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Short communication

# High-performance liquid chromatographic assay of 5-fluorouracil in human erythrocytes, plasma and whole blood

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**Abstract**

A HPLC assay method was modified and validated for the determination of 5-fluorouracil in human red blood cells, plasma and whole blood with a two-fold increased sensitivity (detection limit=10 ng/ml). The assay was linear from 25 to 1500 ng/ml and the accuracy ranged from 96.7 to 103.2% at 25 ng/ml, 94.8 to 99.4% at 500 ng/ml, and 98.9 to 99.5% at 1500 ng/ml. Intra-assay and inter-assay coefficients of variation were less than 8% over the range of concentrations and less than 8% over 10 days of analysis. After intravenous bolus and infusion of 5-fluorouracil in patients with colorectal cancer, the concentrations of 5-fluorouracil in whole blood were 108–111% of plasma concentrations, while packed red blood cells levels were 8–15% of plasma concentrations in the five patients studied. By utilising basic analytical hardware, this represents an accurate, precise, reproducible and affordable method for 5-fluorouracil pharmacokinetics investigation and therapeutic drug monitoring.

*Keywords:* 5-Flourouracil

## 1. Introduction

5-Fluorouracil (5-FU) is being increasingly used in the management of several common malignancies including cancer of the colon, breast and skin [1]. Several assay methods for determining 5-FU in biological fluids have been reported in the literature [1–4]. Most of the current methods use reversed-phase high-performance liquid chromatographic (RP-HPLC) assay techniques. However, the assay procedures described in those reports attain a detection limit of approximately 25 ng/ml and most assay methods were designed and validated only for plasma [1–3]. In another report [4], a sensitivity of 10 ng/ml was achieved in plasma using a HPLC assay with UV detection. However, the concentration range of the assay for 5-FU was limited to 10–500 ng/ml and it required a relatively sophisticated apparatus and a complex extraction procedure. Drug concentrations measured in plasma can not be assumed to be the same as those measured in erythrocytes and whole blood [5]. The differences in pharmacokinetic values might be of importance in clinical investigation as a surrogate measure of intracellular 5-FU concentrations. Determination of drug and metabolite concentrations in erythrocytes and/or white blood cells has provided a useful tool for other antimetabolite cancer chemotherapy agents [6,7]. Application of RP-HPLC assay methodology to 5-FU analysis in human erythrocytes and comparison of the levels of

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5-FU in red blood cells, plasma and whole blood have not been previously reported.

In this study, we report a method for analysis of 5-FU in erythrocytes, plasma and whole blood and its application in patients with colorectal cancer receiving 5-FU treatment. By modifying the widely used method of Christophidis et al. [2], increased sensitivity was obtained with a detection limit of approximately 10 ng/ml. As 5-FU is rapidly eliminated from the systemic circulation, increased sensitivity is an important factor for thorough pharmacokinetic analysis.

## 2. Experimental

### 2.1. Chemicals and reagents

5-Fluorouracil (5-FU) and 5-fluorocytosine (5-FC) were purchased from Sigma (St. Louis, MO, USA). All solvents and other chemicals were of analytical reagent grade. Concentrated solutions were prepared in HPLC water, aliquots stored at −20°C. Dilute solutions were prepared freshly as required, stored at 4°C and used within a week.

### 2.2. Apparatus and chromatographic conditions

The chromatographic system consisted of a Jasco 880-PU Intelligent HPLC pump (Japan Spectroscopic, Tokyo, Japan) equipped with a Rheodyne 7125 injection valve fitted with a 20 μl loop (Cotati, CA, USA) and a stainless-steel column (200 mm× 4.6 mm I.D.) slurry packed with 5 μm ODS Hypersil (Shandon HPLC, Runcorn, UK). Ultraviolet detection was by a Varian 2550 variable λ detector (Japan Spectroscopic) operating at 270 nm and connected to a SE 120 BBC Goerz Metrawatt chart recorder (Belmont instruments, Glasgow, UK) for manual peak height measurement. The mobile phase, $KH_2PO_4$ buffer (50 m*M*, pH 3, adjusted using orthophosphoric acid SG 1.69, Fisons Scientific Equipment, Loughborough, UK), was degassed prior to use by vacuum filtration through a 0.45 μm filter (Type HVLP, Millipore, Milford, MA, USA). The sample extracts were separated at ambient temperature using a flow-rate of 1.0 ml/min. Peak heights were measured at appropriate absorbance ranges, usually at 0.005–0.02 absorbance unit full scale (a.u.f.s.).

### 2.3. Patients and sample collection

Following ethical committee approval, 5-FU pharmacokinetics were investigated in five consenting patients, all of whom had histologically proven colorectal cancer. Patients were treated with 5-FU 375 mg/m$^2$ i.v. bolus over 5 min (two patients) or 600 mg/m$^2$ over 30 min followed by 600 mg/m$^2$ over 22 h (three patients). Blood samples (8 ml) were collected in heparinized tubes prior to the start, at the end, and 30 min from the end of the 5 min i.v. 5-FU bolus or 18 h after the start of the 5-FU infusion. The tubes were immediately placed on ice. Plasma and red blood cell were separated within 20 min to minimize degradation of 5-FU at low concentrations in whole blood [8]. Plasma was separated by centrifugation at 3000 *g* for 10 min at 4°C and the red blood cell fraction was washed twice with a 0.9% NaCl solution. The supernatant was separated by centrifugation as before and discarded. All samples were stored at −20°C until analyzed within 10 days.

### 2.4. Extraction procedure

The extraction procedure used was modified from that of Christophidis et al. [2]. To a 15-ml polypropylene conical tube was added 0.6 ml of sample (red blood cells, plasma or whole blood), 40 μl of a 0.4 m*M* aqueous solution of 5-fluorocytosine (internal standard), 30 μl of sodium acetate buffer (1 *M*, pH 5.3), 0.3 ml of a 1.4 *M* solution of anhydrous sodium sulphate and 5.4 ml of *n*-propanol–diethyl ether (16:84, v/v). The tube was tightly capped and vortex-mixed for 90 s, and centrifuged at 3000 *g* for 5 min. The organic solvent layer was transferred to a second tube and 0.3 ml of phosphate buffer (50 m*M*, pH 11) was added. After vortex mixing and centrifugation as before, the organic solvent phase was aspirated and discarded. The aqueous phase was adjusted to neutral pH by adding 5 μl of 1 *M* $H_2SO_4$, and 20 μl of this mixture injected onto the HPLC column.

Standard calibration curves were prepared by adding pure 5-fluorouracil in concentration of 25, 50,

100, 250, 500, 1000 and 1500 ng/ml to pooled drug-free specimens. Then these samples were treated as described above. The concentrations of 5-fluorouracil were determined from a graph relating peak height ratios of 5-fluorouracil to 5-fluorocytosine, and to the concentrations of known standards. In patient samples where drug concentrations were expected to exceed 1500 ng/ml, a smaller volume was utilized with the difference to 0.6 ml made up with an appropriate volume of blank sample.

## 3. Results and discussion

Chromatograms resulting from the analysis of 5-fluorouracil, using the method described above, are shown in Fig. 1. They show the sharp, distinct peaks produced by 5-fluorocytosine (5-FC, internal standard) and 5-fluorouracil (5-FU), with retention times of 4 and 5.8 min, respectively. The total analysis time required for each run was 12 min. The internal standard (5-FC) contained no detectable 5-FU and was stable under the storage and HPLC conditions used in this study. Folinic acid, which is often used in combination with 5-FU, did not interfere with the assay. The endogenous components that eluted with retention characteristics similar to those of 5-FU also did not interfere with quantitation.

Table 1
Recovery of 5-FU extracted

| 5-FU spiked (ng/ml) | Recovery (S.D.) ($n$=10) (%) | | |
|---|---|---|---|
| | Erythrocytes | Plasma | Whole blood |
| 25 | 97 (9) | 98 (10) | 97 (8) |
| 500 | 96 (4) | 102 (4) | 96 (4) |
| 1500 | 96 (3) | 101 (4) | 97 (5) |

The recoveries of 5-FU extracted from spiked red blood cells, plasma and whole blood, calculated by comparison with a solution of 5-FU in mobile phase at the concentrations of 25, 500 and 1500 ng/ml, are shown in Table 1. The extraction recovery exceeded 96% at all concentrations in red blood cells, plasma and whole blood.

The linearity of this assay was assessed by comparison of calibration curves for each matrix from analyses of spiked solution of 5-FU at 0–1500 ng/ml on six different days. Close correlations with the linear regression equations were observed for all matrices. The results of the linear regression analysis of these data were shown in Table 2. In these equations $y$ represents the ratio of peak heights of 5-FU to 5-FC, $x$ represents the concentration of 5-FU (ng/ml) and $r$ is the correlation coefficient.

The results of accuracy, precision and reproducibility tests of the assay are shown in Table 3. The assay had a high level of accuracy in all three

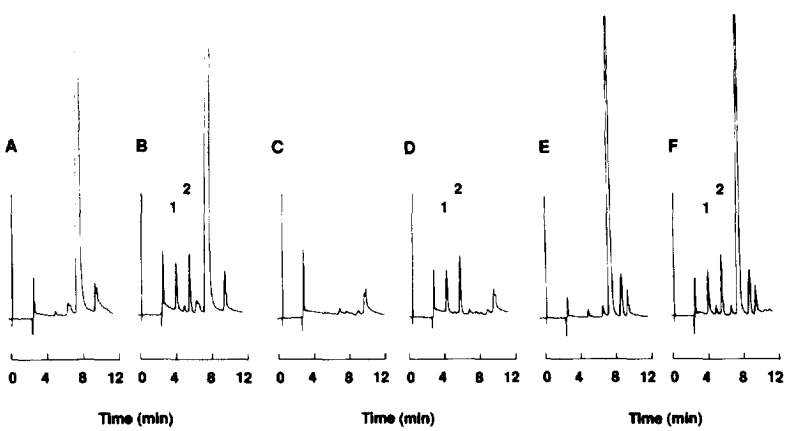


Fig. 1. Typical chromatograms of blank (A) red blood cell, (C) plasma and (E) whole blood, respectively, and spiked (B) red blood cell, (D), plasma and (F) whole blood, respectively, with 500 ng 5-fluorouracil (5-FU)/ml matrix and 5-fluorocytosine (5-FC) as internal standard, at a sensitivity setting of 0.02 a.u.f.s. (1=5-FC peaks; 2=5-FU peaks). For chromatographic conditions and extraction procedure, see text.

Table 2
Calibration curves for the HPLC assay of 5-FU

| Matrix | Linear regression equation ($y=a+bx$) ($n=6$) | | |
|---|---|---|---|
| | Slope (S.D.) | Intercept (S.D.) | $r^2$ |
| Erythrocytes | 0.002414 (0.000066) | −0.003079 (0.013689) | >0.9991 |
| Plasma | 0.002465 (0.000056) | −0.000532 (0.014234) | >0.9997 |
| Whole blood | 0.002555 (0.000101) | −0.019308 (0.015863) | >0.9992 |

biological matrices for repeated analysis of known concentrations, ranging from 96.7 to 103.2% at 25 ng/ml, 94.8 to 99.4% at 500 ng/ml and 98.9 to 99.5% at 1500 ng/ml. The assay was precise with coefficients of variation (C.V.) less than 8% for red blood cells, plasma and whole blood over the range of concentrations tested. The assay also demonstrated at the three concentrations tested reproducibility, with C.V. <8% over ten days of analysis (Table 3).

The detection limit was 10 ng/ml, as estimated by injecting successively lower concentrations until a signal-to-noise ratio of approximately 3 was obtained. This two-fold enhanced sensitivity enabled measurement of the concentrations of 5-FU found in human erythrocytes during infusion of the drug and the concentrations of 5-FU in plasma and whole blood during the decay phase after the end of infusion. Such concentrations were normally found in blood samples of the patients receiving prolonged infusions of 5-FU, but were generally unmeasurable by the previously reported methods [1–3]. The improved sensitivity was mainly the result of using a smaller volume of both the organic solvent for extraction and phosphate buffer (pH 11) for back extraction as well as a change in the sodium acetate buffer from pH 4.8 to 5.3. Sensitivity was also improved by changing the detection wavelength from 254 nm to 270 nm. These improvements allowed the use of a smaller sample volume (0.6 ml) which is important for patient studies with intensive pharmacokinetic sampling.

The assay was then applied to the analysis of 5-FU in the red blood cells, plasma and whole blood after bolus or infusion administration in five patients with colorectal carcinoma (Fig. 2). Concentrations of 5-FU in whole blood were 108–111% of plasma concentrations, while packed red blood cells levels were 8–15% of plasma concentrations in the five patients studied.

In conclusion, this modified method has proved convenient, reliable, sensitive and suitable for routine analysis of 5-FU clinically. In addition, the sample processing and analytical approach used in the assay were relatively inexpensive, allowing implementation in developing laboratories. We found that following bolus and infusion administration in patients

Table 3
Accuracy, precision and reproducibility of the 5-FU HPLC assay

| Matrix | Mean concentrations of 5-FU ($n=10$) (ng/ml)[a] | | | | | |
|---|---|---|---|---|---|---|
| | Intra-day[b] | | | Inter-day[c] | | |
| | 25 ng/ml | 500 ng/ml | 1500 ng/ml | 25 ng/ml | 500 ng/ml | 1500 ng/ml |
| Erythrocytes | 24.79 (7.67) | 493.33 (2.01) | 1484.18 (1.12) | 25.95 (7.74) | 497.53 (1.77) | 1481.96 (3.28) |
| Plasma | 24.17 (7.47) | 497.23 (1.02) | 1487.13 (1.63) | 25.01 (7.12) | 493.91 (1.80) | 1498.28 (2.29) |
| Whole blood | 25.81 (7.46) | 473.82 (1.38) | 1492.27 (3.74) | 27.33 (7.87) | 488.84 (4.38) | 1527.50 (5.93) |

[a] Values in parentheses are coefficients of variation (%).
[b] Intra-day=ten consecutive analyses, using a single calibration procedure.
[c] Inter-day=analyses of the same solution on 10 days.

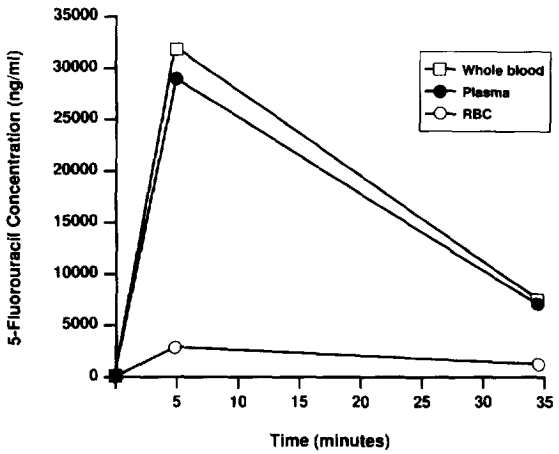


Fig. 2. 5-FU concentrations in the whole blood, plasma and red blood cells from a representative patient receiving 375 mg/m$^2$ over 5 min. for details, see text.

with colorectal carcinoma, the levels of 5-fluorouracil in whole blood were around 10% higher than those in plasma and were much higher than those in packed red blood cells. Further investigations into 5-FU concentrations in intracellular, plasma and whole blood and the relationship between pharmacokinetics and pharmacodynamics of 5-FU are the subjects of ongoing studies using this new analytical method.

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