# ARTICLE

# A Simple and Rapid UPLC-UV Method for Detecting DPD Deficiency in Patients With Cancer

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Detecting patients with dihydropyrimidine dehydrogenase (DPD) deficiency is becoming a major concern in clinical oncology. Monitoring physiologic plasma uracil and/or plasma uracil-to-dihydrouracil metabolic ratio is a common surrogate frequently used to determine DPD phenotype without direct measurement of the enzymatic activity. With respect to the increasing number of patients rquiring analysis, it is critical to develop simple, rapid, and affordable methods suitable for routine screening. We have developed and validated a simple and robust ultraperformance liquid chromatography–ultraviolet (UPLC-UV) method with shortened (i.e., 12 minutes) analytical run-times, compatible with the requirements of large-scale upfront screening. The method enables detection of uracil (U) over a range of 5–500 ng/ml (265 nm) and of dihydrouracil (UH2) over a range of 40–500 ng/ml (210 nm) in plasma with no chromatographic interference. When used as part of routine screening for DPD deficiency, this method was fully able to discriminate nondeficient patients (i.e., with U levels < 16 ng/ml) from deficient patients at risk of severe toxicity (i.e., U > 16 ng/ml). Results from 1 month of routine testing are presented and, although no complete deficits were detected, 10.7% of the screened patients presented DPD deficiency and would thus require s decresed dose. Overall, this new method, using a simple preanalytical solid-phase extraction procedure, and based on use of a standard UPLC apparatus, is both cost- and time-effective and can be easily implemented in any laboratory aiming to begin routine DPD testing.

## Study Highlights

### WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Detecting patients with dihydropyrimidine dehydrogenase (DPD) deficiency is an increasingly critical issue in order to secure the administration of fluoropyrimidine drugs such as 5-fluoropyrimidine (5-FU) or capecitabine.

### WHAT QUESTION DID THIS STUDY ADDRESS?
☑ Determining DPD status is challenging. Herein we present a simple and rapid ultraperformance liquid chromatography–ultraviolet (UPLC-UV) method to determine uracil (U) and dihydrouracil (UH2) levels in plasma as a surrogate for DPD activity.

### WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ This new method has been developed to be both time- and cost-effective, thus meeting the requirements for large-scale prospective screening. Data from one representative month of routine testing from our institute illustrate the performance of this phenotyping method for routine DPD testing.

### HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ This method has been kept as inexpensive, simple, and rapid as possible to allow any laboratory to implement DPD testing as part of routine screening. This could contribute to secure the administration of 5-FU and capecitabine, both widely prescribed in clinical oncology.

Fluoropyrimidine drugs (i.e., 5-FU, oral capecitabine) have been a mainstay to treat a wide range of solid tumors in adults. 5-FU is characterized by extensive liver metabolism leading to inactive compounds, depending on a unique catabolic step driven by dihydropyrimidine dehydrogenase (DPD). DPD is coded by the *DPYD* gene, known to be highly polymorphic, with marked changes in phenotypic status. Consequently, patients exhibit a wide range of DPD activities, leading to a high risk of severe/lethal toxicities in individuals with poor metabolizer (PM) phenotype.[1,2] DPD deficiency accounts for the vast majority of life-threatening toxicities in patients treated with 5-FU or oral capecitabine, as demonstrated by numerous clinical reports and meta-analyses in recent decades.[3] Upfront detection of DPD deficiency is thus critical to customize dosing and ensure optimal treatment without triggering potentially lethal

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toxicities.[4] Toward this end, establishing the DPD phenotype is appealing because genotyping *DPYD* and search for allelic variants usually associated with PM phenotype is highly specific, yet hindered by poor sensitivity.[5–7]

Several methods have been proposed over the last 20 years to establish DPD status on a functional, rather than genetic, basis. Neither direct measurement of DPD activity in peripheral blood mononuclear cells (PBMCs) nor the uracil breath test (UBT) can meet the requirements of routine testing due to cost- and time-effectiveness concerns.[8,9] For instance, isolating PBMCs requires a large volume of blood and time-consuming multistep isolation procedures, whereas the UBT requires a specific apparatus, a infrared spectrometer, which is not available in most hospital laboratories.

Because DPD converts physiologic uracil (U) into dihydrouracil (UH2), monitoring UH2/U ratio or uracil levels in plasma has been proposed as an inexpensive, rapid, and convenient alternative to get an insight on DPD activity using a surrogate test.[5] First methods were mostly based on high-performance liquid chromatography–ultraviolet (HPLC-UV) techniques.[10,11] Because UH2 is better detected at 210 nm, i.e., at a wavelength with little specificity and numerous endogenous signals from plasma compounds, most HPLC methods required long run-times, because several columns had to be used in line to achieve optimal separation of the analytes.

Some alternative HPLC methods have succesfully reduced analytical run-times to about 15 minutes, but then required a large volume of plasma.[12] This makes such methods unsuitable for a large screening campaign, when thousands of patients must be tested quickly for DPD deficiency before starting treatment. For instance, in France, a country where an estimated 80,000 new patients receive 5-FU every year, since 2019 all patients scheduled for fluoropyrimidine treatment are legally required to be DPD tested, primarily through measurement of their U levels in plasma.[13]

A cutoff of 150 ng/ml uracilemia has been identified by French health authorities as a warning signal associated with complete deficiency and risk for toxic death. Patients with U between 16 and 150 ng/ml are considered as moderately/partially DPD-deficient, because several reports have shown that U > 16 ng/ml is a first warning signal associated with increased risk to experience severe toxicities with either 5-FU or capecitabine.[14,15] As a consequence, and with respect to the number of new patients due to be treated with a fluoropyrimidine drug, developing methods with simplified analytics is required to test an increasing number of samples as part of routine detection of DPD deficiency. In this study we present the performances and a direct clinical application of a new ultraperformance liquid chromatography–ultraviolet (UPLC-UV) method with run-times < 12 minutes, thus allowing analysis of several dozens of samples in a single batch and expediting interpretation of the results.

## METHODS
### Stock solutions, calibration standards, and quality control samples
All chemicals and reagents, including suppliers, are presented in **Supplementary Data 1**.

Stock solutions of U, UH2, and internal standard 5-FU were prepared in ultrapure water at concentrations of 500,

500, and 1,000 µg/ml, respectively. Working solutions were further prepared by diluting stock solutions in ultrapure water. All stock solutions were stored at –20°C.

Calibration standards and quality controls (QCs) were prepared by addition of working solution to bovine serum albumin (BSA, 80 g/l). Nominal concentrations of 10, 40, and 200 ng/ml were used for U QC samples (low, middle, and high concentrations, respectively), similarly nominal concentrations of 50, 100, and 300 ng/ml were used for UH2 QC samples. The following levels were used for calibration standards: 5, 10, 20, 40, 100, 200, and 500 ng/ml, and 40, 50, 75, 100, 200, 300, and 500 ng/ml for U and UH2, respectively. To avoid possible operator-dependent biases, QCs, and standards, including stock solutions, were prepared by two different laboratory technicians.

### UPLC
All UPLC experiments were performed using an H-CLASS Acquity UPLC system, consisting of an autosampler, pump, column oven, and TUV detector (Waters, Guyancourt, France). Chromatographic separation was performed on an Acquity UPLC HSST3 analytical column (150 × 2.1 mm, 1.8-µm particle size (Waters, Guyancourt, France)) using a 0.2-µm in-line filter. Details on chromatography conditions are presented in **Supplementary Data 2**.

### Sample preparation
Five hundred microliters of heparinized plasma were transferred in a 5-ml hemolysis tube. Five hundred microliters of a 10-mM phosphate buffer (pH 2) was added before manual mixing (30 seconds). The sample was first underwent solid-phase extraction (SPE) using CX100-Interchim cartridges (Atoll, San Diego, CA) preconditioned with methanol and water. Cartridges was washed with 10-mM formate buffer (pH 5.1), and further eluted using 500 µl methanol. Methanol was then evaporated under a gentle stream of nitrogen. The residue was reconstituted in 100 µl of eluent A, vortexed, and centrifuged for 5 minutes at 20,000*g*. Finally, the solution was transferred to an autosampler vial, with 10 µl of the sample injected into the UPLC system.

### Bioanalytical validation
The bioanalytical method validation was conducted in accordance with International Conferences of Harmonization and European Medicines Agency guidelines.[16] The following validation parameters were assessed: calibration model, accuracy, and precision (lower limit of quantification (LLOQ) and QC), dilution integrity, selectivity, instrument carryover, recovery, and stability. Details of each validation item are described in **Supplementary Data 3**.

### Application in routine DPD testing
Once validated, the method was applied routinely in our hospital laboratory to establish DPD phenotype on U measurement in plasma as part of routine screening for DPD deficiency per French Health Authorities recommendations. UH2 was measured as well, both to evaluate UH2/U ratio as an additional information on DPD status, and as a surrogate for possible analyte degradation after sampling.

Two hundred sixty-three blood samples from 14 different institutions were collected over 1 month of routine analysis and analyzed in single weekly analytical batches (four different batches in total). Phenotype interpretation and dose recommendations were performed according to current guidelines in France; i.e., U > 150 ng/ml was considered as a marker for complete DPD deficiency, whereas U levels between 16 and 150 ng/ml were associated with intermediary deficiency.[13] In addition, monitoring UH2/U ratio has been proposed previously by several groups as an additional marker for DPD deficiency–the lower the value, the deeper the deficiency.[4,11,17]

### Statistical analysis

All statistical analyses were performed using MedCalc version 18.10.2 software (MedCalc Software, Ostend, Belgium). $P < 0.05$ was regarded as statistically significant.

## RESULTS
### Calibration

The means of deviation percentage values for each calibration level are provided in **Table 1**. The mean regression coefficient for the six runs of both U and UH2 was 0.9991 ± 0.00178.

### Accuracy and Precision

**Intrarun tests.** For LLOQ and each QC level, all biases were respectively within ± 20% or ± 15%, and all precision levels were ≤ 20% or ≤ 15%. The mean bias and precision are presented in **Table 2**. Representative blank BSA chromatograms are shown in **Figure 1**.

**Interrun tests.** For LLOQ and each QC level all biases were within ± 20% or ± 15%, and all precision levels ≤ 20% or ≤ 15%, respectively. The means for bias and precision are presented in **Table 2**.

### Dilution integrity

All bias and precision levels were within ± 15% and ≤ 15%, respectively.

**Table 1 Results of calibration testing**

| | Calibration level | Nominal value (ng/ml) | Means of deviation (%) |
|---|---|---|---|
| Uracil | 1 | 5 | −4.02 |
| | 2 | 10 | −10.2 |
| | 3 | 20 | 0.786 |
| | 4 | 40 | −1.66 |
| | 5 | 100 | 4.00 |
| | 6 | 200 | −1.28 |
| | 7 | 500 | 0.059 |
| Dihydrouracil | 1 | 5 | 0.429 |
| | 2 | 10 | −2.03 |
| | 3 | 20 | −1.8 |
| | 4 | 40 | −1.1 |
| | 5 | 100 | 1.98 |
| | 6 | 200 | 0.344 |
| | 7 | 500 | −0.338 |

**Table 2 Results of accuracy and precision testing**

| | Samples | Bias (%) | Precision (%) |
|---|---|---|---|
| **Uracil** | | | |
| Intrarun tests | LLOQ | −6.63 | 8.59 |
| | QC low | 14.9 | 1.91 |
| | QC mid | 0.084 | 13.2 |
| | QC high | 9.75 | 9.59 |
| Interrun tests | LLOQ | −2.85 | 10.2 |
| | QC low | 8.15 | 9.26 |
| | QC mid | −0.238 | 10.9 |
| | QC high | 2.88 | 13.1 |
| **Dihydrouracil** | | | |
| Intrarun tests | LLOQ | 7.93 | 12 |
| | QC low | −7.35 | 12.4 |
| | QC mid | −5.01 | 11.7 |
| | QC high | −2.99 | 13.7 |
| Interrun tests | LLOQ | 0.429 | 13.9 |
| | QC low | −0.779 | 13.7 |
| | QC mid | −4.94 | 10.5 |
| | QC high | 0.938 | 4.05 |

### Selectivity

Analysis of plasma samples spiked with representative drugs (i.e., acetaminophen, morphine, voriconazole, posaconazole, clobazam, metoclopramide, granisetron, oxaliplatin, irinotecan, cisplatin, docetaxel) showed no interfering signals for retention times of U and UH2 (see **Supplementary Data 4**).

### Instrument carryover

The blank to LLOQ response after injection of a concentrated sample (i.e., 1,500 ng/ml) were within ≤ 20% (see **Supplementary Data 5**).

### Recovery

Mean recoveries of three QC levels were 48.9% (U) and 43.7% (UH2), respectively.

### Stability

**Short-term stability.** Short-term stability results are expressed as percentage of baseline levels. All U and UH2 levels were within ± 20% of baseline values.

**Long-term stability.** Long-term stability results are expressed as percentage of baseline levels. All U and UH2 levels were within ± 20% of baseline values.

**Freeze/thaw cycles.** Freeze/thaw cycle stability results are expressed as percentage of the baseline levels. All U and UH2 levels were within ± 20% of baseline values over two freeze/thaw cycles.

### DPD screening in routine patients

Performance of the DPD screening method in real-world conditions was evaluated over 1 month of routine sampling in our laboratory. Mean calibration curves were: $y = 3.21 \times 10^{-3}x + 5.3 \times 10^{-3}$ for UH2 and $y = 6.91 \times 10^{-3}x + 8.1 \times 10^{-3}$ for U. A mean of five sets of internal
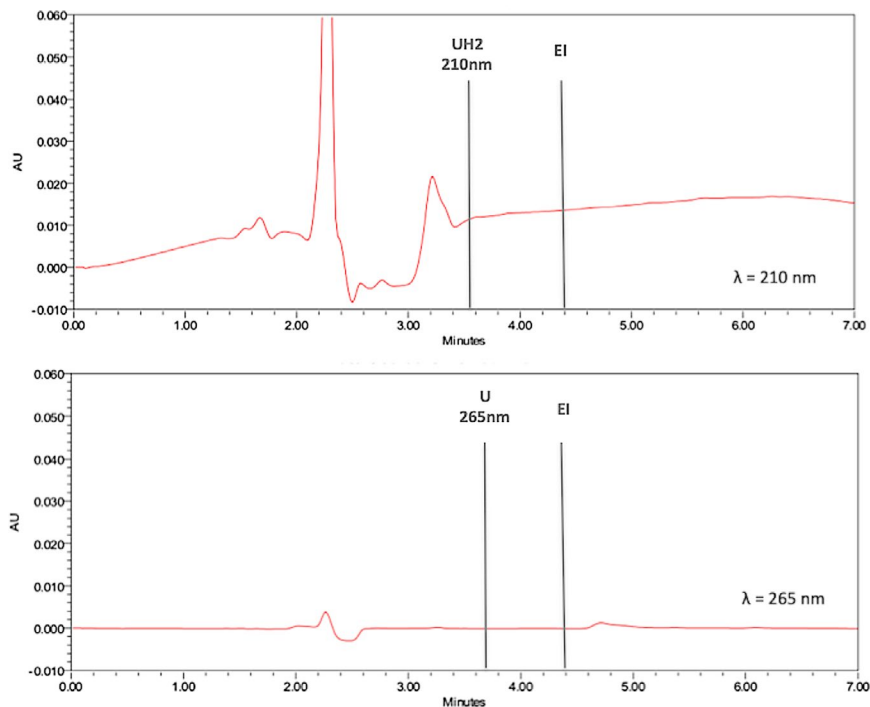
**Figure 1** Blank BSA chromatograms at 210 and 265 nm. BSA, bovine serum albumin.

QCs were included in each analytical batch and were all in the 15% precision range of their respective target values (i.e., 51.3 ± 9.8 ng/ml, 100.9 ± 8.1 ng/ml, and 316.8 ± 21.4 ng/ml for UH2; and 10.0 ± 1.4 ng/ml, 40.6 ± 4.0 ng/ml, and 205.3 ± 8.5 ng/ml for U). A total of 263 adult patients (143 females and 120 males; mean age 65 ± 11.4 years, range 36–88 years) had their blood samples collected over 1 month of routine upfront DPD screening at La Timone University Hospital of Marseille and surrounding general hospitals and cancer centers. Notably, 27 patients had samples with handling/shipping issues (e.g., blood sample sent to the laboratory having been at room temperature for > 90 minutes, blood sample sent to the laboratory at 4°C for > 4 hours, or frozen plasma found thawed upon arrival), thus failing to meet guidelines regarding proper handling for DPD testing.[13] One patient was wrongly sampled on a polymer gel tube and another was wrongly sampled during 5-FU infusion. Therefore, these 29 samples could not be interpreted for DPD status, mostly because of the instability of the analytes upon sampling, leading to an artifactual increase in both U and UH2, potentially generating false-positive results.[12,18] Of the remaining 234 samples fully analyzed (128 females and 106 males; mean age 64.9 ± 11.3 years, range 36–88 years), mean/median U concentrations were 11.4 and 10.2 ng/ml, respectively (range < 5 ng/ml –85.6). In addition, mean/median UH2 concentrations were 138.8 ng/ml and 125.5 ng/ml, respectively (range 34–585 ng/ml). Mean/median calculated UH2/U ratios were 15.2 and 12.6, respectively (range 1.7–40.8 ng/ml). Twenty-five patients (i.e., 10.7%) had plasma U > 16 ng/ml, suggesting potential DPD deficiency, including 12 patients (5.1%) with U values between 16 and 20 ng/ml, 8 patients (3.4%)

between 20 and 30 ng/ml, 2 patients (0.85%) between 30 and 40 ng/ml, 2 patients (0.85%) between 40 and 50 ng/ml, and 1 patient (0.42%) with U = 85.6 ng/ml (**Figure 2**). No difference in gender (*P* = 0.109, chi-square test) or age (*P* = 0.58, *t* test) was seen between patients with U > 16 ng/ml and patients with U < 16 ng/ml (*P* > 0.05, chi-square test). No patient with complete DPD deficiency (i.e., U > 150 ng/ml) was observed over this 1-month screening period. Of note, the patient with U = 85.6 ng/ml displayed as well an UH2/U ratio of 2.6, thus confirming his profoundly DPD-deficient status (**Figure 3**). **Figure 4** shows the distribution in U values among our patients. In addition, we analyzed the 27 samples that failed to be properly shipped to our laboratory. We found that both U and UH2 mean values were significantly higher than the mean values of the samples that were properly shipped (U: 23.6 vs. 11.4 ng/ml, *P* = 0.040 (*t* test); UH2: 201.3 vs. 139.3 ng/ml, *P* = 0.018 (*t* test)), thus confirming that uridine was probably converted to U, then to UH2 after sampling, thus leading to an artificial increase of both analytes and preventing the DPD phenotype from being properly assessed. Consequently, 52% of the samples with improper shipping conditions had U values > 16 ng/mL (range: 18–157 ng/ml), thus wrongly suggesting PM phenotypes.

## DISCUSSION

Determining the best way to evaluate DPD status in 5-FU patients is a decade-old story now in clinical oncology.

Historically, direct DPD activity measurement by radio-HPLC in PBMCs has been proposed—but this method was costly and time-consuming and required radioactive standards, thus failing to meet the requirements of
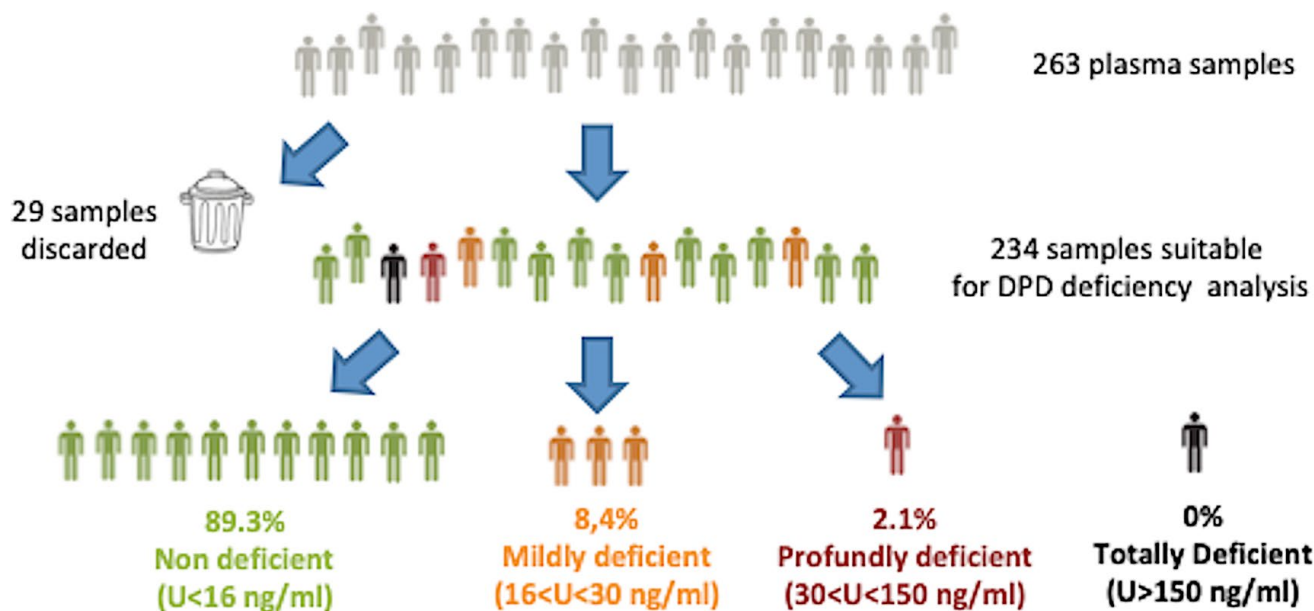
**Figure 2** DPD testing of 1 month of routine analysis. DPD, dihydropyrimidine dehydrogenase.

large-scale screening.[19] Alternate methods developed in the past two decades, such as the UBT, were similarly hard to transpose in routine practice.[8] Conversely, monitoring physiologic U in plasma and its conversion rate to UH2 was proposed as early as the mid-1990s as a convenient surrogate, requiring only basic apparatuses such as HPLC-UV.[20] Many improved HPLC-UV, gas chromatography–mass spectroscopy (GC-MS), or liquid chromatography–tandem mass spectroscopy (LC-MS/MS) methods have been developed since that time.[21–23] Recently, several groups have proposed to assay U and UH2 from saliva samples, so as to facilitate large-scale screening from an easily accessible tissue, but these assays are still based on HPLC-UV or LC-MS/MS analyses.[24–26] Similarly, alternate DPD testing using a loading dose of exogenous U subsequently required MS.[27]

With respect to the increasing number of patients who require screening, developing a simple, rapid, and affordable bioanalytical method that is easily transposable in any laboratory is challenging. In particular, shifting from HPLC to UPLC is critical so as to decrease analytical run-times. Indeed, most published HPLC-UV methods have relied on several columns in line so as to increase the separation of the analytes, especially at 210 nm, with subsequent run-times often exceeding 40 minutes.[11,24] However, to date, all published UPLC-based methods to assay U and UH2 are coupled to MS/MS detection,[17,28] thus limiting the number of platforms or hospital laboratories able to implement the methods at low cost such as with a basic double-wavelength UV detection. Indeed, MS-based analysis, although presenting excellent data, requires an apparatus, labeled standards, and maintenance costs, all much more expensive than basic UPLC-UV analysis. This could be an issue in emerging countries where 5-FU remains the backbone of most anticancer regimens in treating adult patients with solid tumors, and is therefore on the World Health Organization list of Essential

Medicines.[29] Consequently, although more expensive than HPLC, UPLC is a good compromise with regard to time- and cost-effectiveness, which allows DPD testing to be widely implemented, even in underserved countries where *DPYD* genotyping or MS-based phenotyping are too expensive to be part of routine practice.

In this study we have developed and validated, following current bioanalytical guidelines, a new UPLC method to assay both U and UH2 at two different UV wavelengths as a surrogate for DPD phenotyping. Solid-phase extraction was used because it can be easily automated if large batches of samples must be processed. All endpoints regarding method validation were met, making this method suitable for clinical use in patients. Of note, run-times were remarkably short (i.e., 12 minutes), considered a critical point with respect with the ever-increasing numbers of patients in need of routine DPD testing. As a comparison, our previous HPLC-based method used to assay U and UH2 required 45 minutes of run-time.[4] In another study, a 50-minute analysis time was needed.[24]

To illustrate performance of this UPLC-UV method, we have presented results from a representative setting of DPD screening in our laboratory over 1 month of routine testing. Over this 1 representative month, a total of 263 samples were sent to our laboratory (mean 66 samples/week), but only 234 had full final DPD status interpretation due to shipping issues affecting 27 of them (10.2% of entire sample) and other issues for 2 samples. This highlights how rigorous control of shipping conditions (i.e., time and temperature) is critical when developing routine screening for DPD deficiency based on U and/or UH2/U ratio monitoring in plasma. The stability of our blood samples' internal testing showed that U and UH2 were stable for 12 hours at 4°C and 2 hours at ambient temperature (data not shown). However, as per French guidelines, blood samples for DPD
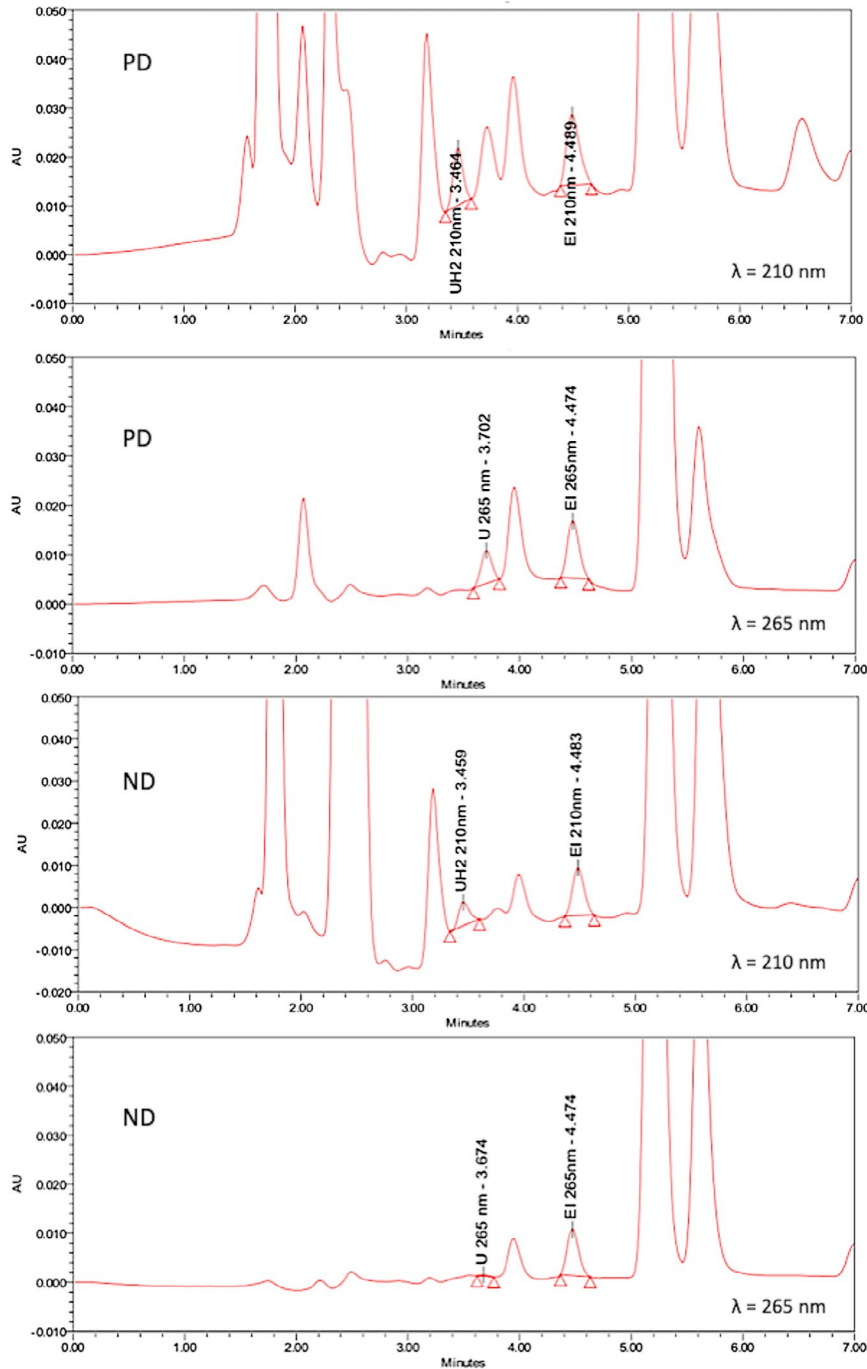
**Figure 3** Chromatograms of representative PD and ND patients at 210 and 265 nm. ND, nondeficient; PD, partially deficient.

testing are to be discarded when time from sampling to centrifugation exceeds 90 minutes with ambient temperature shipping, or 4 hours with 4°C shipping. When samples failing to meet these criteria were analyzed, we found significant differences in both U and UH2 levels when compared with samples with proper handling, thus confirming that these should in fact be discarded, due to elevated risk of false-positive results. In fact, 52% of these samples had U values theoretically associated with a DPD-deficiency syndrome (range 18–157 ng/ml). In addition, unusually

high UH2 values (i.e., up to 612 ng/ml, whereas mean/median value in our patients were 139.3 and 125 ng/ml) assisted in detection of probable degradation of these samples during transfer, which emphasizes how UH2 measurement can be helpful. No chromatographic interference was observed, despite the fact that cancer patients are all heavily treated with a variety of drugs and that 210 nm is far from being a specific wavelength. Overall, 10.7% of patients could be identified as presenting with a variety of DPD-deficiency status because U was > 16 ng/ml, an incidence
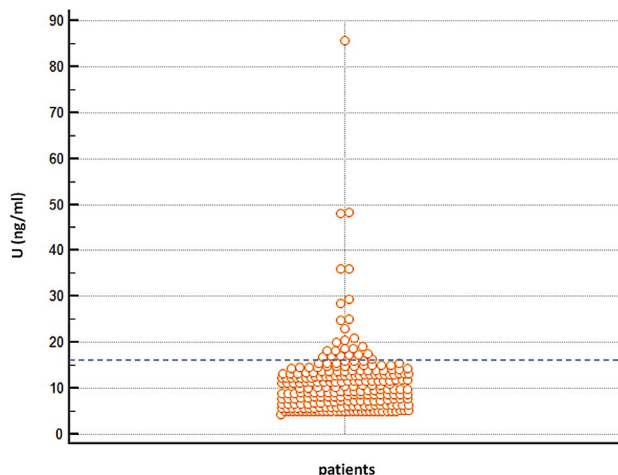
**Figure 4** Uracil distribution in the 234 patients analyzed.

higher than that seen genotype-based studies.[30] Even if the vast majority of our patients (80%) were considered only mildly deficient, with U values between 16 and 30 ng/ml, this figure is slightly higher than the reported incidence (i.e., < 8% at best) of DPD deficiency based on *DPYD* genotyping.[3] This discrepancy is unsurprising as genotyping *DPYD* has been repeatedly characterized as being highly specific but with a poor sensitivity, thus possibly underestimating the actual incidence of DPD deficiency.[31] Consequently, and despite the continuous efforts to better understand the impact of new deleterious variants in *DPYD*[32] or to develop genetic scores to customize 5-FU dose,[33] preemptive genotyping remains unrecognized by both the European Society for Medical Oncology[34] and the US National Comprehensive Cancer Network panel.[35] Patients with U > 150 ng/ml should be precluded for any fluoropyrimidine-based therapy. During this 1-month screening, no such patients were detected. For deficient patients with U levels between 16 and 150 ng/ml, our institutional recommendations range from simple warning of possible impaired elimination with decrease in dose left to the discretion of the oncologist (i.e., for patients with U values between 16 and 30 ng/ml) to proposal for reduction in dose by –25% down to –75%. The extent of this dose reduction depends on the level of DPD deficiency (the higher the U value, the more severe the deficiency and the more drastic the decrease in dose) and other clinical covariates, such as age or performance status as well as initial dose and coadministered anticancer agents. For instance, in the current study, the patient with *U* = 85 ng/mL was considered to be profoundly DPD-deficient, with several comorbidities, and a 75% reduction in 5-FU dose was subsequently proposed.

In conclusion, upfront detection of DPD deficiency is now a major concern in medical oncology when fluoropyrimidine drugs are scheduled. Herein we have presented a rapid, simple, and affordable UPLC-UV method that allows quantification of U and UH2 as a surrogate for DPD activity. Overall, a relatively simple sample preparation step plus reduced run-time can allow 100 patient samples to be run in a single batch over 1 or 2 consecutive working days in our laboratory,

meaning that up to 250 samples can be analyzed per week. Thus, the UPLC-UV method for U and UH2 joint analysis has been shown to be a rapid, robust, and reliable way to evaluate DPD status as part of the routine monitoring of cancer patients scheduled for 5-FU or capecitabine-based therapy.

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