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Simultaneous determination of 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) uracil (FAU) and 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) 5-methyluracil (FMAU) in human plasma by liquid chromatography/tandem mass spectrometry

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ABSTRACT

A liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) assay was developed and validated for simultaneous determination of 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) uracil (FAU) and its active metabolite 1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) 5-methyluracil (FMAU) in human plasma. FAU and FMAU were extracted from plasma samples using solid-phase extraction with Waters Sep-Pak® Vac C_{18} cartridge. Chromatographic separation was achieved on a Waters Atlantis T3 C_{18} column with a gradient mobile phase consisting of methanol and water with 0.45% formic acid (v/v) running at a flow rate of 0.2 ml/min. The analytes were monitored by triple quadrupole mass spectrometer under positive ionization mode. The lower limit of quantitation (LLOQ) was 10 and 2 ng/ml for FAU and FMAU in plasma, respectively. Calibration curves were linear over FAU and FMAU plasma concentration range of 10–2000 and 2–1000 ng/ml, respectively. The intra-day and inter-day accuracy and precision were within the generally accepted criteria for bioanalytical method (<15%). The method has been successfully employed to characterize the plasma pharmacokinetics of FAU and FMAU in cancer patients receiving 1-h intravenous infusion of FAU 50 mg/m^2.

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1. Introduction

Thymidylate synthase (TS) catalyzes the conversion of deoxyuridylate (dUMP) and 5,10-methylenetetrahydrofolate to dTMP and 7,8-dihydrofolate. This reaction is the sole de novo biosynthesis of thymine in DNA and therefore, inhibition of TS blocks DNA synthesis and thereby causing cell death. Given its essential role in DNA synthesis, TS is an important target for chemotherapeutic drugs, such as 5-fluorouracil (5-FU) and capecitabine (orally bioavailable 5-FU prodrug). 5-FU is the mainstay of therapeutic regimens for the treatment of colorectal cancer and other human malignancies. However, tumors can develop resistance to TS inhibitors due to high expression/activity of TS in tumor cells [1,2]. There is an urgent need to develop new treatment strategies for treating tumors that are resistant to TS inhibitors. It has been proposed that, instead of inhibiting TS, the possibility exists of using the high catalytic activity of TS to activate deoxyuridine prodrugs to form toxic byproducts that could be incorporated into DNA thereby causing cell death [3]. For patients whose tumors express a high level of TS, there is no FDA-approved therapeutic drug currently available that targets this pathway. Hence, a therapy that is specifically targeted toward tumors that express high levels of TS could have widespread applicability.

FAU [1-(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl) uracil] is a pyrimidine nucleoside, which acts as a suicide prodrug, taking advantage of high TS activity as a mechanism of activation. FAU has been shown to be readily transported into several cell lines and converted by intracellular thymidine kinase (TK) to its monophosphate, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) uracil monophosphate (FAUMP), which is then methylated by TS in the 5-position to form the methylated product FMAUMP [1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) 5-methyluracil monophosphate] (Fig. 1) [4]. The methylated product FMAU can be incorporated into DNA and thus causing cell death [4]. It has been shown that greater DNA incorporation of FAU in cell lines with high TS activity resulted in increased cytotoxicity compared to cell lines with lower TS activity [3]. FAU is a novel investigational agent that has not been extensively studied in humans and never with therapeutic intent. The Karmanos Cancer Institute (KCI) Phase I service has recently received approval from the National Cancer Institute (NCI) Cancer Therapy Evaluation Program (CTEP) to conduct a Phase I clinical trial of FAU in patients with metastatic or unresectable solid tumors for which standard curative or palliative measures do not exist or are no longer effective. The primary objectives of

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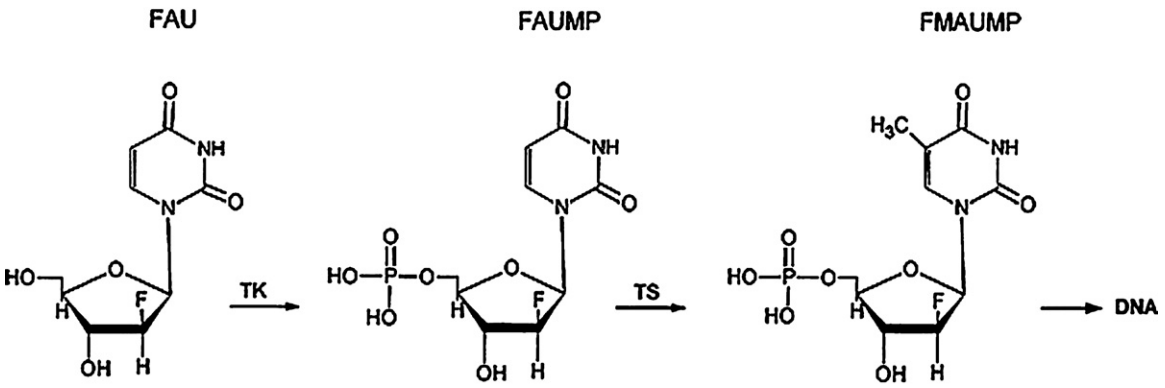

Fig. 1. Metabolic activation pathway of FAU [4]. FAU is first phosphorylated by intracellular thymidine kinase (TK) to form 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) uracil monophosphate (FAUMP), which is then methylated by thymidine synthase (TS) to form methylated product FMAUMP [1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl) 5-methyluracil monophosphate]. The methylated product FMAU can be incorporated into DNA and thus causing cell death.

this Phase I study were to determine the safety profile, dose limiting toxicity, and maximum tolerated dose (MTD) of FAU when it was administered as a 1-h infusion weekly on days 1, 8, 15, and 22 of a 28-day cycle. The secondary objectives include evaluation of plasma pharmacokinetics of FAU and its active metabolite FMAU in patients.

To characterize clinical pharmacokinetics of FAU and FMAU, a specific, sensitive, accurate, and reproducible method for quantitation of FAU and FMAU was critically needed. Here, we described, for the first time, a high-performance liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) method for simultaneous determination of FAU and FMAU in human plasma. Given the high water solubility of FAU, liquid–liquid extraction with organic solvents such as ethyl acetate resulted in low extraction recovery of FAU from plasma sample. Thus, a solid extraction method using Waters Sep-Pak® Vac C_{18} cartridge was optimized for simultaneous extraction of FAU and FMAU from plasma samples. The present method demonstrated sufficient sensitivity, with the lower limit of quantitation (LLOQ) of 10 ng/ml for FAU and 2 ng/ml for FMAU, for applications in clinical pharmacokinetic studies of FAU and FMAU.

2. Experimental

2.1. Chemicals and reagents

The reference standards of FAU and FMAU were provided by the National Cancer Institute Cancer Therapy Evaluation Program (NCI CTEP) (Bethesda, MD). The internal standard, zileuton [N-(1-benzobthien-2-ylethyl)-N-hydroxyurea] was obtained from Rhodia Pharma Solutions Ltd. (Northumberland, UK). All other chemicals and reagents were HPLC grade. Water was filtered and deionized with a US Filter PureLab Plus UV/UF System (Siemens, Detroit, MI, USA) and used throughout in all aqueous solutions. Drug-free (blank) human plasma from six different healthy donors was obtained from Innovative Research Inc. (Novi, MI, USA).

2.2. Stock solutions, calibration standards, and quality control samples

Stock solution of FAU or FMAU was prepared by dissolving an accurately weighed amount of the compound in methanol to obtain a final concentration of 1 mg/ml, and stored in brown glass vials at −20 °C. Working solutions were prepared freshly on each day of analysis as serial dilutions in methanol. The calibration curves were constructed by simultaneously spiking FAU and FMAU in blank human plasma at FAU concentrations of 10, 20, 50, 100, 200, 500, 1000 and 2000 ng/ml and at FMAU concentrations of 2, 10, 20, 50, 100, 200, 500 and 1000 ng/ml. The QC samples were prepared in blank plasma at FAU concentrations of 10 (LLOQ), 30, 800, and 1600 ng/ml and at FMAU concentrations of 2 (LLOQ), 6, 400 and 800 ng/ml. All calibration standards and QC samples were prepared fresh daily. For long-term and freeze–thaw stability, QC samples were prepared as a batch and stored at −80 °C.

2.3. Sample preparation

Sample preparation involved a solid phase extraction by using Waters Sep-Pak® Vac 1 cm^3 (100 mg) C_{18} cartridge (Waters, Milford, MA). The cartridge was conditioned with 1 ml of methanol followed by equilibration with 1 ml of distilled water. Frozen plasma samples were thawed at ambient temperature, and 250-μl aliquot of sample containing FAU and FMAU was loaded onto the Waters Sep-Pak® Vac C_{18} cartridge. Then, the cartridge was eluted with 1.0 ml of methanol followed by elution with 0.5 ml of methanol. A total of 1.5-ml of effluent was collected and evaporated to dryness under a stream of nitrogen in a water bath at 50 ± 2 °C. The residue was reconstituted in 100 μl of mobile phase containing the internal standard (zileuton) at the concentration of 100 ng/ml, vortex-mixed for 30 s, sonicated for 30 s, and centrifuged at 14,000 rpm for 5 min at 4 °C. The supernatant was transferred to an autosampler vial, and 10 μl was injected into the HPLC instrument using a temperature-controlled autosampling device (set at 4 °C).

2.4. Chromatographic and mass-spectrometric conditions

Chromatographic analysis was performed using a Waters Model 2695 HPLC system (Milford, MA, USA). Separation was achieved at 30 °C using a Waters Atlantis T3 C_{18} column (3.0 μm, 100 mm × 2.1 mm i.d.) with a Waters guard column (3.5 μm, 10 mm × 2.1 mm i.d.). The mobile phase, consisting of solvent A (HPLC grade water with 0.45% formic acid, v/v) and solvent B (methanol), was pumped at the flow rate of 0.2 ml/min using the following gradient elution: 0–1 min, 10% B; 1–2 min, the percentage of B increasing from 10% to 100%; 2–8 min, 100% B; 8–8.1 min, the percentage of B decreasing from 100% to 10%; 8.1–15 min, 10% B. The column effluent was monitored using a Waters Quattro Micro™ triple quadrupole mass (Milford, MA, USA). The instrument was equipped with an electrospray ionization source, and controlled by the Masslynx 4.1 software. The analytes were detected in multiple reaction monitoring (MRM) mode using the positive ionization mode operating at a cone voltage of 18 V for FAU, 20 V for FMAU, and 24 V for internal standard, zileuton. Samples were introduced into the ionization source through a heated

nebulized probe (350 °C). The spectrometer was programmed to allow the $[MH]^+$ ions of FAU at *m*/*z* 247.26, FMAU at *m*/*z* 261.18, and zileuton at *m*/*z* 237.13 to pass through the first quadrupole (Q1) and into the collision cell (Q2). The collision energy was set at 12, 12, and 9 eV for FAU, FMAU and zileuton, respectively. The product ions for FAU at *m*/*z* 112.64, FMAU at *m*/*z* 126.70, and zileuton at *m*/*z* 160.79 were monitored through the third quadrupole (Q3). Argon was used as collision gas at a pressure of 0.00172 mBar, and the dwell time per channel was 0.5 s for data collection.

2.5. Method validation

2.5.1. Specificity and selectivity

The presence of endogenous interfering peaks was inspected by comparing the chromatograms of the extracted human plasma samples from 6 different donors and those spiked with FAU and FMAU at the LLOQ (10 ng/ml for FAU and 2 ng/ml for FMAU). The interfering peak area should be less than 10% of the peak area for the analyte at the LLOQ. In addition, potential interference peaks in patient plasma were inspected by analyzing the pre-treatment plasma sample from each patient.

2.5.2. Calibration curve, accuracy, and precision

Linearity was assessed at FAU concentrations ranging from 10 to 2000 ng/ml and FMAU concentrations ranging from 2 to 1000 ng/ml in plasma. Calibration curves were built by fitting the analyte concentrations of the calibrators versus the peak area ratios of the analyte to internal standard using linear regression analysis with a weighting scheme of $1/X^2$.

The intra-day and inter-day accuracy and precision were assessed for the calibrator standards (in duplicate) and QCs (including LLOQ, low, medium, and high QCs, each in quintuplicate) on four days. The accuracy was assessed as the percentage of the determined concentration relative to nominal concentration. The intra- and inter-day precisions were estimated by one-way analysis of variance (ANOVA) using the JMP™ statistical discovery software version 5 (SAS Institute, Cary, NC). The intra-day variance (VAR_{intra}), the inter-day variance (VAR_{inter}), and the grand mean (GM) of the observed concentrations across runs were calculated from ANOVA analysis. The intra-day precision (P_{intra}) was calculated as: $P_{intra} = 100 \times (\sqrt{(VAR)_{intra}}/GM)$. The inter-day precision (P_{inter}) was defined as: $P_{inter} = 100 \times (\sqrt{((VAR_{inter} - VAR_{intra})/n)}/GM)$, where n represents the number of replicate observations within each day.

2.5.3. Matrix effect and extraction recovery

Matrix effect and extraction recovery were assessed in human plasma from 6 different donors, as described previously [5]. Briefly, three sets of QC samples were prepared. Set 1 QCs were prepared by spiking FAU and FMAU (at the low, medium, and high QC concentrations) in human plasma prior to extraction. After extraction, the analytes were reconstituted in a 100-μl aliquot of the mobile phase for injection. Set 2 QCs were prepared by spiking the same amount of FAU and FMAU as Set 1 in a 100-μl aliquot of blank matrix extracts (i.e., post-extraction reconstitution solution of blank plasma). Set 3 QCs were prepared by spiking the same amount of FAU and FMAU as Set 1 in a 100-μl aliquot of the mobile phase to evaluate the detector response. The matrix effect is expressed as the ratio of the mean peak area of an analyte spiked post-extraction (set 2) to that from neat solution (set 3). The extraction recovery is calculated as the ratio of the mean peak area of an analyte spiked prior to extraction (set 1) to that from post-extraction solution (set 2).

2.5.4. Stability

The short-term (bench-top) stability of the FAU and FMAU in methanol (working solution) at the concentration of 1 and 100 μg/ml as well as in plasma at the concentration of 30 and 1600 ng/ml for FAU and 6 and 800 ng/ml for FMAU were tested at ambient temperature (25 °C) for 6 h. The autosampler stability of FAU and FMAU in the reconstitution solution (methanol/0.45% formic acid in water, 60:40, v/v) was examined at 4 °C for 12 h after the low and high QC plasma samples (30 and 1600 ng/ml for FAU and 6 and 800 ng/ml for FMAU) were processed. The freeze–thaw stability of the FAU and FMAU in plasma at the low and high QC concentrations (30 and 1600 ng/ml for FAU and 6 and 800 ng/ml for FMAU) was assessed through three freeze–thaw cycles. The long-term stability of FAU and FMAU in stock solution (1 mg/ml) and in plasma (30 and 1600 ng/ml for FAU and 6 and 800 ng/ml for FMAU) was investigated up to 2 months so far. All QCs were run in triplicate.

2.6. Application of the method to clinical pharmacokinetic study

FAU is currently being evaluated in a Phase I clinical trial (NCI study #7916) at the Karmanos Cancer Institute in patients with advanced solid tumors. The protocol was approved by the Institutional Review Board of the Karmanos Cancer Institute at Wayne State University (Detroit, MI). All the patients provided written informed consent. FAU was administered as 1 h-intravenous infusion weekly on days 1, 8, 15, and 22 of a 28-day cycle, with the lowest starting dose of 50 mg/m^2, and five dose levels (50, 100, 200, 400, and 600 mg/m^2) have been predefined for this study at present. To date, three patients have been treated with FAU at the doses of 50 mg/m^2. The plasma pharmacokinetics of FAU and FMAU were evaluated in the treated patients on days 1 and 22 in cycle 1. Four ml of blood samples were collected on day 1 at pretreatment, at the end of infusion, and following the end of infusion at 15 min, 30 min, 1, 2, 4, 8, and 24 h. In addition, repeat intensive sampling occurred on day 22 at pretreatment, at the end of infusion, and following the end of infusion at 15 min, 30 min, 1, 2, 4, 8, and 24 h.

The blood samples were centrifuged at 4 °C, at 3000 rpm for 10 min, and plasma samples were collected and stored at −80 °C until analysis. The concentrations of FAU and FMAU in patient plasma samples were determined using the described validated method. The pharmacokinetic parameters for FAU and FMAU were estimated using noncompartmental analysis with the computer software program WinNonlin 5.2 (Pharsight Corporation, Mountain View, CA).

3. Results and discussion

3.1. Mass spectrometry and chromatography

In the positive ion mode, FAU and FMAU showed protonated molecule ions (MH^+) at *m*/*z* 247.26 and 261.18, respectively. The major fragments observed were at *m*/*z* 112.64 and 126.70 and were selected for subsequent monitoring in the third quadrupole for FAU and FMAU, respectively (Fig. 2a and b). The internal standard, zileuton, was monitored at the transition of *m*/*z* 237.10 > 160.80. The fragmentation pathways for FAU, FMAU, and zileuton are depicted in Fig. 2.

Fig. 3 shows the representative chromatograms of blank human plasma and plasma samples spiked with 10 ng/ml of FAU and 2 ng/ml of FMAU (LLOQ) as well as a patient plasma sample collected at the end of 1-h infusion of FAU at the dose of 50 mg/m^2. The retention time (expressed as mean ± standard deviation from 15 analytical runs) for FAU, FMAU, and zileuton was 3.18 ± 0.12,

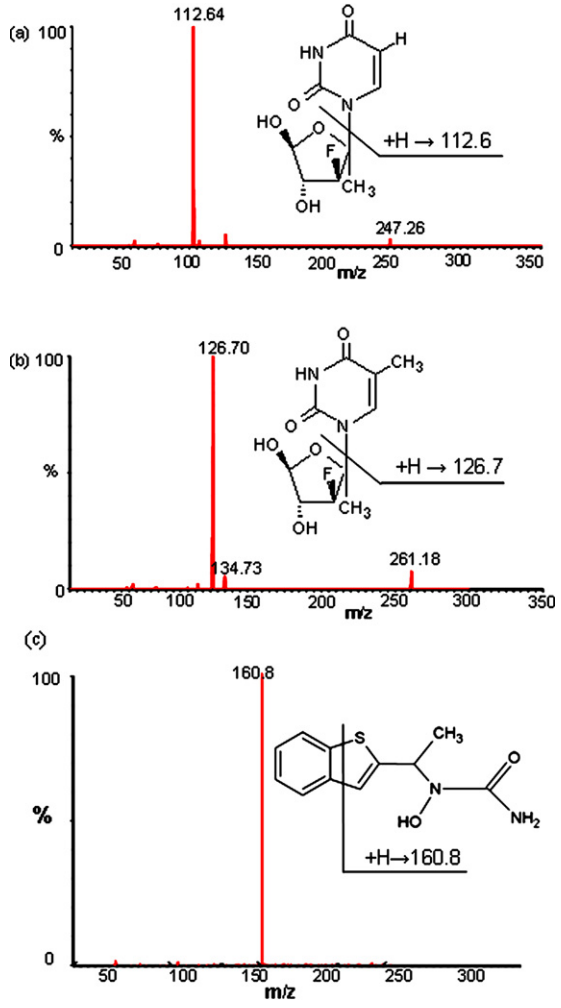

Fig. 2. Product mass spectrum of FAU at m/z 247.26 → 112.64 (a), FMAU at m/z 261.18 → 126.70 (b), and zileuton at m/z 237.13 → 160.79 (c).

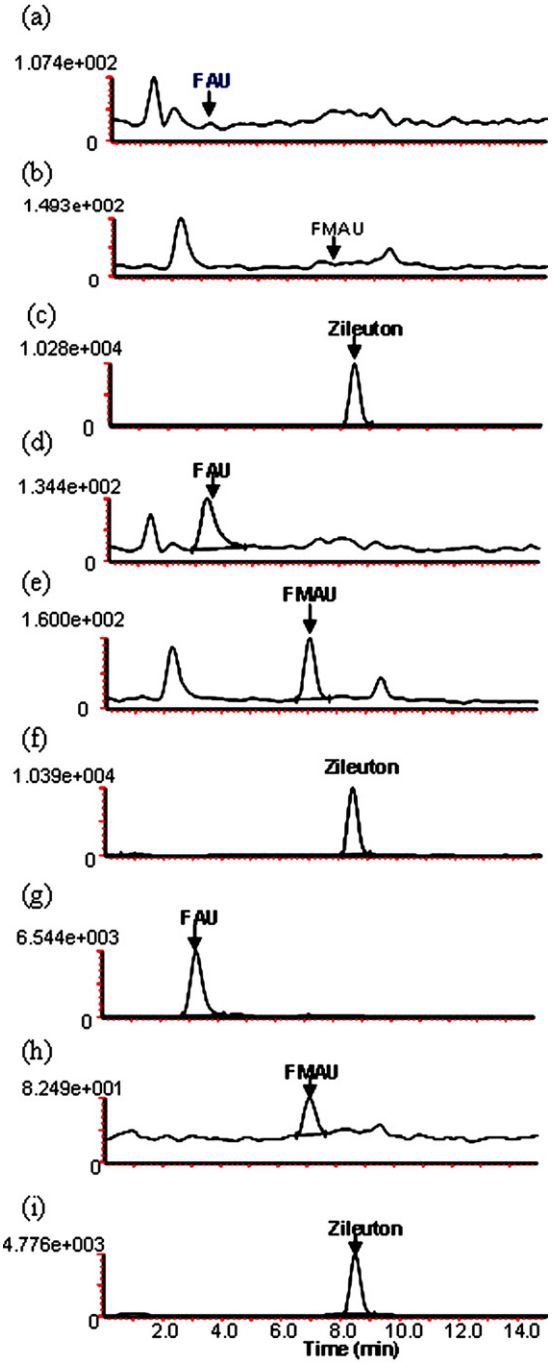

Fig. 3. Chromatograms of blank plasma (a–c), plasma spiked with FAU (10 ng/ml) and FMAU (2 ng/ml) at LLOQ (d–f), and a patient plasma sample collected at the end of 1-h intravenous infusion of FAU at the dose of 50 mg/m^2 (g–i), monitored at m/z 247.26 → 112.64 for FAU, m/z 261.18 → 126.70 for FMAU, and m/z 237.13 → 160.79 for internal standard, zileuton. The retention times for FAU, FMAU, and zileuton were 3.18 ± 0.12, 7.05 ± 0.06, and 8.48 ± 0.02 min, respectively.

7.05 ± 0.06, and 8.48 ± 0.02 min, respectively, with an overall chromatographic run time of 15 min.

Ideally, an isotope-labeled form of the analyte is used as the internal standard. However, an isotope-labeled internal standard is not always available because synthesizing isotope-labeled chemicals can be expensive and time-consuming. Since isotope-labeled FAU and FMAU were not available, zileuton was chosen as the internal standard in the present study for three reasons: (1) this compound is stable in aqueous and organic solvents, (2) it exhibits reproducible mass spectrometric response under electrospray ionization in the positive-ion mode, and (3) it has been successfully used as the internal standard for other studies in our lab [5,6]. One limitation of using zileuton as the internal standard in the present study was that hydrophobic zileuton could not be well extracted simultaneously with less hydrophobic FAU and FMAU by the Waters Sep-Pak® Vac C_{18} cartridge (preliminary data not shown here). To overcome this limitation, zileuton was spiked in the reconstitution solution post the solid-phase extraction while not being spiked in the plasma sample prior to the extraction. Although post-extraction addition of the internal standard could not compensate for potential variations occurring in the extraction process, the present method has demonstrated acceptable intra-day and inter-day accuracy and precision (Table 2).

3.2. Specificity and sensitivity

Blank plasma samples from 6 different donors as well as pre-treatment plasma samples from the patients showed no interference for the analytes and internal standard. The LLOQ for FAU and FMAU were established at 10 and 2 ng/ml in plasma samples, respectively, at which the mean signal-to-noise ratios were 20.9 ± 14.6 (n = 15) and 17.0 ± 6.7 (n = 15), respectively. The intra- and inter-day accuracy and precision of FAU and FMAU at the

Table 1
Accuracy, intra- and inter-day precisions of calibrator standards[a] in the calibration curves of FAU and FMAU.

Nominal concentration (ng/ml)	Determined concentration (ng/ml) [c]	Average accuracy (%)	Intra-day precision (%)	Inter-day precision (%)
FAU				
10 (LLOQ)	10.2 ± 0.3	101.5	3.7	–[b]
20	19.9 ± 1.4	99.6	6.8	2.9
50	46.2 ± 2.9	92.5	6.5	–[b]
100	102.9 ± 11.8	102.9	14.8	–[b]
200	201.7 ± 18.3	100.9	4.8	8.3
500	497.8 ± 48.5	99.6	11.5	–[b]
1000	1035.2 ± 58.9	103.5	7.4	–[b]
2000	2008 ± 188.7	100.4	9.3	1.7
FMAU				
2 (LLOQ)	2.0 ± 0.1	102.3	5.6	–[b]
10	9.5 ± 0.6	95.2	7.0	–[b]
20	20.2 ± 0.8	100.8	3.1	2.3
50	48.6 ± 2.8	97.2	3.6	4.9
100	103.2 ± 4.4	103.2	2.6	3.7
200	211.5 ± 16.8	105.8	5.3	6.4
500	518.6 ± 33.9	103.7	3.9	5.7
1000	1053.6 ± 29.8	105.4	3.2	–[b]

[a] Each calibrator was evaluated in duplicate on four days.
[b] No additional variation was observed as a result of performing assay in different days.
[c] Expressed as mean ± standard deviation from 8 replicates.

Table 2
Accuracy, intra- and inter-day precision for the QC samples[a] of FAU and FMAU.

Nominal concentration (ng/ml)	Determined concentration (ng/ml)[c]	Average accuracy (%)	Intra-day (%)	Inter-day (%)
FAU				
10 (LLOQ)	9.9 ± 0.8	98.8	7.3	2.6
30	27.7 ± 1.9	92.3	6.8	1.9
800	785.2 ± 69.5	98.1	6.2	6.8
1600	1633.9 ± 133.9	102.1	7.8	2.7
FMAU				
2 (LLOQ)	2.1 ± 0.2	103.5	11.2	–[b]
6	5.6 ± 0.4	92.7	6.3	2.4
400	399.3 ± 38.9	99.8	5.4	8.8
800	833.0 ± 59.0	104.1	6.2	3.7

[a] Each QC was performed in quintuplicate on four days.
[b] No additional variation was observed as a result of performing assay in different days.
[c] Expressed as mean ± standard deviation from 20 replicates.

Table 3
Matrix effect and recovery of FAU and FMAU from 6 different sources of human plasma.

Analyte	Nominal concentration (ng/ml)[a]	Mean peak area			Matrix effect (%)[e]	Recovery (%)[f]
		Set 1[b]	Set 2[c]	Set 3[d]		
FAU	30	102.9	164.7	925.4	17.8 (3.5%)	62.7 (12.3%)
	800	3563.0	5991.2	29,761.0	20.1 (7.7%)	59.5 (8.9%)
	1600	7379.5	10,697.4	63,953.3	16.7 (5.3%)	69.0 (9.2%)
FMAU	6	102.7	131.8	234.3	56.3 (5.7%)	77.9 (12.1%)
	400	8752.0	12,535.2	19,426.6	64.6 (7.1%)	70.0 (8.0%)
	800	18,818.8	23,761.4	36,648.5	64.9 (7.3%)	79.3 (4.6%)

[a] The nominal concentrations of the analyte spiked in plasma before extraction (set 1). The same amount of the analyte as in set 1 was spiked in the plasma extract and mobile phase for set 2 and set 3.
[b] The mean peak area of an analyte that was spiked before extraction in plasma from 6 different sources (donors), each source of plasma in triplicate measurements.
[c] The mean peak area of an analyte that was spiked postextraction in plasma extracts from 6 different sources of human plasma, each source of plasma in triplicate measurements.
[d] The mean peak area of an analyte that was spiked in the mobile phase from triplicate measurements.
[e] Matrix effect is expressed as the ratio of the mean peak area of an analyte spiked postextraction (set 2) to the mean peak area of the same amount of analyte spiked in the mobile phase (set 3). Data are shown as the mean (%CV) from six different sources of plasma.
[f] Recovery is calculated as the ratio of the mean peak area of an analyte spiked before extraction (set 1) to the mean peak area of the same amount of the analyte spiked postextraction (set 2). Data are shown as the mean (%CV) from six different source of plasma.

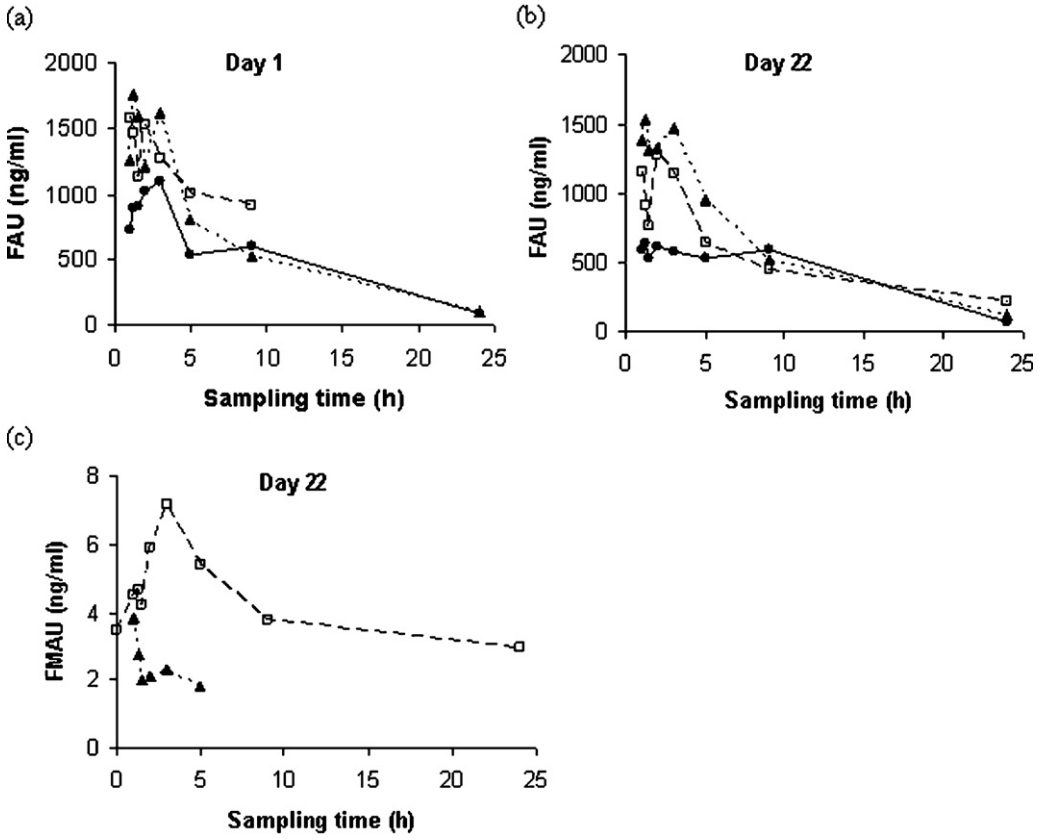

Fig. 4. (a and b) Plasma concentration–time profiles of FAU on days 1 and 22 in patients #001 (shown as ●), #002 (□), and #003 (▲) who received 1-h infusion of FAU 50 mg/m^2. FAU plasma concentration was below the LLOQ (10 ng/ml) at 24 h on day 1 in patient #002 (□). (c) Plasma concentration time profile of FMAU in patients #002 (□) and #003 (▲) on day 22. FMAU plasma concentrations were below the LLOQ (2 ng/ml) on day 1 at all sampling time points except for at the end of infusion in three patients, and on day 22 at all sampling time points except for at the end of infusion in patient #001, and on day 22 at 8- and 24-h sampling time points in patient #003.

LLOQ were within the generally accepted criteria for bioanalytical method (Table 2).

3.3. Linearity, accuracy, and precision

The linear calibration curves were established over the concentration range of 10–2000 ng/ml for FAU and 2–1000 ng/ml for FMAU. A linear correlation coefficient (R^2) of >0.99 was obtained in all analytical runs.

For all calibrator standards (including LLOQ) of FAU and FMAU in plasma, the average accuracy in terms of percent recovery of the back-calculated relative to nominal concentration ranged from 92.5% to 103.5% (n = 8) for FAU and 95.2% to 105.8% (n = 8) for FMAU; the intra- and inter-day precisions were less than 14.8% and 8.3% for FAU and FMAU, respectively (Table 1).

The intra- and inter-day accuracy and precision were assessed for FAU and FMAU at the LLOQ and at the low, medium, and high QC concentrations in plasma samples over 4 days. The average accuracy, expressed as the percent recovery of the back-calculated relative to nominal concentration, ranged from 92.3% to 102.1% for FAU and from 92.7% to 104.1% for FMAU (Table 2). The intra- and inter-day precisions were within 7.8% and 11.2% for FAU and FMAU, respectively (Table 2).

3.4. Matrix effect and extraction recovery

The matrix effect was examined in 6 different sources of human plasma to assess the possibility of ionization suppression or enhancement for FAU and FMAU. The average plasma matrix effects from 6 different sources of plasma were determined ranging from 16.7% to 20.1% for FAU (at 30, 800 and 1600 ng/ml) and from 56.3% to 64.9% for FMAU (at 6, 400 and 800 ng/ml), respectively (Table 3). The variability in plasma matrix effect, as measured by the coefficient of variation from 6 different sources of plasma, was <7.7% for FAU and <7.3% for FMAU (Table 3). These results indicated a consistent matrix effect from different sources of human plasma, despite the fact that ionization suppression was observed from the plasma matrix for both FAU and FMAU.

The average recovery from 6 different sources of human plasma ranged from 59.5% to 69.0% for FAU (assessed at the concentration of 30, 800, and 1600 ng/ml) and from 70.0% to 77.9% for FMAU (assessed at the concentration of 6, 400, and 800 ng/ml) (Table 3). The variability in recovery, as measured by the coefficient of variation from 6 different sources of plasma was <12.3 for FAU and <12.1% for FMAU (Table 3). It should be noted that the recovery of an analyte need not be 100%, but the extent of recovery of an analyte should be consistent, precise, and reproducible (FDA Guidance for Bioanalytical Method Validation: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf). Our data demonstrated that the recovery of FAU or FMAU from human plasma was consistent and reproducible.

3.5. Stability

The short- and long-term stabilities of FAU and FMAU were demonstrated in Table 4. The bench-top stability test suggested that FAU and FMAU were stable in both methanol (at 1 and 100 μg/ml) and in human plasma (at low and high QC concentrations) at ambient temperature (~25 °C) for at least 6 h (Table 4). The autosampler

Table 4
Assessment of stability of FAU and FMAU.[a]

	FAU concentrations (ng/ml)[b]		FMAU concentrations (ng/ml)[b]	
	30	1600	6	800
Bench-top stability (in plasma) (25 °C)				
1.0 h	95.7	98.0	93.6	92.3
2.0 h	93.5	89.0	92.7	87.9
3.0 h	89.0	88.9	90.2	86.9
4.0 h	90.7	89.8	89.2	85.3
6.0 h	91.8	88.3	88.3	86.7
Freeze–thaw stability (in plasma) (−80 °C)				
Cycle 1	96.0	89.3	106.9	108.9
Cycle 2	88.7	97.9	98.5	96.0
Cycle 3	87.0	96.4	98.9	106.5
Long-term stability (in plasma) (−80 °C)				
2 month	95.7	95.7	96.3	102.6

[a] Stability data were expressed as mean percentage of the analyte concentration determined at predefined time point relative to that at time zero.
[b] Each concentration at each time point was assessed in triplicate.

stability test suggested that FAU and FMAU was stable in the reconstitution solution (methanol/0.45% formic acid in water, 60:40, v/v) at 4 °C for at least 12 h, allowing the assay to be performed continuously overnight for a large number of samples. The freeze–thaw stability test suggested that FAU (at 30 and 1600 ng/ml) and FMAU (at 6 and 800 ng/ml) in human plasma showed less than 13% degradation through three full cycles of freeze–thaws (Table 4). The long-term stability tests suggested that the stock solution of FAU and FMAU in methanol at 1 mg/ml was stable at −20 °C for at least 6 months, and FAU (at 30 and 1600 ng/ml) and FMAU (at 6 and 800 ng/ml) were stable in human plasma at −80 °C for at least 2 months (Table 4). The long-term stability for FAU and FMAU in methanol and in human plasma is continuously monitored.

3.6. Clinical application

The present LC–MS/MS method has been applied to study the plasma pharmacokinetics of FAU and FMAU in three cancer patients receiving 1-h intravenous infusion of FAU at the dose of 50 mg/m^2. Fig. 4 shows the plasma concentration–time profiles of FAU and FMAU on days 1 and 22 following weekly 1-h intravenous infusion of FAU 50 mg/m^2 in 3 individual patients. The maximum plasma concentrations (C_{max}) of FAU and FMAU were achieved at the end of 1-h infusion. In the three patients, the C_{max} (expressed as mean ± standard deviation) of FAU were 1479 ± 345 and 1152 ± 458 ng/ml on days 1 and 22, respectively. The plasma concentrations of FMAU were only measurable at the end of infusion on day 1, with the mean C_{max} of 2.1 ± 0.2 ng/ml. Interestingly, the plasma level of FMAU was significantly increased on day 22, with the mean C_{max} of 4.1 ± 2.9 ng/ml, suggesting FMAU (the active metabolite of FAU) was accumulated in systemic circulation following weekly 1-h infusion of FAU. This could be due to the slow blood clearance of FMAU and/or gradual release of FMAU from tissues to the circulation. The exact underlying mechanism is yet to be determined.

4. Conclusion

A sensitive and reliable LC–MS/MS method has been developed and validated for simultaneous determination of FAU and FMAU in human plasma. The LLOQ was established at 10 ng/ml for FAU and 2 ng/ml for FMAU in human plasma. Linear calibration curves were established over the concentration range of 10–2000 ng/ml in plasma for FAU and 2–1000 ng/ml for FMAU. The method has been successfully applied to the study of the plasma pharmacokinetics of FAU and FMAU in cancer patients receiving 1-h intravenous infusion of FAU at the starting dose of 50 mg/m^2 in an ongoing Phase I trial.

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