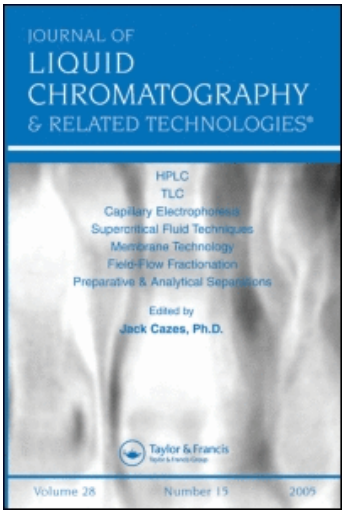

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A SIMPLE CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF PYRIMIDINES AND THEIR DIHYDROGENATED METABOLITES

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ABSTRACT

Fluorouracil (5-FU) is a pyrimidine analog widely used in the treatment of numerous malignancies. One major metabolic pathway is the reduction of its double bond by dihydropyrimidine dehydrogenase (DYPD), whose activity is a strong determinant of 5-FU plasma levels. Recent findings show that deficiencies in DYPD are less rare than generally assumed and play a major role in 5-FU toxic effects. We developped a simple, sensitive and accurate liquid chromatographic method that allows the simultaneous determination of uracil, 5-fluorouracil and their dihydrogenated metabolites in plasma. This method offers a useful tool for the detection of defects in pyrimidine degradation. HPLC was carried out by using Spherisorb ODS1 (10 cm) and ODS2 (25 cm) columns serially mounted, and 10 mM phosphate buffer, pH 3.0, as the mobile phase with UV detection at 205 nm.

Many parameters, such as mobile phase pH, ionic strength, column temperature, were found to have a marked influence on the results. We calculated the ratio dihydrouracil/uracil and could describe a Gaussian distribution of this ratio in a population of 78 healthy volunteers.

INTRODUCTION

Fluorouracil (5-FU) is widely used in the treatment of a large range of tumors and according to various schedules. Recently, the concept of dose-intensity has been applied to 5-FU and high doses of this drug are currently administered, mostly by continuous infusion, over 5 to 21 days.[1] The use of high doses by continuous infusion, and the enlargement of the indications have generated a new profile of toxicity. Several studies have reported a high individual variability of 5-FU metabolism and a close link between its toxicity and its individual pharmacokinetic parameters.[1,2] Moreover, some authors have reported a relationship between 5-FU plasma levels and the response to treatment.[1,2]

5-FU metabolism is predominantly linked to the activity of dihydropyrimidine dehydrogenase (DYPD), a key enzyme of endogenous pyrimidine metabolism, which is submitted to a genetic polymorphism.[1,3-5] Complete deficiencies have been previously reported,[4-6] in which extremely high plasma levels of 5-FU were maintained for a long time after a low dose of 5-FU, with a severe, sometimes fatal, subsequent toxicity.[7,8] There is a genetic polymorphism of 5-FU metabolism, and a large range of DYPD activity, with a Gaussian distribution, has been shown among a large population of patients.[3,5] Moreover, a relationship between the DYPD activity in lymphocytes and 5-FU plasma levels has been reported in several studies.[3-5,9]

These results raise the question of the detection of DYPD deficiencies. A method using a test-dose of 5-FU appears difficult to set in current practice, since the plasma kinetics of 5-FU is complex. Moreover, this test leads to a risk of lethal toxicity in case of complete DYPD deficiency. On the other hand, the determination of DYPD activity in lymphocytes by a radioenzymatic technique, as described by Harris et al.,[4] would be an elegant solution. However, this method is long, tedious, and needs a large quantity of blood for Ficoll separation, as well as radioactive substrate. It cannot be easily and widely used in current practice. Finally, the coefficient of correlation between DYPD activity and 5-FU plasma levels is only 0.34 and it is not known whether the enzyme activity level in lymphocytes is a reliable reflect of the enzyme activity in organs, such as liver, lung, and kidney, where this enzyme is largely

widespread. Some authors have measured uracil, the endogenous substrate, in plasma, but its levels are influenced by several parameters and it does not provide a good detection of DYPD disorders.[10,11]

We have developed a new HPLC method which enables us to measure simultaneously, in plasma, DYPD substrates and their dihydrogenated metabolites. Thus, on a single chromatogram, one can measure both uracil (U) and its reduced metabolite, dihydrouracil (UH_2), and 5-FU and its metabolite, dihydrofluorouracil (FUH_2). This technique is simple, selective, very sensitive, and allows one to determine the ratio UH_2/U, which can be considered as representative of DYPD activity.

MATERIALS AND METHODS

Chemicals

5-FU, U and UH_2 were purchased from Sigma (Saint-Quentin-Fallavier, France). FUH_2 was obtained from Hoffmann-La Roche AG (Basel, Switzerland). Ammonium sulfate, potassium dihydrogen phosphate, phosphoric acid, and all other chemicals used to prepare buffers, as well as isopropanol, chloroform, and ethyl acetate, were of HPLC grade (Cofralab, Gradignan, France). The water used was of Milli-Q grade (Millipore) and was degassed with helium before use.

Extraction

To 500 µl plasma samples, 2.5 µg of 5-bromouracil (internal standard), dissolved in 25 µL water, were first added, followed by 200 µL of 10 mM phosphate buffer, pH 3.0, and 100 µL of chloroform, and the solution was vortex-mixed. Plasma proteins were precipitated with 1,800 mg ammonium sulfate. The tubes were then vortex-mixed for 1 min and 7 mL isopropanol in ethyl acetate (15/85), as extraction solvent, were added. The tubes were gently mixed for 15 min in a rotatory stirrer (45 turns per min) and centrifuged for 4 min at 4°C (max 8,000 *g*). The supernatant was transferred to a glass tube and the solution was evaporated at 56°C for 20 min under a stream of nitrogen. The dry extract was reconstituted with 200 µL of 10 mM phosphate buffer, pH 3.0, mixed for 1 min, and 50 µL of chloroform were added. The solution was centrifuged at 4°C for 3 min and the supernatant was recovered, vortex-mixed, and 40 µL of it were injected onto the column.

Chromatography

All reversed phase analyses were performed with a Kontron chromatograph (Kontron, Montigny-le-Bretonneux, France), equipped with an autosampler (model 465), a pump (model 422 S), a variable-wavelength UV detector (model 430), set at 260 or 205 nm. A thermostated oven was used (Peltier's effect, 5°C to 100°C, from B.A.E.I., Phase Sep, Pessac, France). Peak areas were determined by electronic integration (MT2, Kontron).

The following analytical reversed phase columns, purchased from Waters (Saint-Quentin-en-Yvelines, France), were evaluated for separation : Lichrocart C_{18} (250 x 25 mm, 5 µm particle size), Symmetry C_{18} (250 x 4.6 mm, 5 µm particle size), Spherisorb ODS1 (250 or 150 x 4.6 mm, 5 µm particle size), Spherisorb ODS1 (100 x 4.6 mm, 3 µm particle size), Spherisorb ODS2 (250 x 4.6 mm, 5 µm particle size), Spherisorb ODS2 (150 x 4.6 mm, 3 µm particle size).

The mobile phase consisted in 0.01 M potassium phosphate buffer adjusted at pH 3.0 with phosphoric acid and was used routinely at a flow rate of 0.6 mL/min. The total analysis time required in these conditions for each run was 90 minutes (Figure 1). Final results could be given within 2 hours after reception of the blood sample. At least 12 plasma samples could be extracted and analyzed per day.

Sample extraction was usually done in the morning and the samples were injected in the afternoon and overnight, so as to treat the data in the next morning.

Resolution of the Peaks on the Chromatograms

The resolution factor R was calculated by using equation :

$$R = 2\frac{T_2 - T_1}{W_1 + W_2}$$

T and W being the retention times and base widths of the peaks, respectively. Two peaks were regarded as reasonably well separated when $R \leq 1$, since at this value only 2 % of peak overlap occurs.[12] Larger values of R reflect better separation.

Method Validation

Linearity

5-FU, FUH_2, U, UH_2 and 5-bromouracil were dissolved in milli-Q water at a concentration of 1 mg in 10 mL and stored at - 20°C. Standard solutions were prepared by further dilution of the appropriate standard into milli-Q water. Plasma standards were prepared in a series of polypropylene mini-Eppendorf tubes, by the addition of 25 µL standard solution of the selected compounds, 25 µL of the internal standard and 475 µL of human plasma for a final total volume of 525 µL. The dilutions were 1/1600, 1/800, 1/400, 1/200, 1/100, 1/50, 1/20, 1/10, 1/5, 1/2, giving concentrations of 6.25, 12.5, 25, 50, 100, 200, 500, 1000, 2000, 5000 µg/L, respectively. Each of the compounds was injected directly onto the column and peak area data were recorded. 5-bromouracil solutions were prepared by dilution from a 1 mg/mL solution in milli-Q water with sonication until complete dissolution.

Calibration graphs were obtained using the least-squares method. Peak area ratios between each analyte and the internal standard were used to construct the least-squares regression curves. We determined the concentrations of selected compounds in unknown plasma samples by interpolation between known concentrations.

Precision

Repeated injections (n = 5) were performed on a single day to establish the within-day coefficient of variation (precision). The between-day coefficient of variation was determined similarly. Carry-over between injections was minimal. Before each run, the syringe was rinsed and the injector loop was back-flushed with mobile phase at a flow-rate of 1.3 mL/min. Samples were run in order of increasing concentration.

Accuracy

Repeated injections (n = 5) were performed on a single day to establish the mean accuracy. The accuracy was expressed as the ratio (x 100) of the concentration measured to the concentration added.

Mean analytical recovery

5-FU, FUH_2, U and UH_2 recoveries were evaluated using similarly prepared standards. Different concentrations were studied, whereas concentrations of 5-bromouracil were maintained constant. The peak areas

measured were then compared to those recorded without extraction. Recovery was calculated by reference to unextracted aqueous solutions to which identical quantities of internal standard had been added.

Detection limit

The limit of quantitation (LOQ) and the limit of detection (LOD) of the selected compounds in plasma were determined.

RESULTS

Wavelength Selection

The absorption spectra of 5-FU, U, and 5-bromouracil exhibited two absorbance maxima at 205 and 260 nm. The wavelength 260 nm does not permit to visualize some catabolites of 5-FU and U, especially the hydrogenated ones, UH_2 and FUH_2, which have an absorbance maxima at 205 nm. We selected this wavelength for the simultaneous determination of these analytes.

Influence of Various Parameters on the Extraction Yield

Ammonium sulfate

Sample pretreatment procedures involve a deproteination step that can be accomplished by using either a precipitating agent, a membrane ultrafiltration, or an ion-exchange column. We chose a precipitating agent to avoid the use of a preparative cartridge[13] and to perform at the same time that step and the liquid-liquid extraction. We selected ammonium sulfate because it did not affect recovery in plasma samples, and the quality of the chromatograms was excellent, according to the literature and our own experience.[14]

The protein precipitation usually performed with trifluoro- or trichloroacetic acid was unfavorable, because 5-FU coprecipitated with these chemicals. Ice-cold ethanol had no effect on recovery, but it made the use of an internal standard impossible, due to overlap with interferent peaks.[15]

We tested several amounts of ammonium sulfate, from 600 to 2,000 mg. The recovery was stable but 1,800 mg permitted a better separation of the aqueous and organic phases and, thus, provided an easier withdrawal of the aqueous phase.

Extraction Solvent

We compared different systems for extraction of the compounds from plasma. The recovery was calculated by reference to the unextracted aqueous solution to which identical quantities of internal standard had been added. We selected a solution of 15 % isopropanol in ethyl acetate that provided excellent results for 5-FU extraction in previous studies and in our own experience.[16-17] We tested different volumes of this solution, especially, the ratio of organic and aqueous phase and we found that the best combination was 7 mL isopropanol-ethyl acetate, 200 µL phosphate buffer (10 mM, pH 3.0) and 500 µL of plasma. This addition of 200 µL of mobile phase improved the recovery, compared to either nothing or 200 µL water, maybe because of the light acidification it produced. The addition of chloroform for extraction was used for eliminating interfering compounds and did not affect the extraction of the compounds.

We also tested an acidic extraction with sulfuric or phosphoric acid. They did not give good results, because they simultaneously increased the extraction of interfering compounds. The use of diethylether decreased the extraction yield and led to the occurrence of a compound which interfered with 5-FU.

Mixing appeared to be an essential step in the extraction. We tested the type of mixing by comparing vortex-mixing and gentle mixing in a rotative stirrer (45 turns per min). They provided equivalent extraction yield, but gentle mixing gave more reproducible results and was not operator-dependent.

Choice of the Internal Standard

Several compounds were tested : 5-fluorocytosine, 5-chlorouracil, 5-bromouracil, and 5-fluorouridine. We compared their extraction yield with isopropanol/ethyl acetate and the resolution of their peaks on the chromatogram. We selected 5-bromouracil that had an excellent recovery (90 %) and appeared isolated on the chromatogram. Unfortunately, its retention time is relatively long, 45 to 50 min.

Influence of Different Parameters on the Retention Times

Column type

Table 1 describes the columns used and shows the resolution factors for FUH_2 and UH_2 (R1), UH_2 and U (R*2*), U and 5-FU (R*3*), as an estimate of the performance of each system. The results with each column are described

Table 1

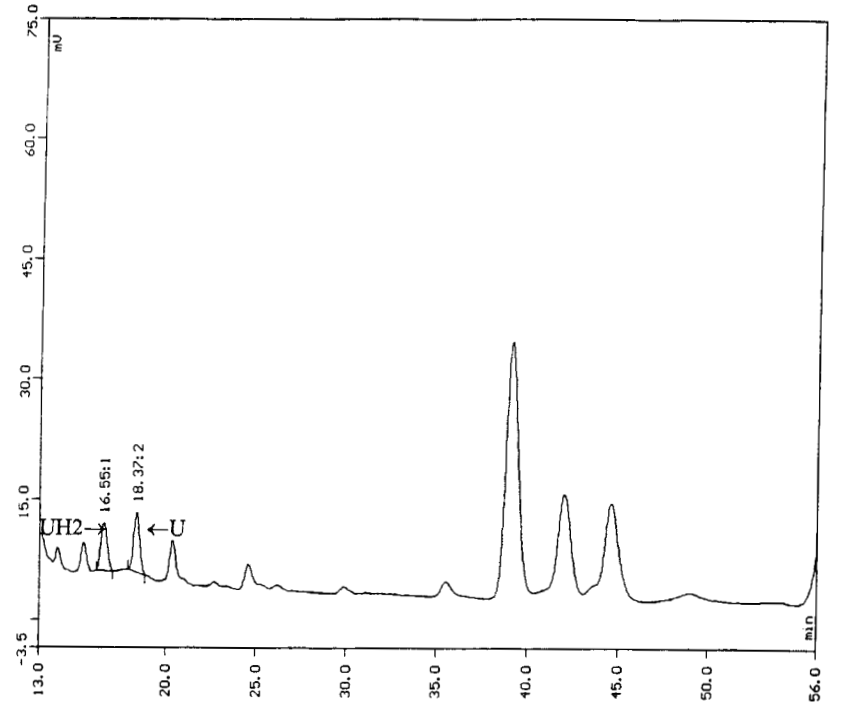
Influence of Column Characteristics on the Resolution Factor

Column	Length (mm)	Particle Size FUH_2 (μm)	Resolution Factor (R) UH_2 R1	U R2	5-FU R3
ODS1	100	3	3.3	0.34	0.64
ODS1	250	5	7.43	0.4	1.9
ODS2	150	3	<1	<1	1.5
ODS2	250	5	0.18	1.6	2.5
ODS1-ODS2	100+150	3+5	2.9	1	1
ODS1-ODS2	100+150	3+3	2.9	0.6	0.78
ODS1-ODS2	150+250	5+5	3.2	1.7	2
ODS1-ODS2	100+250	3+5	2.3	1.6	2
ODS1-ODS2	250+250	5+5	4.4	1.5	0.8
Symmetry	250	5	<1	<1	1.2
Lichrocart	250	5	0.38	0.93	1.33

below. ODS1 columns gave a good resolution of dihydrogenated compounds, but they were less efficient for separating U and 5-FU. On the other hand, ODS2 columns separated 5-FU and U well, but not dihydrogenated metabolites. Using both types of columns serially mounted, we could obtain a good separation of the 4 compounds. The best results were obtained with the combination of ODS1 (100 mm length, 3 μm particle size) and ODS2 (250 mm length, 5 μm particle size) columns.

Other columns, such as Symmetry and Lichrocart, have been tested but they did not provide a good resolution of FuH_2 and UH_2. A typical chromatogram obtained in the optimal conditions as described here is presented on Figure 1.

Figure 1 (right). A typical chromatogramme obtained with a plasma extract containing pyrimidines and dihydrogenated metabolites; *above*: blank chromatogram, retention times: UH_2 = 16.6 min, U = 18.4 min. There is no peak at 5-FU, 5-FUH_2 and 5-BU retention times; *below*: 5-FuH_2, 5Fu and 5-BU retention times are 14.59 min, 19.48 min and 53.20 min, respectively. Conditions as follows : 2 columns serially mounted, ODS1 (150, 5μm) and ODS2 (250, 5 μm); flow rate = 0.6 mL/min; column temperature = 12°C.



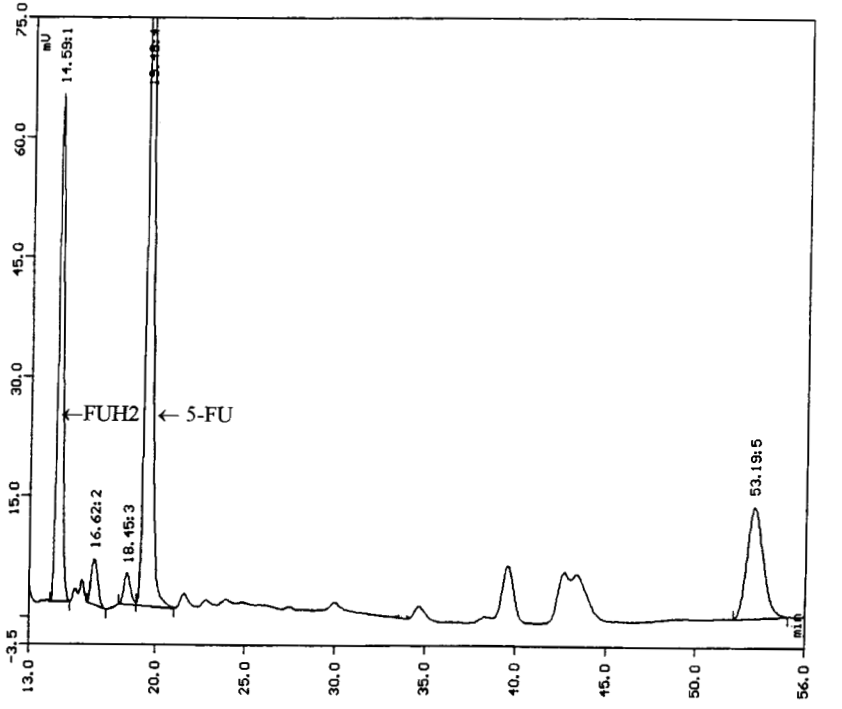


Table 2

Influence of Flow Rate on the Resolution Factor

	Resolution Factor (R)		
Flow Rate (mL/min)	**FUH_2 R1**	**UH_2 R2**	**U R3**
0.6	2.7	1.94	3.03
0.7	2.5	1.86	3.06
0.9	2.5	1.85	2.9

Table 3

Influence of Column Temperature on the Resolution Factor

	Resolution Factor (R)		
Temperature	**FUH_2 R1**	**UH_2 R2**	**U R3**
24°C	2.7	1.94	3.03
15°C	3.44	2.23	1.77
12°C	3.73	2.53	1.58
8°C	4.07	2.85	1.3

Flow-rate

We tested several flow rates (Table 2) and selected 0.6 mL/min because it provided the better resolution, and avoided too high column pressure (> 170 bars).

Temperature

The column temperature influences greatly the retention time of each analyte in different ways. Table 3 shows the resolution factors at three different temperatures. We selected 12°C which provided the best results for the separation of the dihydrogenated metabolites and pyrimidines.

Method Validation

Linearity

Quantitations of 5-FU, U, FUH_2 and UH_2 were obtained from calibration curves in which the peak area ratio drug/internal standard was plotted against the drug concentration. There was a linear relationship between the peak area ratios of the selected compounds over the concentration range 12.5-5,000 μg/L. The correlation coefficients for the calibration curves were all > 0.9999 (n = 5) for each compound (Figure 2).

We tested different concentrations of 5-FU and FUH_2 on one hand, and U and UH_2 on the other. New schedules use very high doses of 5-FU, according to the concept of dose-intensity, and high FUH_2 concentrations in plasma can be expected, whereas the plasma concentrations of the natural substrates are much lower, rarely over 200 μg/L.

Precision

The data for the validation of the within-day and between-day precisions are presented in Table 4. The results show very low coefficients of variation, even for low plasma levels.

For all compounds, the within-day and the between-day reproducibilities were always lower than 2% and 4%, respectively.

Accuracy

The accuracy, expressed as the ratio of compound added to that measured, is also presented in Table 4. It remains in the range 2-4% for low concentrations and was generally around 1% for higher concentrations.

Detection limits

The limit of quantitation (LOQ) of U, 5-FU, UH2, and FUH2 was 6 μg/L.

Mean analytical recovery

Table 5 shows that the recoveries ranged between 75% for FUH_2 and 90% 5-bromouracil.

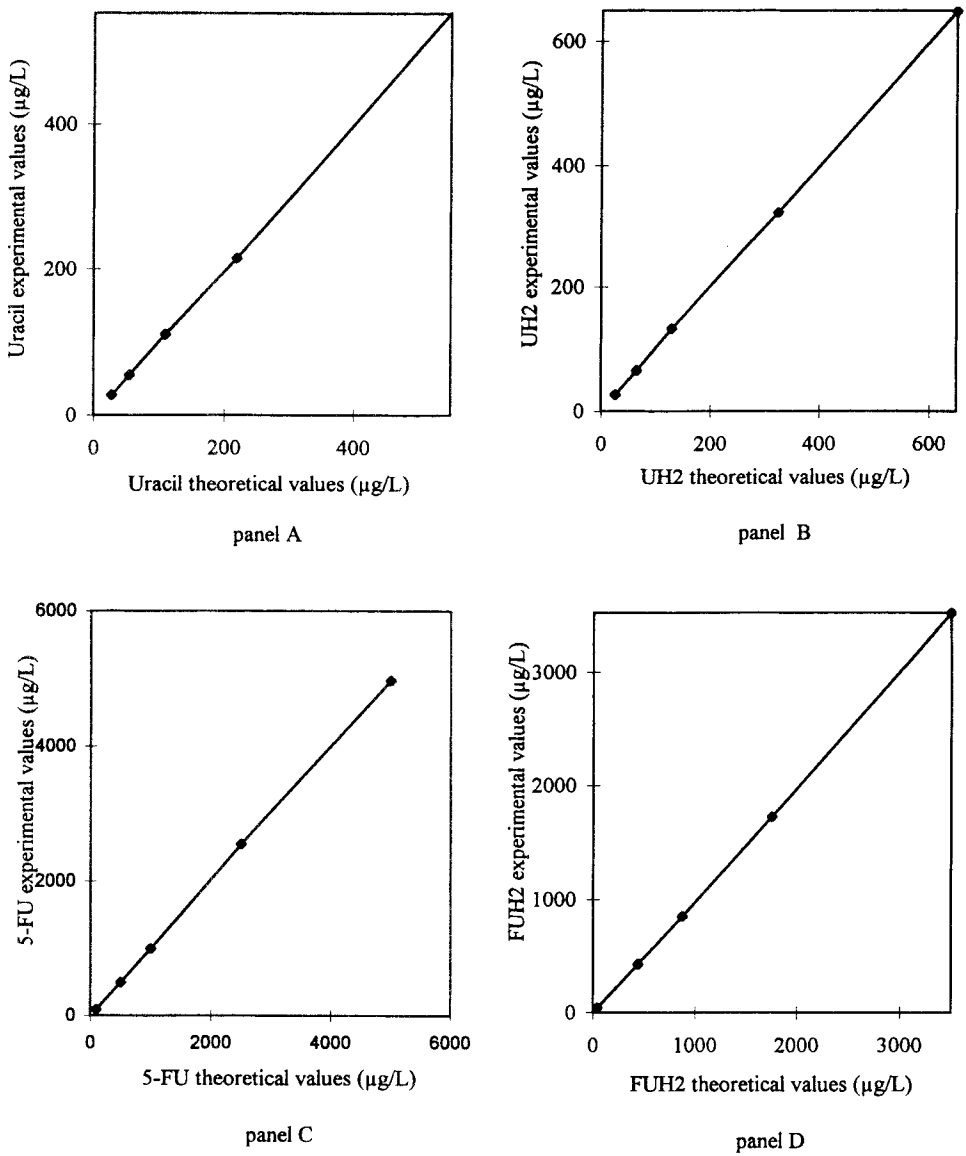


Figure 2. Study of the linearity of pyrimidines and their metabolites extractions. Regression slopes and coefficients of correlation were as follows : U (panel A) : y=0.9999x , R^2=0.9998 ; UH_2 (panel B) : y=x; R^2=0.999 ; 5-FU (panel C) : y=0.9999x , R^2=0.9998 ; FUH_2 (panel D) :y=0.9999x , R^2=0.9997.

Table 4

Reproducibility and Accuracy of the Determinations of U, UH_2, 5-FU, and FUH_2 in Plasma

Dihydrofluorouracil

	Within-Day (n=5)			Between-Day (n=5)		
Conc μg/L	Mean±SD	CV%	Accuracy	Mean±SD	CV%	Accuracy%
43.75	42.44±0.8	1.8	3	45.58±9.1	2	4.1
437.5	427.1±5.5	1.3	2.4	435.14±11	2.6	0.6
875	860.4±1.5	1.75	1.67	871.3±12	1.4	0.43
1750	1744±8.4	0.5	0.35	1732.8±26	1.5	1
3500	3496±23.5	0.7	0.12	3495±37.4	1	0.13

Dihydrouracil

	Wthin-Day (n=5)			Between-Day (n=5)		
Conc μg/L	Mean±SD	CV%	Accuracy	Mean±SD	CV%	Accuracy%
26	25.86±0.2	0.2	0.54	25.9±0.34	0.85	0.47
65	66.53±0.6	0.95	2.35	66.9±1	1.53	2.8
130	132.7±1.5	1	2.08	133.1±1.6	1.19	2.4
325	324.7±1.5	0.46	0.01	324.5±1.4	0.43	0.15
650	652.7±7.5	1.6	0.41	653.7±8.9	1.4	0.56

Uracil

	Wthin-Day (n=5)			Between-Day (n=5)		
Conc μg/L	Mean±SD	CV%	Accuracy	Mean±SD	CV%	Accuracy%
27.5	26.9±0.5	1.85	2.22	27±1	3.8	1.86
55	55.95±0.7	1.16	1.7	55.45±1	1.9	0.8
110	108.2±1.2	1.13	1.7	108.8±2.3	2.1	1.14
220	220.5±2.3	1.02	0.22	220±1.8	0.8	0.04
550	550.7±6.5	1.17	0.12	550.8±4.9	0.89	0.15

5-Fluorouracil

	Wthin-Day (n=5)			Between-Day (n=5)		
Conc μg/L	Mean±SD	CV%	Accuracy	Mean±SD	CV%	Accuracy%
290	286.9±2.5	0.87	1.06	286.3±3.3	1.14	1.25
540	530.45±11	2.07	1.77	533.6±13	2.41	1.19
1040	1034±16.7	1.61	0.56	1034±15.3	1.48	0.56
2040	2056±23	1.11	0.78	2052±18	0.88	0.6
4040	4017.4±50	1.25	0.57	4030±43	1.07	0.22

Table 5

Mean Analytical Recovery (Mean ± SD) of the Four Compounds

n=8	FUH_2	UH_2	U	5-FU	5-BU
Mean Recovery	85±1.66	75.6±1.7	79.25±2.6	84.75±2.33	90.75± 2.33

Study of Dihydrouracil/Uracil Ratio in Healthy Volunteers

We measured plasma concentrations of U and UH_2 in a population of 47 healthy volunteers. Blood samples were collected in heparinized tubes and centrifuged within the next hour for 5 min at 8,000 *g*. The resulting plasma was then treated in a way identical to that described as final method, or immediately stored at -20°C and later transferred to a -70°C deep freezer until analysis. The values of the ratio of the concentrations of UH_2 to those of U are presented in Figure 3.

DISCUSSION

Pyrimidine metabolism disorders have recently been the focus of considerable attention. DYPD activity presents a large dispersion among individuals. Partial deficiencies may be rendered responsible for the toxic events encountered in the population of patients treated with 5-FU, which are related precisely to the 5-FU steady-state plasma levels.[6-8]

Complete deficiencies seem to be rather exceptional, but are responsible for the occurrence of an extremely severe toxicity of 5-FU, frequently lethal in patients as early as the first course of treatment.[6-8,10,11] Such patients generally present no signs other than this extreme sensitivity to 5-FU, but may also present clinical symptoms such as mental retardation or neurologic disorders.[10,11]

Several authors have developed techniques for the determination of DYPD activity in lymphocytes using a radiolabelled substrate; this method is accurate, but difficulties arise when dealing with large populations, owing to the complex procedures used for measuring enzyme activity.[3-5,9] In addition, the DYPD activity in lymphocytes may not relect the enzyme activity present in the organs, which are in charge of the major part of the metabolic transformation of 5-FU, especially the liver.

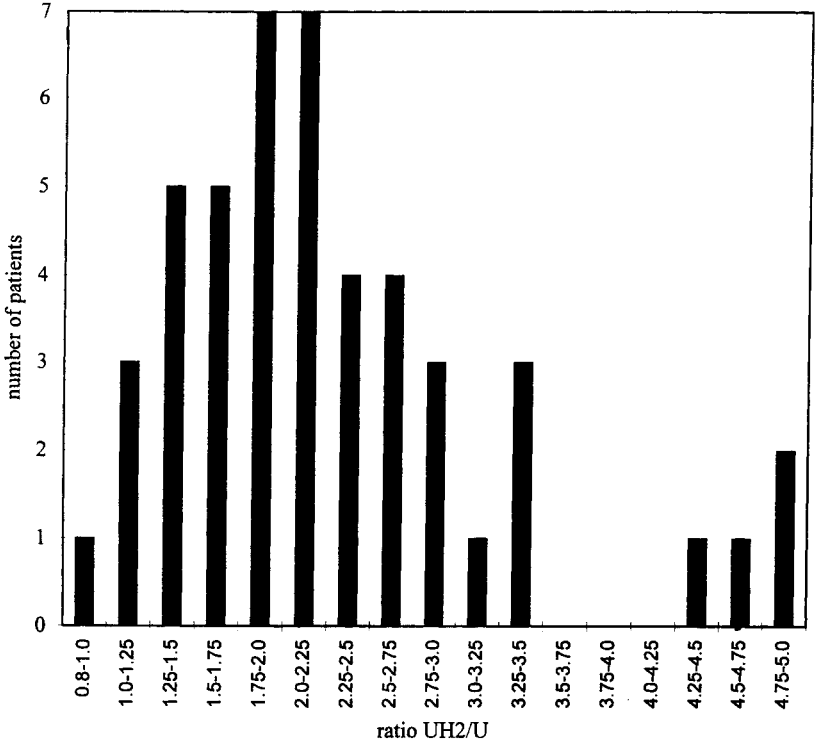


Figure 3. Distribution of UH_2/U ratios in a population of 47 healthy volunteers. This distribution is Gaussian.

It could be possible to approach this problem by the evaluation of the circulating levels of the dihydro derivatives, either of a naturally occurring pyrimidine, uracil, or of 5-FU itself. Methods have been previously reported for the measure of uracil, thymine, and their metabolites in urine.[10,18,19]

However, none of them reported the normal values of excretion of thymine and uracil in healthy populations, and no reference values for the ratios of dihydrometabolites to original pyrimidines in urine.

The method we propose allows the determination of both U and UH_2/U ratio in plasma. More than the single measurement of U concentration in plasma, it gives a reflect of the endogenous DYPD substrate and of its metabolite. It appeared to be sensitive, linear, and therefore, very suitable for

the routine studies. A further advantage of the method over previously HPLC published methods is the simplicity of extraction.[16,20,21] Sample preparation is rapid, but the time required for chromatography is rather long, since the total analysis time required for each run is 90 minutes.

Final results can be given within two hours after blood sampling. Therefore, about 12 samples can be handled daily, enabling injections and calculations to be done overnight, making this technique quite adapted to routine applications.

In a population of 47 healthy volunteers, the UH_2/U ratio followed a Gaussian curve that can be compared to that of DYPD activity, as reported previously by Etienne et al.[3] We are now currently exploring the relationship between this ratio and the plasma levels of 5-FU in patients treated with this drug, in order to evaluate the predictibility of this parameter on 5-FU toxicity.

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