Dihydropyrimidine dehydrogenase phenotyping in human volunteers and a DPD deficient patient by assessing uracil pharmacokinetics after an oral uracil test dose. A preliminary report

Jan Gerard Maring¹, Barbara N. Theeuwes-Oonk¹, André B.P. van Kuilenburg², Donald R.A. Uges³, Elisabeth G.E. de Vries⁴, Geke A.P. Hospers⁴

¹Department of Pharmacy, Diaconessen Hospital Meppel and Bethesda Hospital Hoogeveen; ²Department of Clinical Chemistry, Academic Medical Center Amsterdam; Departments of ³Pharmacy and ⁴Medical Oncology, University Hospital Groningen, The Netherlands
Chapter 5

DPD phenotyping with an oral uracil test dose

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Abstract

**Aim** Objective To develop an oral Uracil Challenge Test for dihydropyrimidine dehydrogenase (DPD) phenotyping in patients scheduled to receive 5-fluorouracil (5-FU).

**Methods** The pharmacokinetics of uracil and its metabolite 5,6-dihydrouracil (DHU) were studied after oral administration of an uracil challenge dose in 12 human volunteers and 1 patient with DPD deficiency. All subjects ingested 500 mg/m² uracil as an oral solution on an empty stomach. Blood sampling was carried out during 4 h after oral intake. Plasma uracil and DHU levels were quantified by high performance liquid chromatography. DPD activity in peripheral blood mononuclear (PBM) cells was measured by the conversion rate of radiolabeled thymine. The DPD deficiency in the patient was proven by DNA sequencing of the dihydropyrimidine dehydrogenase gene (DPYD).

**Results** All volunteers (6 male and 6 female, age 27-53) had DPD activities within normal range (mean ± SD; 7.0 ± 1.3 nmol/mg/h). The DPD activity in the patient (female, age 72) was reduced (2.1 nmol/mg/h) due to heterozygosity for missense mutation D949V in exon 22 and I543V polymorphism in exon 13 of the DPYD gene. All subjects had normal liver and renal function parameters. Uracil plasma concentrations at 1 and 2 h were 17.20 and 6.94 mg/L in the patient and increased (P<0.05) compared to concentrations (mean ± 2SD) of respectively 8.07 ± 4.78 and 1.74 ± 3.16 mg/L in the volunteers. The AUC was 30.77 mg.h/L in the patient and higher compared to mean 15.72 ± 10.08 mg.h/L in the volunteers.

**Conclusions** The clearance of uracil was reduced in the DPD deficient patient. Plasma levels at 1 and 2 h were discriminating between normal and DPD deficient. The oral Uracil Challenge Test needs to be further investigated in a larger number of previously characterized, DPD deficient patients to determine its use in pre-chemotherapy DPD-phenotyping for early detection of DPD deficiency.

Introduction

Fluorouracil (5-FU) is widely used in chemotherapeutic regimens for the treatment of breast-, colorectal- and head and neck cancer. The cytotoxic mechanism of 5-FU is complex, requiring intracellular bioconversion of 5-FU into cytotoxic nucleotides. The cytotoxicity, however, is caused by only a small part of the administered 5-FU dose, as the majority of 5-FU is rapidly metabolized into inactive metabolites [1]. The initial and rate-limiting enzyme in the catabolism of 5-FU is dihydropyrimidine dehydrogenase (DPD), catalyzing a reduction of 5-FU into 5,6-dihydrofluorouracil (DHFU). Unfortunately, in patients with DPD enzyme deficiency, 5-FU chemotherapy is associated with severe, life-threatening toxicity [2]. A markedly prolonged elimination half-life of 5-FU has been observed in patients with partial and complete deficiency of DPD enzyme activity [3,4]. The frequency
of DPD deficiency has been estimated to be as high as 1-2% [5,6]. So far, more than 31 mutations in the DPYD gene have been described. The most common mutation is the IVS14+1G→A splice site mutation, comprising around 40% of all detected mutations in patients experiencing excessive 5-FU toxicity [7].

Several methods have been proposed for detection of DPD deficiency [7]. High-throughput DNA sequencing procedures have been developed, but only about 60% of patients with a deficient phenotype appear to have a molecular basis for their reduced DPD activity [2, 8-10]. Thus, measuring the DPD activity in peripheral blood mononuclear cells is currently considered as “the reference method,” but less suitable for screening of large sample volumes on a routine basis [7]. Furthermore, the analysis of the uracil/dihydouracil ratio in plasma has been suggested as a diagnostic tool, but the sensitivity of this test remains to be established [11]. Monitoring of 5-FU plasma levels using a limited sampling strategy may be helpful in early detection of DPD deficiency, but requires rapid analysis immediately after the first 5-FU administration and is therefore not feasible [4]. Thus, a cheap, fast, and easy screening method for DPD deficiency is not yet available.

Uracil and 5-FU are chemically almost alike and both substances are substrates for DPD. Uracil is an endogenous pyrimidine involved in RNA synthesis and, accordingly, an excellent candidate for DPD phenotyping. Uracil is part of the commercial preparation UFT®, which contains the 5-FU prodrug tegafur combined with uracil in a molar proportion of 1:4. Its role in UFT® is to diminish 5-FU catabolism by DPD. To our knowledge a detailed description of uracil pharmacokinetics has never been published before.

The current preliminary pharmacokinetic study was performed with the aim to develop an oral Uracil Challenge Test for DPD phenotyping in patients scheduled to receive 5-FU. The study was performed in human volunteers to assess uracil pharmacokinetics after oral ingestion. These values were compared to the pharmacokinetic data of uracil in a DPD deficient patient.

Materials and Methods

Study subjects

Healthy human volunteers, aged 18 years and older, and a patient with previously diagnosed DPD deficiency were asked to participate. Creatinine, ALAT and gamma-GT levels had to be below 1.5 times the upper limit of normal (ULN), which corresponded to <150 µmol/L creatinine, < 73 U/L ALT and < 75 U/L gamma GT. Volunteers were not allowed to take any medication in the week preceding the experiment (an exception was made for oral contraceptives). All subjects had to abstain food during the entire experiment. Uracil (purity >99.9%; Sigma Chemicals Co, Zwijndrecht, The Netherlands) was administered as an oral solution in a dose of 500 mg/m² and ingested on an empty stomach (last food intake > 8 h earlier) between 9.00 and 10.00 am. This standardization was introduced to diminish the
influence of circadian fluctuations in DPD activity on uracil pharmacokinetics. The study was approved by the Medical Ethics Review Boards of the Diaconessen Hospital Meppel and the University Hospital Groningen and written informed consent was obtained from all subjects.

**Collection of blood samples**
For pharmacokinetic sampling, a canule was placed intravenously in one arm of the subject. Blood samples of 5 mL were collected in EDTA containing tubes just before, and 15, 30, 45, 60, 80, 100, 120, 150, 180, and 220 min after uracil ingestion. Collected samples were immediately placed on ice and subsequently centrifuged at 2,500 g for 10 min at 4 °C and stored at –20 °C analysis. The plasma samples were analyzed for uracil and DHU concentrations by high-performance liquid chromatography (HPLC) within 1 week after sample collection.

**Reversed phase HPLC analysis**
Uracil and DHU concentrations were measured by HPLC analysis using a modification of the method described by Ackland et al. [12]. In a plastic Eppendorf microfuge cup 200 µL of plasma sample was mixed with 100 µL chlorouracil (Sigma Chemical Co, Zwijndrecht, The Netherlands) solution (80 mg/L) as internal standard. Then 200 µL of acetonitril was added, followed by vortex mixing. At 11,000 g the samples were centrifuged for 5 min. The supernatant was transferred into a glass centrifuge tube and 1.5 mL of a 0.1 M phosphate buffer pH 5.5 was added. After vortex mixing 5 mL ethyl acetate was added and the samples were mixed for 15 min in a rotary mixer. The samples were then centrifuged at 3,000 g for 5 min. The organic upper layer was transferred into a conical tube and evaporated to dryness under a gentle stream of nitrogen at ambient temperature. The samples were reconstituted in 100 µL mobile phase and transferred into a glass insert for autosampler vials. 50 µL of sample was injected into the chromatographic system. Calibration samples were prepared by spiking human heparinized plasma (Red Cross Blood Bank, Groningen, the Netherlands) with appropriate amounts of uracil and 5,6-dihydrouracil (Sigma Chemical Co, Zwijndrecht, the Netherlands).

The chromatographic system consisted of a Waters 616 pump equipped with a Waters 717+ autosampler. The separations were performed on an Atlantis dC18 (5 µm, 4.6 x 250 mm) column (Waters, Etten-Leur, the Netherlands), equipped with a Phenomenex C18 (5 µm, 4.0 x 3.0 mm) Security Guard column (Bester, Amstelveen, The Netherlands). The column was used at ambient temperature. The mobile phase consisted of 990 mL 1.5 mM phosphate buffer (pH 5.8) mixed with 10 mL of methanol. The flow during the analysis was 0.8 mL/min. Drug detection was performed using a Waters 996 Photo Diode Array UV detector interfaced with a Millenium 2010 Chromatography Manager Workstation. Spectra were acquired in the 201-300 nm range. Uracil was monitored at 266 nm and DHU at 205 nm. The internal standard chlorouracil was monitored at both wavelengths. The
limit of quantification in plasma was 0.04 mg/L for both uracil and DHU.

**Pharmacokinetic analysis**
The pharmacokinetic analyses were performed in both the MW\Pharm (version 3.5, Mediware, Groningen, The Netherlands) and ADAPT II (version 4.0; University of Southern California, Los Angeles, CA) packages. Variance for the observations was assumed to be proportional to the measured values and set at 10%. The area under the curve (AUC$_{0\rightarrow220\text{min}}$) of uracil and DHU were calculated using the trapezoidal rule. The terminal elimination half-life of DHU was determined by non-compartmental analysis in MW\Pharm.

**Determination of dihydropyrimidine dehydrogenase activity**
The activity of DPD was determined in peripheral blood mononuclear (PBM) cells. PBM cells were isolated from 15 mL EDTA anticoagulated blood and the activity of DPD was determined according previously described methods [13]. In brief, the sample was incubated in a reaction mixture containing 35 mM potassium phosphate pH 7.4, 1 mM di-thiothreitol, 2.5 mM magnesium chloride, 250 µM NADPH and 25 µM [4-14C] thymine. After an appropriate incubation time, the reaction catalyzed by DPD was terminated by adding 10 % (v/v) perchloric acid. The reaction mixture was centrifuged at 11,000 g for 5 min to remove protein. The separation of radiolabeled thymine and the reaction products was performed by reversed phase HPLC.

**Statistical analysis**
Subject data were analyzed for descriptive statistics in the SYSTAT 7.0 statistical program (SPSS inc. 1997). Each value, measured in the DPD deficient patient, was compared to the mean ± 2 S.D. range of the corresponding parameters in the volunteers control group. Values outside this range, which comprises 95% of all individuals in a normal distribution, were considered abnormal (p<0.05).

**Results**

**Patients**
Between March 2003 and February 2004, 12 volunteers and 1 DPD deficient patient were included. All volunteers (6 male and 6 female, age 27-53) had DPD activities within normal range (mean±SD; 7.0±1.3 nmol/mg/h). The DPD activity in the patient (female, age 72) was reduced (2.1 nmol/mg/h) due to heterozygosity for missense mutation D949V in exon 22 and I543V polymorphism in exon 13 of the DPYD gene. All subjects had normal liver and renal function parameters. An overview of patient and volunteers characteristics is represented in table 1.
Tabel 1  Characteristics of human volunteers and the DPD deficient patient, participating in the study.

<table>
<thead>
<tr>
<th></th>
<th>Volunteers (n=12)</th>
<th>Patient</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>39 ± 9</td>
<td>72</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>6/6</td>
<td>female</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>74 ± 10</td>
<td>77</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>178 ± 9</td>
<td>170</td>
</tr>
<tr>
<td>Body surface area (m²)</td>
<td>1.91 ± 0.16</td>
<td>1.88</td>
</tr>
<tr>
<td>PBMC DPD (nmol/mg/h)</td>
<td>7.0 ± 1.3</td>
<td>2.1</td>
</tr>
<tr>
<td>Serum-creatinine (µmol/L)</td>
<td>82 ± 12</td>
<td>106</td>
</tr>
<tr>
<td>Serum-ALT (U/L)</td>
<td>20 ± 8</td>
<td>23</td>
</tr>
<tr>
<td>Serum-gamma-GT (U/L)</td>
<td>16 ± 6</td>
<td>21</td>
</tr>
</tbody>
</table>

Values are depicted as mean ± SD

Pharmacokinetics
The mean pharmacokinetic curves of uracil and DHU are represented in figure 1. The model, used for calculating uracil and DHU pharmacokinetics was similar to the model that was previously described for 5-FU pharmacokinetics [14]. A three compartment model was applied with absorption from a peripheral (gut) compartment after bolus administration and subsequent distribution over a central and deep peripheral compartment. Michaelis-Menten elimination was modelled from the central compartment. The calculated model parameters

![Figure 1](image-url)  Mean (± s.d) concentration-time profiles of uracil and DHU in human volunteers (n=12) and the individual concentration-time curves of uracil and DHU in a patient with DPD deficiency after oral intake of 500 mg/m² uracil solution.
DPD phenotyping with an oral uracil test dose

The interindividual variation in uracil clearance was considerable, resulting in uracil AUCs ranging from the lowest to the highest value over a factor 4. However, despite this variation, the uracil AUC of the DPD deficient patient was still larger than the mean plus two times the standard deviation AUC value of the reference population.

Discussion

This preliminary study shows that low DPD activity, due to a mutation in the DPYD gene, results in an increased uracil plasma AUC after oral intake of uracil. Current data suggest that further development of a Uracil Challenge Test is at least warranted. We applied an uracil dose of 500 mg/m². Enzyme saturation is a prerequisite for adequate discrimination between normal and deficient subjects. A comparable dose of 672 mg/m² uracil is employed for DPD enzyme saturation in the commercial preparation UFT (in combination with 300 mg/m² of the 5-FU analogue tegafur) for treatment of colorectal cancer [15]. Our uracil dose is also in the range of commonly applied 5-FU bolus doses of 400-600 mg/m² in the treatment of colorectal and breast cancer [16-19].

The uracil pharmacokinetics was described in a three compartment Michaelis-Menten model. Michaelis-Menten pharmacokinetics is characterized by first-order elimination at low substrate concentrations, and gradually transforms into zero-order elimination when substrate concentrations rise above levels causing enzyme saturation. This pattern is distinct in the concentration-time plasma curve of the DPD deficient patient compared to the mean curve of the volunteers in figure 1. During the first 2 h, the uracil plasma levels in the DPD deficient patient rise above 2 SD from the mean in volunteers, due to reduced elimination. After 2 h, plasma levels appear to drop below the enzyme saturation level, since the terminal elimination half-lifes are almost equal in both groups. As a result, uracil

Table 2. Pharmacokinetic parameters of uracil and DHU in volunteers and the DPD deficient patient.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Volunteers (n=12)</th>
<th>Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{uracil} t=1 h (mg/L)</td>
<td>8.07 ± 4.78</td>
<td>17.20*</td>
</tr>
<tr>
<td>C_{uracil} t=2 h (mg/L)</td>
<td>1.74 ± 3.16</td>
<td>6.94*</td>
</tr>
<tr>
<td>Ratio C_{uracil/DHU} t=1 h</td>
<td>4.44 ± 5.98</td>
<td>8.56</td>
</tr>
<tr>
<td>Ratio C_{uracil/DHU} t=2 h</td>
<td>0.69 ± 1.56</td>
<td>2.31*</td>
</tr>
<tr>
<td>AUC_{uracil} 0→&lt;220 min (mg.h/L)</td>
<td>15.72 ± 10.08</td>
<td>30.77*</td>
</tr>
<tr>
<td>V_{max} (1/h)</td>
<td>815 ± 370</td>
<td>472</td>
</tr>
<tr>
<td>T_{1/2 elimination} (h)</td>
<td>0.42 ± 0.32</td>
<td>0.50</td>
</tr>
</tbody>
</table>

The values are depicted as mean ± 2. SD # outside 95% confidence interval, P<0.05

are listed in table 2. The interindividual variation in uracil clearance was considerable, resulting in uracil AUCs ranging from the lowest to the highest value over a factor 4. However, despite this variation, the uracil AUC of the DPD deficient patient was still larger than the mean plus two times the standard deviation AUC value of the reference population.
plasma levels at 1 and 2 h are most discriminating between normal and deficient, whereas later levels are not. Fortunately, early discriminating time points favor the applicability of a Uracil Challenge Test in clinical practice.

Based on the pharmacokinetic parameters, derived from the Michaelis-Menten model, we also analyzed whether the sensitivity of the test should improve when higher uracil doses are administered. Simulations showed that a higher uracil dose might indeed increase the sensitivity, however also results in a prolonged absorption phase and therefore indirectly leads to a shift in optimal sampling times to later moments. Recently, Mattisson et al. developed a simple, uracil breath test for DPD phenotyping, based on the release of $^{13}$CO$_2$ from 2-$^{13}$C uracil in the presence of intact DPD [20]. Expired air was collected 5-90 min after oral ingestion of 6 mg/kg 2-$^{13}$C uracil. Partially deficient DPD breath profiles were well differentiated from normal profiles. This approach is very interesting. Unfortunately 2-$^{13}$C uracil is a very expensive compound, making costs a major drawback for routine implementation of this test. The price of commercially available 2-$^{13}$C uracil is about 1 US$ per mg, which implies that a routine test in a 70 kg adult costs at least 420 US$. Additionally, IR spectroscopic breath analyzers are not generally available in most hospitals. The analysis of a single plasma sample by HPLC after ingestion of normal uracil may be more cost-efficient, as the price of 1000 mg uracil is only about 1US$. HPLC equipment is also quite common in most hospitals for therapeutic drug monitoring purposes.

The reduced DPD activity in the patient appeared to be due to heterozygosity for missense mutation D949V in exon 22 and I543V polymorphism in exon 13 of the DPYD gene. Both mutations have been observed previously in partially DPD deficient patients and were both related to increased 5-FU toxicity [2,7,21].

The current method has to be tested in a larger panel of DPD deficient patients, including deficiencies due to different kinds of gene mutations, to establish the predictive value of our test.

We conclude that preliminary results of the oral Uracil Challenge Test are very interesting, but that more research is needed to establish the full potential of this cheap and easy test.
References

14. Maring JG, Piersma H, Van Dalen A, Groen HJM, Uges DRA, De Vries EGE. Extensive hepatic re-
placement due to liver metastases has no effect on 5-flourouracil pharmacokinetics. Cancer Chemother Pharmacol 2003;51:167-173


