

Validation of an Ultra-High Performance Liquid Chromatography Tandem Mass Spectrometric Method for Quantifying Uracil and 5,6-Dihydrouracil in Human Plasma

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A simple, rapid, sensitive and specific ultra-high-performance liquid chromatography–tandem mass spectrometry method (Waters UPLC–MS–MS) is developed and validated for the quantification of uracil (U) and 5,6-dihydrouracil (UH2) levels in human plasma. Analytes are extracted using ethyl acetate and isopropanol after deproteination, and separated by high-performance liquid chromatography (HPLC) (Acquity UPLC BEH C18 column) in a binary mobile phase system under gradient elution conditions at a flow rate of 0.4 mL/min. 5-Bromo-uracil (UBr) is used as the internal standard. The detection is performed on a triple-quadrupole mass spectrometer via electrospray positive ionization. Multiple reaction monitoring mode using the transitions m/z 112.82 → 70.05, m/z 114.88 → 55.04 and m/z 190.83 → 117.86 is used to quantify U, UH2 and UBr, respectively. The method is linear in the concentration range of 0.625–160.0 ng/mL. The total run time is 4.5 min per injection. Nine-point calibration curve and four-points quality controls are used. Excellent linearity and precision are observed with correlation coefficient (r^2) > 0.9999. The intra-batch and inter-batch precisions are ≤ 7.3% and ≤ 8.6%, and accuracy is ≤ 17%. The developed method is shown to be suitable for routine quantitative determination of U, UH2 and 5,6-dihydrouracil-to-uracil ratio in clinical practice.

Introduction

Uracil (U) is catabolized to 5,6-dihydrouracil (UH2) by the enzyme dihydropyrimidine dehydrogenase (DPD). DPD has the highest activity in liver and peripheral blood mononuclear cells. This enzyme also metabolizes 5-fluoro-uracil (5-FU), a first-line antimetabolic chemotherapy drug commonly used to treat digestive, breast, head and neck cancer (1). However, DPD is subject to genetic polymorphism and its activity shows a broad range of individual variation. DPD activity thus ranges from partial (3–5% of the population) to complete loss (0.2% of the population), resulting in severe polyvisceral 5-FU-induced toxicity (2, 3). The determination of the UH2/U ratio in biological fluids can pinpoint a DPD deficiency (4–8). The 2-¹³C-uracil breath test (9) can be performed for evaluation of the pyrimidine catabolic pathway, but it is difficult to implement, requires equipment installed in only a few specialized units, and 2-¹³C-uracil is not currently available in many countries.

The most frequently used methods for determining U and UH2 are classical liquid chromatography (LC) with ultraviolet (UV) detection (10–15). However, these methods have low detection sensitivity and are time-consuming with a solid-phase extraction (SPE) pretreatment (12–15) combined with a long run-time of up to 45 min (10, 11). Tandem mass spectrometric (MS) detection reduces the lower limit of quantification in biological samples (5, 15, 16). The ultra-high-performance liquid chromatography (UHPLC) system, using sub-2 μm hybrid columns and with the ability to deliver mobile phase at high pressures (> 8,000 psi) with low dispersion, provides better chromatographic peak resolution, more sensitive analyses and reduced run-time and solvent consumption. To the best of our knowledge, UHPLC–MS–MS has not been used for the quantification of U and UH2 levels in plasma.

In this study, we developed a UHPLC system coupled with tandem mass spectrometric detection to determine U and UH2 simultaneously in human plasma with high specificity, improved resolution, increased sensitivity (0.625 ng/mL) and shorter analysis times (4.5 min) than conventional liquid chromatographic systems.

Experimental

Chemicals and reagents

Acetonitrile, ethyl acetate and 2-propanol, all of LC–MS quality grade, uracil, 5,6-dihydrouracil, 5-bromo-uracil (UBr) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). Ammonium sulfate was obtained from Merck (Darmstadt, Germany). The water was produced locally by a Milli-Q system (Millipore; Saint-Quentin-en-Yvelines, France). All drug-free plasma samples were obtained from the Etablissement Français du Sang (Hôpital Européen Georges-Pompidou; Paris, France). All plasma samples were stored at –20°C and protected from light until assayed. The mobile phases were filtered through a 0.22-μm filter (Millipore) and degassed in an ultrasonic bath (Selecta; Barcelona, Spain).

Instrumentation

The Waters Acquity TQD system consisted of an Acquity UPLC system (Waters; Milford, MA) with a cooling autosampler, column oven and an Acquity triple-quadrupole tandem mass spectrometer with an electrospray ionization (ESI) interface

(Waters). All data were acquired and processed by MassLynx 4.1 software with the QuanLynx program (Waters).

UHPLC–MS–MS conditions

The mobile phase was a mixture of water with 0.5% acetic acid (solvent A) and acetonitrile with 0.5% acetic acid (solvent B). The solvents were delivered at a flow rate of 0.4 mL/min in the following linear gradient mode: 100% of A from 0 to 2 min, followed by 100 to 0% of A from 2 to 2.5 min and 0 to 100% of A from 2.5 to 4 min. The column (Acquity UPLC BEH C18 column (100 × 2.1 mm, i.d., 1.7 μm), obtained from Waters Corporation (Wexford, Ireland), was equilibrated from 4 to 4.5 min with 100% of A. The column temperature was maintained at 25°C. The autosampler was conditioned at 10 ± 0.5°C and the sample volume injected was 20 μL.

Injection wash solvents were water containing 0.5% acetic acid (v/v) and acetonitrile containing 0.5% acetic acid (v/v) for weak and strong wash, respectively.

The mass spectrometer was operated with an ESI interface in positive ionization mode. The cone and desolvation gas flow rates were 50 and 900 L/h, respectively, and were obtained from in-house nitrogen. High purity argon was used as collision gas at a flow rate of 0.12 mL/min.

The optimal MS parameters were as follows: capillary voltage of 3 kV, source temperature 120°C and desolvation temperature 350°C. Cone potential was 35 V and collision energy was 15 eV. The scan time was set at 0.07 s per transition.

Following Svobaitė *et al.* (15) and Jiang *et al.* (16), we did not use costly labeled internal standards. We chose UBr as internal standard (IS) for its chemical structure, similar to that of the two assayed compounds, and its low cost.

Preparation of standard and quality control samples

Standard stock solutions of U, UH2 and IS (200 μg/mL) were prepared separately in water, further diluted and stored at –20°C. Standard solutions of U, UH2 (1 μg/mL) and IS (0.1 μg/mL) were prepared by diluting stock solution with water. All solutions were stored at –20°C and brought to room temperature immediately before use.

Following Jiang *et al.* (16), calibration curves were plotted by spiking 3% BSA with various quantities of U and UH2 standard solutions. The final concentrations in the calibration samples were 0.625, 1.25, 2.5, 5, 10, 20, 40, 80 and 160 ng/mL for U and UH2. The quality control (QC) samples were prepared in the same way as the calibration samples from the standard stock solutions prepared by different investigators. The QC sample concentrations were 2.5, 16, 64 and 100 ng/mL.

Extraction procedure

In 5-mL glass tubes, 300 mg of ammonium sulfate (to precipitate the proteins), 500 μL of each sample (standard samples, QC samples or human plasma) and 5 μL of IS solution (0.1 μg/mL) were added and vortexed for 1 min; 2.5 mL of ethyl acetate–isopropanol (85:15, v/v) were added, mixed for 10 min on a cyclomixer and centrifuged for 15 min at 3,200 g at 4°C. The organic layer (2 mL) was separated and evaporated to dryness under vacuum in a Speedvac concentrator system

(Savant Instrument, Farmingdale, NY). The residue was reconstituted with 200 μL of the solvent A, vortexed for 1 min and centrifuged for 15 min at 3,200 g. The supernatant was transferred into an autosampler vial and injected (20 μL) into the UHPLC–MS–MS system.

Method validation

A full validation was performed according to the US Food and Drug Administration (FDA) guidance on bioanalytical method validation (17). Following Remaud *et al.* (14) and Jiang *et al.* (16), standard samples and QC samples were not prepared in plasma because this biological matrix is not analyte-free. All results are expressed as mean ± standard deviation (SD).

Linearity

Each calibration standard was prepared in 3% BSA samples and analyzed on seven different days. The regression equations for U and UH2 underwent unweighted linear regression analysis of peak area ratios to internal standard against the spiked concentrations. Slope, Y-intercept and correlation coefficient were calculated using MassLynx 4.1 software.

Precision and accuracy

Intra-day accuracy and precision were determined by replicate analysis ($n = 6$) of each of the four QC samples (2.5, 16, 64 and 100 ng/mL) performed on the same day. Inter-day accuracy and precision were determined by analysis of the same QC concentrations on seven different days. The precision of the method was determined by the relative standard deviation (RSD). Accuracy was calculated as the percent deviation of the mean calculated concentration from the nominal concentration (RE: relative error). Accuracy and precision had to be below 15%.

Lower limit of quantification and limit of detection

The lower limit of quantification (LLOQ) should be at least five times the blank response. The response should be reproducible with an accuracy and precision below 20%. The limit of detection (LOD) was determined by serial dilutions of working solutions to obtain a signal/noise ratio of $\approx 3:1$ (17). Each point was measured six times.

Extraction efficiency and matrix effect

Three individual extracts per calibration standard and three diluted replicates in water at the same concentration were injected onto the column. The assay efficiency was determined as follows: (absolute peak area of extract/absolute peak area of unextracted samples) × 100.

Two common methods are used to assess matrix effect: the post-column infusion method (18) and the post-extraction spiked method (19). In our study, ion suppression attributable to matrix effect was investigated by a post-column infusion experiment, providing a qualitative assessment with identification of chromatographic regions most likely to experience a matrix effect. Each U, UH2 or IS solution (10 μg/mL) was continuously infused post-column (20 μL/min) and mixed with the column effluent before entering the mass spectrometer. Extracted blank drug-free BSA and plasma samples were

injected into the UHPLC–MS–MS and eluted as previously described.

Stability

The stability of stock solutions of U and UH2 kept at -20°C for three months was evaluated after diluting each with mobile phase and comparing their peak areas with those of freshly prepared samples at the same nominal concentrations.

The stability was determined in spiked 3% BSA solutions by comparing the peak area ratios of freshly prepared samples with those obtained after stability testing at low (16 ng/mL), medium (64 ng/mL) and high (100 ng/mL) U and UH2 concentrations. The stability was evaluated under five different conditions: freeze and thaw stability for three cycles, short-term temperature stability at room temperature for 6 h, long-term temperature stability at -20°C for 30 days in spiked 3% BSA and ready-to-inject stability in the autosampler at 10°C for 6 h.

The stability was also evaluated in human blood samples from 10 anonymous healthy individuals stored at room temperature or maintained at $+4^{\circ}\text{C}$ for 72 h.

Clinical application

The method was used to quantify U and UH2 plasma levels in 26 anonymous patients with advanced or recurrent cancer, before undergoing 5-FU therapy. All human blood samples were collected in EDTA tubes, centrifuged promptly after collection (< 30 min) at 3,200 g for 10 min, and two aliquots of 0.5 mL of plasma were transferred to Eppendorf polypropylene tubes. Plasma samples were then stored at -20°C until analysis.

Results and Discussion

Choice of sample: urine or plasma?

The concentration of U and UH2 in urine is influenced by drugs with pharmacological effects (20). Jiang *et al.* (16) and van Kuilenburg *et al.* (21) showed that U and UH2 were more concentrated in blood than in urine and that the detection limit was 10 to 100 times lower in plasma than in urine. Also, the most recent studies (3, 15, 16, 22, 23) conducted to investigate the predictive value of U concentration and/or the UH2/U ratio for the prediction of adverse side effects were performed on plasma. For these reasons, and as members of a national network to develop external QC and implement better monitoring of patients, we opted to work on human plasma.

Method development

Sample preparation

Extraction and validation procedures were developed using 3% BSA because human plasma is not analyte-free and no analyte-free samples of the authentic matrix were available. It is reported in the literature that the use of SPE procedures reduces the matrix effect, but increases overall time and cost of analysis (24).

Extraction was carried out according to Jiang *et al.* (16) using a small amount of ammonium sulfate before extraction and purification by ethyl acetate–isopropanol (85:15, v/v). We used this simple liquid–liquid extraction procedure, which was fast enough for high-throughput analysis. In addition, the use of UBr as IS helped us make quantification independent of the matrix.

Chromatography

C18, silica, Hilic and Bridged Ethylene Hybrid (BEH) columns were tested and the best results were obtained with BEH. The classical water–acetonitrile–acetic acid mobile phase was retained for this separation.

The developed UHPLC–MS–MS method, owing to the very high pressure applied ($> 8,000$ psi), was rapid and convenient, with a single run-time less than 4.5 min, and displayed a high sensitivity for both U and UH2. The specific physical properties and retention mechanisms of the 1.7- μm BEH particles increase the chemical stability of the column, explaining the very good reproducibility of the retention time (RT) between the beginning and end of column life, with over 1,250 samples on the same column (U and UH2, RT = 1.01 ± 0.02 min; UBr, RT = 2.65 ± 0.02 min; $n = 30$, randomly selected). The reproducibility was also excellent between three different columns from three different batches (U and UH2, RT = 1.01 ± 0.02 min; UBr, RT = 2.64 ± 0.03 min; $n = 30$, randomly selected). All of these characteristics obviate tedious adjustments of the MS–MS acquisition parameters.

Mass spectrometry

Measurements of U, UH2 and IS levels in samples of human plasma were made in multiple reaction monitoring (MRM) scan mode. Solutions of U and UH2 were directly infused into the mass spectrometer. Mobile phase and MS parameters were optimized to obtain maximum sensitivity for respective product ions.

Protonated molecular ions for U, UH2 and IS were at m/z 112.82, 114.88 and 190.83, respectively. To use the most intense product ion of each compound, we selected the following transitions for MRM acquisition: U m/z 112.82 \rightarrow 70.05; UH2 m/z 114.88 \rightarrow 55.04; and IS m/z 190.83 \rightarrow 117.86 (Figure 1). The product ion of $[\text{U} + \text{H}]^+$ (m/z 113) could be explained by the loss of HNCO (m/z 43), while that of $[\text{UH2} + \text{H}]^+$ (m/z 115) could be explained by the loss of NH_2CONH_2 (m/z 60). Typical MRM chromatograms obtained from the drug-free BSA, 3% BSA spiked with standard U and UH2, and human plasma samples are shown in Figure 2, indicating that 3% BSA was suitable for preparing standard curve samples for quantification (Figure 2A), and obtaining an adequate signal/noise ratio for low plasma levels of analytes (Figure 2B), allowing the determination of U and UH2 concentration in human plasma.

Method validation

All the previously described conditions enabled us to improve parameters such as analysis time, sensitivity, precision and accuracy compared with previously published methods (10–15)

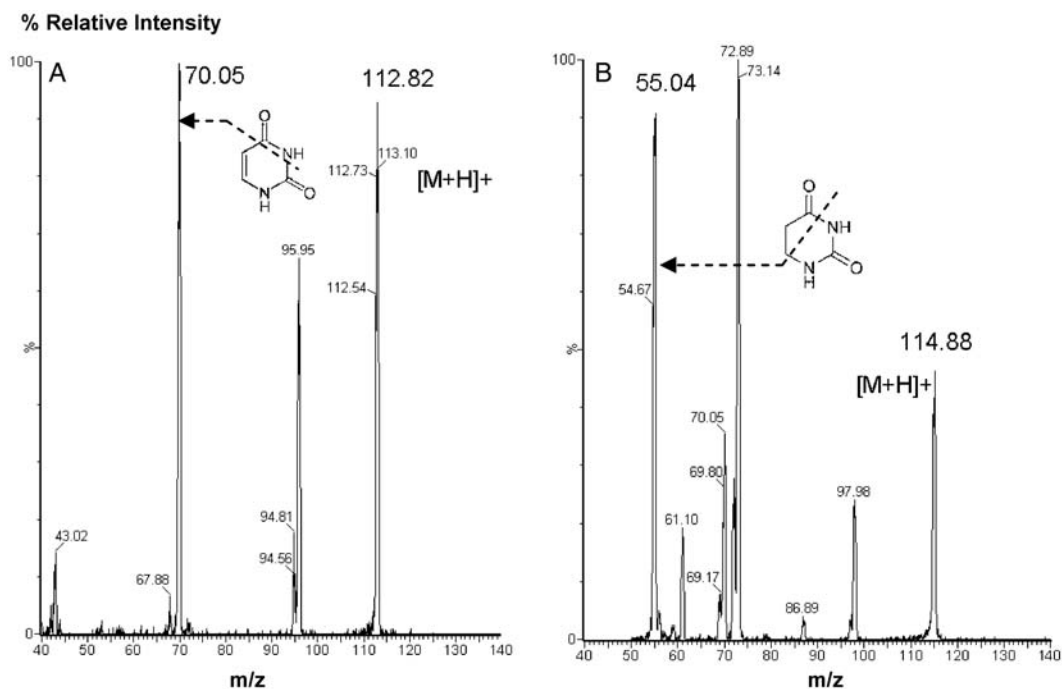


Figure 1. Product ion mass spectra for uracil (A); 5,6-dihydrouracil (B).

MS-MS detection was chosen to ensure high sensitivity and to avoid endogenous and exogenous interference (i.e., coadministered drugs) as currently observed with UV detection and sometimes in single-ion monitoring mode MS.

The range of concentrations in the calibration samples, which were the same for U and UH2, made the procedure easier and spanned the physiological values of the two compounds of interest.

Linearity

The standard calibration curves were linear in the concentration range 0.625–160 ng/mL ($r^2 > 0.9999$). Representative regression equations for the calibration curve ($n = 7$) were $y = 0.0269x + 0.0478$ ($r^2 = 0.9994$) and $y = 0.0077x + 0.0344$ ($r^2 = 0.9988$) for U and UH2, respectively.

Precision and accuracy

Data for intra-day and inter-day precision and accuracy of the method are given in Table I. For U, the inter-day and intra-day ranged from 0.7 to 6.7% and 2.7 to 4.4%, respectively, and RE ranged from -4.9 to +2.7 and -4.2 to +4.1, respectively. For UH2, the inter-day and intra-day ranged from 2.7 and 16.5% and 6.0 to 9.8%, respectively, and RE ranged from -13.2 to -1.7 and -13.8 to +13.4, respectively. The results indicated that all values were within the acceptable ranges, and the method exhibited good precision and accuracy.

LLOQ and LOD

The LLOQ was 0.625 ng/mL for both U and UH2. Estimated with a signal-to-noise ratio of 3:1, the LOD was approximately 0.2 ng/mL.

These results are markedly better than those obtained by UV detection (11–14). On the other hand, our levels are lower

than those reported by Svobaite *et al.* (15) at 2.5 and 3.1 ng/mL for U and UH2, respectively, and by Jiang *et al.* (16) for UH2 (5.0 ng/mL); our LOQ for U determination is close to that of Jiang *et al.* (0.625 versus 0.500 ng/mL).

Extraction recovery and matrix effect

Classically, the solvent front and the end of the elution gradient are most strongly affected by interferences. Here, such interference had no impact on the assay accuracy; the RT values of our compounds (1.01 and 2.64 min) were in the safe chromatographic window (with no matrix effect between 0 and 4 min).

At each concentration between 0.625 and 160 ng/mL in 3% BSA, recovery values were $41.2 \pm 5.9\%$, $47.0 \pm 5.2\%$ and $67.7 \pm 2.8\%$ for U, UH2 and UBr, respectively.

The concentration of BSA can be very variable. Two concentrations, 80 and 30 g/L, were proposed and validated by Svobaite *et al.* (15) and Jiang *et al.* (16). Our own tests at both levels showed no significant difference (data not shown). We selected a concentration of 30 g/L for convenience, lower cost and a concentration near physiological values of albumin in human plasma, because U, UH2 and 5-FU are primarily bound to albumin.

Stability

The stock solutions of U, UH2 and IS were stable at -20°C for at least three months. Table II summarizes the results of all the stability studies, which all met the criteria for stability measurements under the conditions indicated. Table III shows the variations of U and UH2 concentrations when human blood samples are either maintained at room temperature ($+22^\circ\text{C}$) or stored at $+4^\circ\text{C}$ before the centrifugation.

Unexpectedly, there was no degradation of either compound. Both U and UH2 concentrations increased with time

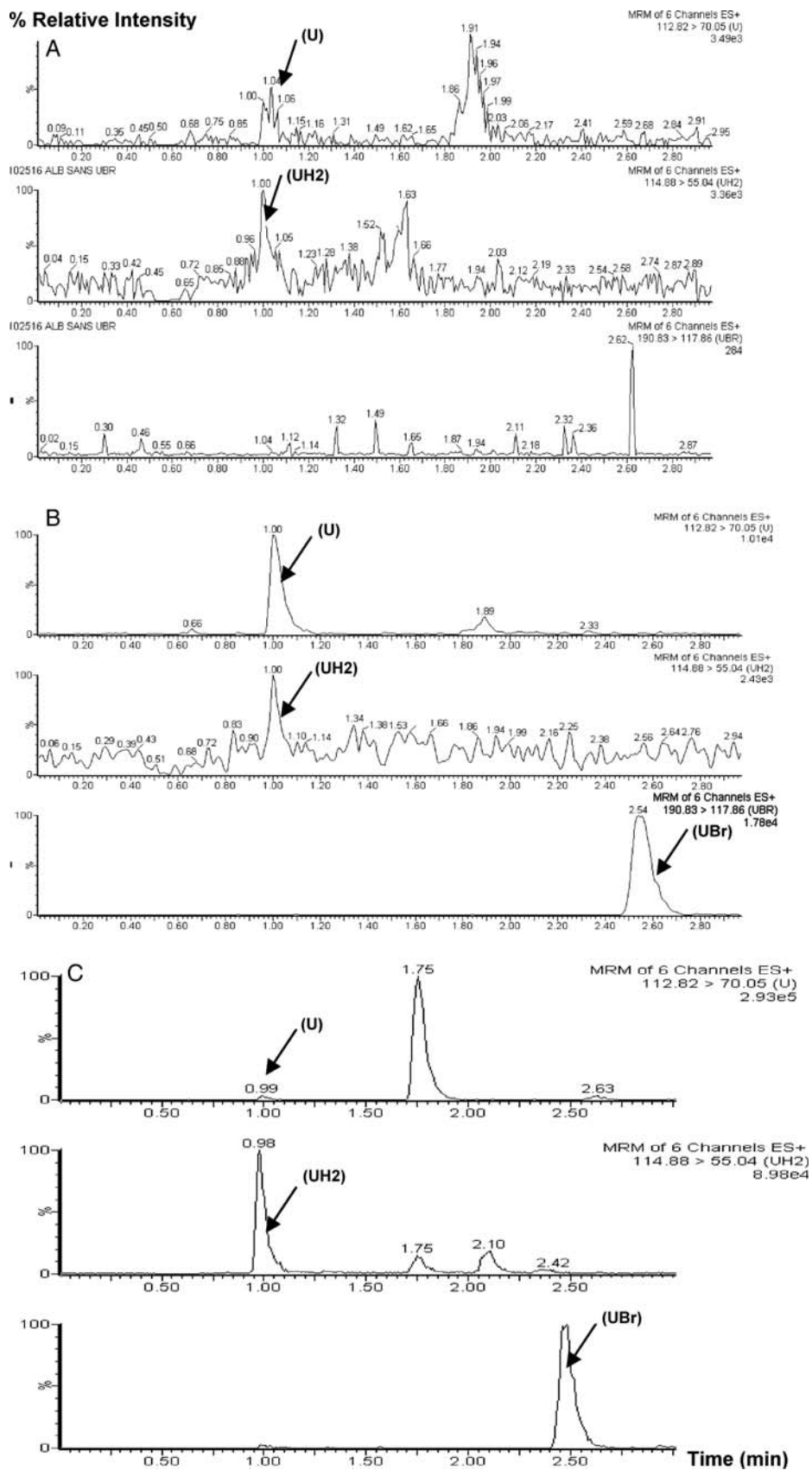


Figure 2. MRM chromatograms of U, UH2 and (respective transitions: m/z 112.82 \rightarrow 70.05; m/z 114.88 \rightarrow 55.04; m/z 190.83 \rightarrow 117.86): a blank 3% BSA sample without UBr (A); a sample spiked with U and UH2 (2.5 ng/mL each) and UBr (B); a human plasma sample containing U (8.4 ng/mL) and UH2 (157.0 ng/mL) (C).

Table I
Intra-Day and Inter-Day Precision and Accuracy for the Quantification of Uracil and 5,6-Dihydrouracil

Spiked concentration (ng/mL)		RSD (%)	RE (%)
Intra-day (n = 5)			
U	2.5	3.5	+ 1.5
	16	4.4	+ 2.1
	64	2.7	-4.8
	100	3.1	+ 4.1
UH2	2.5	9.8	+ 13.4
	16	7.3	-11.8
	64	6.6	-13.8
	100	6.0	-12.6
Inter-day (n = 7)			
U	2.5	3.2	-1.2
	16	6.7	-4.9
	64	4.9	-1.2
	100	0.7	+ 2.7
UH2	2.5	16.5	-7.9
	16	3.5	-1.7
	64	8.6	-13.2
	100	2.7	-6.5

both at ambient temperature and at +4°C with a rapid increase in concentrations after 120 min (+27.2% ± 15.1 and +10.1% ± 10.8, respectively, for U and UH2 at ambient temperature) and a high inter-individual variability. This increase, which could be very marked (up to +13,77% for U at 72 h), was probably due to an *in situ* production of U and UH2, possibly by a process of release from their transport protein, albumin, or from DNA and/or pyrimidines degradation.

In addition, because of the difference in percent variation of U and UH2 concentrations, especially at +4°C (+42% versus +384% at 72 h for U and UH2, respectively), the use of the UH2/U ratio does not address these variations.

Our results agree with those of Déporte *et al.* (12) and Remaud *et al.* (14), who observed that both U and UH2 concentrations increased with time, with a rapid increase in U

Table III
Variation in U and UH2 Values During Storage under Controlled Conditions

Time	Mean U variation ± SD (n = 10)	
	Ambient temperature	+ 4°C
15 min	+ (4.6 ± 11.7)%	
30 min	+ (7.4 ± 10.0)%	
60 min	+ (11.3 ± 11.1)%	
90 min	+ (16.8 ± 10.4)%	
2 h	+ (27.2 ± 15.1)%	
5 h	+ (163 ± 26.8)%	+ (2.2 ± 10.1)%
24 h	+ (169 ± 9.1)%	
72 h	+ (1,377 ± 69.6) %	+ (383 ± 98.2)%
Mean UH2 variation ± SD (n = 10)		
	Ambient temperature	+ 4°C
15 min	+ (4.3 ± 6.1)%	
30 min	+ (9.9 ± 11.3)%	
60 min	+ (10.0 ± 9.9)%	
90 min	+ (10.2 ± 12.5)%	
2 h	+ (10.1 ± 10.8)%	
5 h	+ (46.1 ± 21.5)%	-(1.1 ± 5.9)%
24 h	+ (108 ± 7.8)%	
72 h	+ (867 ± 30.7)%	+ (42 ± 8.3)%

concentrations from day to day at +4°C and at ambient temperature, and nevertheless, a relative stability of UH2 concentrations for 14 days.

Finally, we recommend blood collection between 08:00 h and 10:00 h (in the fasted state) to minimize the influence of DPD circadian variations (25), immediate centrifugation of samples (3,200 g and +4°C for 10 min) and storage of plasma at -20°C until analysis.

Clinical Application

The developed LC-ESI-MS-MS method was successfully applied to determine UH2-to-U ratio (UH2/U) in 26 anonymous healthy subjects.

Table II
Stability of Uracil and 5,6-Dihydrouracil in Samples at Three Different Concentrations, Low (L), Medium (M) and High (H), Exposed to Various Storage Conditions (Mean ± SD, n = 3)

		Three freeze/thaw (spiked BSA)		Room temperature for 6 h (spiked BSA)		-20°C for 30 days (spiked BSA)		Sample rack for 6 h at 10°C after extracting and reconstitution (spiked BSA)	
		U	UH2	U	UH2	U	UH2	U	UH2
Before	L	15.7 ± 0.1	15.8 ± 0.2	16.6 ± 1.3	15.8 ± 1.4	16.3 ± 0.7	14.1 ± 1.0	14.4 ± 0.3	14.9 ± 0.2
	M	63.0 ± 0.5	58.5 ± 0.2	64.4 ± 4.4	63.3 ± 3.1	60.9 ± 1.6	55.2 ± 3.6	55.2 ± 0.6	56.2 ± 0.3
	H	109.0 ± 0.5	106.6 ± 3.5	99.5 ± 1.0	93.9 ± 6.0	107.1 ± 1.5	108.2 ± 4.7	111.2 ± 1.3	101.7 ± 1.5
After	L	16.6 ± 0.7	15.6 ± 1.9	17.1 ± 0.8	16.6 ± 0.8	15.4 ± 2.0	15.2 ± 1.5	14.9 ± 0.2	13.7 ± 0.5
	M	62.9 ± 1.4	56.9 ± 101.5	65.7 ± 1.2	58.9 ± 1.3	62.1 ± 6.0	56.7 ± 5.5	59.9 ± 0.2	59.2 ± 1.9
	H	113.0 ± 0.6	101.5 ± 2.9	100.1 ± 1.1	93.7 ± 3.8	113.2 ± 2.9	97.1 ± 8.6	116.3 ± 7.7	100.6 ± 2.3
RSD	L	4.3%	11.8%	4.4%	4.9%	13.0%	10.1%	13.0%	3.5%
	M	2.2%	2.7%	1.8%	2.2%	9.7%	9.7%	0.3%	3.1%
	H	0.5%	1.9%	1.1%	4.1%	2.8%	8.8%	6.6%	4.1%
RE	L	-5.7%	+ 1.0%	+ 3.4%	-5.8%	+ 3.9%	-4.6%	-3.2%	+ 8.6%
	M	+ 0.2%	+ 2.7%	-2.2%	+ 6.9%	-3.0%	+ 2.4%	-8.6%	-5.3%
	H	-3.6%	+ 2.6%	-0.6%	+ 0.1%	+ 3.2%	+ 11.9%	-4.6%	+ 0.7 %

Table IVMean \pm SD Plasma Concentrations (ng/mL) of U, UH2 and UH2/U Ratio in 26 Patient Samples

	Mean	SD	Median	Min.	Max.
U	9.7	7.7	8.4	0.6	43.3
UH2	100.3	35.9	110.9	13.6	146.3
UH2/U	13.1	5.0	13.4	0.4	26.2

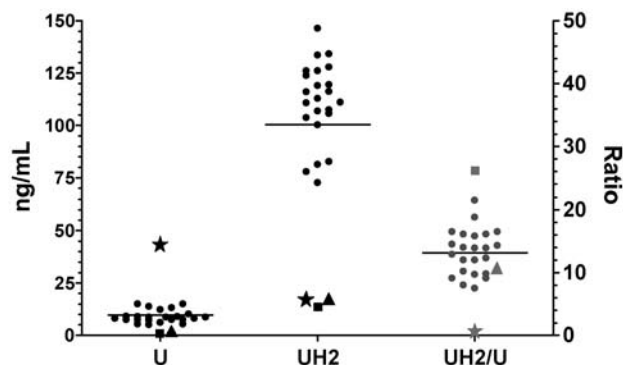


Figure 3. Distribution of U and UH2 concentrations (ng/mL) and UH2/U ratio in plasma of 26 healthy subjects. Three subjects present a very low UH2 level. One of these subjects (Subject A, designated by a star) with high U level and low UH2 level, is DPD deficient. The two others (Subject B, designated by a square; and Subject C, designated by a triangle), despite their low UH2 levels, have normal UH2/U levels, because of a very low U level.

The estimated values are illustrated in Table IV and the distribution in Figure 3. Mean, median and range of plasma U concentrations were 9.7 (SD = 7.7), 8.4 and 0.6–43.3 ng/mL, respectively. Mean, median and range of plasma UH2 concentrations were 100.3 (SD = 35.9), 110.9 and 13.6–146.6 ng/mL, respectively. We observed a large inter-individual variation in the UH2/U ratio.

Among these 26 subjects, 23 presented normal human physiological UH2/U ratio as defined by Jiang *et al.* (16), Remaud *et al.* (14), Boisdron-Celle *et al.* (3) and Déporte *et al.* (12), using LC–MS or LC–UV methods and showing UH2/U ratio ranges of 0.14–6.88 ($n = 123$), 5.8–13.2 ($n = 8$), 0.0002–17.3 ($n = 252$) and 1.9–29.4 ($n = 165$). Only 3 subjects presented U and UH2 concentrations significantly different from the others. They are highlighted in Figure 3. One (Subject A) showed a very low DPD activity and a high U concentration, prompting a recommendation to avoid 5-FU treatment. The other two values (Patients B and C) were linked to a decrease in both U and UH2 concentrations, maintaining a physiological UH2/U ratio, which does not contra-indicate the 5-FU treatment. This highlights the importance of determining the UH2/U ratio.

Using the remaining 23 values, we thus may estimate the reference values: 9.0 ± 3.0 , 111.0 ± 19.0 and 13.0 ± 4.0 ng/mL for U, UH2 and UH2/U, respectively. These data are close to those of the literature (3, 12, 14, 16), but given that this study was conducted on a very small sample, a prospective confirmatory study on a larger population is now necessary.

Conclusion

With the wider availability of LC–MS–MS in routine laboratory analysis, this validated sensitive and specific LC–MS–MS method offers a rapid routine test for the precise diagnosis of the rare dihydropyrimidine dehydrogenase enzyme deficiency that leads to very high U concentrations in plasma and may increase 5-FU-induced toxicity. In addition, the calculation of the UH2/U ratio allows easier identification of patients at risk of adverse effects after 5-FU infusion.

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