

Short communication

Validated liquid chromatographic determination of 5-fluorouracil in human plasma

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Abstract

A sensitive, reproducible, selective and accurate high performance liquid chromatographic (HPLC) method for the quantitative determination of 5-fluorouracil in plasma has been developed and validated using isocratic elution and UV detection. The method provides a selective quantifications of 5-fluorouracil without any interference of the endogenous uracil. The assay is performed after a double extraction of 5-fluorouracil and thymine (internal standard) from human plasma using ethyl acetate. The drug and the internal standard were eluted from a Genesis C₁₈ analytical column at ambient temperature with mobile phase consisting of methanol:water (10:90, v/v) adjusted to pH 3.2 with perchloric acid at a flow rate of 1.0 ml/min. The effluent was monitored with an ultraviolet detector at 260 nm. Quantification was achieved by the measurement of the peak-height ratios and the limit of quantification for 5-fluorouracil in plasma was 30 ng/ml. The retention times for 5-fluorouracil, uracil, and thymine were 4.5, 6.0, and 9.0, respectively. The intra-day coefficient of variation (CV) ranged from 1.35 to 4.53% at three different concentrations and the inter-day CVs varied from 1.29 to 4.98%. The relative and absolute recoveries varied from 96 to 101%. Stability tests showed that 5-fluorouracil is stable for at least 72 h in plasma after freezing. The simple method may permit the assessment of 5-fluorouracil plasma concentrations for pharmacokinetic studies in combination with clinical trials.

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1. Introduction

5-Fluorouracil (5-FU) is a commonly applied antineoplastic agent that has been widely used for the treatment of solid tumors [1]. Colon cancer is one of the major diseases that is frequently treated using this chemotherapeutic agent [2].

5-FU (Fig. 1A) is not easily separated from structurally related compounds such pyrimidines, especially uracil (Fig. 1B) which has a retention time close to that of 5-FU. Thus, making the separation between the two compounds a troublesome task. [3–5]. Several analytical methods have been previously reported for the assay of 5-FU in biological fluids, including those methods that were based on solid phase extraction [6], gas chromatography (GC) [7], gas chromatography combined with mass spectroscopy (GC–MS) [8], liquid–liquid extraction using four serial columns [9], and LC–MS/MS [10]. These methods required

high sophisticated equipment and are not amenable to rapid and routine clinical assay [11]. The described techniques needed a relatively large plasma volume (2.0 ml) which may not be always available, and involving tedious extraction and derivatization steps. More importantly, some of the formerly published methods were not appropriately validated according to the current FDA guidelines for biological method validation [12].

A successful analytical technique should be cost saving and easily available in most laboratories with minimal tedious work. Therefore, the aim of this present study was to develop a simple, rapid, and properly validated HPLC method coupled with ultraviolet detection for quantitative determination of 5-FU in human plasma utilizing a single column and using isocratic elution. Analytical validation, including linearity, sensitivity, precision, and accuracy were discussed. The stability of drug in plasma after freezing was established. The total chromatographic run time was 10.0 min which is approximately a quarter period of time of what other methods have previously described. The

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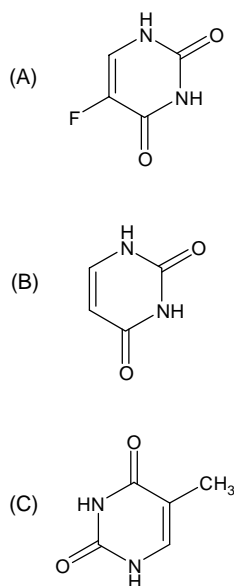


Fig. 1. Chemical structures of: (A) 5-fluorouracil, (B) uracil, and (C) thymine.

proposed method takes also into accounts the possible interference of 5-FU with uracil and would allow a relatively reasonable resolution of 5-FU from uracil.

2. Experimental

2.1. Chemicals and reagents

5-FU (lot # 008004, purity > 99%) was generously donated by Roche (Basle, Switzerland). Uracil (lot # 39203), perchloric acid and the internal standard, thymine (lot # 39188), were all purchased from Riedel Dehaën (Darmstadt, Germany). Methanol and ethyl acetate were of HPLC grade and were obtained from BDH Laboratory Supplies (BDH Chemicals Ltd., Poole, UK). Distilled and deionized water was obtained by passage through ELGA® (a trade name of Vivendi Water Systems Ltd., Wycombe, Bucks, UK) and was further filtered through a 0.22 µm membrane filter (Millipore, Bedford, MA, USA). Normal Human plasma collected from healthy volunteers was obtained from the blood bank at King Khalid University Hospital (Riyadh, Saudi Arabia). Stock solutions were prepared by dissolving the compounds in water. The standard solutions were prepared every day, stored in the dark and refrigerated.

2.2. HPLC apparatus and conditions

The HPLC system consisted of a Waters Model 515 HPLC pump, a Waters autosampler Model 717 plus (Waters Inc., Bedford, MA, USA), a Shimadzu Model SPD-10A variable-wavelength UV detector (Shimadzu Corporation, Kyoto, Japan) governed by a microcomputer running

Millennium® version 32 software, and vortex mixer (Scientific Industries Inc., New York, USA). The detector wavelength was set at 260 nm. Separation was achieved by isocratic elution with a mobile phase methanol:water (10:90, v/v) with the pH adjusted to pH 3.2 (perchloric acid), delivered at a flow-rate of 1.0 ml/min at ambient temperature through a Genesis C₁₈ analytical, 300 mm × 3.9 mm i.d., 10 µm particle size (Jones, CO, USA), protected by a guard column (Upchurch Scientific, Oak Harbour, WA, USA) of the same material.

2.3. Stocks solutions and standards

Stock solutions of 5-FU were prepared in triplicate by dissolving 10.0 mg 5-FU in 100 ml water, resulting in a solutions containing 100 µg/ml. A stock solution of the internal standard at a concentration of 100 µg/ml was prepared by dissolving 10.0 mg of thymine in 100 ml water. Working solutions of 5-FU (10 and 1 µg/ml), and 25 µg/ml of thymine were prepared by dilution of the stock solutions in water.

Standards were prepared with the following concentration of 0, 30, 40, 50, 100, 150, 200, 400, 500 and 1000 ng/ml for 5-FU. Plasma samples (500 µl) were supplemented with internal standard (50 µl of 25 µg/ml thymine). Three pools of quality control (QC) samples for 5-FU were prepared in human plasma at concentrations of 30, 200 and 1000 ng/ml, by addition of small volumes of the 5-FU working solutions to human plasma.

2.4. Extraction procedure

Different concentrations of 5-FU were first added to thawed plasma samples (500 µl) which were vortex-mixed for 2 min. Then, 50 µl internal standard were added. A solution of 5 ml of ethyl acetate, as an extracted solvent, was added to the plasma samples. Samples were vortex-mixed for 7 min and then centrifuged (4000 g, 10 min) (Jouan, GR 412, Saint Mazaire Cedex, France). The supernatant was transferred to 100× mm borosilicate glass tubes (James A. Jobling and Company, Ltd., Sunderland, UK). The organic extraction process was repeated collecting organic supernatant into the same glass tube. Samples were evaporated to dryness under a stream of nitrogen at 60 °C and reconstituted in 200 µl of water, vortex mixed. The supernatants were transferred to glass tubes with caps (Waters Inc., Millipore, MA, USA) and a 50 µl volume was injected into the HPLC system.

2.5. Assay validation

Validation of this assay was in compliance with the current FDA guidelines for biological method validation [12]. A validation run included a set of calibration samples, lower limit of quantification (LLQ) samples and QC samples at three levels performed on six separate occasions. The precisions

were calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable.

2.5.1. Quantification

Plasma samples were obtained from healthy volunteers and spiked with 5-FU and thymine stock solutions. These samples were then subjected to the extraction and HPLC separation. The peak-height ratios of 5-FU to that of thymine were calculated. Standard curves were constructed by plotting the peak-height ratios versus the plasma concentrations of 5-FU using least-squares regression lines. Plasma standards were analyzed concurrently with set of unknown samples. Controls containing drug-free samples were also included in each batch and were spiked with known amounts of 5-FU and the internal standard.

2.5.2. Precision, accuracy and limit of quantitation

Repeated injections ($n = 6$) were performed on a single day to establish the intra-day coefficient of variation (precision). The inter-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 1.0 ml/min. Standards were assayed in order of increasing concentration. The accuracy was expressed as the ratio of the compound added to that measured (mean value/nominal value) $\times 100$. The LLQ was 30 ng/ml and the limit of detection (LOD) of 5-FU was 10 ng/ml.

2.5.3. Recovery

Extraction efficiency of 5-FU and thymine was determined, using similarly prepared standards, by spiking 500 μ l aliquots of plasma and water with 30, 200 and 1000 ng/ml 5-FU and thymine ($n = 6$). Absolute recoveries of 5-FU and thymine in plasma were determined by assaying the samples as described above and comparing the peak-heights ratios of extracted plasma with those obtained from direct injection of the compounds dissolved in aqueous supernatant of processed blank plasma. Relative analytical recovery of 5-FU was determined by comparing the measured concentration with the ones added using three different quality control samples.

2.5.4. Stability and specificity

Stability studies in heparinized plasma spiked with three QC (30, 200 and 1000 ng/ml) through three freeze/thaw cycles were (72 h period at $-20 \pm 5^\circ\text{C}$ to room temperature) have been performed. Samples were removed from freezer, allowed to stand on the bench top, under room lighting, for 30 min to thaw, which is an optimum period of time for withdrawing blood for human subject, then assayed for 5-FU content. The specificity of the analytical method was evaluated by analyzing drug-free blank plasma from several healthy volunteers.

3. Results and discussion

5-FU is most conveniently assayed by HPLC with UV detection [13–15]. However, the retention times of 5-FU and uracil are very close, making the quantitation of 5-FU and uracil difficult [9]. We have developed a new and sensitive assay to accurately measure 5-FU in plasma. The assay was validated in terms of linearity, accuracy, precision, lower limit of quantitation, detection of unknown concentration and recovery. The alternatives to using this method include GC–MS (the most popular) and column switching [16]. The advantages of mentioned method include high sensitivity (1 ng/ml). The disadvantages, however, is the limited accessibility to a GC–MS system and complex extraction procedures [9].

The HPLC behavior of 5-FU extracted from human plasma was isocratically examined by using Genesis C₁₈ analytical column. We have tested a number of mobile phases and found that (methanol:water, 10:90%, v/v) gave excellent separation of the compounds of interest in a run time of 10 min. The mobile phase reported herein (methanol:water, 10:90%, v/v) was optimized for a rapid and interference-free chromatogram. The selected chromatographic conditions provided optimum resolution of 5-FU and the internal standard. The retention times for 5-FU, uracil, and thymine were 4.5, 6.0, and 9.0, respectively.

A typical HPLC chromatogram of human plasma without spiking 5-FU and internal standard and a chromatogram of an extract of human plasma spiked with 5-FU (30 ng, LOQ) and thymine (50 ng, I.S.) are shown in Fig. 2A and B, respectively. A comparison of the chromatograms shown in Fig. 2 demonstrates that there is no interference from endogenous components in the analysis of plasma uracil.

The calibration curves were linear from 30 to 1000 ng/ml. The mean (\pm S.D.) regression equation for six replicated calibration curves constructed using 500 μ l of plasma samples on different days was $(0.0043 \pm 0.0002)C + (-0.000148 \pm 0.0000331)$, $r^2 = 0.999913 \pm 0.0000761$. Standard curves were constructed over a 8-week period to determine the variability of the slopes and intercepts. The results showed little day-to-day variability in the slopes and intercepts. Analysis of variance of the slopes, intercepts and correlation coefficients of the six standard plots from plasma indicated non-significant differences ($P > 0.05$). The coefficient of variation for the slopes was 4.7% which indicates a high stability and precision for the assay. The results confirm the linearity of the standard curves and the excellent reproducibility of the assay method. The limit of quantification for this method was attained with plasma samples containing 30 ng/ml of 5-FU.

The intra-day precision (random analytical variation) was evaluated by replicate analysis of pooled plasma samples containing 5-FU uracil at three different concentrations (low, medium, and high). The intra-day precision showed a coefficient of variation of 1.35–4.53% (Table 1). The inter-day was similarly evaluated on several days up to 8 weeks. The

Table 1

Intra-day and inter-day accuracy and precision of 5-FU in human plasma (mean \pm S.D., $n = 6$)

C_{nominal} (ng/ml)	Intra-day			Inter-day ^a		
	C_{est} (ng/ml)	Precision as CV (%)	Accuracy (%)	C_{est} (ng/ml)	Precision as CV (%)	Accuracy (%)
30	30.1 \pm 1.36	4.53	100.3	29.5 \pm 1.47	4.98	98.3
200	200.2 \pm 3.89	1.94	100.1	199.0 \pm 3.74	1.88	99.5
1000	999.5 \pm 13.45	1.35	99.9	1003.4 \pm 12.99	1.29	100.3

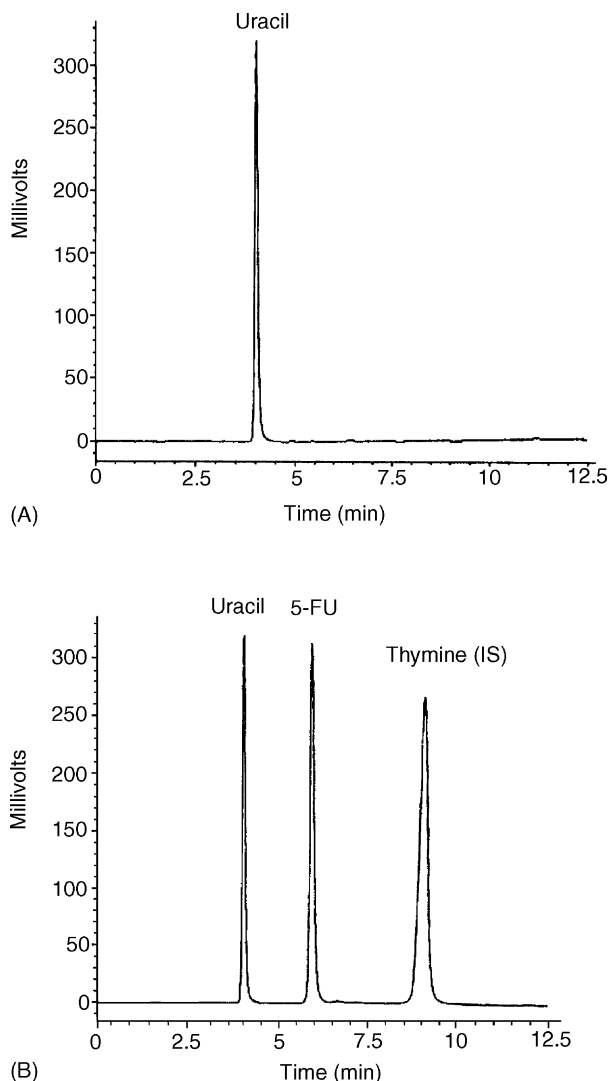
 C_{nominal} : nominal concentration; C_{est} : estimated concentration.^a Inter-day reproducibility was determined from six different runs over a 8-week period at three concentrations. The concentration of each run was determined from a single calibration run on the first day of the study.

Fig. 2. (A) A typical HPLC chromatogram of human plasma without spiking 5-FU and internal standard. (B) Chromatogram of an extract of human plasma spiked with 5-FU (30 ng, LOQ) and thymine (50 ng, I.S.).

inter-day CVs varied from 1.29 to 4.98% (Table 1). The absolute recoveries of 5-FU and the internal standard ranged from 99.2 to 96.0% and 98.3 to 99.5%, respectively. The relative recovery of 5-FU was calculated by comparing the concentrations obtained from drug-supplemented plasma to the actual added concentrations. The relative recovery ranged

from 98.82 to 101.44%. Both accuracy and precision values throughout the concentration range (30–1000 ng/ml) were acceptable [12].

5-FU is a photolabile, and consequently care was taken to protect the samples against photo-oxidation. All manipulations with the samples were made in a dark room. Furthermore, wrapping the samples in aluminum foil improved the stability dramatically. The stability of 5-FU in plasma was investigated using spiked samples at three different concentration levels (20, 200 and 100 ng/ml). Spiked samples were analyzed after different storage conditions: immediately, after three freeze/thaw cycles and after 7272 h stored at -20°C . Under the test conditions, no significant change was observed in the analytical results. The mean percentage recovery ($100 \times (\text{measured concentration}/\text{added concentration})$) ranged from 94.06 ± 2.69 to 100.37 ± 6.91 and the CV% was less than 7.0%. The results of this investigation demonstrate that 5-FU added to plasma is stable at different conditions without a significant degradation. Processed human plasma samples containing 5-FU and thymine in mobile phase were also found to be stable at room temperature in the autosampler. No interference from endogenous plasma constituent was observed at the retention times of 5-FU and the internal standard.

4. Conclusion

The present HPLC method provides a rapid, reproducible, and thoroughly validated assay for the determination of 5-FU in plasma. This assay provides a selective quantifications of 5-fluorouracil without any interference of the endogenous uracil. The reported method was sensitive enough to detect a very low concentration, which makes it a potentially valuable tool in many applications, such as therapeutic drug monitoring bioavailability and bioequivalence studies with sufficient specificity, simplicity, and sensitivity.

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