Background: Patients with a partial dihydropyrimidine dehydrogenase (DPD) deficiency have an increased risk of developing severe 5-fluorouracil–associated toxicity. We developed a rapid and specific method to measure the DPD activity in peripheral blood mononuclear cells (PBMC) using HPLC tandem-mass spectrometry (HPLC-MS/MS).

Methods: The activity of DPD was measured with thymine as the substrate, followed by reversed-phase HPLC combined with electrospray ionization MS/MS and detection of the product dihydrothymine with multiple-reaction monitoring. Stable-isotope labeled dihydrothymine was used as the internal standard.

Results: Dihydrothymine was measured within an analytically run of 10 min, with a lower limit of quantification of 54 μmol/L (0.4 μmol/L). The intraassay and interassay variations of the DPD activity assay were both <7%. A linear correlation (R² = 0.980; P < 0.001) was observed between the HPLC-MS/MS data and those obtained with a reference method using radiolabeled thymine. There were no systematic differences between the 2 methods, and both methods yielded similar results.

Conclusion: The analysis of the DPD activity with HPLC-MS/MS is rapid, accurate, and sufficiently sensitive to be used as a screening method for patients with a DPD deficiency.

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of the internal standard (IS). The IS was prepared in water containing 250 μmol/L 5,6,6-2H₂-Me²H₂-DHT. The reaction mixture was centrifuged in a microcentrifuge (11 000 g for 5 min) to remove the protein, and the supernatant was saved for further analysis by HPLC-MS/MS.

Samples (50 μL) were introduced into the mass spectrometer via reversed-phase HPLC. Ion suppression at the retention time of the analyte and IS was <10%, and corrected for by use of a labeled IS. DHT was separated, at ambient temperature, on a Phenomenex Aqua analytical column (250 × 4.6 mm, 5-μm particle size), protected by a guard column (Phenomenex SecurityGuard C₁₈ ODS; 4 × 3.0 mm). Solvent A consisted of 50 mmol/L acetic acid, adjusted to pH 4.0 with 25% ammonia, and solvent B consisted of 100% methanol. Elution was performed at a flow rate of 1 mL/min with a linear gradient of 0–5 min, 75% solvent A to 50% solvent A; 5–5.1 min, 50% solvent A to 75% solvent A, and equilibration with 75% solvent A from 5.1 to 10 min. A splitter between the HPLC column and the mass spectrometer was used to introduce the eluent into the mass spectrometer at a flow rate of 50 μL/min. The eluent from 2.5 to 6 min was introduced into the mass spectrometer with an electronically operated valve. A TSQ Quantum AM tandem mass spectrometer (ThermoFinnigan) was used in the positive electrospray ionization mode. We used nitrogen as the sheath gas, the collision gas was argon, the cell pressure 0.13 Pa, capillary voltage 4.0 kV, and the spray voltage was maintained at 4.0 kV. Multiple-reaction monitoring was used to detect DHT and 2H₆-DHT, using the specific m/z transitions of 129→69 and 135→74, respectively (9) (see Fig. 2 in the online Data Supplement). The collision energy for both compounds was optimized at 18 eV. The calibration curve of DHT standards prepared in water was linear from 0.42 to 100 μmol/L (R² = 0.998). The concentration of DHT in each unknown sample was calculated by comparing the DHT/IS area ratio for the sample to the DHT/IS area ratio for a single 25 μmol/L calibrator.

The DPD assay was observed to be linear, with reaction times up to 3 h and protein concentrations between 0.1 and 1 g/L (see Fig. 3 in the online Data Supplement). The HPLC-MS/MS chromatogram of a reaction mixture showed the identification of DHT and the IS 2H₂-DHT (see Fig. 4 in the online Data Supplement). The retention time of DHT and 2H₆-DHT was 4.3 min, and the concentration of DHT (3.1 μmol/L) produced by DPD from PBMC, corresponding with a specific activity of 3.1 nmol/mg/h, was readily detectable.

The intraassay and interassay variations of the analysis of DHT, added to a reaction mixture at low, normal, and high concentrations, ranged from 1.0% to 3.2% and from 1.7% to 4.7%, respectively (Table 1). In addition, an excellent apparent recovery was observed for the detection of DHT (Table 1). Furthermore, the intraassay and interassay variations of the entire procedure for measuring DPD activity by HPLC-MS/MS, including the analysis of the protein concentrations, were <7%. Thus, the precision and apparent recovery of the HPLC-MS/MS procedure were comparable to those observed for assays using radiolabeled substrates or nonradiolabeled 5FU (4–7). The detection limit for 5,6-DHT in the HPLC-MS/MS system, defined as a signal-to-noise ratio of 3, was 0.6 ng (5 pmol). The detection limit for 5,6-DHT in a sample, defined as a signal-to-noise ratio of 3, was 16 μg/L (0.13 μmol/L), which corresponds to a DPD activity of ~0.13 nmol/mg/h. The limit of quantification (LOQ) for 5,6-DHT in a sample, defined as a signal-to-noise ratio of 10, was 54 μg/L (0.42 μmol/L), which corresponds to a DPD activity of ~0.42 nmol/mg/h.

The DPD activity values [mean (SD)] in controls and individuals heterozygous for a mutation in the DPD gene (DPYD; dihydropyrimidine dehydrogenase) were 9.9 (2.8) nmol/mg/h and 4.8 (1.7) nmol/mg/h, respectively (10). To date, various threshold limits have been proposed for DPD activity to identify patients with a risk of developing severe 5FU-associated toxicity (1, 2). The most stringent threshold for DPD activity of patients at risk is the lower limit of the 95% distribution range of the DPD activity in the normal population, i.e., a DPD activity <4.4 nmol/mg/h. Thus, the lower LOQ of 0.42 nmol/mg/h for our

Table 1. Validation parameters for the analysis of dihydrothymine and the DPD activity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Precision</th>
<th>Apparent recovery, %</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Intra assay</td>
<td>Inter assay</td>
</tr>
<tr>
<td></td>
<td>(n = 10)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Dihydrothymine (1 μmol/L)</td>
<td>80.6 (2.7)</td>
<td>3.3</td>
</tr>
<tr>
<td>Dihydrothymine (5 μmol/L)</td>
<td>91.9 (2.8)</td>
<td>3.0</td>
</tr>
<tr>
<td>Dihydrothymine (10 μmol/L)</td>
<td>96.7 (3.0)</td>
<td>3.2</td>
</tr>
<tr>
<td>DPD activity (nmol/mg/h)</td>
<td>6.13 (0.43)</td>
<td>7.1</td>
</tr>
<tr>
<td>DPD activity (nmol/mg/h)</td>
<td>6.48 (0.25)</td>
<td>3.9</td>
</tr>
<tr>
<td>DPD activity (nmol/mg/h)</td>
<td>6.36 (0.27)</td>
<td>4.3</td>
</tr>
</tbody>
</table>

* Recovery of dihydrothymine, without correction with the internal standard.
* The apparent recovery was defined as the percentage of the ratio of the calculated concentration and the nominal concentration.
* The reaction mixtures and the samples were incubated for 5 min; the amount of dihydrothymine produced by DPD was near the LOQ level.
* The reaction mixtures and the samples were incubated for 90 min.
* The reaction mixtures and the samples were incubated for 180 min.
HPLC-MS/MS procedure is sufficiently sensitive to identify patients at risk.

HPLC-MS/MS, with its high specificity obtained by using selective precursor-ion—fragment-ion transitions, also enables the use of stable-isotope labeled ISs, allowing optimal compensation for losses during sample preparation and intensity fluctuations resulting from matrix effects. As a result, the HPLC-MS/MS-based assay allows for a short run time and high precision and accuracy. The analysis time of the HPLC-MS/MS-based assay is considerably shorter than that of HPLC-ultraviolet procedures and those using radiolabeled substrates (4, 6, 7). In addition, the detection limit of the HPLC-MS/MS assay is 5 times lower than that of the reversed-phase HPLC-ultraviolet procedure, in which the product 5-fluorodeoxyuracil is detected at 205 nm (4). Nevertheless, because of the superior sensitivity of radiochemical assays, the presence of a complete DPD deficiency can be established only with the DPD assays that use radiolabeled substrates (5–7).

We used the HPLC-MS/MS procedure and a radiolabeled thymine reference method to measure DPD activity in PBMC. For this purpose, blood samples were obtained from 28 cancer patients suffering from severe 5FU-associated toxicity and 1 pediatric patient with a verified mutation in DYPD. In the radiochemical assay, the activity of DPD was determined in a reaction mixture containing 35 mmol/L potassium phosphate (pH 7.4), 2.5 mmol/L MgCl2, 1 mmol/L dithiothreitol, 250 μmol/L NADPH, and 25 mmol/L [14C]thymine (7). Separation of radiolabeled thymine from radiolabeled DHT was performed isocratically [50 mmol/L NaH2PO4 (pH 4.5) and 75 mL/L methanol] at a flow rate of 1 mL/min by HPLC on a reversed-phase column (Phenomenex Aqua 125A C18; 250 × 4.6 mm, 5-μm particle size) and a guard column (Phenomenex Security Guard C18 ODS; 4 × 3.0 mm inner diameter) with online detection of the radioactivity. A linear correlation (R² = 0.98, P < 0.001) existed between the HPLC-MS/MS data and those obtained with the reference radiochemical assay (Fig. 1A). The paired t-test showed no significant differences between the 2 analytical methods (P = 0.97). Furthermore, the Bland-Altman plot showed that no systematic differences existed between the 2 methods and that both methods yielded comparable results (Fig. 1B). Thus, determination of DPD activity using HPLC-MS/MS is a fast, accurate, and sufficiently sensitive method that can be used as a screening procedure to identify patients with a DPD deficiency before the start of treatment with fluoropyrimidines.

References
10. van Kuijlenburg ABP, Meinsma JR, Zoetekouw L, van Genipp AH. Increased risk of grade IV neutropenia after administration of 5-fluorouracil due to a dihydropyrimidine dehydrogenase deficiency: high prevalence of the IVS1+1g>a mutation. Int J Cancer 2002;101:253–8.