

*Journal of Chromatography*, 344 (1985) 385–390

*Biomedical Applications*

Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2751

## Note

---

### **Sensitive high-performance liquid chromatographic method for the determination of 5-fluorouracil in plasma**

P.L. STETSON\*, U.A. SHUKLA and W.D. ENSMINGER

*Upjohn Center for Clinical Pharmacology, University of Michigan, Ann Arbor, MI 48109 (U.S.A.)*

(First received March 18th, 1985; revised manuscript received June 12th, 1985)

The fluorinated pyrimidine, 5-fluorouracil (FU), has been used in the treatment of a wide variety of solid tumors for more than two decades [1]. It is frequently used in the treatment of early and advanced colorectal carcinomas. Since the drug has a short biological half-life, the clinical pharmacokinetic studies require a sensitive assay procedure which can quantitate low ng/ml FU levels in plasma.

Several gas chromatographic (GC) and high-performance liquid chromatographic (HPLC) methods for determining plasma FU levels have been reported in the literature [2–19]. The GC methods have employed either packed columns [2] or capillary columns [3] and utilized the nitrogen–phosphorus-selective detector. The sensitivity of the capillary GC method was reported to be 150 ng/ml FU. Another GC procedure described in the literature requires derivatization of FU before analysis [9]. Several gas chromatographic–mass spectrometric (GC–MS) assays for FU have also been reported [4, 16–19] which are both sensitive and specific, but the complexities of the assays and associated instrumentation prohibit their routine use. A HPLC method reported in the literature suffered from poor reproducibility [5], which was probably due to adsorption of FU on glass surfaces [8]. Modification of this assay has been reported to improve its reproducibility [6] but the assay employs thymidine as the internal standard which is endogenous in plasma. Another HPLC assay employed back-extraction of the organic extract into phosphate buffer (pH=11), followed by neutralization with sulfuric acid, but the internal standard, 5-fluorocytosine, coeluted with an endogenous peak in blank (control) plasma [7].

The assay procedure described in this report can attain detection limits around 25 ng/ml and is routinely used in the FU concentration range of 50–

2000 ng/ml. Both internal standard (5-chlorouracil, CU) and drug (FU) show no interference by coeluting endogenous or exogenous compounds in the several hundreds of patient samples already analyzed using this procedure. Although primarily designed for plasma assays, this procedure has been used for the determination of FU levels in peritoneal fluid as well (see Fig. 2).

## EXPERIMENTAL

### *Reagents*

Ethyl acetate and methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), monobasic ammonium phosphate (J.T. Baker, Phillipsburg, NJ, U.S.A.), potassium hydroxide and ammonium sulfate (Mallinckrodt, St. Louis, MO, U.S.A.), 5-chlorouracil (Sigma, St. Louis, MO, U.S.A.), and 5-fluorouracil (Roche Labs., Nutley, NJ, U.S.A.) were used.

### *Stock solutions*

A 5-fluorouracil solution (0.10 mg/ml in methanol), and a 5-chlorouracil solution (1.0 mg/ml in methanol) were prepared and stored at  $-20^{\circ}\text{C}$ . Saturated ammonium sulfate solution and 0.5 M potassium hydroxide solution were prepared and stored at room temperature.

### *Standards*

The FU stock solution was diluted 1:10 with water to yield the FU standard solution containing 10  $\mu\text{g}/\text{ml}$ . The CU stock solution was diluted with water to yield the internal standard solution containing 10  $\mu\text{g}$  CU per ml.

Quality-control samples at two concentrations (200 and 2000 ng/ml FU) were prepared by spiking blank plasma with the appropriate volumes of the FU standard. After mixing, 1.0-ml aliquots were transferred to glass tubes, tightly capped, and stored frozen at  $-30^{\circ}\text{C}$ . Three quality-control samples of each concentration were included with every group of experimental samples to be analyzed.

### *Chromatographic conditions*

The HPLC unit included a Waters Assoc. Model 6000A solvent delivery system, a Waters Assoc. Model 440 UV-VIS absorbance detector with a 254-nm filter, a Waters Assoc. Model 710B WISP autosampler and a Hewlett-Packard Model 3390A integrator. The column used was a Dupont Zorbax  $\text{C}_8$  reversed-phase column (25 cm  $\times$  4.6 mm I.D., spherical, 6  $\mu\text{m}$  particle size). The mobile phase was a 0.05 M ammonium phosphate buffer (pH 6.8)—methanol mixture with a final methanol concentration of 2% (v/v). The flow-rate was set at 1.0 ml/min.

Under these conditions the retention times of FU and CU were 4.4 and 7.6 min, respectively.

### *Sample preparation*

To glass tubes (15 ml capacity) were added 1.0 ml plasma, 100  $\mu\text{l}$  internal standard solution (1000 ng CU), 2.0 ml saturated ammonium sulfate solution, 100  $\mu\text{l}$  ammonium phosphate buffer, pH 6.7 and 8.0 ml ethyl acetate. The tubes were tightly capped and shaken for 15 min at room temperature. In un-

known plasma samples where drug concentrations were expected to exceed 2000 ng/ml, a smaller volume was utilized with the difference to 1.0 ml made up with an appropriate volume of blank plasma. After centrifugation (room temperature) for 10 min at 1200 *g*, the ethyl acetate phase was transferred to clean conical-bottomed glass tubes and concentrated to approximately 1.0 ml by evaporation in a water-bath (45°C) under a stream of air. To each sample were added 400  $\mu$ l of 0.5 *M* potassium hydroxide and the drug and internal standard back-extracted by shaking for 15 min at room temperature. After centrifugation (room temperature) at 1000 *g* for 10 min, the ethyl acetate phase was removed by aspiration. Volumes of 3.0–30.0  $\mu$ l of the alkaline aqueous phase were injected for HPLC analysis.

#### Standard calibration curve

Blank plasma samples were spiked in duplicate with the appropriate volumes of the FU standard solution to concentrations ranging from 0 to 2000 ng/ml and subjected to the sample preparation procedure described above. Calibration curves were run (in duplicate) with each set of experimental samples.

#### Calculations

Calibration curves were constructed by plotting the ratio of the peak height of FU to that of the internal standard as a function of the plasma drug concentration. The best-fit straight line was determined using the method of least squares. The FU concentrations of unknown samples were calculated from the least-squares regression line of the calibration curve.

### RESULTS AND DISCUSSION

Under the described conditions, the retention times of FU and CU were 4.4 and 7.6 min, respectively. Fig. 1 illustrates typical chromatograms for control plasma (no internal standard added), a quality-control sample containing 200 ng/ml FU in plasma, and a patient plasma sample containing 750 ng/ml FU. The plasma samples elicit no interfering peaks. The total analysis time required

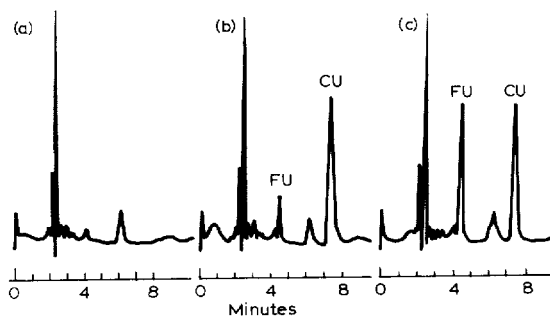


Fig. 1. Typical chromatograms of (a) control plasma without added internal standard, (b) a quality-control sample containing 200 ng/ml FU in plasma, and (c) a plasma sample obtained from a patient containing 750 ng/ml FU. Peaks: FU = 5-fluorouracil; CU = 5-chlorouracil (internal standard).

TABLE I

FLUOROURACIL CONCENTRATIONS IN CONTROL SAMPLES SEEDED WITH 200 AND 2000 ng/ml ASSAYED OVER A THREE-DAY PERIOD

Day	Concentration (ng/ml)	
	200 ng/ml	2000 ng/ml
1	200.4	2125.5
	240.6	2062.3
	238.3	2207.4
2	202.2	2014.5
	229.7	1955.4
	234.6	1855.5
3	200.2	1935.0
	225.1	1977.7
	203.3	2015.8
Mean $\pm$ S.D. (ng/ml)	219.4 $\pm$ 17.5	2016.6 $\pm$ 105.0
Coefficient of variation (%)	8.0	5.2
Percentage from theoretical	+9.7	+0.8

for each run was 15 min. The validity of the assay procedure was established through a careful study of the linearity of response, reproducibility, accuracy and precision.

The peak-height ratio was directly proportional to the FU concentrations over the range 50–2000 ng/ml. The best-fit lines were obtained using linear regression analysis. To test the reproducibility of this procedure, calibration samples were prepared in triplicate on three successive days. Triplicate quality-control samples at each concentration (200 and 2000 ng/ml) were run in parallel. The results of the linear regression analyses of this data were:  $y = (0.00125 \pm 0.00005)x + (0.043 \pm 0.015)$ , ( $r=0.998 \pm 0.001$ ).

The accuracy and precision of the method were assessed by seeding plasma at 5-FU concentrations of 200 and 2000 ng/ml. Triplicate quality-control samples at each concentration were assayed on each of three consecutive days. Table I gives the results of this experiment. The precision of the assay was found to have coefficients of variation (C.V.) ranging from 5.2 to 8.0%. The concentration means for the seeded control samples were found to be within 0.8–9.7% of the theoretical values for FU.

Application of the method developed was demonstrated by measuring the plasma and peritoneal fluid FU levels in a patient following the intraperitoneal infusion of 1.0 g FU per day for four days. Peripheral venous and peritoneal fluid samples were withdrawn at regular intervals and the samples obtained were used for drug analysis. Fig. 2 illustrates the plasma and peritoneal fluid FU concentrations versus time plots from this experiment. As shown in Fig. 2, the peritoneal fluid FU steady-state levels attained in this infusion were approximately 200-fold greater than the corresponding peripheral venous plasma FU levels.

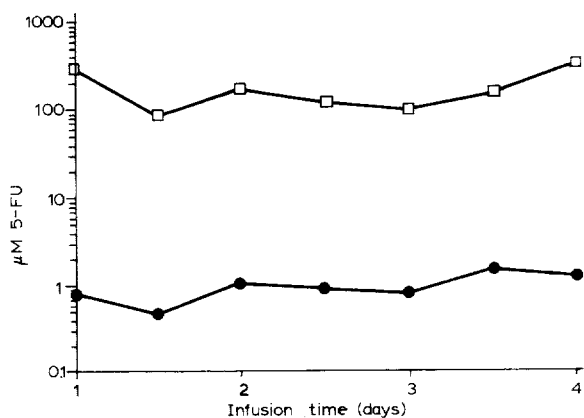


Fig. 2. The plasma and peritoneal fluid FU concentration — time profile of a patient following intraperitoneal infusion of 1.0 g FU per day for four days. (□) Peritoneal fluid levels; (●) plasma levels.

This HPLC assay method for plasma 5-FU has been utilized in our laboratory in the determination of FU levels in several hundred patient and animal samples. It has proved dependable, rapid, accurate and sensitive. Unlike previously published HPLC 5-FU procedures [5–7, 10–15], it does not suffer from sensitivity limitations [11], it does not employ as an internal standard a compound endogenous to plasma [6], it does not necessitate derivatization of the FU before analysis [12], it is not hampered by incomplete chromatographic resolution of the FU peak from surrounding endogenous peaks [7, 13, 15], it does not necessitate the use of radiolabelled drug as internal standards [14], and it does not include extra steps in the sample preparation procedure to neutralize the final solution to be injected for HPLC analysis [7, 10]. This procedure has been successfully utilized in numerous pharmacokinetic studies involving this important antineoplastic agent.

#### ACKNOWLEDGEMENT

This work was supported in part by NIH Grant No. 33825.

#### REFERENCES

- 1 R.T. Dorr and W.L. Fritz, *Cancer Chemotherapy Handbook*, Elsevier/North Holland, Amsterdam, 1980, p. 435.
- 2 O. Driessen, D. de Vos and P.J.A. Timmermans, *J. Chromatogr.*, 162 (1979) 451.
- 3 E.A. de Bruijn, O. Driessen, N. van den Bosch, E. van Strijen, P.H.Th.J. Slee A.T. van Oosterom and U.R. Tjaden, *J. Chromatogr.*, 278 (1983) 283.
- 4 T. Marunaka, Y. Umeno, K. Yoshida, M. Nagamachi, Y. Minami and S. Fujii, *J. Pharm. Sci.*, 69 (1980) 1296.
- 5 A.T. Wu, J.L. Au and W. Sadee, *Cancer Res.*, 38 (1978) 210.
- 6 C.L. Hornbeck, R.A. Floyd, J.C. Griffiths and J.E. Byfield, *J. Pharm. Sci.*, 70 (1981) 1163.
- 7 N. Chrostophidis, G. Mihaly, F. Vajda and W. Louis, *Clin. Chem.*, 25 (1979) 83.
- 8 O. Driessen, D. de Vos and P.J.A. Timmermans, *J. Pharm. Sci.*, 67 (1978) 1494.

- 9 N. Chrostophidis, F.J.E. Vajda, I. Lucas, O. Drummer, W.J. Moon and W.J. Louis, *Clin. Pharmacokinet.*, 3 (1978) 330.
- 10 D.C. Sampson, R.M. Fox, M.H.N. Tattersall and W.J. Hensley, *Ann. Clin. Biochem.*, 19 (1982) 125.
- 11 G.J. Peters, I. Draal, E. Laurensse, A. Leyva and H.M. Pinedo, *J. Chromatogr.*, 307 (1984) 464.
- 12 M. Iwamoto, S. Yoshida and S. Hirose, *J. Chromatogr.*, 310 (1984) 151.
- 13 W.E. Wung and S.B. Howell, *Clin. Chem.*, 26 (1980) 1704.
- 14 A.R. Buckpitt and M.R. Boyd, *Anal. Biochem.*, 106 (1980) 432.
- 15 L.S.F. Hsu and T.C. Marrs, *Ann. Clin. Biochem.*, 11 (1980) 272.
- 16 B.H. Min and W.A. Garland, *Res. Commun. Chem. Pathol. Pharmacol.*, 22 (1978) 145.
- 17 D.B. Lakings, R.H. Adamson and R.B. Diasio, *J. Chromatogr.*, 146 (1978) 512.
- 18 J.P. Cano, J.P. Rigault, C. Aubert, Y. Carcassanne and J.F. Seitz, *Bull. Cancer (Paris)*, 1 (1979) 67.
- 19 C. Finn and W. Sadee, *Cancer Chemother. Rep.*, 59 (1975) 279.