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Automatic quantification of uracil and dihydrouracil in plasma.

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Abstract:

Fluoropyrimidines-based chemotherapies are the backbone in the treatment of many cancers. However, the use of 5-fluorouracil and its oral pre-prodrug, capecitabine, is associated with an important risk of toxicity. This toxicity is mainly due to a deficiency of dihydropyrimidine dehydrogenase (DPD). This deficiency may be detected by using a phenotypic approach that consists in the measurement of uracilemia or the calculation of dihydrouracil (UH_2)/uracil (U) ratio. For uracilemia, a threshold value of 16 ng/ml has been proposed for partial deficiency, while a value of 150 ng/ml has been proposed for complete deficiency

We have developed a rapid, accurate and fully-automated procedure for the quantification of U and UH_2 in plasma. Sample extraction was carried out by a programmable liquid handler directly coupled to a liquid chromatography - tandem mass spectrometry (LC-MS/MS) system. The method was validated according to the EMA guidelines and ISO 15189 requirements and was applied to real patient samples (n=64).

The limit of quantification was 5 and 10 ng/ml for U and UH_2 respectively. Imprecision and inaccuracy were less than 15% for inter and intra-assay tests. Comparison with dedicated routine method showed excellent correlation.

An automated procedure perfectly fulfills the need of low inaccuracy and CVs at the threshold values (less than 5% at 16 ng/ml) and is highly suitable for the characterization of DPD deficiency.

Automatization should guaranty reliable and robust performances by minimizing the sources of variation such as volume inaccuracies, filtration or manual extraction related errors.

Keyword : LC-MS/MS, automated sample preparation, 5-FU, U, UH_2, DPD deficiency

1. Introduction

Fluoropyrimidines-based chemotherapies remain important antineoplastic agents since first approval for testing in humans about 60 years ago [1, 2]. Indeed, 5-fluorouracil and its oral pre-prodrug capecitabine are the backbone in the treatment of colorectal, pancreatic, gastric, breast, head and neck cancers [3, 4]. However, the use of fluoropyrimidines is associated with an important risk of toxicity, including a painful skin condition known as hand-foot syndrome, diarrhea, mucosal ulceration and immune suppression [5-7]. As a result, this toxicity limits or delays the administration of optimal or successive courses, which impacts the clinical outcome of patients with cancer. The mechanisms of fluoropyrimidines-based chemotherapies toxicity and the different strategies to prevent this toxicity are of great importance and numerous works have been published [5-10]. The dihydropyrimidine dehydrogenase (DPD), a rate-limited enzyme, is responsible of 80% of the catabolism of fluoropyrimidines. Most of the toxicities under fluropyrimides treatment can be explained by an inherited deficiency in DPD activity. Up to 15% of patients could exhibit a partial deficit exposing them to moderate or even severe adverse effects. However, in 0.1 to 0.5% of patient, the complete DPD deficiency could lead to lethal toxicity. This deficiency may be detected by using genotyping that detect the DPYD gene inactivating polymorphisms or by using a phenotyping by measuring uracilemia. The calculation of the dihydrouracil (UH_2)/uracil (U) ratio to determine the phenotypes has been also used but it has raised some controversies.

Currently, there is no clear European regulatory obligation for DPD deficiency screening in patients given a fluoropyrimidine-based chemotherapy. However, based on the levels of evidence from the literature, the Group of Clinical Pharmacology in Oncology (GPCO)-UNICANCER and the French Network of Pharmacogenetics (RNPGx) has recently established the following recommendations [5] : (i) to explore DPD deficiency before initiating any chemotherapy containing fluoropyrimidines-based chemotherapies by measuring plasma U concentrations (possibly associated with UH_2/U ratio); (ii) to genotype DPYD by exploring different variants; (iii) to reduce the initial dose, when partial deficiency is diagnosed or to proscribe fluoropyrimidines-based chemotherapies when the deficiency is complete. For uracilemia, a threshold value of 16 ng/ml has been proposed for partial deficiency, while a value of 150 ng/ml has been proposed for complete deficiency [11-13]. No consensus has been defined for the interpretation of UH_2/U ratio, due to a great interlaboratory variability in the measurements [13-16].

In the past years, many labs have developed methods with UV detection [17-26] and more recently some have reported the use of mass spectrometry (MS) detection [26-32]. All these methods were based on classical manual sample preparations with protein precipitation, solid-phase or liquid-liquid extractions. With regards to the rapidity needed to report results and the increase in the number of patients to be monitored, the time dedicated to sample extraction may be a limiting step and its automation will offer a great added value. To our knowledge, no such automated extraction procedure coupled to LC-MS has been proposed yet for the measurement of U and UH_2. Most recent extraction procedures involving any automated step of sample preparation (for the measurements of drugs or toxic compounds) were extensively reviewed by Vogeser and Kirchhoff [33], Zheng et al [34], Prabhu and Urban [35] or Wei [36]. Their main drawback is that they were not fully automated as the extracts needed at least to be transferred into the autosampler. In this context, we recently published a very high throughput method for the measurement of illicit drugs where extraction procedure was fully synchronized to LC-MS and where no human intervention was necessary after the primary tube was loaded on the system [37]. The main objective of this study was to develop a LC-MS method for the measurement of U and UH_2 in plasma with a fully-automated sample preparation.

2. Material and methods

2.1 Chemicals and reagents:

Dihydrouracil, dihydrouracil-$^{13}C^{15}N_2$, uracil and uracil-$^{13}C^{15}N_2$ were supplied by LGC Standards (Molsheim, France). These analytes were stable in stock solution for 6 months at -20°C [29]. Acetonitrile and isopropanol were purchased from Carlo Erba Réactifs (Val de Reuil, France). Bovin serum albumin was purchased from Sigma Aldrich (ref. A7030) (Saint-Quentin Fallavier, France). Formic acid and acetic acid were supplied from Fisher Chemical (Illkirch, France). Pure water was obtained using a Millipore Direct Q purification system (Saint-Quentin-en-Yvelines, France).

2.2 Sample preparation:

Extraction was performed by an automated sample preparation system, the CLAM-2030 (Shimadzu Corporation, Marne-la-Vallée, France), coupled to a LC-MS/MS system (see below). Briefly, once the primary (or secondary) tube was loaded onto the automated system, no further human intervention was required.

Blood samples were collected in heparinized tubes and plasma was separated within 1h30 by centrifugation at +4°C and stored at -20°C according to the recommendations [6, 38].

Once on board, 20 µL plasma (store at +4°C) were automatically pipetted in a pre-conditioned tube containing a filter, to which reagents were then added, mixed and filtered. Precisely: 40 µL of acetonitrile/acid formic (1%) was added to a PTFE (polytetrafluoroethylene) filter vial (0.45 µm pore size) previously conditioned with 20 µL of isopropanol. Then, the 20 µL of plasma and 10 µL of isotopically labelled internal standards (at 1500 ng/ml: namely, 167 ng/mL in the tube) were added. The mixture was agitated for 120 seconds (1900 rpm) then filtered by application of vacuum pressure (-60 to -65 kPa) for 120 seconds into a collection vial. Finally, the extract was diluted with 550µL of water and 50 µL were injected in the LC-MS/MS system.

2.3 LC-MS/MS conditions:

The chromatographic system consisted of two Shimadzu LC-30 AD pumps (Nexera X2), a CTO 20AC oven and a SIL-30 AC-MP autosampler (Shimadzu Corporation, Marne-la-Vallée, France). Chromatographic separation was performed using a Hypercarb 3 µm, 150*2.1 mm column (ThermoFisher, Illkirch, France), and a gradient of (A) water containing 0.5 % acetic acid, and (B) acetonitrile containing 0.5 % acetic acid as mobile phase at a flow-rate of 250 µL/min, as follows: 0.00-3.00 min, 5% (B); 3.00-5.00 min, 5 to 25% (B); 5.00-9.1 min, 25% (B); 9.1-9.2 min, 25 to 100% (B); 9.2-10.5 min, 100% (B); 10.5-10.6, 100 to 5% (B); 10.6-15 min, 5% (B). Oven temperature was set at 25°C.

LC-MS/MS analysis was performed on an LCMS-8060 triple quadrupole mass spectrometer (Shimadzu Corporation, Marne-la-Vallée, France) used in the positive electrospray ionization modes. The interface parameters and common settings were as follows: interface voltage: 1 kV; nebulizing gas flow: 3 L/min; heating gas flow: 14 L/min; drying gas flow: 10 L/min; interface temperature: 380°C; DL (desolvation line) temperature: 300 °C; heat block temperature: 500°C; collision gas pressure 170 kPa.

All compounds were measured by scheduled-MRM (Multiple Reaction Monitoring) using 1 ms pause time and 30 ms dwell time.

2.4 Validation procedure:

The calibration range was 5 to 320 ng/mL for U concentration and 10 to 640 ng/ml for UH_2. The calibration standards and the 3 quality controls (QC) prepared in BSA at 80 g/L were used:

(i) QC1 13 and 23 ng/ml, (ii) QC2 16 and 32 ng/ml and (iii) QC3 150 and 300 ng/ml for U and UH_2, respectively.

The laboratory of Pharmacology-toxicology of the Limoges University Hospital activities has been accredited following the International Standards Organization (ISO) 15189 standard (accreditation number: 8-2607). The methods validation protocol respects also the EMA guidelines and examines the following criteria:

- The lower limit of quantitation (LLOQ) was defined as the lowest concentration that could be measured with intra-assay and inter-assay imprecision (CV%), and inaccuracy (bias) all < 20%.
- The intra-assay precision and accuracy (n=6) and the inter-assay precision and accuracy (n=6) were assessed by analysis of the 3 different QC. To access the intra-assay precision and accuracy 6 replicates of each QC were analyzed in one day. To assess the inter-assay precision and accuracy, each QC were analyzed each day for 6 days. The CV% values and bias values had to be less than 20% at the LLOQ and less than 15% for the other levels.
- Calibration standards were prepared by adding appropriate working standard solutions to 100 µL of BSA prior to extraction in order to obtain the following 6 concentration levels for uracil: 5, 10, 15, 20, 80 or 320 ng/mL; and the following for dihydrouracil: 10, 20, 30, 40, 160 and 640 ng/mL. Calibration curves of the compounds-to-internal standard peak-area ratios of the quantification transition versus expected compound concentrations were constructed using a linear with 1/x weighting regression analysis.
- Recoveries were determined for the 3 QC by comparing analyte / internal standard peak area ratios for sample spiked either before or after the automated sample preparation.
- The carry-over effect was evaluated by analyzing the blank samples that were systematically analyzed after the injection of calibration standards at the upper limit of quantification (respectively 320 and 640 ng/ml for U and UH_2). According to EMA guidelines [39] carry-over should not be greater than 20% of the LLOQ for the 2 analytes and 5% of their internal standards.
- Matrix effects were evaluated using deuterated internal standards. In fact, U and UH_2 are endogenous compounds. Six different human plasma and 6 different water samples were extracted and secondary spiked with internal standards at 100 ng/ml. Potential ion suppression or enhancement was evaluated by comparing the average peak intensity obtained in the two different conditions. The difference had to be less than 15%.
- Selectivity examination is not applicable for U and UH2 since the analytes are naturally present in human plasma at concentrations of approximately 10 ng/mL and 100 ng/mL, respectively. However, we examined the selectivity of IS by evaluating the interference in 6

different blank plasmas. Selectivity of IS was proven by the absence of any peaks at the same retention time.

- The stability of U and UH_2 were studied by others [25-31]. According to Jacobs et al [31] the U and UH_2 are stable in whole blood for 4 hours at +4°C at maximum. Noteworthy, 13% increases in the U concentration after 4h has been observed. Both U and UH_2 were stable in plasma at -20°C after long term storage. This is why all samples have to be centrifuged within 1h30 and stored at -20°C*2.5*

Application of the whole procedure to real patient samples:

The automated sample preparation and LC-MS/MS analysis procedure was tested using 64 patient samples by comparing its quantitative results to those of a pre-existing and validated quantitative LC-MS/MS method routinely used in our lab and considered as a reference here after. Briefly, this method was based on a liquid-liquid extraction of 500µL of plasma with ethyl acetate/2-propanol preceded by a protein precipitation with ammonium sulfate. The chromatographic separation was performed using a XSelect®CSH^{TM} C18, 2.5 µm (100×2.1 I.D.) column and mass detection and quantification were performed on an AB-Sciex QTRAP®4500MD triple quadrupole mass spectrometer (Sciex, Villebon sur Yvette) operated in the positive electrospray ionization (ESI+) with selected reaction monitoring mode.

3. Results and discussion

As DPD deficiency screening in patients receiving a fluoropyrimidine-based chemotherapy is an obligation in France and highly recommended in other countries, most labs in charge of the measurement of U (and UH_2) will or are already facing a great increase of this activity. We propose here a fully-automated solution able to ensure an accurate and robust measurement without requiring precious manpower and allowing high throughput by running samples continuously when necessary. The method was validated according to EMA guidelines. A summary of the results of validation and the MRM conditions are reported in Table 1 and in Table 2.

- Selectivity

Among validation criteria, the specificity examination is challenging since U and UH_2 are both endogenous compounds in human plasma and that most determination methods use BSA to build matrix matched calibrations. We examined the selectivity of the IS by evaluating

interferences in 6 different plasma. No significant signal (<5%) were observed at the RT of the IS.

An elegant approach has been proposed by Jacobs et al [31] where control human plasma was spiked with U-$^{15}N_2$ and UH_2-$^{13}C_4$-$^{15}N_2$ and quantified using U and UH_2 as internal standards for examination of the selectivity, the recovery and the matrix effects. However, for selectivity, only the selectivity of the IS was examined but any interference from possible co-administered medications could not be verified. As patients receiving fluoropyrimidines-based chemotherapies are generally poly-medicated, this verification is necessary, though. Therefore, we examined, in our in-house built library, a list of 900 drugs and metabolites of interest to identify a potential interfering compound. None of these drugs presented a mass of 114.0 or 112.0. Collision induced dissociation ions, produced in the ESI source, with these m/z could be another source of interference. But it is still unlikely that such a compound would have the same retention time and transitions as U and HU_2. In addition, the use of a column allowing a significant retention of very polar molecules, such Hypercarb, contributes to improve the selectivity of the method. Table 3 resumes the published methods assessing U and UH_2.

- Linearity and limits of quantification:

Correlation coefficients values were greater than 0.99 for each standard calibration curves in the concentration range from 5 to 320 ng/ml for U and from 10 to 640 ng/ml for UH_2. The LLOQs were 5 ng/ml for U and 10 ng/ml for UH_2. All LLOQs were below the physiological concentrations and were quantifiable with an accuracy and a precision within 20%. ULOQs were above the concentrations observed in DPD deficient patients, namely: 320 and 640 ng/ml for U and UH_2 respectively.

- Carry over, recovery and matrix effect:

By inspecting blank samples used during the validation procedure, no carry over was observed after the injection of calibration standards prepared at the ULOQ. A typical example of chromatogram is presented in Figure 1 for U and UH_2. The mean recoveries were very stable for all the tested concentrations: 65 to 70% for UH_2 and 75 to 80% for U, with low CV% values for the 2 molecules. The mean matrix effects were 10.5 and 10.2% for U $^{13}C^{15}N_2$ and UH_2 $^{13}C^{15}N_2$, respectively.

- Accuracy and precision

Intra and inter-assay precisions (%RSD) varied from 2.8 to 8.8 complying with EMA validation guidelines. Intra and inter-assay accuracies varied from 85.3 to 104.6 and were also within these acceptance criteria. The inaccuracy and CV were less than 5% for intra and inter-assay tests for the threshold value 16 ng/ml.

Recent recommendations proposed threshold values of 16 and 150 ng/ml for uracilemia to characterize a partial or a complete DPD deficiency, respectively. This implies that analytical methods have to be the most accurate, reliable and robust as possible. Indeed, a suboptimal quantification, in particular around threshold values may change or delay patient's care and medical decisions. Generally, inaccuracy and CV less than 15% are the common acceptance criteria to validate each parameter of a method [39]. Unfortunately, the French recommendations do not mention any acceptable inaccuracies for the 2 thresholds. In this case, a range of quantification from 13.6 to 18.4 ng/ml could be expected for the threshold value set at 16 ng/ml. When the recommendations are strictly respected, for patient's care, such range may have different consequences: (i) Wrongly measured less than 16 ng/mL, a patient could be considered as having no DPD deficiency. The clinician may not reduce the dose of 5-FU and the patient could be overexposed with a risk of toxicity;(ii) Wrongly measured more than 16 ng/mL, a patient could be considered as having a partial DPD deficiency. The 5-FU dose may be reduced and the patient could be underexposed with a suboptimal treatment. Practically, to prevent these risks it may be recommended to re-analyze the sample or to explore the deficiency by genotyping. Both solutions delay the treatment whereas it's highly recommended that patients benefit from it as soon as they are diagnosed.

Obviously, a method with very low inaccuracy is needed to explore DPD deficiency. Even if they can provide such performances, the analytical methods reported up to now for the measurement of U and UH_2 are particularly long and tedious (Table 3). Most of these methods required 200 to 500 µL of plasma (except for [33]) and a preparation of the sample with a protein precipitation was always necessary before the extraction. Each also needed a liquid/liquid extraction (LLE) or solid phase extraction (SPE) with multiple manual steps including centrifugation, filtration and evaporation. In this study, we have automated the sample preparation in order to insure and maintain low accuracy. The automated sample preparation required 8 minutes. Then, after the automated transfer of the extract, the chromatographic separation was performed in 15 minutes. About 23 minutes were needed to obtain the first

result. Then, as extraction and separation were performed in parallel, the system produced a result every 15 minutes.

Once the sample is on board of the system, this approach drastically decreases the manpower dedicated to sample preparation. In addition, automation is an ideal solution allowing the analysis of patients' samples rapidly/immediately in labs willing to perform this analysis every day/at all times.

- Clinical practice

The relevance of UH2/U ratio was already review by French group (ref HAS). The UH2/U ratio as a biomarker for severe toxicities under fluoropyrimidines treatment is still controversial. In addition, no consensual value could be set between the different laboratories. The main reason is the presence of interfering compounds of UH2 when analyzed by LC-UV. The genotyping approach for the diagnosis of DPD deficiency focuses on only 4 variants leading to a lack of sensitivity (false negatives). Therefore, the use of U concentrations as phenotypic approach of DPD deficiency is recommended currently by the French health authorities. The validated method here is now used for routine analysis and allowed the comparison of results obtained for 64 patients with a reference method. Our results show reliable quantification. The concentrations of U in 63 patients ranged between 4.9 and 36.6 ng/mL. Seventeen patients have concentrations between 14 and 18 ng/mL. Among these, the 2 methods would have proposed different diagnosis for 5 patients (based on the threshold of 16 ng/ml). The difference varied from 1.8 to 5.6 ng/ml. In this study, 100% and 93.7% of the Bland Altman plots were within the ±2SD interval for or U and UH2 concentrations, respectively. A summary of this study is presented in Figure 2.

4. Conclusion

To our knowledge, a fully-automated LC-MS/MS method for accurate and robust quantification of U and UH_2 has not been published yet. This method could be used in routine labs facing a great increase in this activity since it allows high throughput by running samples continuously when necessary. It is application in routine clinical for the characterization of DPD deficiency is an ideal solution to reduce incidence of errors and thus insure best reproducibility, robustness and reliability [33, 35].

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Table 1: Results of the validation study

Compound	Associated IS	Recovery (%) (%RSD) (n=3)			Intra-assay precision (%) (%RSD) (n=6)			Inter-assay precision (%) (%RSD) (n=6)			Matrix effect
		QC 1	QC 2	QC 3	QC 1	QC 2	QC 3	QC 1	QC 2	QC 3	
Dihydrouracil	Dihydrouracil $^{13}C^{15}N_2$	64.8 (7.7)	68.9 (6.7)	69.6 (4.2)	85.3 (7.7)	86.5 (5.7)	101.3 (8.8)	89.5 (3.0)	93.8 (2.8)	100.6 (4.8)	10.2 (4.9)
Uracil	Uracil $^{13}C^{15}N_2$	76.5 (14.2)	72.0 (5.6)	78.8 (8.9)	104.6 (4.7)	96.6 (4.4)	98.4 (3.1)	100.8 (6.6)	97.1 (3.8)	98.3 (6.0)	10.5 (2.2)

RSD: relative standard deviation; QC: quality control

Table 2: Optimised mass spectrometry parameters

Compounds	Precursor ion		Product ion						Retention time (min)
	m/z	Q1 pre-bias (V)	Quantitation			Reference			
			m/z	Collision energy (V)	Q3 (V)	*m/z*	Collision energy (V)	Q3 (V)	
Dihydrouracil	114.9	-12.0	30.1	-18.0	-13.0	55.0	-23.0	-21.0	4.26
Dihydrouracile $^{13}C^{15}N_2$	118.3	-10.0	55.0	-25.0	-20.0	76.1	-14.0	-12.0	4.28
Uracil	113.1	-14.0	70.1	-19.0	-27.0	96.0	-22.0	-17.0	8.71
Uracil $^{13}C^{15}N_2$	116.2	-10.0	71.1	-19.0	-28.0	98.0	-20.0	-18.0	8.71

Table 3: State of the art for methods assaying U and UH_2 by LC-MS/MS or LC-UV

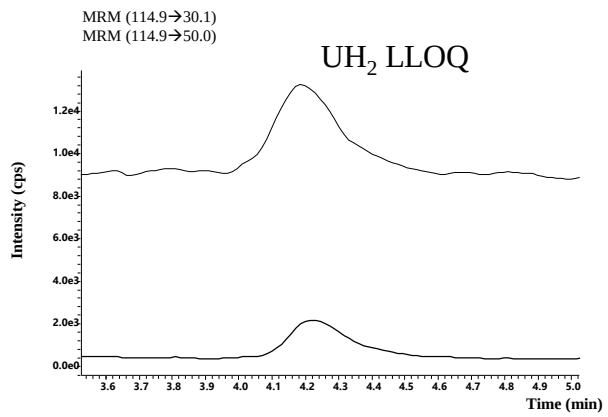
Compound	Matrix	ISTD	Sample preparation	Extraction	Column	LC run (min)	Mobile phase	Detection mode	Limit of quantification (ng/ml)	Reference
U, UH_2, 5FU, $5FUH_2$, FUPA, Fβala	Plasma 50 µL	U-$^{13}C^{15}N_2$ UH_2-$^{13}C^{15}N_2$ 5FU-$^{13}C^{15}N_2$ FUH_2-$^{13}C^{15}N_2$ FUPA-$^{13}C_3$ Fβala-$^{13}C_5$	Prot prec: 100 µL $ZnSO_4$ 1M	L/L (*2): 600 µL 95% Acetonitrile/5% Methanol 50 µL Acetic acid 0.25 M 400 µL 15% Isopropanol/85% Ethyl acetate	Luna-PFP	8	0.25% Acetic acid/0.05% Formic acid/99.7% Water Acetonitrile	LC-MS/MS ESI+	U = 5 UH_2 = 10 5FU = 50 FUH_2 = 50 FUPA = 50 FBala = 50	32
U, UH_2	Plasma 300 µL	U-$^{15}N_2$ UH_2-$^{13}C_4^{15}N_2$	Prot prec: 900 µL 50% Acetonitrile/50% Methanol	Dryness, recovery and centrifugation	Acquity UHPLC HSS T3	5	0.1% Formic acid Acetonitrile/0.1% Formic acid	LC-MS/MS ESI : U : - UH2: +	U = 1 UH_2 = 10	31
U, UPA, βala	Plasma 300 µL	U-$^{13}C_4^{15}N_2$ UPA βala U-$^{13}C_3^{15}N$	Dilution: 4 times Prot prec: 1.8 mL Ethanol	Centrifugation and ultrafiltration	Synergi Hydro-RP	18	50 mM Ammonium acetate (pH 2.8)/10% Methanol 50 mM ammonium acetate (pH 2.8)/50% Methanol	LC-MS/MS ESI +	U = 73 UPA = 616 Bala = 1157	30
U, UH_2, 5FU, $5FUH_2$	Plasma 200 µL	U-$^{13}C_2$ UH_2-$^{13}C_4^{15}N_2$ 5FU-$^{15}N_2$	Prot prec : 200 µL $H_2SO_4NH_3$	L/L (*2) : 10% Isopropanol/90% Ethyl acetate	C18 Atlantis	10	1 mM Ammonium acetate/0.5 mM formic acid/3.3% Methanol	LC-MS/MS ESI+	U = 4.1 UH_2 = 12.1 5FU = 13 FUH_2 = 100	29
U, UH_2	Plasma 500 µL	U-Cl	Prot prec: 50 µL phosphonic acid	L/L: 40% Ethyl acetate/30% Dichloromethane/30% Methyl-ter-butyl-ether	C18X-Terra	5	Methanol/0.1% Sodium hydroxide (15:85)	LC-MS/MS ESI-	U=5 UH_2 =10	28
U, UH_2	Plasma 500 µL	U-Br	Prot prec : 300 mg $H_2SO_4NH_3$	L/L : 15% Isopropanol/85% Ethyl acetate and derivation	Acquity UPLC BEH	4.5	0.5% Acetic acid 99.5% Acetonitrile/0.5% acetic acid	LC-MS/MS ESI +	U = 0.625 UH_2 = 0.625	27
U, UH_2	Plasma 500 µL	5FC $5B_8C$	Prot prec: 500 µL KH_2PO_4 10 mM	SPE: Atoll Xtrem 500 µL Methanol for elution, dryness, 200 µL mobile phase A	C18 Atlantis	12	Water/0.1% Acetic acid Acetonitrile	LC-MS/MS ESI+ LC-UV	U = 4.1 UH_2 = 12.1	26
U, UH_2	Plasma 200 µL	U-Br	Prot prec: 150 mg $(NH_4)_2SO_4$	L/L : 5 mL 85% Ethyl acetate/15% Isopropanol.	Discovery Amide C16		3% Methanol	LC-MS/MS ESI -	U = 0.5 UH_2 = 5	25
U, UH_2	Plasma 500 µL	5FU	Prot prec: 600 mg $(NH_4)_2SO_4$	Centrifugation, dryness, 120 µL water, filtration.	Hypercarb	42	Water Acetonitrile	LC-UV	U = 1.25 UH_2 = 0.675	24
U, UH_2	Plasma 500 µL	5FU	Prot prec: 500 mg $(NH_4)_2SO_4$	L/L : 4 mL 85% Ethyl acetate/15% Isopropanol	Hypercarb	30	Water Acetonitrile	LC-UV	U = 0.2 UH_2 = 0.2	23
U, UH_2	Plasma 1 mL	5-bromo-2′-deoxyuridine	Prot prec: 1 mL perchloric acid 1 M	SPE: non polar high capacity polymeric sorbent	RP-18 Supelcosil		50% Acetonitrile 0.1% trifluoroacetic acid	LC-UV		22

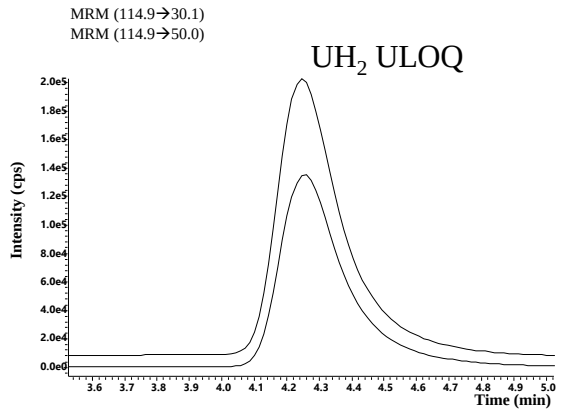
				One column volume Methanol for elution. Dryness.150 µL water						
U, UH_2	Plasma 500 µL	5FC	Prot prec: 500 µL KH_2PO_4 10 mM	SPE: Atoll Xtrem 1000 µL ammonium formate buffer (10 mM, pH 5.0) for elution, dryness, 200 µL mobile phase A	dC18 Atlantis	15	10 mM potassium phosphate buffer (pH 3.0)	LC-UV	U = 2.5 UH_2 = 6.75	21
U, UH_2	Plasma 500 µL	FUH_2	Prot prec: 600 mg $(NH_4)_2SO_4$	L/L : 4 mL 85% Ethyl acetate/15% Isopropanol	Hypercarb	77	Water Acetonitrile	LC-UV	U = 1.25 UH_2 = 0.675	20
U, UH_2, 5FU	Plasma 500 µL	U-Br	Prot prec: 20 µL orthophosphorique acid (5%)	L/L : 6 mL 16% Propanol/84% Ether	RP-18X Terra	35	50 mM potassium phosphate, 0.1% triethylamaine	LC-UV	5FU = 5	19
U, UH_2	Plasma 500 µL	U-Cl	Prot prec: 200 µL $(NH_4)_2SO_4$	L/L : 6 mL 85% Ethyl acetate/15% Isopropanol	Spherisorb ODS2	45	10 mM potassium phosphate buffer (pH 3.0)	LC-UV	U = 2 UH_2 = 10	18
U, 5FU	Plasma 500 µL	U-Br U-Cl	Prot prec: 600 mg $(NH_4)_2SO_4$	L/L : 1200 µL 85% Ethyl acetate/15% Isopropanol	Spherisorb ODS2 Phase Sep	25	10 mM potassium dihydrogen phosphate (pH 3.0)	LC-UV	5FU = 6	17

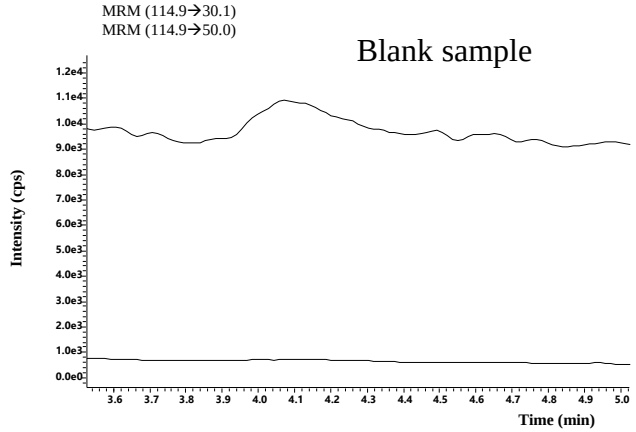
Prot prec: protein precipitation; L/L: liquid/liquid; SPE : solid phase extraction ; LC : liquid chromatography ; MS/MS : tandem mass spectrometry; UV: ultraviolet; ESI: electrospray ionization

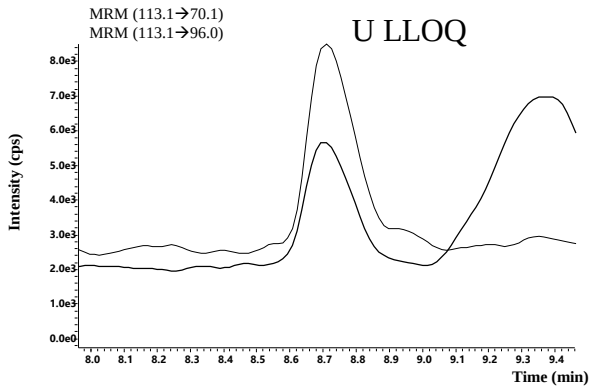
Figure 1: Typical MRM chromatograms of uracil (bottom) and dihydrouracil (top) at the lower and upper limits of quantification (LLOQ and ULOQ). Blank samples were obtained after ULOQ.

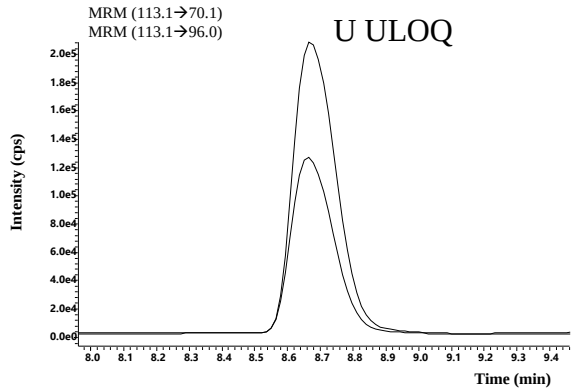
Figure 2: Bland Altman analysis for uracil and dihydrouracil quantified with the newly procedure and a validated LC-MS method with manual sample preparation.
d: mean difference
ssd: standard deviation

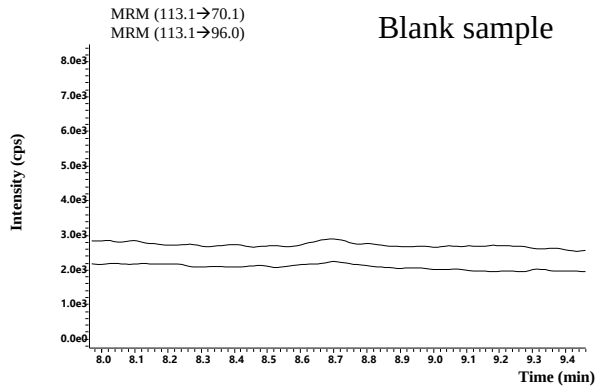

MRM (114.9→30.1)
MRM (114.9→50.0)
UH2 LLOQ
Intensity (cps)
Time (min)

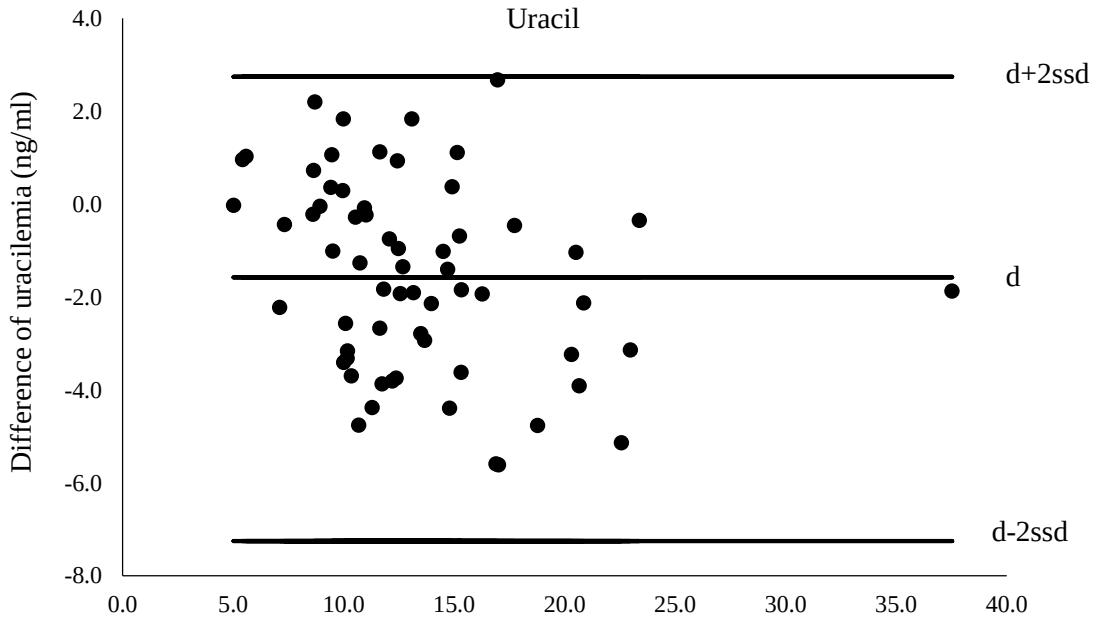

MRM (114.9→30.1)
MRM (114.9→50.0)
UH2 ULOQ
Intensity (cps)
Time (min)

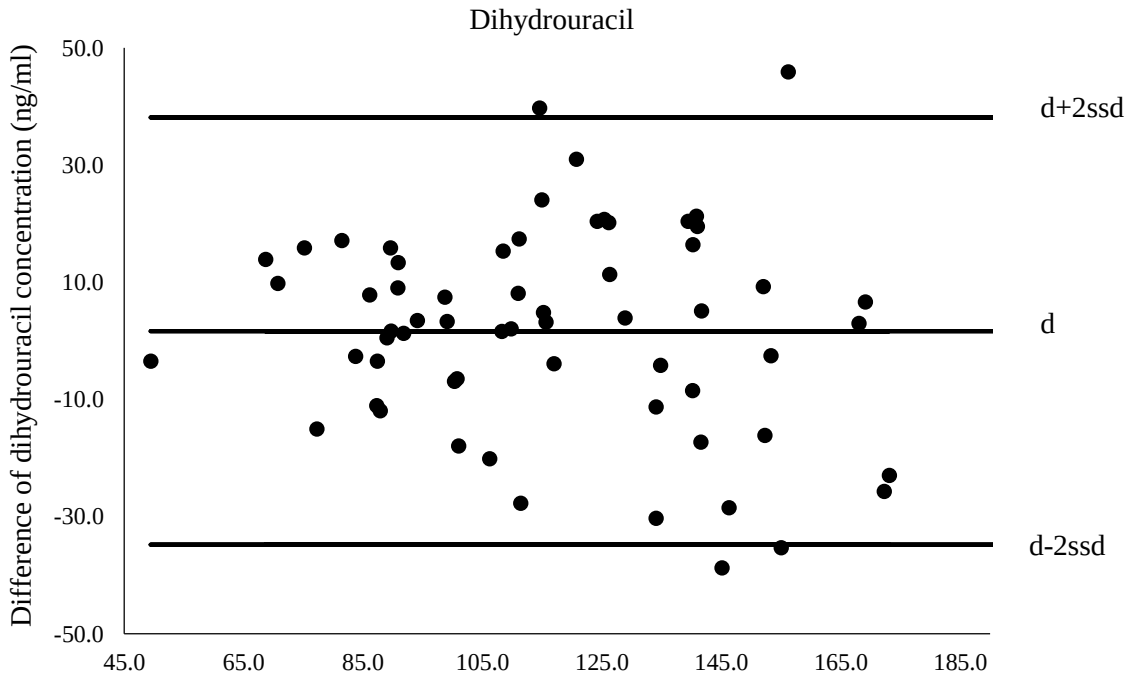

MRM (114.9→30.1)
MRM (114.9→50.0)
Blank sample
Intensity (cps)
Time (min)


MRM (113.1→70.1)
MRM (113.1→96.0)
U LLOQ
Intensity (cps)
Time (min)


MRM (113.1→70.1)
MRM (113.1→96.0)
U ULOQ
Intensity (cps)
Time (min)


MRM (113.1→70.1)
MRM (113.1→96.0)
Blank sample
Intensity (cps)
Time (min)


Uracil
Difference of uracilemia (ng/ml)
4.0
2.0
0.0
-2.0
-4.0
-6.0
-8.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
d+2ssd
d
d-2ssd


Dihydrouracil
Difference of dihydrouracil concentration (ng/ml)
50.0
30.0
10.0
-10.0
-30.0
-50.0
45.0
65.0
85.0
105.0
125.0
145.0
165.0
185.0
d+2ssd
d
d-2ssd