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Short communication

Determination of 5-fluorouracil in microvolumes of human plasma by solvent extraction and high-performance liquid chromatography ☆

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Abstract

In the present study, a new reversed-phase HPLC method has been developed and validated for the quantitative determination of 5-fluorouracil (5-FU) in human plasma using only 100-μl samples. The sample extraction and clean-up procedure involved a simple liquid–liquid extraction after addition of 5-chlorouracil (5-CU), used as internal standard, with 5 ml ethyl acetate. Chromatographic separations were performed on an Inertsil ODS-3 column (250×4.6 mm ID; 5 μ*M* particle size), eluted with a mobile phase composed of acidified water (pH 2.0). The column effluent was monitored by UV absorption measurement at a wavelength of 266 nm. The calibration curves were constructed over a range of 0.20–50.0 μ*M* and were fitted by weighted ($1/x$) linear regression analysis using the ratio of peak heights of 5-FU and 5-CU versus concentrations of the nominal standards. Extraction recoveries over the total range averaged 92 and 93% for 5-FU and 5-CU, respectively. The lower limit of quantitation was established at 0.20 μ*M* (~26 ng/ml), with within-run and between-run precisions of 4.2 and 7.0%, respectively, and an average accuracy of 109.3%. The within-run and between-run precisions at four tested concentrations analyzed in quintuplicate over a time period of four days were <1.4 and <4.4%, respectively. The accuracy at the tested concentrations ranged from 98.4 to 102.3%. Compared to previously described validated analytical methods for 5-FU, our present assay provides equivalent to superior sensitivity using only microvolumes of sample.

Keywords: 5-Fluorouracil

1. Introduction

The pyrimidine analogue 5-fluorouracil (5-FU, Fig. 1) has been widely used in the treatment of solid tumors, and still remains an essential component for the treatment of various human malignancies, including colorectal, head and neck, and breast cancers [1,2]. The drug is used as single agent or in combination therapy, mostly by continuous infusion, over 5 or 21 days, or by a combination of bolus and prolonged i.v. infusion [1]. The pharmacokinetics and metabolism of 5-FU have been extensively investigated. Although as much as 80% of the

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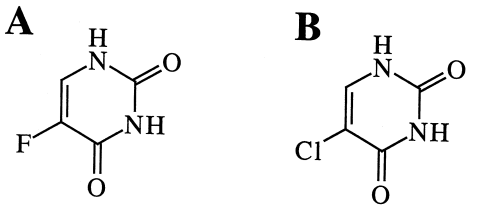

Fig. 1. Chemical structures of 5-fluorouracil (**A**) and 5-chlorouracil (**B**).

administered 5-FU dose is metabolized, current therapeutic adjustment may still be best made on the basis of parent drug kinetics and its systemic exposure. There is a good relationship between plasma levels of 5-FU and the subsequent toxicities and response rates, but several studies showed a high individual variability in 5-FU pharmacokinetics, primarily due to polymorphism of enzymes involved in drug metabolism [3–5]. Genetic deficiencies of the key enzyme of the catabolic pathway, dihydropyrimidine dehydrogenase (DPD), has been shown to result in increased plasma concentrations of the parent drug, and altered toxicological profiles. Pharmacokinetic studies revealed rapid distribution and elimination phases, resulting in short plasma disposition half-lives, with considerable intra- and interpatient variation that is associated with variability of DPD activity [6].

Several analytical methods have been previously developed for the determination of 5-FU in biological matrices, including assays based on GLC [7], GC–MS [8], straight-phase HPLC and, most widely applied, RP–HPLC [9–20]. Most of the earlier analytical methods were not properly validated according to current requirements [21–23], and/or made use of large sample volumes (500–1000 μl), that are problematic in case of pharmacokinetic studies in which combination therapy with 5-FU is given. It was the aim of the present study to develop a simple and rapid method for the quantitative determination of 5-FU in small volumes of human plasma with at least equal absolute sensitivity as compared to the previously reported methods. The procedure is based on single solvent extraction prior to RP–HPLC with UV detection, and has been subjected to a rigorous validation procedure as described [21].

2. Experimental

2.1. Chemicals and reagents

5-FU (lot 86H0818; purity: >99.0% by RP-HPLC) and the internal standard 5-CU (lot 40H7700) were obtained from Sigma (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Rathburn (Walkerburn, UK), and aqueous perchloric acid (70–72%, v/v) and ethyl acetate were supplied by Baker (Deventer, The Netherlands). All water used in the study was filtered and deionized with a Milli-Q-UF system (Millipore, Milford, MA, USA). Drug-free human plasma for construction of the calibration curves and quality control samples originated from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

2.2. Stock solutions and standards

Stock solutions of 5-FU were prepared in triplicate by dissolving 20.00 mg 5-FU in 20.00 ml water, resulting in a solution containing 1.00 mg/ml. A stock solution of the internal standard at a concentration of 10.00 mg/ml was prepared by dissolving 200.0 mg 5-CU in 20.00 ml DMSO. Working solutions of 5-FU and 5-CU, containing 1250 and 500 μ*M* respectively, were prepared by dilution of the stock solution in water.

Spiked plasma samples used as calibration standards were prepared daily in duplicate by addition of 10 μl of serial dilutions of the working solution of 5-FU in water to 240 μl drug-free human plasma, resulting in calibration standards of 0.20, 0.50, 2.50, 10.0, 25.0 and 50.0 μ*M* 5-FU. Three pools of quality control (QC) samples for 5-FU were prepared in human plasma at concentrations of 1.00, 20.0 and 40.0 μ*M*, by addition of small volumes or dilutions of the 5-FU working solution to human plasma. A fourth quality control sample, containing 400 μ*M* 5-FU, used for small volume injections, was prepared by adding the 5-FU stock solution directly to blank plasma.

2.3. HPLC instrumentation and conditions

The HPLC equipment was composed of a constaMetric 4100 solvent delivery system (LDC Ana-

lytical, Riviera Beach, FL, USA), a Waters 717 plus autosampling device (Bedford, MA, USA) and a UV-2000 detector (Spectra Physics, San Jose, CA, USA). Separations were achieved on a stainless-steel analytical column (250×4.6 mm ID, 5 μm particle size) packed with Inertsil ODS-3 material delivered by Alltech Applied Science (Breda, The Netherlands). The mobile phase was composed of water with the pH adjusted to pH 2.0 (perchloric acid), delivered at a flow-rate of 1.00 ml/min. The column was maintained at 35°C, using a Mistral column oven (Spark Holland, Meppel, The Netherlands), and the eluent was monitored at a wavelength of 266 nm.

Peak recording and integration was performed with the Chrom-Card data analysis system (Fisons, Milan, Italy). Calibration curves were fitted by weighted ($1/x$) linear regression analysis by using the ratios of 5-FU to the internal standard 5-CU versus the concentrations of the nominal standard.

2.4. Sample pretreatment

A volume of 10 μl of the internal standard (500 μ*M* 5-CU in water), and 5 ml ethyl acetate were added to 100 μl human plasma in a glass tube with PTFE-lined screw caps. The sample was mixed vigorously for 1 min on a multi-tube vortex mixer, followed by centrifugation for 5 min at 4000 *g* at ambient temperature. The organic layer was collected in a glass tube and evaporated to dryness at 60°C for 45 min under a gentle stream of nitrogen. Finally, the residue was redissolved in 50 μl mobile phase, transferred to a low volume insert of glass, and a volume of 25 μl (2.5 μl for the QC sample containing 400 μ*M*) was injected into the HPLC system.

2.5. Validation

A validation run included a set of calibration samples assayed in duplicate, lower limit of quantitation (LLQ) samples and QC samples at four levels in quintuplicate, and was performed on four separate occasions. Precisions were calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable.

The LLQ was determined by spiking blank plasma samples from five different individuals with 0.20 μ*M* 5-FU, as described for the standards of the calibration curve. The LLQ samples were also analyzed on four separate occasions along with a standard curve run in duplicate, and the calculations were performed as described. The same blank plasma samples were used to demonstrate the lack of endogenous interferences around the retention times of 5-FU and the internal standard.

The absolute analytical recovery of 5-FU and 5-CU was calculated in four analytical runs, by comparing peak heights obtained by direct injection of a standard solution containing 50 μ*M* 5-FU and 100 μ*M* 5-CU in mobile phase, to those obtained in extracted plasma samples of the calibration curve.

The stability of 5-FU in human plasma was established with the four QC samples during three consecutive freeze-thawing cycles, where samples were put at room temperature for 15 min after each thawing. The stability was also tested at 37°C by incubation of the QC samples for a time period of 18 h. The long-term storage stability of 5-FU at −80°C was tested at the same concentrations for up to 12 months.

The following potentially co-administered drugs were tested for interference with 5-FU or 5-CU: acetaminophen (2 mg/ml), paroxetine (0.4 mg/ml), alizapride and ranitidine (0.2 mg/ml), and codeine, domperidon, morphine, leucovorin, metoclopramide, lorazepam and dexamethasone (0.04 mg/ml each).

3. Results and discussion

Chromatograms of a blank plasma sample and two spiked human plasma samples containing 0.50 and 25.0 μ*M* 5-FU, respectively, are shown in Fig. 2. No interfering peaks were found at the retention time of 5-FU or 5-CU in the drug-free human plasma delivered by the Central Laboratory of the Blood Transfusion Service. However, blank heparinized plasma from five healthy volunteers showed a small interfering peak for 5-FU. The interference was not caused by the presence of the anticoagulant heparin, since serum and plasma collected in glass tubes containing EDTA also showed small interfering peaks for 5-FU. Also some other additional peaks were found, which did not interfere with 5-FU or 5-CU, in the plasma of the healthy volunteers that were absent in plasma delivered by the Blood

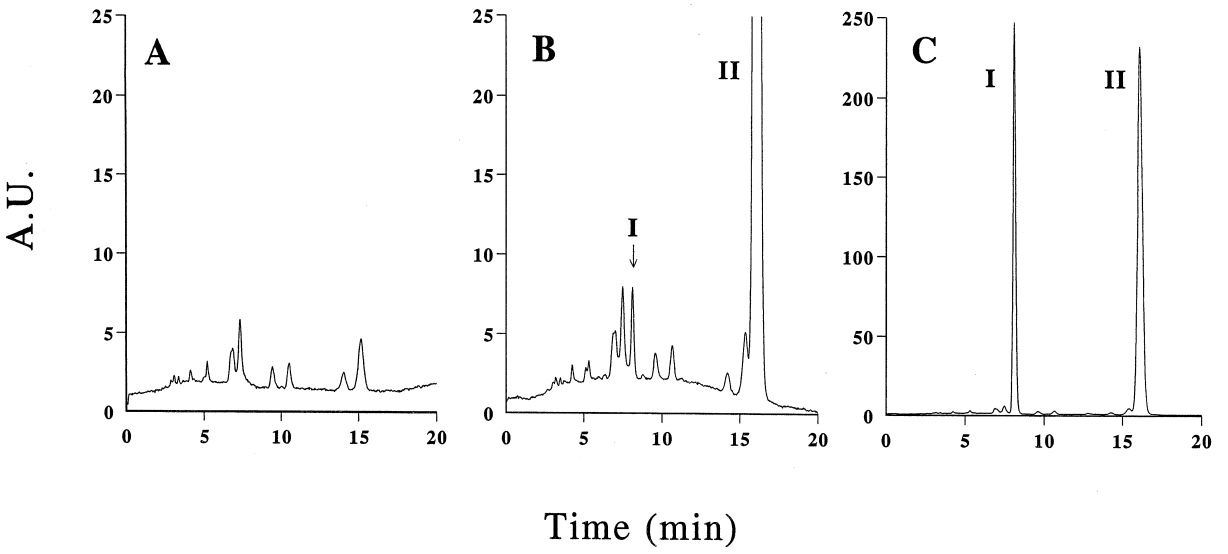

Fig. 2. Chromatograms of a blank plasma sample (**A**), and plasma samples spiked with 0.50 μ*M* (**B**) and 25.0 μ*M* (**C**) of 5-FU. Chromatographic peaks labeled I and II represent 5-FU and 5-CU, respectively.

Transfusion Service. Eventually, a large analytical column with a mobile phase lacking any organic modifier was selected in order to optimize the separation of 5-FU and 5-CU from endogenous material, resulting in retention times of 8 and 16 min for 5-FU and 5-CU, respectively. The overall chromatographic run time was established at 20 min.

The calibration curves were linear, with regression correlation coefficients >0.999, in a 5-FU concentration range of 0.20–50.0 μ*M*, using weighted ($1/x$) linear least-squares regression analysis. The deviations of the interpolated concentrations of all standards in the daily calibration curves of 5-FU in drug-free human plasma were always within the acceptable range of 85–115% [21].

All the tested drugs potentially co-administered with 5-FU did not give interfering peaks for 5-FU and 5-CU in the assay. The LLQ for 5-FU was established at 0.20 μ*M*, due to the small interfering peaks present in heparinized plasma. At this concentration 80% of the samples were within the acceptable accuracy range of 80% to 120% [21]. The within-run and between-run precisions of these samples were 4.2% and 7.0%, respectively, with an average accuracy of 109.3% (Table 1). The within-run and between-run precisions at the concentrations of the four QC samples were ≤1.4 and ≤4.4%, respectively, and the average accuracy showed values ranging within 98.4 and 102.3% of the nominal values (Table 1).

The extraction recoveries of 5-FU and 5-CU were independent of the spiked concentrations, resulting in overall recoveries of 92±5.1 and 91±2.8% for 5-FU and 5-CU, respectively ($n=40$).

5-FU was found to be stable during three freeze–thaw cycles and during incubation for 18 h at 37°C. The long term stability of 5-FU in plasma samples stored at −80°C was at least 12 months (data not shown). Processed human plasma samples containing 5-FU and 5-CU in mobile phase were also found to be stable at room temperature in the autosampler,

Table 1
Calculations of the between-run and within-run precisions and the average accuracy of the LLQ and QC samples[a]

Spiked (μ*M*)	GM (μ*M*)	WRP (%)	BRP (%)	ACC (%)	*n*
0.20	0.219	4.2	7.0	109.3	4
1.00	1.022	1.4	3.4	102.2	5
20.0	20.46	1.3	4.4	102.3	5
40.0	40.36	1.0	4.1	100.9	5
400	393.4	1.1	4.4	98.4	5

[a] Abbreviations: GM, grand mean; WRP, within-run precision; BRP, between-run precision; ACC, average accuracy; *n*, number of replicate observations on each analysis day.

allowing the pretreatment of a large number of samples in each analytical run.

In conclusion, a thoroughly validated assay for the quantitative determination of 5-FU in microvolumes of human plasma has been described, which meets the current requirements as to validation of bioanalytical methodologies [21]. The described procedure is based on a single solvent extraction, thereby eliminating the need of laborious extraction procedures, and demonstrated sufficient sensitivity, with excellent accuracy and precision and a high extraction recovery. Taking into account the small sample volume used, our method has equivalent absolute sensitivity as compared to the procedure described by Gamelin et al. [14]. The method permits the analysis of patient samples in studies with combination therapy without taking additional blood samples, and will be implemented in future clinical and pharmacokinetic studies.

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