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Rapid and Accurate Determination of 5-Fluorouracil in Human Plasma by LC-UV Method

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Abstract A sensitive and specific LC-UV method for the analysis of 5-Fluorouracil in human plasma has been developed and validated. 5-Fluorouracil was extracted from plasma by liquid-liquid method and separated on C18 reverse phase column using an elution gradient with a total run time of 11 min and detected by UV at 275 nm. For quantitative analysis of 5-Fluorouracil, 5-Bromouracil was used as internal standard. Calibration curve ranging from 9ng/mL to 2400ng/mL was obtained by enriching blank plasma pool with standard solution of 5-Fluorouracil. Within-day and between-day imprecision, as CV, ranged from 0.6% to 3.3% and from 4.4% to 13.6%, respectively. The extraction method is able to recover on average 96.8% of 5-Fluorouracil from plasma. Finally, plasma levelsof 5-Fluorouracilweredetermined in 10patients treated with 5-Fluorouracil. Results from calibration curves, quality controls and patient samples reveal that the method is suitable to analyze plasma 5-Fluorouracilfor therapeutic drug monitoring of treated patients.

Keywords LC-UV, 5-FU, chemotherapy monitoring

Introduction

5-Flurouracil (5-FU) is an antimetabolite drug with a pyrimidine-like structure that acts in the thymine biosynthesis pathway leading to a depletion of deoxythymidine triphosphate in the nucleotide pool and, consequently, to the inhibition of DNA synthesis [1]. In addition, it has been observed that 5-FU treatment induces apoptosis in cancer cells [2]. For this reason, 5-FU represents a standard chemotherapy drug also for metastatic cancers. This drug can be applied alone or with other chemotherapeutic agents, for the treatment of solid tumors such as colorectal and gastric cancers [3].

Normally, in humans 80-90% of standard administered doses of 5-FU are rapidly metabolized in the liver to 5,6-dihydro-5-fluorouracil by dihydropyrimidine dehydrogenase, the key enzyme of 5-FU catabolism, and only a small fraction can exert its action [4,5]. Therefore, the drug is administrated at high-doses to maintain the therapeutic concentration.

Response to 5-FU treatment is highly variable among patients, of whom 10–30% experience severe to life-threatening adverse effects at standard doses, such as mouth sores, swallowing difficulties, diarrhea, nausea and vomiting, low white blood cells count and anemia [6, 7]. 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.

Different methods have already published for 5-FU dose monitoring by liquid chromatography (LC) [8]. Some LC methods coupled with mass spectrometry are very sensitive, but are time-consuming and rather expensive. Moreover, many extraction procedures are too complex to be compatible for a rapid routine analysis.

Herein, we report the development and validation of a simple, sensitive, fast, and economical LC-UV method for determination of 5-FU in human plasma. In addition, 5-FU levels have been determined by this new method in plasma samples collected from 10 treated patients before 5-FU administration and after 2 h and 44 h of treatment.

Materials and Methods

Reagents and standard solutions

5-Fluorouracil (5-FU), 5-Bromouracil (5-BrU) and ethyl-acetate were obtained from Sigma-Aldrich (St Louis, MO, USA). Ammonium sulfate and isopropyl alcohol were purchased from Carlo ErbaReagenti (Milano, Italy). The analytical solvent of HPLC grade, methanol and acetic acid were obtained from J.T BAKER (Deventer, Netherlands) and Carlo Erba Reagenti. Deionized water was generated by a deionized system (Elix^R Essential, Merk Millipore, Germany).

Stock standard solutions were prepared in methanol/water (50/50; v/v) to provide the concentrations of 50 µg/mL for 5-BrU (internal standard) and 24 µg/mL for 5-FU. Working solution of internal standard was prepared by dilution (1:5) of the stock solution. The solutions were stored protected from light at room temperature.

Patients and samples

Ten patients with metastatic colorectal cancer were treated with bevacizumab plus chemotherapy regimen, mFOLFOX6 (oxaliplatin85 mg/m^2, leucovorin 200 mg/m^2, 5-FU bolus 400 mg/m^{2}followed by a continuous intravenous infusion of 5-FU 2400 mg/m^{2}over 46 h).

Plasma samples were collected from patients before drug administration and after 2 h and 44 h of treatment, all plasma samples were stored at -80°C until the analysis.

5-FU-free plasma (blank plasma) was prepared from not treated subjects at the Diagnostic Laboratory of the Hospital (I.N.T. Giovanni Pascale - Naples). Five blank plasma pools were prepared, aliquoted, and stored at -80°C until use. Each pool was prepared from at least 15 patient's plasma samples. All blank plasma pools were testedto ascertain the presence of signals at the retention time of 5-FU.

The study was undertaken in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice. The study was approved by the Ethics Committee of the National Cancer Institute – G. Pascale Foundation (CE: Protocol 7/15, April 29th 2015). All patients provided written informed consent before participating.

Preparation of calibrators and quality controls

The calibrators were prepared by enriching a blank plasma pool with 5-FU stock solution to obtain a pool at concentration of 2400ng/mL and by serial dilutions of this pool with a blank plasma to obtain the following 5-FU concentrations: 9.38, 18.75, 37.5, 75, 150, 300, 600, and 1200 ng/mL. The calibrators were prepared freshly for each analytical batch.

Three levels of quality controls (QCs) were prepared by enriching blank plasma pool with 5-FU stock solution to obtain 5-FU concentrations of 25, 100 and 400ng/mL. All QC samples were aliquoted and stored at -80°C until analysis.

Extraction procedure

Calibrators, QCs, and plasma samples from 5-FU-treated patients were analyzed using 300 µL of sample with the addition of internal standard (50 µL). A saturating amount (about 200 mg) of ammonium sulfate was added to precipitate plasma protein and the sample was vigorously mixed for 1 min. 5-FU was extracted two times with 1 mL of isopropanol/ethyl acetate (15/85; v/v)that was vigorously mixed with the sample for 1 min and centrifuged at

10,000 rpm for 3 min; the supernatant solution was transferred in a glass test tube. Then, the pooled supernatants were dried under a stream of nitrogen at 40°C, the dried residue was dissolved in 200μL of deionized water, transferred in a micro test tube, and centrifuged at 10,000 rpm for 3 min. The supernatant solution was transferred in HPLC vial for the analysis.

LC-UV analysis

Fifteen microliters of specimen were injected into an HPLC system (Agilent 1260 Infinity) equipped with autosampler. 5-FU separation was performed using an analytical column (Agilent Eclipse Plus C18 3.5 μm, 4.6 x 100 mm) maintained at room temperature without guard column. The separation has been optimized using an elution gradient (**table 1**) of mobile phase constituted by solution A (10 mM acetic acid) and solution B (methanol) with an increasing flow rate from0.8 to 1.2mL/min and a column head pressure from 105 to 190bar, respectively. The total run time was of 11 min, including column washing and equilibration (3.5 min). 5-FU detection was acquired at 275 nm with UV/Vis Detector (BIO-RAD UV-1806, Bio-Rad Laboratories, Segrate - Italy).

Table 1: Elution gradient and flow rate of mobile phase in LC-UV analysis of 5-FU

Time (min)	Solution A[a] (%)	Solution B[b] (%)	Flow (mL/min)
0	98	2	0.8
3	98	2	0.8
6	96	4	1
6.1	20	80	1
7	20	80	1.2
8.6	20	80	1.2
8.7	98	2	1.2
11	98	2	1.2

[a]Solution A: 10 mM acetic acid.

[b]Solution B: methanol.

Method validation

The method was validated for selectivity, linearity, extraction efficiency, imprecision, inaccuracy, carry-over, stability, LOD and LOQ.

The selectivity of the method was evaluated by comparing the profiles obtained from 5 different blank plasma pools without internal standard with the profile obtained from the calibrator containing 5-FU at the concentration of 37.5 ng/mL and mixed with internal standard as described in the extraction procedure. The samples were extracted and analyzed for potential interfering compounds on the retention time of 5-FU and internal standard. In addition, potential interferences from endogenous compounds was also evaluated by enriching a blank plasma, a hemolytic plasma (hemoglobin = 1.1 g/dL) and an icteric plasma (total bilirubin = 11 mg/dL) with 5-FU standard solution to obtain the concentration of 800 ng/mL and measuring the peak area ratios of 5-FU to internal standard (5-FU/IS).

Calibration curves were prepared by analyzing the plasma calibrators at 9 concentration levels and in 10 separate days. The linearity of each calibration curve was evaluated by linear regression analysis, plotting 5-FU/IS (y) *versus* the 5-FU nominal concentration (x).

LOD and LOQ were defined as the lowest concentrations that provide S/N higher than 3 and 10, respectively.

To evaluate the extraction efficiency of 5-FU from plasma, 5-FU/IS values obtained from the analysis of plasma calibrators of 10 calibration curves were compared to those obtained from the analysis of corresponding saline dilutions of 5-FU standard solution without extraction.

Imprecision and inaccuracy of the method were evaluated by analyzing the three QC levels. Within-day imprecision and inaccuracy were calculated analyzing 5 times each level of QC samples in the same analytical run. For evaluation of between-day imprecision and inaccuracy, each level of QC samples was analyzed once a day for 10 working days along two months.

Carry-over was evaluated by three consecutive chromatographic runs of the highest calibrator (2400 ng/mL) followed by three runs of a blank plasma pool. The percentage of carry-over was calculated for 5-FU and internal standard as ratio between the average area of analyte obtained from the blank plasma pool and the highest calibrator.
5-FU stability was evaluated analyzing two calibrators at the concentrations of 300 and 1200 ng/mL stored at –80°C for a variable time, ranging from 2 days to 2 months.

Method comparison

The plasma samples collected from the patients after 2 h (n=10) and 44 h (n=10) of 5-FU administration were simultaneously analyzed b your LC-UV method and by the standardized Saladax My5-FU™ immunoassay method for 5-FU (Saladax Biomedical, USA).The immunoassay method was performed on a Cobas 6000 analyzer (Roche Diagnostic, S.p.A. Italy) with a quantification range of 85–1800ng/mL.

Statistical Analysis

Data are reported as average and SD. The statistical significance of differences between groups was evaluated using the appropriate Student's t-test and the significance was accepted at the level of $p < 0.05$. The method comparison was performed by linear regression analysis.

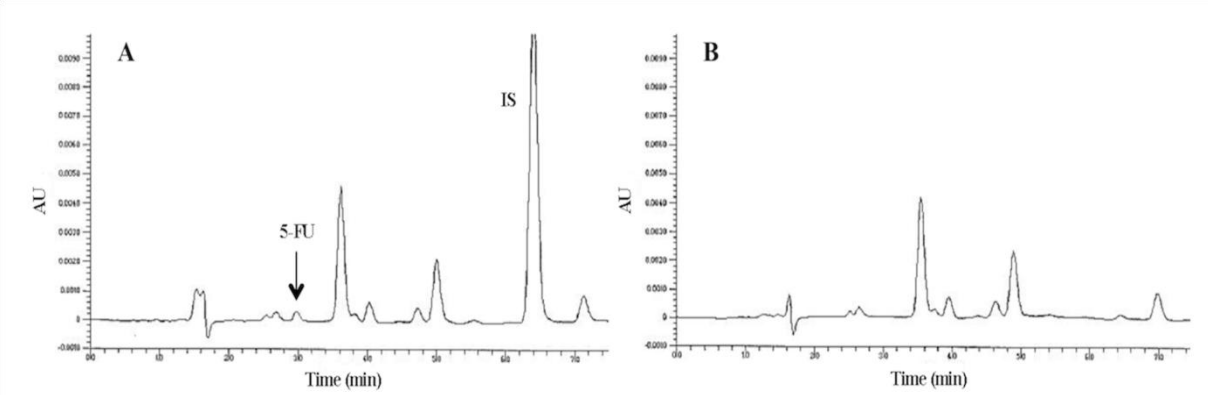
Results and Discussion

Assay performance

Fig. 1 shows LC-UV chromatograms obtained using our method from a calibrator at 37.5 ng/mL of 5-FU plus internal standard (panel A) and from 5 different blank plasma pools without internal standard (panels B-F). All analyzed blank plasma pools show six main unidentified peaks, while the profile of calibrator shows two additional peaks corresponding to 5-FU and internal standard. The retention times (RT ± SD, n = 20) of 5-FU and internal standard were3.01 ± 0.03min and 6.41 ± 0.03 min, respectively. Analytical interferences were not observed on both 5-FU and internal standard in hemolytic and icteric plasma compared to blank plasma. In particular, the percentage bias values of 5-FU/IS in the spiked hemolytic and icteric plasma were 0.5%and 1.3%, respectively.

The 5-FU is an anti-neoplastic drug and it is administered at high doses, therefore calibration points, ranging from 9.38 to 2400ng/mL, were chosen based on the therapeutic doses and pharmacokinetic range of this drug [9-11]. The average ± SD of slope and intercept of 10 different calibration curves were 0.00060 ± 0.00001and -0.00015 ± 0.00046, respectively. The linearity estimated by the correlation coefficient (r) was 0.9995(SD = 0.0005).

The LOD and LOQ were evaluated analyzing in triplicate two blank plasma samples enriched with 5-FU stock solution at the concentrations of 2.34 and 9.38 ng/mL, respectively. The S/N ratios obtained from the analysis of the samples with 2.34 and 9.38 ng/mL of 5-FU were 3.6 and 10.8, respectively. Based on these results, the LOD and LOQ of this method for 5-FU were of 2.34 ng/mL and 9.38 ng/mL, respectively. In addition, the imprecision, as CV%, at calibration level of 9.38 ng/mL was less than 10% (n = 10). These results are in agreement with that previously reported [10, 12-14].



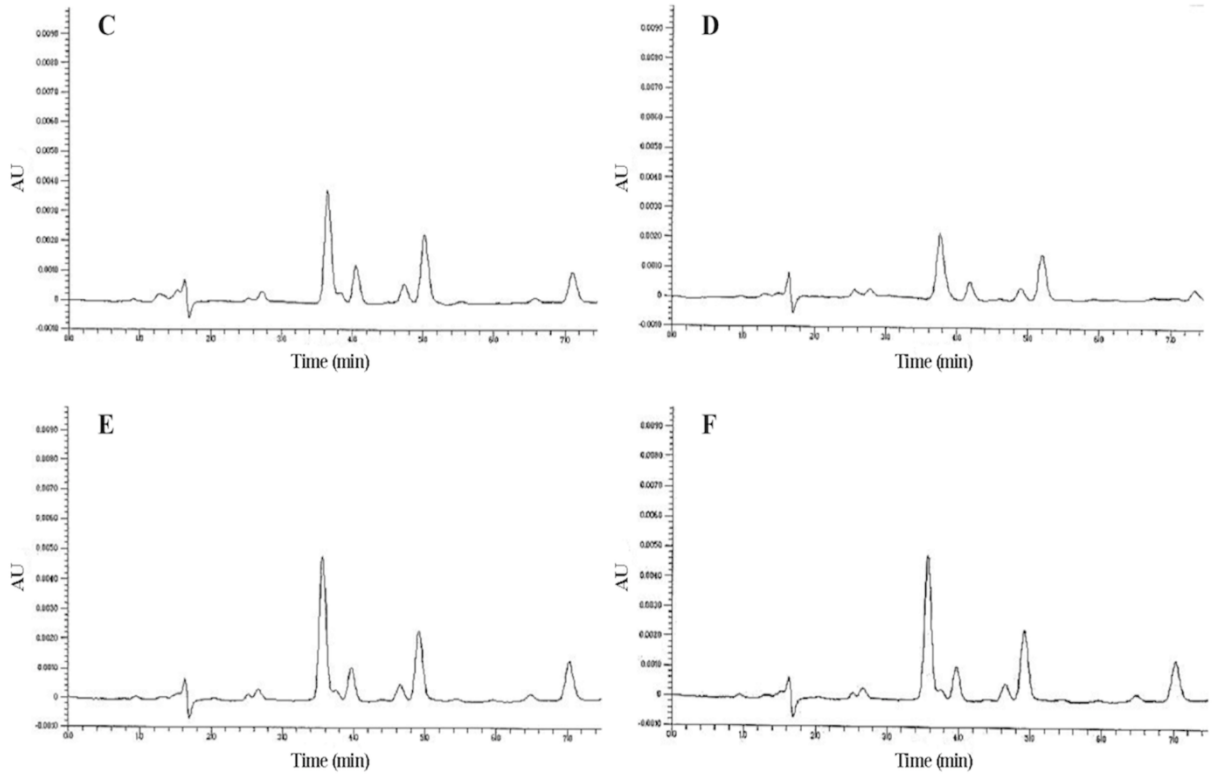


Figure 1: LC-UV chromatograms obtained from a calibrator at 37.5 ng/mL of 5-FU plus internal standard (A) and from 5 different blank plasma pools without internal standard (B-F).
5-FU: 5-fluorouracil; IS: internal standard.

As shown in **table 2**, the within-day imprecision (CV%) and inaccuracy (%)for 5-FU were less than 3.3% and 14.9%, respectively. The between-day imprecision and inaccuracy were less than 13.6% and 12.4%, respectively.

Table 2: Within- and between-day imprecision and inaccuracy study of 5-FU in the three QC levels

	Within-day (n = 5)				***Between-day (n = 10)***		
	Expected value (ng/mL)	**Measured value (ng/mL)**	**Imprecision (CV%)**	**Inaccuracy (%)**	**Measured value (ng/mL)**	**Imprecision (CV%)**	**Inaccuracy (%)**
QC low	25	28.7	3.3	14.9	28.1	13.6	12.4
QC medium	100	99.5	1.5	-0.5	98.6	4.4	-1.4
QC high	400	413.2	0.6	3.3	403.7	4.6	0.9

The evaluation of extraction efficiency, calculated as ratio between 5-FU/IS values in plasma calibrators extracts and in corresponding dilutions of 5-FU standard solution without extraction, showed that the extraction method is able to recover on average 96.83% (SD = 12.95; range from 80% to 115%; n = 10) of 5-FU from plasma.

The percentage of carry-over, calculated as ratio between the average areas of compounds found in blank plasma pool and in the highest calibrator, was 0.32% for 5-FU and 3.4% for internal standard.

The analysis of the two calibrators at 300 and 1200 ng/mL of 5-FU during the storage time up to two months at -80°C showed on average 5-FU concentrations of 307.8 ng/mL (CV=11.9%) and 1131.3 ng/mL (CV=7.0%), respectively.

Method comparison

Linear regression analysis was performed to compare the 5-FU plasma levels determined by LC-UV method with those obtained by immunoassay method (n = 20). The comparison between the two groups of values showed a linear correlation coefficient r of 0.977, a slope of 0.893 and an intercept of 23 ng/ml (**Fig. 2A**).

The results obtained from LC-UV method presented here were on average of 4-5% lesser than that obtained from immunoassay method.

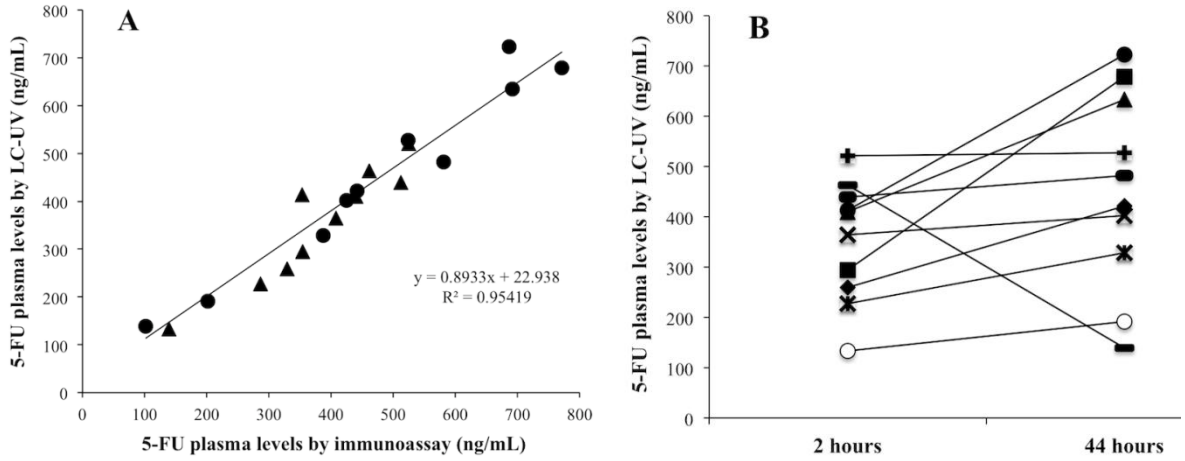


Figure 2: A) Comparison of LC-UV and My5-FU™ immunoassay methods for the determination of 5-FU concentrations in patient plasma collected after 2 h (black triangles; n = 10) and 44 h (black circles; n = 10) of 5-FU administration. B) Plasma 5-FU monitoring of the patients (n = 10) after 2 and 44 hours of drug administration.

Therapeutic drug monitoring of patients treated with 5-FU

The method here presented has been used to analyze plasma samples from 10 patients treated with 5-FU. **Fig. 2B**shows plasma levels of 5-FU in the treated patients after 2 h and 44 h of drug administration. Before the treatment, 5-FU was absent in all patient plasma samples. After 2 h, the average level of 5-FU in patient plasma was 352ng/mL (SD=121; CV=34%; range from 133to 521ng/mL). After 44 h, the average level of compound was 453ng/mL (SD=197; CV=43%; range from 139 to 723ng/mL). The between-group comparison of levels obtained at 2 hours and 44 hours was not statistically different (Student's t-test: p=0.138).Although the number of subjects evaluated in this study was low, the 5-FU plasma levels after 2 and 44 hours and the inter-patient variability were in agreement with the data previously published [4, 9-11, 15].

Several studies have reported a high individual variability in 5-FU pharmacokinetics due to a genetic polymorphism of its metabolism [16]. The alterations in the catabolic pathway of 5-FU are suggested to influence its efficacy and the risk of adverse effects. Patients with reduced dihydropyrimidine dehydrogenase activity present with extremely high plasma levels of 5-FU maintained for long periods and the subsequent 5-FU toxicity owing to massive cell death in rapidly proliferating body tissues, such as mucous membranes or bone marrow [17]. In such cases the monitoring of 5-FU plasma levels is fundamental to avoid toxicity.

Measurements of systemic exposure of 5-FU, such as the area under the concentration-time curve, have been shown to correlate with toxicity, tumor response, and survival outcomes [18, 19].

Therefore, we tried to evaluate the area under the concentration-time curve between 2 hours and 44 hours of 5-FU administration in the ten patients monitored in this study (**Fig. 2B**). The results showed that the area values(average ± SD) of9 patients were18 ± 4 mg • h/L and in agreement with the literature [20] and just 1 patient showed an area value of 7 mg • h/L, that may suggest an elevated 5-FU catabolism.

Conclusion

We implemented this LC-UV method to quantify 5-FU in plasma from patients treated with the cytotoxic drug as well we compared this method with the immunoassay procedure already used in our lab. The sensitivity obtained by

LC-UV method presented here was significantly higher than that obtained using the immunometric assay (9.38 ng/mL *vs* 85 ng/mL). Furthermore, the LC-UV method is also more sensitive or comparable to other previously described LC-UV methods [10, 14]. In addition, it shows a shorter run time (11 min), a turnaround-time of 2 h, an extraction efficiency of 96.8%, a lower plasma volume (0.3 mL) and a comparable solvent volume (1 x 2 mL) [10, 14].

The validation study of LC-UV method developed here showed a very good analytical performance, with an analytical CV lower than 14% and a wide quantification range to monitor5-FU in plasma of treated patients.

This method is suitable for all laboratories provided by a LC-UV instrument, as well as it can be automated and miniaturized to routine application with an estimated productivity of at least 54 samples/day/instrumentation.

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