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Quantification of uracil, dihydrouracil, thymine and dihydrothymine for reliable dihydropyrimidine dehydrogenase (DPD) phenotyping critically depend on blood and plasma storage conditions

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ABSTRACT

Establishing dihydropyrimidine dehydrogenase (DPD) activity is highly important in determining the correct starting dose of fluoropyrimidines such as 5-fluorouracil and capecitabine. The concentration ratio of endogenous uracil with its metabolite dihydrouracil (DHU) is a well-known parameter that is linked to DPD activity. Concentration ratios such as thymine over its DPD-converted metabolite dihydrothymine (DHT) is less described and may serve as an alternative diagnostic biomarker for DPD activity.

In this study, we describe the development and validation of an ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) assay for the quantification of uracil, DHU, thymine, and DHT in human plasma. In addition, stability experiments were performed.

Uracil and thymine were quantified up to 80.0 ng/mL and DHU and DHT up to 800 ng/mL. Intra- and inter-assay precision were maximum 8.0 % and 7.6 %. respectively. Also, recovery was adequate and significant matrix-effects and carry-over were excluded. Stability experiments showed that uracil concentrations increased with 27–52 % when stored for 1 or 2 h at ambient temperatures compared to cold storage. Thymine, DHU, and DHT concentrations remained stable, thymine after 1 h in plasma excluded, showing the DHT:T ratio might be a more robust marker for DPD activity than DHU:U.

In conclusion, we present here a novel assay capable of quantifying uracil, thymine, DHU and DHT in a single analytical run. We provide additional data showing increased stability for DHU, thymine and DHT compared to uracil. This assay may be used as a diagnostic test in future studies, establishing the association of these endogenous biomarker concentrations with DPD activity and safety to treatment with fluoropyrimidines. In addition, future research should also be focused on reducing pre-analytical instability. Standardization in this field is essential to set proper reference values and to allow inter-study comparison on clinical outcomes.

1. Introduction

Fluoropyrimidines are widely used anticancer drugs for the treatment of different types of cancer such as colon, breast, head, neck, and stomach cancer [1,2]. The group of fluoropyrimidines includes 5-fluorouracil (5-FU) and its prodrugs capecitabine and tegafur. The cytotoxic effect of 5-FU is explained by the inhibition of both RNA and DNA synthesis. The key enzyme responsible for the deactivation of more than

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80 % of the administered amount of 5-FU is dihydropyrimidine dehydrogenase (DPD). Next to the metabolism of 5-FU, the DPD enzyme is responsible for the endogenous metabolism of the nucleotide bases uracil (U) and thymine (T) [3–5] (Fig. 1). The conversion of 5-FU to fluoro-dihydrouracil (FDHU) by DPD is the major metabolic pathway of the drug [4].

Polymorphism in the *DPYD* gene, the gene encoding for the DPD enzyme, can result in decreased DPD-enzyme activity. Various genetic polymorphisms have been identified and are associated to elevated drug concentrations when using standard dosages [6–8]. It has been shown that 15–30 % of the patients are overdosed, resulting in severe toxicity (grade 3 and 4) [3,5,9,10]. In 0.5–1 % of cases, this toxicity can even lead to treatment-related death, illustrating the high clinical need for personalized therapy regimens to prevent severe toxicity [3,9].

Dose-individualization based on general patient characteristics, such as sex, age, Body Surface Area (BSA), and renal function, are of limited use to improve clinical outcome [11,12]. The most commonly applied method to individualize dosing is based on genetic polymorphism within *DPYD* [6,13,14]. DPYD genotyping is easily implemented in routine care and has been shown to reduce the incidence of toxicity [15–17]. Although DPYD-genotype-guided dosing is nowadays considered standard of care in most European countries, and clearly shown to prevent (lethal) 5-FU-induced toxicity, pretherapeutic *DPYD* genotyping may not identify all DPD-deficient patients. Namely, its sensitivity may be limited since not all mutations resulting in DPD deficiency are known or are screened for, resulting in only a partial reduction of toxicity cases [18]. To further improve pretherapeutic identification of DPD-deficient patients, it is important to introduce additional diagnostic techniques, such as the assessment of DPD phenotype. Using phenotyping techniques, it is expected that more patients with reduced DPD enzyme activity may be identified, compared to using genotyping only. One method is the direct measurement of the 5-FU concentration in plasma after a 5-FU test dose. However, this method still lacks prospective validation and has the disadvantage that it requires additional time of the patient [19]. In addition, DPD activity may also be determined in peripheral blood monocular cells (PBMCs) and is currently considered the golden standard for phenotypic determination of DPD enzyme activity. However, this method is not suitable for implementation in routine clinical care, since this assay is complex, time-consuming, laborious, and costly to perform [20–22]. Moreover, no studies have been conducted on the safe and effective dosing of 5-FU based on DPD enzyme activity measurements.

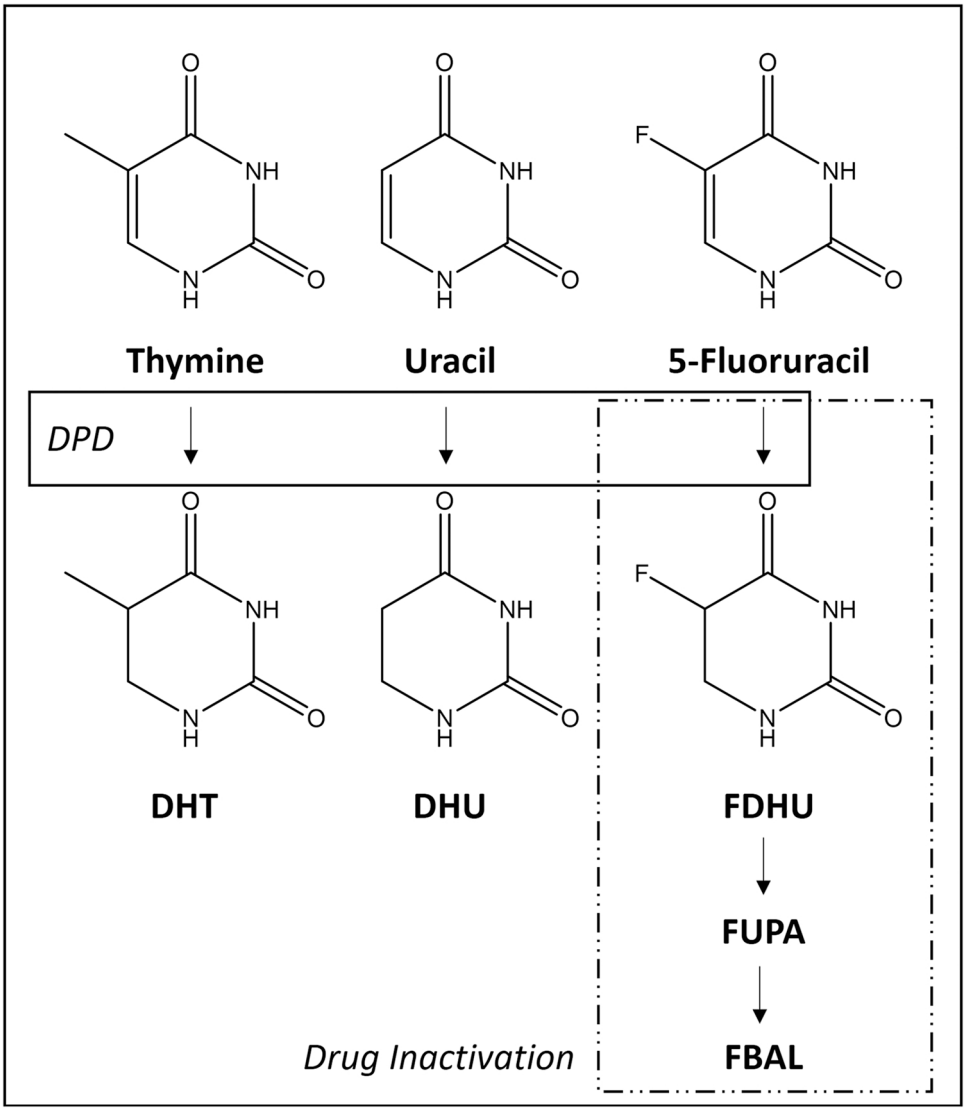

Fig. 1. Dihydropyrimidine dehydrogenase (DPD) is responsible for the metabolism of uracil, thymine, and 5-fluorouracil into dihydrouracil (DHU), dihydrothymine (DHT), and fluoro-dihydrouracil (FDHU), respectively. FDHU is further metabolized into fluoro-β-ureidopropionate (FUPA) and subsequently into α-fluoro-β-alanine (FBAL), which is cleared by the kidneys.

An alternative way to determine DPD activity is the quantification of the plasma concentrations of endogenous uracil and thymine and their respective metabolites dihydrouracil (DHU) and dihydrothymine (DHT), and to establish the DHU:U and DHT:T ratios (Fig. 1). Multiple studies have already investigated the predictive value of uracil and DHU concentrations as marker for DPD deficiency, however, contradictory results exist within clinical trials with no or minimal correlation. [23–26]. One of the major drawbacks in the analysis of endogenous uracil and DHU concentrations is the instability of these analytes in non-frozen plasma conditions. Especially uracil concentrations tend to increase a lot during short-time storage, resulting is significant variations in DHU:U ratios. The short-term stability of uracil and DHU was determined by various other studies, with uracil stability outcomes that varied highly [3,23,27–33]. The stability of thymine and DHT was not investigated previously. Using the method described in this paper additional research on stability and influence on both DHU:U and DHT:T ratios can be performed. In general, uracil concentrations increase rapidly in plasma after venipuncture until the sample is centrifuged, frozen or processed chemically. A possible explanation is the conversion of the uracil precursor uridine to uracil by ex vivo metabolism [34,35]. The stability of DHU was less affected by short-term storage, although DPD present in plasma can convert uracil in DHU [36]. The outcome of clinical trials studying these markers varies highly, probably due to the variation in pre-analytical conditions [37]. Although various studies reported variation in uracil concentration after short-term storage, standardized protocols are not available and cross validation between the methods and laboratories have not been performed. This greatly hinders the definition and use of validated cut-off and/or reference values.

Unlike the determination of uracil and DHU levels and the derived DHU:U ratio, which have been described in previous work, the determination of thymine and DHT levels and the derived DHT:T ratio is a relatively unexplored parameter. Although based on theory, these levels and ratios potentially have higher diagnostic accuracy than uracil concentration alone and can serve as a solution to uracil instability. Thus, the added clinical value of the addition of thymine and DHT quantification in plasma needs to be established. To our knowledge, the short-term stability of thymine and DHT in plasma and whole blood were not determined previously. The short-term stability of thymine and DHT is expected to be comparable to uracil and DHU since these compounds follow similar metabolic pathways [38].

The aim of this study was to develop and validate an LC-MS/MS method for the simultaneous determination of uracil, DHU, thymine, and DHT in a single analytical run. This method was combined with tightly controlled pre-analytical conditions to investigate the effects on compound levels and DHU:U ratios and to establish DHT:T ratios.

2. Materials and methods

2.1. Chemicals and reagents

Uracil (> 99.0 %) and thymine (97 %) were purchased from Sigma-Aldrich (Saint-Louis, MO, USA). Dihydrouracil and dihydrothymine (> 98.0 %) were purchased from Alfa Aesar (Ward Hill, MA, USA). [^{13}C,$^{15}N_2$]-uracil (98.0 %), [^{13}C,$^{15}N_2$]-dihydrouracil (98.0 %) and [^{13}C,$^{15}N_2$]-thymine (98.0 %) were purchased from Alsachim (Illkirch Graffenstaden, France) and 5,6-dihydrothymine [5,6,6-D_3, methyl-D_3] (98.0 %) was purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). UPLC-grade acetonitrile (ACN), formic acid (FA) and ULC/MS grade water were purchased from Biosolve (Valkenswaard, the Netherlands).

2.2. Blank plasma

Blank plasma was prepared by transferring 15 mL pooled human EDTA plasma into a 3.5 K MWCO 30 mL Slide-A-Lyser™ Dialysis Cassette (Thermo Fisher Scientific, Waltham, MA, USA). The dialysis cassette was then placed in 1 liter PBS (pH: 7.4) at ambient temperature under continuous stirring. PBS was refreshed after 0.5, 1, 2, and 4 h, followed by an overnight dialysis step.

2.3. Quality Control samples calibrators and stock solutions

Quality Control (QC) samples were prepared in blank plasma at 6.00, 12.0 and 50.0 ng/mL for uracil and thymine, and at 60.0, 120, and 500 ng/mL for DHU and DHT, respectively. Calibration samples were prepared at 2.50, 5.00, 10.0, 20.0, 40.0 and 80.0 ng/mL for uracil and thymine and 25.0, 50.0, 100, 200, 400 and 800 ng/mL for DHU and DHT in blank plasma, respectively. Internal standard stock solution was prepared by dissolving 2 000 ng/mL of isotopically labelled uracil and thymine and 20,000 ng/mL of isotopically labelled DHU and DHT into ULC/MS grade water with 0.1 % (v/v) formic acid. Calibrators, QC samples and internal standards were stored in 200 μL aliquots at − 80 °C until analysis.

2.4. Sample preparation

Blood samples were collected by venipuncture using 4 mL K2-EDTA vacutainer vials by Becton Dickinson (BD) (Franklin Lakes, NJ, USA). After immediate centrifugation (4 °C, 2 500 g, 10 min), plasma samples were stored at − 80 °C until analysis. Samples were prepared by adding 20 μL internal standard stock solution to 200 μL of the collected EDTA plasma followed by the addition of 600 μL acetonitrile. Samples were mixed and centrifuged for 10 min at 20,000*g*. The supernatant was transferred to a new tube where the solvents were evaporated for circa 45 min at 40 °C with a gentle stream of air. The dried sample was then dissolved in 100 μL MilliQ + 0.1 % (v/v) FA and mixed. Lastly, the redissolved sample was centrifuged for 15 min at 20,000*g* at 4 °C and the supernatant was transferred to a 96-wells plate for analysis. When samples exceeded concentrations of the used calibration curves, re-analysis using dilution was performed. Only anonymized leftover plasma was used for the study. None of the patients actively disproved the use of their leftover material for validation purposes, according to the Dutch legislation.

2.5. UPLC-MS/MS conditions

UPLC-MS/MS was performed on a Waters (Milford, MA, USA) Acquity UPLC system coupled to a Waters Acquity Xevo TQ-S triple quadrupole mass spectrometer. The devices were controlled by MassLynx Software (version 4.1, Waters). Multiple Reaction Monitoring (MRM) in the positive electrospray ionization (ESI+) mode was used as MS acquisition mode. A 3 μm, 100 × 2.1 mm Hypercarb™ porous graphitic carbon UPLC column (Thermo Fishers Scientific, Waltham, MA, USA) was placed inside a column oven at 40 °C. Flowrate was set at 0.3 mL/min, and a gradient of water containing 0.1 % (v/v) formic acid (A) and acetonitrile containing 0.1 % (v/v) formic acid (B) was set as follows (all displayed as % v/v): 0.0–2.0 min (5 % B), 2.0–7.0 min (5–50 % B), 7.0–8.0 min (50 % B), 8.0–8.5 min (50–90 % B), 8.5–13.5 min (90 % B), 13.0–14.0 min (90–5 % B), 14.0–15.0 min (5 % B). After every series the column was flushed using methanol and acetonitrile (90:10) + 0.1 % (v/v) formic acid for 10 min. Mass Spectrometry settings were set as follows: capillary voltage: 0.5 kV, cone voltage: 32 V, desolvation temperature: 200 °C, source temperature: 150 °C, desolvation gas: 800 L/h, nebulizer gas: 7.0 bar, collision gas: 0.15 mL/min, dwell time: 0.263 s

2.6. Method validation

The method validation procedures, including acceptance criteria, were adapted from the ICH guideline M10 on bioanalytical method validation (2019) of the European Medicines Agency (EMA) [39].

The linearity was determined by measuring two separately prepared

calibration curves of uracil, thymine, DHU and DHT at the previously described concentration levels for 6 days. Linear weighted regression analysis (1/x) was used to construct calibration curves between analyte-to-internal standard peak area ratio and expected concentrations of the compounds. Acceptance criteria were as follows: $R^2 \geq 0.995$ for each analyte and relative bias on the back calculated concentrations < 20 % at the lower limit of quantification (LLoQ) and < 15 % for the other concentrations.

Intra- and inter-assay precision and accuracy were determined by analysis of the QC samples. The intra- and inter-assay precision were determined by the analysis of 6 replicates of each QC for 6 days. The overall accuracy should be within ± 15 % of the nominal concentration and the imprecision (%CV) should not exceed 15 % at each concentration level above the LLoQ. At the LLoQ, accuracy and precision should not exceed 20 %.

The lower limit of detection (LLoD) and LLoQ were determined by preparing and analyzing 0.10 ng/mL U and T, 20.0 ng/mL DHU and 1.00 ng/mL DHT in blank plasma 20 times. The LLoD and LLoQ were calculated as follows: LLoD = 3.3 x the standard error of the y-intercept (σ) / slope and LLoQ = 10 x σ / slope.

The matrix effect of the plasma was determined by comparing a calibration curve prepared in blank plasma to a calibration curve prepared in water with 0.1 % (v/v) FA, in duplicate. Matrix effect was calculated by dividing the average slope of the calibration curve in water by the average slope of the calibration curve in plasma multiplied by 100 %. Acceptable relative standard deviations (RSDs) of the matrix effects were ≤ 15 %.

The carry-over was determined by analyzing a water sample after the analysis of a standard sample at the ULoQ (80.0 ng/mL of both uracil and thymine and 800 ng/mL of both DHU and DHT). Carry-over should not be greater than 20 % of the analyte response at the LLoQ and 5 % of the internal standard response.

Recovery of the sample preparation was determined by analyzing an extra calibration curve where internal standards were added after sample preparation. The recovery was calculated by dividing the AUC of the internal standard before sample preparation by the AUC of the internal standard added after sample preparation multiplied by 100 %. Acceptable RSDs of the recovery were ≤ 15 %.

2.7. Stability

Blood samples for stability testing were collected by venipuncture using EDTA-tubes from 10 healthy individuals. Samples were stored and processed at five different conditions. One sample was placed on ice directly after collection and centrifuged immediately at 4 °C to obtain plasma, which was immediately frozen at − 80 °C. Two samples were centrifuged to obtain plasma and stored at room temperature for one and two hours. Two other samples were stored at room temperature as whole blood for one and two hours before further processing. All plasma samples were finally stored at − 80 °C before sample preparation and analysis. Normal distribution of the data was tested using a Shapiro-Wilk test. A non-parametric two-tailed Wilcoxon signed ranked test was used to determine the significance of the data, with $p < 0.05$ being considered statistically significant.

3. Results

3.1. Optimized LC-MS/MS conditions

The optimization of precursor ion *m/z*, product ion *m/z*, collision energy, desolvation temperature and cone voltage was performed by flow injection analysis (FIA). For all compounds, a solution of 100 ng/mL was injected directly into the source with a continuous flow of 0.3 mL/min. The results for the optimized precursor ion *m/z*, product ion *m/z* and collision energy are shown in Table 1. The optimal desolvation temperature was 200 °C; higher temperatures resulted in reduced peak intensity. The cone voltage was set at 32 eV to achieve adequate intensity for all compounds. Typical chromatograms at the lowest calibration points are shown in Fig. 2. Chromatograms of dialyzed blank plasma can be found in the supplementary information (SI1).

3.2. Method validation

3.2.1. Linearity

The assay was found to be linear using weighted linear regression (1/x) over the analyzed concentration range of 2.50–80.0 ng/mL for both uracil and thymine and 25.0–800 ng/mL for DHU and DHT. All correlation coefficients (R^2) were equal to or greater than 0.998 for each of the standard calibration curves within the tested range. The linearity acceptance criteria were met for all four compounds. Representative calibration curves are displayed in Fig. 3.

3.2.2. Accuracy and precision

Precision was expressed as intra- and inter-assay coefficient of variation (CV), and both varied from 2.5 to maximally 8.0 %. The accuracy varied from 93.6 % to 101.4 %. The results of the accuracy and precision experiments are displayed in Table 2 and met the predefined criteria.

3.2.3. Limits of quantification, matrix effect, carry-over, and recovery

The LLoQs for this method 2.41, 21.5, 0.95 and 6.55 ng/mL for uracil, DHU, thymine, and DHT, respectively. The lowest calibration standards were therefore always higher in concentration than the LLoQ. The mean matrix effects were between 3.6 % and 5.2 % for the four compounds. The carry-over following the highest calibration standards were 0.14 %, 0.06 %, 0.10 %, and 0.03 % for the analytes uracil, DHU, thymine, and DHT, respectively, and 0.07 %, 0.04 %, 0.08 %, and 0.01 % for the internal standards of uracil, DHU, thymine, and DHT, respectively. The mean recoveries were in between 70.8 % and 84.1 % for all four compounds. RSD values of the recoveries were in between 3.0 % and 8.3 %. The tested validation parameters are displayed in Table 3. The acceptance criteria for matrix effect, carry-over and recovery were met.

Table 1
Retention times, optimized precursor ion *m/z*, product ion *m/z*, collision energies and selected quantifier and qualifier ions for uracil, dihydrouracil (DHU), thymine, dihydrothymine (DHT) and their corresponding isotopically labelled internal standards.

Compound	Retention time (min)	Precursor Ion (*m/z*)	Quantifier		Qualifier	
			Product Ion (*m/z*)	Collision Energy (eV)	Product Ion (*m/z*)	Collision Energy (eV)
Uracil	4.34	113.0	70.0	18	96.1	21
Uracil-$^{13}C^{15}N_2$	4.33	116.0	71.0	18		
DHU	1.95	115.0	29.9	12	72.2	11
DHU-$^{13}C^{15}N_2$	1.95	118.0	31.1	12		
Thymine	5.39	127.0	110.1	18	54.0	23
Thymine-$^{13}C^{15}N_2$	5.38	130.0	112.0	20		
DHT	3.42	129.1	86.0	11	112.0	10
DHT-D_3	3.30	135.0	90.0	11		

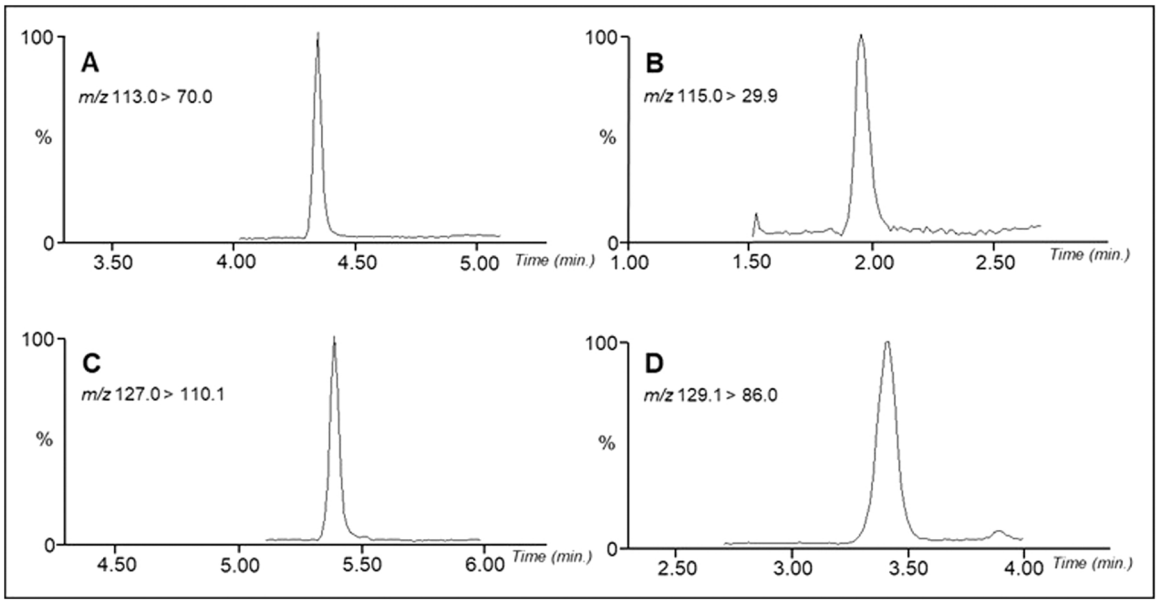

Fig. 2. Chromatograms obtained at the lowest plasma calibration level, 2.50 ng/mL for uracil (A) and thymine (C), 25.0 ng/mL for DHU (B) and DHT (D). Retention times are displayed on every x-axis, relative intensities are displayed on each y-axis.

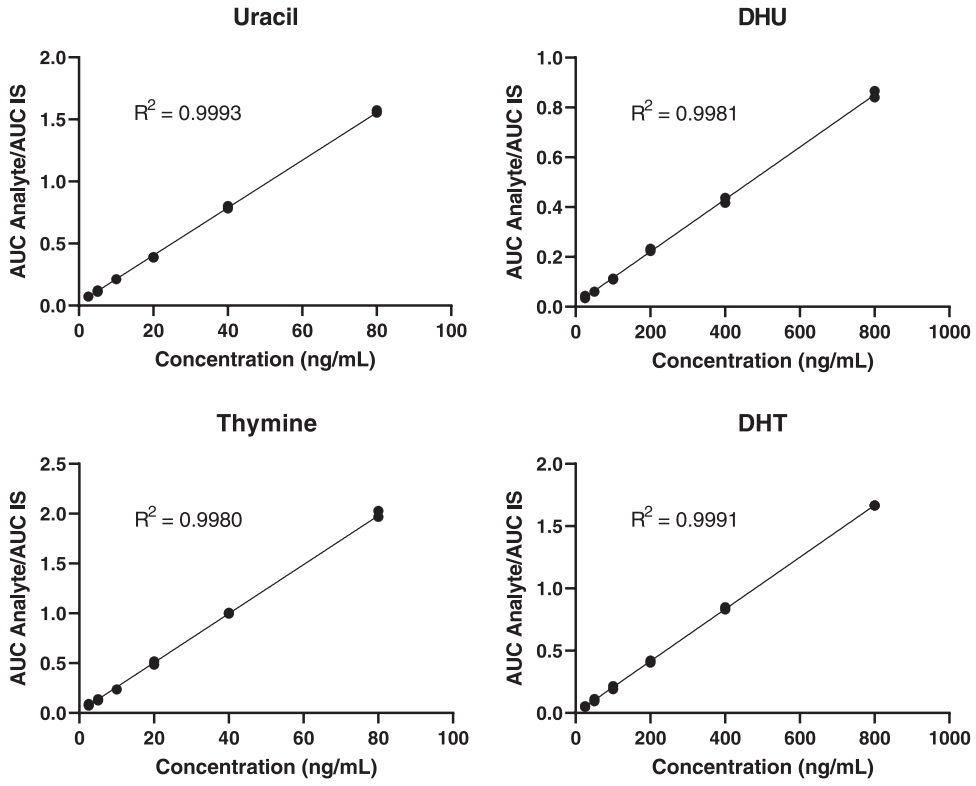

Fig. 3. Calibration curves of uracil, dihydrouracil (DHU), thymine and dihydrothymine (DHT) with corresponding R^2 all $\geq$ 0.998, curves are based on two separately prepared calibration curves on one validation day and are representative curves for the overall validation.

3.3. Stability

Fig. 4 shows the variation in concentrations of uracil, DHU, thymine and DHT after the different storage conditions. Storage conditions were selected as representative waiting times in a clinical laboratory. Compared to immediate collection followed by cold centrifugation and storage, the uracil concentration increased significantly by 27 % and 52 % when plasma was stored at ambient temperature for one and two hours, respectively. When stored as whole blood, the plasma uracil concentration increased significantly after two hours by 34 %. The plasma concentrations of DHU and DHT did not change at these storage conditions. Thymine concentrations did only change significantly after storage, except for a significant decrease when plasma was stored for one hour. With DHU levels remaining constant and uracil levels increasing, DHU:U ratios are inversely affected, reducing the ratios significantly with 30–40 %. The DHT:T ratios were not significantly

Table 2
Assay performance data for the analysis of uracil, dihydrouracil, thymine and dihydrothymine at three quality control concentration levels with six replicates each, determined at six days.

Compound	QC	Theoretical concentration (ng/mL)	Average measured concentration (ng/mL)	Accuracy	Intra-assay variation (%)	Inter-assay variation (%)
Uracil	Low	6.00	5.80	95.9	8.0	7.6
	Medium	12.0	11.9	99.1	4.7	4.7
	High	50.0	49.5	99.2	3.9	3.9
Dihydrouracil	Low	60.0	56.2	93.6	5.4	5.3
	Medium	120	118	98.1	4.1	4.1
	High	500	507	101.4	4.0	4.0
Thymine	Low	6.00	5.90	97.9	5.5	5.4
	Medium	12.0	11.7	97.1	4.1	4.1
	High	50.0	49.4	98.9	2.5	2.5
Dihydrothymine	Low	60.0	58.9	98.1	3.2	3.2
	Medium	120	118	97.3	3.5	3.5
	High	500	493	98.5	2.9	3.0

Table 3
Lower quantification limits, matrix effect, recovery, and carry-over for uracil, dihydrouracil (DHU), thymine and dihydrothymine (DHT).

Compound	LLoD (ng/mL)	LLoQ (ng/mL)	Matrix effect (%)	Recovery (%)	Recovery (%RSD)	Carry-Over (%)	Carry-Over (% of IS)
Uracil	0.79	2.41	5.0	78.2	5.0	0.14	0.07
DHU	7.10	21.5	5.2	70.8	7.9	0.06	0.04
Thymine	0.31	0.95	3.6	84.1	8.3	0.10	0.08
DHT	2.16	6.55	4.9	82.2	3.9	0.03	0.01

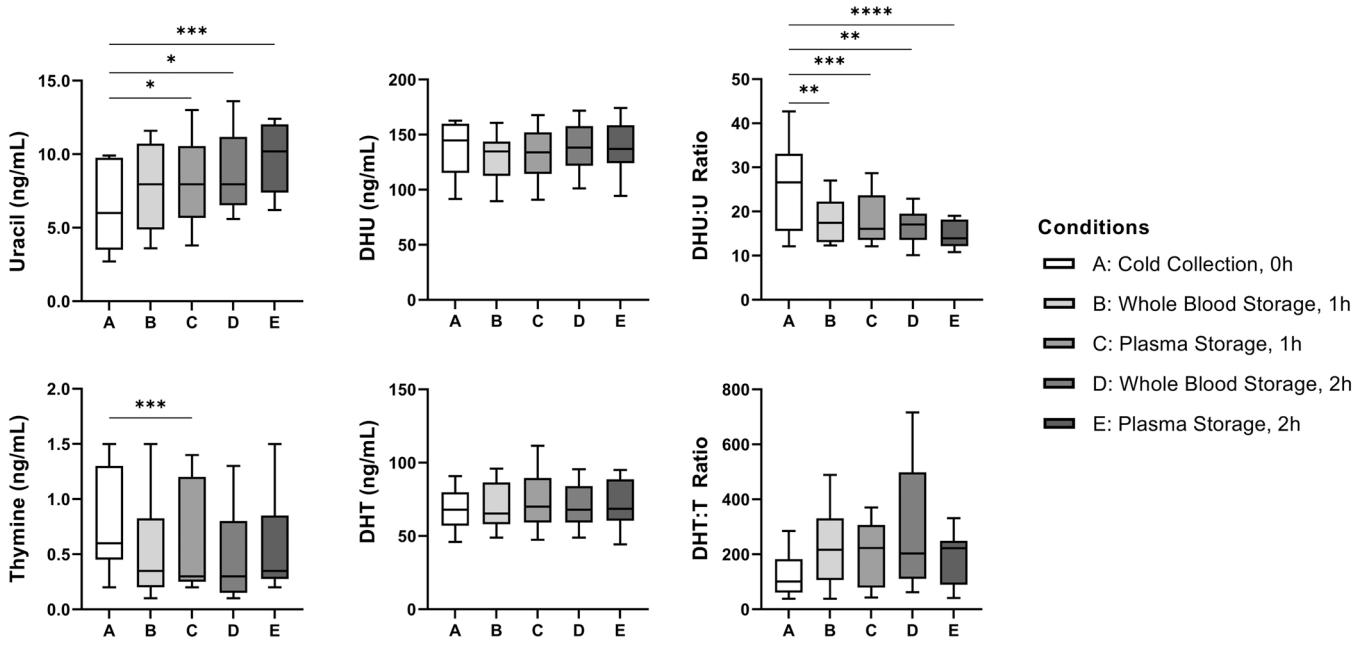

Fig. 4. Concentration of uracil, dihydrouracil (DHU), DHU:U ratio, thymine, dihydrothymine (DHT) and DHT:T ratio at five different storage conditions, in plasma and in whole blood. Data is plotted with the median as the middle line and the box extending from the 25th to 75th percentiles with minimal and maximal observed levels as outer bars (* $p < 0.05$, ** $p < 0.025$, *** $p < 0.01$, **** $p < 0.005$).

affected.

4. Discussion

In this paper, we have described the development and validation of an LC-MS/MS method for the simultaneous quantification of the endogenous concentrations of uracil, dihydrouracil, thymine and dihydrothymine in plasma. Ratios of both uracil and thymine and their corresponding metabolites may be used as phenotypic marker of the DPD enzyme activity. To the best of our knowledge, this is the first report that demonstrates the combined quantification of these four compounds in a single analytical run with adequately low LLoQ, good reproducibility, and within an acceptable analytical run time. One of the main inherent pitfalls in the pre-analysis is the instability of these analytes when stored *ex vivo*, especially uracil in plasma. In this study, we aimed to further objectify this instability and to extend this knowledge to thymine and DHT.

Uracil and DHU instability in biological matrices is a known issue which has been reported previously [3,23,27–33]. However, the magnitude of this instability differs highly between studies, ranging from no significant difference to the 27 % increase in uracil reported in the present study. Table 4 shows an overview of the available literature.

Table 4
Overview of the scientific literature with respect to the short-term storage stability, storage time, and temperature for uracil and dihydrouracil (DHU) when stored as plasma and when stored as whole blood. To best compare the literature with our own study, time points of one and two hours were selected from the reviewed studies. When these time points were not available in the reported studies, the earliest time point available were used. Hence, not all data and time points that were available in every study are shown.

Reference	Compound	Storage Matrix	Storage Temperature	Storage Time	Number of samples	Results	Reported Findings
Remaud et al. [23]	Uracil	Plasma	Ambient	24 h	2	–	Increased quickly from day to day especially at ambient temperature, no earlier time point. DHU was found to be stable for 14 days at both conditions.
			4 °C	24 h	2	–	
	DHU		Ambient	24 h	2	–	
			4 °C	24 h	2	–	
Büchel et al. [27]	Uracil	Plasma	Ambient	6 h	4	+ 13.8 %[1] & + 6.62 %[2]	
	DHU		Ambient	6 h	4	+ 12.5 %[1] & + 11.5 %[3]	
César et al. [28]	Uracil	Plasma	Ambient	6 h	5	- 8.7 %	There was no significant difference between the analyte responses at time zero and after 6 h at room temperature, 6 h is the earliest timepoint
	DHU		Ambient	6 h	5	- 0.5 %	
Jacobs et al. [3]	Uracil	Plasma	Ambient	4 h	3	0.0 %	No significant change reported, stability determined based on stability of plasma spiked with corresponding stable isotopes.
	DHU		Ambient	4 h	3	- 4.4 %	
Chavani et al. [29]	Uracil	Plasma	Ambient	6 h	6	- 3.3 % to + 3.5 %[4]	It was concluded uracil and DHU were stable in plasma for short-term storage
	DHU		Ambient	6 h	6	+ 2.2 % to + 9.1 %[5]	
Robin et al. [30]	Uracil	Plasma	4 °C	4 h	n.a.	+ 13 %	
Tafzi et al. [31]	Uracil	Plasma	Ambient	8 h	n.a.	–	A not specified increase was reported after 8 h.
	DHU		Ambient	8 h	n.a.	–	
Marin et al. [41]	Uracil	Plasma	Ambient	1.5 h	n.a.		Significant differences were found in both uracil and DHU levels when samples were analyzed that were shipped and exceeded 1.5 h at ambient temperature or 4 h at 4 °C before handling compared to proper handled samples.
			4 °C	4 h	n.a.		
	DHU		Ambient	1.5 h	n.a.		
			4 °C	4 h	n.a.		
Capiau et al. [32]	Uracil	Plasma	Ambient	1 h	10	+ 5 %[6]	Increase in uracil after 1 h at ambient temperature was found to be within acceptable limits (between ± 15 %). DHU increase was within acceptable limits after 1 and 2 h.
				2 h	10	+ 22 %[6]	
	DHU		Ambient	1 h	10	+ 2 %[6]	
				2 h	10	+ 12 %[6]	
Findings of this paper	Uracil	Plasma	Ambient	1 h	10	+ 27.2 %	
				2 h	10	+ 52.2 %	
	DHU		Ambient	1 h	10	- 2.6 %	
				2 h	10	+ 1.6 %	
Coudoré et al. [33]	Uracil	Whole blood	Ambient	1 h	10	+ 11.3 %	Results agree with results of Deporte et al. (2005) and Remoud et al. (2005). Rapid uracil increases at both ambient temperature and + 4 °C.
				2 h	10	+ 27.2 %	
	DHU		Ambient	1 h	10	+ 10.0 %	
				2 h	10	+ 10.1 %	
Jacobs et al. [3]	Uracil	Whole blood	Ambient	1 h	3	+ 9 %[6]	
				2 h	3	+ 27 %[6]	
	DHU		Ambient	1 h	3	- 5 %[6]	
				2 h	3	- 3 %[6]	
	Uracil		2–8 °C	1 h	3	+ 7 %[6]	
				2 h	3	+ 15 %[6]	
	DHU		2–8 °C	1 h	3	- 4 %[6]	
				2 h	3	- 7 %[6]	
Findings of this paper	Uracil	Whole blood	Ambient	1 h	10	+ 24.0 %	
				2 h	10	+ 34.1 %	
	DHU		Ambient	1 h	10	- 5.9 %	
				2 h	10	+ 0.7 %	

[1] At a concentration of 0.1 μM, [2]At a concentration of 10 μM, [3] at a concentration of 1 μM, [4] Concentration range from 15 to 150 ng/mL at 4 levels, [5] Concentration range from 30 to 300 ng/mL at 4 levels, [6] Results adapted from graphs.

Possible explanations for the contrary findings of these studies could be the use of inappropriate surrogate matrices, the spiking of samples using either endogenous or isotopically labelled compounds, or the limited sample size varying from 3 to 6 samples [3,28,29,40]. In our experiments we used five paired samples of human EDTA-plasma from ten volunteers and determined the naturally occurring uracil, DHU, thymine, and DHT levels without any additional spiking. Recently, Capiau et al. (2022) performed similar stability measurements to the one we show here [32]. In their study, the observed uracil concentration-increase after one hour in plasma at ambient temperatures was only 5 %. Their conclusion that samples are stable if kept at room temperature for less than an hour could therefore be a relevant underestimation [32]. This has also been confirmed by other studies that showed an increase in plasma uracil concentration. Remaud et al.

(2005), Büchel et al. (2012), Robin et al. (2020), and Tafzi et al. (2020) all reported an increase in plasma uracil concentration from 7 % to 14 % after different time points and storage temperatures [23,27,30,31]. In addition to this, Marin et al. (2020) reported significant differences in both uracil and DHU levels when samples were shipped and not centrifuged within 1.5 h at ambient temperature, or 4 h at 4 °C compared to samples with proper handling [41].

We also studied the effects of short-term storage in whole blood. The reported increases in uracil concentration found in the literature were in line with the findings of the present study but were not significantly

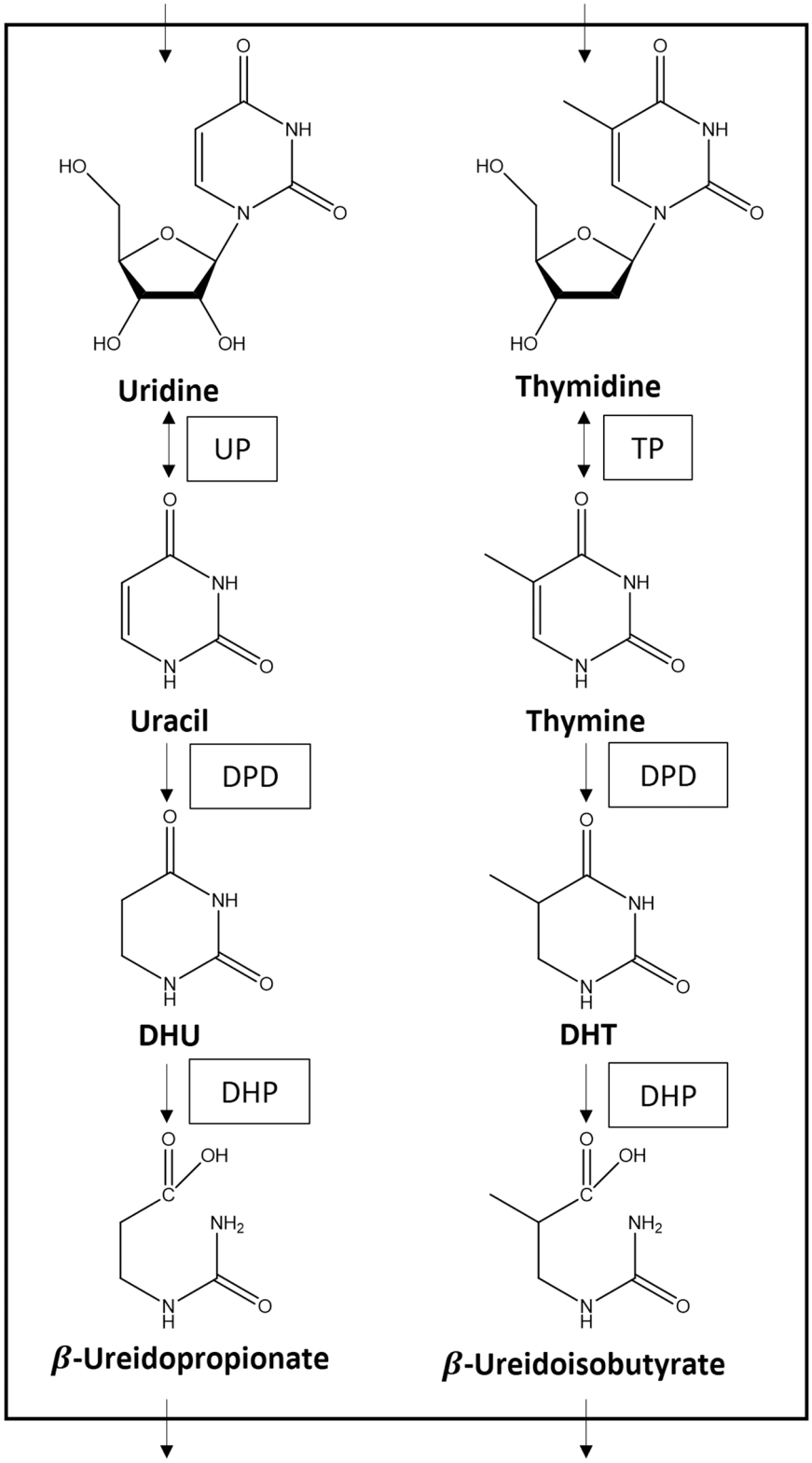

Fig. 5. Uridine and thymidine are metabolised by uridine phosphorylase (UP) and thymidine phosphorylase (TP) into uracil and thymine, respectively. Both uracil and thymine are subsequently metabolized by dihydropyrimidine dehydrogenase (DPD) into dihydrouracil (DHU) and dihydrothymine (DHT), respectively. DHU and DHT are both further metabolized into β-Ureidopropionate and β-Ureidoisobutyrate by dihydropyrimidase (DHP).

different from those found in plasma [3,33]. Since the instability in whole blood and plasma was comparable, it is unlikely that the cellular compartment influences the increase in uracil concentration. The overarching conclusion of this study and others must be that the pre-analytical phase should be carefully controlled if the analysis of nucleotide bases is to be applied clinically as a pretherapeutic diagnostic test. This was elegantly shown by a recent report by De With et al. (2022), describing the relevant influence of inter-laboratory variation on clinical management in a large multi-center trial. These variations were such that with uncontrolled sample handling, pre-treatment uracil concentrations may not be used as biomarker for DPD deficiency and to guide safe and effective dosing of 5-FU-based chemotherapy [37].

A possible explanation for the observed uracil increase in both plasma and whole blood is the continued *ex vivo* metabolism of uridine to uracil by the enzyme uridine phosphorylase (UP) (Fig. 5) [3,32,33]. UP and other enzymes in the purine pathway may remain active until the plasma has been chemically processed. Various strategies can be applied to inhibit these enzymes: First, samples can be collected, transported, and centrifuged at 4 °C followed by aliquoting and freezing. Second, sample preparation can be performed immediately after venipuncture by protein precipitation and spiking with internal standard, but this is difficult to implement in a routine laboratory. Third, specific enzyme inhibitors can be added to blood samples immediately after, or possibly even before, the blood draw. These inhibitors should target DPD, but also uridine phosphorylase (UP), thymidine phosphorylase (TP) and dihydropyrimidinase (DHP) to make sure none of the metabolites of interest can be formed or degraded during sample workup (Fig. 5).

Additional areas of future work could be the use of urine or saliva as alternatives to plasma, and the importance of circadian rhythm. Also, it is advised to collect blood samples from patients between 8.00 am and 10.00 am in fasting state, although based on uracil and DHU we expect similar effects for thymine and DHT [32,40]. The observation that thymine and DHT are more stable than uracil and DHU may be an argument to investigate these compounds further, prospective clinical testing is needed to examine the predictive value. Also, the addition of DHU:U and DHT:T ratios in clinical workflows may be beneficial when the pre-analysis is controlled, cross validated and standardized between clinical laboratories.

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Sebastian A.H. van den Wildenberg: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Alexander S. Streng:** Conceptualization, Methodology, Formal analysis, Writing – review & editing. **Renske van den Broek:** Methodology, Validation, Investigation. **Maarten A.C. Broeren**: Resources, Data curation. **Maarten J. Deenen:** Funding acquisition, Conceptualization. **Joost L.J. van Dongen**: Methodology, Investigation, Resources. **Maarten A. Hanrath:** Funding acquisition, Conceptualization. **Chyara Lapré:** Methodology, Validation, Investigation. **Luc Brunsveld:** Supervision, Data curation, Funding acquisition. **Volkher Scharnhorst**: Supervision, Data curation, Funding acquisition. **Daan van de Kerkhof:** Supervision, Formal analysis, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2022.115027.

References

[1] U. Amstutz, et al., Clinical pharmacogenetics implementation consortium (CPIC) guideline for dihydropyrimidine dehydrogenase genotype and fluoropyrimidine dosing: 2017 update, Clin. Pharmacol. Ther. 103 (2) (2018) 210–216.
[2] J.H. Beumer, et al., Therapeutic drug monitoring in oncology: international association of therapeutic drug monitoring and clinical toxicology recommendations for 5-fluorouracil therapy, Clin. Pharmacol. Ther. 105 (3) (2019) 598–613.
[3] B. Jacobs, et al., Development and validation of a rapid and sensitive UPLC-MS/MS method for determination of uracil and dihydrouracil in human plasma, J. Pharm. Biomed. Anal. 126 (2016) 75–82.
[4] M. Launay, et al., Upfront DPD deficiency detection to secure 5-FU administration: Part 1 – where do we stand? Clin. Cancer Drugs 4 (2) (2018).
[5] M.C. van Staveren, H. Jan Guchelaar, A.B.P. van Kuilenburg, H. Gelderblom, J. G. Maring, Evaluation of predictive tests for screening for dihydropyrimidine dehydrogenase deficiency, Pharm. J. 2013 135 13 (5) (2013) 389–395.
[6] L.M. Henricks, et al., Translating DPYD genotype into DPD phenotype: using the DPYD gene activity score, Pharmacogenomics 16 (11) (2015) 1277–1286.
[7] N. Zhang, Y. Yin, S.-J. Xu, W.-S. Chen, 5-Fluorouracil: Mechanisms of Resistance and Reversal Strategies Mol. 2008, 13, Pages 1551–1569, 13, no. 8, pp. 1551–1569, Aug. 2008.
[8] M. Del Re, et al., Dihydropyrimidine dehydrogenase polymorphisms and fluoropyrimidine toxicity: ready for routine clinical application within personalized medicine? EPMA J. 1 (3) (2010) 495.
[9] D. Meulendijks, A. Cats, J.H. Beijnen, J.H.M. Schellens, Improving safety of fluoropyrimidine chemotherapy by individualizing treatment based on dihydropyrimidine dehydrogenase activity – ready for clinical practice? Cancer Treat. Rev. 50 (2016) 23–34.
[10] A.F.A. Galarza, et al., Endogenous plasma and salivary uracil to dihydrouracil ratios and DPYD genotyping as predictors of severe fluoropyrimidine toxicity in patients with gastrointestinal malignancies, Clin. Biochem. 49 (16–17) (2016) 1221–1226.
[11] G. Milano, et al., Influence of sex and age on fluorouracil clearance, J. Clin. Oncol. 10 (7) (1992) 1171–1175.
[12] D. Meulendijks, et al., Renal function, body surface area, and age are associated with risk of early-onset fluoropyrimidine-associated toxicity in patients treated with capecitabine-based anticancer regimens in daily clinical care, Eur. J. Cancer 54 (2016) 120–130.
[13] M.J. Deenen, et al., Upfront genotyping of DPYD*2A to individualize fluoropyrimidine therapy: A safety and cost analysis, J. Clin. Oncol. 34 (3) (2016) 227–234.
[14] D. Meulendijks, et al., Clinical relevance of DPYD variants c.1679T>G, c.1236G>A/HapB3, and c.1601G>A as predictors of severe fluoropyrimidine-associated toxicity: a systematic review and meta-analysis of individual patient data, Lancet Oncol. 16 (16) (2015) 1639–1650.
[15] G.A. Brooks, S. Tapp, A.T. Daly, J.A. Busam, A.N.A. Tosteson, Cost-effectiveness of DPYD genotyping prior to fluoropyrimidine-based adjuvant chemotherapy for colon cancer, Clin. Colorectal Cancer (2022).
[16] F.K. Martens, D.W. Huntjens, T. Rigter, M. Bartels, P.M. Bet, M.C. Cornel, DPD testing before treatment with fluoropyrimidines in the Amsterdam UMCs: an evaluation of current pharmacogenetic practice, Front. Pharmacol. 10 (2020) 1609.
[17] C. White et al., Dihydropyrimidine Dehydrogenase Deficiency and Implementation of Upfront DPYD Genotyping Clin. Pharmacol. Ther., Jun. 2022.
[18] J.E. Knikman, H. Gelderblom, J.H. Beijnen, A. Cats, H.J. Guchelaar, L.M. Henricks, Individualized dosing of fluoropyrimidine-based chemotherapy to prevent severe fluoropyrimidine-related toxicity: what are the options? Clin. Pharmacol. Ther. 109 (3) (2021) 591.
[19] G. Bocci, et al., Comparative pharmacokinetic analysis of 5-fluorouracil and its major metabolite 5-Fluoro-5,6-dihydrouracil after conventional and reduced test dose in cancer patients, Clin. Cancer Res. 6 (8) (2000).
[20] M. Chazal, M.C. Etienne, N. Renée, A. Bourgeon, H. Richelme, G. Milano, Link between dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells and liver, Clin. Cancer Res. 2 (3) (1996).
[21] A. Di Paolo, et al., Relationship between 5-fluorouracil disposition, toxicity and dihydropyrimidine dehydrogenase activity in cancer patients, Ann. Oncol. 12 (9) (2001) 1301–1306.
[22] A.M. Lostia, et al., A liquid chromatography-tandem mass spectrometry method for the determination of 5-fluorouracil degradation rate by intact peripheral blood mononuclear cells, Ther. Drug Monit. 31 (4) (2009) 482–488.
[23] G. Remaud, M. Boisdron-Celle, C. Hameline, A. Morel, E. Gamelin, An accurate dihydrouracil/uracil determination using improved high performance liquid chromatography method for preventing fluoropyrimidines-related toxicity in clinical practice, J. Chromatogr. B 823 (2) (2005) 98–107.

[24] M.H. Kristensen, P. Pedersen, J. Mejer, The value of dihydrouracil/uracil plasma ratios in predicting 5-fluorouracilrelated toxicity in colorectal cancer patients, J. Int. Med. Res. 38 (4) (2010) 1313–1323.
[25] Y. Nakayama, et al., Correlation between the urinary dihydrouracil-uracil ratio and the 5-FU plasma concentration in patients treated with oral 5-FU analogs, Anticancer Res. 26 (5 B) (2006) 3983–3988.
[26] J. Sistonen, et al., Predicting 5-fluorouracil toxicity: DPD genotype and 5,6-dihydrouracil:uracil ratio, Pharmacogenomics 15 (13) (2014) 1653–1666.
[27] B. Büchel, P. Rhyn, S. Schürch, C. Bühr, U. Amstutz, C.R. Largiadèr, LC-MS/MS method for simultaneous analysis of uracil, 5,6-dihydrouracil, 5-fluorouracil and 5-fluoro-5,6-dihydrouracil in human plasma for therapeutic drug monitoring and toxicity prediction in cancer patients, Biomed. Chromatogr. 27 (1) (2013) 7–16.
[28] I.C. César, G.F. Cunha-Júnior, R.M. Duarte Byrro, L.G. Vaz Coelho, G.A. Pianetti, A rapid HPLC-ESI-MS/MS method for determination of dihydrouracil/uracil ratio in plasma: evaluation of toxicity to 5-flurouracil in patients with gastrointestinal cancer, Ther. Drug Monit. 34 (1) (2012) 59–66.
[29] O. Chavani, B.P. Jensen, R.M. Strother, C.M. Florkowski, P.M. George, Development, validation and application of a novel liquid chromatography tandem mass spectrometry assay measuring uracil, 5,6-dihydrouracil, 5-fluorouracil, 5,6-dihydro-5-fluorouracil, α-fluoro-β-ureidopropionic acid and α-fluoro-β-alanine in human plasma, J. Pharm. Biomed. Anal. 142 (2017) 125–135.
[30] T. Robin, F. Saint-Marcoux, D. Toinon, N. Tafzi, P. Marquet, S. El, Balkhi, Automatic quantification of uracil and dihydrouracil in plasma, J. Chromatogr. B 1142 (2020), 122038.
[31] N. Tafzi, J.B. Woillard, A. Fleytoux, N. Picard, P. Marquet, Phenotyping of uracil and 5-fluorouracil metabolism using LC-MS/MS for prevention of toxicity and dose adjustment of fluoropyrimidines, Ther. Drug Monit. 42 (4) (2020) 540–547.
[32] S. Capiau, A. Van Landschoot, T. Reyns, H. Stepman, Pre-analytical considerations for the analysis of uracil and 5,6-dihydrouracil in heparin plasma, Clin. Chem. Lab. Med. 0 (0) (2022).
[33] F. Coudoré, et al., Validation of an ultra-high performance liquid chromatography tandem mass spectrometric method for quantifying uracil and 5,6-Dihydrouracil in human plasma, J. Chromatogr. Sci. 50 (10) (2012) 877–884.
[34] T.W. Traut, Physiological concentrations of purines and pyrimidines, Mol. Cell. Biochem. 140 (1) (1994) 1–22.
[35] O.H. Temmink, et al., The role of thymidine phosphorylase and uridine phosphorylase in (fluoro)pyrimidine metabolism in peripheral blood mononuclear cells, Int. J. Biochem. Cell Biol. 38 (10) (2006) 1759–1765.
[36] A.B.P. Van Kuilenburg, H. Van Lenthe, M.J. Blom, E.P.J. Mul, A.H. Van Gennip, Profound variation in dihydropyrimidine dehydrogenase activity in human blood cells: major implications for the detection of partly deficient patients, Br. J. Cancer 79 (3–4) (1999) 620–626.
[37] M. de With, et al., Dihydropyrimidine dehydrogenase phenotyping using pretreatment uracil: a note of caution based on a large prospective clinical study, Clin. Pharmacol. Ther. (2022).
[38] Y.Y. Elamin, S. Rafee, N. Osman, K.J. O'Byrne, K. Gately, Thymidine phosphorylase in cancer; enemy or friend? Cancer Microenviron. 9 (1) (2016) 33–43.
[39] European Medicines Agency, ICH guideline M10 on bioanalytical method validation 4 Step 2b 5 2019.
[40] H. Jiang, J. Jiang, P. Hu, Y. Hu, Measurement of endogenous uracil and dihydrouracil in plasma and urine of normal subjects by liquid chromatography–tandem mass spectrometry, J. Chromatogr. B 769 (1) (2002) 169–176.
[41] C. Marin, et al., A simple and rapid UPLC-UV method for detecting DPD deficiency in patients with cancer, Clin. Transl. Sci. 13 (4) (2020) 761–768.