection of the covariates to be tested was based on their physiological and clinical relevance. Age, weight, height, gender, body mass index, body surface area[1], co-medication, renal function (creatinine clearance)[2], hepatic function (ALT, AST, γ-GT, bilirubin), total protein, smoking status, DPD activity (UH_2/U), and the genotyping of DPD, TS and MTHFR were considered as covariates.

According to the literature, the DPD activity could be reduced by the mutations found in the patients of this study. Based on this assumption, the patients in this population pharmacokinetic study were divided into 3 groups depending on the presence of at least one single-nucleotide polymorphisms (SNP) in the *DPYD* gene: wild-type (no mutation), heterozygote (at least one heterozygous mutation), and homozygote mutants (at least one homozygous mutation) with respect to different mutations.

Covariates that are continuous in nature (e.g. BSA, age) were centred on their medians. The continuous covariates were entered into the population pharmacokinetic model

[1] BSA = (body weight in kg/70 kg)$^{0.73}$ x 1.73 m^2
[2] CL_{Cr} (male) (mL/min)=[140–age (yrs)][weight (kg)]/(72)[Cr_{serum} (mg/dL)], CLcr (female)=0.85x[140–age][weight]/(72)[Cr_{serum}]

according to the following equation: $P = \theta_1*[1 + (COV - C\bar{O}V)*\theta_2]$, in which *P* is the individual estimate of the parameter, COV is the value of the covariate of respective patient from the study population, and $C\bar{O}V$ is the median value of the covariate in the study population. θ_1 is the typical value of the parameter in individuals with the "median covariate" and θ_2 is the percentage change in the typical parameter with each unit change from the median covariate value.

Categorical covariates (e.g. sex) were included in the model by using the following equation: $P = \theta_1*\theta_2$, where *P* is the individual estimate of the parameter, θ_1 is typical value of the parameter when the covariate is not present, and θ_2 is the fractional change in the value of *P* when the covariate is present. For some categorical covariates (e.g. MTHFR C667T genotype), a typical value for respective pharmacokinetic parameter was estimated separately for each subgroup of study subjects.

Once the selected covariates were included in the base model, the selected covariates were tested for statistical significance one by one in NONMEM using a forward addition development procedure. In this step, the ΔOFV was also used to evaluate the GOF upon inclusion of each covariate. The ΔOFV was obtained by comparing a model in which the tested covariate was absent to a model in which the covariate was included. The ΔOFV (decrease in OFV) of ≥ 3.84 (corresponding to a *p*-value < 0.05) was required to identify a covariate as being significant. The most significant model, i.e. the model with one covariate and lowest OFV, was kept in the next step and into this model; each of the remaining significant covariates was again included, one at a time. This continued until no additional covariate was judged to change the model significantly. All significant covariates were then forced into a multivariate intermediate model, and each was eliminated in a backward stepwise approach to determine if its exclusion was statistically significant. The OFV obtained in multivariate intermediate model was again used as reference value to evaluate the decrease in GOF obtained upon independent deletion of each covariate: the ΔOFV (increase in OFV) of ≥ 6.63 with p <0.01, was required to achieve the level of significance and to retain a covariate. The significant remaining covariates represented the final model.

The change in the extent of random variability in the presence of covariate(s) was used as a further indicator of the GOF. A reduction in random variability and a less systematic distribution of weighted residuals are generally accepted signs for model refinement.

3.8.1.4 Model assessment

The stability of final parameter estimates was assessed by the jack-knife method. The method assumes fitting model to the study population lacking one individual. This was repeated until every individual had been excluded once from the analysis, which resulted in 30 analyses. Unbalanced results were expected if the parameter estimates of the model were highly determined by a single individual.

In order to identify the sensitivity of the final model for individuals that alone are responsible for (driving) a covariate relationship, the final covariate model was re-run ten times using only nine-tenth of each dataset at a time.

3.8.1.5 Calculation of additional pharmacokinetic parameters

The individual pharmacokinetic parameters estimated directly with the combined and final pharmacokinetic model specifications were CL, V_c, Vp and Q of 5-FU, and elimination rate constant and fraction of metabolite for 5-FUH_2. Bayesian pharmacokinetic estimates for individual subjects were obtained by specification of the POSTHOC option to NONMEM. These POSTHOC Bayesian estimates of the pharmacokinetic parameters were further used to calculate the additional pharmacokinetic parameters of 5-FU and 5-FUH_2 in the combined and final model. The following parameters were calculated for 5-FU: metabolic CL of 5-FU converted to 5-FUH_2, residual elimination clearance, terminal elimination half-life ($t_{1/2,\lambda z}$), total volume of distribution (V_d), and area under the plasma concentration vs. time curve from time point zero extrapolated to infinity [$AUC_{(0-\infty)}$]. For 5-FUH_2 the total clearance, $t_{1/2,\lambda z}$ and $AUC_{(0-\infty)}$ were calculated. The following equations were used:

For 5-FU:

$$t_{1/2,\lambda z} = \frac{\ln 2}{K_{el}}$$

$$AUC_{(0-\infty)} = \frac{Infused\ dose}{CL}$$

Where K_{el} is the overall elimination rate constant of 5-FU (1/h), CL is the total clearance (L/h) and $t_{1/2,\lambda z}$ is terminal elimination half-life.

For 5-FUH$_2$:

$$t_{1/2,\lambda z} = \frac{\ln 2}{K_{30}}$$

$$CL_{5-FUH_2} = K_{30} * V_m$$

$$AUC_{5-FUH_2} = Dose * \frac{F_m}{CL_{5-FUH_2}}$$

Where $t_{1/2,\lambda z}$ is the terminal elimination half-life (h), K_{30} is elimination rate constant (1/h), V_m is volume of distribution of 5-FUH$_2$ (100 L), and F_m is the fraction of the initial dose of 5-FU converted to 5-FUH$_2$ divided by V_m of 5-FUH$_2$

3.8.2 Statistical analysis

Clinical data of all patients were listed. Summary statistics of demographic data, laboratory values, vital signs etc. were performed. A listing of all adverse events reported was set up together with the clinical assessment of these adverse events. All other data relevant to the clinical course of the study was reported either as listing of individual findings or as summary statistics. Values were described by the arithmetic mean and the relative standard deviation for continuous and normally distributed data as well as by the median and range. The one-sample Kolmogorov-Smirnov test was applied to check whether a parameter was normally distributed. A *p*-value < 0.05 indicated a significant difference between two distributions.

Concerning toxicity, since only few adverse events were observed and since all of them were relatively mild, therefore adverse events were evaluated in term of the occurrence of adverse events, regardless of the type, clustered into 2 groups: no adverse event (grade 0) and 5-FU adverse event (grade 1-2). Additionally, due to hematological toxicity being the main adverse effect, changes in blood cell counts for leukocyte,

erythrocyte and thrombocyte was calculated as the safety test by using the following equation:

$$\frac{\left[\left(\textit{pretreatment blood count} - \textit{nadir blood count}\right) \times 100\right]}{\textit{pretreatment blood count}}$$

The following statistical analyses were evaluated: a) First, the search for factors of influence on pharmacokinetics which pharmacokinetic variables were the dependent variables, and genotypes and phenotype were the independent ones. To test the relevant influence of DPD phenotype on pharmacokinetics, simple linear regression analysis was used. The relevant influences of genotypes (DPD, TS, and MTHFR) on pharmacokinetics were tested by using Kruskal-Wallis-test. b) Then, factors of influence on adverse events which the pharmacokinetics were independent variables as genotypes and phenotype were. Again, the simple linear regression was applied to describe the functional relationship between the pharmacokinetics and the relative decrease in blood cell counts as well as the DPD phenotype and the relative decrease in blood cell counts. While Kruskal-Wallis-test was used to test the influence of genotypes on the relative decrease in blood cell counts. Univariate analysis was carried out using binary logistic regression for each individual genotype in DPD, TS, and MTHFR as a predictor of adverse event. Parametric statistical tests using the pharmacokinetic variables and DPD phenotype were performed to identify differences between subgroups of patients categorized according to adverse event.

The pharmacokinetics used in statistical analyses were AUC of 5-FU (mg·h/L), AUC of 5-FUH_2 (mg·h/L) and AUC ratio of 5-FU/5-FUH_2.

For the association of *DPYD*-genotyping with any variable, each informative nucleotide was tested separately assuming an additive allele effect.

Statistical significance was set at the $p < 0.05$ level. Statistical analysis was performed using SPSS for Windows version 11.0 (SPSS Inc. Chicago, IL, U.S.A.).

4. Results

4.1 Patient characteristics

A total of 33 patients with colorectal or oesophageal carcinoma were enrolled in the study of whom 30 completed the pharmacokinetic study. Three patients were removed from the study for unrelated medical problems before the 5-FU started due to technical problem in blood collection (2 patients) and personal reasons not related to the study (1 patient). The first patient entered the study in April 2003 and the last in April 2005. The study finish lasted in October 2005. Baseline patient characteristics and pretreatment values of relevant hematologic and biochemical parameters are listed in table 17. The study included 25 male and 5 female patients with a median age of 59.5 years (range 37-73 years). The mean Karnofsky performance status was 100% (100-100). The most prevalent primary tumor site was the oesophagus. The majority of patients had stage III disease.

Table 17 Characteristics of patients (*n* = 30)

Characteristics	Value
Tumor primary site	
Oesophagus	15
Rectal	1
Colorectal	13
Anus	1
Disease stage	
0	1
I	5
II	6
III	9
IV	6
Median height, m (range)	1.8 (1.6-1.9)
Median weight, kg (range)	75.5 (46-111)
Median body mass index (BMI), kg/m^2 (range)	24.3 (18.7-33.2)
Median body surface area (BSA), m^2 (range)	1.91 (1.48-2.35)
Median baseline laboratory values (range)	
Hemoglobin (g/dL)	13.7 (10.1-16.6)
Platelets (x1E12/L)	277 (48-426)
Erythrocytes (x1E9/L)	4.6 (3.8-5.5)
Leukocytes (x1E9/L)	6.90 (4.68-11.28)
Albumin (g/dL)	42 (35-47)
Serum glutamic ocaloacetic transaminase (SGOT) (U/L)	18 (9-50)
Serum glutamic pyruvic transaminase (SGPT) (U/L)	15 (8-90)
Gamma-glutamyl transferase (γ-GT= (U/L)	24 (13-81)
Total bilirubin (mg/dL)	0.45 (0.4-0.5)
Creatinine (mg/dL)	0.85 (0.44-1.06)

4.2 Adverse events

All 30 patients were evaluable for adverse events. Four patients had no adverse event throughout the duration of treatment. The most frequently observed adverse events during 5-FU therapy are listed in table 18. A total of 69 adverse events were recorded during the course of the study, 54 of which were classified as grade 1 (78.26%), and 15 as grade 2 (21.74%). 5-FU-associated gastrointestinal adverse event were the most common side effects observed. None of the 26 patients who experienced adverse event had adverse event above grade 2.

Table 18 Adverse effects during 5-FU therapy

Toxicity	Grade I		Grade II	
	Events	Patients (n)	Events	Patients (n)
Hematologic				
Leukopenia	1	1	1	1
Gastrointestinal				
Nausea	11	10	3	2
Nausea with emesis	2	2	1	1
Emesis	6	5	1	1
Diarrhea	1	1	1	1
Soft stool	1	1	1	1
Blood in stool	1	1	0	0
Mucositis	3	3	2	2
Metal-like taste on the tongue	2	2	0	0
Gastric discomfort	4	4	0	0
Loss of appetite	2	2	0	0
Thirst	1	1	0	0
Abdominal pain	2	2	0	0
Pain				
Rectal pain	0	0	4	1
Pain in hemorrhoids	1	1	0	0
Constitutional				
Tiredness/fatigue/weakness	4	4	0	0
Sickness	1	1	1	1
Orthostatic symptoms	1	1	0	0
Cardiovascular				
Tachycardia	1	1	0	0
Neurologic				
Headache	4	3	0	0
Dizziness	1	1	0	0
Depressive mood	1	1	0	0
Feeling of warmness	1	1	0	0
Increased body temperature	1	1	0	0
Feeling of redhead	1	1	0	0

In this study, 69 total events were observed and 30 patients were evaluated

4.3 Changes in blood cell counts

Hematological changes were evaluated for 29 patients. For one patient no blood cell count data were available. The hematological changes were defined as percentage decrease in leukocyte, erythrocyte and thrombocyte counts. A median duration of

follow-up for the hematological changes was 1 month (range 2-12 weeks). Mild leukopenia (grade 1-2) was observed in 2 patients. One patient presented leukopenia during the 5-FU infusion with a duration of adverse event of 1 month: the leukocyte counts at that time were 2.46-4.13 $x10^9$/mL (baseline 5.01 $x10^9$/mL). In another patient, leukopenia occurred on post-study day (day 10 after start of 5-FU infusion) with duration of this adverse event of 2 weeks: the leukocyte counts at that time were 2.30-3.22 $x10^9$/mL (baseline 6.87 $x10^9$/mL). The hematological characteristics for all patients are listed in table 19. The leukocyte, erythrocyte and thrombocyte counts decreased by less than 50% (table 19).

Table 19 Hematological changes characteristic

Hematological characteristics	Blood cell counts type	Mean	Range	Median	SD
Baseline (n = 30)	Leukocyte ($x10^9$/L)	6.94	4.00-13.66	7.22	1.95
	Erythrocyte ($x10^{12}$/L)	4.63	3.8-5.3	4.7	0.37
	Thrombocyte ($x10^9$/L)	273.43	147-395	279	80.72
Nadir (n = 29)	Leukocyte ($x10^9$/L)	3.80	1.72-8.11	3.30	2.02
	Erythrocyte ($x10^{12}$/L)	4.16	2.50-5.00	4.30	0.39
	Thrombocyte ($x10^9$/L)	167.79	22-280	170	64.92
Percent decrease in blood cell count	Leukocyte (%)	48.78	20.69-75.22	50.70	16.29
	Erythrocyte (%)	13.98	6.00-49.02	10.42	4.86
	Thrombocyte (%)	39.32	2.00-74.00	42.74	17.84

SD = standard deviation

4.4 DPD phenotype

4.4.1 DPD phenotype distribution

DPD phenotype was assessed using the urinary ratio of dihydrouracil/uracil (UH_2/U). Concentrations of U and UH2 in urine samples of 30 patients were determined, and their concentration ratios were calculated (table 20). One patient had unquantifyable UH_2 levels in urine. The UH_2/U ratio was normally distributed as assessed by the Kolmogorov-Smirnov test (p >0.05) with a mean ± SD of 1.18 ± 0.5 (table 20).

Table 20 Concentrations of U (µM), UH_2 (µM) and the UH_2/U ratio in urine

Analyte	n	Medium	Minimum	Maximum
Uracil	30	29.63	4.14	102.21
Dihydrouracil	29	32.85	12.90	62.49
Dihydrouracil/Uracil ratio	29	1.18	0.37	2.68

4.5 Genotyping

4.5.1 *DPYD* polymorphisms

Analysis of the *DPYD* gene of 30 patients revealed the presence of 6 polymorphisms in 22 of 30 patients (table 21). In eight patients, no polymorphism was detected in any of the 23 exons investigated. The mutation 85T>C (*DPYD**9A) was detected in 10 patients (33.3%), with homozygous alleles in 2 patients and heterozygous alleles in 8 patients: allelic frequency of 20% (12/60). The 1627A>G (*DPYD**5) was detected in 11 patients (36.7%), with homozygous alleles in 2 patients and heterozygous alleles in 9 patients: allelic frequency of 21.7% (13/60).

Other mutations were only present heterozygously (table 21): the allelic frequency of these mutations was 20% (12/60). Of these polymorphisms, the mutations 1236G>A and 1601G>A (*DPYD**4), and 2194G>A (*DPYD**6) were found in one and two patients, respectively, whereas 496A>G was found in 8 patients. The *DPYD* polymorphisms found in 22 patients are presented in table 22. In these patients, eight had multiple mutations in the coding region of the *DPYD* gene.

Table 21 Allelic frequencies of polymorphisms in the *DPYD* gene in 30 patients

***DPYD* polymorphism**		**Effect (nucleotide change)**	**Wild-type (*n*)**	**Heterozygous mutant (*n*)**	**Homozygous mutant (*n*)**	**Allelic frequency (%)**
nomenclature	**Exon**					
*DPYD*9A*	2	Cys29Arg (85T>C)	20	8	2	12/60 (20%)
	6	Met166Val (496A>G)	22	8	0	8/60 (13.33%)
	11	Glu412Glu (1236G>A)	29	1	0	1/60 (1.67%)
*DPYD*4*	13	Ser534Asn (1601G>A)	29	1	0	1/60 (1.67%)
*DPYD*5*	13	Ile543Val (1627A>G)	19	9	2	13/60 (21.67%)
*DPYD*6*	18	Val732Ile (2194G>A)	28	2	0	2/60 (3.33%)

DPYD = dihydropyrimidine dehydrogenase gene, *n* = number of patients

Table 22 *DPYD* polymorphisms detected in patients

Patient no. [sex, age]	Primary cancer	DPD genotype	Effect	Exon
1 [M, 68]	Oesophageal	1627A>G	Ile543Val	13
3 [M, 37]	Rectal	496A>G 1627A>G	Met166Val Ile543Val	6 13
4 [M, 46]	Colon	85CC 496A>G 1236G>A	Cys29Arg Met166Val Glu412Glu	2 6 11
5 [F, 58]	Oesophageal	85CC 496A>G	Cys29Arg Met166Val	2 6
6 [M, 45]	Oesophageal	1627A>G	Ile543Val	13
9 [F, 50]	Rectal	85T>C 496A>G 1627GG	Cys29Arg Met166Val Ile543Val	2 6 13
10 [M, 52]	Oesophageal	1601G>A	Ser534Asn	13
11 [M, 46]	Oesophageal	85T>C 496A>G 1627A>G	Cys29Arg Met166Val Ile543Val	2 6 13
13 [M, 62]	Oesophageal	1627GG	Ile543Val	13
14 [M, 50]	Rectal	85T>C	Cys29Arg	2
15 [F, 60]	Rectal	1627A>G	Ile543Val	13
16 [M, 51]	Rectal	85T>C	Cys29Arg	2
17 [F, 70]	Rectal	85T>C 496A>G	Cys29Arg Met166Val	2 6
18 [M, 61]	Rectal	1627A>G	Ile543Val	13
19 [M, 58]	Rectal	85T>C	Cys29Arg	2
20 [M, 66]	Oesophageal	85T>C 496A>G	Cys29Arg Met166Val	2 6
22 [M, 66]	Oesophageal	85T>C 496A>G	Cys29Arg Met166Val	2 6
24 [M, 64]	Rectal	1627A>G	Ile543Val	13
26 [M, 52]	Rectal	2194G>A	Val732Ile	18
28 [M, 51]	Oesophageal	2194G>A	Val732Ile	18
29 [M, 66]	Rectal	1627A>G	Ile543Val	13
30 [M, 73]	Anal	1627A>G	Ile543Val	13

Age in year, 85T>C = *DPYD**9A, 1601G>A = *DPYD**4, 1627A>G = *DPYD**5, 2194G>A = *DPYD**6

4.5.2 Thymidylate synthase polymorphism

Among 30 patients analyzed for the TS genotype, 5 (16.70%) were homozygous for the triple repeat (3R/3R), 19 (63.30%) were heterozygous (2R/3R), and 6 (20.00%) were homozygous for the double repeat variant (2R/2R) within the human TS promoter region.

4.5.3 Methylenetetrahydrofolate reductase (MTHFR) polymorphism

The MTHFR 677CT and MTHFR 677CC genotypes occurred with almost the same frequency [43% (n = 13) and 40% (n = 12), respectively], while the MTHFR 677TT genotype was only observed in 17% (n = 5) of the patients.

4.6 Pharmacokinetics of 5-FU and 5-FUH_2

4.6.1 Raw concentration-time data

The pharmacokinetic evaluation was carried out for all 30 patients. Plasma concentrations of 5-FU and 5-FUH_2 in 30 patients are presented in figures 10 and 11. A large variability in 5-FU and 5-FUH_2 concentrations were observed both between the patients (median %CV = 60.43, range 17.61-76.12) and in the same patient (median %CV = 34.70, range 3.40-93.85) during infusion (fig. 10). As shown in figure 11, 5-FUH_2 concentrations were consistently greater than those of 5-FU. The 5-FU plasma concentrations decreased rapidly when the infusion was stopped, which may be interpreted as that 5-FU accumulated during infusion, and was eliminated very fast, while the concentrations of 5-FUH_2 decreased more slowly (fig 11).

Figure 10 Plasma concentrations of 5-FU (a) and 5-FUH_2 (b) in 30 patients

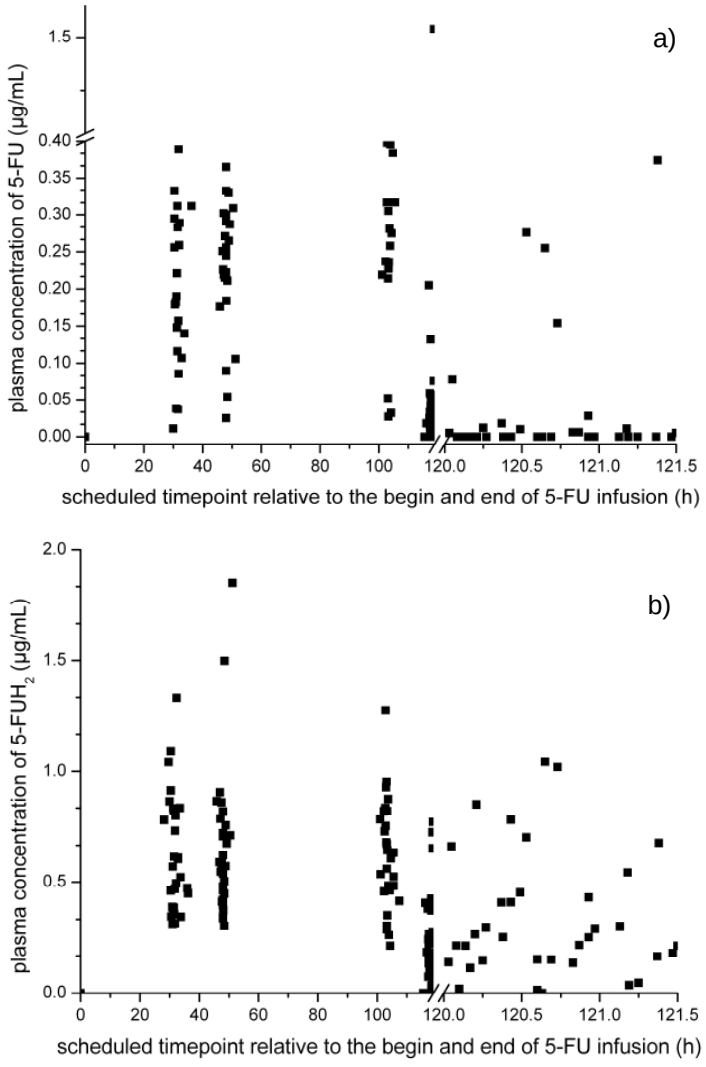
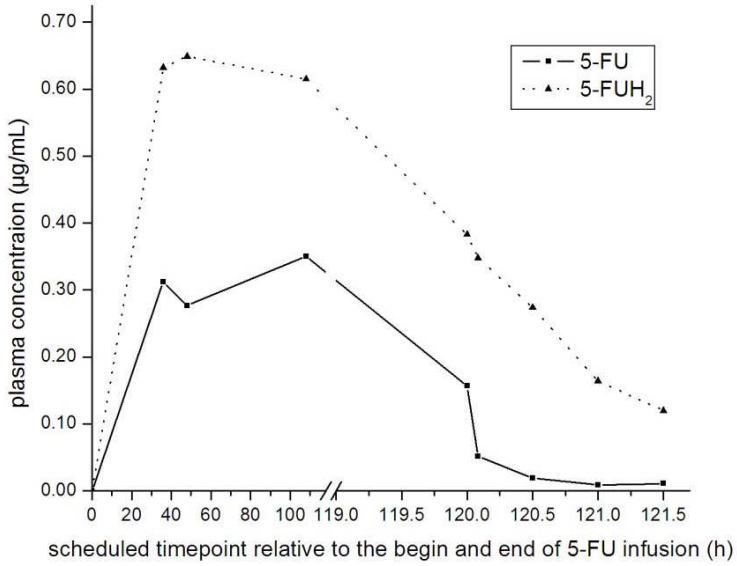


Figure 11 Mean plasma concentrations of 5-FU and 5-FUH_2 in 30 patients



4.6.2 Population pharmacokinetic analysis

4.6.2.1 Base model or covariate-free model

The population pharmacokinetic model was developed based on 199 and 251 plasma concentrations of 5-FU and 5-FUH_2, respectively, obtained from 30 patients. First, a model for 5-FU pharmacokinetics alone was developed. The model best describing 5-FU concentration-time data was an open two-compartment model with first-order elimination (subroutine ADVAN3 TRANS3 in NONMEM), with interindividual variability in total clearance. The mean population pharmacokinetic parameter estimates for 5-FU and these 95% confidence intervals (95% CI) obtained from this 5-FU model were the total clearance (CL_{tot}) 238 L/h (198-278), central volume of distribution (V_c) 11.4 L (5.5-17.3), intercompartment clearance (Q) 12.9 L/h (4.9-20.9), and total volume of distribution (V_d) 30.4 L (17.8-43.0). Interpatient variability in CL_{tot} of 5-FU was about 9-fold, the coefficient variation (CV) was 44.3%.

For the development of a combined pharmacokinetic model of 5-FU and 5-FUH_2, the two-compartment model with first-order elimination was used to describe 5-FU concentration-time data, whereas a one-compartment model gave the best fit for the 5-FUH_2 concentration-time data. These two models were then combined to describe the pharmacokinetics of both analytes within one model (subroutine ADVAN5 TRANS1 in NONMEM, general linear model).

The combined pharmacokinetic model was parameterized in terms of CL_{tot}, Q, central and peripheral volume of distribution (V_c and V_p) for 5-FU, the fraction of the initial 5-FU dose converted to 5-FUH_2 divided by the volume of distribution of the 5-FUH_2, (F_m), and the elimination rate constant of 5-FUH_2 (K_{30}). By simultaneously analysis of the 5-FU and the 5-FUH_2 data, the model with inclusion of the interindividual variability for the CL_{tot} of 5-FU and elimination rate constant of 5-FUH_2 gave the best fit. By inclusion of interindividual variability (θ) on such parameters as Q, V, F_m, the fitting did not get better and the point estimate for θ was very small. Because it is important to keep the model simple as possible, the respective θ(s) were not included in the model. The population mean estimate for CL_{tot} of 5-FU was found to be 237 L/h (95% CI, 197-227 L/h), the interpatient variability (expressed as %CV) was 44%. The population mean estimate for K_{30} of 5-FUH_2 was 1.17 1/h (95% CI, 0.90-1.43 /h) with an interpatient variability of 31.8% (expressed as %CV). Other population pharmacokinetic parameters are shown in table 23.

Table 23 Population pharmacokinetics of 5-FU and 5-FUH_2 (*combined base model*)*

Population estimate of PK parameter (unit)	Point estimate	SE of estimate	95% CI	Interindividual CV (%)	Jack-knife validation	
					Median	Range
5-FU						
CL_{tot} (L/h)	237	20.6	197 – 227	44.0	236	230 – 251
V_c (L)	10.8	2.7	5.5 – 16.1	n.s.[a]	10.8	9.8 – 12.0
V_p (L)	19.4	5.6	8.5 – 30.3	n.s.[a]	19.4	16.4 – 21.5
Q (L/h)	12.2	3.9	4.5 – 19.9	n.s.[a]	12.5	9.8 – 13.1
5-FUH_2						
F_m/V_m (1/L)[b]	0.863	0.111	0.647 – 1.078	n.s.[a]	0.859	0.827 – 0.897
K_{30} (1/h)	1.17	0.13	0.90 – 1.43	31.8	1.17	1.11 – 1.21

* The values for the pharmacokinetic parameters represent estimates for a typical individual in the population.
[a] n.s. = inclusion of this element of inter-individual variation did not improve the model significantly
[b] F_m/V_m = the fraction of the initial 5-FU dose converted to 5-FUH_2 divided by the volume of distribution of the 5-FUH_2
PK = pharmacokinetic, SE = standard error, CL_{tot} = total clearance, V_c = central volume of distribution, V_p = peripheral volume of distribution, Q = intercompartmental clearance, K_{30} = elimination rate constant of 5-FUH_2

The population pharmacokinetic parameter estimates of 5-FU in the combined model were essentially equal to those previously calculated based on 5-FU concentration-time data only (subroutine ADVAN3 TRANS3) (0.4% to 5.4% deviation), and their 95% CI included the mean parameter estimates obtained from the former model during step 1 (see above).

The additional pharmacokinetic parameters of 5-FU and 5-FUH_2 were calculated from the individual patient parameter estimates obtained by using NONMEM. These parameters are presented in table 24. The mean values for CL_{tot} of 5-FU and of 5-FUH_2 were 237 (range 50-443) and 117 (range 67-217) L/h, respectively. The mean value of 5-FU metabolic clearance converted to 5-FUH_2 (CL_{met}) was 205 L/h (range 43-383), indicating that approximately 86.3% of 5-FU was catabolized to 5-FUH_2. Additionally, the $AUC_{0-\infty}$ of 5-FU and the terminal elimination half-life ($t_{1/2\lambda z}$) of 5-FU and of 5-FUH_2 were also calculated for all patients (table 24).

Table 24 Pharmacokinetic parameters of 5-FU and 5-FUH_2 calculated from individual patient pharmacokinetic parameter estimates (*combined base model*)

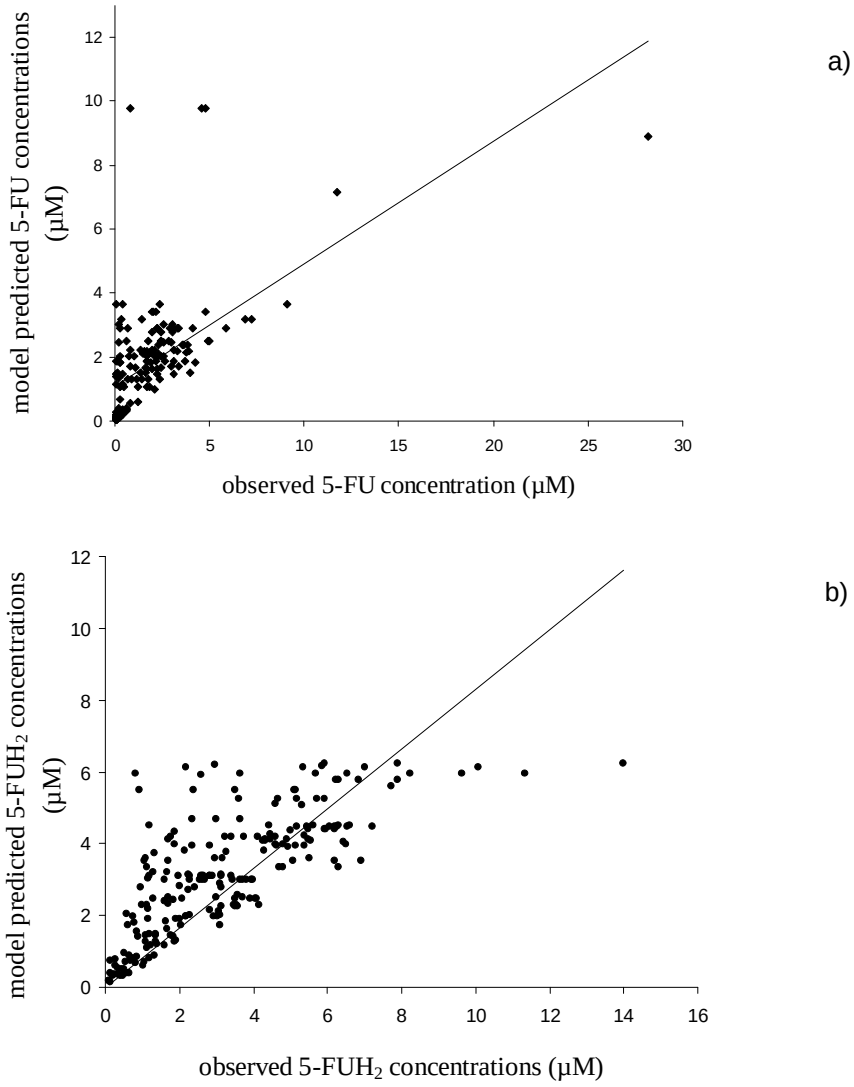
PK parameter (unit)	Formula	Geometric mean ± SD	Median	Range
5-FU				
CL_{met} (L/h)	$CL_{met} = K_{13}*V_c$ $K_{13} = F_m*(CL_{tot}/V_c)$	205 ± 75	225	43 – 383
CL_{res} (L/h)	$CL_{res} = CL_{tot} - CL_m$	33 ± 12	36	7 – 61
CL_{tot} (L/h) - estimated	n.a.	238 ± 87	261	50 – 443
$t_{½,\lambda z}$ (h)	$t_{½,\lambda z} = \ln(2)/ K_{el}$ $K_{el} = (1 - F_m)*(CL_{tot}/V_c) + K_{13}$	0.032 ± 0.024	0.029	0.017 – 0.15
$AUC_{(0-\infty)}$ (mg·h/L)	$AUC_{(0-\infty)} = Dose/CL_{tot}$	34.49 ± 23.62	33.56	16.61 – 150.80
5-FUH_2				
CL_m (L/h)	$CL_m = K_{30}*V_m$	117 ± 39	116	67 – 217
$AUC_{(0-\infty)}$ (mg·h/L)	$AUC_{(0-\infty)} = Dose * F_m/CL_m$	60.40 ± 19.61	62.88	30.73 – 97.51
$t_{½,\lambda z}$ (h)	$t_{½,\lambda z} = \ln(2)/ K_{30}$	0.59 ± 0.19	0.60	0.32 – 1.04

PK = pharmacokinetic, SD = standard deviation, CL_{met} = metabolic clearance of 5-FU converted to 5-FUH_2, CL_{res} = residual elimination clearance of 5-FU, CL_{tot} = total clearance of 5-FU, $t_{½,\lambda z}$ = terminal elimination half-life, $AUC_{(0-\infty)}$ = area under the concentration-time curve from time 0 to infinity, CL_m = total clearance of 5-FUH_2, K_{el} = overall elimination rate constant of 5-FU, K_{13} = formation rate constant of 5-FUH_2, V_c = central volume of distribution, K_{30} = elimination rate constant of 5-FUH_2, V_m = volume of distribution of 5-FUH_2, F_m = fraction of the initial 5-FU dose converted to 5-FUH_2 divided by the volume of distribution of the metabolite (1/L); point estimate for F_m = 0.863

The appropriateness of the combined base model was evaluated graphically by goodness of fit (GOF) plots: the points on the model-predicted vs. observed plots for 5-FU and 5-FUH_2 concentrations and on the weighted residuals vs. predicted concentrations plots (fig. 12-13) did not show distributions suggesting systematic deviations.

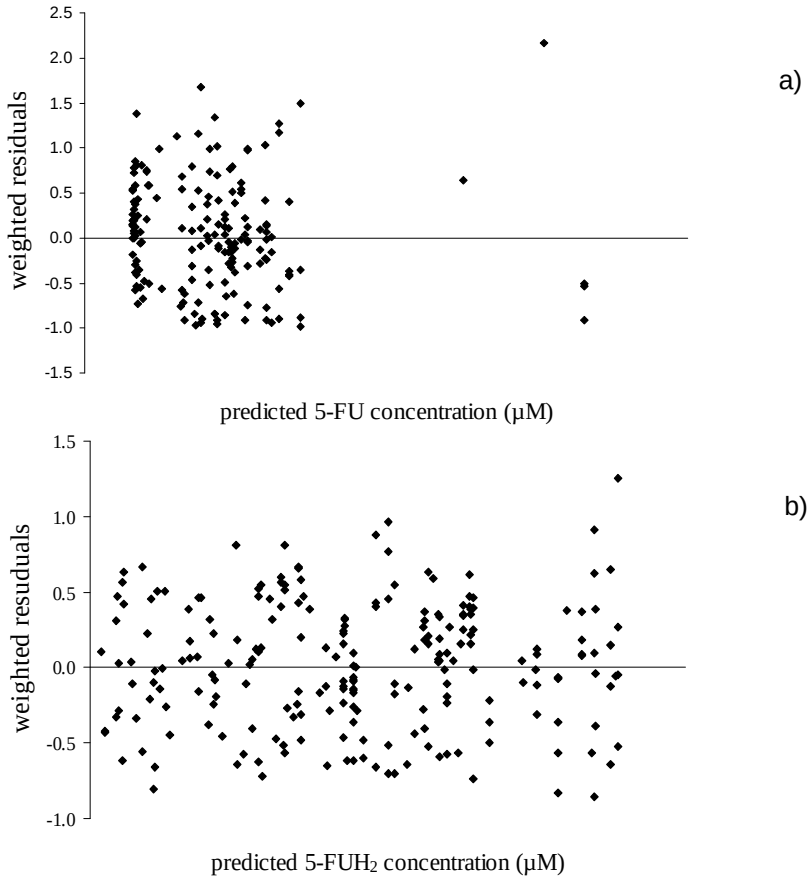
Scatter plot of the individual predicted vs. the observed 5-FU and FUH_2 plasma concentrations, illustrated in fig. 12, indicated that the model adequately described the concentration-time data of 5-FUH_2, however, there was a tendency to overestimate 5-FU plasma concentrations.

Figure 12 Individual predicted vs. observed plasma concentrations of the base model for 5-FU (a) and 5-FUH_2 (b)



Residual variability in the combined base model was calculated separately for 5-FU and 5-FUH_2. The proportional error model for both substances yielded the lowest objective function value and the best residual plots (fig. 13). The residual variability in the base model was 64% and 43% for 5-FU and 5-FUH_2, respectively. A combined proportional and additive error models resulted in a significantly worse fit, and an additive error model was also not superior to the proportional error model.

Figure 13 Weighted residuals vs. predicted concentrations of the base model for 5-FU (a) and 5-FUH$_2$ (b) plasma concentrations



4.6.2.2 Covariate analysis and final model

The effect of the covariates (see section 3.8 data analysis) on the pharmacokinetic parameters of 5-FU and 5-FUH$_2$ were tested in the *combined* pharmacokinetic base model.

The preliminary separate testing of covariates in the model with 5-FU concentration-time data developed in step 1 suggested that only some patient characteristics could potentially explain the interindividual variability in the pharmacokinetics of 5-FU, particularly, the CL_{tot} of 5-FU was associated with BSA, BMI, body weight and height, sex (2-fold lower clearance in females), while Q was affected by MTHFR C677T and TS genotype and hepatic function represented by ALT, AST, and γ-GT values in the model.

Using the addition development procedure and the elimination approach, a covariate model for *5-FU* data alone was obtained, in which the estimated value for 5-FU CL_{tot}

was affected by BSA and MTHFR C677T genotype. The effect of MTHFR C677T genotype on CL has been modelled as a sum of clearance fractions, namely, a typical value of CL_{tot} (95% CI) for subjects with two T-alleles and increase in CL_{tot} in the presence of C-allele were 169 L/h (112- 226) and 72 L/h (21-123), respectively. Thus, the point estimates for 5-FU CL_{tot} were 169, 241 and 313 L/h for individuals with TT-, CT-, and CC-haplotype of *MTHFR* gene, respectively. Addition of other covariates, which have been significant factors of influence in univariate analysis, did not result in significant improvements in the structural model in multivariate analysis.

In univariate analysis, the *combined* pharmacokinetic model was significantly improved with the separate inclusion of the following covariates: CL_{tot} was influenced by BSA, body weight, BMI, sex, MTHFR and DPD genotype; Q was influenced by hepatic function (γ-GT value), MTHFR C677T genotype, and age. The 5-FU CL_{tot} increased with increasing value of BSA, body weight, BMI, whereas Q increased with increasing γ-GT values, but decreased with age. However, some of these models gave unstable models, particularly, models describing the influence of body weight, and BMI on CL_{tot} as well as models describing the effect of MTHFR genotype, and γ-GT values on Q. Therefore, these covariates were not included in the multivariate analysis.

The results of testing the influence of groups of *DPYD* genotypes, divided into 3 groups depending on the presence of at least one single-nucleotide polymorphisms (SNP) in the *DPYD* gene (homozygous wild-types, heterozygotes, and homozygotes mutants with respect to different mutations), on pharmacokinetic parameters showed an impact on 5-FU CL_{tot}: i.e. point estimates were 263 L/h (95% CI, 231-295) and 175 L/h (95% CI, 87-263) in patients with wild-type genotype and homozygous mutations, respectively. The OFV decreased significantly ($p < 0.05$) by inclusion of patient's DPD genotype as co-factor in the combined model; however, the confidence intervals for estimations on the CL_{tot} were very broad with overlapping the values between the groups. Thus, the covariance DPD genotype was not included in the final model because of the lacking statistical significance and due to a very small number of mutations in the *DPYD* gene found in the study population that would lead to difficulties in interpreting the results.

The inclusion of age as a covariate for Q in multivariate analysis resulted in a significant reduction of the objective function value (OFV), but the level of significance was not achieved in the backward elimination procedure. Therefore, this cofactor was not included in the final pharmacokinetic model.

In multivariate analysis, the influence of BSA and sex on the CL_{tot} in combination with MTHFR genotype was tested in separate models because of the co-linearity of these parameters. Inclusion of the parameters sex *or* BSA yielded a significant reduction in the objective function value and in the random variability. The correlation between BSA and CL_{tot} was slightly stronger than the correlations between sex and CL_{tot}. The significant impact of BSA and MTHFR genotype on CL_{tot} of 5-FU was confirmed by multivariate analysis and by backward elimination. The remaining covariates after having carried out the backward elimination procedure represented the final model.

The final equation including BSA and MTHFR C667T genotype as covariates, which best described the CL of 5-FU in this study, was as follows:

BSA alone:

$$CL = 250*[1 + (BSA - 1.91)*0.85]$$

$$= 250*(0.85*BSA - 0.62) \ (p = 0.008, r^2 = 0.224)$$

BSA and MTHFR genotype:

$$CL = 145*[1 + (BSA - 1.91)*0.85]$$

$$= 145*(0.85*BSA - 0.62), \text{ for patient with TT-haplotype}$$

$$CL = 276*[1 + (BSA - 191)*0.85]$$

$$= 276*(0.85*BSA - 0.62)], \text{ for patient with CC- and CT-haplotype}$$

$$(r^2 = 0.415, p = 0.025)$$

The population pharmacokinetic parameter estimates obtained from this final covariate model are summarized in table 25. As it has been done for the parameters obtained with the base model, additional pharmacokinetic parameters of 5-FU and $5\text{-}FUH_2$ were calculated from individual patient parameter estimates and are presented in table 26. Results of the jack-knife analysis, that was used to assess the stability of final parameter estimates are detailed in table 23. Median values for each parameter obtained from

jack-knife analysis were in the same range as the parameter estimates obtained from the model using the complete dataset. Similarly, the testing of final covariate model for its sensitivity did not yielded any shortcoming of the model

Table 25 Population pharmacokinetics of 5-FU and 5-FUH$_2$ (*final covariate model*)*

Population estimate of PK parameter (unit)	Point estimate	SE of estimate	95% CI	CV (%)
CL_{tot} of 5-FU in patients with two T-alleles in *MTHFR* gene (L/h)	145[a]	31.5	83 – 207	29.6
CL_{tot} of 5-FU in patients with one- or two C-alleles *in MTHFR* gene (L/h)	276[a]	13.4	250 – 302	29.6
Central volume of distribution of 5-FU (L)	11.1	2.8	5.7 – 16.5	n.s.[b]
Peripheral volume of distribution of 5-FU (L)	21.8	6.7	8.7 – 34.9	n.s.[b]
Intercompartmental clearance of 5-FU (L/h)	13.2	5.0	3.4 – 23.0	n.s.[b]
Fraction of metabolite (5-FUH$_2$) (1/L)	0.863 [c]	0.110	0.647 – 1.079	n.s.[b]
Elimination rate constant of 5-FUH$_2$ (1/h)	1.17	0.13	0.90 – 1.43	31.6

* values for the PK parameters represented estimates for the typical individual in the population. PK = pharmacokinetic, SE = standard error, CI = confidential interval, CV = coefficient of variation
[a] CL_{tot} was estimated separately for 2 subgroups of patients: patients with TT-alleles and patient with CT or CC in *MTHFR* gene
[b] n.s., inclusion of this element of inter-individual variation did not improve the model significantly
[c] fraction of the initial 5-FU dose converted to 5-FUH$_2$ divided by the volume of distribution of the metabolite

Table 26 Pharmacokinetic parameters of 5-FU and 5-FUH$_2$ calculated from individual patient parameter estimates (*final covariate model*)

PK parameter (unit)	Formula	Geometric mean ± SD	Median	Range
5-FU				
CL_{met} (L/h)	$CL_{met} = K_{13}*V_c$ $K_{13} = F_m*(CL/V_c)$	207 ± 75	230	43 – 381
CL_{res} (L/h)	$CL_{res} = CL - CL_m$	33 ± 12	36	7 – 60
CL_{tot} (L/h) - estimated	n.a.	240 ± 87	266	49 – 441
$t_{½,\lambda z}$ (h)	$t_{½,\lambda z} = \ln(2)/ K_{el}$ $K_{el} = (1-F_m)*(CL/V_c)+K_{13}$	0.032 ± 0.025	0.029	0.017 – 0.155
$AUC_{(0-\infty)}$ (mg·h/L)	$AUC_{0-\infty} = Dose/CL$	34.15 ± 23.98	32.74	17.52 – 152.46
5-FUH$_2$				
CL_m (L/h)	$CL_m = K_{30}*V_m$	117 ± 38	116	67 – 213
$AUC_{(0-\infty)}$ (mg·h/L)	$AUC_{0-\infty} = Dose*F_m/CL_m$	60.29 ± 19.53	62.85	30.67 – 97.29
$t_{½,\lambda z}$ (h)	$t_{½,\lambda z} = \ln(2)/ K_{30}$	0.59 ± 0.18	0.60	0.32 – 1.04

PK = pharmacokinetic, CL_{met} = metabolic clearance 5-FU converted to 5-FUH$_2$, CL_{res} = residual elimination clearance, CL_{tot} = total clearance, $t_{½,\lambda z}$ = terminal elimination half-life, $AUC_{(0-\infty)}$ = area under the concentration-time curve at time 0 to infinity, CL_m = total clearance of 5-FUH$_2$, F_m = 0.863, K_{13} = formation rate constant of 5-FUH$_2$ (1/h), K_{30} = elimination rate constant of 5-FUH$_2$, K_{el} = overall elimination rate constant of 5-FU, V_c = central volume of distribution of 5-FU, V_m = volume of distribution of 5-FUH$_2$

4.7 Factors influencing of additional pharmacokinetic parameters

The 5-FU AUC ($AUC_{5\text{-}FU}$) and 5-FUH_2 AUC ($AUC_{5\text{-}FUH2}$) were calculated from the base model, using the POSTHOC option of NONMEM. The mean AUC of 5-FU and of 5-FUH_2 were 38.22 ± 23.62 mg·h/L (range 16.16-150.80) and 63.40 ± 19.61 mg·h/L (range 30.73-97.51), respectively. The $AUC_{5\text{-}FU}/AUC_{5\text{-}FUH2}$ ratio (AUC ratio) was also calculated which was 0.62 ± 0.28 (range 0.31-1.55).

4.7.1 DPD phenotype

Simple linear regression analysis showed that no correlations between the DPD phenotype, expressed in term of UH_2/U ratio, and $AUC_{5\text{-}FU}$ (adjusted r^2 = 0.041, p = 0.149) or the $AUC_{5\text{-}FUH2}$ (adjusted r^2 = - 0.016, p = 0.465). There were also no statistically significant correlation between DPD phenotype and AUC ratio (adjusted r^2 = -0.14, p = 0.432).

4.7.2 *DPYD* polymorphisms

Using Kruskal-Wallis-test, neither significant correlation was found between $AUC_{5\text{-}FU}$ nor $AUC_{5\text{-}FUH2}$ and these *DPYD* polymorphisms. The same was true for the AUC ratio. Statistical analysis is showed in table 27.

Table 27 Statistical analysis of the associations of DPYD and pharmacokinetics

***DPYD* polymorphisms**	**AUC of 5-FU (mg·h/L)**		**AUC of 5-FUH_2 (mg·h/L)**		**AUC ratio**	
	χ^2	***p*-value**	**χ^2**	***p*-value**	**χ^2**	***p*-value**
85T>C	4.460	0.108	0.967	0.617	4.540	0.103
496A>G	0.079	0.778	0.372	0.542	0.317	0.574
1236G>A	0.564	0.453	0.404	0.525	1.472	0.225
1601G>A	1.205	0.272	1.472	0.225	0.164	0.686
1627A>G	0.459	0.498	0.403	0.525	0.790	0.374
2194G>A	0.333	0.561	0.249	0.618	0.000	1.000

4.7.3 Thymidylate synthase polymorphism

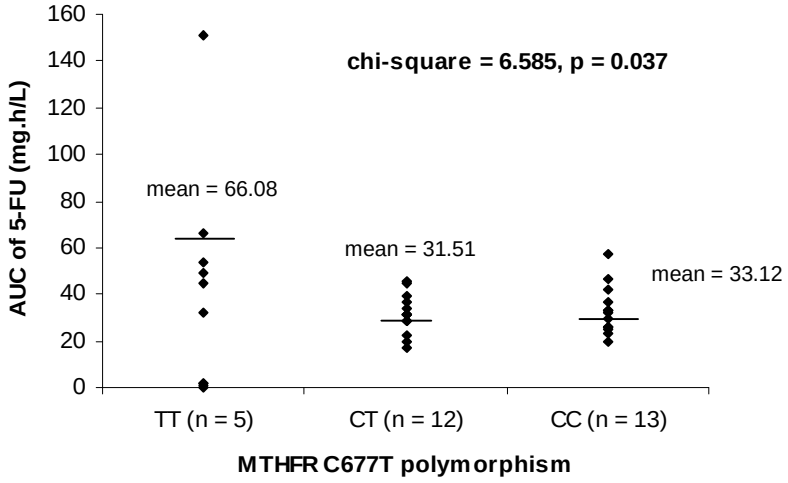
A possible influence of TS polymorphisms on the pharmacokinetics was tested using Kruskal-Willis-test. There was no relationship between the TS polymorphism and the

$AUC_{5\text{-}FU}$ (χ^2 = 2.015, p = 0.365) or the $AUC_{5\text{-}FUH2}$ (χ^2 = 2.586, p = 0.274) or the AUC ratio (χ^2 = 0.280, p = 0.869)

4.7.4 Methylenetetrahydrofolate reductase polymorphism

Using Kruskal-Willis-test, a significant correlation was observed between the $AUC_{5\text{-}FU}$ and the MTHFR C677T polymorphism (χ^2 = 6.585, p = 0.037; fig. 14). The $AUC_{5\text{-}FU}$ was higher in patients homozygote for MTHFR 677TT than in patients with the wild-type MTHFR 677CC or heterozygous carriers of the MTHFR 677CT allele. However, no relationship was identified between the MTHFR polymorphism and the $AUC_{5\text{-}FUH2}$ (χ^2 = 1.154, p = 0.565) and the AUC ratio (χ^2 = 4.202, p = 0.122).

Figure 14 The association of MTHFR C677T polymorphism and AUC of 5-FU



4.8. Factors influencing of adverse events and changes in blood cell counts

4.8.1 DPD phenotype

Four patients (13.33%) were defined as having no adverse event and 26 patients (86.67%) had adverse events (grade 1-2). Of these 26 patients, one patient who had unquantifyable UH_2 levels in urine presented U concentrations of 4.14 µM. This patient had mild adverse event (grade 1; nausea and vomiting).

The influence of the UH_2/U ratio on the occurrence of adverse event was tested for 29 patients. The UH_2/U ratio was not significantly different in patients with adverse event

compared to patients who tolerated the treatment [1.17 ± 0.51 (range 0.37-2.68) vs. 1.29 ± 0.40 (0.83-1.69), respectively, $p = 0.650$].

An additional test was carried out to evaluate whether the DPD phenotype was associated with changes in blood cell counts. The changes in blood cell counts for leukocyte, erythrocyte, and thrombocyte served as the dependent variables here. Simple linear regression analysis revealed no correlation between DPD phenotype and the changes in leukocyte count (adjusted $r^2 = 0.05$, $p = 0.264$), erythrocyte count (adjusted $r^2 = 0.07$, $p = 0.182$), and thrombocyte count (adjusted $r^2 = 0.01$, $p = 0.637$).

4.8.2 *DPYD* polymorphisms

In 19 of the 26 patients who experienced adverse events, *DPYD* mutations were detected; whereas no mutations were observed in 7 patients with adverse events, table 28. Three of four patients without adverse event had *DPYD* mutations. The frequency of haplotypes was too small to reliably assess differences within and between haplotype (fig. 15).

Figure 15 Haplotypes of the *DPYD* gene and adverse events of 5-FU

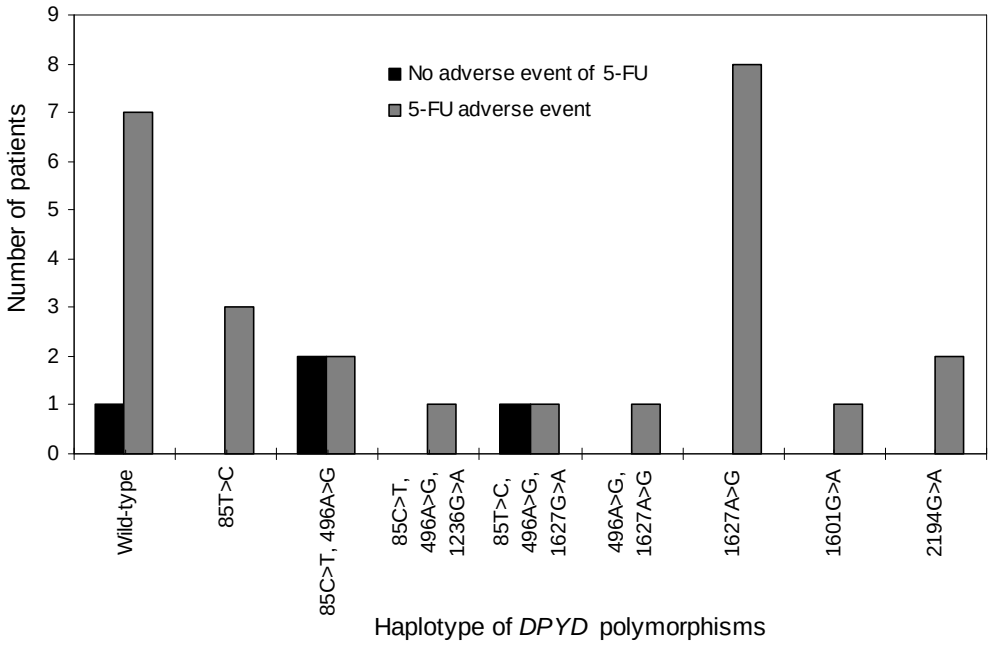


Table 28 *DPYD* mutations in patients exhibiting adverse events

Patient no. [sex, age]	Primary cancer	UH_2/U	*DPYD* genotype	adverse events (grade)
1 [M, 68]	Oesophageal	n.d.	1627A>G	nausea (1), emesis (1)
3 [M, 37]	Rectal	0.77	496A>G, 1627A>G	gastric discomfort (1)
4 [M, 46]	Colon	0.61	85CC, 496A>G, 1236G>A	diarrhea (1), apthous oral (1), meterorism (1), weakness (1), mucositis (2)
5 [F, 58]	Oesophageal	0.58	85CC, 496A>G	leukopenia (1)
6 [M, 45]	Oesophageal	0.76	1627A>G	dizziness (1), red head feeling (1), nausea and emesis (1)
7 [M, 41]	Oesophageal	1.45	-	tachycardia (1), nausea (1)
8 [M, 60]	Rectal	1.09	-	increased body temperature (1)
9 [F, 50]	Rectal	1.20	85T>C, 496A>G, 1627GG	gastric pain (1), tiredness (1), mucositis (2), nausea and emesis (1)
10 [M, 52]	Oesophageal	1.23	1601G>A	nausea (1)
12 [M, 64]	Rectal	2.68	-	Meterorism (1), nausea (1), mucositis (1)
13 [M, 62]	Oesophageal	0.99	1627GG	emesis (1), nausea (1), headache (1)
14 [M, 50]	Rectal	1.41	85T>C	depressive mood (1), soft stool (1), metal-like taste on tongue (1), abdominal pain (1)
15 [F, 60]	Rectal	1.39	1627A>G	headache (1)
16 [M, 51]	Rectal	0.86	85T>C	leukopenia (2)
18 [M, 61]	Oesophageal	0.37	1627A>G	nausea (1)
19 [M, 58]	Oesophageal	0.75	85T>C	tiredness (1), nausea (1)
20 [M, 66]	Oesophageal	1.17	85T>C, 496A>G	nausea (1), emesis (1), mucositis (1)
21 [M, 71]	Rectal	2.37	-	rectal pain (2), nausea (2)
23 [M, 68]	Oesophageal	0.95	-	emesis (2), nausea (2),
24 [M, 64]	Rectal	1.37	1627A>G	orthostatic symptoms
25 [M, 70]	Oesophageal	0.95	-	warmness feeling (1), tiredness (1), sickness (1)
26 [M, 52]	Rectal	1.17	2194G>A	nausea (1), emesis (1), loss of appetites (1)
27 [F, 40]	Rectal	1.20	-	headache (1), diarrhea (2), thirst (1)
28 [M, 51]	Oesophageal	1.67	2194G>A	nausea (1), metal-like taste on tongue (1)
29 [M, 66]	Rectal	1.43	1627A>G	blood in stool (1)
30 [M, 73]	Anal	0.92	1627A>G	loss of appetite (1)

M = male, F = female, U = uracil, UH_2 = dihydrouracil, age in years, DPD phenotype expressed as UH_2/U ratio

The polymorphisms 1236G>A and 1601G>A were found in only 1 patient each (but suffering from adverse event), and 2194G>A was found in 2 patients who both presented adverse events (table 28). Thus, the number of patients with these mutations was too low to identify an association with adverse event using logistic regression analysis. For the polymorphisms 85T>C, 496A>G and 1627A>G, the evaluation of associations between adverse event and allele frequencies showed that the 496A>G was significantly correlated to the occurrence of adverse event [$\chi^2 = 4.84$, df = 1, $p = 0.028$, odds ratio = 0.079 (95% CI = 0.007-0.933)]. Patients with the wild-type 496AA had more frequently adverse event than carriers of the mutation. However, after correction for multiple testing [by the Bonferroni correction technique; with 3 tests performed, a

p-value must be < 0.05/3 (i.e., 0.016) to be statistically significant] it is did not prove to be significant (p = 0.02). No influence on adverse event was seen for 85T>C (χ^2 = 5.035, df = 2, p = 0.081) and for 1627G>A (χ^2 = 8.39, df = 2, p = 0.658)

To evaluate the effect of the 6 polymorphisms detected in the study population on the changes in blood cell counts, the Kruskal-Wallis-test was performed. None of the polymorphisms showed an effect on changes in blood cell counts (table 29).

Table 29 The association between *DPYD* and changes in blood cell counts

***DPYD* polymorphisms**	**Changes in blood cell counts**					
	leukocyte		**erythrocyte**		**thrombocyte**	
	χ^2	***p*-value**	**χ^2**	***p*-value**	**χ^2**	***p*-value**
85T>C	1.503	0.472	1.891	0.389	0.403	0.818
496A>G	0.117	0.733	0.467	0.494	0.021	0.884
1236G>A	1.729	0.189	0.515	0.473	2.414	0.120
1601G>A	0.700	0.403	1.159	0.282	2.057	0.151
1627A>G	4.288	0.117	2.292	0.318	0.376	0.828
2194G>A	0.896	0.344	0.364	0.547	0.741	0.389

4.8.3 Thymidylate synthase polymorphism

In 26 patients experiencing adverse events, the 2R/2R and the 3R/3R were equally distributed 15.38% (n = 4), and 2R/3R occurred in 69.20% (n = 18) of the patients (table 30).

Table 30 TS polymorphism and adverse events in 30 patients

TS polymorphism	**No adverse event**		**5-FU adverse event**		**Total no. of patients**
	No. of patients	**%**	**No. of patients**	**%**	
2R/2R	2	33.3	4	66.7	6
2R/3R	1	5.3	18	94.7	19
3R/3R	1	20	4	80	5

A possible influence of TS polymorphisms on the occurrence of adverse events was tested using logistic regression analysis. No influence of TS genotype was observed on the occurrence of adverse events (χ^2 = 3.083, df = 2, p = 0.214).

A link between TS mutation and the changes in blood cell counts was tested with Kruskal-Wallis-test. No association was observed for the change in leukocyte count (χ^2

= 0.390, p = 0.823), for the change in erythrocyte count (χ^2 = 2.845, p = 0.241) and for the change in thrombocyte count (χ^2 = 0.995, p = 0.608).

4.8.4 Methylenetetrahydrofolate reductase polymorphism

All patients without adverse event (n = 4) were found to be heterozygous for the MTHFR C677T polymorphism (CT). Patients experiencing adverse events were homozygous (TT), heterozygous (CT), and wild-type (CC) for the MTHFR polymorphism in 5, 8 and 13 cases, respectively (table 31).

Table 31 MTHFR C677T polymorphism and adverse events in 30 patients

MTHFR C677T polymorphism	No adverse event		5-FU adverse event		Total no. of patients
	No. of patients	%	No. of patients	%	
CC	0	0	13	100	13
CT	4	33.3	8	66.7	12
TT	0	0	5	100	5

Analysis of the association between the MTHFR C677T and the occurrence of adverse events by logistic regression showed that MTHFR C677T was not linked to the occurrence of adverse events during 5-FU therapy (χ^2 = 4.381, df = 2, p = 0.112).

Using Kruskal-Wallis-test, for the MTHFR mutation, there was no a significant effect of MTHFR mutation on the changes in blood cell counts, either with respect to the leukocyte (χ^2 = 2.429, p = 0.297), or with respect to the erythrocyte (χ^2 = 2.133, p = 0.344), or with respect to the thrombocyte (χ^2 = 0.963, p = 0.618).

4.9 Influence of pharmacokinetics on adverse events and changes in blood cell counts

The $AUC_{5\text{-}FU}$ and the $AUC_{5\text{-}FUH2}$ generated from the final model were 37.93 ± 23.98 mg·h/L and 63.30 ± 19.54 mg·h/L, respectively. The AUC ratio was 0.62 ± 0.30. Comparisons of the $AUC_{5\text{-}FU}$, the $AUC_{5\text{-}FUH2}$, and the AUC ratio were performed according to adverse event groups of patients (table 32). No significant difference between the $AUC_{5\text{-}FU}$ and the $AUC_{5\text{-}FUH2}$ in the group of patients experiencing adverse events compared to the group of patients not reporting adverse events was observed. Similarly, no significant difference in the AUC ratio between these patient groups was observed (p = 0.319).

Table 32 Pharmacokinetic parameters categorized to the adverse event

Parameter	Group	n	Mean ± SD	Minimum	Maximum	t-test (*p*)
AUC of 5-FU (mg·h/L)	No 5-FU adverse event	4	22.70 ± 6.00	17.52	31.50	0.177
	5-FU adverse event	26	40.28 ± 24.89	20.00	152.46	
AUC of 5-FUH_2 (mg·h/L)	No 5-FU adverse event	4	51.23 ± 21.34	30.68	73.43	0.190
	5-FU adverse event	26	65.13 ± 19.01	35.86	97.31	
AUC ratio	No adverse event	4	0.48 ± 0.12	0.35	0.64	0.319
	5-FU adverse event	26	0.64 ± 0.31	0.32	1.57	

Using linear correlation analysis, for the pharmacokinetic parameters, no relationships to the changes in blood cell counts was observed (fig. 16-18). Additionally, there were also no significant correlation between other pharmacokinetic parameters (CL and $t_{1/2}$ of 5-FU, CL and $t_{1/2}$ of 5-FUH_2) and the changes in blood cell counts in this study population (all p-value > 0.05).

Figure 16 The relationship between pharmacokinetics and the change in leukocyte count

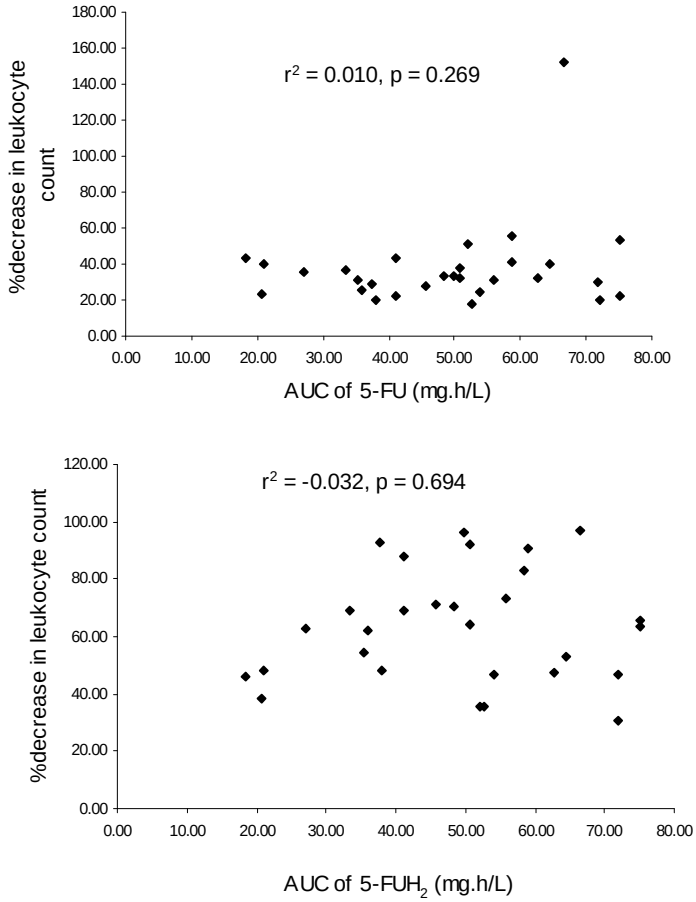


Figure 16 Cont.

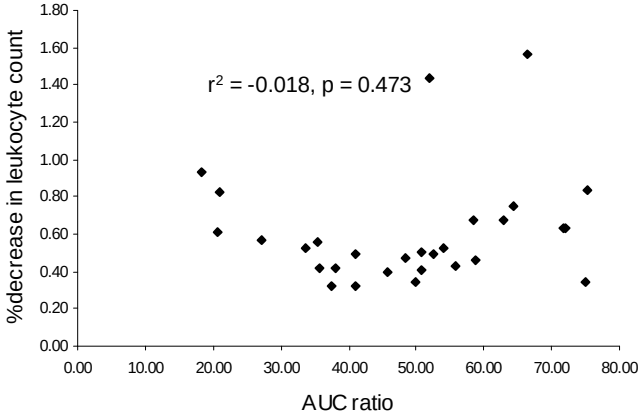
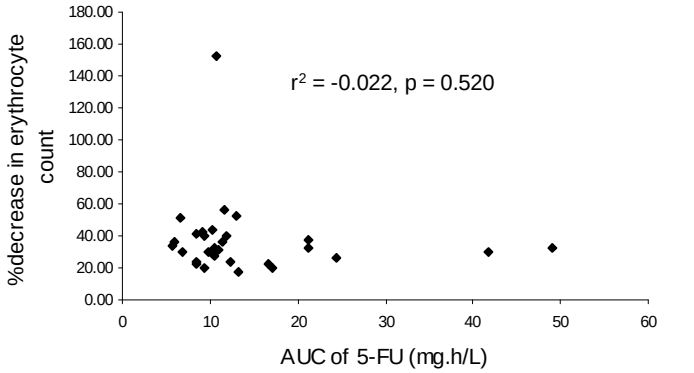
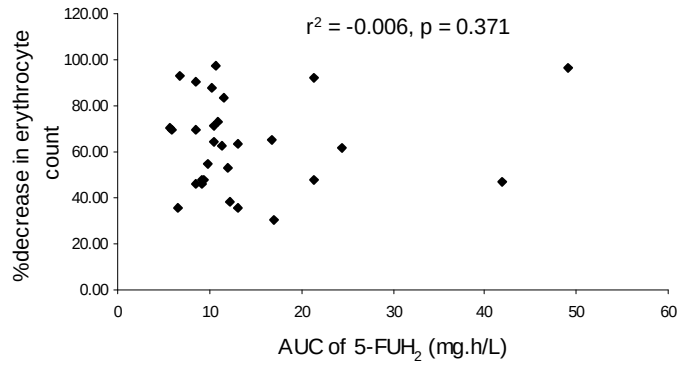


Figure 17 The relationship between pharmacokinetics and the change in erythrocyte count





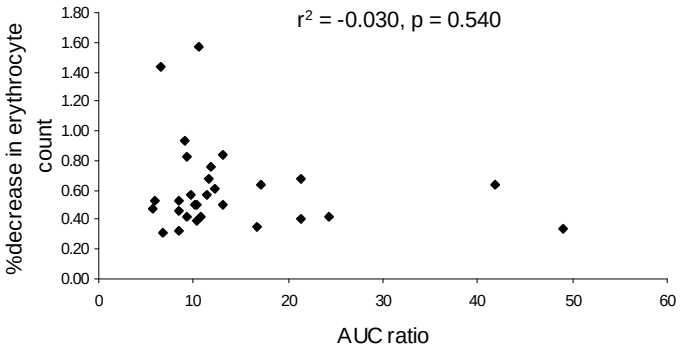
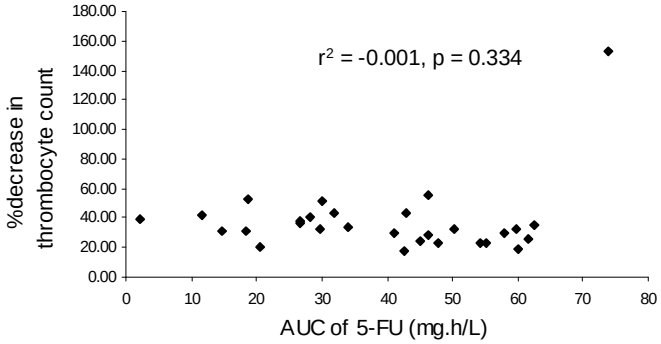
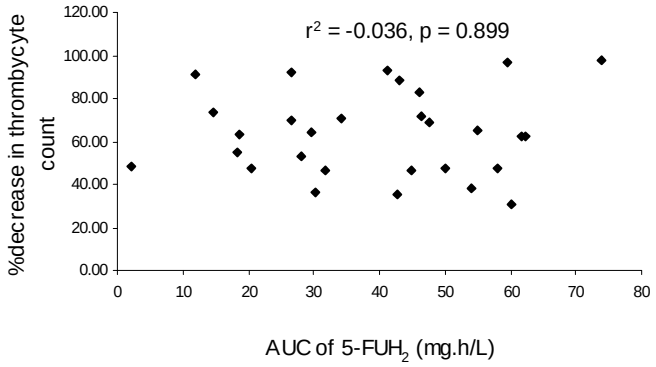
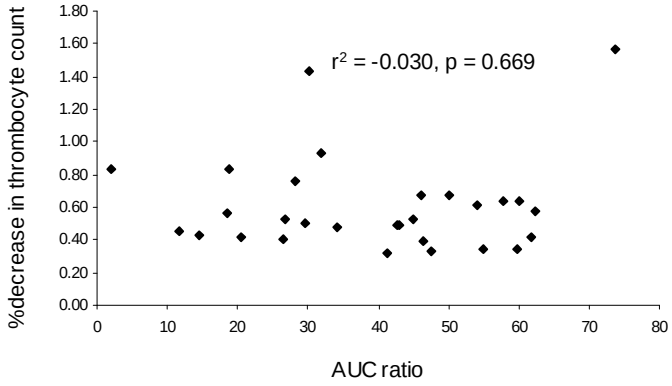


Figure 18 The relationship between pharmacokinetics and the change in thrombocyte count







5. Discussion

In the present study, the pharmacokinetics of 5-FU and 5-FUH_2 were investigated in 30 previously untreated colorectal or oesophageal cancer patients. The three main aims of this study were hence to 1) identify factors of influence on 5-FU pharmacokinetics, 2) identify factors influencing adverse events and blood cell count changes, and 3) evaluate the influence of pharmacokinetics on adverse events and blood cell count changes. These three aspects will be discussed separately.

The most common side effects observed in this study were gastrointestinal, consistent with the know toxicity of 5-FU. Leukopenia, the most common hematologic toxicity associated with 5-FU (Grem, 2000), was observed in only two patients with mild intensity. In addition, other commonly occurring symptoms during i.v. 5-FU therapy including mucositis, nausea, vomiting, anorexia, and diarrhea (Grem, 2000) also observed in this study were tolerable with CDC common toxicity grading of ≤ 2. The lack of serious toxicity of 5-FU may be attributable to the 5-FU regimen used. In comparison to 5-FU bolus administration, the long-term 5-day continuous infusion has a different and more favourable spectrum of adverse events. The pattern of toxicity seems to be dependent on the velocity of administration. An intravenous bolus typically causes depression of both white blood cell and platelet counts, while a continuous intravenous infusion often leads to more severe stomatitis, diarrhea, and hand-foot syndrome (Gamelin et al., 1996; Meta-Analysis Group in Cancer, 1988; Thyss et al., 1986). However, the sample of 30 patients may have been too small to observe more and also severe adverse events.

5.1 Methodological aspect

It is important to realize that the result presented here should only apply when 5-FU is administered as long-term continuous infusion for 5 days because of the relationship suggested between 5-FU pharmacokinetics and both clinical response and toxicity (Diasio & Harris, 1989; Gamelin et al., 996; Grem, 2000).

5.1.1 Analytic methods

5.1.1.1 Determination of DPD phenotype

DPD expression can be phenotypically assessed by determining enzyme activity in an accessible site such as peripheral blood mononuclear cells (PBMC), or by monitoring catabolite formation of endogenous uracil in body fluids. Although determination of DPD activity in PBMC is regarded as a global method to estimate the total DPD activity (Etienne et al., 1994; Harris et al., 1990; Johnson et al., 1997; Lu et al., 1993), it is time-consuming, requires a large volume of blood and radioactive labeled materials, making the test difficult to be used in clinical routine. Since 5-FU and uracil have the same metabolic pathway via DPD due to their similar chemical structures, the concentrations of the naturally occurring pyrimidines in urine or plasma differ between patients with normal and reduced DPD activity, respectively (Tuchman et al., 1985; Gamelin et al., 1999). So analyzing endogenous uracil (U) and dihydrouracil (UH_2) as well as their concentration ratio (UH_2/U) in biological fluids (plasma, urine) before administering 5-FU may be a tool to prospectively identify patients with DPD deficiency. In this study, a simple LC-MS/MS method was developed to determine simultaneously the urinary concentration of uracil and its dehydrogenated metabolite (UH_2). However, the purpose of the measurement of UH_2/U concentration ratios is not to determine the precise DPD activity. The ratio serves as a tool to identify patients with a complete DPD deficiency, and to get an impression of the overall pyrimidine metabolism, including other factors besides DPD activity.

5.1.1.2 Assay method for 5-FU and 5-FUH_2

A number of assays have been developed to measure 5-FU and its main metabolite, 5-FUH_2 in plasma. The assay used most often today in clinical pharmacokinetic studies is HPLC with reverse phase column. Several different sample preparation techniques are used with the HPLC and permit determination of 5-FU and of 5-FUH_2 in the nanogram range (6.5-75 ng/mL for 5-FU, 75-100 ng/mL for 5-FUH_2, Ackland et al., 1997; Bocci et al., 2000; Casale et al., 2002; Findlay et al., 1996; Maring et al., 2005). Thus, HPLC methods are useful for measuring 5-FU and 5-FUH_2 concentrations in the range observed in patients receiving the drug via continuous infusion. In the present study, a HPLC

method was developed to determine simultaneously the plasma concentration of 5-FU and 5-FUH_2, which was an easily accessible technology in laboratory and allowed low 5-FU (5 ng/mL) and 5-FUH_2 (10 ng/mL) plasma concentrations to be determined.

5.1.2 The raw genotyping results

In the present study, the 23 coding exons of the *DPYD* gene were analyzed for the presence of mutations/polymorphisms in order to associate the mutations and adverse events. Analysis of the *DPYD* gene in 30 patients revealed 6 different polymorphisms with an allelic frequency ranging from 1.67 to 21.67%. No unknown mutations were identified. Five of the observed sequence variations have been described as common polymorphisms in previous studies: (a) 85T>C (*DPYD**9A, Cys29Arg); (b) 496A>G (Met166Val); (c) 1601G>A (*DPYD**4, Ser534Asn); (d) 1627A>G (*DPYD**5, Ile543Val); and (e) 2194G>A (*DPYD**6, Val732Ile) (van Kuilenburg, 2004). One rare exonic alteration has also been reported before: 1236G>A (Seck et al., 2005).

Additionally to the *DPYD* gene, far more information is available regarding polymorphism of target enzyme TS. There 3 predominant genotypes of TS: (a) a homozygous with double-tandem repeat (2R/2R), (b) homozygous with triple-tandem repeat (3R/3R), and (c) heterozygous with both alleles (2R/3R). In the present study, among 30 patients analyzed for the TS genotype, 5 (16.70%) were 3R/3R, 19 (63.30%) were 2R/3R, and 6 (20.00%) were 2R/2R. The allele frequency of 3R/3R genotype in 30 Caucasian patients presently investigated was higher than that in reports of other studies with Caucasian patients [32% (28 of 90 patients), Lecomte et al., 2004; 23.84% (21 of 88 patients), Jakobsen et al., 2005]. The difference in allele frequency of 3R/3R may be due to the number of patients enrolled in the study.

Directly linked to the 5-FU-mediated inhibition TS is the presence of intracellular folate. A polymorphism that may influence the efficacy of 5-FU by influencing folate pools is that of methylenetetrahydrofolate reductase gene (MTHFR). The major nucleotide 677 polymorphism (C to T) at codon 22 (exon 4) is the most commonly linked with altered enzyme activity (Maring et al., 2005). In the present study, the MTHFR 677CT and MTHFR 677CC genotype occurred with almost the same frequency [43% (n = 13) and 40% (n = 12), respectively], while the MTHFR 677TT genotype was only observed in

17% (n = 5) of the patients. The frequency distributions of MTHFR 677TT genotype observed in the present Caucasian patients were in agreement in the Caucasians [11.4% (9 of 79 patients), Jakobsen et al., 2005; 18.4% (18 of 98 patients), Etienne et al., 2004].

5.2 Pharmacokinetics of 5-FU and 5-FUH_2

5.2.1 Raw concentration-time data

In the thirty cancer patients studied here, plasma concentrations of 5-FU and 5-FUH_2 varied considerably between and within patients during the 5-day infusion (fig. 10). Variations in 5-FU and 5-FUH_2 plasma concentrations were observed according to the time of day (fig. 11). Similar observations were found in most studies on pharmacokinetics of 5-FU administered by continuous infusion (Erlichman et al., 1986; Hillcoat et al., 1978; Jiang et al., 2004b; Schneider et al., 1995; Vokes et al., 1996). The circadian variations in plasma concentrations of both substances can be explained by the existence of a circadian rhythm of the DPD activity, which has been suggested from both human and animal investigations (Harris et al., 1990; Jaing et al., 2004a; Petit et al., 1988). Petit et al. (1988) reported a 2.2-fold difference in 5-FU concentration at steady-state (C_{ss}) during a 5-day infusion of 1,000 mg/m^2/day (with i.v. cisplatin on day 1); the peak value averaged 560 ng/mL and occurred at 1:00 a.m., whereas the minimum value averaged 249 ng/mL and occurred at 1:00 p.m. With protracted continuous 5-FU infusions of 300 mg/m^2/day, Harris et al. (1990) observed a comparable variability, but the time when the peaks occurred was different, with peak values (27.4 ng/mL) occurring at 11 a.m. and trough values (5.6 ng/mL) at 11 p.m. The discrepancy between the times of day at which peak and trough 5-FU levels occurred in these two studies suggests that other factors, perhaps geographic, seasonal, individual sleep and wake habits, administration of other drugs, or a combination of the four, and presumably other unknown factors, may influence 5-FU clearance. There is evidence of consistency in the timing of highest and lowest DPD activity within a given individual (Grem et al., 1997; Harris et al., 1990) and it seems likely that each individual exhibits his or her own circadian rhythm. This may lead to the variation in plasma 5-FU and 5-FUH_2 concentrations from patient to patient. Sampling at different times of the day will therefore yield different results. Since plasma samples in the present study were drawn

randomly around few predefined time points relative to the start of the infusion, it was likely that circadian variability in plasma concentrations would be visible (fig. 11).

It has to be noted, however, that in the present study, circadian differences could not be detected, because a limited sampling scheme was used during the infusion which allowed to get an impression of the overall intraindividual variability, but sampling was not intense enough to determine the individual peaks and troughs.

The 5-FUH_2 concentrations in the present study were consistently greater than of the corresponding 5-FU plasma concentrations (fig. 11), which is in agreement with a small study (n = 11) conducted by Ackland et al. (1997). These results may depend on the volume of distribution of 5-FU which is relate to 5-FUH_2.

5.2.2 Population pharmacokinetic analysis

Population pharmacokinetic approach is increasingly recognized as a valuable tool in drug therapy optimization. The major advantages of this technique include the ability to describe complex pharmacokinetic models, to quantify interindividual and intraindividual variability, and to identify the quantitative relationships between patient characteristics (covariates) and these variabilities. Furthermore, sparse and dense data, multiple dose levels, and different treatment schedules can be analyzed simultaneously.

Data concerning the pharmacokinetic profile of 5-FUH_2, which is dependent on systemic (mainly hepatic) DPD activity rather than on the enzymatic activity in PBMCs, are still needed. This investigation may add to that issue, because it investigates more extensively the pharmacokinetics of this primary 5-FU metabolite and it is the first covariate-model analysis that takes into account genetic polymorphisms in *DPYD*, *TS*, and *MTHFR* genes. In addition, additional information on plasma concentrations after the end of the long-term infusion is provided, which has not yet been investigated extensively in literature.

A population pharmacokinetic model was developed that described the plasma concentrations of 5-FU and its metabolite, 5-FUH_2, in 30 cancer patients receiving long-term 5 days continuous infusion. In the present study, 5-FU plasma pharmacokinetics were well described by a two-compartment open model with first-order elimination. The point estimate for 5-FU clearance (238 L/h) was close to values previously reported

during a 5-day continuous infusion (181 L/h: Milano et al., 1992, 287.71 L/h: Fleming et al., 1992) or during a 3-day continuous infusion (270 L/h: Grem et al., 1993b), by non-compartmental analysis. Interestingly, despite different models used in data interpretation, numerical values of clearance were also similar in a study reported by Etienne et al. (1998): a clearance value of 235 L/h was derived from a 1-compartment model with first-order elimination. In addition, the point estimate values obtained for 5-FU clearance in the present study were consistent with the mean values of the half-saturating plasma concentration (K_m) and the maximum rate of elimination (V_{max}) previously observed in a study in which a bolus of 400 mg/m^2 was followed by a 22-hour infusion of 600 mg/m^2 for 2 consecutive days (Terret et al., 2000). Indeed, the ratio V_{max}/K_m = 250 L/h approximates the clearance when plasma 5-FU concentrations are far below K_m, which is the case for this schedule of administration.

The combined pharmacokinetics of 5-FU and 5-FUH_2 were well described by an open two-compartment model with first-order elimination for 5-FU and a one-compartment model for 5-FUH_2. This combined model proved to be robust: the median values obtained during jack-knife analysis were in the same range as the respective model estimates for every parameter analyzed (table 25). Besides, the parameters' point estimates of 5-FU in the combined pharmacokinetic model (table 25) were essentially equal to those generated with the concentration versus time data of 5-FU only (0.4-5.4% deviation), and the 95% confidence intervals included the mean parameters' estimates obtained from the former model of 5-FU. However, the model tended to overestimate plasma concentrations of 5-FU (fig. 12a), indicating that the model had some limitations in describing the variability of 5-FU concentrations observed in the study. Because of the limited amount of concentration data, further model refinement was not possible. The pharmacokinetic model was on average unbiased in predicting 5-FUH_2 concentrations (fig. 12b).

The pharmacokinetics of 5-FU have been extensively studied, while the pharmacokinetics of 5-FUH_2 in human plasma are less well investigated, especially during long-term continuous infusion. There are some studies published which investigated 5-FUH_2 pharmacokinetics after 5-FU i.v. bolus injection (Bocci et al., 2000; Heggie et al., 1987; Di Paolo et al., 2001; Di Paolo et al., 2002). In the present study, the pharmacokinetics of 5-FUH_2 were modeled as a metabolite compartment connected to the central compartment (fig. 9), and a one-compartment model best described 5-FUH_2 pharmacokinetics.

The parent-metabolite pharmacokinetic model was used to estimate pharmacokinetic parameters for the individual patients (table 23). The total clearance of 5-FUH_2 was smaller than that of 5-FU, mean total clearance values were 117 L/h and 238 L/h for the metabolite and the parent compound, respectively. These differences in clearance translated to a longer elimination half-life ($t_{1/2, \lambda z}$) of 5-FUH_2: the $t_{1/2, \lambda z}$ of 5-FU averaged 2 min, whiles the $t_{1/2, \lambda z}$ of 5-FUH_2 was on average 35.4 min. The longer half-life of 5-FUH_2 could be one explanation for the higher plasma concentrations of the metabolite during continuous infusion (fig. 11). It is well known that the availability of 5-FU for anabolism is regulated primarily by catabolism (Diasio & Harris, 1989). The average of the metabolic clearance of 5-FU converted to 5-FUH_2 was 205 L/h, indicating that approximately 86% of 5-FU was catabolized to 5-FUH_2. This result is in agreement with previous studies which showed that most individuals eliminate around 85% of a 5-FU dose via the catabolic pathway (Diasio & Harris, 1989). Additionally, up to 10% of the dose is excreted unchanged by the kidneys (Diasio & Harris, 1989).

The estimate for the coefficient of variation of interindividual variability in total clearance of 5-FU was high both in the base pharmacokinetic model for 5-FU (CV = 44.3%) and in the base combined pharmacokinetic model for 5-FU and 5-FUH_2 (CV = 44.0%). Correspondingly, most studies on 5-FU pharmacokinetics demonstrated that the interpatient variability in 5-FU pharmacokinetics was relatively large, with CV 20-31% (Etienne et al., 1998; Sandström et al., 1996; Terret et al., 2000). Individual factors like genotypes, sociodemographic variables, disease-related variables, concomitant treatments, etc., could be sources of this interindividual variability. In the present study, besides patient's characteristics and other factors, mutations in three genes related to the metabolism and the activity of 5-FU were tested as covariates for 5-FU pharmacokinetic parameters in order to explain at least parts of the interindividual variability in 5-FU pharmacokinetics.

5.2.3 Factors of influence on pharmacokinetic parameters

Among the variables that were tested by the NONMEM approach, it was found that the full predictive model (final covariate model) included two independent variables which were body surface area and the mutation at position 677 in the MTHFR gene. The final model for covariables indicated that total clearance of 5-FU tended to increase with

increasing body surface area and tended to be higher in patients harbouring the MTHFR C677C (wild-type) or MTHFR C667T (heterozygous) genotypes. Other pharmacokinetic parameters were not statistically significantly influenced by individual factors.

The present result shows that the total 5-FU clearance increases to 0.85% with 1% increase in body surface area. Although several studies could not substantiate an influence of body surface area on 5-FU clearance (Climente-Martí et al., 2003; Etienne et al., 1998; Porta-Oltra et al., 2004), but a study supporting the present finding, pointed out that this covariate can be the predictor of 5-FU clearance (Port et al., 1991). These suggest that body surface area is, at least to certain extent that can be quantified, useful for dose individualization.

If a relationship between body surface area and clearance of 5-FU is not surprising, an opposite relationship would be more expected with MTHFR genotype. There is no clear reason known at present which may serve as a scientific explanation of this finding. One reason may be that the finding has arisen by chance. Recent studies suggested an influence of the MTHFR C677T polymorphism on the folate pool in cancer tissue (which influences the formation and stability of inhibitory ternary complexes between 5,10-methylentetrahydrofolate, TS and 5FdUMP) and response to treatment. However, the exact mechanisms by which an influence on 5-FU clearance may be explained are unknown to date, and cannot be deduced from what is known about the enzymes and metabolic pathways to date. Further in vitro studies to clarify a possible relationship and in vivo studies to verify the present result are needed.

For the polymorphisms in the TS gene, no influence of the mutations studied here on the pharmacokinetics of 5-FU and 5-FUH_2 was observed. Thus, the possible impact of TS polymorphisms on 5-FU pharmacology may rather occur on a pharmacodynamic than on a pharmacokinetic level.

The significant interpatient variability in 5-FU clearance may be explained in part by genetic differences in the enzyme activity of DPD (Milano & Etienne, 1994). Plasma clearance of 5-FU is reported to be dependent on the catabolic pathway which is closely linked to the activity of DPD (Diasio & Harris, 1989). To date, there are only very few reports combining results of DPD genotyping with pharmacokinetics of 5-FU. It is known that patients with complete deficiency of DPD activity demonstrate minimum catabolism

of 5-FU, with a 10-fold longer half-life of 5-FU compared with patients with a normal DPD activity (Diasio et al., 1988). In a patient with a partial deficiency of DPD, due to heterozygosity for the IVS14+1G>A mutation (exon 14 skipping), the clearance of 5-FU was 2.5 times lower and the AUC of 5-FU (24.1 mg.h/L) was 2.5 times higher, compared with controls (Maring et al., 2002). However, the mutation reportedly leading to more pronounced changes in 5-FU pharmacokinetics was not observed in the population study here.

The present study is the first systemic analysis in which the results of a sequencing of the *DPYD* gene were combined to pharmacokinetics of 5-FU. Since the small number of mutations in the *DPYD* gene was observed in the present study population and since the incidence of some DPD mutations was too low to reliably identify a possible relationship, thus patients were divided into three groups depending on the presence of at least one single-nucleotide polymorphisms (SNP) in the *DPYD* gene: wild-type genotype, heterozygote, and homozygote mutants with respect to different mutations. The effect of DPD genotype on the total clearance of 5-FU in our model were treated with that respect. Although an apparent influence of DPD genotype on total clearance of 5-FU was also observed in the present study: i.e. point estimates were 263 L/h (95% CI, 231-295) and 175 L/h (95% CI, 87-263) in patients with wild-type and homozygous mutations, respectively, the confidence intervals for estimations of the total clearance of 5-FU were very broad with overlapping values between the groups. Thus, the DPD genotype was not included in the final model because of the lacking statistical significance to improve the model. Accordingly, there was a lack of effect of the most mutations detected in the present population study on pharmacokinetics of 5-FU (AUC of 5-FU) also in other reports (Zhu et al., 2004).

No effect of DPD activity determined by the urinary UH_2/U concentration ratio on any pharmacokinetic parameters of 5-FU or 5-FUH_2 was found in the present study. The lack of correlation between 5-FU pharmacokinetics and the urinary UH_2/U concentration ratio suggests that this ratio may not predict systemic clearance of the drug. The urinary UH_2/U concentration may not be representative of the enzyme activity of DPD in the body, particularly the liver.

Other factors including sex, weight, and body mass index could potentially affect the total clearance of 5-FU. Most of the models which described the influence of these covariables, especially weight and body mass index, on 5-FU pharmacokinetics were not stable, leading to an exclusion of these factors in multivariate analysis. In another study in 27 cancer patients who received 5-FU 450 mg/m^2, 1 day per week for 48 doses, weight was defined as a predictor of 5-FU clearance (Climente-Martí et al., 2002). However, the authors indicated that adding this covariate to the basic model of 5-FU, interindividual variability of clearance were reduced only from 76% to 71%, suggesting that weight could not explain totally the interindividual variability in 5-FU clearance.

Contradictory results have also been reported on the influence of gender on 5-FU clearance. A study of Milano et al. (1992) indicated that women showed median 5-FU clearance values that were 10% lower than those found in men (p = 0.0005). In another study, the average clearance in males was by 0.22 L/min higher than that in females (Port et al., 1991). Correspondingly, DPD activity in women was about 15% lower than in men (Milano & Etienne, 1994). In the present study, the separate testing of covariates in the base pharmacokinetic model for 5-FU revealed a 2-fold lower 5-FU clearance in females. However, this influence of sex on the 5-FU clearance was not retained in the final covariate model for 5-FU. Similarly, in the parent-metabolite pharmacokinetic model (combined pharmacokinetic model) sex was not included in the final covariate model because in combination with other covariates, the body surface area was found to be a statistically significantly better predictive factor for 5-FU clearance than sex. It should be noted that the discrepancy with those previous studies can be consequences of a) the relatively small patient population (n = 30), b) differences in 5-FU administration, since 5-FU pharmacokinetics are strongly dependent on the administration schedule (Larsson et al., 1996), and c) the small number of women in our study population (female/male ratio = 1:6). Clearly, the present study therefore did not have the power to detect small gender differences. Moreover, this apparent discrepancy may also be caused by differences in both the statistical approaches and the way of determining 5-FU clearance. In addition, the previously found difference in 5-FU clearance between men and women was rather small, since average values were 172 and 155 L/h/m^2, respectively (Port et al., 1991). However, the final result of the present study, i.e. that gender does not have an influence on 5-FU pharmacokinetics, is corroborated by a large population analysis (n = 104). This

analysis showed that gender was not an independent parameter in predicting 5-FU clearance (Etienne et al., 1998).

Literature is contradictory concerning the influence of age on 5-FU pharmacokinetics. As in the present study, age did not appear to influence the elimination of 5-FU (Climente-Martí et al., 2002; Port et al., 1991; Porta-Oltra et al., 2004), while other studies suggested that increased patient age lead to a moderate decrease in 5-FU clearance (Etienne et al., 1998; Milano et al., 1992). This discrepancy may reflect different methods of statistical analysis: age was considered as a continuous variable in most studies (as in the present study), while age classes were examined both alone and in covariance matrices in others. Additionally, in some studies, the age range examined may not have included sufficiently patients with advanced age where the influence may have been more pronounced.

Likewise, as the present study reflects, other authors did not find a significant influence of hepatic function and renal function tests on 5-FU pharmacokinetics (Climente-Martí et al., 2002; Fleming et al., 1992; Porta-Oltra et al., 2004). These results are in agreement with the findings obtained in two recent studies which enrolled patients with liver metastases from gastrointestinal cancer (Maring et al., 2003) and patients with mild organ dysfunction (hepatic or renal) (Fleming et al., 2003). The influence of liver metastases on the pharmacokinetics of 5-FU and 5-FUH_2 after bolus injection of 5-FU was studied in a comparison between 16 patients with metastatic and 18 patients with nonmetastatic gastrointestinal cancer. The patients in both groups displayed similar pharmacokinetics. No effect of liver metastases on 5-FU clearance was observed (Maring et al., 2003). Similarly, a trial on 24-h continuous infusion of 5-FU with leucovorin in patients with elevated serum bilirubin or mild renal dysfunction (n = 64) showed that no association between 5-FU clearance and either serum bilirubin (p = 0.517) or serum creatinine (p = 0.396) was present (Fleming et al., 2003). In a patient with colorectal carcinoma and end-stage renal insufficiency on maintenance hemodialysis therapy, pharmacokinetic parameters of 5-FU and 5-FUH_2 were in the ranges reported in literature for patients with normal renal function (Rengelshausen et al., 2002). Because renal elimination of unchanged 5-FU accounts for only 10% of the injected dose (Diasio & Harris, 1989), renal abnormalities should have, a priori, a minimal effect at least on pharmacokinetic of parent compound 5-FU. Thus, these findings may be the explanation of why the influences of hepatic and renal function test were not observed in the present study.

Finally, we have shown that by using a statistical approach based on NONMEM analysis, it is possible to identify several independent patient characteristics which have a significant influence on 5-FU clearance during a 5-day continuous infusion. Previous reports by others (Milano et al., 1994; Vokes et al., 1996) have identified target 5-FU plasma concentrations at steady state or AUC values of 5-FU which were related to an optimal 5-FU therapeutic index during 5-day continuous infusion. Given these findings, there is a potential clinical interest for 5-FU dose tailoring. Although the two covariables identified in the present investigation were significantly correlated to 5-FU clearance and decreased the remaining interindividual variability from 44.0% to 29.6%, the so far unexplained interindividual variability in 5-FU clearance remained high. Additionally, the individual factors tested in the present study could not explain the interindividual variability in the elimination rate constant of 5-FUH_2 (CV = 31.8%). These may be explained by the fact that the residual variability (64% for 5-FU and 43% for 5-FUH_2) included many sources of random variability, i.e. intraindividual variability, analytical error, pre-analytical errors such as recording of sampling times, different ways of handling the blood samples, and unidentified factors of potential influence such as further mutations in genes. Finally, it is the hope of the present study to stimulate such future investigations to clarify the influence of other factors on the pharmacokinetics of 5-FU.

5.3 Factor of influence adverse events and changes in blood cell counts

5.3.1 DPD phenotype

DPD is generally considered the rate-limiting step in the catabolism of the 5-FU (Diasio & Harris, 1989). The activity of DPD may be an important determinant for predicting the toxicity of 5-FU. Individuals can be screened for alterations in DPD activity by phenotyping and/or genotyping before the first administration of 5-FU. Studies have documented that patients with absent or reduced DPD activity show severe hematological, neurological, and gastrointestinal toxicity upon standard 5-FU administration (reviewed in Ploylearmsaeng et al., 2006) due to a reduced 5-FU clearance (Maring et al., 2002; van Kuilenburg et al., 2000). In the present study, no impact of DPD phenotype on adverse events and on changes in blood cell counts was seen. Since only mild adverse events were observed, and no dramatically decreased DPD activity was present in the present study population, thus no such relationship was found.

The UH_2/U concentration ratios in urine collected before the 5-FU infusion was started were 1.18 ± 0.50 (mean ± SD) (n = 29). One patient had undetectable UH_2 concentrations in urine which made exact quantitative assessment of DPD activity impossible. This patient experienced grade 1 toxicity (nausea and vomiting). With respect to the concentrations of urinary uracil in patients with DPD deficiency, most of the DPD deficient patients show extreme increases in urinary uracil concentrations (Milano & Etienne, 1994). The uracil concentration in urine of this patient (4.14 µM) appeared to be lower compared to the entire study population (median = 29.63 µM), which may have caused that the UH_2 concentrations were too low to be quantified.

Gamelin et al. (1999) showed in 152 patients that a low plasma UH_2/U concentration ratio (< 1.8), as expected in DPD deficiency, is associated with toxic effects after the first weekly course of 5-FU treatment. In this study, toxic effects were observed only in patients with initial UH_2/U concentration ratios of less than 1.8. No adverse effects were noted in patients with UH_2/U concentration ratios greater than 2.25. Based on these results, the authors speculated that plasma UH_2/U concentration ratios could help to identify patients with metabolic deficiency and to predict the occurrence of toxic side effects of 5-FU, and thereby reduce the risk of 5-FU toxicity. In contrast, the present study did not corroborate the existence of an association between the (urinary) pretreatment UH_2/U concentration ratios and the risk of developing side effects of 5-FU. In the present study, the average pretreatment UH_2/U concentration ratios were lower in patients who presented 5-FU-related toxicity (1.17) than in patients who tolerated the treatment (1.29) but this difference did not reach statistical significance ($p = 0.65$). In addition, the risk of developing side effects was not linked to the pretreatment UH_2/U concentration ratios ($p = 0.646$). This may be due to the small number of patients in this study ($n = 30$) and the fact that most patients ($n = 26$) experienced mostly mild adverse events. Besides, the matrix (urine versus plasma), the method of quantification (LC-MS/MS versus HPLC with diode array detection) and the 5-FU treatment regimen (5-day continuous infusion versus 8-hour infusion) investigated differed from the published study (Gamelin et al., 1999). To date, no studies directly comparing the UH_2/U ratio in plasma and urine have been performed.

Because blood dyscrasias, especially leukopenia, are the most common adverse effects of 5-FU therapy (Grem, 2000), the relationship between the pretreatment urinary UH_2/U concentration ratios and the relative decreases in blood cell counts (leukocyte,

erythrocyte, and thrombocyte) were also investigated. No correlation between the pretreatment UH_2/U concentration ratios and the relative decreases in blood cell counts were found. This indicated that different responses among patients were not associated with the differences in the UH_2/U concentration ratios in urine. In another clinical study in 40 gestational trophoblastic tumor (GTT) patients treated with 30 mg/kg of 5-FU or prodrug floxuridine (FUDR) during 10 days per cycle, correlation analysis showed that the pretreatment UH_2/U concentration ratio in plasma were significantly correlated with the absolute neutrophil count (r = 0.768, p < 0.01). The authors concluded that pretreatment UH_2/U concentration ratios were a key factor associated with the interpatient variability in toxicity and efficacy (Jiang et al., 2004b). It may be speculated that the absolute neutrophil count is a more sensitive parameter than the relative decrease in white blood cells used in the present study or that the UH_2/U ratio in plasma is more reliable than the ratio determined in urine, however, there is no scientific endorsement for these hypotheses.

These findings suggest that methods to estimate the DPD activity by using endogenous pyrimidines may be imperfect to predict adverse effects caused by 5-FU.

5.3.2 *DPYD* polymorphisms

The mutations detected in our patients who presented 5-FU adverse events were 85T>C, 496G>A, 1236G>A, 1601G>A, 1627A>G and 2194G>A. Analysis of these 6 mutations in relationship to adverse events revealed that only the 496A>G mutation was associated with 5-FU adverse events ($p < 0.028$). Zhu et al. (2004) studied the association between *DYPD* mutations and toxicity of 5-FU in 17 patients with colorectal adenocarcinoma. Seven mutations in the *DPYD* gene, including 85T>C, 496A>G, 1627A>G, 3351T>C, 3649G>A, 3844A>G, and 3856T>C, were correlated to the 5-FU-related toxicity grouped as grade 0-2 (n = 13) and grade 3-4 (n = 4). In this study, each informative nucleotide was tested separately for an additive allele effect on the binary toxic response. None of the nucleotides showed a statistically significant additive allele effect on the toxicity outcome (all $p > 0.05$), either with respect to cycle 1 toxicity or with respect to the worst toxicity during the entire study. The authors indicated that a combination of mutations may explain a part of the toxicity, while each single mutation did not explain toxicity. It

remains unclear which mutations exerted a particular influence on the occurrence of severe toxicity.

The 496A>G mutation has been described in 3 patients who were suffering from severe 5-FU toxicity but who also carried other variants including 85T>C (*n* = 1; van Kuilenburg et al., 2000), 85T>C and 2846A>T (*n* = 1; van Kuilenburg et al., 2000), and IVS14+1G>A (*n* = 1; Johnson et al., 2002). In the present study the 496A>G mutation was found in 7 patients, who also carried other variants (fig. 20), with an allele frequency similar to those found in other populations (Sech et al., 2005; Zhu et al., 2004), however, three of these patients did not experience adverse events. Hence, patients who were heterozygous for 496A>G mutation had a decreased risk for the occurrence of adverse events [odds ratio = 0.079 (95% CI, 0.007-0.933)]. It has to be emphasized that there was no patient homozygous for the 496GG mutation in the present study. In addition, the odds ratio showed a very broad confidence interval which almost included 1 and 0 that made the result questionable. Several mutations were tested in this way in this study, thus the result may have arisen by chance, but it may also be a hint that this mutation may have some effect on the occurrence of adverse events. However, after a correction for multiple testing by the Bonferroni correction technique; with 3 tests performed, a p-value must be < 0.05/3 (i.e., 0.016) to be statistically significant, the result is no longer statistically significant, so that the findings in the present study are lastly not contradicting literature findings (van Kuilenburg et al., 2000; Johnson et al., 2002). This correction is very conservative as it is not adjusted for linkage disequilibrium between the polymorphisms. However, the present results also do not support the literature findings, because there was no a statistically significant influence of the mutation in the direction that more adverse events occur when the mutation is present (van Kuilenburg et al. 2000; Jonhson et al., 2002). Since the numbers were too small to draw a definitive conclusion, since a homozygous carrier of the mutation was not present in the study population, and since the present results does not support the observed effect of this mutation in the literatures, it is unlikely that such a relationship could be corroborated in a larger study.

Although the *DPYD* 85T>C and 1627A>G mutations have been identified in patients who presented severe 5-FU toxicity (reviewed in Ploylearmsaeng et al., 2006), in the present study, no association between these two mutations and adverse events could be assessed ($p > 0.05$). The association between the presence of the 1601G>A and of 2194G>A

mutations with 5-FU adverse events could not be investigated in our study, because these mutations were found in only 1 and 2 patients, respectively. At the present, no study has reported that patients carrying the 1236G>A mutation were at risk for 5-FU toxicity. Since this mutation does not lead to an amino acid exchange, and the DPD activity is reported to be normal (Seck et al., 2005), it is not expected that carriers of this mutation differ from the general population in terms of 5-FU associated events. In our study, one patient who experienced adverse events carried this mutation, but the toxicity was mild (grade 1).

In 7 patients who experienced adverse events during the present study, no mutations in the coding sequences of the *DPYD* gene could be detected. In 14 patients with severe 5-FU toxicity, ten *DPYD* exons containing most of the known mutations were analyzed using gene sequencing techniques, and in 3 patients, no mutations were detected at all (Collie-Duguid et al., 2000). It cannot excluded, however, that genetic factors not identified in the present study like a mutation in the promoter region of the *DPYD* gene may have been present in the patients with adverse events who did not carry a mutation in coding regions. Recently, Hasegawa et al. (2005) investigated polymorphisms in the 5´-flanking region of *DPYD* gene, which are considered to control expression of *DPYD* gene, in genomic DNA extracted from 37 kinds of human cancer cells. As the results, in DLD-1 cells, which have C-insertion polymorphism in 5´-flanking region of the *DPYD* gene, the DPD activity was below detection limit (≤ 0.5 pmol/min/mg protein). Furthermore, 50% of cytosine residue on the CpG site generated by the C insertion was methylated at the 5 position which might be associated with loss of the sequence of binding site for transcription factors. The authors indicated that the prevention of binding a transcription factor with a methylated newly generated CpG site that probably affects *DPYD* gene regulation (Hasegawa et al., 2005). Considering the patients without 5-FU adverse events during the present study (3 of 4 patients), mutations were also detected in the *DPYD* gene. This finding indicates that screening for coding mutations alone cannot unambiguously identify all patients at risk.

The most often described mutation in the *DPYD* gene associated with 5-FU-related toxicity is a G to A point mutation within the 5´-splicing site of intron 14 (IVS14+1G>A, known as *DPYD**2A), which leads to skipping of exon 14 and consequently to DPD enzyme deficiency (Raida et al., 2001; van Kuilenburg et al., 2001). In this study, we did not find this mutation in any patient. However, the presence of IVS14+1G>A is not the

only reason for severe 5-FU-related toxicity. Indeed, studies on populations of individuals phenotypically deficient in DPD activity and of patients with 5-FU-related toxicity detected the IVS14+1A mutation in only 14 of 22 (64%) (van Kuilenburg et al., 2002) and in 6 of 25 (24%) subjects (van Kuilenburg et al., 2001). In a prospective study carried out in 351 patients receiving 5-FU to assess the impact of *DPYD* mutations on 5-FU toxicity, there was no evidence for a pivotal role of the exon 14 skipping mutation (Kollmannsberger et al., 2001). However, although the precise role of this mutation as a prognostic factor in the 5-FU-related toxicity still has to be fully established, there is some evidence to suggest that patients with the IVS14+1G>A mutation are at increased risk for the development of severe 5-FU-related toxicity (Raida et al., 2001; van Kuilenburg et al., 2002). Some authors suggested to advise clinicians to screen all patients for the IVS14+1G>A mutation prior to 5-FU therapy. If the IVS14+1G>A mutation is present, dose reductions should be performed or alternative cytotoxic agents should be considered (Omura, 2003; van Kuilenburg, 2004).

5.3.3 Thymidylate synthase polymorphism

The controversy regarding the association between the mutant genotype of *DPYD* gene and 5-FU toxicity suggests a role for additional genomic variants or an influence of epigenetic events. Polymorphism in drug targets are an important area in pharmacogenetic studies, as interindividual differences in the expression of drug targets could lead to resistance or toxicity towards standard chemotherapy regimens. Thymidylate synthase (TS) is considered to be the main intracellular target of 5-FU. In addition to *DPYD* gene, the TS gene (called *TYMS*) also contains a genetic polymorphism that may be also involved in efficacy or toxicity of 5-FU-based chemotherapy.

Genetic polymorphisms in the *TYMS* gene in the promoter enhancer region have been shown to influence toxicity of 5-FU based therapy (Lecomte et al., 2004; Pullarkart et al., 2001), although protein expression levels were not linked to 5-FU pharmacodynamics (Tomiak et al., 2001, Westra et al., 2005). In these studies, individuals who were homozygous for the double repeat in the *TYMS* promoter region (2R/2R) had more severe side effects to 5-FU ($p < 0.05$). In contrast, our results showed that no influence of the *TYMS* tandem repeat promoter polymorphism on 5-FU adverse events ($p = 0.214$) and on the changes in blood cell counts (all $p > 0.05$). Since only few toxic cycles and no serious

toxicity was observed in the present study and since the sample size of 30 patients may be too small, the statistical power of the investigation of the association may have been insufficient. However, the present results are comparable to a previous study in 17 patients which also did not confirm an effect of the *TYMS* polymorphism in the promoter region on 5-FU-related toxicity ($p = 0.242$; Zhu et al., 2004).

The difference in the correlation between *TYMS* promoter genotype and 5-FU adverse events might be obscured by the fact that patients were treated with the different dose and regimen. It should be noted that 5-FU may act as two different drugs according to mode of administration (Grem, 2000). Bolus 5-FU may exert its major effect on RNA, whereas continuous infusion may have a preferential effect on TS. The predictive value of TS gene polymorphism may vary with mode of administration. However, analysis by type of 5-FU-based therapy showed that the rate of toxicity is dependent on the number of patient with a favorable *TYMS* promoter genotype included in each group rather than the specific 5-FU-based therapy. Furthermore, no significant heterogeneity concerning the toxicity risk associated with the 2R/2R genotype was observed according to the different 5-FU-based therapy regimens (Lecomte et al., 2004). However, to extend the understanding of the relationship between polymorphism/mutation in *TYMS* gene and toxicity of 5-FU, it will likely be necessary to take into account functional polymorphism outside the promoter regions and haplotypes.

5.3.4 Methylenetetrahydrofolate reductase polymorphism

The *MTHFR* gene is highly polymorphic in the general population. A common C677T transition in exon 4 of the *MTHFR* gene results in a thermolabile enzyme variant with lower specific activity (reviewed in Maring et al., 2005). Since a loss in MTHFR activity, due to the MTHFR C677T mutation, may theoretically favor an increase in intracellular CH_2FH_4 concentrations, it can be hypothesized that patients exhibiting mutated MTHFR genotype may be more sensitive to 5-FU cytotoxicity than patients with wild-type genotype, and become at a risk of 5-FU-associated toxicity. Accordingly, MTHFR may also be an important predictive factor of the toxicity of 5-FU.

To date, few data exist on the possible importance of *MTHFR* gene polymorphisms, and most of them focused on the response rate of 5-FU. In the present study, the C667T polymorphism in the *MTHFR* gene was correlated to adverse events and to the changes in

blood cell counts. The MTHFR C677T mutation had no influence on adverse events and on the changes in blood cell counts. Literature reports on the association of the MTHFR C677T mutation and adverse outcomes of 5-FU based chemotherapy are conflicting. Toffoli et al. (2000) reported that 5 of 6 patients who developed severe acute toxicity (grade 4 leukopenia, mucositis, granulocytopenia, thrombocytopenia) in the first cycle of adjuvant CMF (cyclophosphamide, methotrexate, and 5-FU) had the variant homozygous genotype T677T (83%) and one patient had the homozygous C677C phenotype. The authors suggested that patients with 677TT genotype in the MTHFR gene could have an increased risk of developing severe acute toxicity. However, those serious toxicities may result from the additive effect of adjuvant chemotherapy, especially from methotrexate because the activity of both drugs is dependent of a competitive interaction with folate metabolism. In addition, a combined effect of methotrexate and reduced activity of MTHFR resulting from C677T mutation could occur, leading to toxicity.

In conclusion, further sufficiently powered clinical trials and additional mechanistic investigations may be needed to elucidate the role of the polymorphisms in *DPYD*, *TYMS* and *MTHFR* gene in explaining so far unexpected outcomes of treatments containing 5-FU. Larger populations will be needed to clarify the effect of the mutations in these genes on the 5-FU adverse events.

5.4 Influence of pharmacokinetics on adverse events and changes in blood cell counts

Previous investigations have indicated a positive association between the 5-FU exposure (AUC) and the toxicity of a 5-day continuous infusion (Milano et al., 1988; Santini et al., 1989; Thyss et al., 1986). In the present study, the AUC of 5-FU tended to be increased in patients with adverse events which may have been addressed as 5-FU adverse events (grade 1-2, table 32), although this relationship did not reach statistical significance. The AUC of 5-FUH_2 was almost identical in these two groups of patients. Some patients who suffered from toxicity had a 5-FU AUC value similar to that of subjects belonging to non-toxicity group.

In this study, the apparent lack of sensitivity of the AUC in detecting the subjects at risk for 5-FU adverse events was compensated by the 5-FU/5-FUH_2 AUC ratio. The results were also not in agreement with the degree of adverse events. Additionally, no statistically significant relationship between the AUC of 5-FU, the AUC of 5-FUH_2 and

the AUC ratio, respectively, and the changes in blood cell counts (fig. 16-18) was present in the population studied here. The present results are in agreement with the findings conducted with 18 patients (Casale et al., 2004), but do not confirm the results of two previous studies who showed an association between an increased AUC ratio and severe toxicity (mucositis, diarrhea, nausea, vomiting and hand-foot syndrome) (Di Paolo et al., 2001, 2002). These observations may have been influenced by the small number of subjects and/or the variability of data, and because the treatment was quite well tolerated ($\leq$ 2 toxicity grade).

5.5 Conclusion

The present study represents a further step toward a detailed modeling of the kinetics of 5-FU and its main metabolite 5-FUH_2. This study confirms the high individual variability of 5-FU pharmacokinetics administered in long-term infusion for 5 days. In addition, this study suggests the influence of body surface area and MTHFR gene polymorphism in position 677 on 5-FU clearance. To extend the present understanding of the influence of this polymorphism on 5-FU pharmacokinetics, further studies aimed at confirming the influence of this polymorphism on 5-FU pharmacokinetics in the larger population would be useful.

Concerning the adverse events of 5-FU, although there was no overall relationship between the polymorphisms in the *DPYD*, *TYMS* and *MTHFR* gene and adverse events of 5-FU, further investigations with a larger number of patients will be necessary to assess the effect of the mutations in *DPYD*, *TYMS* and *MTHFR* gene on the 5-FU adverse events.

6. Summary

The aims of this study were to characterise pharmacokinetics of 5-FU and its main metabolite (5-FUH_2) and to quantify factors of influence on pharmacokinetics as well as to identify factors influencing adverse events and blood cell count changes. Additionally, the relationship of pharmacokinetics on adverse events and blood cell count changes was evaluated.

Thirty patients (5 females, 25 males) with colorectal or oesophageal cancer receiving 5-FU 650 or 1000 mg/m^2/day as 5-day continuous infusion were entered in this study. A HPLC method was used for the simultaneous assay of 5-FU and 5-FUH_2 in plasma samples obtained at baseline and at multiple time points during infusion and after the end of infusion. DPD phenotype was assessed as the UH_2/U urinary concentration ratio using LC-MS/MS. Genotyping assays were developed for *DPYD* gene (23 exons), the 5′ promoter region of *TYMS* gene and the *MTHFR* gene in position 677. Population pharmacokinetics of 5-FU and 5-FUH_2 were tested with NONMEM using 199 and 251 quantifiable plasma concentrations of 5-FU and 5-FUH_2, respectively. Patients' covariables were included in the modelling process after the best basic model had been identified.

An open two-compartment model with first-order elimination was found to best describe 5-FU concentration-time data, and a one-compartment model was suitable for 5-FUH_2 data. The data showed that total clearance of 5-FU tends to increase with body surface area and to be higher in patients with MTHFR 677CC or 667CT genotype in the *MTHFR* gene. Point estimates for clearance (95% CI) were 145L/h (83-207) and 276L/h (250-302) in patients with the 667TT and the 677CT or 667CC, respectively. No mutation in the *DPYD, TYMS* or *MTHFR* gene was significantly associated with 5-FU adverse effect and with the changes in blood cell counts served as parameters for adverse event, respectively. There was also no significant impact of the pharmacokinetics (AUC of 5-FU, AUC of 5-FUH_2 and 5-FU/5-FUH_2 AUC ratio) on the occurrence of adverse events and changes in blood cell counts.

In conclusion, the present study provides pharmacokinetic data on long-term 5-FU infusion and suggest a role of the MTHFR C677T polymorphism for 5-FU clearance, which however needs to be further investigated.

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Plasma concentrations of 5-FU

Patient #	Plasma concentrations at time point relative to 5-FU infusion (μg/mL)								
	Predose	+36h	+48h	+108h	+120h	+120.05h	+120.30h	+121h	+121.30h
1	0	0.389	0.330	0.027	n.a.	n.a.	0.018	n.d.	n.d.
2	0	0.116	0.09	0.305	0.042	0.026	n.d.	n.d.	n.d.
3	0	0.764	0.291	0.532	n.a.	n.a.	n.d.	n.d.	n.d.
4	0	0.312	0.256	0.396	0.057	0.025	0.037	0.005	n.d.
5	0	0.622	0.1057	0.593	3.668	1.527	0.109	0.028	n.d.
6	0	0.256	0.292	0.219	n.d.	n.d.	n.d.	n.d.	n.d.
7	0	0.506	0.217	0.429	0.093	0.054	0.276	0.046	0.016
8	0	0.148	0.184	0.227	0.034	0.012	n.d.	n.d.	n.d.
9	0	0.648	0.365	0.644	0.083	0.038	0.578	0.036	0.007
10	0	0.333	0.025	0.398	n.a.	n.a.	n.a.	n.a.	0.016
	121.45h = 0.009								
11	0	0.038	0.245	0.552	0.036	0.019	2.329	0.103	0.105
12	0	0.462	0.302	0.497	0.475	0.104	0.018	0.006	n.d.
13	0	0.037	0.222	0.275	0.027	0.021	0.012	0.006	n.d.
14	0	0.140	0.211	0.317	n.a.	n.a.	n.a.	n.d	n.d
15	0	0.439	0.404	0.431	n.a.	n.a.	n.a	n.a	0.016
16	0	0.190	0.251	0.236	0.492	0.076	0.010	n.d.	n.d.
17	0	0.157	0.219	0.237	0.059	0.024	0.007	n.d.	n.d.
18	0	0.179	0.271	0.401	0.205	0.025	0.011	0.008	0.007
19	0	1.180	0.309	0.052	0.008	0.006	n.d.	n.d.	n.d
20	0	0.107	0.437	0.384	0.021	0.014	0.007	0.005	0.005
21	0	0.520	0.176	0.214	0.011	0.007	0.005	n.a.	n.a.
22	0	0.289	0.054	0.407	0.013	0.009	0.005	n.d.	n.d
23	0	0.011	0.486	0.407	0.021	0.008	1.985	0.057	0.008
24	0	0.259	0.300	0.228	0.195	0.075	0.009	n.d.	n.d
25	0	0.182	0.937	0.897	0.0043	0.012	0.122	0.011	n.d.
26	0	0.295	0.333	0.317	0.132	0.05	0.009	0.005	n.d.
27	0	0.283	0.265	0.258	0.625	0.276	0.028	0.005	n.d.
28	0	0.086	0.287	0.032	0.374	0.077	0.022	0.202	0.025
29	0	0.221	0.215	0.281	0.345	0.078	0.010	n.d.	n.d
30	0	0.312	0.226	0.317	0.255	0.154	0.011	n.d.	n.d.

n.a = no plasma available, n.d = not detectable

Plasma concentrations of 5-FUH$_2$

Patient #	Plasma concentrations at time point relative to 5-FU infusion (μg/mL)								
	Predose	+36h	+48h	+108h	+120h	+120.05h	+120.30h	+121h	+121.30h
1	0	0.732	0.573	0.560	n.a	n.a	0.018	n.d	n.d
2	0	0.385	0.398	0.288	0.097	0.102	0.087	0.074	0.014
3	0	0.473	0.464	0.415	n.a	n.a	0.019	0.019	0.013
4	0	0.449	0.342	0.262	0.152	0.127	0.112	0.099	0.087
5	0	1.041	1.849	0.784	0.393	0.772	0.684	-	0.295
6	0	0.462	0.538	0.536	0.075	0.082	0.034	0.027	0.019
7	0	1.331	0.708	0.925	0.289	0.341	0.849	-	0.300
8	0	0.371	0.374	0.303	0.157	0.265	0.159	0.110	0.064
9	0	0.833	0.622	0.634	0.150	0.170	0.265	0.149	0.036
10	0	1.089	0.479	0.753	n.a	n.a	n.a	n.a	0.021
	121.45h = 0.246								
11	0	0.310	0.397	0.483	-	0.158	0.414	0.410	0.251
12	0	0.781	0.786	0.819	0.725	0.709	0.409	0.216	0.164
13	0	0.356	0.336	0.212	0.298	0.224	0.146	0.135	0.046
14	0	0.345	0.303	0.483	n.a	n.a	n.a	0.059	0.054
15	0	0.522	0.503	0.524	n.a	n.a	n.a	n.a	0.032
16	0	0.190	0.251	0.236	0.492	0.076	0.010	n.d	n.d
17	0	0.469	0.547	0.458	0.132	0.152	0.115	0.093	0.066
18	0	0.346	0.412	0.352	0.381	0.243	0.181	0.090	0.078
19	0	0.612	0.712	0.680	0.310	0.283	0.226	0.178	0.175
20	0	0.605	0.858	0.632	0.248	0.175	0.148	0.142	0.138
21	0	0.829	0.864	0.951	0.247	0.236	0.169	n.a	n.a
22	0	0.494	0.448	0.607	0.426	0.373	0.274	0.245	0.159
23	0	0.862	1.498	1.274	0.110	0.123	0.224	0.415	0.253
24	0	0.800	0.721	0.820	0.683	0.589	0.321	0.213	0.150
25	0	0.389	0.420	0.730	0.142	0.139	0.472	0.271	0.212
26	0	0.911	0.818	0.672	0.226	0.220	0.151	0.069	0.047
27	0	0.615	0.757	0.478	0.782	0.703	0.431	0.212	0.409
28	0	0.313	0.676	0.463	0.678	0.606	0.567	0.489	0.516
29	0	0.831	0.584	0.872	0.741	0.663	0.454	0.290	0.179
30	0	0.821	0.903	0.833	1.042	1.019	0.544	0.490	0.373

n.a = no plasma available, n.d = not detectable, - = plasma not enough

DPD phenotype (UH_2/U urinary concentration ratio)

Patient #	concentration of U (µM)	Concentration of UH_2 (µM)	UH_2/U ratio
1	4.14	n.d	-
2	16.93	26.65	1.57
3	43.84	33.59	0.77
4	54.31	33.01	0.61
5	28.49	16.54	0.58
6	38.55	29.15	0.76
7	38.21	55.29	1.45
8	28.82	31.55	1.09
9	27.60	33.11	1.20
10	25.25	31.13	1.23
11	35.73	60.46	1.69
12	8.27	22.19	2.68
13	52.95	52.38	0.99
14	20.53	28.62	1.39
15	43.39	37.35	0.86
16	27.22	29.30	1.08
17	102.21	38.12	0.37
18	34.15	25.76	0.75
19	28.14	32.85	1.17
20	17.05	40.45	2.37
21	57.73	45.80	0.79
22	15.68	21.50	1.37
23	30.44	28.92	0.95
24	32.77	38.40	1.17
25	10.73	12.90	1.20
26	37.51	62.49	1.67
27	22.42	32.01	1.43
28	40.31	37.02	0.92
29	25.90	36.47	1.41
30	34.24	28.38	0.83

U = uracil, UH_2 = dihydrouracil

DPD genotype in patient number 1-10

exon	Patient #									
	1	2	3	4	5	6	7	8	9	10
1	x	x	x	x	x	x	x	x	x	x
2	x	x	x	85CC	85CC	x	x	x	85CC	x
3	x	x	x	x	x	x	x	x	x	x
4	x	x	x	x	x	x	x	x	x	x
5	x	x	x	x	x	x	x	x	x	x
6	x	x	496AG	496AG	496AG	x	x	x	496AG	x
7	x	x	x	x	x	x	x	x	x	x
8	x	x	x	x	x	x	x	x	x	x
9	x	x	x	x	x	x	x	x	x	x
10	x	x	x	x	x	x	x	x	x	x
11	x	x	x	1236GA	x	x	x	x	x	x
12	x	x	x	x	x	x	x	x	x	x
13	1627AG	x	1627AG	x	x	1627AG	x	x	1627GG	1601GA
14	x	x	x	x	x	x	x	x	x	x
15	x	x	x	x	x	x	x	x	x	x
16	x	x	x	x	x	x	x	x	x	x
17	x	x	x	x	x	x	x	x	x	x
18	x	x	x	x	x	x	x	x	x	x
19	x	x	x	x	x	x	x	x	x	x
20	x	x	x	x	x	x	x	x	x	x
21	x	x	x	x	x	x	x	x	x	x
22	x	x	x	x	x	x	x	x	x	x
23	x	x	x	x	x	x	x	x	x	x

DPD genotype in patient number 11-20

exon	Patient #									
	11	12	13	14	15	16	17	18	19	20
1	x	x	x	x	x	x	x	x	x	x
2	x	x	x	85CC	x	85TC	85TC	x	85TC	85TC
3	x	x	x	x	x	x	x	x	x	x
4	x	x	x	x	x	x	x	x	x	x
5	x	x	x	x	x	x	x	x	x	x
6	496AG	x	x	x	x	x	496AG	x	x	496AG
7	x	x	x	x	x	x	x	x	x	x
8	x	x	x	x	x	x	x	x	x	x
9	x	x	x	x	x	x	x	x	x	x
10	x	x	x	x	x	x	x	x	x	x
11	x	x	x	x	x	x	x	x	x	x
12	x	x	x	x	x	x	x	x	x	x
13	1627AG	x	1627GG	x	1627AG	x	x	1627AG	x	1601GA
14	x	x	x	x	x	x	x	x	x	x
15	x	x	x	x	x	x	x	x	x	x
16	x	x	x	x	x	x	x	x	x	x
17	x	x	x	x	x	x	x	x	x	x
18	x	x	x	x	x	x	x	x	x	x
19	x	x	x	x	x	x	x	x	x	x
20	x	x	x	x	x	x	x	x	x	x
21	x	x	x	x	x	x	x	x	x	x
22	x	x	x	x	x	x	x	x	x	x
23	x	x	x	x	x	x	x	x	x	x

DPD genotype in patient number 21-30

exon	Patient #									
	21	22	23	24	25	26	27	28	29	30
1	x	x	x	x	x	x	x	x	x	x
2	x	85TC	x	x	x	x	x	x	x	x
3	x	x	x	x	x	x	x	x	x	x
4	x	x	x	x	x	x	x	x	x	x
5	x	x	x	x	x	x	x	x	x	x
6	x	496AG	x	x	x	x	x	x	x	x
7	x	x	x	x	x	x	x	x	x	x
8	x	x	x	x	x	x	x	x	x	x
9	x	x	x	x	x	x	x	x	x	x
10	x	x	x	x	x	x	x	x	x	x
11	x	x	x	x	x	x	x	x	x	x
12	x	x	x	x	x	x	x	x	x	x
13	x	x	x	1627AG	x	x	x	x	1627AG	1627AG
14	x	x	x	x	x	x	x	x	x	x
15	x	x	x	x	x	x	x	x	x	x
16	x	x	x	x	x	x	x	x	x	x
17	x	x	x	x	x	x	x	x	x	x
18	x	x	x	x	x	2194GA	x	2194GA	x	x
19	x	x	x	x	x	x	x	x	x	x
20	x	x	x	x	x	x	x	x	x	x
21	x	x	x	x	x	x	x	x	x	x
22	x	x	x	x	x	x	x	x	x	x
23	x	x	x	x	x	x	x	x	x	x

TS and MTHFR genotype in 30 patients

Patient #	TS genotype			MTHFR genotype		
	2R/2R	2R/3R	3R/3R	C677C	C677T	T677T
1	x			x		
2		x			x	
3			x			x
4		x		x		
5		x				x
6		x		x		
7		x		x		
8		x		x		
9		x			x	
10		x			x	
11	x				x	
12			x		x	
13	x				x	
14	x			x		
15		x			x	
16		x		x		
17	x				x	
18	x				x	
19		x		x		
20		x		x		
21			x	x		
22			x		x	
23		x			x	
24		x				x
25		x				x
26		x			x	
27			x			x
28		x		x		
29		x		x		
30		x		x		

Patient characteristics

#ID	Amt (mg)	Rate (mg/h)	Sex	Age (yr.)	Ht (cm)	Wt (kg)	BMI (kg/m^2)	BSA (m^2)	Co-med	CL_{cr}	GGT (U/L)	ALT (U/L)	AST (U/L)
1	8804.07	76.17	M	68	170	76	26.3	1.79	1	95	38	27	19
2	6342.96	53.93	M	59	180	79	24.4	1.99	1	123.44	44	13	15
3	8856.94	74.36	M	37	181	79	24.1	1.91	0	144.89	13	13	16
4	8855.17	75.31	M	46	186	75	21.7	1.98	0	108.8	74	90	36
5	7535.4	63.44	F	58	162	49	18.7	1.502	1	81.78	13	16	15
6	9105.72	77.38	M	45	182	105	31.7	2.259	1	153.94	30	40	24
7	9004.36	75.27	M	41	186	68	19.7	1.91	1	108.72	16	15	18
8	8820.97	75.12	M	60	180	82	25.3	1.996	0	96.93	24	10	13
9	7848.13	65.58	F	50	161	57	22	1.594	0	99.28	21	22	23
10	9003.88	77.8	M	52	183	80	23.9	2.021	1	113.7	15	14	20
11	7944.56	66.52	M	46	172	53.5	18.1	1.609	1	114.5	31	8	18
12	7907.88	65.97	M	64	169	54	18.9	1.614	0	53.77	22	15	17
13	8929.91	74.57	M	62	184	111	32.8	2.358	0	93.22	28	19	17
14	8940.83	75.88	M	50	179	93	29	2.12	0	110.71	50	45	20
15	7944.23	67.9	F	60	164	59	21.9	1.591	0	76.33	24	14	16
16	8640.33	72.81	M	51	176	76	24.5	1.911	0	110.52	17	11	24
17	4688.15	39.93	F	70	165	46	16.9	1.482	0	86.4	33	8	10
18	8801.76	75.21	M	61	174	84	27.7	1.989	1	119.7	24	19	9
19	8681.78	71.38	M	58	167	75.8	27.2	1.786	1	90.59	24	14	14
20	8843.28	74.61	M	66	175	70	23	1.85	1	74.17	16	16	18
21	8860.5	75.23	M	71	173	99	33.1	2.124	0	111.62	16	12	10
22	8828.39	74.65	M	66	178	77	24.3	1.95	1	94.21	38	13	23
23	8844.7	74.4	M	68	176	66	21.3	1.81	1	76.74	22	10	19
24	8877.42	74.55	M	64	176	103	33.18	2.17	0	124.97	45	34	50
25	5816.28	49.03	M	70	172	68	23	1.8	1	74.28	23	15	18
26	8742.91	74.3	M	52	175	65	21.2	1.79	0	149.9	24	20	20
27	8368.03	69.48	F	40	165	62	22.8	1.68	0	82.24	16	14	19
28	9157.61	75.44	M	51	173	91	30.4	2.05	1	133.91	20	13	15
29	7645.053	63.72	M	66	180	89	27.5	2.09	0	120.36	18	24	18
30	7650	63.41	M	73	183	81	24.2	2.03	2	100.5	81	31	32

Amt = real dose (without remaining volume in device), rate = infusion rate (dose/duration of infusion), calculated from real dose, sex: F = female, M = male, Ht = height, Wt = weight, BMI = body mass index, BSA = body surface area, Co-med = co-mediation: 0 = without cisplatin, 1 = with cisplatin, CL_{cr} = creatinine clearance, GGT = gamma-glutamyl transaminase, ALT = alanine transaminase (SGPT, Serum glutamic pyruvic transaminase), AST = aspartate transaminase (SGOT, Serum glutamic ocaloacetic transaminase)

Pharmacokinetic data of 5-FU and 5-FUH_2 for each patient (combined base model)

ID	CL	K_{30}	K_{10}	K_{13}	CL_{res}	CL_{met}	K_{EL}	AUC of 5-FU	$t_{1/2}$ of 5-FU	CL_m	$t_{1/2}$ of 5-FUH_2	AUC of 5-FUH_2
1	238.3	1.2	3.0	19.0	32.7	205.6	22.0	36.94	0.031	121	0.571	62.581
2	317.9	1.8	4.0	25.4	43.6	274.3	29.4	19.95	0.024	178	0.389	30.730
3	198.1	2.2	2.5	15.8	27.2	170.9	18.3	44.71	0.038	217	0.320	35.299
4	209.2	1.6	2.7	16.7	28.7	180.5	19.3	42.34	0.036	159	0.437	48.145
5	50.0	0.7	0.6	4.0	6.9	43.1	4.6	150.80	0.150	67	1.039	97.507
6	363.7	2.1	4.6	29.0	49.9	313.9	33.6	25.03	0.021	206	0.336	38.075
7	266.3	0.8	3.4	21.2	36.5	229.8	24.6	33.81	0.028	80	0.863	96.754
8	443.4	1.6	5.6	35.4	60.8	382.6	41.0	19.89	0.017	158	0.437	48.034
9	200.8	1.3	2.5	16.0	27.5	173.2	18.6	39.09	0.037	128	0.543	53.042
10	198.1	0.9	2.5	15.8	27.2	170.9	18.3	45.45	0.038	86	0.810	90.804
11	281.8	0.9	3.6	22.5	38.6	243.2	26.0	28.19	0.027	93	0.748	74.012
12	214.1	1.0	2.7	17.1	29.4	184.8	19.8	36.93	0.035	98	0.706	69.504
13	282.5	1.6	3.6	22.5	38.7	243.7	26.1	31.62	0.027	164	0.423	47.077
14	348.7	1.7	4.4	27.8	47.8	300.9	32.2	25.64	0.022	166	0.417	46.473
15	178.9	1.5	2.3	14.3	24.5	154.4	16.5	44.40	0.042	148	0.467	46.230
16	259.4	1.2	3.3	20.7	35.6	223.8	24.0	33.31	0.029	116	0.600	64.509
17	282.2	1.1	3.6	22.5	38.7	243.5	26.1	16.61	0.027	114	0.608	35.509
18	260.1	1.6	3.3	20.7	35.7	224.4	24.0	33.84	0.029	159	0.437	47.854
19	150.7	1.2	1.9	12.0	20.7	130.0	13.9	57.62	0.050	119	0.584	63.184
20	338.5	1.2	4.3	27.0	46.4	292.1	31.3	26.12	0.022	123	0.565	62.178
21	387.4	1.1	4.9	30.9	53.1	334.3	35.8	22.87	0.019	110	0.629	69.351
22	395.0	1.2	5.0	31.5	54.2	340.9	36.5	22.35	0.019	116	0.597	65.629
23	309.1	0.8	3.9	24.6	42.4	266.7	28.6	28.62	0.024	82	0.846	93.158
24	274.4	1.1	3.5	21.9	37.6	236.7	25.4	32.36	0.027	109	0.637	70.467
25	118.5	0.9	1.5	9.4	16.2	102.2	10.9	49.09	0.063	90	0.774	56.041
26	281.7	1.4	3.6	22.5	38.6	243.0	26.0	31.04	0.027	139	0.500	54.470
27	156.6	0.9	2.0	12.5	21.5	135.1	14.5	53.43	0.048	86	0.803	83.707
28	198.8	0.9	2.5	15.9	27.3	171.6	18.4	46.06	0.038	90	0.774	88.283
29	261.3	0.9	3.3	20.8	35.8	225.5	24.1	29.26	0.029	92	0.749	71.349
30	194.8	0.7	2.5	15.5	26.7	168.0	18.0	39.28	0.039	72	0.967	92.076
min	50.0	0.7	0.6	4.0	6.9	43.1	4.6	16.61	0.017	67	0.320	30.730
mean	255.3	1.2	3.2	20.4	35.0	220.3	23.6	38.22	0.035	123	0.619	63.401
mean*	237.7	1.2	3.0	19.0	32.6	205.1	22.0	34.49	0.032	117.1	0.592	60.397
median	260.7	1.2	3.3	20.8	35.8	224.9	24.1	33.56	0.029	115.8	0.598	62.882
max	443.4	2.2	5.6	35.4	60.8	382.6	41.0	150.80	0.150	216.5	1.039	97.507
SD	86.7	0.39	1.10	6.91	11.89	74.80	8.01	23.62	0.024	39.1	0.187	19.607

* = geometric mean, min = minimum value, max = maximum value, SD = standard deviation

CL = total clearance of 5-FU, K_{30} = elimination constant of 5-FUH_2, K_{10} = residual elimination constant of 5-FU, K_{13} = metabolic constant of 5-FU to 5-FUH_2, CL_{res} = residual elimination clearance of 5-FU, CL_{met} = metabolic clearance of 5-FU to 5-FUH_2, CL_m = clearance of 5-FUH_2

Pharmacokinetic data of 5-FU and 5-FUH_2 for each patient (final covariate model)

ID	CL	K_{30}	K_{10}	K_{13}	C_{Lres}	CL_{met}	$t_{1/2}$ of 5-FU	$t_{1/2}$ of 5-FUH_2	CL_m	AUC of 5-FU	AUC of 5-FUH_2
1	246.9	1.22	3.05	19.26	33.8	213.1	0.031	0.570	121.6	35.66	62.50
2	324.7	1.78	4.01	25.33	44.4	280.3	0.024	0.388	178.5	19.53	30.68
3	172.0	2.13	2.13	13.41	23.5	148.4	0.045	0.325	213.2	51.51	35.86
4	222.7	1.59	2.75	17.37	30.5	192.2	0.034	0.436	159.0	39.77	48.08
5	49.4	0.67	0.61	3.86	6.8	42.7	0.155	1.037	66.8	152.46	97.31
6	392.9	2.07	4.86	30.65	53.7	339.2	0.020	0.335	206.6	23.18	38.05
7	272.6	0.80	3.37	21.27	37.3	235.3	0.028	0.861	80.5	33.03	96.58
8	441.0	1.59	5.45	34.40	60.3	380.7	0.017	0.435	159.2	20.00	47.84
9	196.4	1.28	2.43	15.32	26.9	169.5	0.039	0.542	127.9	39.96	52.96
10	216.5	0.86	2.68	16.89	29.6	186.9	0.035	0.810	85.6	41.59	90.81
11	255.0	0.93	3.15	19.90	34.9	220.2	0.030	0.742	93.4	31.15	73.43
12	215.1	0.98	2.66	16.78	29.4	185.7	0.036	0.704	98.4	36.76	69.36
13	299.6	1.64	3.70	23.37	41.0	258.6	0.026	0.423	163.9	29.81	47.04
14	368.1	1.66	4.55	28.71	50.3	317.7	0.021	0.417	166.3	24.29	46.41
15	185.0	1.48	2.29	14.43	25.3	159.7	0.041	0.467	148.4	42.95	46.21
16	266.2	1.16	3.29	20.77	36.4	229.8	0.029	0.598	115.8	32.46	64.40
17	267.7	1.14	3.31	20.88	36.6	231.1	0.029	0.606	114.3	17.52	35.41
18	272.1	1.59	3.36	21.23	37.2	234.9	0.028	0.435	159.2	32.34	47.74
19	164.1	1.19	2.03	12.80	22.4	141.7	0.047	0.585	118.5	52.91	63.23
20	344.2	1.23	4.26	26.85	47.1	297.1	0.022	0.563	123.1	25.69	62.03
21	393.8	1.10	4.87	30.72	53.9	339.9	0.019	0.628	110.4	22.50	69.28
22	390.4	1.17	4.83	30.46	53.4	337.0	0.020	0.595	116.5	22.61	65.42
23	300.6	0.82	3.72	23.45	41.1	259.5	0.026	0.844	82.1	29.43	92.94
24	266.5	1.09	3.29	20.79	36.4	230.0	0.029	0.636	108.9	33.32	70.37
25	111.7	0.90	1.38	8.71	15.3	96.4	0.069	0.766	90.4	52.06	55.51
26	285.8	1.39	3.53	22.30	39.1	246.7	0.027	0.500	138.6	30.59	54.46
27	150.0	0.87	1.85	11.70	20.5	129.5	0.051	0.797	86.9	55.79	83.08
28	209.8	0.90	2.59	16.37	28.7	181.1	0.037	0.773	89.6	43.65	88.22
29	272.5	0.93	3.37	21.26	37.3	235.3	0.028	0.748	92.6	28.05	71.27
30	204.4	0.72	2.53	15.94	28.0	176.4	0.038	0.965	71.8	37.44	91.96
min	49.4	0.67	0.61	3.86	6.8	42.7	0.017	0.325	66.8	17.52	30.68
mean	258.6	1.23	3.20	20.17	35.4	223.2	0.036	0.618	122.9	37.93	63.28
mean*	240.1	1.17	2.97	18.73	32.8	207.2	0.032	0.591	117.4	34.15	60.30
median	266.3	1.16	3.29	20.78	36.4	229.9	0.029	0.597	116.2	32.74	62.87
max	441.0	2.13	5.45	34.40	60.3	380.7	0.155	1.037	213.2	152.46	97.31
SD	88.8	0.39	1.10	6.93	12.2	76.7	0.025	0.186	38.8	23.98	19.53

* = geometric mean, min = minimum value, max = maximum value, SD = standard deviation

CL = total clearance of 5-FU, K_{30} = elimination constant of 5-FUH_2, K_{10} = residual elimination constant of 5-FU, K_{13} = metabolic constant of 5-FU to 5-FUH_2, CL_{res} = residual elimination clearance of 5-FU, CL_{met} = metabolic clearance of 5-FU to 5-FUH_2, CL_m = clearance of 5-FUH_2

Hematology response in 30 patients

ID #	Leukocyte count			Erythrocyte count			Platelet count		
	baseline ($x10^9$/L)	nadir ($x10^9$/L)	Change in leukocyte count (%)	baseline ($x10^{12}$/L)	nadir ($x10^{12}$/L)	Change in erythrocyte count (%)	baseline ($x10^9$/L)	nadir ($x10^9$/L)	Change in platelet count (%)
1	6.93	5.05	27.13	4.4	3.9	11.36	189	71	62.43
2	10.31	2.88	72.07	4.1	3.4	17.07	393	157	60.05
3	8.47	4.06	52.07	4.6	4.3	6.52	242	169	30.17
4	5.59	4.42	20.93	5.4	4.9	9.26	150	147	2.00
5	6.87	2.3	66.52	4.7	4.2	10.64	278	73	73.74
6	6.09	4.83	20.69	4.9	4.3	12.24	48	22	54.17
7	6.24	3.13	49.84	5.1	2.6	49.02	285	115	59.65
8	6.94	4.3	38.04	4.3	3.9	9.30	245	195	20.41
9	7.74	2.75	64.47	4.2	3.7	11.90	274	197	28.10
10	5.25	2.16	58.86	4.7	4.3	8.51	280	247	11.79
11	12.64	8.11	55.86	4.6	4.1	10.87	321	280	14.64
12	7.34	4.88	33.51	5.0	4.7	6.00	322	236	26.71
13	8.32	2.34	71.88	4.3	2.5	41.86	349	147	57.88
14	11.28	5.19	53.99	4.7	4.3	8.51	309	170	44.98
15	7.15	5.84	18.32	4.4	4	9.09	302	206	31.79
16	4.99	2.46	50.70	4.8	4.3	10.42	249	175	29.72
17	9.02	4.27	52.66	3.8	3.3	13.16	365	209	42.74
18	7.55	2.81	62.78	4.7	3.7	21.28	223	111	50.22
19	6.94	1.72	75.22	4.6	4	13.04	293	238	18.77
20	4.74	3.04	35.86	4.1	3.1	24.39	277	106	61.73
21	7.13	4.2	41.09	4.7	4.3	8.51	344	180	47.67
22	7.2	1.8	75.00	4.3	3.58	16.74	256	115	55.08
23	6.71	4.19	37.56	4.4	4.1	6.82	364	214	41.21
24	6.39	3.3	48.36	5.3	5	5.66	258	170	34.11
25	13.66			5.0			215		
26	6.00	3.88	35.33	5.1	4.6	9.80	293	239	18.43
27	7.36	3.05	58.56	4.3	3.8	11.63	426	229	46.24
28	3.99	2.35	41.10	4.9	4.4	10.20	172	98	43.02
29	6.24	3.39	45.67	4.8	4.3	10.42	375	201	46.40
30	6.09	3	50.74	4.7	3.7	21.28	147	108	26.53