DPD screening to prevent toxicity in fluoropyrimidine treated patients

Staveren, M.C. van

Chapter 4

Pharmacokinetics of orally administered uracil in healthy volunteers and in DPD-deficient patients, a possible tool for screening of DPD deficiency

Maurice van Staveren
Barbara Theeuwes-Oonk
Henk-Jan Guchelaar
André van Kuilenburg
Jan Gerard Maring

Cancer Chemother.Pharmacol.,
2011. **86**(6): p. 1611-1617

ABSTRACT

Purpose: Dihydropyrimidine dehydrogenase (DPD) deficiency can lead to severe toxicity in patients treated with standard doses of 5-fluorouracil (5-FU). Oral uracil administration and subsequent measurement of uracil and dihydrouracil (DHU) plasma concentrations might detect patients with DPD deficiency. This study compares the pharmacokinetics (PK) of uracil and DHU after oral uracil administration in subjects with normal and deficient DPD status.

Methods: Five hundred milligrams of uracil per metre square was administered orally to 11 subjects with normal DPD status and to 10 subjects with reduced DPD activity. Repeated administration (n = 3) of this dose was performed in 4 subjects and 1,000 mg uracil/m^2 was administered to 4 subjects to assess intra-individual variation and linearity of pharmacokinetics.

Results: In subjects with normal DPD status, 500 mg/m^2 uracil resulted in uracil C_{max} levels of 14.4 ± 4.7 mg/L at T_{max} = 30.0 ± 11.6 min and in DPD-deficient subjects, 20.0 ± 4.5 mg/L at 31.5 ± 1.1. The uracil $AUC_{0>180}$ was 31.2 ± 5.1 mg L/h in DPD-deficient subjects which was significantly higher ($p < 0.05$) than in the subjects with normal DPD status (13.8 ± 3.9 mg L/h). Repeated uracil dosing showed reproducible uracil PK in subjects with normal DPD status and dose elevation of uracil suggested linear pharmacokinetics.

Conclusion: The PK of uracil differs significantly between subjects with a normal DPD activity and those with a deficient DPD status. The AUC and C_{max} of uracil can be useful as a diagnostic tool to differentiate patients with regard to DPD status.

INTRODUCTION

5-Fluorouracil and its prodrug capecitabine are commonly used chemotherapeutic drugs in the treatment of colorectal, breast and head and neck cancer. The intracellular metabolism of 5-FU is complex, requiring conversion into cytotoxic nucleotides. The main cytotoxic metabolite is 5-fluoro-2'-deoxyuridine 5'-monophosphate that inhibits thymidylate synthase [1]. However, only a small proportion of the administrated 5-FU dose is converted to cytotoxic metabolites. Within a few hours after parenteral administration, 70–90% of the 5-FU dose is metabolized into inactive metabolites. DPD is the initial, and rate-determining enzyme in the catabolism of 5-FU [2-5]. Patients with a partial or complete DPD deficiency have a strongly reduced capacity to degrade 5-FU [6, 7] or its oral prodrug capecitabine [8, 9]. As a consequence, treatment with 5-FU and/or capecitabine in patients with reduced DPD activity can cause severe or life-threatening toxicity such as neutropenia, diarrhea and mucositis [10]. It was initially estimated that in 3–5% of Caucasians the activity of DPD is strongly reduced due to (epi)genetic variations in the gene encoding DPD. However, this percentage can be disputed because the incidence of DPD deficiency depends on the method that is used to detect it [11] and the cutoff level chosen or determined to define DPD deficiency [12]. A recent study found DPD deficiency in 40% of the patients included using the uracil/dihydrouracil ratio in plasma, an observation higher than the outcome expected with DPYD genetic polymorphism [13].

Several methods have been developed to detect patients with reduced DPD activity, such as genotyping [14], determination of DPD activity in peripheral blood mononuclear cells (PBMCs) [4, 10], phenotyping with a breath test using [2-^{13}C] uracil [15-17], the administration of a 5-FU test dose [7, 18] and assessment of the endogenous uracil/DHU plasma ratio [19-27]. A major drawback of many of these methods is that they are costly and/or laborious or in the case of a 5-FU test dose potentially toxic, thus precluding the routine implementation in clinical practice for prospective screening of DPD deficiency. To achieve the most accurate and simple method to predict DPD deficiency prior to 5-FU- or capecitabine-based treatment, a combined testing strategy has been proposed [12, 21]. However, oral administration of an uracil test dose and subsequent measurement of uracil and its metabolite DHU in plasma might be a cheap, fast, and simple method for screening for DPD deficiency prior to 5-FU or capecitabine containing therapy, which can be used clinically. So far, only the pharmacokinetics of orally administered ^{13}C-uracil has been reported using low doses of 50, 100, and 200 mg uracil. The major drawback of using low doses of uracil

concerns the lower chance of reaching adequate plasma concentrations. The use of higher uracil doses is expected to result in a more adequate discrimination between normal and deficient individuals due to a prolonged DPD enzyme saturation in DPD-deficient subjects compared with lower doses of uracil. Moreover, this situation may better reflect the DPD enzyme dynamics in the clinical situation when 5-FU doses of 1,000–2,000 mg are being used. From previous work with 5-FU, we estimated that a uracil plasma level of at least 10 mg/l would be needed for proper discrimination between DPD-deficient and normal DPD subjects with high sensitivity and specificity [6].

The main objective of this study was to compare the pharmacokinetics of orally administered uracil between healthy volunteers with normal DPD activity and patients with DPD deficiency due to heterozygosity for a *DPYD* gene mutation. Secondary objectives involved the investigation of linearity of uracil pharmacokinetics at increased uracil dose and the intra- and interday variation in uracil pharmacokinetics.

MATERIALS AND METHODS

Study subjects

Eleven subjects with a normal DPD status and ten DPD-deficient subjects, aged 18 years and older, participated in this study. The eleven subjects were all healthy volunteers and the ten DPD-deficient subjects were colorectal and breast cancer patients who suffered CTC grade III or IV side effects following a 5-FU or capecitabine containing drug schedules and had DPD activity < 5 nmol/mg protein/hr. DPD activity was measured in PBMCs, and the DPD status was considered normal or deficient when the DPD activity in PBMCs was > 5 nmol/mg protein/h, or < 5 nmol/mg protein/h respectively [11]. In all patients, DPD deficiency was confirmed by sequence analysis of *DPYD* showing heterozygosity for a pathological mutation. Heterozygosity for the c.1905 + 1G>A (IVS14 + 1G>A), c.2846A>T, c.1129 - 5923C>G, and the novel c.2579delA mutation was detected in 5, 2, 2, and 2 patients, respectively. One of the patients was heterozygous for both the c.1905 + 1G>A mutation and the c.1129 - 5923C>G mutation.

Prior to uracil administration, blood samples were taken to measure creatinine, alanine transaminase (ALAT), and gamma-glutamyl transpeptidase (gamma-GT) as markers for renal and liver function.

The study was approved by the local Medical Ethics Committee of Diaconessen Hospital Meppel, the Netherlands. Informed consent was obtained from each subject.

Uracil administration

Uracil (Pharmorgana GmbH, Raubling/Rosenheim, Germany) was administered orally at a test dose of 500 mg/m^2 body surface area, calculated by the DuBois and DuBois formula, after an overnight fast (last food intake > 8 h earlier). All subjects had to abstain food during 2 hours after ingesting the uracil. All the test doses were administered between 08:00 a.m. and 09:00 a.m. to avoid circadian effects. The uracil powder was mixed with 100–200 ml tap water, and immediately after preparation, the suspension was ingested within a few minutes. In addition, repeated administration on subsequent days (n = 2 in 3 subjects and n = 1 in one subject) with 500 mg/m^2 was performed in 4 volunteers to assess intra-individual variation and 1,000 mg uracil/m^2 was administered to 4 volunteers to assess linearity of pharmacokinetics, respectively.

Collection of blood samples

A cannula was placed intravenously in one arm of each subject. Blood samples of 5 ml were collected in heparin-containing tubes. In an intensive sampling schedule, blood samples were collected just before and at t = 15, 30, 45, 60, 80, 100, 120, 150, 180, and 220 min (500 mg/m^2) or 240 min (1,000 mg/m^2) after uracil intake. Samples were immediately placed on ice and subsequently centrifuged at 2,500 x g for 10 min at 4°C and stored at -20°C until analysis.

For the repeated uracil administration in the 4 subjects, blood samples were collected according to a limited sampling schedule. This schedule is based on results from an interim analysis on intensive schedule results from both volunteers and patients, in which t = 60 min and t = 120 min were selected as optimal sampling points for the limited sampling strategy.

DPD activity

The activity of DPD was determined in PBMCs using radiolabeled thymine followed by the separation of radiolabeled thymine from radiolabeled dihydrothymine using reversed-phase HPLC, as described before [28].

Analytical method for uracil and dihydrouracil

Uracil and DHU plasma concentrations were measured by a validated HPLC method described by Maring et al. [29]. Calibration samples were prepared by spiking human heparinized plasma (Red Cross Blood Bank, Groningen, the Netherlands) with appropriate amounts of uracil and 5,6-DHU (Sigma Chemical Co, Zwijndrecht, the Netherlands). Uracil was quantified at 266 nm and DHU at 205 nm. The internal standard chlorouracil was quantified at both wavelengths. The limit of quantification in plasma was 0.004 mg/l for both uracil and DHU.

Pharmacokinetic and statistical analysis

The pharmacokinetic parameters T_{max}, C_{max}, $AUC_{0\text{-}180min}$ were calculated with Phoenix™ Winnonlin® Version 6 (Pharsight® Products, CA) using noncompartmental analysis. Mean plasma clearance was calculated with the formula Dose/AUC. Statistical analysis was performed by using SPSS version 16.0 (SPSS inc, Chicago, IL). Normality of data was tested by performing a Kolmogorov-Smirnov test.

To examine whether the uracil and DHU plasma concentrations and derived pharmacokinetic parameters differed between subjects with a normal DPD status and those with DPD deficiency, an unpaired Student's t test was performed on data obtained from the 500 mg/m^2 dose. To investigate whether the distribution of gender in both groups differs between the DPD-deficient and normal individuals, chi-square statistic was used. One-way ANOVA analysis was performed on the uracil and DHU values measured in plasma at t = 60 and 120 min in the four volunteers after repeated 500 mg/m^2 doses to study the inter- and intrasubject variability.

RESULTS

The characteristics of the subjects included in this study are displayed in Table 4.1. No differences between the two groups were observed ($p > 0.05$) except for age, DPD activity, and disease status.

The HPLC method that was used in this study revealed fully separated peaks for uracil and DHU in the chromatogram as is depicted in Figure 4.1.

Table 4.1 Patient and volunteer characteristics

	Normal DPD activity (n = 11)	DPD-deficient (n = 10)	p value
Age (years)	38 ± 9	62 ± 12	< 0.001
Sex (male/female)	5/6	4/6	Chi square = 0.002
Weight (kg)	74 ± 10	76 ± 15	0.636
Height (cm)	177 ± 9	173 ± 5	0.154
PBMC DPD (nmol/mg/l)	7.2 ± 1.3	3.6 ± 0.8	< 0.001
Serum creatinine (µmol/l)	80 ± 10	78 ± 18	0.707
Serum ALT (U/l)	20 ± 8	25 ± 5	0.104
Serum gammaGT (U/l)	16 ± 6	39 ± 40	0.160

Values are displayed as mean ± SD.

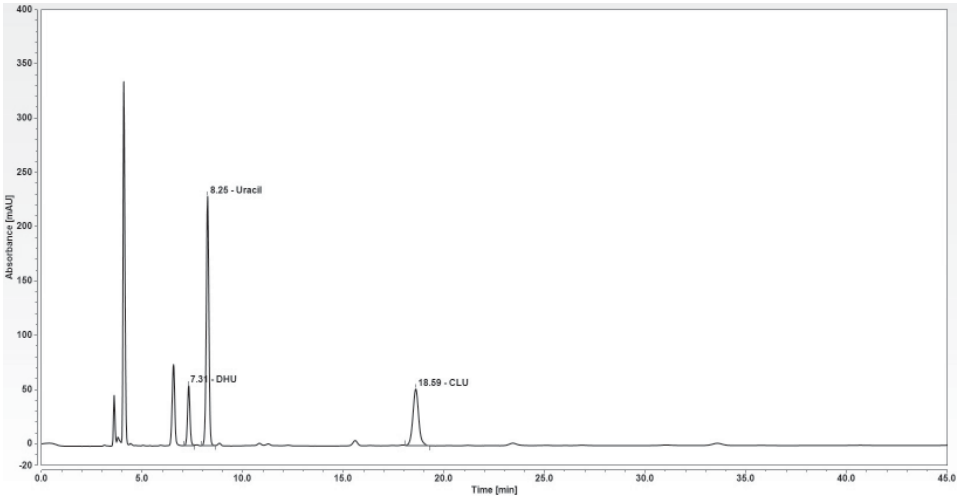

Figure 4.1 Representative chromatogram obtained from a blood sample of a patient with normal DPD status at t = 60 min after oral intake of 1000 mg uracil.
The chromatogram was recorded at 205 nm. The uracil and dihydrouracil concentrations were estimated as 9.5 mg/L and 3.4 mg/L, respectively.

The mean uracil and DHU plasma concentrations in the 11 subjects with normal DPD and in the 10 DPD deficient subjects following a uracil dose of 500 mg/m^2 are depicted in Figure 4.2 and the estimated pharmacokinetic parameters T_{max}, C_{max}, $AUC_{0\text{-}180min}$ and the mean Cl are displayed in Table 4.2.

Table 4.2 Pharmacokinetic parameters of 500 mg/m2 and 1,000 mg/m2 in volunteers and 500 mg/m² in patients

	Normal DPD activity		DPD deficient		p value (500 mg/m²)		Normal DPD activity	
	Uracil 500 mg/m² (n = 11)	DHU 500 mg/m² (n = 11)	Uracil 500 mg/m² (n = 10)	DHU 500 mg/m² (n = 10)	Uracil	DHU	Uracil 1,000 mg/m² (n = 4)	DHU 1,000 mg/m² (n = 4)
T_{max} (min)	30.0 ± 11.6	104.5 ± 23.4	31.5 ± 11.1	166.0 ± 55.6	0.765	0.007	33.8 ± 18.9	195.0 ± 57.4
C_{max} (mg/l)	14.4 ± 4.7	3.0 ± 0.9	20.0 ± 4.5	2.2 ± 0.9	0.011	0.046	24.1 ± 11.9	5.2 ± 1.1
$AUC_{0\text{-}180min}$ (mg h/l)	13.8 ± 3.9	5.9 ± 1.9	31.2 ± 5.1	4.4 ± 1.7	< 0.001	0.074	36.0 ± 14.4	8.0 ± 2.3
Cl_{mean} (l/min)	1.3 ± 0.5	3.1 ± 1.8	0.5 ± 0.1	4.0 ± 1.6	< 0.001	0.230	1.1 ± 0.6	4.5 ± 1.7

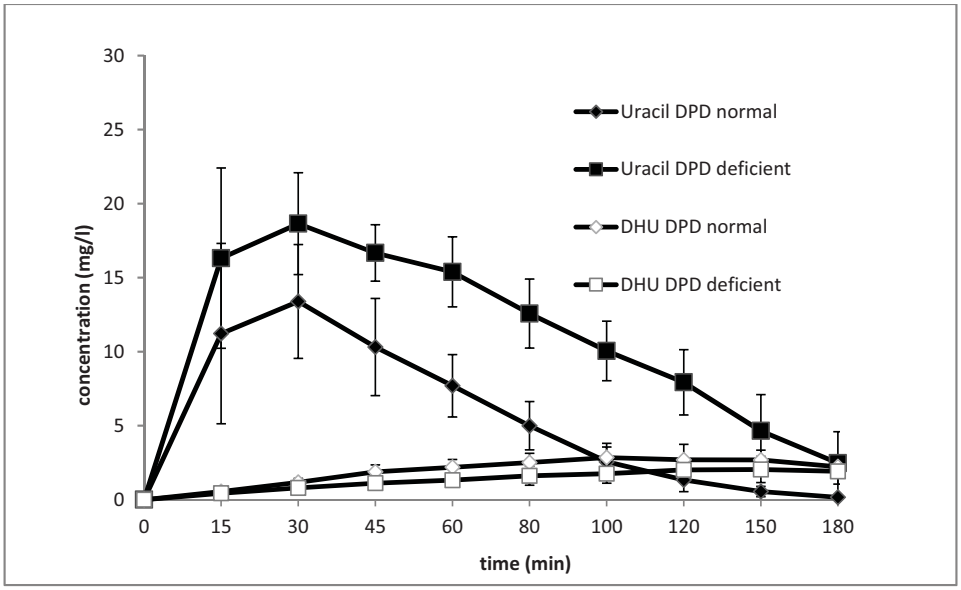

Figure 4.2 Concentration-time profile of uracil and DHU in subjects with normal DPD (n = 11) and DPD-deficient subjects (n = 10) after oral intake of 500 mg/m² uracil suspension.
The results shown are the mean ± SD.

T_{max} of uracil, $AUC_{0\text{-}180min}$ and Cl of DHU did not differ between the two groups ($p > 0.05$). All the other displayed parameters differed significantly ($p < 0.05$). After reaching T_{max}, the decline of uracil concentration in both groups followed zero-order kinetics, which suggests that the DPD enzyme is fully saturated at the administered dose in both groups. In the subjects with normal DPD, after t = 100 min, the elimination changed gradually from zero order to first order, which resulted in an exponential decline. The same phenomenon occurred in DPD-deficient subjects, although at a later stage (after t = 150 min).

In subjects with normal DPD activity, uracil was completely eliminated within approximately 180 min, whereas in DPD-deficient subjects the uracil plasma concentration was still 2.5 ± 2.1 mg/l indicating that in DPD-deficient individuals the clearance of uracil was decreased. In addition, due to the reduced DPD activity, the reaction rate of the formation and the absolute amount of DHU were reduced in the group of DPD-deficient subjects. The uracil and DHU plasma concentrations measured in DPD-deficient subjects differed significantly ($p < 0.05$) as compared to the individuals with normal DPD except for uracil at t = 15 min ($p = 0.071$) and for DHU levels at t = 15, 120, 150 and 180 min ($p = 0.149$, $p = 0.111$, $p = 0.087$, and $p = 0.363$, respectively).

Comparable uracil concentrations at t = 60 and 120 min were observed in subjects with normal DPD status who were tested multiple times. However, significant differences were observed for DHU levels at t = 60 and 120 min (p = 0.012 resp. p = 0.001).

Figure 4.3 shows the plasma concentrations of uracil and DHU after administration of 1,000 mg uracil/m^2 compared to 500 mg uracil/m^2 in subjects with normal DPD status. The C_{max} of both curves is reached after approximately 30 min (T_{max}). Only after 45 min, the uracil levels differed significantly between both dosages.

For DHU, the concentrations after ingestion of both doses differed not significantly the first 100 minutes, but after this point, the DHU concentrations measured in the group of 1,000 mg/m^2 were significantly higher. The Cmax values of uracil and DHU (Table 4.2) at 1,000 mg/m^2 are, respectively 1.7 ± 0.3 and 1.5 ± 0.4 times higher compared with those after 500 mg/m^2.

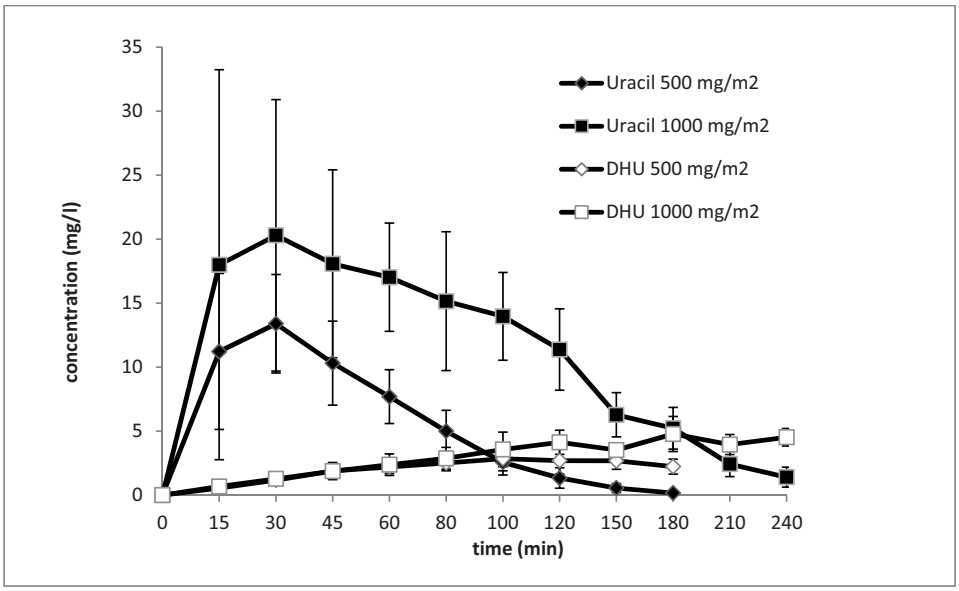

Figure 4.3 Concentration-time profile of uracil and DHU in subjects with normal DPD activity after oral administration of a dose of 500 mg/m² (n = 11) and 1,000 mg/m² (n = 4) uracil suspension.
The results shown are the mean ± SD.

DISCUSSION

In this study, it is shown that the pharmacokinetics of uracil after an oral uracil dose of 500 mg/m^2 was significantly different in subjects with DPD deficiency as compared to those with a normal DPD status suggesting that oral uracil administration may be useful as a test to determine patients with DPD deficiency.

The patient characteristics of the two groups in which we studied uracil and DHU pharmacokinetics were comparable except for DPD status, age, and disease state. The subjects with normal DPD consisted of young healthy individuals, in contrast to the subjects with DPD deficiency, who all were colorectal and breast cancer patients. Aging involves progressive impairments in the functional reserve of multiple organs, which might also affect drug metabolism and pharmacokinetics [30]. With age, the liver mass and its perfusion decreases causing a diminished first pass effect of highly cleared drugs, and renal clearance of drugs is reduced by the loss of kidney function [31]. The liver contains a high amount of DPD, so increasing age might lead to reduced first pass effect and metabolism of uracil in the liver causing higher plasma concentrations in elderly compared to younger individuals. In addition, in cancer patients, metastases of the liver and steatosis caused by systemic chemotherapy [32] can reduce liver drug metabolism, which may also lead to changes in uracil metabolism. However, Maring et al. [33] described that extensive hepatic replacement due to liver metastases had no effect on 5-fluorouracil pharmacokinetics, indicating that the amount of DPD is probably not influenced by reduction in liver function. In addition, in our study population of DPD-deficient patients receiving systemic chemotherapy, no significant differences were observed in liver function or serum creatine compared with the group of healthy individuals. As a result of these findings, we consider it unlikely that the 2.6-fold decrease in clearance of uracil can be ascribed to differences in age or disease state between the two groups. The differences between the two uracil curves representing both the groups with and without DPD deficiency at 500 mg/m^2 are caused by the amount of DPD available in deficient subjects, which is lower than in subjects with normal DPD activity causing reduces clearance of uracil. Mattison et al. [15] evaluated fixed doses of 2-^{13}C-uracil of 100, 200, and 300 mg as well as doses adjusted to body weight (1, 3, 6 and 12 mg/kg). They demonstrated with their uracil breath test that an administered dose of 6 mg/kg 2-^{13}C-uracil generated less variable C_{max} and T_{max} than single fixed doses of 100, 200, or 300 mg. The dose of 500 mg uracil/m^2 used in our study is in range with the 2-^{13}C-uracil dose of 12 mg/kg that was also used by Mattison et al. and with the commonly applied

5-FU bolus doses of 400–600 mg/m^2 in the treatment of colorectal and breast cancer [34-37]. The gastrointestinal absorption of uracil is a pharmacokinetic first order process and the elimination follows a reversible and saturable Michaelis-Menten kinetics [17]. When DPD is saturated the elimination of uracil follows zero-order kinetics. The formation of DHU depends on the Km of DPD and the amount of uracil and enzyme present. When all the present DPD enzyme is saturated, the metabolism of uracil will depend on the absolute amount of DPD present and not on the amount of uracil, i.e., if the same dose of uracil is administered to two individuals with different enzyme levels, the individual with the highest amount of enzyme will have the highest "zero-order" reaction rate and the lowest C_{max} of both individuals. So, in order to discriminate between individuals with a normal DPD activity and those with a DPD-deficiency the uracil dose used needs to be high enough to saturate the DPD enzyme both in individuals with and without DPD-deficiency in order to achieve significant different plasma levels. For oral uracil to be used as a diagnostic test, the pharmacokinetics of uracil between patients with and without DPD deficiency has to be clearly discriminating. Mattison et al. [15] describe that the AUC following a dose of 6 mg/kg 2-^{13}C uracil is significantly different in subjects with normal DPD activity versus partial DPD-deficiency. This is in line with the results we found using a dose 500 mg/m^2 uracil. For a broad clinical use, a diagnostic test has to be simple, cheap, sensitive, and specific. The uracil breath test is expensive because of the use of 2-^{13}C uracil and breath bags, and the technique of IR spectrophotometry for analysis of exhaled samples is not available in every hospital. Our test might be more cost effective and might lead to quick test results since the price of 1 gram uracil is about 1 US$ and the HPLC equipment that is used for analysis is common in most hospitals for therapeutic drug monitoring purposes. If the HPLC equipment is not available at the testing site, the plasma samples have to be stable enough to be transported. Prior to this study, the stability of U and DHU in whole blood and plasma were determined. The results show that uracil in whole blood can be stored at 4°C for up to 4 hours. The degradation of U and DHU in plasma was less than 2% during 24 hour at room temperature. We therefore concluded that the stability in plasma is sufficient enough to perform the analytical extraction procedure without further precautions and that the plasma samples are suitable for transportation within 24 hours. As a result, our test can be incorporated broadly into common clinical practice. However, a disadvantage of the current setup of the test is the intensive blood sampling scheme that takes 4 hour to perform and makes it patient intense. The test has to be further optimized into a limited sampling strategy to be more patient friendly. Based on a limited sampling strategy, the parameters

AUC, clearance, and C_{max} are less suitable for discriminating, but the uracil and/or DHU concentrations or U/DHU ratio at selected time points (e.g. 120 min after administration) might be. At this stage, it is unclear if monitoring DHU concentrations can be useful in a limited sampling strategy setting since we found only slightly different values in DHU pharmacokinetic parameters in both groups. This might be explained by the fact that the DHU levels are not only determined by the degradation of uracil by DPD but also by its volume of distribution and the subsequent hydrolysis of DHU into N-carbamyl-β-alanine by dihydropyrimidinase. We conclude that uracil administration at a single dose of 500 mg/m^2 leads to significant and reproducible differences in pharmacokinetics of uracil and DHU between volunteers with a normal DPD activity and DPD-deficient patients. $AUC_{0\text{-}180min}$ and C_{max} might be useful to detect partial DPD deficiency. In addition, uracil doses above 500 mg/m^2 have no discriminating benefits but will only result in a right shift of the uracil and DHU concentration curve and unnecessary longer exposition to high uracil levels. The results presented here points toward a promising development of an oral uracil challenge as a diagnostic test for DPD deficiency. The sensitivity and specificity of this test are currently investigated in a larger population of cancer patients with and without DPD deficiency.

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