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Gas chromatographic–mass spectrometric method for routine monitoring of 5-fluorouracil in plasma of patients receiving low-level protracted infusions

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ABSTRACT

A gas chromatographic–mass spectrometric (GC–MS) method is described which quantitates 5-fluorouracil (5-FU) plasma levels ranging from 0.5 to 50 ng/ml. The analysis uses two internal standards, 1,3-[$^{15}N_2$]-5-fluorouracil and 5-chlorouracil. Extraction and derivatization of the pyrimidine bases were accomplished in a single step using acetonitrile. Compounds were analyzed as their 1,3-dipentafluorobenzyl derivatives by electron-impact MS, and the GC–MS analysis was automated with respect to sample injection and data reduction. Stability of the analysis was demonstrated by continuous unattended analysis of 5-FU in human plasma for periods of up to three days with no deterioration of the quantitative results. The method is applicable to quantitating 5-FU plasma levels in patients receiving protracted infusions of the drug for colorectal cancer or other malignancies.

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INTRODUCTION

5-Fluorouracil (5-FU), a rationally designed analogue of uracil introduced over 30 years ago as a chemotherapeutic agent, is widely used in the management of cancer including colon, breast, skin, and ovarian tumors [1]. The development of ambulatory pumps including mechanical syringe, peristaltic, elastomeric balloon, and implantable types [2–4] have made it possible to explore long-term infusions of 5-FU continuing for weeks. Despite the interest in long-term infusions, pharmacokinetic data obtained during treatment by this route are limited. Measurement of 5-FU in plasma during prolonged drug administration requires analytical methodology capable of quantitation at high pg/ml to low ng/ml levels [5].

High-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) are the most widely used instrumental techniques for quantitating 5-FU in biological fluids. In most cases, GC–MS methods are one to two orders of magnitude more sensitive than those reported for HPLC [6]. Surprisingly, less emphasis has been placed on developing "routine" methods for quantitating 5-FU in plasma using GC–MS even though the mass spectrometer is more sensitive and highly specific [7–10]. This may be due in part to the perception that GC–MS techniques are complex and expensive. However, GC–MS instruments are currently available which can be used for routine automated analysis of drugs in biological fluids and are competitive with some automated HPLC systems in terms of cost and simplicity of operation.

We have developed a GC–MS method which quantitates 5-FU in a 1-ml plasma aliquot over a concentration range of 0.5–50 ng/ml using a bench-top GC–MS system equipped with an automatic injector and data system for data reduction. 5-FU was quantitated using both 1,3-[$^{15}N_2$]-5-fluorouracil ([$^{15}N_2$]-5-FU) and 5-chlorouracil (5-ClU) as internal standards. Compounds were analyzed by electron-impact (EI) GC–MS as their 1,3-dipentafluorobenzyl (PFBz) derivatives.

EXPERIMENTAL

Chemicals

5-FU, 5-fluorouridine (5-FUrd), 5-fluorodeoxyuridine (5-FdUrd) and 5-ClU were purchased from Sigma (St. Louis, MO, USA). [$^{15}N_2$]-5-FU (99 atom % ^{15}N) was obtained from MSD Isotopes (Montreal, Canada). The supplier for pentafluorobenzylbromide (PFBzBr) was Pierce (Rockford, IL, USA). Solvents for HPLC analysis were HPLC reagent grade and all other chemicals used were analytical reagent grade.

Sample preparation for GC–MS analysis

Human plasma standards were prepared containing 5-FU ranging from 0.5 to 50 ng/ml. Aliquots of each plasma standard (1 ml) or control plasma (1 ml) were placed in 15-ml polypropylene centrifuge tubes and 0.05 ml of an internal standard solution containing 1 μg/ml each of 5-ClU and [$^{15}N_2$]-5-FU were added. The compounds were extracted from plasma after adding 1 ml of acetonitrile. The sample was mixed with a vortex-mixer for 30 s, centrifuged at 5000 *g* for 10 min, and the supernatant transferred to 4-ml screw-top glass vials. Formation of the PFBz derivatives was accomplished by adding 0.1 ml of 1 *M* K_2HPO_4, pH 11.0 and 20 μl of PFBzBr. The sample was reacted at 100°C for 1 h in a Reacti-Therm heating module (Pierce). Samples were then centrifuged at 5000 *g* for 5 min and the supernatant (approximately 2 ml) applied to prewashed (1 × 3 ml of methanol followed by 3 × 3 ml of water) individual 200-mg, 3.0-ml solid extraction C_{18} columns (Bond-Elut, Varian Sample Preparation Products, Harbor City, CA, USA). After loading the sample, the column was washed with 3 × 3 ml of water and the compounds were eluted with 2 × 0.5 ml of absolute ethanol into 1.3-ml glass vials (Sunbrokers, Wilmington, NC, USA). Finally, solvent was removed from the sample using a Speed Vac evaporator (Savant Instruments, Farmingdale, NY, USA) and the residue was dissolved in 0.05 ml of acetone and 0.1 ml of hexane for analysis by GC–MS.

Optimization of derivatization procedure

Plasma samples (1 ml) were spiked with 50 μg of 5-FU and 50 μg 5-ClU. The compounds were then extracted using acetonitrile as described above. Derivative formation was optimized by monitoring product formation by HPLC after varying the reaction time, reaction temperature, and quantity of derivatizing agent, PFBzBr. Maximum product was formed after reaction for 1 h at 100°C using 20 μl of PFBzBr. Since no reference PFBz-5-FU was available, product formed represents relative conversion of 5-FU to PFBZ-5-FU. The PFBz-pyrimidine derivatives were stable for more than two weeks at room temperature.

Recovery of compounds during sample preparation for GC–MS analysis

Plasma and water standards were prepared containing 50 μg/ml 5-FU and 50 μg/ml 5-ClU. A 1-ml volume of acetonitrile was added to 1-ml aliquots of the water standards and the samples derivatized as described. Concentrations of PFBz-5-FU and PFBz-5-ClU were determined in a 20-μl aliquit of the sample by HPLC. Similarly, the plasma standards were extracted, derivatized, and analyzed by HPLC as described. Comparison of HPLC results obtained for the water and plasma standards gave plasma extraction efficiencies (mean ± S.D.) of 87 ± 2% for 5-FU and 85 ± 2% for 5-ClU.

The water and plasma standard extracts were then applied to individual C_{18} extraction columns and eluted with ethanol as previously described. Aliquots (20 μl) of each sample analyzed for PFBz-5-FU and PFBz-5-ClU by HPLC revealed an additional loss of 10% during this step, giving an overall analytical recovery (mean ± S.D.) of 76 ± 3% for 5-FU and 79 ± 1% for 5-ClU.

Gas chromatography–mass spectrometry

Quantitation of the derivatized bases was performed on a 5970B mass-selective detector equipped with a Series II 5890 gas chromatograph, a Model 7673 autosampler, and a Model 59970C ChemStation using 59974k (REV 3.2) GC–MS operating software (Hewlett-Packard, Palo Alto, CA, USA). Instrument operation was monitored with weekly Autotunes, and samples were analyzed with a multiplier voltage set at 200 V above the Autotune value.

Compounds were separated on an 18 m × 0.25 mm, 0.25 μm stationary phase thickness SPB-20 capillary column (Supelco, Bellefonte, PA, USA). Helium at a flow-rate of 20 cm/s was used as the carrier gas. Aliquots of the sample (2 μl) were introduced into the GC column by automatic injection via a split–splitless injector with the purge valve off. The purge valve was turned on 0.3 min after injection and the split vent flow-rate maintained at 30 ml/min throughout the analysis. The injection port temperature was at 294°C, and the transfer line was maintained at 290°C. The column temperature was initially set at 130°C. The oven temperature was programmed 1 min after injection as follows: 10°C/min to 190°C, 190°C for 1 min, 2°C/min to 230°C, 12°C/min to 290°C, and finally maintained at 290°C for 5 min.

Data were obtained from the GC–MS system by selected-ion monitoring of the pyrimidine base derivatives. At 16 min after start of analysis, ions at m/z 490–494 were monitored and at 24 min the instrument was switched to monitor the ions at m/z 506–509. Area ratios obtained for 5-FU (m/z 490) to [$^{15}N_2$]-5-FU (m/z 492) and 5-FU (m/z 490) to 5-ClU (m/z 506) were used for quantitation of 5-FU.

The total GC–MS analysis time for a single analysis in sequence mode including data reduction and turnover time was about 45 min. The GC–MS analysis was fully automated and left unattended during the runs.

Conversion of 5-FdUrd and 5-FUrd to 5-FU

Chemical conversion of 5-FdUrd or 5-FUrd to 5-FU during the GC–MS analysis was evaluated. Reference pyrimidine nucleosides, 5-FdUrd (200 ng) and 5-FUrd (200 ng) containing less than 0.5% contamination with 5-FU, were added to individual 1-ml aliquots of plasma and analyzed by our GC–MS method for 5-FU. The results of this analysis showed that 4.5 ± 0.2% (mean ±

S.D.) of the original FdUrd was converted and that no FUrd was converted to 5-FU during the analytical procedure.

Stability of 5-FU in plasma and whole blood

Whole blood and plasma standards were prepared containing 5-FU at a concentration of 50 ng/ml. The samples were placed on ice or left at room temperature for 0, 0.5, 2, or 4 h. At the specified times, 5-FU remaining in the samples was quantitated by GC–MS.

Patient 5-FU administration

After giving informed consent, patients entered on Protocol GWCC8191 at the George Washington University Cancer Center (Washington, DC, USA) received intravenous infusions of 5-FU by external pump. Blood samples were obtained at various times after start of infusion and 1-ml aliquots of plasma were analyzed for 5-FU by GC–MS.

RESULTS

Gas chromatography–mass spectrometry

EI mass spectra of PFBz-5-FU (Fig. 1A), PFBz-[$^{15}N_2$]-5-FU (Fig. 1B), and PFBz-5-ClU (Fig. 1C) each show prominent molecular ions at *m/z* 490, 492, and 506, respectively. Loss of a PFBz group from the molecular ion gave rise to the most intense ion in each spectrum at *m/z* 181. Fragment ions observed at *m/z* 309 (Fig. 1A), *m/z* 311 (Fig. 1B), and *m/z* 325 (Fig. 1C) suggested that some charge was retained in the pyrimidine base moiety after loss of a PFBz group. A *retro* Diels–Alder type reaction produced fragment ions at *m/z* 266 for PFBz-5-FU, at *m/z* 267 for PFBz-[$^{15}N_2$]-5-FU, and at *m/z* 282 for PFBz-5-ClU.

Selected-ion monitoring (SIM) was used for quantitation of the pyrimidine compounds. Fig. 2A shows a typical SIM tracing of a 10 ng/ml 5-FU plasma standard containing both internal standards, 5-[$^{15}N_2$]-FU and 5-ClU. Fig. 2B and C are SIM tracings obtained for a 0.5 ng/ml 5-FU plasma standard and control plasma, respectively. A signal-to-noise ratio of 8:1 was deter-

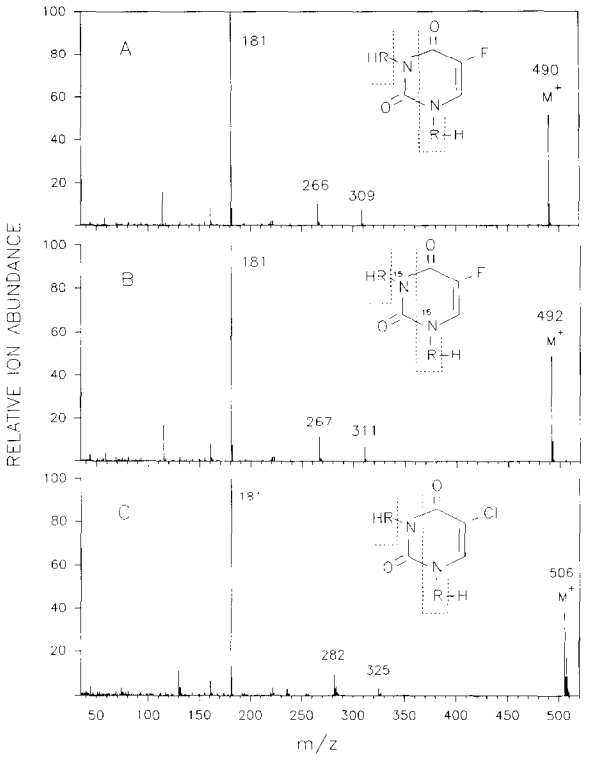

Fig. 1. Electron-impact mass spectra of the PFBz derivatives of reference: (A) 5-FU; (B) [$^{15}N_2$]-5-FU; and (C) 5-ClU.

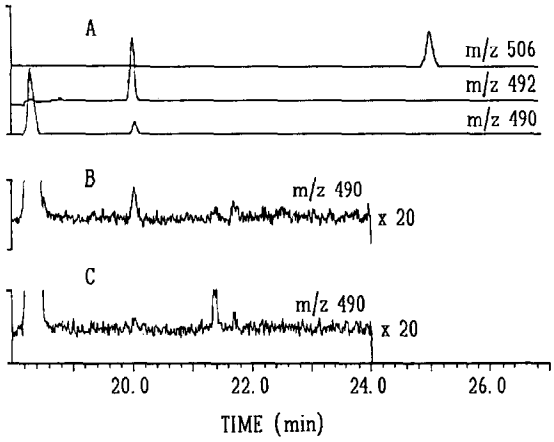

Fig. 2. Selected-ion monitoring tracings of plasma extracts obtained from the following plasma standards: (A) 10 ng/ml 5-FU, 50 ng/ml [$^{15}N_2$]-5-FU, and 50 ng/ml 5-ClU; (B) 0.5 ng/ml 5-FU, 50 ng/ml [$^{15}N_2$]-5-FU, and 50 ng/ml 5-ClU; (C) 50 ng/ml [$^{15}N_2$]-5-FU and 50 ng/ml 5-ClU. 5-ClU and [$^{15}N_2$]-5-FU tracings are not included in B or C.

mined by the HP 59970C ChemStation for the 0.5-ng 5-FU standard. No measurable signal was observed in control plasma for the m/z 490 ion at the retention time for 5-FU (Fig. 2C).

Stability of 5-FU in plasma and whole blood

Stability of 5-FU in while blood and plasma was determined in order to establish conditions for sample work-up and storage. 5-FU was stable in both blood and plasma when kept on ice for 4 h and was unstable in both blood and plasma left at room temperature (Fig. 3), similar to observations by Schaaf *et al.* [11] and Murphy *et al.* [12]. 5-FU levels in blood and plasma held at room temperature for 4 h decreased by 60 and 10%, respectively. Plasma standards kept at −20°C were stable for at least three months (data not shown). These findings indicate that blood samples should immediately be placed on ice and plasma separated from the whole blood to ensure accurate measurements of 5-FU in plasma samples.

Quantitation of 5-FU by GC–MS

Plasma standards prepared as described in the Experimental section were analyzed by GC–MS to assess the precision and accuracy of the method. FU plasma standards ranging from 0.0 to 50 ng/ml were analyzed in triplicate by GC–MS. Standard curves were generated and regression analysis [13] of the data indicated linearity over the range of the analysis for both internal standards. The regression equations determined for each internal standard were:

For $[^{15}N_2]$-5-FU: $y = 0.0212x + 0.0016$
($r^2 = 0.999$, S.E. of slope = ±0.0002) (1)

For 5-ClU: $y = 0.0306x - 0.004$
($r^2 = 0.999$, S.E. of slope = ±0.0002) (2)

The correlation coefficients and standard errors of the slopes determined for each internal standard were essentially the same. The overall precision of measurement as reflected by the mean coefficient of variation (C.V.) for all standards was 3.2 and 6.5%, respectively for the internal standards $[^{15}N_2]$-5-FU and 5-ClU, suggesting that either internal standard is suitable for the analysis.

Long-term variation in the analysis

Long-term variation in the analysis was eval-

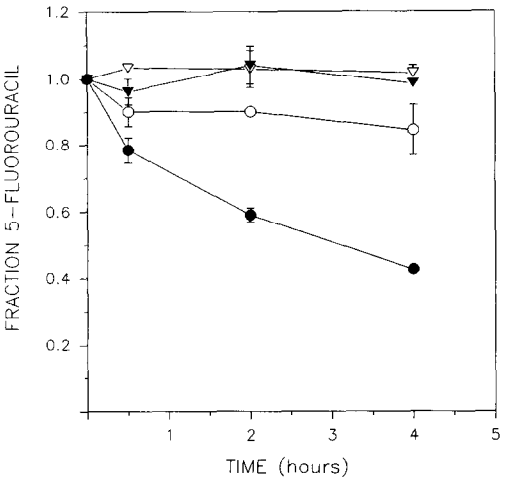

Fig. 3. Stability of 5-FU after incubation in (○) plasma at room temperature, (▽) plasma on ice, (●) whole blood at room temperature, and (▼) whole blood on ice.

TABLE I

LONG-TERM PRECISION OF THE GC–MS METHOD

Values in parentheses are coefficients of variation (%).

Standard (ng/ml)	$[^{15}N_2]$-5-FU[a] (mean ± S.D.) (ng/ml)	5-ClU[a] (mean ± S.D.) (ng/ml)
0.5	0.44 ± 0.11 (21)	0.42 ± 0.25 (59)
1	0.93 ± 0.12 (11)	1.03 ± 0.11 (10)
2	2.06 ± 0.19 (9.7)	1.90 ± 0.28 (14)
4	4.13 ± 0.31 (7.5)	3.94 ± 0.28 (7.0)
6	6.13 ± 0.46 (7.5)	6.00 ± 0.27 (4.6)
8	8.09 ± 0.64 (7.9)	7.89 ± 0.29 (3.7)
10	10.4 ± 0.61 (5.9)	9.99 ± 0.83 (8.4)
15	14.0 ± 0.80 (5.7)	14.1 ± 1.7 (12)
20	20.7 ± 1.8 (8.7)	22.1 ± 2.2 (10)
30	29.5 ± 1.9 (6.5)	30.2 ± 2.2 (7.4)
40	41.1 ± 3.3 (7.9)	40.5 ± 2.9 (7.1)
50	49.4 ± 5.7 (11)	50.5 ± 2.8 (5.5)

[a] Calculated from the respective calibration curves eqn. 1 and eqn. 2. Values represent the mean ± S.D. of 9 determinations.

uated over a three-month period to determine the between-day stability of the analysis including the slope and intercept. During this period, at least nine plasma standards at each concentration were analyzed by GC–MS. The expected 5-FU plasma concentrations were determined using the regression equations determined for the two internal standards, $^{15}N_2$-5-FU (eqn. 1) and 5-ClU (eqn. 2). Results of these multiple analyses were pooled and the expected values are presented in Table I. The only significant difference between the two sets of data was an approximately two-fold decrease in precision for multiple measurements of individual plasma standards over the three-month period.

Patient samples

Table II lists data obtained on three patients receiving 5-FU for their malignancies by protracted intravenous infusions. 5-FU was not detected in control plasma samples obtained before the start of infusion. Plasma levels of 5-FU ranging from 7 to 74 ng/ml were determined in plasma samples taken at various times during infusion of the drug.

DISCUSSION

Numerous analytical methods for quantitating 5-FU plasma levels have appeared in the literature including both HPLC and GC–MS techniques. Few HPLC methods [14,15] or GC–MS methods [7–10] have the required sensitivity to quantitate steady-state 5-FU plasma levels ranging from 4 to 28 ng/ml reported by Slavik *et al.* [5] in patients administered 5-FU at 200 mg/m^2 per day by portable pump. Only the method described by Kinberg *et al.* [15] included between-day variability of their analysis. The two GC–EI-MS methods [7,9] both analyzed 5-FU as its silyl derivative, however, we and others [16] have experienced carry-over of sample when using silyl derivatives for quantitation of 5-FU.

We have described a GC–EI-MS method and demonstrated the long-term stability of the analysis. Automatic injection and unattended analysis of 5-FU in human plasma samples for periods of up to three days have been accomplished with no deterioration of the quantitative results.

Although the primary goal of this study was to develop a method for quantitating low 5-FU plasma levels, addition of two internal standards at different concentrations could be used to expand the range of analysis and should be useful for obtaining pharmacokinetics data in patients following bolus administration of the drug where plasma concentrations can vary over 1000-fold [17].

We are presently using the GC–MS method to study the correlation of 5-FU plasma levels with response and toxicity in patients receiving protracted infusions of 5-FU for colorectal cancer or other malignancies.

TABLE II

5-FU PLASMA LEVELS IN PATIENTS RECEIVING CONTINUOUS PROLONGED INFUSIONS OF THE DRUG

Patient sample	Infusion rate (mg/m^2 per day)	Infusion time (days)	5-FU (ng/ml)
P1	0	0	<0.5
P1	250	23	7.0
P2	176	26	46
P3	0	0	<0.5
P3	300	4	74[a]

[a] 1:2 dilution used for analysis of the sample.

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