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Assay performance and stability of uracil and dihydrouracil in clinical practice

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Abstract

Purpose Measurement of endogenous uracil (U) is increasingly being used as a dose-individualization method in the treatment of cancer patients with fluoropyrimidines. However, instability at room temperature (RT) and improper sample handling may cause falsely increased U levels. Therefore we aimed to study the stability of U and dihydrouracil (DHU) to ensure proper handling conditions.

Methods Stability of U and DHU in whole blood, serum, and plasma at RT (up to 24 h) and long-term stability (≥ 7 days) at -20°C were studied in samples from 6 healthy individuals. U and DHU levels of patients were compared using standard serum tubes (SSTs) and rapid serum tubes (RSTs). The performance of our validated UPLC-MS/MS assay was assessed over a period of 7 months.

Results U and DHU levels significantly increased at RT in whole blood and serum after blood sampling with increases of 12.7 and 47.6% after 2 h, respectively. A significant difference ($p=0.0036$) in U and DHU levels in serum was found between SSTs and RSTs. U and DHU were stable at -20°C at least 2 months in serum and 3 weeks in plasma. Assay performance assessment fulfilled the acceptance criteria for system suitability, calibration standards, and quality controls.

Conclusion A maximum of 1 h at RT between sampling and processing is recommended to ensure reliable U and DHU results. Assay performance tests showed that our UPLC-MS/MS method was robust and reliable. Additionally, we provided a guideline for proper sample handling, processing and reliable quantification of U and DHU.

Keywords Uracil · Stability · Serum · Fluoropyrimidines · Dihydropyrimidine dehydrogenase (DPD) · Assay performance

Introduction

Fluoropyrimidines, including 5-fluorouracil (5-FU), and its oral prodrugs capecitabine, tegafur and S-1, play a vital part in the treatment of several solid tumors and are estimated to be used in two million patients annually [1–3]. Although fluoropyrimidines have been used for several decades and are reasonably well tolerated by patients, severe toxicity remains a substantial clinical problem that can result in early treatment discontinuation, hospital admission and even death [1–5]. Severe fluoropyrimidine-related toxicity is often caused by a deficiency of the main catabolic enzyme dihydropyrimidine dehydrogenase (DPD) resulting in high exposure to 5-FU due to less capacity to convert active 5-FU into inactive metabolites [2, 6]. DPD is encoded by the gene *DPYD* and single nucleotide polymorphisms in *DPYD* have been related to reduced DPD enzyme activity and increased risk of severe fluoropyrimidine-related toxicity [7, 8]. Pre-therapeutic screening of the *DPYD* gene and subsequent

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dose reductions in *DPYD* variant allele carriers have importantly reduced the risk of severe fluoropyrimidine-related toxicity [9, 10]. Despite this success, severe fluoropyrimidine-related toxicity still occurs in approximately 23% of patients who are non-carriers for the four *DPYD* variants currently being screened for [10]. Alongside *DPYD*-genotyping several other methods have been studied to establish the presence of DPD deficiency, mainly aimed toward the measurement of endogenous uracil (U) and dihydrouracil (DHU) levels [11–13]. Considering that U is converted by DPD into DHU, similar to 5-FU, it is hypothesized that the concentration of U or the DHU/U-ratio can be used as a surrogate for the DPD enzyme activity with high levels of U or low ratio's being indicative of DPD deficiency and predictive of severe fluoropyrimidine-induced toxicity [11–14]. In line with this thought, it has been shown that pretreatment U levels are associated with an increased risk of severe fluoropyrimidine-related toxicity [11, 15, 16]. However, U is an endogenous substance and large variability in measured U concentrations have been found between cohorts and hospitals, most likely as a result of pre-analytical factors [17]. Previous research has also shown that U levels are influenced by food intake, circadian rhythm, fluoropyrimidine therapy and renal impairment [14, 18–21]. In addition, stability experiments performed during the development of quantification methods for U and DHU have shown that both U and DHU are highly unstable at room temperature (RT) with substantial increases in concentration, indicating that both U and DHU are also being formed after blood sampling [22–25]. This phenomenon could be the result of enzymes involved in uracil metabolism which are still active in whole blood after sampling at RT. U is formed from uridine and deoxyuridine by uridine phosphorylase and thymidine phosphorylase, respectively [18, 22, 24]. Activity of these enzymes after blood sampling could lead to ex vivo formation of U resulting in an increase of the measured U concentration. This increase in concentration, indicates that specific and standardized sample handling and processing are required to ensure reliable results and subsequently accurate assessment of DPD deficiency [22–26]. However, the extent to which U and DHU levels are impacted at RT differ between studies ranging from +5.0 to +27.2% after 1 h and +22.0 to +52.2% after 2 h dependent on the matrix [24]. The main conclusion from these studies is that blood samples taken for the measurement of pre-treatment U levels should be processed quickly, although strict guidelines are scarce. Interestingly, DPD phenotyping by measurement of pretreatment U levels is nowadays mandatory for anyone treated with fluoropyrimidines in France and is also used in Belgium where both also have provided guidance for sample handling (Supplementary Table 1) [27, 28]. Furthermore, recently Maillard et al. recommended to reduce the time between sampling and centrifugation to 1 h as after 1.5 h the uracil concentration

significantly exceeded $\pm 15\%$ accepted bioanalytical variation (+23.4%) [29]. The aim of our study was to further establish a strict and extensive guideline for sample handling and processing to ensure reliable pretreatment U levels. Afterwards, this guideline was implemented in support of a large clinical trial (The Alpe2U-study, NCT04194957) in which dosing was based on pretreatment U levels.

Methods

Stability experiments were performed on blood samples taken from both healthy individuals and from cancer patients treated with fluoropyrimidines in the Netherlands Cancer Institute–Antoni van Leeuwenhoek in the Alpe2U-study. [22] Data have been collected from patients participated in the Alpe2U study from February 2020 to August 2022.

Stability of uracil and dihydrouracil in whole blood, serum, and plasma

Blood samples were collected from 6 healthy individuals to study the stability of U and DHU in different matrices and under different storage conditions (Table 1) to mimic situations that could occur in routine clinical care. Stability of U and DHU was studied in whole blood, serum and plasma. Stability in whole blood was assessed by collecting 5 blood samples of 3.5 mL using standard serum tubes (BD Vacutainer® SST™ Tubes) and storing these blood samples at RT for 0.5, 1, 2, 4, and 24 h to resemble the situation in which a sample was left at RT after sampling. Of note, whole blood coagulates within 0.5 h when using serum tubes, forming a blood clot resulting in a whole blood/serum matrix which will be referred to as whole blood. Stability in serum was assessed by collecting two blood samples per individual ($n=6$) using SSTs of 3.5 mL which were processed after 0.5 h at RT. One of these serum samples was kept at RT after which aliquots of 300 μ L serum were taken at 0.5, 1, 2, 4, and 24 h to resemble the situation in which a sample was centrifuged according to protocol but afterwards left at RT. The other serum sample was divided over 5 Eppendorf tubes of 2.0 mL from which one sample was analyzed directly after processing and the other four after 7 days, 3 weeks, 2 months and 21 months stored at $-20\text{ }^{\circ}\text{C}$ to assess long-term stability. Stability in plasma was assessed by taking one blood sample of 10 mL of each individual using a lithium heparin tube (BD Vacutainer® Heparin Tubes) which was directly centrifuged after sampling. The obtained plasma was divided over 5 Eppendorf tubes of 2.0 mL of which one was directly analyzed after processing. The four other samples were analyzed after storage of 7 days, 1 month, 2 months, and 6 months at $-20\text{ }^{\circ}\text{C}$

Table 1 Overview of samples taken per healthy individuals and storage conditions for stability testing of U and DHU

	Matrix	Type of sampling tube	N (per individual)	Amount of blood sampled per tube	Storage condition	Time points
Short-term stability (max. 24 h)	Whole blood	BD vacutainer® SST™	5	3.5 mL	RT	0.5, 1, 2, 4, 24 h
	Serum	tubes	1	8.5 mL		
Long-term stability (≥ 7 days)	Serum	BD vacutainer® SST™	1	8.5 mL	– 20 °C	0.5 h, 7 days, 3 weeks, 2 months, 6 months, 21 months
	Plasma	BD vacutainer® heparin tubes	1	10 mL		
Standard serum tubes vs. rapid serum tubes	Serum	BD vacutainer® SST™	1	3.5 mL	RT	Min 0.5 h–max 60 min
	Serum	BD vacutainer® Rapid Serum Tube	1	8.5 mL		

DHU dihydrouracil, *h* hours, *RT* room temperature, *SST* serum separator tube, *U* uracil

to assess long-term stability. All blood samples were centrifuged at 4 °C at 3300 rpm (1960 g) after which serum or plasma was obtained for further analyses.

Standard serum tubes vs. rapid serum tubes

The stability of U and DHU in patients treated with fluoropyrimidines was assessed by comparing concentrations in standard serum tubes (BD Vacutainer® SST™ Tubes) and rapid serum tubes (BD Vacutainer® Rapid Serum Tubes) (Table 1). Two blood samples per patient were collected from 31 patients treated with fluoropyrimidines before the start of treatment of which the standard serum tube (3.5 mL) which was processed after at least 0.5 h and a maximum of 1 h at RT and one rapid serum tube (8.5 mL) which was processed after at least 5 min and a maximum of 15 min at RT. Samples were centrifuged at 3300 rpm (1960 g) at 4 °C after which serum was obtained and directly frozen at – 20 °C. Aliquots of 300 µl serum were taken to measure U and DHU concentrations.

Sample analysis

Aliquots of 300 µl serum and plasma were taken to measure U and DHU concentrations. Analytes were extracted using protein precipitation by adding 900 µl methanol:acetonitrile (50:50, v/v). After a 10 s vortex spin, samples were shaken for 10 min in an automatic shaker. Subsequently, samples were centrifuged at 14,000 rpm (18,626 g) at room temperature. Clear supernatants were collected and evaporated under a stream of nitrogen gas at 40 °C for 45 min. Afterwards, dry extracts were obtained and reconstituted with 100 µl of 0.1% formic acid in water, vortex mixed, and centrifuged at 14,000 rpm for 10 min at 4 °C. All samples were measured in duplicate using a validated rapid and sensitive

ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay. A volume of 5 µl was injected into the UPLC-MS/MS system. Analytes were chromatographically separated using an Acquity UPLC system (T3 column with gradient elution) (Waters, Milford, MA, USA) and analyzed with QTrap 5500 triple quadrupole spectrometer (Sciex, Framingham, MA, USA) equipped with an electrospray ionization source as described in detail by Jacobs et al.[22]

In addition to the stability experiments, the analytical performance of the used quantification method for U and DHU during was assessed over a period of 7 months (March 2022 to August 2022). This was done by performing and evaluating a system suitability test (SST) before starting an analytical run or a check run. SST solution was prepared by adding 10 µl of DHU working solution (100,000 ng/mL), 10 µl U working solution (10,000 ng/mL) and 100 µl internal standard working solution DHU/U (10,000 ng/mL stable isotope labeled (SIL) DHU-¹³C₄¹⁵N₂ and 1,000 ng/mL SIL U-¹³C₄¹⁵N₂) to 10 mL water in a polypropylene (PP) tube of 15 mL resulting in final concentrations of 100 ng/mL DHU, 10 ng/mL U, 100 ng/mL SIL DHU, 10 ng/mL SIL U. To ensure adequate system suitability the covariance of variation (CV) should be less than or equal to 10.0% of the area ratio analyte/IS and the signal to noise ratio (S/N) of all analytes should be greater than 10. Furthermore, calibration standards, spiked QC samples in dialyzed plasma and a QC sample in the biomatrix were measured in every run to ensure adequate assay performance. Calibration standards consist of five non-zero standards, a standard spiked with only IS and a blank sample and were prepared in formic acid 0.1% in water and were validated over a range of 4–20 ng/mL. QC samples were prepared in dialyzed human plasma to remove the endogenously present U and DHU. The dialysis of the control human plasma is described in Jacobs et al.

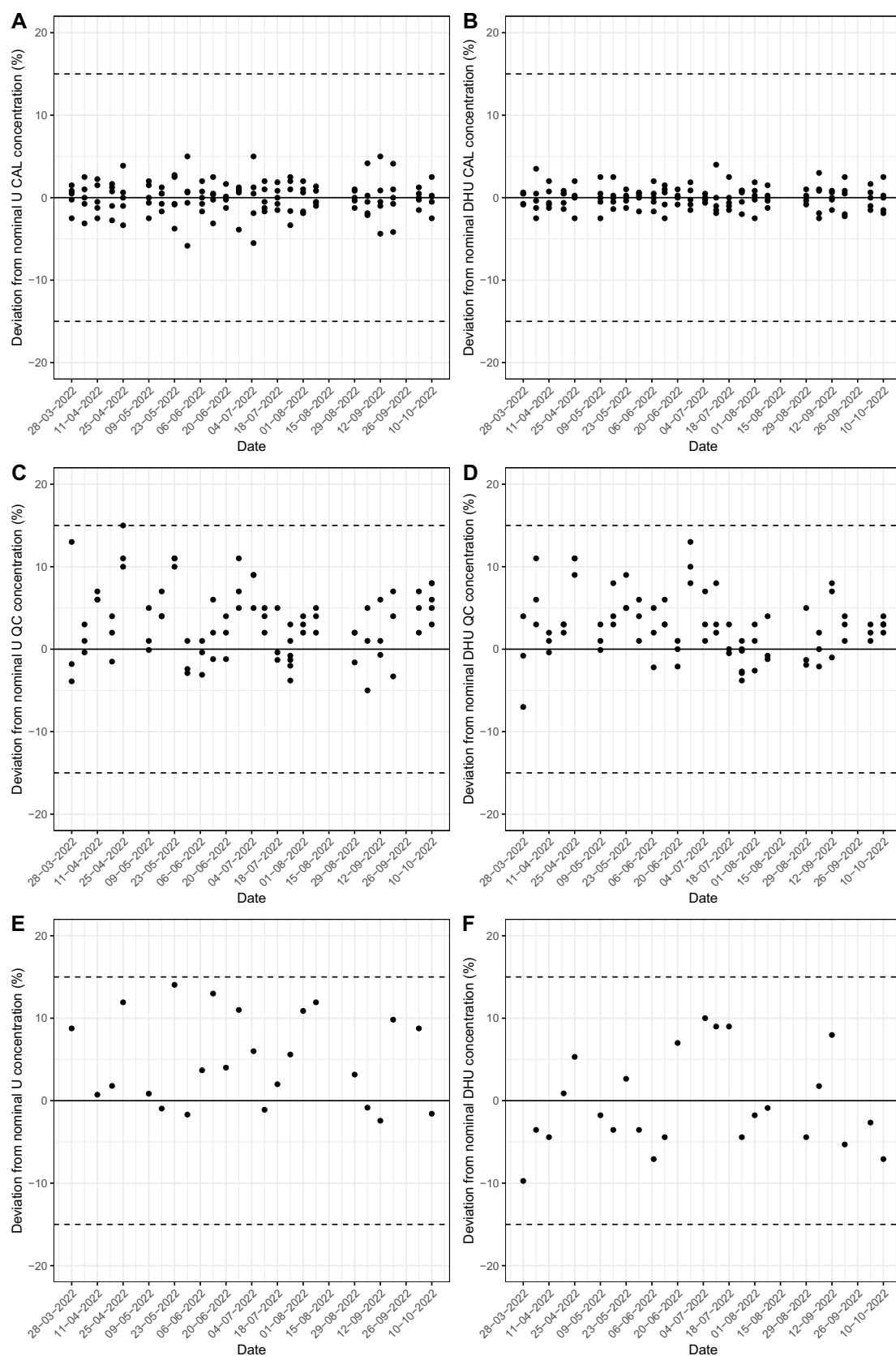


Fig. 1 Overview of the deviation from the nominal U and DHU concentration of the calibration standards (A and B), spiked QC mid samples in dialyzed plasma (C and D), and the QC sample in biomatrix (E and F). *CAL* calibration standards, *DHU* dihydrouracil, *U* uracil, *QC* quality control

[22]. Thereupon, QC samples were spiked with concentrations of U and DHU at 10 and 100 ng/mL and measured in triplo, respectively. The QC sample in biomatrix was prepared by obtaining a blood sample from 2 healthy individuals using standard serum tubes (BD Vacutainer® SST™ Tubes). These samples were centrifuged after 0.5 h at RT at 3300 rpm (1960 g) at 4 °C for 10 min to obtain serum which was pooled. Aliquots of 300 µL were taken and measured 5 times to determine the measured concentration. The remaining serum was filled in aliquots of 300 µL and frozen at – 20 °C. During every run, an aliquot of the reference standard was also measured to compare to the measured concentration. To meet the acceptance criteria 4 of 5 calibration samples, 2 of 3 spiked QC mid samples in dialyzed plasma, and the QC sample in biomatrix should be within $\pm 15\%$ of the measured.

Statistical analysis

Descriptive statistics including median, mean, and standard deviation (SD) were used to describe the change in concentration of U and DHU under the tested condition. To assess the stability of the analytes the ratio of measured concentrations at the stated time points and the reference concentration ($T=0$ for plasma or $T=0.5$ h for serum and whole blood) were calculated and multiplied by 100 to obtain a percentage. An interval of $\pm 15\%$ was considered an acceptable variation in concentration from the reference concentration. After testing for normality of distribution according to the Shapiro–Wilk normality groups were statistically compared using either the Wilcoxon signed-rank test of medians (non-parametric) or student's *t* test (parametric). When samples of the same patient were compared tests were paired. For analyses, *p* values < 0.05 were considered statistically significant. All statistical analyses were performed using R v3.6.3 [30].

Results

Assay performance

The CVs of the system suitability tests ranged from 0.7 to 8.9% for U and 1.6–5.5% for DHU and were within the acceptance criteria of 10.0% and signal-to-noise ratio was > 10 for all runs. The deviations of the calibration standards, the spiked mid-QC samples in dialyzed plasma and the QC sample in biomatrix are shown in Fig. 1. The deviations

of the calibration standards ranged from – 5.83 to 5.0% and – 2.5 to 4.0% for U and DHU, respectively. The deviation of the spiked mid QCs in dialyzed plasma ranged from – 5.0 to 15.0% and – 7.0 to 13.0% for U and DHU, respectively. Both Calibration standards and spiked mid QCs of both U and DHU fulfilled the acceptance criteria with no deviations larger than 15% at all concentration levels during all runs. The U and DHU concentrations of the QC sample in biomatrix deviated more than $\pm 15.0\%$ once during this time period for both analytes (on separate occasions).

Stability of uracil and dihydrouracil in whole blood, serum and plasma

The measured concentrations of U and DHU are shown in supplementary Table 2. The relative concentrations in % compared to $T=0.5$ h are depicted in Fig. 2 and Table 2. The mean U concentration increased rapidly at RT when stored as whole blood and even more pronounced when stored as serum, with increases of 12.7 and 47.6% after 2 h, respectively. However, one individual (S1) in the serum stability test had a substantially lower U (2.47 and 56.05 ng/mL) and DHU concentration at $T=0.5$ h compared to the following time points (Table 2 and supplementary Table 3). DHU concentration also increased over time, however, less substantial when compared to U, with increases of 50 and 30% after 24 h stored as whole blood and serum, respectively. Interestingly, U and DHU concentration stored in both whole blood and in serum at $T=0.5$ h were markedly lower compared to the concentration in plasma at $T=0$ (supplementary table 2). DHU/U-ratio slowly decreased during the first 4 h and significantly decreased after 24 h 4 h (Supplementary Table 3).

Long term stability

Long-term stability was assessed by storing plasma and serum at – 20 °C for prolonged periods of time (≥ 7 days, supplementary Table 4). Both U and DHU concentration increased over time, however, less pronounced when kept at RT. In addition, mean deviations were within the accepted deviation of $\pm 15\%$ from baseline after 2 months except for plasma with a deviation of + 20.4% (Supplementary table 4).

Standard serum tube vs. rapid serum tube

Samples taken with standard serum tube and rapid serum tube were available from 31 patients who were treated with fluoropyrimidines. U concentration was significantly lower in the rapid serum tube samples compared to the standard serum tube samples with mean U levels of 10.14 and 10.51 ng/mL ($p=0.0036$, Fig. 3), respectively. Mean DHU levels in SSTs and RSTs were 111.7 and 108.5 ng/mL ($p=0.012$, Fig. 3), respectively.

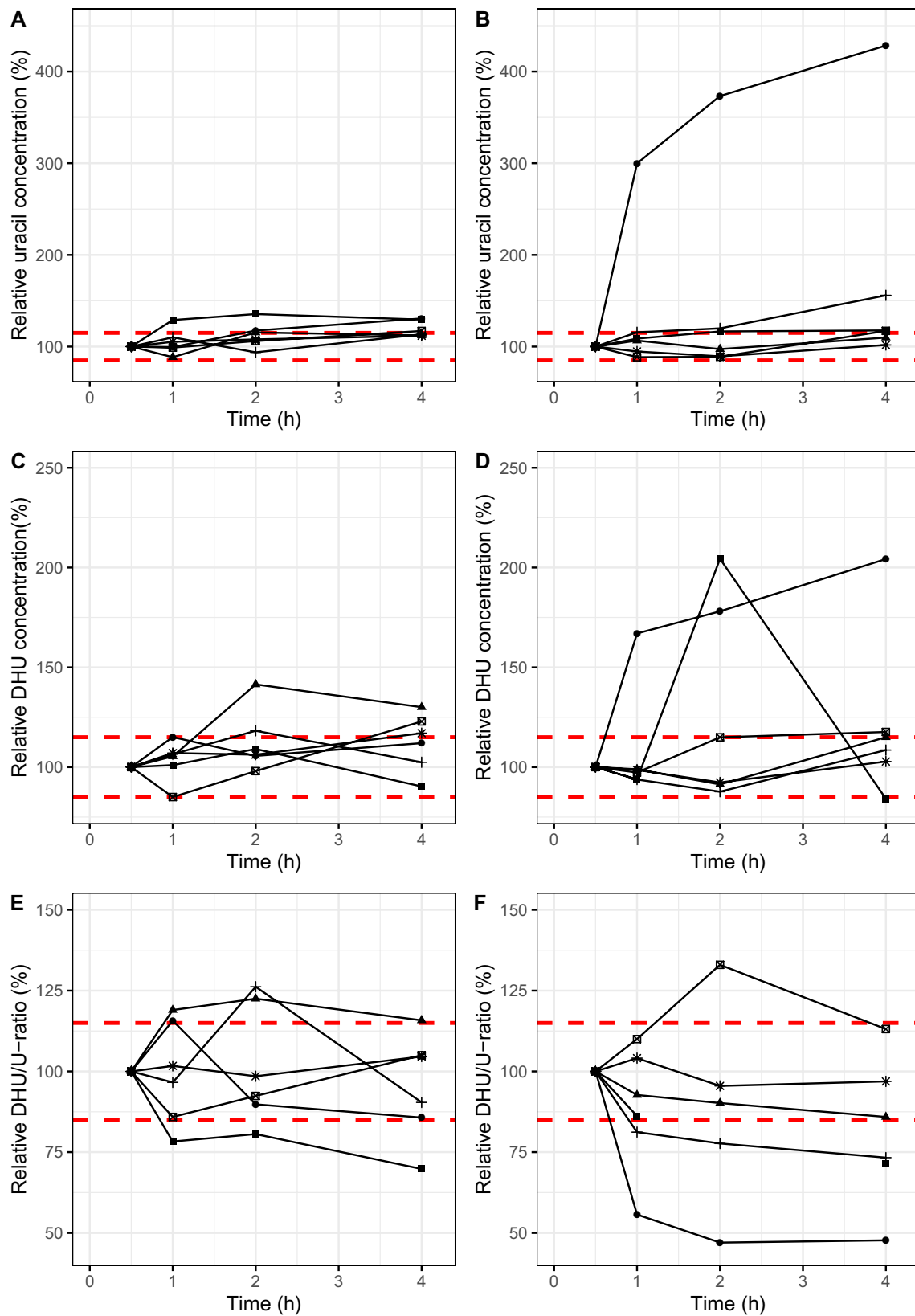


Fig. 2 Concentrations of uracil (U) and dihydrouracil (DHU) and the DHU/U-ratio in % compared to $T=0.5$ h in both whole blood and serum between $T=0.5$ h to $T=4$ h. Red dotted line shows accepted $\pm 15\%$ variation. **A** Relative uracil concentration (%) in

whole blood, **B** Relative uracil concentration in serum, **C** Relative DHU-concentration in whole blood, **D** Relative DHU concentration in serum, **E** Relative DHU/U-ratio in whole blood, **F** Relative DHU/U-ratio in serum. DHU dihydrouracil, h hours, U uracil

Table 2 Concentration of uracil (U) and dihydrouracil (DHU) over time in % to $T=0.5$ h in whole blood and serum at room temperature

Analyte	Matrix	Time point	Concentration (% of $T=0.5$ h)						Mean of concentration (% of $T=0.5$ h)	Mean deviation (SD)	Relative standard deviation (RSD)
			S1	S2	S3	S4	S5	S6			
U	Whole blood	0.5 h	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1 h	99.47	88.48	129.04	110.16	98.93	105.26	105.22	13.75	13.06
		2 h	117.53	115.45	135.48	93.73	106.07	107.89	112.69	13.98	12.40
		4 h	130.73	112.26	129.49	113.44	117.08	111.84	119.14	8.70	7.31
		24 h	391.33	308.32	380.70	289.02	357.68	229.82	326.15	61.96	19.00
	Serum	0.5 h	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1 h	299.80	106.59	108.75	115.63	88.49	94.69	135.66	81.01	59.72
		2 h	373.28	97.28	116.56	119.90	88.90	89.38	147.55	111.38	75.49
		4 h	428.34	109.85	117.67	155.94	117.19	101.77	171.79	127.06	73.96
		24 h	971.66	341.71	320.19	295.47	270.52	177.43	396.17	287.65	72.61
DHU	Whole blood	0.5 h	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1 h	115.07	105.39	101.01	106.46	85.02	107.05	103.33	10.06	9.74
		2 h	105.60	141.46	109.08	118.22	98.00	106.22	113.10	15.35	13.57
		4 h	112.02	130.03	90.31	102.42	122.89	117.01	112.45	14.35	12.76
		24 h	175.15	154.52	151.26	141.29	148.73	132.37	150.55	14.43	9.59
	Serum	0.5 h	100.00	100.00	100.00	100.00	100.00	100.00	100.00	0.00	0.00
		1 h	166.99	98.89	93.83	93.80	97.29	98.60	108.23	28.87	26.68
		2 h	178.06	91.32	204.40	87.71	114.95	92.31	128.12	50.51	39.42
		4 h	204.28	114.99	84.16	108.55	117.62	102.80	122.07	42.00	34.41
		24 h	216.77	107.47	142.94	95.34	119.74	98.95	130.20	45.77	35.15

DHU dihydrouracil, S subject, SD standard deviation, U uracil

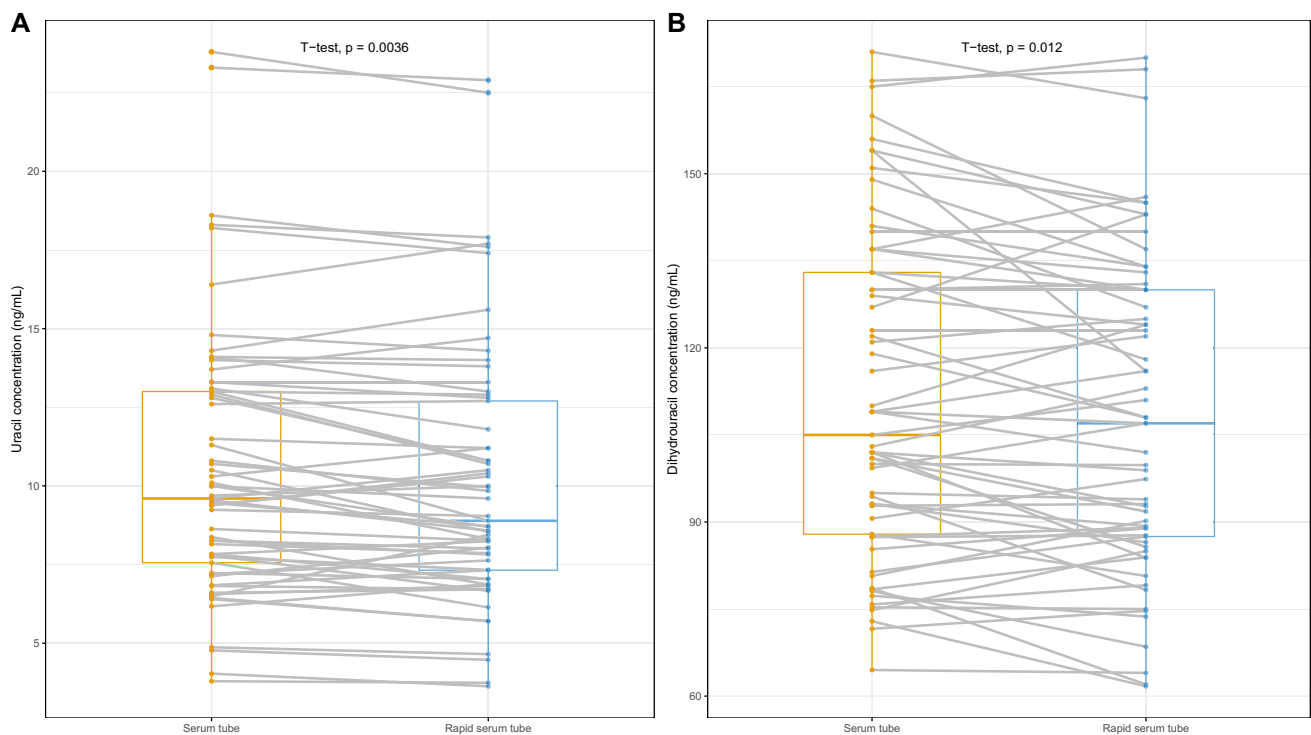


Fig. 3 Comparison of uracil (A) and dihydrouracil (B) concentrations measured using standard serum tubes and rapid serum tubes in the same patient. Data have plotted the median as the middle line and the

box extending from the 25th to 75th percentiles. Grey lines indicate the paired samples

Discussion

Since April 2020, the EMA has included phenotype testing based on U concentrations as a suitable method to identify patients with DPD deficiency before treatment with fluoropyrimidines [31]. However, previous studies have shown that U is unstable in biological matrices after blood sampling and strict guidelines for sample handling are scarce. We, therefore, conducted this study to further assess the stability of U and DHU and to provide a manageable guideline for sample handling, processing and quantification to ensure reliable results.

Our results showed that U and DHU (although less pronounced) are highly unstable at RT regardless of the biological matrix, indicating the importance of proper sample handling to generate reliable concentrations and to support optimal dosing of fluoropyrimidines. One individual (S1) had substantial lower U and DHU concentrations when measured in serum (2.47 and 56.05 ng/mL) compared to whole blood (7.5 and 98.2 ng/mL) at $T=0.5$ h, resulting in a substantial increase in concentration after 1 h significantly affecting the relative U concentration (% to $T=0.5$ h) in serum and could be considered an outlier. Therefore, the U and DHU concentration were also assessed without S1 (supplementary table 5) which significantly reduced the mean relative change of U concentration to +2.4% after two hours at RT. Nonetheless, a maximum of 1 h between blood sampling and processing is recommended to minimize the *ex vivo* formation of U and DHU. Interestingly, the mean U and DHU concentrations were lower when measured in serum after 0.5 h of storage at RT compared to plasma which was directly processed after blood sampling. However, this difference was still within the accepted interval of $\pm 15\%$ and could also be attributed to bioanalytical variation. In addition, U and DHU concentrations remained stable over prolonged periods when stored at $-20\text{ }^{\circ}\text{C}$ of time indicating that samples can easily be transported to other laboratories when frozen at $-20\text{ }^{\circ}\text{C}$. Of note, DHU/U-ratio seemed to be more stable over time with deviations of only 1.7 and 3.2% after 2 h in whole blood and serum compared to both U and DHU, respectively. Notably, recently it was shown that U levels were less stable when centrifuged at $4\text{ }^{\circ}\text{C}$ compared to RT. However, no clear explanation was given and further research is needed [32]. Comparison of SSTs with RSTs has shown that the U concentration was significantly lower in RSTs. This was expected as these tubes can be processed almost immediately after sampling, preventing *ex vivo* conversion of uridine and deoxy-uridine to U. However, the absolute difference in concentration was small (~ 0.36 ng/mL) which suggests that the advantage of

using RSTs compared to SSTs is limited. Especially, considering that immediate sample processing is not always possible in routine clinical care. Notwithstanding, when pretreatment U levels are utilized as a dose-individualization method this small absolute difference could result in the misclassification of patients as being DPD deficient when their U level is close to the threshold of 16 ng/mL described in literature [11, 17].

In addition to the performed stability experiments we also assessed the performance of our UPLC-MS/MS assay over a period of 7 months in support of a large clinical trial. In this study blood samples for quantification of U and DHU were taken using serum tubes and were processed within 1 h after sampling and were frozen immediately after processing as was concluded from our stability experiments. Samples from other hospitals were sent on dry ice and were measured within 1 week of sampling. Assay performance assessment fulfilled the acceptance criteria and showed that the quantification method was robust and reliable. Therefore, we have no reason to believe that possible differences in U and DHU were caused by bioanalytical errors.

A potential way to overcome the instability of U and DHU at RT could be the use of inhibitors of enzymes involved in the metabolism of U. The increase of uracil could potentially be halted by adding inhibitors of uridine phosphorylase and thymidine phosphorylase to prevent the conversion of uridine and deoxy-uridine to U and an inhibitor of DPD to prevent the conversion from U to DHU, resulting in stable uracil levels. However, this warrants further research.

Conclusion

We can conclude from these experiments that U concentrations increase rapidly over time when kept at RT showing the difficulty of clinical implementation of U DPD deficiency testing. Preferred is to process these samples directly after blood sampling to minimize the increase in U, and to a lesser extent DHU, concentration. A maximum of 1 h at RT between sampling and processing is recommended. Based on our experiments and previous research we provided a guide (Table 3) in which critical pre-analytical factors have been taken into account to ensure proper sample handling, processing, and reliable quantification and could be immediately used in clinical practice. This is, to our knowledge, the first extensive guideline for sample handling, processing and quantification of U and DHU samples and could potentially reduce the number of patients being wrongly classified as DPD deficient and subsequently reduce sub-optimal treatment.

Table 3 Guideline for handling and processing of blood samples for the quantification of uracil and dihydrouracil

Steps	Recommendation	Specification
Step 1 Sampling tube/matrix	BD vacutainer® rapid serum tube BD vacutainer® SST™ tubes BD vacutainer® heparin tubes	Rapid serum tubes are recommended if quick processing (within 15 min of blood sampling) of the sample is possible Otherwise standard serum tubes will suffice Lithium heparin tubes can be used however, higher concentrations were found at $T=0$ compared to standard serum tubes at $T=0.5$ h
Step 2 Blood sampling	Between 08:00 and 10:00 am In fasted state (>8 h since last food-intake)	To minimize influence of circadian rhythm To minimize influence of food-effect
Step 3 Sample handling & processing	Maximum of 1 h at ambient temperature in whole blood between blood sampling and centrifugation Centrifuge at 3300 rpm (1960 g) for 10 min at 4 °C Transfer at least 1.0 mL of the supernatant (serum or plasma) to a 2.0 mL eppendorf tube	To minimize ex vivo forming of uracil and dihydrouracil Preferably centrifuge at 4 °C to minimize ex vivo formation of uracil and dihydrouracil
Step 4 Storage conditions	Freeze immediately at a maximum of -20°C Maximum storage time at -20°C Serum: 2 months Plasma: 3 weeks	To minimize ex vivo formation of uracil and dihydrouracil
Step 5 Thawing	Thaw samples on ice-water (maximum of 2 h) and vortex-mix each sample for approximately 10 s	To minimize ex vivo formation of uracil and dihydrouracil
Step 6 Sample processing	Centrifuge at 3000 rpm (855 g) for 3 min at 4 °C	Preferably centrifuge at 4 °C to minimize ex vivo formation of uracil and dihydrouracil
Step 7 Sample processing	Transfer a aliquot of 300 μL into 2.0 mL reaction tubes and add 20 μL of internal standard working solution [22]	
Step 8 Sample pretreatment & quantification	Extract analytes using protein precipitation by adding methanol:acetonitrile (50:50) Remaining sample preparation and quantification according to method published by Jacobs et al. using a rapid and sensitive UPLC-MS/MS assay [22]	

Rpm rounds per minute, *Sec* seconds, *SST* serum separator tube, *UPLC-MS/MS* ultra-performance liquid chromatography-tandem mass spectrometry

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00280-023-04518-7>.

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Data availability All data generated or analysed during this study are included in this published article [and its supplementary information files].

Declarations

Conflict of interest The authors declare no conflict of interest in the relationship with this work. Jos Beijnen is a (part-time) employee, stock- and patent holder of Modra Pharmaceuticals, a spin-out company developing oral taxane formulations; not related to the manuscript.

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