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An accurate dihydrouracil/uracil determination using improved high performance liquid chromatography method for preventing fluoropyrimidines-related toxicity in clinical practice

Gaëlle Remaud[b], Michèle Boisdron-Celle[b], Catherine Hameline[b], Alain Morel[b], Erick Gamelin[a,b,*]

[a] *Department of Medical Oncology, INSERM U564, Anticancer Centre Paul Papin, 2 rue Moll, 49933 Angers Cedex 9, France*
[b] *Oncopharmacology-Pharmacogenetics, INSERM U564, Anticancer Centre Paul Papin, 2 rue Moll, 49933 Angers Cedex 9, France*

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Abstract

An accurate and improved HPLC method was set up to measure both dihydrouracil ($UH_{2)}$ and uracil (U) in plasma, and to assess their ratio.

Analytes retention time, separation and peak purity were greatly optimized with a Hypercarb column and a diode array detector. U and UH_2 limits of quantification were 1.25 and 0.625 ng/mL. U and UH_2 within-day precisions were 0.9–2.3% and 0.7–5.6%. Between-day precisions were 1.3–5.3% and 1.3–7.1%. Accuracy was 0.1–6.1%.

UH_2/U ratio between-day variability was low, but ratio decreased from 02:00 p.m.

This method is now used in practice to detect patients at risk of fluoropyrimidine toxicity and to individually adapt the dosage.

Keywords: Fluoropyrimidines; Uracil; Dihydrouracil; HPLC

1. Introduction

5-Fluorouracil (5-FU) is one of the most commonly anticancer agents used in the treatment of solid tumors, especially in digestive tract, head and neck and breast cancers. The metabolic pathways of 5-FU have been extensively investigated and several studies have reported a high individual variability of 5-FU metabolism [1–3]. Moreover, a close relationship has been demonstrated between individual pharmacokinetic parameters and toxicity or response rate [2–4].

Severe side effects, including hematological, mucosal and gastrointestinal toxicity, have been encountered during conventional 5-FU based treatments and have been attributed to a genetic deficiency of dihydropyrimidine dehydrogenase (DPD) activity, the initial enzyme of 5-FU catabolism pathway [5,6]. The frequencies of this catabolism deficiency in the population reach 3–5% for major ones and 3‰ for complete ones. Patients presenting complete deficiency of DPD enzyme activity are the most likely to undergo life-threatening toxicity. Given the high number of patients receiving a 5-FU based treatment, detection of deficient individuals in the population seems essential. In order to reduce the toxic side effects of 5-FU, an individual dose adjustment can be proposed to adapt the dosage to the patient's metabolic capacities [7,8]. However, this method does not allow to prevent the severe, sometime lethal, toxicity that occurs at the first course of treatment [9,10].

Several approaches have been developed in an attempt to detect patients at high risk of 5-FU toxicity prior to treatment. Determination of DPD enzyme activity in peripheral blood mononuclear cells has been established but this method, though elegant, remains unsuitable for current practice because of the use of radiolabelled materials [5,11].

* Corresponding author.
E-mail address: e.gamelin@unimedia.fr (E. Gamelin).

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Moreover, there is only a weak correlation between 5-FU plasma levels and DPD enzyme activity determined in peripheral blood mononuclear cells and this activity level may not well reflect that in important tissues, such as liver, lung or gastrointestinal tract [12].

The genetic approach by detecting DYPD gene single nucleotide polymorphism (SNP) is promising but not presently suitable since more than 30 SNP have been reported with different and not fully understood impacts on DPD enzyme activity [13].

The measurement of plasma or urine concentration of uracil (U), the endogenous substrate of DPD enzyme, may not be a reliable method because uracil level can be influenced by many other factors [14,15]. These factors can be strongly lessened by measuring simultaneously the plasma concentrations of U and its dihydrogenated metabolite 5,6-dihydrouracil (UH_2), formed by reduction of U by DPD, and by calculating the plasma UH_2/U ratio [16].

We were the first to develop UH_2/U ratio in plasma pretherapeutic determination and used it to prevent high risk of toxicity [17], but this HPLC method was complicated by the use of two columns in series and was aimed to detect four compounds at the same time: U, UH_2, 5-FU and 5,6-dihydro-5-fluorouracil (FUH_2) [17]. Therefore, our purpose was to set up a new simple and reliable HPLC method for simultaneously measuring U and UH_2 in plasma usable in clinical practice. The column was strictly selected for optimizing retention time and separation, and a diode array detector allowed us to establish the analyte peak purity at low wavelength.

Using this method, we first evaluated the impact of the time between sample collection and centrifugation on the concentrations of U and UH_2 and on the UH_2/U ratio. We then studied the stability along the time of these concentrations in plasma samples stored at different temperatures.

In order to evaluate UH_2/U ratio during the day and between day variations, blood samples from eight healthy volunteers were collected at several hours of the day and between 09:00 a.m. and 10:00 a.m. on nine different days.

2. Experimental

2.1. Chemicals

U, UH2, 5-FU, 5-chlorouracil, 5-bromouracil, 5-fluorocytosine, 5-fluorouridine, 5-bromocytosine and 2-chloropyrimidine were purchased from Sigma (Saint Quentin Fallavier, France). FUH_2 was obtained from Roche (Basel, Switzerland). Ammonium sulfate, acetonitrile, ethyl acetate and isopropanol were of HPLC grade (VWR International, Pessac, France). Phosphoric acid was purchased from VWR International and was of analytical grade. The water used was of Milli-Q grade (Millipore, Molsheim, France) and was degassed with helium before use.

2.2. Sample extraction

This extraction procedure is derived from that used by Gamelin et al. for the extraction of 5-FU and has been improved [17]. First, 25 μL of internal standard (FUH_2, 1 μg/mL) were added to 500 μL plasma samples and vortex-mixed. Plasma proteins were then precipitated with 600 mg ammonium sulfate. After vortex mixing for 1 min, 4 mL isopropanol–ethyl acetate (15:85, v/v) were added. The samples were gently mixed for 5 min in a rotatory stirrer (45 rpm) and centrifuged for 15 min at 3500 × *g*. The supernatant was transferred to a glass tube and evaporated at 56 °C for 20 min under a stream of nitrogen. The dry extract was reconstituted with 200 μL of water and a 150-μL volume was injected onto the column after filtration through a 0.45-μm vinylidene polyfluorure membrane plate (Millipore, Saint Quentin en Yvelines, France).

2.3. Instrumentation and chromatographic conditions

The HPLC system consisted of Perkin-Elmer Series 200 pump, autosampler and diode array detector (Perkin-Elmer, Courtaboeuf, France).

Several analytical columns, with their corresponding guard columns, were evaluated to achieve separation of the compounds. Three were reverse phase columns purchased from Waters (Saint-Quentin-en-Yvelines, France): Spherisorb ODS2 (250 mm × 4.6 mm; 5 μm), Symmetry Shield RP8 (150 mm × 4.6 mm; 5 μm) and XTerra RP18 (100 mm × 3 mm; 3.5 μm) and one was a porous graphitic carbon phase column purchased from ThermoElectron (Courtaboeuf, France): Hypercarb (200 mm × 3 mm, 5 μm).

Mobile phase was optimized and differed according to the column tested. For Spherisorb ODS2, Symmetry Shield RP8 and XTerra RP18, an isocratic elution was performed using 10 mM potassium phosphate buffer adjusted at pH 3 with phosphoric acid at a flow rate of 1 mL/min. For Hypercarb column, separation was achieved by gradient elution using a mobile phase delivered at a flow rate of 0.28 mL/min and consisting in water and acetonitrile. The gradient composition was optimized to achieve the best separation of all the compounds.

The detector wavelengths were set at 210 nm for UH_2 and FUH_2 and 260 nm for U. Peak recording and integration were performed using the Perkin-Elmer TotalChrom software.

2.4. Method validation

2.4.1. Linearity

U, UH_2 and FUH_2 were dissolved in Milli-Q water at a concentration of 1 mg/mL and stored at −20 °C. Standard solutions were prepared by further dilution of the appropriate compound into Milli-Q water. Calibration curves of U and UH_2 were prepared by adding 25 μL standard solution of each compound and 25 μL of the internal standard to 450 μL of Milli-Q water. The final generated concentra-

tions for U and UH_2 were 6.25, 12.5, 25, 50, 100 ng/mL and 12.5, 25, 50, 100, 200 ng/mL, respectively, with a concentration of 50 ng/mL for FUH_2. These samples were then treated according to extraction and HPLC procedures.

Calibration graphs were obtained using the least-squares method. Standard curves for UH_2 and U were generated by plotting the peak area ratio of UH_2 or U to that of the internal standard versus the concentration of each compound.

2.4.2. Detection and quantitation limits

The limits of detection (LOD) and quantitation (LOQ) were determined for U and UH_2. For both, LOD was determined at a signal/noise = 5 and LOQ was calculated as the minimum concentration that gave a relative standard deviation less than 10%.

2.4.3. Analytical recovery

Similar samples as those used for generating calibration curves were prepared ($n = 15$). Five different concentrations for each compound were studied, whereas concentration of FUH_2 was maintained constant. The recovery of U, UH_2 and FUH_2 was evaluated by comparing peak areas obtained for these extracted samples to those obtained by direct injection of standard solutions of the same concentration.

2.4.4. Precision and accuracy

For the determination of within-day precision and accuracy, five samples of each concentration (U: 6.25–12.5–25–50–100 ng/mL and UH_2: 12.5–25–50–100–200 ng/mL) were extracted and injected on the same day. For between-day precision and accuracy, one sample of each concentration was analyzed per day on five consecutive days.

2.5. Importance of the time between the sample collection and centrifugation

Blood samples were collected in two healthy volunteers and centrifuged (10 min, 3500 × *g*) at 0.5, 1, 2, 3, 5 and 8 h after collection. Plasma samples were then analyzed according to extraction and HPLC procedures.

2.6. Stability of the concentrations of U and UH_2 in plasma

Stabilities of the concentrations of U and UH_2 and of the UH_2/U ratio in plasma were evaluated in two healthy volunteers during several days at three different temperatures. Thus, after collection and centrifugation of the blood samples, discarded plasma was stored at −20 °C, +4 °C or ambient temperature during 14 days.

2.7. Measurement of the UH_2/U ratio from day to day

Blood samples of eight healthy volunteers were collected into heparinized tubes at nine different days distributed on 5 weeks. All samples were collected between 09:00 a.m. and 10:00 a.m. and were centrifuged for 10 min at 3500 × *g*. Plasma concentrations of U and UH_2 were then measured in each sample to evaluate the stability of the UH_2/U ratio from day to day.

2.8. Measurement of the UH_2/U ratio during the day

Blood samples of four healthy volunteers were collected in heparinized tubes at five different hours of the day: 9.30 a.m., 12:00 a.m., 02:00 p.m., 04:00 p.m. and 06:00 p.m. All samples were immediately centrifuged for 10 min at 3500 × *g*. After extraction, plasma concentrations of U and UH_2 were measured in each sample to evaluate the stability of the UH_2/U ratio during the day.

3. Results

3.1. Internal standard

Several compounds have been tested: 5-FU, FUH_2, 5-chlorouracil (5-CU), 5-bromouracil (5-BU), 5-fluorocytosine, 5-fluorouridine, 5-bromocytosine and 2-chloropyrimidine. 5-FU, 5-CU, 5-BU, 5-fluorouridine and 5-bromocytosine had very high retention times (Table 1), and thus would have required a modification of the elution gradient, resulting in an increase of the analysis time. Despite suitable retention times, 5-fluorocytosine and 2-chloropyrimidine were not chosen because of weak recoveries (about 40%) after extraction from plasma. FUH_2 had a recovery of 80% and a retention time of 23 min and was at last selected as internal standard for the analysis.

3.2. HPLC procedure

3.2.1. Analytical column

Retention times and resolution of U and UH_2 were determined for the four different analytical columns tested. On classical reverse phase columns, U and UH_2 were poorly retained, with retention times close to 2 min on XTerra column and close to 7 min on Spherisorb ODS2 and Symmetry Shield RP8 columns. This poor retention on traditional silica gel stationary phases, even when the mobile phase used is totally aqueous, can be explained by the high polarity of

Table 1
Retention times of the different internal standards tested in the final chromatographic conditions

Internal standard	Retention time (min)
FUH_2	23
2-Chloropyrimidine	26
5-Fluorocytosine	33
5-Bromocytosine	44
5-FU	45
5-Fluorouridine	48
5-Bromouracil	>50
5-Chlorouracil	>50

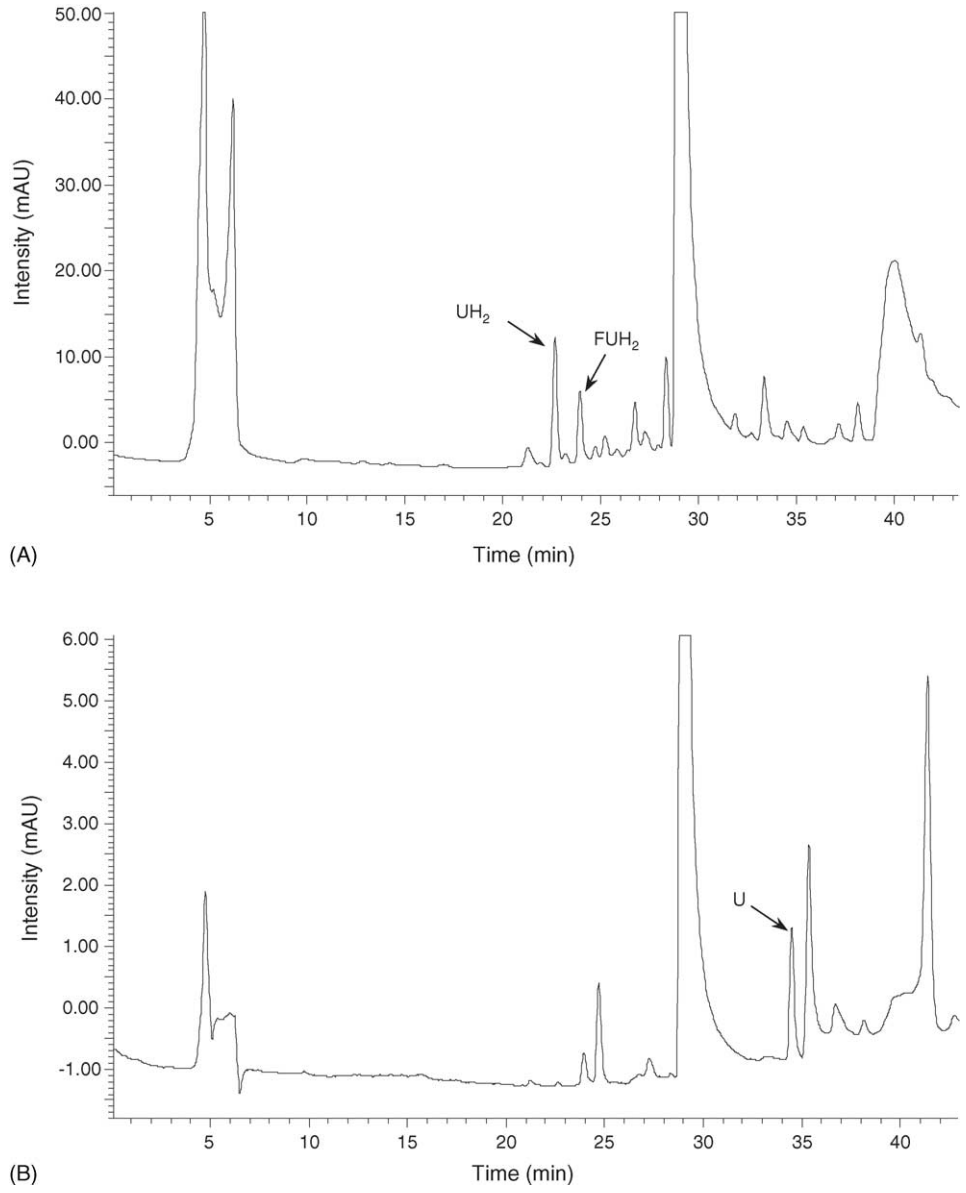

Fig. 1. HPLC chromatograms of a healthy volunteer plasma sample at 210 nm (A) and 260 nm (B) containing 9.9 ng/mL U and 112.1 ng/mL UH_2 and added internal standard FUH_2.

U and UH_2 and results in laborious separation from other compounds present in plasma.

The peculiar physical properties and retention mechanism of the Hypercarb column, consisting in stationary phase with a non-derivatised porous graphitic carbon surface, have allowed us to develop a method where U and UH_2 were eluted at 22 and 34 min, respectively.

3.2.2. Elution gradient optimization

The elution gradient, consisting in water and acetonitrile, was optimized for the Hypercarb column maintained at 19 °C. The different steps were set to achieve the best separation of U, UH_2 and FUH_2 from other compounds in plasma. Elution was performed by applying a linear gradient as follows: from 1% to 11% acetonitrile in 15 min, then from 11% to 16% acetonitrile in 15 min and from 16% to 75% acetonitrile in 10 min. A 75% acetonitrile phase was applied for 10 min followed by a linear gradient from 75% to 1% acetonitrile in 2 min. The column was then equilibrated with 1% acetonitrile for 25 min before next analysis.

The peak purity for U, UH_2 and FUH_2 was checked with the Perkin-Elmer Turboscan 200 software. All Purity Index values were found between 1 and 1.5, thus confirming the purity of the chromatogram peaks. A plasma extract chromatogram of a healthy volunteer is presented in Fig. 1.

3.3. Analytical recovery

The mean analytical recoveries for U, UH_2 and FUH_2 were homogenous, $73 \pm 2\%$, $67 \pm 2\%$ and $82 \pm 3\%$, respec-

Table 2
Precision and accuracy of the method

Concentration (ng/mL)	Within-day			Between-day		
	Mean ± S.D.	R.S.D. (%)	Accuracy (%)	Mean ± S.D.	R.S.D. (%)	Accuracy (%)
Uracil						
6.25	6.37 ± 0.13	2.0	1.9	6.62 ± 0.35	5.3	6.0
12.5	12.80 ± 0.11	0.9	2.4	12.62 ± 0.36	2.8	1.0
25	25.60 ± 0.25	1.0	2.4	25.15 ± 0.60	2.4	0.6
50	51.34 ± 1.16	2.3	2.7	50.47 ± 0.93	1.8	0.9
100	101.24 ± 1.17	1.2	1.2	101.80 ± 1.34	1.3	1.8
Dihydrouracil						
12.5	12.39 ± 0.35	2.9	0.9	11.73 ± 0.83	7.1	6.1
25	23.90 ± 1.34	5.6	4.4	24.37 ± 0.62	2.5	2.5
50	49.58 ± 1.01	2.0	0.8	49.22 ± 1.05	2.1	1.6
100	101.85 ± 2.44	2.4	1.8	98.92 ± 1.29	1.3	1.1
200	202.43 ± 1.42	0.7	1.2	200.07 ± 3.98	2.0	0.1

tively. To improve the impact of the biological matrix on the extraction, we also studied the analytical recovery of FUH_2 in plasma samples ($n = 5$). The result was very similar to that obtained with water extracts as the recovery was 81 ± 2%.

3.4. Linearity, detection and quantitation limits

The calibration curves for both compounds were linear, with coefficients of determination $r^2 > 0.9998$, in the physiological concentration ranges tested (6.25–100 ng/mL for U and 12.5–200 ng/mL for UH_2).

The LOD obtained for U and UH_2 were 0.625 and 0.25 ng/mL, respectively and the LOQ calculated were 1.25 ng/mL for U (1.64 ± 0.11 ng/mL, CV: 6.48%, accuracy: 30.9%) and 0.625 for UH_2 (0.77 ± 0.03 ng/mL, CV: 3.44%, accuracy 23.2%) (Fig. 2).

3.5. Precision and accuracy

The precision and accuracy were determined with five samples per concentration. All the values are presented in Table 2. The within-day precision (R.S.D.) varied between 0.9% and 2.3% for U and between 0.7% and 5.6% for UH_2. The between-day precision was found between 1.3% and 5.3% for U and between 1.3% and 7.1% for UH_2.

The accuracy, expressed as the ratio of compound added to that measured, remained in the range 0.1–6.1% and was around 1% or 2% for higher concentrations.

Table 3
Influence of the time between sample collection and its centrifugation on plasma concentrations of U and UH_2 and on UH_2/U ratio

Time (min)	Subject C					
	U		UH_2		UH_2/U	
	Concentration (ng/mL)	Variation (%)	Concentration (ng/mL)	Variation (%)	Ratio	Variation (%)
0	10.4	0.0	87.9	0.0	8.4	0.0
0.5	10.6	2.0	89.9	2.2	8.4	0.2
1	11.9	14.0	90.7	3.1	7.6	−9.5
2	11.6	10.1	97.3	10.6	8.4	0.2
3	11.1	7.0	102.9	17.0	9.2	9.4
5	12.1	16.1	113.3	28.8	9.4	11.0
8	15.4	47.4	126.1	43.4	8.2	−2.7
Time (min)	Subject I					
	U		UH_2		UH_2/U	
	Concentration (ng/mL)	Variation (%)	Concentration (ng/mL)	Variation (%)	Ratio	Variation (%)
0	14.1	0.0	61.1	0.0	4.3	0.0
0.5	12.6	−10.9	61.1	0.1	4.8	12.3
1	12.3	−12.6	60.6	−0.8	4.9	13.5
2	15.2	7.4	62.8	2.9	4.1	−4.2
3	13.5	−4.6	60.6	−0.8	4.5	3.9
5	14.8	4.5	64.4	5.4	4.4	0.8
8	15.7	11.0	67.8	10.9	4.3	−0.1

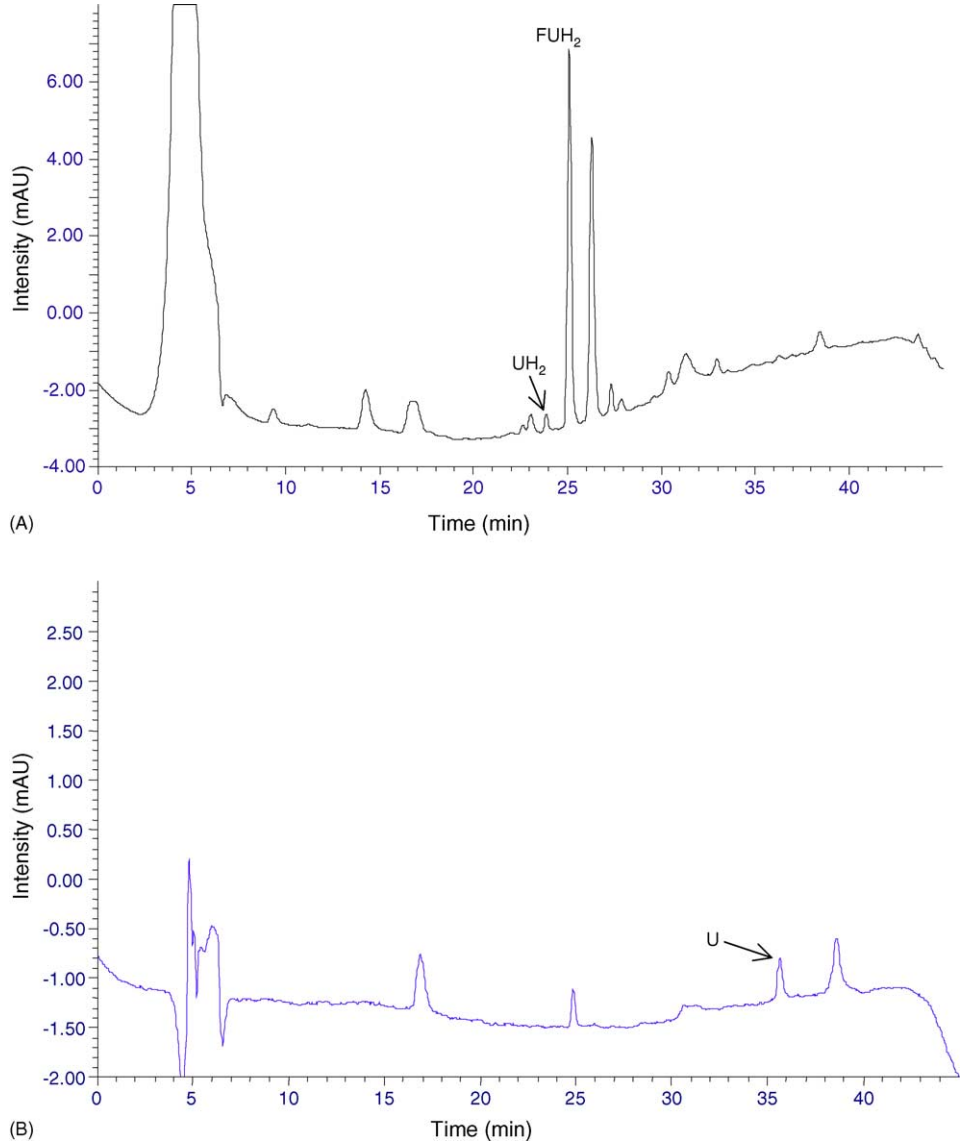

Fig. 2. HPLC Chromatograms of sample at 210 nm (A) and 260 nm (B) containing 1.25 ng/mL U and 0.625 ng/mL UH_2 and added internal standard FUH_2.

3.6. Importance of the time between the sample collection and centrifugation

In both healthy volunteers, the plasma concentrations of U and UH_2 increased with the time between the sample collection and its centrifugation (Table 3). That augmentation seemed to differ between individuals as the increase of the plasma concentrations of U and UH_2 was higher in volunteer C than in volunteer I: 40% versus 11% after 8 h for individuals C and I, respectively.

3.7. Stability of the concentrations of U and UH_2 in plasma

In both healthy volunteers, the plasma concentrations of U and UH_2 remained constant at −20 °C until 14 days leading to a stability of UH_2/U ratio with variations always above 11% compared to the initial ratio (Fig. 3).

At +4 °C and at ambient temperature, UH_2 was stable for 14 days in both healthy volunteers. The mean concentrations (averages of the seven concentrations observed during the 14 days) were 129.8 ± 8.7 ng/mL and 63.3 ± 3.1 ng/mL at +4 °C and 121.4 ± 6.1 ng/mL and 64.5 ± 2.6 ng/mL at ambient temperature, for volunteer C and E, respectively.

Counter to UH_2, U was not stable at these temperatures. In the two cases, especially at ambient temperature, U concentration increased quickly from day to day.

Because of the difference of variation of U and UH_2 concentrations, UH_2/U ratio showed an important decrease as early as the first day after the sample collection. For volunteer C, the ratio, initially of 13.4, was found at 10.1 after only 1 day at ambient temperature. After 14 days, a decrease of

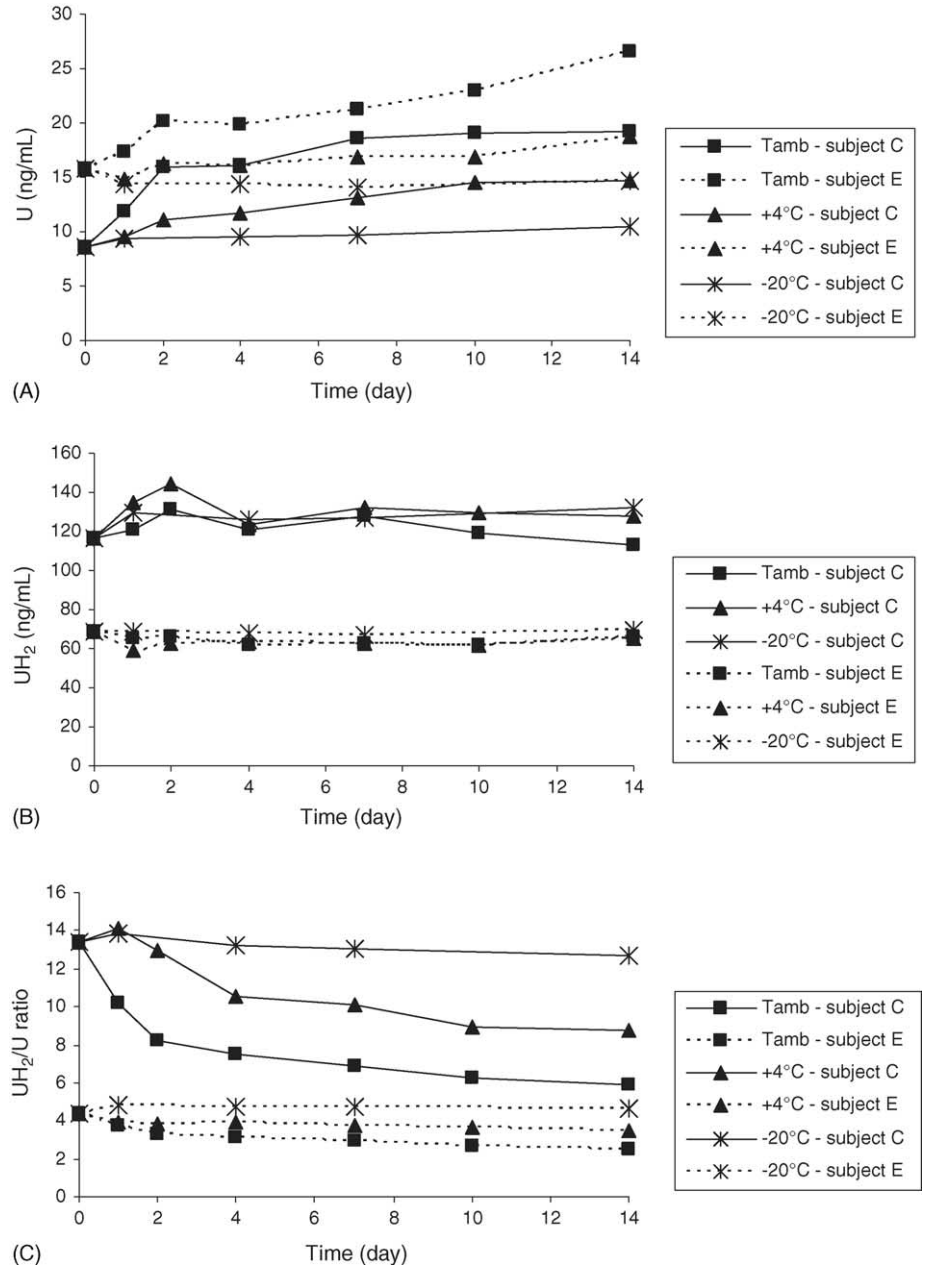

Fig. 3. Stability in two healthy volunteers of plasma concentrations of U (A), UH_2 (B) and of UH_2/U ratio (C) at three different temperatures (−20 °C, +4 °C and ambient temperature).

56% and 35% of the initial ratio was observed at ambient temperature and at +4 °C, respectively, leading to UH_2/U ratios of 8.7 and 5.9. The results observed for volunteer E were similar to those of volunteer C, but with a lower diminution of the UH_2/U ratio as the augmentation of U concentration occurred more slowly.

3.8. Measurement of the UH_2/U ratio from day to day

Results obtained for the 8 volunteers are summarized in Table 4. The relative standard deviations (R.S.D.) obtained varied between 13.0% and 24.2% and between 11.4% and 19.8% for the concentrations of U and UH_2, respectively. The variation of the UH_2/U ratio was less important as the relative standard deviations calculated for each healthy subject ranged between 8.3% and 13.1%, suggesting that the ratio remained relatively constant from day to day when blood samples were collected at the same hour of the day.

3.9. Measurement of the UH_2/U ratio during the day

As shown in Fig. 4, UH_2/U ratio seemed to vary during the day. Between 9.30 a.m. and 12:00 a.m., the ratio increased of

Table 4
Measurement of concentrations of U and UH_2 and of UH_2/U ratio from day to day in plasma of height healthy volunteers collected between 9.00 and 10.00 during 9 days

Subject identification	UH_2 (ng/ml)		U (ng/ml)		UH_2/U	
	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)	Mean ± S.D.	R.S.D. (%)
A	97.2 ± 12.1	12.4	8.4 ± 1.1	13.2	11.6 ± 1.0	9.0
B	63.9 ± 12.2	19.2	8.1 ± 1.9	22.9	8.0 ± 0.8	9.8
C	117.8 ± 13.5	11.4	9.0 ± 1.4	15.8	13.2 ± 1.1	8.3
D	83.1 ± 13.5	16.2	10.2 ± 2.5	24.2	8.3 ± 0.9	11.1
E	78.9 ± 14.9	18.9	13.7 ± 2.5	18.4	5.8 ± 0.6	10.0
F	90.0 ± 17.5	19.4	12.8 ± 3.0	23.4	7.1 ± 0.9	13.1
G	75.7 ± 11.5	15.1	7.4 ± 1.8	24.0	10.5 ± 1.3	12.2
H	66.4 ± 13.1	19.8	8.5 ± 1.9	22.6	7.9 ± 0.9	11.5

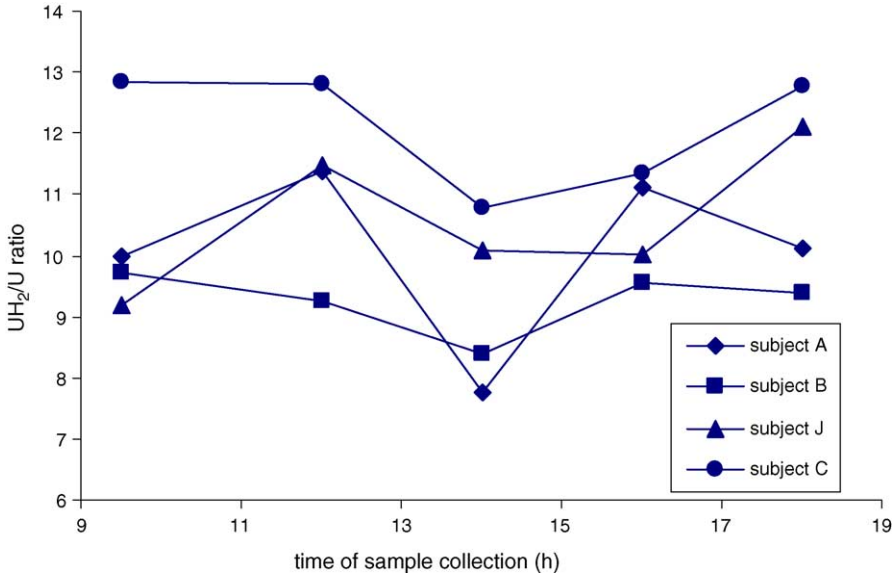

Fig. 4. Measurement of plasma UH_2/U ratio during the day in four healthy volunteers.

14% and 25% for subjects A and J while it remained constant for subjects B and C. For all the healthy volunteers, a diminution of 9–32% of the ratio was observed at 02:00 p.m. compared to the ratio at 12:00 a.m. After 02:00 p.m., the ratio increased to reach equivalent value as that found at 9.30 a.m., except for subject J for which the ratio at 06:00 p.m. was upper than the value found at 9.30 a.m. (+31%).

4. Discussion

Twenty five to thirty percent of patients develop grade III–IV toxic side effects when treated with 5-FU. Some of them occur very early, sometimes at the first cycle and are related to a lowered 5-FU catabolism. 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 pyrimidine catabolism defects is a priority. Several techniques have been proposed.

The direct measurement of DPD enzyme activity in blood mononuclear cells requires a complex procedure using radiolabelled compounds and is therefore difficult to apply to a large population of patients [5,11]. Quantitation of uracil or thymine concentration, either in urine or plasma, has also been tested but its level can be influenced by many other factors such as diet or even disease. Moreover, the measurement of urinary uracil excretion requires the simultaneous determination of creatinine level because of variation of urine concentration [15].

We developed another approach consisting of measuring the plasma concentrations of DPD substrates (U and 5-FU) and their dihydrogenated metabolites (UH_2 and FUH_2) [17]. The metabolite/substrate ratio then calculated can be considered as representative of DPD activity. We first showed the interest of this method in term of prediction of 5-FU toxicity and in term of correlation with 5-FU plasma clearance [16]. Then other authors developed techniques using mass spectrometry as a detection system after separation

by gas chromatography (GC/MS) or liquid chromatography (LC/MS) [19–21]. The main advantage of both techniques is the specificity of detection, especially in complex matrices like plasma where numerous endogenous compounds can co-elute with U or UH_2. However, they are less amenable than HPLC/UV for routine use and furthermore GC/MS requires a time-consuming derivatization procedure.

A few authors have developed techniques allowing the quantitation of the FUH_2/5-FU ratio [18,19]. However, the choice of these compounds may not be the most relevant because of the necessity of a first administration of 5-FU. Then this approach does not allow to prevent the toxicity encountered at the first course of treatment while they can be very severe and sometimes lethal.

We developed simple extraction and HPLC method for measuring U and UH_2 simultaneously in plasma. The use of a diode array detector instead of a simple UV detection system allowed us to verify the chromatographic peak purity. The extraction procedure was an improved adaptation of that used by Gamelin et al. for the extraction of 5-FU and allowed to obtain suitable and reproducible recovery of each compound [17]. Separation was optimized with a Hypercarb column, packed with porous graphitic carbon stationary phase, leading to a better separation and retention of polar compounds than other conventional columns. U and UH_2 retention times thus obtained were 22 and 34 min, respectively.

Because U and UH_2 are endogenously present in human plasma, calibration curves had to be prepared using another matrix. We tried to use albumin solution at a concentration of 10 or 40 g/L but this choice was discarded because of compounds with the same retention time as U and UH_2. As recoveries of FUH_2 in water and plasma were very similar, we finally used water extracts for the calibration curves. Linearity was validated in the concentration range studied for each compound. Sensitivity was adequate for the analysis of the low concentrations of U and UH_2 usually found in human plasma.

Using this method, we studied the stability of U and UH_2 under different conditions. As the collections are often performed in a ward far from the laboratory where blood samples are analyzed, the influence of the time between sample collection and its centrifugation was evaluated. We demonstrated that U and UH_2 plasma concentrations increased in parallel, with no impact on UH_2/U ratio.

Stability of U and UH_2 in plasma and of the UH_2/U ratio at different temperatures was also investigated. All compounds were stable at $-20\,^{\circ}C$ for 14 days, but at $+4\,^{\circ}C$ and at ambient temperature, the uracil concentration gradually increased as early as the first day while the concentration of UH_2 remained stable, inducing a slump of UH_2/U ratio.

We demonstrated in eight healthy volunteers that the UH_2/U ratio between-day variability remained relatively low provided that blood samples were collected between 09:00 a.m. and 10:00 a.m. However, the ratio varied in the day, especially with a decrease at 02:00 p.m. When blood samples collection was performed only between 9.30 a.m. and 06:00 p.m., we can guess that this variation of the UH_2/U ratio was due to a circadian rhythm, as Jiang et al. observed a circadian variation of this ratio in a study with 12 healthy subjects [22]. Thus, when using the UH_2/U ratio as a potential biomarker to prevent the toxicity in cancer patients about to receive 5-FU, blood samples have to be always collected at the same moment of the day, for example between 08:00 a.m. and 10:00 a.m.

The method described here permitted us to correlate UH_2/U ratio and both 5-FU pharmacokinetic parameters and tolerance to treatment (data not shown). It is now applied in practice to detect, before the first course of treatment, the patients at high risk of severe toxicity and to individually adapt 5-FU dosage to the patient.

References

[1] G. Milano, P. Roman, R. Khater, M. Frenay, N. Renee, M. Namer, Int. J. Cancer 41 (1988) 537.
[2] E.C. Gamelin, E.M. Danquechin-Dorval, Y.F. Dumesnil, P.J. Maillart, M.J. Goudier, P.C. Burtin, R.G. Delva, A.H. Lortholary, P.H. Gesta, F.G. Larra, Cancer 77 (1996) 441.
[3] J.L. Grem, L.K. Yee, D.J. Venzon, C.H. Takimoto, C.J. Allegra, Cancer Chemother. Pharmacol. 40 (1997) 117.
[4] G. Milano, M.C. Etienne, N. Renee, A. Thyss, M. Schneider, A. Ramaioli, F. Demard, J. Clin. Oncol. 12 (1994) 1291.
[5] M.C. Etienne, J.L. Lagrange, O. Dassonville, R. Fleming, A. Thyss, N. Renee, M. Schneider, F. Demard, G. Milano, J. Clin. Oncol. 12 (1994) 2248.
[6] Z. Lu, R. Zhang, R.B. Diasio, Cancer Res. 53 (1993) 5433.
[7] J. Santini, G. Milano, A. Thyss, N. Renee, P. Viens, P. Ayela, M. Schneider, F. Demard, Br. J. Cancer 59 (1989) 287.
[8] E. Gamelin, M. Boisdron-Celle, R. Delva, C. Regimbeau, P.E. Cailleux, C. Alleaume, M.L. Maillet, M.J. Goudier, M. Sire, M.C. Person-Joly, M. Maigre, P. Maillart, R. Fety, P. Burtin, A. Lortholary, Y. Dumesnil, L. Picon, J. Geslin, P. Gesta, E. Danquechin-Dorval, F. Larra, J. Robert, J. Clin. Oncol. 16 (1998) 1470.
[9] R.A. Fleming, G.A. Milano, M.H. Gaspard, P.J. Bargnoux, A. Thyss, R. Plagne, N. Renee, M. Schneider, F. Demard, Eur. J. Cancer 29A (1993) 740.
[10] C.H. Takimoto, Z.H. Lu, R. Zhang, M.D. Liang, L.V. Larson, L.R. Cantilena Jr., J.L. Grem, C.J. Allegra, R.B. Diasio, E. Chu, Clin. Cancer Res. 2 (1996) 477.
[11] M.R. Johnson, J. Yan, L. Shao, N. Albin, R.B. Diasio, J. Chromatogr. B Biomed. Sci. Appl. 696 (1997) 183.
[12] B.E. Harris, R. Song, S.J. Soong, R.B. Diasio, Cancer Res. 50 (1990) 197.
[13] E.S. Collie-Duguid, M.C. Etienne, G. Milano, H.L. McLeod, Pharmacogenetics 10 (2000) 217.
[14] J.A. Bakkeren, R.A. De Abreu, R.C. Sengers, F.J. Gabreels, J.M. Maas, W.O. Renier, Clin. Chim. Acta 140 (1984) 247.
[15] R. Berger, S.A. Stoker-de Vries, S.K. Wadman, M. Duran, F.A. Beemer, P.K. de Bree, J.J. Weits-Binnerts, T.J. Penders, J.K. van der Woude, Clin. Chim. Acta 141 (1984) 227.
[16] E. Gamelin, M. Boisdron-Celle, V. Guerin-Meyer, R. Delva, A. Lortholary, F. Genevieve, F. Larra, N. Ifrah, J. Robert, J. Clin. Oncol. 17 (1999) 1105.
[17] E. Gamelin, M. Boisdron-Celle, F. Larra, J. Robert, J. Liq. Chrom. Relat. Technol. 20 (1997) 3155.

[18] T. Maeda, S. Sumi, K. Hayashi, K. Kidouchi, T. Owaki, H. Togari, S. Fujimoto, Y. Wada, J. Chromatogr. B Biomed. Sci. Appl. 731 (1999) 267.
[19] S.P. Ackland, M.B. Garg, R.H. Dunstan, Anal. Biochem. 246 (1997) 79.
[20] T. Kuhara, C. Ohdoi, M. Ohse, J. Chromatogr. B Biomed. Sci. Appl. 758 (2001) 61.
[21] H. Jiang, J. Jiang, P. Hu, Y. Hu, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 769 (2002) 169.
[22] H. Jiang, J. Lu, J. Ji, Br. J. Pharmacol. 141 (2004) 616.