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High-performance liquid chromatographic assay with UV detection for measurement of dihydrouracil/uracil ratio in plasma

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Abstract

A rapid, robust and sensitive HPLC method for analysis of uracil (U) and dihydrouracil (UH2) in plasma was developed using solid phase extraction and ultraviolet detection. Separation was achieved with a SymmetryShield RP18 column and an Atlantis dC18 column using a 10 mM potassium phosphate buffer as mobile phase. Compounds were eluted within 15 min without interference. Recovery was 80.4 and 80.6% for U and UH2. Calibration curves were linear from 2.5 to 80 ng/mL for U and 6.75 to 200 ng/mL for UH2. The LLQ was, respectively, 2.5 ng/mL for U, and 6.75 ng/mL for UH2. Within-run and between-run precision were less than 5.94% and inaccuracy did not exceed 7.80%. The overall procedure has been applied to correlate UH2/U ratio with dihydropyrimidine dehydrogenase activity in 165 cancer patients.

Keywords: DPD activity; Uracil; Dihydrouracil; HPLC

1. Introduction

Dihydropyrimidine dehydrogenase (DPD) deficiency with a defect of the pyrimidine degradation pathway has become the focus of considerable attention, due to severe 5-fluorouracil (5-FU) toxicities occurring in DPD deficiency patients [1]. 5-FU and its derivatives remain the most widely used anticancer agents in the treatment of breast, head and neck and colorectal cancers [2]. The catabolic route plays a significant role as more than 80% of the administered 5-FU is catabolized by DPD, making it an important regulator for this commonly used anticancer drug. Several reports have described an inherited disorder in which patients with absent or significantly decreased DPD activity develop life-threatening toxicity following exposure to 5-FU. Due to alteration of catabolic pathway in DPD-deficient patients, administration of standard doses of 5-FU results in altered 5-FU pharmacokinetics and severe toxicities including mucositis, granulocytopenia, neuropathy and sometimes death. Population studies of DPD activity in peripheral blood mononuclear cells (PBMC) were reported in healthy volunteers and cancer patients to evaluate the incidence of complete or partial DPD deficiency [3–7]. In these studies, a large degree of variation was observed, and the frequency of partial or complete DPD deficiency was estimated to be 3–5% and 0.1%, respectively.

Various methods for the detection of DPD deficiency have been developed. Direct methods are based on the determination of the precise DPD enzymatic activity in PBMC [8,9]. These methods require PBMC isolation and high-performance liquid chromatography (HPLC) analysis of 5,6-dihydrofluorouracil (5-FUH2) formed from 5-FU, making the procedure time-consuming and labor-intensive. The development of more convenient methods to discriminate, prior treatment, between healthy, partially or profound DPD-deficient patients is necessary. Since 5-FU and uracil (U) are metabolized by the same pathways, with DPD as the key rate limiting enzyme, the measurement of plasma ratio of U and its dehydrogenate metabolite 5,6-dihydrouracil (UH2) would be theoretically a sensitive marker for indirect evaluation of DPD enzyme activity and therefore for prevention of high risk of toxicity. Gamelin et al. were the first to show a close correlation between UH2/U ratio, 5-FU plasma levels and toxic effects [10]. More recently, significant linear correlation was demonstrated between UH2/U ratio and PBMC DPD levels [11,12].

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In the last few years, numerous HPLC methods have been described for the determination of U and its metabolite, but most have limitations which restrict their usefulness [11,13–18]. Some of these methods employed mass spectrometry detection which limit its use for routine drug monitoring, and most have relied upon liquid phase extraction procedure for sample clean-up which hampered the prospect for development of automated technique for analysis of large series of samples.

In the present study, we described the characteristics of a new validated HPLC method with ultraviolet (UV) detection and solid phase extraction (SPE) for measuring U and UH2 in plasma. The requirements of this study were to find an analytical procedure that is rapid, robust, sensitive and which can be performed in most laboratory equipped for HPLC analysis.

Using this method, a series of 165 patients were assayed for quantification of plasma U and UH2, determination of UH2/U ratio, and correlation with PBMC DPD activity.

2. Experimental

2.1. Chemicals and reagents

U, UH2 and the internal standard 5-fluorocytosine (5-FC) were purchased from Sigma (Saint Louis, MO, USA). The water used was of Milli-Q grade (Millipore, Molsheim, France). Methanol and phosphoric acid were obtained from Carlo Erba (Gradignan, France). Potassium dihydrogenophosphate, ammonium formate and bovine serum albumine (BSA) were obtained from Sigma (Saint Louis, MO, USA), and formic acid from Acros Organics (New Jersey, USA). All reagents were of HPLC grade or equivalent purity.

2.2. Instrumentation and chromatographic conditions

The assay was developed using a high-performance liquid chromatograph (Waters, France) equipped with a 600 HPLC pump, an automatic injector (Model 717) and an UV detector (Model 2487). Data acquisition and processing were accomplished using the Millenium 2010 software (Version 3.2, Waters). UV detection was monitored at 205 nm. Chromatographic separation was achieved at room temperature using a SymmetryShield RP18 column (5 μm, 4.6 mm × 250 mm, Waters, France) connected with an Atlantis dC18 column (5 μm, 4.6 mm × 100 mm, Waters, France). Elution was carried out isocratically at a flow rate of 0.6 mL/min with a mobile phase consisting in 10 mM potassium phosphate buffer (pH 3.0).

2.3. Preparation of calibration standards

Two stock solutions ($C = 100$ μg/mL) for generating the U and UH2 calibration curves and quality controls (QC) were prepared by dissolving an accurately weighed amount of U and UH2 in purified water. The concentration of the internal standard (5-FC) stock solution was 100 μg/mL. The solutions were stored in 1 mL aliquots at −20 °C. An internal standard working solution (10 μg/mL) was freshly prepared on the day of analysis by dilution in water.

The calibration curve of U and UH2 were prepared in BSA (80 mg/mL) by adding the required amount of stock solution to obtain final concentrations of 2.5, 5.0, 10, 20, 40 and 80 ng/mL U and concentrations of 6.75, 12.5, 25, 50, 100 and 200 ng/mL UH2. BSA solution was used to avoid measurement of physiological U and UH2 in conditions near plasma matrix (70–80 mg/mL of protein). Calibration curves of U and UH2 were combined in the same sample.

2.4. Sample preparation

An aliquot of 500 μL of plasma was mixed with 30 μL internal standard working solution and 500 μL potassium phosphate buffer KH_2PO_4 (10 mM, adjusted to pH 2.0 with phosphoric acid). Samples were mixed 10 s and centrifuged at 2500 × *g* for 5 min at +4 °C. The mixture was applied on a SPE Atoll Xtrem Capacity cartridge (Interchim, France) connected to a vacuum manifold system and conditioned with 1 mL of methanol and 1 mL of purified water. The mixture was allowed to run through by gravity. Plasma constituents were then eluted with 1 mL of ammonium formate buffer (10 mM, pH 5.0). Pressure was applied (10 mmHg) until the cartridge sorbent was dried. Elution of analytes was performed with 500 μL of methanol. The eluates were evaporated to dryness in a vacuum centrifuge at 45 °C during 30 min. The dried residues were reconstituted in 200 μL of mobile phase and transferred into a glass insert for autosample vials. A 50 μL aliquot was injected into the HPLC.

2.5. Validation procedure

A full validation procedure was performed consisting of the following experiments, selectivity, linearity, within-run and between-run precision and accuracy, recovery of the analytes, stability after sample preparation, limit of detection (LOD) and lower limit of quantification (LLQ).

For validation of the assay, calibration standards of seven levels (including the blank) and sets of QC samples (four levels) were prepared in BSA by adding required amounts of U and UH2 stock solutions. The results of the tests were evaluated against acceptance criteria described by Lang et al. [19].

2.5.1. Linearity

Six separate sets of standard curve calibration were prepared and analyzed separately by HPLC. The peak area ratio of UH2 and U with internal standard (5-FC) were plotted versus the nominal concentrations of the calibration standards. The linearity of the six calibration curves was tested with the *t*-test for lack of fit with a weight factor of 1/concentration. The deviation from the nominal concentration was calculated for each calibration level.

2.5.2. Accuracy and precision

To determine the accuracy and precision of the assay, BSA samples were spiked with U and UH2 to obtain four levels of concentration as follow: one low level of 2.5 ng/mL U plus 6.75 ng/mL UH2, two medium levels of 8.0 ng/mL U plus 15.0 ng/mL UH2 and 25.0 ng/mL U plus 75.0 ng/mL UH2, and

one high level of 50 ng/mL U plus 150 ng/mL UH2. Each quality control was analyzed in quadruplicate with the six separate sets of standard calibration curves. The U and UH2 concentrations were determined using the standard calibration curve previously validated, and the results were plotted as measured concentrations versus nominal values. The accuracy was defined as the percentage of the ratio of the observed concentration and the nominal concentration. Within-run and between-run precision were calculated using a one-way analysis of variance (ANOVA) with the analytical run as the group variable. From the ANOVA analysis, the run mean square (RunMS), error mean square (ErrMS) and grand mean (GM) were obtained. Within-run and between-run precision were calculated by the following formulas:

$$\text{Within-run precision}\,(\%) = 100 \times \sqrt{\text{ErrMS}/\text{GM}}$$

$$\text{Between-run precision}\,(\%) = 100 \times \frac{\sqrt{(\text{RunMS} - \text{ErrMS})/N}}{\text{GM}}$$

N is the number of replicates

2.5.3. Recovery

The absolute recoveries were evaluated in plasma and BSA by comparing the peak areas of U, UH2 and 5-FC obtained after extraction with unextracted aqueous solutions with the same amounts of U, UH2 and 5-FC.

2.5.4. Stability of U and UH2 after samples preparation

Stability during storage in the sample compartment was studied at room temperature by analyzing samples over a period of 24 h. The calculated response at $t = 0$ h was compared with the calculated response at $t = 12$ h and 24 h. Samples were considered stable if the decrease was less than 10%.

2.5.5. Limit of quantification and limit of detection

The limit of detection (LOD) was determined using a signal-to-noise of 3. The lower limit of quantification was defined as the lowest concentration with an accuracy and precision below 20%.

2.6. Patients plasma analysis

The population analysis consisted of 165 patients whose PBMC DPD activity was determined prior 5-FU based treatment according to our routine practice to detect patient with DPD deficiency.

Five milliliters blood sample were collected in heparinized tube at the same times blood was drawn for DPD activity measurement. According to the recently published results from Remaud et al. [18], plasmas were immediately separated by centrifugation at +4 °C and stored at −70 °C, in order to avoid changes in U concentration. PBMC DPD activity was measured according to the method of Johnson et al. [8] modified by Deporte et al. [9]. PBMC cells were isolated from 15 mL heparinized anticoagulant blood. In brief, the sample was incubated in a reaction mixture containing 35 mM sodium phosphate buffer pH 8.0, 250 μM NADPH, 2.5 mM magnesium chloride and 20 μM 5-FU. After incubation during 40 min, the reaction was stopped by addition of 125 μl of sulphate sodium saturated solution and acidified by 10 μL of 2 M sulphuric acid. The sample was centrifuged (10 min, 10,000 rpm) to remove proteins. Hundred microlitres of the supernatant was injected in the HPLC system for quantification of 5-FUH2. Separation was performed at 262 nm using a SymmetryShield RP18 column. 5-FUH2 was eluted by an isocratic phase consisting of distilled water at a flow rate of 0.6 mL/min.

To determine the distribution pattern of U concentrations, UH2 concentrations, UH2/U ratios in the population, we tested the hypothesis that the sample population distribution followed a normal distribution using the Kolmogorov–Smirnov test. Mean, standard deviation (S.D.), median and range were calculated. Correlation between UH2/U ratio and PBMC DPD activity was assessed by simple regression analysis.

3. Results

3.1. Assay validation of U and UH2 determination

3.1.1. Selectivity

The identity of the peak corresponding in retention time to that of U and UH2 reference standard was established by the UV spectrum of the putative plasma U and UH2 peak matched to that of the reference standard. Blank human plasma showed a peak at the retention time of 14.41 and 12.37 min, which corresponded to the physiological concentration of U (11.1 ng/mL) and UH2 (135 ng/mL), respectively (Fig. 1). Late eluting peaks were detected after 18 min until 26 min. Interference with these strongly retained components in subsequent chromatogram was prevented by injection of 100 μL of methanol and by introduction of a 15 min delay after each run.

To exclude possible interference by other hydrophilic compounds, such as other pyrimidines, standard samples spiked with thymine and cytosine were injected into the HPLC. Retention time were 32.4 min and 7.0 for thymine and cytosine, respectively.

Selectivity of the assay was investigated by the analysis of different blank plasma samples, especially with plasma from patients treated with 5-FU regimen. On the chromatograms of these patients, two peaks corresponding to 5-FU and 5-FUH2 were observed at 16.46 and 13.29 min, respectively (Fig. 2).

3.1.2. Linearity

All calibration curves proved to be linear over the concentration range of 2.5–80 ng/mL for U and 6.75–200 ng/mL for UH2. Tables 1 and 2 shows the mean deviation (RE) and the relative standard deviation (R.S.D.) at each calibration levels calculated using data obtained on six consecutive runs. The criterion for accepting any curve was that all data points should have a RE and a R.S.D. of less than 15%. For U, these criteria were met by the results of the analysis of the lowest calibration standard (2.5 ng/ml) where 8.0% and −1.57% were achieved for R.S.D. and RE, respectively. Same results were obtained for UH2 (lowest calibration standard point: 6.75 ng/mL) were

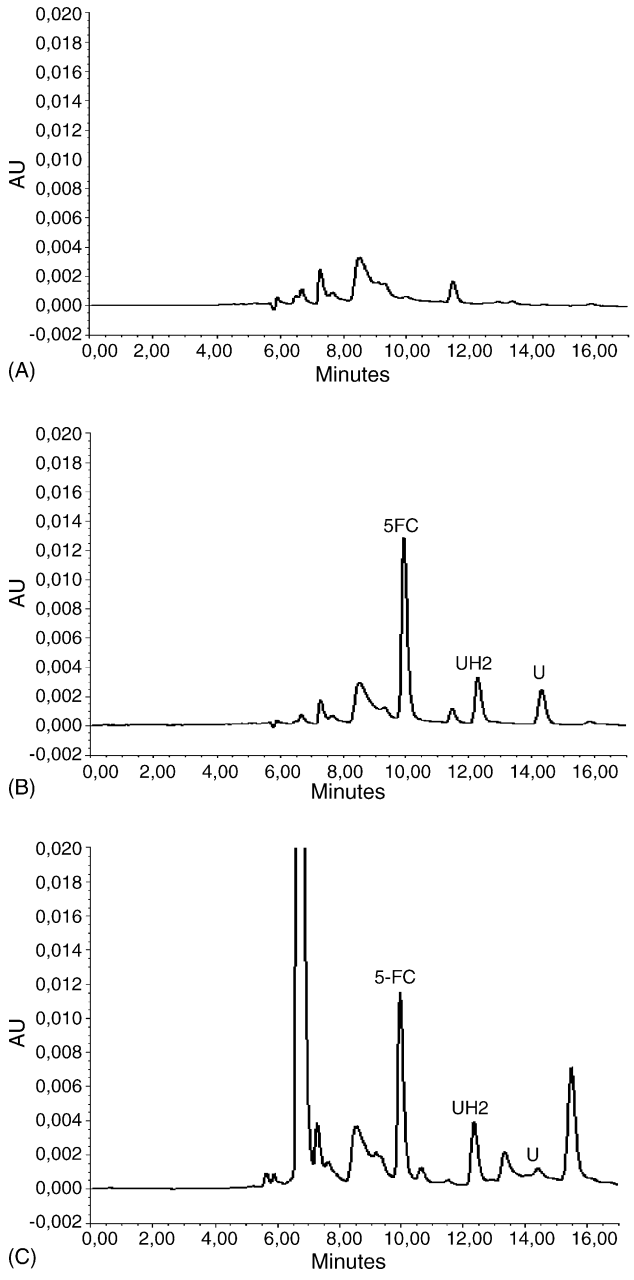

Fig. 1. Representative chromatograms of a blank bovine serum albumine sample (A), a bovine serum albumin sample (B) spiked with U ($C = 50$ ng/mL), UH2 ($C = 100$ ng/mL) and internal standard 5-FC ($C = 600$ ng/mL), and a patient plasma sample (C) containing 11.1 ng/mL U and 135 ng/mL, spiked with internal standard 5-FC ($C = 600$ ng/mL).

11.58 and 0.38% were achieved for R.S.D. and RE, respectively. The regression coefficients (r^2) for each calibration curves were >0.9959 for U and UH2.

3.1.3. Within- and between-run accuracy and precision

For U, within-run precision ranged between 2.44 and 5.06%, between-run precision ranged between 1.85 and 3.68%, and the range of the accuracy was −3.36 to 6.08%. For UH2, within-run precision ranged between 2.30 and 5.94%, between-run precision ranged between 3.38 and 5.17%, and the range of the accuracy was 1.15–3.87% (Table 3).

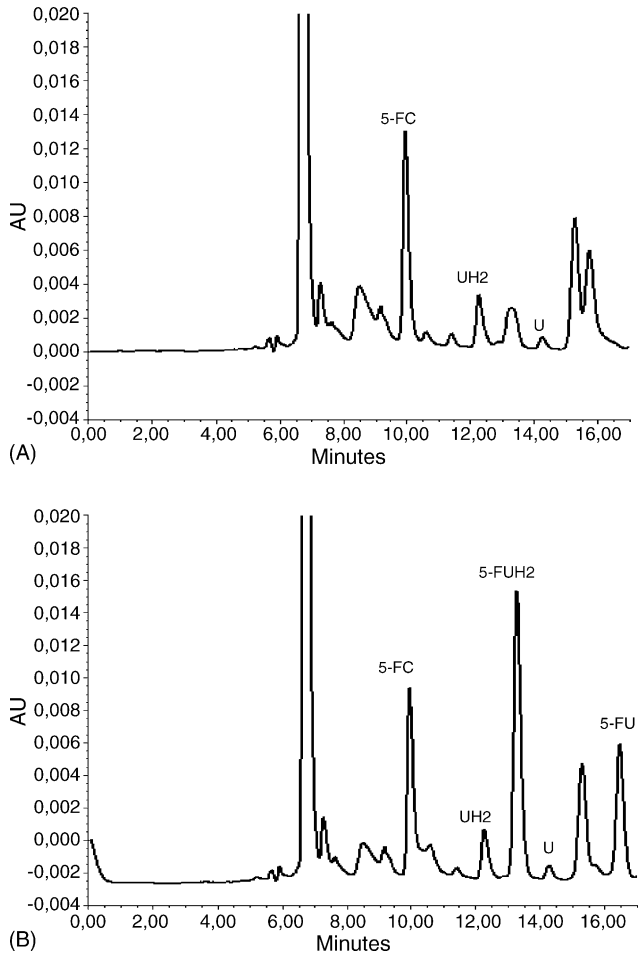

Fig. 2. Representative chromatograms of a patient plasma sample before (A) and during (B) treatment with continuous 5-FU infusion (1000 mg/m^2 per day).

3.1.4. Recovery

The mean recoveries of U in plasma (80.4% ± 1.7, $n = 5$) differed from BSA (85.2% ± 1.6, $n = 5$) by 4.8%. In order to demonstrate that analytical procedure is able to normalise this difference, precision and accuracy of the measurement of U were calculated in plasma sample spiked to obtain U concentrations of 2.5, 8.0, 25 and 50 ng/mL and compared with results obtained in BSA (Table 4). Lower accuracy was obtained with plasma samples (range −7.80 to −5.50%) compared to BSA (range −3.36 to 6.08%). However, values are in the normal acceptance criteria described by Lang et al. [19].

For UH2, the values were 80.6% ± 1.1 ($n = 5$) for plasma and 81.5% ± 1.6 ($n = 5$) for BSA. For 5-FC, recoveries of 23.4% ± 0.4 ($n = 5$) and 23.3% ± 1.0 ($n = 5$) were found for plasma and BSA, respectively.

3.1.5. Stability of U and UH2 after samples preparation

Three QCs were prepared and processed in duplicate at time T0 h with the calibration row. The stability of U and UH2 in the autosampler at room temperature was investigated by re-analyzing samples 12 and 24 h with a new calibration row. In these conditions, concentrations of U and UH2 remained constant over the 24 h period tested.

Table 1
Assayed concentrations of calibration standards of U

Nominal concentration (ng/mL)	Assayed concentration (ng/mL)						Mean	R.S.D. (%)	RE (%)
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6			
2.5	2.39	2.54	2.22	2.54	2.55	2.53	2.46	8.00	−1.57
5.0	5.23	5.00	5.22	4.98	4.63	5.12	5.03	6.23	0.60
10.0	10.58	9.62	10.85	9.75	9.64	9.15	9.93	7.11	−0.69
20.0	20.00	19.79	20.00	19.83	20.42	21.41	20.24	5.22	1.21
40.0	39.26	42.16	42.83	40.97	39.72	40.92	40.98	3.64	2.44
80.0	80.26	78.39	76.61	79.41	80.17	78.50	78.89	4.07	−1.39
Slope	0.00522	0.00525	0.00520	0.00506	0.00556	0.00540			
r^2	0.9995	0.9992	0.9959	0.9996	0.9997	0.9979			

Table 2
Assayed concentrations of calibration standards of UH2

Nominal concentration (ng/mL)	Assayed concentration (ng/mL)						Mean	R.S.D. (%)	RE (%)
	Run 1	Run 2	Run 3	Run 4	Run 5	Run 6			
6.75	6.85	6.50	7.16	6.80	6.79	6.55	6.78	11.58	0.38
12.5	12.52	13.36	12.90	13.53	13.06	13.11	13.08	8.89	4.64
25.0	24.53	25.15	22.93	24.49	24.89	24.51	24.42	8.10	−2.33
50.0	50.77	49.95	48.10	50.10	50.19	52.90	50.34	5.84	0.67
100.0	98.25	106.21	108.42	101.84	98.95	100.38	102.34	4.95	2.34
200.0	201.36	193.93	195.13	198.52	200.93	197.40	197.88	4.38	−1.06
Slope	0.00336	0.00347	0.00325	0.00326	0.00357	0.00333			
r^2	0.9997	0.9978	0.9959	0.9997	0.9999	0.9991			

Table 3
Within-run and between-run precision and accuracy of the quantification of U and UH2

Nominal QCs concentrations (ng/mL)		Within-run precision (%)		Between-run precision (%)		Accuracy (%)		95% CI of accuracy[a] (%)	
UH2	U	UH2	U	UH2	U	UH2	U	UH2	U
6.75	2.5	3.14	3.55	3.38	3.27	1.61	−3.36	−4.08–7.29	−8.97–2.25
15.0	8.0	5.94	5.06	3.82	1.85	1.25	0.56	−7.22–9.73	−5.76–6.88
75.0	25.0	3.56	2.05	4.49	2.44	1.15	2.07	−5.93–8.23	−1.89–6.02
150.0	50.0	2.30	2.44	5.17	3.68	3.87	6.08	−3.44–11.19	0.32–11.84

[a] 95% CI: = 95% confidence interval.

3.1.6. LOD and LLQ

The criteria for precision and accuracy at the LLQ were R.S.D. < 20% and RE < 20%. The criteria were met for U and UH2 by the results of the analysis of the lowest calibration levels (2.5 and 6.75 ng/mL, respectively, Tables 1 and 2).

3.2. Population analysis

Mean, median and range of plasma U concentrations were 11.8 (S.D. = 10.6) ng/mL, 10.0 ng/mL, and 3.0–109.4 ng/mL, respectively. Mean, median and range of plasma UH2 concentrations were 108.9 (S.D. = 47.2) ng/mL, 99.7 ng/mL and 35.0–386.4 ng/mL, respectively (Table 5).

UH2/U ratio in the 165 patients showed a normal distribution ($p = 0.079$, Fig. 3A) and globally followed a Gaussian distribution. A large degree of inter individual variation was observed; mean and median values were 10.8 and 10.5, respectively; minimum and maximum UH2/U ratio were 1.9 and 29.4, respectively, with a S.D. value of 4.4 (Table 5).

Table 4
Precision and accuracy of the quantification of U in plasma and bovine serum albumin solution (BSA)

Nominal QCs concentrations (ng/mL)	Measured QCs concentrations (ng/mL)		Precision (%)		Accuracy (%)	
	Plasma	BSA	Plasma	BSA	Plasma	BSA
2.5	2.30	2.42	10.52	4.36	−7.80	−3.36
8.0	7.56	8.04	4.69	4.72	−5.50	0.56
25.0	23.36	25.52	4.60	2.91	−6.58	2.07
50.0	47.08	53.04	6.48	4.08	−5.85	6.08

Table 5
Plasma concentrations of U, UH2, UH2/U and PBMC DPD activity in 165 cancer patients

	U concentration (ng/mL)	UH2 concentration (ng/mL)	UH2/U ratio	PBMC DPD activity (nmol/min/mg protein)
Patient number	165	165	165	165
Mean ± S.D.	11.8 ± 10.6	108.9 ± 47.2	10.8 ± 4.4	0.292 ± 0.123
Median	10.0	99.7	10.8	0.287
Minimum	3.0	35.0	1.9	0.054
Maximum	109.4	386.4	29.4	0.784

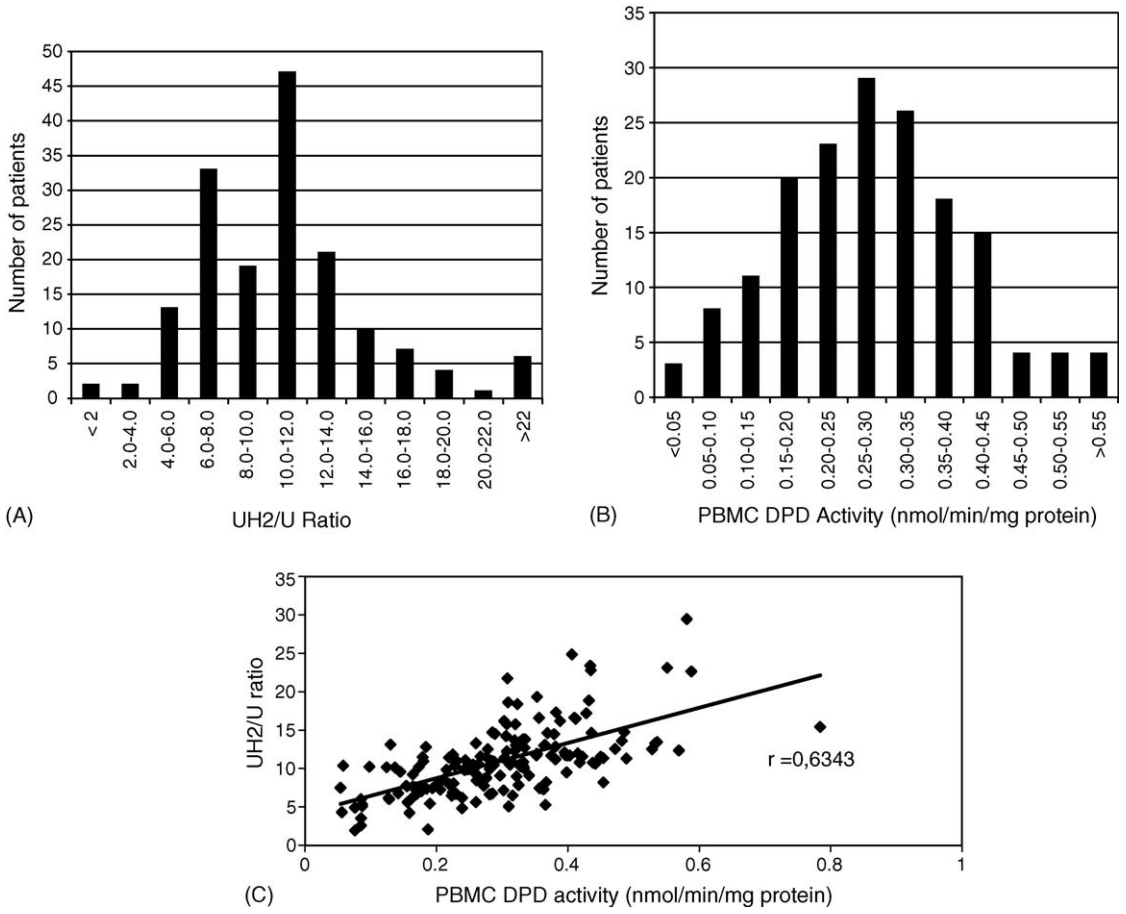

Fig. 3. (A) Normal distribution of UH2/U ratios ($p = 0.079$); (B) normal distribution of peripheral blood mononuclear cells (PBMC) DPD activity ($p = 0.710$); (C) correlation between UH2/U ratios and PBMC DPD activity ($r = 0.6343$, $p < 0.01$) in 165 cancer patients.

The frequency histogram of the PBMC DPD activity for the entire population of patients showed a normal distribution ($p = 0.710$, Fig. 3B). Mean and median PBMC DPD activity were 0.292 and 0.287 nmol/min/mg of protein, respectively. Wide inter-patient variability was observed (S.D. = 0.123, range 0.054–0.784 nmol/min/mg of protein, Table 5).

A significant correlation was observed between UH2/U ratio and PMBC DPD activity (Pearson correlation coefficient $r = 0.6343$, $p < 0.01$, Fig. 3C).

4. Discussion

Due to the wide use of 5-FU in cancer patients, the detection of those at high risk of 5-FU related toxicity because of catabolism defect is a priority. Many reports have shown that individual variations in PBMC DPD enzyme activity resulted in marked differences in clinical toxicities because DPD determines the degradation of more than 80% of administered 5-FU [1,6,20]. Alternative methods to the complex procedure used for evaluation of DPD enzyme activity in PBMC, have been described including, quantitation of uracil or thymine in plasma or urine [21], detection of DYPD gene single nucleotide polymorphism (SNP) [22], 2-^{13}C-uracil breath test for evaluation of the pyrimidine catabolic pathway [23], quantification of 5-FU/5-FUH2 ratio [14,15,24], assessment of uracil pharmacokinetics after oral uracil test dose [25] and measurement of plasma concentrations of U and UH2 with evaluation of UH2/U ratio. Among these various techniques, we chose calculation of UH2/U ratio, since different studies revealed that UH2/U ratio could be a potential biomarker in reflecting DPD activity

level [11,12] and detecting patients with high risk of toxicity [10].

The analytical method described here for measurement of U and UH2 in plasma relies on a HPLC assay with direct ultraviolet detection and a solid-phase (SPE) extraction procedure for sample clean-up.

The difficulty in the determination of U and UH2 in plasma with UV detection is mainly a result of the hydrophilicity of analytes. To get them retain on reversed phase, the use of column with a packing that is compatible with high aqueous condition was necessary. We managed to enhance retention of U and UH2 by using SymmetryShield RP18 and Atlantis dC18 columns connected in series. Their reversed-phase particles with embedded polar groups water-wettable allowed better interaction with our analytes. U and UH2 were eluted at 14.41 and 12.37 min, respectively, with a good resolution.

As far as we know, several methods using HPLC for U and UH2 measurement in plasma have been published [11,13–18], but no SPE has been reported for the simultaneous extraction of U and UH2. Usually, clean-up of the sample was performed by protein precipitation following by liquid–liquid extraction. In recent years, SPE has replaced traditional liquid–liquid extraction in many instances. Specific advantages of SPE include quicker sample processing, higher specificity of sorbent–analyte interactions resulting in a good pre-purification of complex samples, economical use of solvent and procedural simplicity potentially reducing the risks of manipulation errors in routine assays. All these advantages were met in the present study: sample preparation and chromatography time run were short (less than 2 h) allowing to give results on the day of sampling; U and UH2 were eluated without interference with endogenous substances and the pyrimidine-like drug 5-FU; extraction of the analytes was performed with only 1.5 mL of methanol. It is actually possible to handle 50 samples daily.

With the aim to simplify the resolution of the problem associated with the physiological U and UH2 concentrations in plasma, we analyzed the possibility to substitute plasma with bovine serum albumin (BSA) solution. Matrix effect was evaluated by comparison of absolute recovery measured in BSA and plasma. Although recovery was similar in BSA and plasma for UH2, a 4.8% difference was found for U. However, this difference involved no significant change of the accuracy and precision of the measurement of U in the two matrices. Consequently, BSA solution was used for preparation of standard calibration curves and quality controls. Standard solutions were treated using SPE extraction as patient samples after pH adjustment with phosphate buffer 10 mM to pH 2.0 since pH within the matrices varied between 7.4 and 6.0 for subject plasma and BSA, respectively.

Assay performances were assessed both on the basis of statistical characteristics of individual calibration curves and the results of quality control samples. The assay meet the current requirement of the validation of a bioanalytical assay. The method validated for concentration of U and UH2 ranging, respectively, from 2.5 to 80 ng/mL and 6.75 to 200 ng/mL has a good precision and accuracy. The LLQ of 2.5 and 6.75 ng/mL for U and UH2, respectively, are adequate for the analysis of U and UH2 in plasma. Within-run and between-run precision and accuracy are very close to the results of the recently published method for UH2/U determination in plasma using liquid–liquid extraction [18].

The described analytical method was applied to a population of 165 patients for assessment of the UH2/U ratio, and measurement of U and UH2 concentrations in plasma. Our results demonstrated a wide inter-patient variability (range: 3.0–109.4 ng/mL, 35.0–386.4 ng/mL, 1.9–29.4 for U, UH2 and UH2/U, respectively). These data are in accordance with those previously published [10–13], and are very close to the ranges of U, UH2 and UH2/U reported in a population of 10 patients recently published by Remaud et al. [18].

A significant correlation between UH2/U ratio and PBMC DPD ($p < 0.01$) was found, but the correlation is quite moderate ($r = 0.634$). Interestingly, similar results were obtained between PBMC DPD activity and indices of uracil metabolism evaluated after oral administration of 2-^{13}C-uracil [23]. Thus, DPD activity from PBMC cannot be directly compared with UH2/U ratio. This is likely because DPD activity determination in a single tissue (PBMC) does not take into account other factors that can interfere with uracil metabolism, such as anabolic pathway and catabolism by other tissues (especially liver and tumors), which are known to have various levels of DPD [26].

In patients with colorectal carcinoma, Gamelin et al. [10] proved that the UH2/U ratio in plasma was highly correlated to 5-FU clearance and tolerance to treatment and recommended greater precaution when UH2/U ratio was less than 1.8. Jiang et al. [12] in patients with gestational trophoblastic tumors concluded that patients with UH2/U ratio beyond 1 should be advised to applied other chemotherapy strategy because of unexpected severe toxicity. Thus, large population study should be now investigated to demonstrate the utility of UH2/U ratio as predictive marker of toxicity for patient treated with 5-FU based chemotherapy.

In conclusion, the HPLC method developed and validated in the present work proved to be sufficiently rapid, robust and sensitive for clinical use performed to define UH2/U ratio threshold predictable for patient at risk of severe toxicity.

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