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Journal of Chromatography B, 695 (1997) 409–416

JOURNAL OF CHROMATOGRAPHY B

Rapid and sensitive high-performance liquid chromatographic analysis of halogenopyrimidines in plasma

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Received 27 November 1996; received in revised form 7 March 1997; accepted 24 March 1997

Abstract

Recent studies have stressed the need for individual adjustment of 5-fluorouracil (5-FU) dosage. Most of the techniques previously reported are not well adapted to routine application. We describe a sensitive, selective and simple HPLC technique under isocratic conditions for the quantitation of 5-FU and other halogenopyrimidines. The proportion of reagents and internal standard were optimised to allow the use of minitubes, particularly adapted to large series of plasma assays. High extraction yield, 75% for 5-FU and 90% for 5-bromouracil and 5-chlorouracil, was obtained using 1.2 ml isopropanol–ethyl acetate (15:85, v/v) following precipitation of plasma proteins with 300 mg ammonium sulfate. The mobile phase was 0.01 *M* phosphate buffer (pH 3.0). Uracil and 5-fluorouracil were fully resolved with Spherisorb ODS2 column. The limits of quantitation and detection in human plasma were 6 ng ml^{-1} and 3 ng ml^{-1}, respectively, for all compounds studied. The total analysis time required for each run was 25 min. Final results could be given within 90 min of blood sampling. At least 50 plasma samples could be analysed per day. This method has been successfully used for monitoring 5-FU-based treatments. © 1997 Elsevier Science B.V.

Keywords: Halogenopyrimidines; 5-Fluorouracil; 5-Bromouracil; 5-Chlorouracil; 5-Fluorocytosine; 5-Methylcytosine

1. Introduction

After 30 years of usage, 5-fluorouracil (5-FU) remains widely used in the treatment of a large range of tumors and according to various schedules. High doses of this drug are currently administered, mostly by continuous infusion, over 5 to 21 days. Some authors have reported a relationship between 5-FU plasma levels and its toxicity and the response to the treatment [1,2]. Several studies have reported a high individual variability in 5-FU pharmacokinetics due to a genetic polymorphism of its metabolism [3]. Complete deficiencies in dihydropyrimidine dehydrogenase (DPD), the key enzyme of 5-FU catabolism, have been reported [4]. In such cases, extremely high plasma levels of 5-FU were maintained for long periods and the subsequent toxicity was severe, sometimes fatal [5]. Since toxicity and response rates clearly correlate to 5-FU plasma levels in different administration schedules, drug levels measurement in plasma can help to monitor 5-FU dose and to develop an optimum procedure for its administration. Some authors have already reported promising results in 5-FU dose monitoring by pharmacokinetic

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PII S0378-4347(97)00211-9

follow-up with liquid chromatography [1,2]. The high aqueous solubility of 5-FU makes it difficult to extract into organic solvents. Numerous HPLC assays reported previously are relatively sensitive, but are time-consuming (see Zakaria and Brown for review [6]). Sample pretreatment procedures vary a lot and most of them involve several steps such as deproteination of plasma either by a precipitating agent, a membrane ultrafiltration or an ion-exchange column and extraction. Many extraction procedures are too complex to be compatible for a rapid routine analysis. Besides, it is difficult to resolve 5-FU from the internal standard and uracil with the commonly used reversed-phase columns [7]. Since 5-FU and uracil have relatively high pK_a values, high pH values could improve their separation [8], but most reversed-phase columns restrict the use to eluents with pH values of less than 7 [9,10]. Moreover, the limit of detection and quantitation of most methods is high, 100–300 $\mu g\,l^{-1}$ [11]. We have developed a new, simple, sensitive and fast method, perfectly adapted to routine application. The procedure of 5-FU extraction from plasma avoids the back extraction commonly used and permits use of a micromethod. Moreover, it allows full resolution of 5-FU from uracil.

2. Experimental

2.1. Chemicals and hplc reagents

5-Fluorouracil (5-FU), 5-chlorouracil (5-CU), 5-fluorocytosine (5-FC), 5-bromouracil (5-BU), uracil (U) and 5-methylcytosine (5-MC) were purchased from Sigma (St Louis, MO, USA). Ammonium sulfate, potassium dihydrogen phosphate, phosphoric acid were of HPLC grade (Normapur, supplied by Prolabo, Gradignan, France). Isopropanol, methanol, ethyl acetate were of HPLC grade. The water used was of Milli-Q grade and degassed with helium. The mobile phase consisted of phosphate buffer at concentrations varying between 0.01 and 0.1 *M*. We also studied mobile phase pH values between 2.2 and 6.0, the adjustment being done with 1 *M* phosphoric acid. Addition of an ion-pairing reagent (heptane sulfonate) was tested at concentrations of 1 and 2 m*M*.

2.2. HPLC instrumentation

Separation of compounds was carried out with a thermostat, on different C_{18} reversed-phase columns (see below). A matching guard column Spheri-RP 18 5 μm (Brownlee, Touzart et Matignon, France) or Spherisorb 5 μm 1 cm (Phase Sep, Pessac, France) preceded the column. A 422 S pump from Kontron Instruments (Montigny-le-Bretonneux, France) delivered the mobile phase, at a flow-rate of 1.3 $ml\,min^{-1}$. A HPLC Autosampler 460 automatic injector (Kontron), equipped with a variable volume loop injected the samples onto the column. Detection was carried out at 260 nm with a 332 variable-wavelength UV detector (Kontron). A MT2 computer (Kontron) monitored the system and recorded the peak data.

2.3. Chromatographic conditions

The mobile phase was 0.01 *M* potassium dihydrogen phosphate (pH 3.0) adjusted with 1 *M* orthophosphoric acid. The flow-rate was 1.3 $ml\,min^{-1}$. The column was Spherisorb ODS2 (250× 4.6 mm with 5 μm particles, 12% C). The final internal standard was either 5-BU or 5-CU.

2.4. Influence of different parameters on the retention times

The effects of the major parameters were studied for optimizing the resolution of the different compounds: the pH and the ionic strength of the mobile phase and a ion-pairing reagent (heptane sulfonate). The resolution factor (R) was calculated by the equation: $R=2\,(T_2-T_1)/\,W_1+W_2$, T and W being the retention times and base widths of the peaks. The two peaks were regarded as reasonably well separated when $R=1$, since at this value only 2% of peak overlap occurs [12]. Larger R values reflect better resolution.

2.4.1. Column type

Different C_{18} reversed-phase columns were tested: Spherisorb 5 ODS1 (250×4.6 mm, packed with 5 μm particles, 7% C) purchased from Biochrom (Champniers, France), Spherisorb 5 ODS1 (250×4.6 mm with 5 μm particles, 7% C) purchased from

Phase Sep). Spherisorb 5 ODS2 (250×4.6 mm with 5 μm particles, 12% C) purchased from Phase Sep, Micro-Bondapak C_{18} (250×5 mm, 5 μm particle size) purchased from Waters (Saint Quentin-en-Yvelines, France), Symmetry C_{18} (250×4.6 mm with 5 μm particles, 19.5% C) purchased from Waters, and LiCrospher 100 RP-18 (250×4.6 mm with 5 μm particles, 21.5% C) from Merck (Nogent-sur-Marne, France).

2.4.2. Column temperature

Different temperatures of the columns were tested from 15°C to 40°C to ensure a better separation of the compounds.

2.5. General extraction procedure

The internal standard was first added to 500 μl plasma samples which were vortex-mixed. Plasma proteins were precipitated by the addition of 300 mg ammonium sulfate and the tubes were vortex-mixed for 1 min and centrifuged for 5 min (≤8000 *g*). Then, 1200 μl isopropanol–ethyl acetate (15:85) were added. The tubes were vortex-mixed for 3 min and centrifuged for 15 min (≤8000 *g*). The supernatant was separated from the pellet and transferred to a mini-Eppendorf tube. The solution was evaporated at 56°C for 20 min under a stream of nitrogen. The dry extract was diluted in 200 μl mobile phase and it was transferred into conical tubes.

This solution was briefly vortex-mixed and 40 μl injected onto the column. The total analysis time required for each run was 25 min. Final results could be given in 90 min. At least 50 plasma samples could be analysed per day. Sample extraction was usually done in the afternoon and the samples were injected overnight so as to obtain the data the next morning.

2.6. Method validation

2.6.1. Linearity

Fluorouracil, uracil, and the different potential internal standards 5-CU, 5-FC, 5-MC, 5-BU, were dissolved in deionised milli Q water at a concentration of 2 mg in 10 ml and stored at −20°C. Standard solutions were prepared by further dilution of the appropriate standard into 10 m*M* potassium dihydrogen phosphate (pH 3). Plasma standards were prepared in a series of polypropylene mini-Eppendorf tubes, by the addition of 25 μl of the 5-FU standard solution, 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 for concentrations of 6.25, 12.5, 25, 50, 100, 200, 500, 1000, 2000, 5000 $\mu g\,l^{-1}$, respectively. Each of the compounds was injected directly onto the column and peak area data were recorded. 5-CU and 5-BU solutions were prepared by dilution from a 1 $mg\,ml^{-1}$ 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 its corresponding internal standard were used to construct the least-squares regression lines. We determined the concentrations of 5-FU in plasma by interpolation from the graphs using the peak-area ratios obtained from unknown samples.

We prepared 5-FU solutions in plasma at 5 different concentrations based on the expected range of concentrations: (6.25, 12.5, 25, 50 and 100 $\mu g\,l^{-1}$), (50, 100, 200, 500 and 1000 $\mu g\,l^{-1}$), (200, 500, 1000, 2500 and 5000 $\mu g\,l^{-1}$).

In routine practice, plasma standards were analysed concurrently with each set of unknown samples. Two controls were included in each batch. They contained a drug-free sample spiked with known amounts of 5-FU and the internal standard. Samples contained 500 μl of patient's plasma plus 25 μl of internal standard solution.

2.6.2. 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 by the same way. Carry-over between injections was minimal. Before each sample run, the syringe was rinsed and the injector loop was back-flushed with mobile phase at a flow-rate of 1.3 $ml\,min^{-1}$. Standards were assayed in order of increasing concentration.

2.6.3. Accuracy

Repeated injections ($n=5$) were performed on a single day to establish the mean accuracy. The

accuracy was expressed as the ratio of the compound added to that measured.

2.6.4. Mean analytical recovery

Fluorouracil recovery was investigated using similarly prepared standards. Different concentrations were analysed, whereas concentrations of two possible internal standards, 5-CU and 5-BU, were constant. The peak areas measured were then compared to those recorded when the plasma was replaced by an equal volume of mobile phase. The recovery was calculated by reference to the non-extracted aqueous solution to which identical quantities of internal standard had been added.

2.6.5. Detection limit

The limit of quantitation (LOQ) was 6.25 ng ml^{-1} and the limit of detection (LOD) of 5-FU was 3 ng ml^{-1}.

3. Results and discussion

3.1. Method optimisation

3.1.1. UV wavelength selection

The absorption spectra of 5-FU, U, 5-FC, 5-CU and 5-BU exhibited two absorbance maxima at 200 and 260 nm. The wavelength of 260 nm was selected for the simultaneous determination of the analytes. It eliminated a lot of interfering peaks of other compounds.

3.1.2. Influence of pH

Variations of pH of the mobile phase had a very limited effect on 5-FU, 5-CU, 5-BU and U retention times and did not help to resolve them. It had a marked effect on 5-FC retention. A pH value of 3.0 was chosen to respect the reversed-phase column.

3.1.3. Influence of ionic strength

The ionic strength of the phosphate buffer (potassium dihydrogen phosphate) (0.01 to 0.10 *M*) had a large influence on 5-FC retention time (increasing strength resulting in decreasing retention) but much less important on that of 5-FU, 5-CU and 5-BU. Table 1 shows that a low ionic strength of the mobile phase improved 5-FU and U resolution. Therefore, we selected ionic strength 0.01 *M* for a better resolution of 5-FU and U.

Table 1
Influence of column type, column temperature and ionic strength of the mobile phase on 5-FU and U resolution[a]

Parameters	Peak resolution factor (*R*)
Column type	
ODS1 (250 mm, 5 μm)	1.9
ODS2 (250 mm, 5 μm)	2.5
Column temperature	
25°C	3
15°C	1.8
12°C	1.6
8°C	1.3
Ionic strength	
0.1 *M*	1.3
0.5 *M*	1.5
0.01 *M*	1.8

[a] When testing one parameter, the other ones were constant. ODS2 column appeared more appropriate, increased temperature and lower ionic strength gave better U and 5-FU resolution. The best conditions were ODS2 column, temperature >20°C, 0.01 *M* ionic strength.

3.1.4. Influence of ion-pairing

We tested the presence of a counter ion. It was only useful to resolve 5-FU from 5-FC but it had no effect on 5-FU, 5-CU, 5-BU and U retention times.

3.1.5. Column type

Fluorouracil is not easily separated from structurally related compounds, especially U, and other pyrimidines with commonly used reversed-phase columns [7]. Miller et al. tested several such columns for their ability to separate 5-FU from other components and found that they poorly resolved 5-FU from U [12].

We tested six C_{18} columns. Micro-Bondapack and Spherisorb ODS1 from Biochrom provided good quality chromatograms but could not resolve 5-FU and U. Spherisorb ODS1 from Phase Sep could separate the 2 peaks, 5-FU and uracil at 17°C, but did not enable precise measurement of either of them. Symmetry column provided an excellent separation of 5-FU and U as pure standards but chromatograms from plasma extracts were not interpretable because of too many peaks. Spherisorb ODS2

from Phase Sep separated completely 5-FU and U from both pure solutions and plasma and enabled an easy interpretation of the chromatograms (Fig. 1).

3.1.6. Column temperature

The column temperature greatly influenced retention times of 5-FU, U, CU and BU, increasing or lowering them, depending on the type of the column. The best temperature for 5-FU and U resolution with Spherisorb ODS2 column, was >20°C, if 5-CU was used as internal standard. However, at this temperature, if 5-BU was used as internal standard, it could not be separated from an interfering peak and the best temperature was then 25°C (Table 1). These results stress the need for a thermostat for stable retention times.

3.1.7. Influence of parameters on the extraction ratio

3.1.7.1. *Ammonium sulfate.* Deproteination can be accomplished by using a precipitating agent, a membrane ultrafiltration or an ion-exchange column. A precipitating agent avoids the use of a preparative column chromatography [13] and can be used simultaneously with the liquid–liquid extraction. The protein precipitation usually performed with trifluoro- or trichloroacetic acid lowered the recovery because 5-FU coprecipitated with these chemicals. Ice-cold ethanol had no repercussion on recovery but it made the use of an internal standard impossible owing to overlap with interfering peaks [11]. In contrast, ammonium sulfate did not affect recovery in plasma samples, and the quality of the chromato-

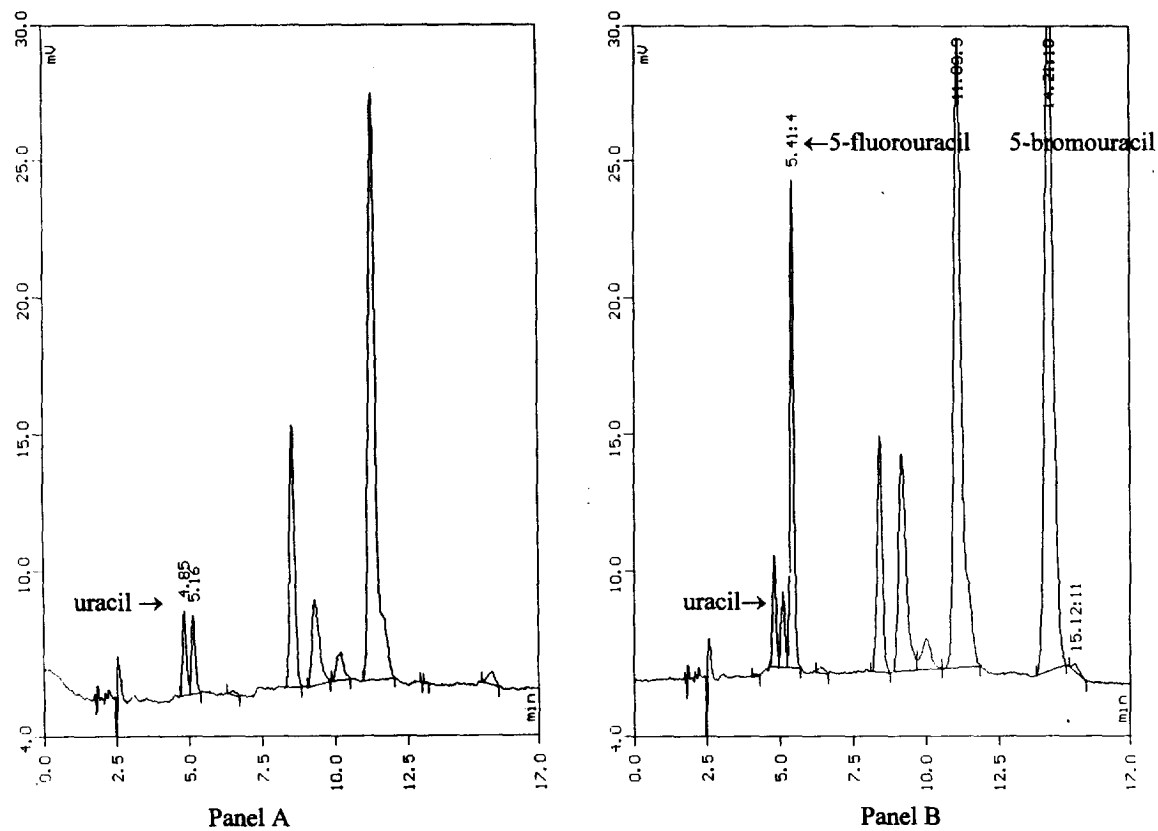

Fig. 1. Panel A: chromatogram obtained with Spherisorb 5 ODS2 column, 5-BU (1050 ng ml^{-1}) used as internal standard. Its retention time is 14.21 min. 5-FU (200 ng ml^{-1}) and U retention times are 5.41 min and 4.85 min, respectively. Panel B: blank chromatogram showing the absence of endogenous peaks interfering with both 5-FU and 5-BU.

grams is excellent according to the literature and our own experience [8]. Compared to the ammonium sulfate solution, the powder presentation gave reduced extraction volume. We tested several amounts of ammonium sulfate. The gain of recovery was very small beyond 300 mg (Fig. 2), and that quantity was the best compromise, for a good recovery and a minimal volume.

3.1.7.2. *Extraction solvent.* Different systems were tested for 5-FU extraction from aqueous solutions. The internal standard was added immediately before extraction. The recovery was calculated by reference to the non-extracted aqueous solution to which identical quantities of internal standard had been added. Different solutions and volumes were tested: chloroform, 16% isopropanol in diethyl ether [14,15], 16% isopropanol in ethyl acetate [16,17], 20% diethyl ether in ethyl acetate. Sixteen percent isopropanol in ethyl acetate gave the best percentage of extraction. Chloroform provided poor recoveries, 20% for 5-FU and <10% for 5-FC. Propanol–diethyl ether gave 40% recovery for 5-FU and 10% for 5-FC although it was the solvent the most widely used in the literature [11,14,15]. Fluorouracil recovery depended on the volume of the extraction solvent and 1200 μl was the minimum volume to reach a 5-FU recovery >70%, from 500 μl of plasma. Over 1400 μl, the recovery gain was negligible. Vortex-mixing was an essential step in the extraction.

Fluorouracil presents a high aqueous solubility that makes it difficult to extract into organic solvents. In many methods previously described, either 5-FU recoveries were mediocre and fluctuant, or detectable amounts of interfering substances hampered the technique and quantitation was difficult at lower levels of 5-FU. The authors adopted different ways to overcome this obstacle: (1) they used a sequential cationic and anionic procedure [18]; (2) they passed the plasma through an anion-exchange column before the extraction procedure [19]; (3) they used a double or a back extraction [7,8,14,16]; (4) they added a clean-up procedure by Sep-Pack after the extraction [17]; (5) they used large volumes of both patients' plasmas (1 to 10ml) [7,17,19,20] and extraction solvent (6–15 ml) [7,16,17]. This last solution required 15-ml tubes and thus prevented from doing 5-FU measurement in plasma in large series.

The main advantage of the present extraction procedure is to combine at the same time, ammonium sulfate protein precipitation and extraction with isopropanol–ethyl acetate. This method provides a very rapid and sensitive method with a high recovery ratio (75% for 5-FU and 90% for 5-CU and 5-BU), barely obtained previously (60%) [17]. It avoids the use of solid-phase extraction cartridges and/or extraction clean-up procedures [13,15]

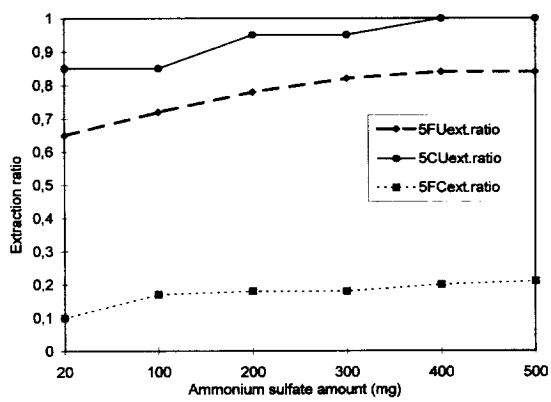

Fig. 2. 5-FU, 5-FC and 5-CU extraction ratios according to the amount of ammonium sulfate, with 1.3 ml IP–AE 5-FU extraction depended on the amount of ammonium sulfate. 5-FC recovery was low because of the low volume of IP–AE.

3.1.8. The choice of the internal standard

Extraction ratios of 5-FC, 5-CU, 5-BU, 5-MC were compared in isopropanol–ethyl acetate as solvent extraction. The extraction output of 5-FC and 5-MC were below 20% for volumes <1.5 ml. A recovery of 70% required volumes >5 ml. In contrast, 5-CU and 5-BU recoveries were very high, whatever the volume of extraction solvent, in the range of 1200 and 1500 μl, 90±1.8% (Fig. 3). Therefore, 5-CU or 5-BU were preferred to 5-FC as internal standard.

The effect of the amount of ammonium sulfate on the yield after extraction has been examined. 5-FC recovery required a large amount of ammonium sulfate, incompatible with low volumes. In contrast, 5-CU and 5-BU extractions were excellent with the

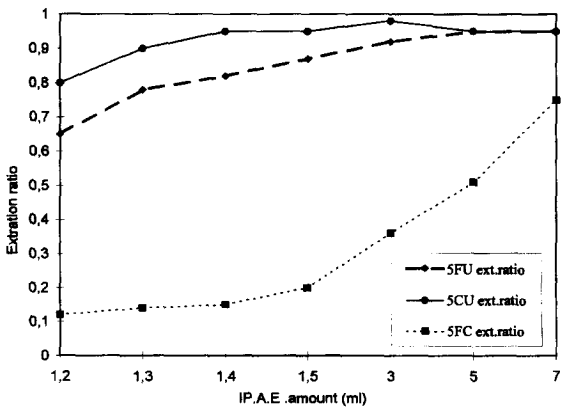

Fig. 3. 5-FU, 5-CU and 5-FC extraction ratios according to the amount of isopropanol–ethyl acetate (IP–AE) (ml). At least 1.2 ml IP–AE is necessary for 5-FU extraction-ratio ≥0.70.

300 mg necessary for a good 5-FU extraction (Fig. 2).

3.2. Method validation

3.2.1. Linearity

Quantitation of 5-FU was 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 5-FU over the concentration range 12.5–10,000 μg l^{-1}. The correlation coefficients for the calibration curves were all >0.9999 (n=5). High concentrations have been tested because new schedules use very high doses of 5-FU, according to the concept of dose-intensity. The least-square regression curve was y=0.00844x and the correlation coefficient for the calibration curves was 0.9995 (n=5).

3.2.2. Precision, accuracy

The data for the validation of the within day and between-day precisions are presented in Table 2. The results show very low coefficients of variation, even for low plasma levels. Accuracy, expressed as the ratio of compound added to that measured is also presented in Table 2.

3.2.3. Mean analytical recovery

The mean recovery was 75±1.5% (n=10) for 5-FU and 90±1.8% (n=10) for 5-CU and 5-BU.

3.2.4. Detection limits

The limit of quantitation (LOQ) of 5-FU in plasma was 6 μg l^{-1}. The detector could identify and integrate 5-FU peaks until 3 μg l^{-1}, with Spherisorb ODS2 column at 25°C.

3.3. Pharmacokinetics

The pharmacokinetics of 5-FU were investigated in patients treated with this drug. Blood samples

Table 2
Within-day and between-day precision and accuracy of the HPLC determination of 5-FU in human plasma

Concentration (ng ml^{-1})	Within-day precision (n=5)			Between-day precision (n=13)		
	Mean±S.D.	C.V. (%)	Accuracy (%)	Mean±S.D.	C.V. (%)	Accuracy (%)
6.25	6.6±0.46	7	5.6	6.75±0.3	4.45	8
12.5	12.4±0.99	8	0.8	12.4±0.53	4.28	0.8
25	24.62±0.46	1.87	1.5	24.33±0.27	1.1	2.7
50	50.55±0.78	1.56	1.1	50.27±0.78	1.55	0.5
100	100.95±0.4	0.4	0.9	100.03±1.23	1.2	0.03
200	202.3±1.8	0.89	1.15	202.48±2.6	1.1	1.24
500	502.0±1.07	0.02	0.4	507.6±4.86	1.29	1.5
1000	1035±34.8	3.35	3.39	1008±33.5	3.3	0.8
2000	2024±19.1	2.27	1.2	2019±40	3.07	0.95
2500	2451±24.2	0.98	2	2465±87	3.5	1.4
5000	4877±19.5	0.4	2.46	4849±125.5	2.58	3.02

were withdrawn at various times during and after the infusion. A blank sample was taken before treatment. The samples were collected in heparinised tubes and centrifuged for 5 min at 8000 *g* within 30 min. The resulting plasma was then treated as described for final method or immediately stored at −20°C and later transferred to a −70°C deep freezer.

Plasma collected before the infusion revealed a small endogenous peak of uracil. This peak could interfere with 5-FU under the current chromatographic conditions with the Spherisorb 5 ODS1 reversed- phase column. The ODS2 Phase Sep column perfectly resolved the two compounds.

Sample preparation and chromatography time were short: the total analysis time required for each run is 25 min. Final results could be given within 90 min of blood sampling. Therefore about 50 samples could be handled daily, enabling injections and calculations to be done overnight, making this technique quite adaptable to routine applications.

Acknowledgments

The authors wish to thank Mrs. J. Houdebine, M.C. Crepeau and A. Lecourt for their technical assistance. This work was funded in part by the Ligue contre le Cancer, Comité départemental de Maine et Loire.

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