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ORIGINAL ARTICLE

Quantitative impact of pre-analytical process on plasma uracil when testing for dihydropyrimidine dehydrogenase deficiency

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Aims: Determining dihydropyrimidine dehydrogenase (DPD) activity by measuring patient's uracil (U) plasma concentration is mandatory before fluoropyrimidine (FP) administration in France. In this study, we aimed to refine the pre-analytical recommendations for determining U and dihydrouracil (UH_2) concentrations, as they are essential in reliable DPD-deficiency testing.

The authors confirm that there is no principal investigator in this study because this study only includes biological data from standard clinical practice data.

Methods: U and UH_2 concentrations were collected from 14 hospital laboratories. Stability in whole blood and plasma after centrifugation, the type of anticoagulant and long-term plasma storage were evaluated. The variation induced by time and temperature was calculated and compared to an acceptability range of ±20%. Inter-occasion variability (IOV) of U and UH_2 was assessed in 573 patients double sampled for DPD-deficiency testing.

Results: Storage of blood samples before centrifugation at room temperature (RT) should not exceed 1 h, whereas cold (+4°C) storage maintains the stability of uracil after 5 hours. For patients correctly double sampled, IOV of U reached 22.4% for U (SD = 17.9%, range = 0–99%). Notably, 17% of them were assigned with a different phenotype (normal or DPD-deficient) based on the analysis of their two samples. For those having at least one non-compliant sample, this percentage increased up to 33.8%. The moment of blood collection did not affect the DPD phenotyping result.

Conclusion: Caution should be taken when interpreting U concentrations if the time before centrifugation exceeds 1 hour at RT, since it rises significantly afterwards. Not respecting the pre-analytical conditions for DPD phenotyping increases the risk of DPD status misclassification.

KEYWORDS
dihydrouracil, dihydropyrimidine dehydrogenase, intra-individual variability, pre-analytical practices, uracil

1 | INTRODUCTION

Fluoropyrimidines (FP, 5-fluorouracil [5-FU] and its prodrug capecitabine) are anticancer drugs indicated for various types of the disease. Despite their widespread use, 10–30% of patients may experience Grades 3–4 gastrointestinal and haematological toxicities that can be life-threatening or even lethal in 0.1–1% of cases.[1] In the liver, more than 80% of 5-FU is metabolized into dihydrofluorouracil (5-FUH_2) by dihydropyrimidine dehydrogenase (DPD) that physiologically reduces endogenous uracil (U) into dihydrouracil (UH_2).[2] Approximately 3–5% of the Caucasian population has a partial reduction of DPD activity, and less than 0.1% has a complete deficiency, explaining the overall 20–60% of severe FP-related toxicities.[3–6] In France, determining DPD activity prior to FP administration has been mandatory since 2018[5] and is recommended in Europe.[7] The gold-standard approach, although unsuitable for daily care, consists in measuring DPD activity in peripheral blood mononuclear cells (PBMCs).[8] Targeted genotyping of germline polymorphisms of the *DPYD* gene has been shown to decrease FP-related toxicity in a prospective study, but with only a low positive predictive value.[9,10] Phenotyping by measuring plasma U (with or without UH_2) is the method approved by the French Health Authorities (Institut National du Cancer/Haute Autorité de Santé, INCa/HAS).[5] A threshold U value set at 16 ng/mL helps discriminate between partially deficient patients (U between 16 and 150 ng/mL) and completely deficient patients (U above 150 ng/mL).[11,12] The UH_2/U ratio may be used as an additional marker for DPD deficiency.[12]

What is already known about this subject

- Uracil and dihydrouracil are highly unstable in biological matrices.
- According to current sample handling recommendations, the time period before whole blood centrifugation should not exceed 1.5 hours if the sample is stored at room temperature (RT) and 4 hours if it is stored at +4°C. However, this statement is based on limited published data.

What this study adds

- The stability study shows that a 1 hour time period between blood sampling and centrifugation at RT is more appropriate than 1.5 hours, while storage of whole blood at +4°C maintains the stability of uracil concentrations for up to 5 hours.
- Failure to adhere to pre-analytical recommendations for sample handling significantly increases the risk of concluding a false DPD phenotype.
- The intra-individual variability of uracil, assessed by the coefficient of variation of samples from patients double sampled for DPD testing, is 22.4% (range from 0% to 99%).

The value of U levels in predicting FP-related severe toxicity has recently been questioned, as it is prone to pre-analytical errors and analytical variability.[13] Indeed, analytical development studies have reported that U and UH_2 are unstable in biological matrices as the concentrations of U and UH_2 increase with time between sampling and centrifugation.[14–22] In clinical practice, it is recommended separating plasma and blood cells immediately.[5] If this is not possible, samples are allowed to be stored at room temperature (RT) no longer than 1.5 hours or be placed at +4°C for up to 4 hours, based on a few analytical studies[14,15,17–20] and laboratories' own experience.

Adhesion to pre-analytical recommendations is even more important as failure may cause misinterpretation of the DPD phenotype and administration of a suboptimal FP dosage. However, strict respect of recommendations may be hampered by daily hospital and laboratory activity (e.g., schedule constraints, unclear information about sample handling and staff turnover) and can lead to unreliable results with significant consequences for the patient.

To address the lack of experimental support for these pre-analytical recommendations, we conducted a study under real-life conditions to quantify the impact of pre-analytical procedures on the interpretation of DPD phenotyping results. Our aim was to refine blood sample transport and storage conditions to ensure stable U and UH_2 plasma concentrations. Furthermore, we assessed intra-individual variability for U and UH_2 to interpret concentration discrepancies for any given patient.

2 | METHODS

2.1 | Data collection and ethics

This study was an initiative of the French Clinical Oncopharmacology group (GPCO-Unicancer) and included 14 hospital laboratories (13 French and one Belgian) routinely performing DPD phenotyping by measuring plasma U. Data were collected from clinical practice over 2 years (July 2019–September 2021). Since UH_2 and UH_2/U can each be used to assess DPD deficiency, both parameters were investigated by certain laboratories. The first objective of the study was to evaluate the stability of U and UH_2 concentrations in whole blood before centrifugation and in plasma after centrifugation with temperature as a covariate (storage at RT or at 4°C). All stability assays were performed on residual aliquots of patients' blood samples where exact times of blood sampling, centrifugation and freezing were reported. The second part of our study targeted the analysis of intra-individual variability (or IOV, inter-occasion variability) of U and UH_2 in a real-life setting. U and UH_2 concentrations were collected from patients undergoing two DPD phenotyping analyses as part of standard clinical practice. Each laboratory routinely assessed plasma U and UH_2 by liquid chromatography coupled with a variable detection technique (i.e., UV, quadripolar mass spectrometry or high-resolution mass spectrometry). Some of these methods have been published.[14,23,24] Furthermore, all centres participated in an external quality assessment programme (EQAS) to ensure reliability and homogeneity of analytical methods (Asqualab, Paris, France[25]). This study was approved by the ethics committee of the Assistance Publique—Hôpitaux de Paris (CERAPHP Centre, Paris, France, Institutional Review Board registration number: 00011928).

2.2 | Stability study

All stability assays were conducted as part of the daily DPD phenotyping activity of each laboratory and performed on residual aliquots from patients' blood samples drawn in clinical services. To assess the stability of concentrations in whole blood, samples were divided into multiple aliquots upon reception in the laboratory (within 30 min after sampling). To obtain a reference sample, one aliquot was immediately centrifuged, and plasma was stored at −80°C or −20°C for further analysis. Residual ('test') blood sample aliquots were kept at RT (mean RT = 21°C) or +4°C and were centrifuged at different times (from 30 min to 24 h later). Plasma was frozen immediately after centrifugation. For the post-centrifugation plasma stability study, plasma was split into several aliquots after separation from blood cells. As before, one aliquot was immediately stored at −80°C or −20°C for further analysis (reference sample) and residual 'test' samples were kept at RT or +4°C and were frozen at different timepoints (from 30 min to 24 h later). Both test and reference samples were analysed simultaneously, respecting standards and internal quality control in each laboratory. Stability of U and UH_2 was assessed by calculating the deviation of the concentration from the reference at each timepoint: deviation (%) = concentration in test sample (C_{test})/concentration in reference ($C_{reference}$) × 100. A ±20% variation in the concentration was considered acceptable. Long-term freezing was assessed by measuring the concentrations of U, UH_2 and the UH_2/U ratio in plasma stored at −20°C or −80°C for 7 days to about 3 years. The bias from the reference value (first measurement before freezing) was determined after thawing (bias [%] = (C_{test} − $C_{reference}$)/$C_{reference}$ × 100). The quantitative impact of the type of anticoagulant (ethylenediaminetetraacetic acid, EDTA or lithium heparinate) on U and UH_2 stability was tested using two different collection tubes for each patient.

2.3 | Data modelling and simulation analyses

U concentrations, obtained for the stability study, were analysed longitudinally as a function of time with a nonlinear mixed-effect approach with NONMEM software (7.4, ICON Development Solutions, Ellicott City, MD, USA). To overcome the fact that the U reference value was not necessarily obtained after immediate centrifugation (rather within 30 min after blood draw), the dataset was built in order to take into account, for each measured value of U, the time spent as whole blood (i.e., the time between blood draw and centrifugation) and the time as plasma (i.e., the time between centrifugation and freezing). This enabled us to longitudinally describe the course of U as a function of time and pre-analytical conditions and to

estimate the 'real' reference U value called U_0 in this analysis. A Gompertz growth model was applied to fit the increase in U (additional methods and NONMEM code are detailed in the Supporting Information). Covariate analysis was limited to the study of a laboratory effect as the U data were provided by six different hospitals. The objective was to identify the acceptable time thresholds for whole blood centrifugation and for freezing centrifuged plasma. A simulation study based on our model was conducted as described in the Supporting Information to calculate the time period for which 95% of U profiles increased less than 20% compared to U_0.

2.4 | Evaluation of intra-individual variability

Thorough research in the laboratory information system in each centre made it possible to retrospectively identify 573 individuals double sampled for DPD testing. Samples were classified as non-compliant or compliant by the laboratory according to whether they satisfied the pre-analytical process currently recommended by INCA/HAS (i.e., time before centrifugation <1.5 h, if blood sample was kept at RT, or <4 h, if kept at +4°C).[5] Intra-individual variability was assessed by the inter-occasion variability (IOV) between the two occasions (O_1 and O_2) by calculating the coefficient of variation (CV) for U, UH_2 and the ratio (CV [%] = standard deviation [SD]/mean of O_1 and $O_2 \times 100$). Determining DPD phenotype was based on the U value of 16 ng/mL, with patients being classified as normal phenotype (U < 16 ng/mL) or DPD deficient (U > 16 ng/mL). Two samples could be concordant (the same phenotype determined) or discordant (different phenotypes).

The distribution of individual U values according to time of day was evaluated to check if DPD circadian activity has relevant consequences on U interpretation. Mean and SD were determined for each 1-hour interval containing individual data.

2.5 | Statistical analyses

All statistical tests were two-sided (type I error rate of 5%) and were chosen according to data distribution. For the stability study in whole blood and plasma, the mean ratio (test/reference) determined for each timepoint was statistically compared to the value of 1 (i.e., no variation from reference). Test pairing was used when comparing samples from a same patient. All statistical tests were computed for a two-sided type I error rate of 5%. Analyses were performed using R software (version 4.1.0, R Foundation for Statistical Computing, Vienna, Austria). Plots were created with GraphPad Prism (v.5.0, La Jolla, CA, USA).

3 | RESULTS

3.1 | Stability of uracil and dihydrouracil in whole blood

A total of 573 U concentrations measured in 186 patients from six hospitals were included in the stability study and were tested for matrix and temperature quantitative impact: whole blood stored at either RT ($n = 170$) or +4°C ($n = 49$) and post-centrifugation plasma at either RT ($n = 168$) or +4°C ($n = 186$). The mean relative deviations of U in whole blood are depicted in Figure 1A. The *P*-values for comparison to the reference value and the number of samples per dual set of time and temperature (RT or +4°C) are detailed in Table S1. U rapidly increased in whole blood stored at RT and differed from the mean reference value from 1.5 hours: Mean increase was 21.0% (95% confidence interval [CI]: 10.7%; 31.3%) ($P < .001$). However, U remained constant for up to 5 hours at +4°C (+1% [−15.9%; 17.9%]). Conversely, UH_2 seemed less sensitive to storage temperature than U in whole blood (Figure 2A): +4.2% [10.9%; 19.3%]) after

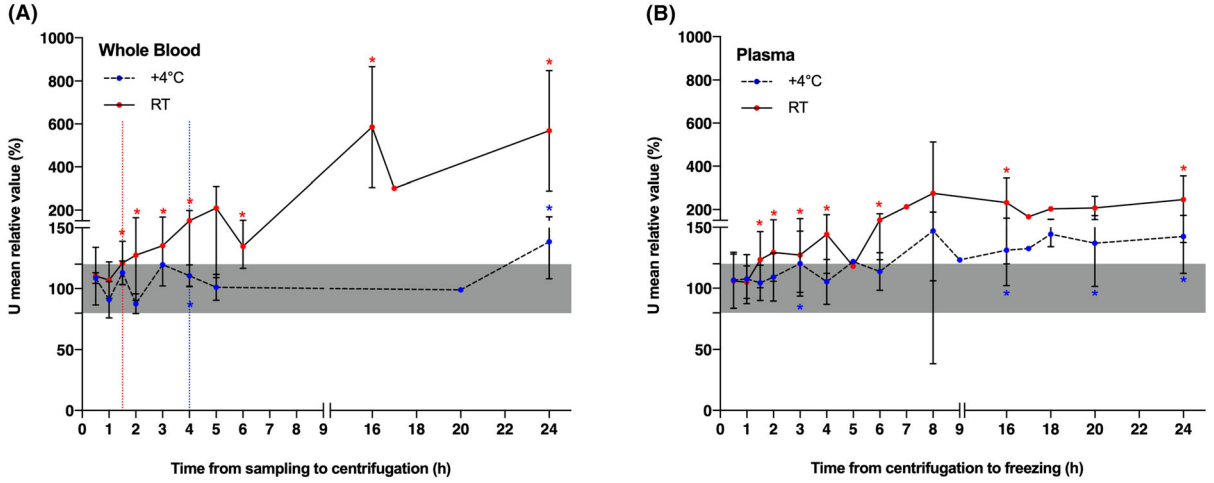

FIGURE 1 Mean relative deviations (± standard deviation) of the uracil concentrations in (A) whole blood and (B) plasma samples kept at room temperature (RT, red points) or +4°C (blue points). Vertical dotted lines represent the current recommended maximal time to keep whole blood samples at RT (1.5 h, in red) and +4°C (4 h, in blue). ±20% acceptance area is in grey. *$P < .05$ (Wilcoxon signed rank paired test)

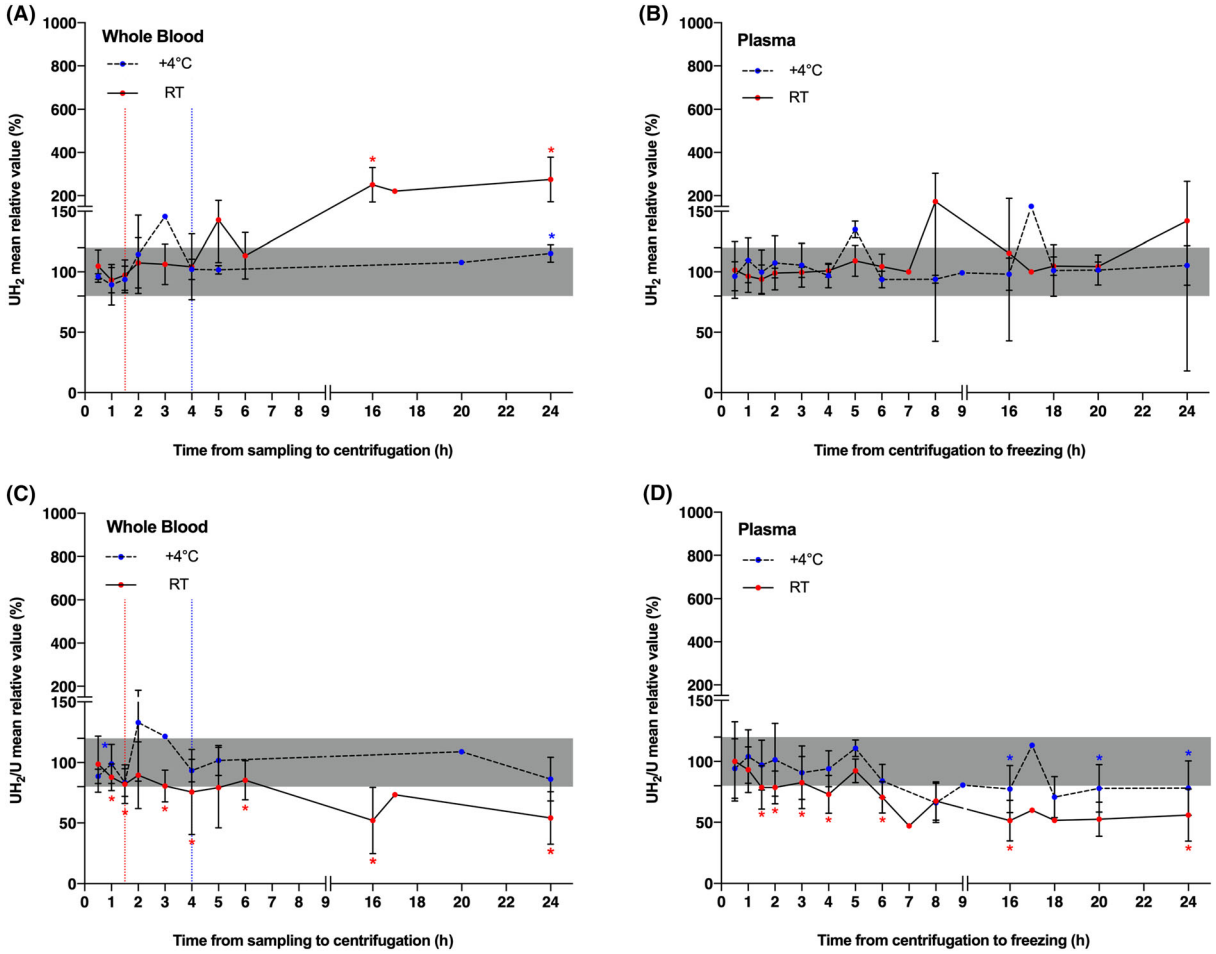

FIGURE 2 Mean relative deviations (± standard deviation) of UH_2 concentrations and UH_2/U ratio in whole blood (left panel) and plasma samples (right panel) kept at room temperature (RT, red points) or +4°C (blue points). Vertical dotted lines represent the current recommended maximal time to keep whole blood samples at RT (1.5 h, in red) and +4°C (4 h, in blue). ±20% acceptance area is in grey. *P < .05 (Wilcoxon signed rank paired test)

4 hours, at RT, +2.1% [−5.0%; 9.1%] at 4°C, both non-significant. As a result of poor U stability in whole blood, the UH_2/U ratio showed high variability at RT (−17.9% [−27.1%; −8.6%]) after 1.5 hours, P < .001), but this was limited by cold storage for 5 hours (+1.7% [−18.0%; 21.4%]) (Figure 2C).

3.2 | Stability of uracil and dihydrouracil in post-centrifugation plasma

For post-centrifugation stability, U and UH_2 concentrations were measured after keeping plasma samples at either RT or 4°C before freezing. At RT, U significantly exceeded the validity threshold value after 1.5 hours (+23.4 [6.9%; 40.0%], P = .004, Figure 1B and Table S2). However, cold storage helped to stabilize the concentrations for up to 6 hours at +4°C (+13.7%, non-significant). Like in whole blood, the UH_2 value was not affected by RT (+4.4% [−1.8%; 10.6%] for 6 hours and −6.2% [−12.6%; 0.1%] at +4°C, Figure 2B). For the UH_2/U ratio, maintaining plasma at 4°C was safe for up to 6 hours at +4°C (−16.1% [−28.7%; −3.4%]) but not beyond 4 hours at RT as seen in the significant decrease (−27.0% [−36.9%; −17%], P = .002, Figure 2D).

3.3 | Modelling and simulation of U stability in biological matrices

The U concentrations of the stability study were modelled simultaneously (Figure 3 and Table S3). Regarding the stability in whole blood at RT, simulations found that the time before centrifugation to maintain 95% of the samples with an increase of U < 20% (compared to the estimated reference U_0) was 47 minutes [CI 90%: 25; 67 min]. The stability of U in plasma at RT depended on the time before whole blood centrifugation: U concentrations did not increase by more than 20% in 95% of the patients over 58 minutes [27; 97 min], if blood was immediately centrifuged after being drawn and plasma was stored at RT. Notably, stability in plasma decreased to 30 minutes [−4; 60 min] if blood was centrifuged

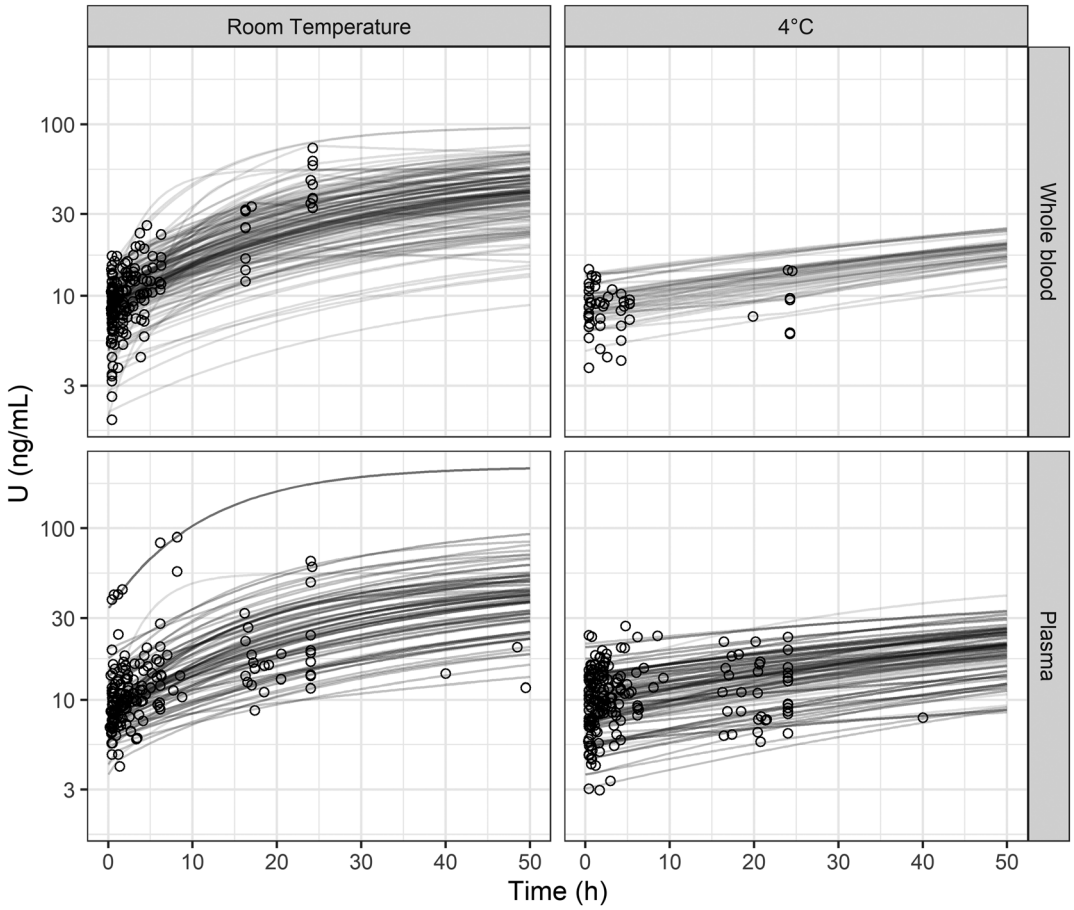

FIGURE 3 Observed (dots) and individual predicted (lines) uracil (U) concentrations as a function of time (in h), stratified by storage conditions. The Gompertz model adequately captures the saturation in U increase for samples stored at room temperature.

30 minutes after sampling and was null if centrifugation took place later than 47 minutes. Storage at 4°C preserved the uracil concentrations, either in whole blood or in post-centrifugation plasma. Indeed, if centrifugation was performed 30 minutes after blood sampling, U in post-centrifugation plasma remains stable for an additional 2.9 hours [1.4; 5.2 h]. The small amount of data available for whole blood at +4°C and at early times did not allow us to accurately simulate a time before centrifugation, for this situation. Of note, the inclusion of the laboratory as a covariate in the model did not result in clinically relevant differences in the simulated U profiles, which demonstrated a negligible effect of the laboratory in the U variability (data not shown).

3.4 | Quantitative impact of anticoagulants

As indicated in the best pre-analytical practices, whole blood should be collected in a tube with the anticoagulant, either EDTA or lithium heparin. U concentrations were compared in 80 samples (76 for UH_2 and UH_2/U) collected simultaneously in two different tubes (EDTA and lithium heparinate). As shown in Table 1, a significant difference was observed for U with a mean deviation between the two samples of 14.2% (±25.1%) in favour of EDTA. This difference was also significant for the UH_2/U ratios but not for UH_2.

3.5 | Long-term conservation of plasma samples

Long-term conservation (over 1 year) was assessed in plasma stored at −20°C or −80°C according to individual laboratories' standard practices. After 1 year, the deviation of samples stored at −20°C from the reference value of U or UH_2 was extremely low (average of 0.4%, SD = 9.3% and 2.1%, SD = 10.4%, respectively, for U and for UH_2, see Table S4). Comparable results were observed for samples stored at −80°C for 36 months: The deviation was still less than 10% with U and UH_2 values not differing significantly from the initial value (6.1%, SD = 14.9% for U and −0.5%, SD = 2.3% for UH_2).

3.6 | Intra-individual variability

A cohort of 573 patients double sampled for DPD-deficiency testing was constituted retrospectively. The median time between the two samples was 21 days (min–max = [0–672 days]). Sample pairs were

TABLE 1 Impact of blood anticoagulant on U, UH_2 concentrations and UH_2/U ratio

	Paired samples (*n*)	Samples in lithium heparinate [min–max] (ng/mL)	Samples in EDTA [min–max] (ng/mL)	Mean deviation (SD) (%)	*P*[a]
U	80	9.25 [3.8–29.4]	10.5 [5.0–36.7]	14.2 (25.1)	<.0001
UH_2	76	92.9 [16.9–226.9]	93.5 [10.8–217.4]	−0.60 (21.3)	.872
UH_2/U	76	8.7 [1.4–41.9]	8.1 [1.0–25.6]	−10.2 (23.0)	<.0001

Note: Median, minimum and maximum values were measured in samples collected in EDTA or lithium heparinate (reference) tube at a single moment for any given patient.

[a]*P*-value calculated with the paired Wilcoxon signed rank test.

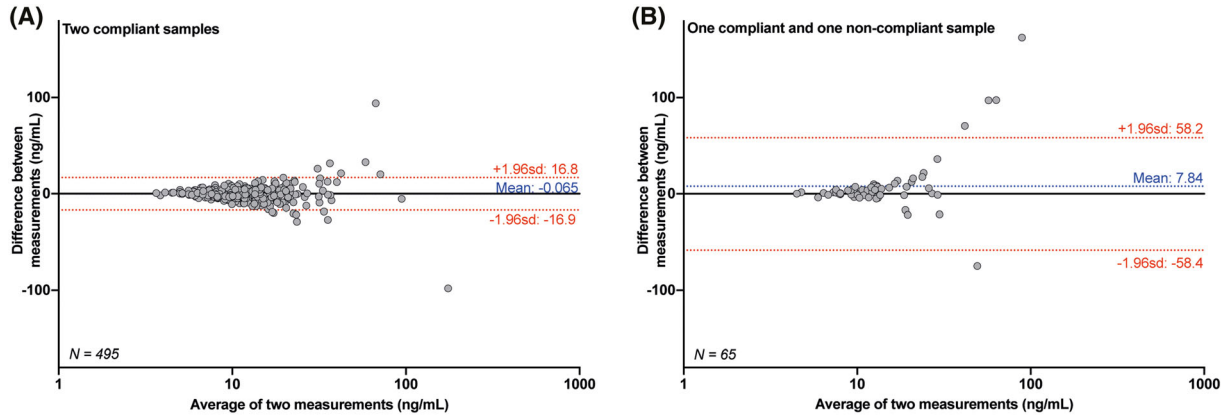

FIGURE 4 Bland–Altman plot showing differences between two samples for a given patient (*Y* axis), against the mean U concentration of the two samples (*X* axis, log scale). Patients were separated into two categories: (A) those having two compliant samples ($n = 495$, first sample chosen as the reference, Uref) and (B) those having only one compliant sample ($n = 65$, compliant sample chosen as the reference, Uref). Difference between the two samples was calculated as follows: U test − Uref. The central dotted blue line represents the mean difference in U of repeated sampling, and the dotted red lines represent the 95% confidence interval.

grouped into three categories based on the respect of pre-analytical practices: pairs of two compliant samples ($n = 495$), pairs with one non-compliant sample ($n = 65$) and pairs with two non-compliant samples ($n = 13$).

U values were subjected to IOV as shown by the differences between samples pairs displayed in Figure 4. Most of the sample pairs (495/573, 86.4%) were correctly handled in the cohort which is illustrated in Figure 4A by an absence of bias and a limited variability (narrow 95% CI) for the difference between samples pairs. The mean CV of this subgroup was 22.4% (SD = 17.9%, range = [0–99%]). UH_2 showed great variability, as seen in the large 95% CI in Figure S1. UH_2/U reflected reduced variability with a narrow 95% CI and limited bias around the mean of the differences between the two measurements. For both variables, mean CVs were comparable (CV = 20.6%, SD = 15.9% [0–81.4%] for UH_2; CV = 21.5%, SD = 18.8% [0–97.5%] for UH_2/U).

As expected, non-respect of the pre-analytical recommendations increased this variability, as reflected in the wide 95% CI and positive bias observed for the group of patients with one non-compliant sample [−58.4; 58.2 ng/mL] (Figure 4B). Mean the CV value increased significantly to as high as 34.3% (range = [1.05–129%], $P = .006$, Wilcoxon signed rank test). In contrast, IOV of UH_2 was not affected (CV = 24.3% [1.29–95.5%]). If both samples were non-compliant, IOV was more limited for U and CV% = 25.6%, [0.6–65.4%]).

Finally, among the 495 patients with two compliant samples, 17% (84/495) had a discordant DPD phenotype based on the U value of their two samples. Variability was high in this subgroup of patients (CV = 34.9%, SD = 20.6%, range = [4.9–86.8%]). For 14 of them, U concentrations were within the ±15% interval around the 16 ng/mL threshold on either side, which leads to a discordant phenotype despite a quite similar U value. Of note, the rate of patients with a discordant phenotype increased significantly when either one sample (22/65, 33.8%) or both (3/13, 23.1%) did not meet pre-analytical recommendations (chi-square test with reference group, $P = .005$).

3.7 | Effect of sample timing on uracil and dihydrouracil concentrations

We evaluated the variation of U concentration during the daytime by plotting individual U concentrations of patients having been correctly double sampled (the first sample was arbitrarily selected, Figure 5A). No specific trend was observed at any time of the day. After selecting patients sampled at two different moments of the day (morning

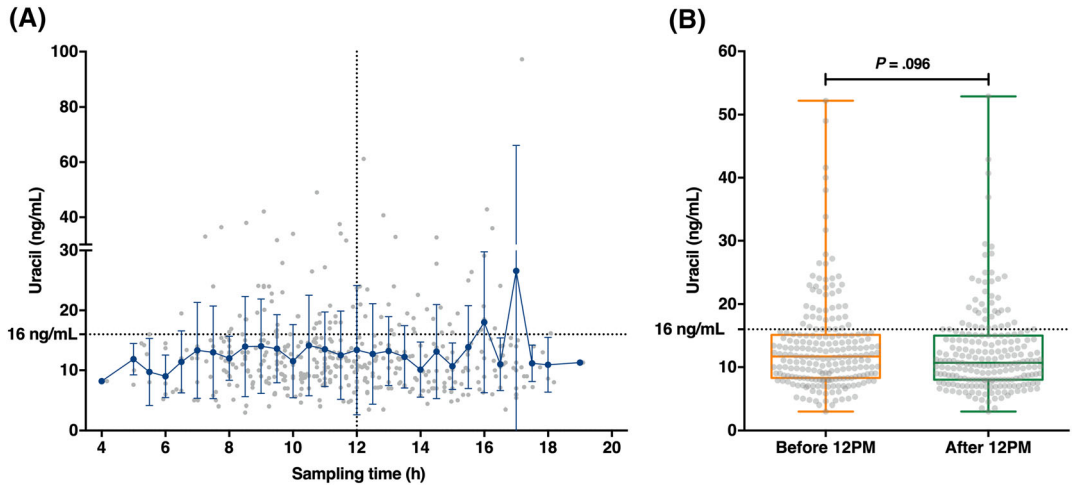

FIGURE 5 Effect of sampling time on uracil concentrations. (A) The individual values (grey symbols) of U from patients with compliant samples were plotted as a function of their sample timing. Means and standard deviations of the corresponding 1-hour interval appear in blue. (B) The U concentrations were compared for patients double sampled at two different moments of the day (before or after 12 pm, Wilcoxon paired comparison test).

versus afternoon), we found no statistical difference (U_{AM} = 13.2 ± 7.3 ng/mL, SD = 7.3 ng/mL; U_{PM} = 12.4 ng/mL, SD = 6.8 ng/mL, see Figure 5B). Equivalent results were obtained for UH_2 ($UH_{2\text{-}AM}$ = 109.9 ng/mL, SD = 44.2 ng/mL; $UH_{2\text{-}PM}$ = 113.6 ng/mL, SD = 42.7 ng/mL, see Figure S2). Additionally, the risk of phenotype discordance between the two U analyses for any given patient was not higher in patients having their two samples taken in a different half of the day (morning vs. afternoon) compared to those with samples taken the same half of the day (chi-square P = .21).

4 | DISCUSSION

Since the publication of the INCa/HAS guidelines in 2018, determining patients' DPD status by measuring plasma U levels has been mandatory before FP administration and is performed daily in hospital laboratories.[5] However, the poor stability of U and its metabolite UH_2 in the biological matrices raises the question of the reliability of the method for predicting DPD deficiency.

Respect of pre-analytical practices is even more important in evaluating DPD phenotyping because it determines the dose to administer to the patient.

After confirming the high variability of U in whole blood samples stored at RT, our most important finding was that the actual recommended time limit of 1.5 hours is associated with a risk of U overestimation (+21%, P < .001). Importantly, storage of whole blood at +4°C helped to delay the increase of U for up to 5 hours, a result supported by a previous stability study.[14] We observed a poor stability of U in post-centrifugation plasma samples stored at RT, as well. The modelling analysis showed this stability to depend on the time period before centrifugation: The longer the time before centrifugation, the shorter the stability in post-centrifugation plasma, as the increases in U in both matrices add up together. Overall, our results suggest that the maximum recommended time to handle the blood samples (from drawing to plasma freezing) should be 1 hour, confirming Capiau et al.'s recent observations.[26]

Numerous factors have been proposed to explain U instability. Launay et al.[27] attempted to stabilize blood samples by adding gimeracil, a potent DPD inhibitor, immediately after drawing blood. U consistently increased in the presence of the inhibitor (+14- to +74-fold the baseline value) suggesting that DPD is still physiologically active in the PBMCs with no inhibitor. However, in the absence of gimeracil and despite DPD remaining activity, U still increased (+3.5- to +9.2-fold). This suggests that U is more likely to be generated from additional sources such as the degradation of uridine by the uridine phosphorylase[28] or from the digestion of plasma circulating ribonucleic acids from food intake.[29]

We observed that U tends to be higher if the whole blood is drawn in a tube with EDTA instead of lithium heparinate possibly because of a pH variation that can affect the stability of endogenous metabolites.[30,31] This difference (+14.2%) was expected to be of limited clinical impact in determining DPD status because it is lower than the deviation that we tolerate for analytical variability (set at ±15% of the reference value). However, for a patient with a U value close to the 16 ng/mL cut-off, this difference may lead to a different DPD phenotype classification. A limitation of our study is that we did not include serum tubes whereas the Dutch study that has contributed to the choice of the 16 ng/mL threshold used uracil levels determined in serum.[32] Our current results should encourage the homogenization of the sampling tube for uracil analysis across hospitals and studies.

The circadian activity of DPD was first highlighted in healthy volunteers by Jiang et al.,[33] and was suggested to influence phenotype-guided FP dosing, with a maximal activity around 1 a.m. and a nadir around 1 p.m.[34] While we sought to determine whether the time of sampling (i.e., morning or afternoon) influenced interpretation of the

U concentration, no trend was observed in U plasma values that would require a specific collection schedule.[5]

Finally, this study is the first to provide IOV data of U collected in cancer patients according to the current INCa/HAS recommendations. Double sampling is often necessary to confirm a pathological value of U, due to non-compliant pre-analytical handling of the first sample or to various organizational issues (unavailable results, test prescribed by another clinician, etc.). When considering compliant samples, IOV was 22.4%, and the rate of patients with discordant phenotypes (and possibly different prescribed FP dose) was 17%. This rate underlines the limitation of using a numeric cut-off to assign a DPD phenotype for a biological parameter (such as U) that is prone to many sources of variabilities. In case of U values close to the 16 ng/mL cut-off, the use of UH_2/U and *DPYD* genotyping can be helpful to identify DPD-deficient patients. We believe that deciphering the sources of U variability will help us to control them to optimize the use of U in clinical routine, as it has been proved to have a better sensitivity and positive predictive value than genotyping alone.[32] For instance, performing the blood pretreatment within 1 hour instead of 1.5 hours may decrease this intra-individual variability. Additionally, various factors such as renal[35] or hepatic functions,[36] tumour lysis syndrome[37] or samples being drawn during FP treatment[38] have been shown to interfere with endogenous concentrations of U. Importantly, we showed that the non-respect of pre-analytical practices increased the risk of misclassifying patients (from 17% to 34%).

Despite the multicentre nature of our study, the impact of interlaboratory variability is thought to be limited as each test sample and corresponding reference were analysed in the same laboratory, and the simulated model-based U profiles were not different if the 'laboratory' covariate was taken into account.

Overall, we showed that phenotype testing based on plasma U is conditioned by the strict respect of pre-analytical practices. However, the predictive value of U on severe FP-related toxicities was not evaluated in this study and could not be compared to targeted *DPYD* genotype or the measurement of DPD activity in PBMCs. A prospective multicentre study combining the different approaches – performed with standardized protocols and validated methods – is even more necessary to determine the best way to identify DPD-deficient patients and adapt FP dose accordingly.

5 | CONCLUSION

This original work provides clinicians and biologists with important pre-analytical basics to interpret U, UH_2 and UH_2/U when testing for DPD deficiency. Although the current recommended time between sampling and centrifugation is 1.5 hours at RT, we strongly encourage not exceeding 1 hour to ensure reliable U value interpretation. Cold storage of whole blood samples may help extend this time up to 5 hours and we encourage shipping samples at +4°C. Attention should be paid to samples centrifuged 1 hour after being drawn, especially if the U value is higher than 16 ng/mL. In that case, a control sample should be required to confirm DPD deficiency. Importantly, besides the pre-analytical process, U is prone to significant IOV that must be considered when interpreting the discrepancies between two samples from any given patient in routine practice.

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COMPETING INTERESTS

The authors have no conflicts of interest to declare.

CONTRIBUTORS

F.T., C.N. and M.-C.E.-G. were responsible for the study design. M.M., M.L., B.R., J.G., E.G.-V., S.B., C.T., J.C., D.R., H.A., V.H., N.T. and A.S. were involved in the data collection. F.L.L. did the simulation study. M.M. and F.T. analysed and interpreted the data. M.M. and F.T. wrote the first draft of the report. All the authors contributed to the final editing of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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