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Development and utilization of a combined LC–UV and LC–MS/MS method for the simultaneous analysis of tegafur and 5-fluorouracil in human plasma to support a phase I clinical study of oral UFT®/leucovorin

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ABSTRACT

Tegafur is a 5-fluorouracil (5-FU) prodrug widely used outside the United States to treat colorectal cancer as well as cancers of the head and neck. The resulting plasma concentrations of tegafur are much higher than those of 5-FU; thus, analytical methods are needed that are sensitive enough to detect low plasma concentrations of 5-FU and robust enough to simultaneously analyze tegafur. Previous LC–MS/MS methods have either failed to demonstrate the ability to simultaneously measure low 5-FU and high tegafur plasma levels, or failed to be applicable in clinical studies. Our goal was to develop a method capable of measuring low concentrations of 5-FU (8–200 ng/ml) and high concentrations of tegafur (800–20,000 ng/ml) in human plasma and to subsequently evaluate the utility of the method in patient samples collected during a phase I clinical study where oral doses of either 200 mg or 300 mg UFT®/LV (uracil and tegafur in a 4:1 molar ratio plus leucovorin) were administered. A combined LC–MS/MS and LC–UV method was developed utilizing negative ion atmospheric pressure ionization (API). The method provides an accuracy and precision of <10% and <6%, respectively, for both analytes. Material recoveries from the liquid–liquid extraction technique were 97–110% and 86–91% for tegafur and 5-FU, respectively. Utilization of this method to determine tegafur and 5-FU plasma concentrations followed by noncompartmental pharmacokinetic analyses successfully estimated pharmacokinetic parameters (C_{MAX}, t_{MAX} and $AUC_{0-10\,h}$) in the clinical study patients. Overall, this method is ideal for the simultaneous bioanalysis of low levels of 5-FU and relatively higher levels of its prodrug, tegafur, in human plasma for clinical pharmacokinetic analysis.

1. Introduction

5-Fluorouracil (5-FU) is a commonly prescribed antineoplastic drug often used alone or in combination with other chemotherapeutic agents to treat esophageal [1,2], gastric [3], and colorectal cancers [4]. 5-FU is thought to produce its anticancer effects by inhibiting thymidylate synthase through incorporation of anabolic, cytotoxic nucleotide metabolites of 5-FU thereby blocking DNA replication [5,6]. The majority of 5-FU, when delivered orally, is rapidly catabolized in the liver by dihydropyrimidine dehydrogenase (DPD) [7], which poses a pharmacokinetic hurdle in efficient and convenient systemic delivery of 5-FU. In order to increase efficacy and 5-FU exposure while potentially minimizing related toxicities, alternative formulations of 5-FU have been developed. One particular oral formulation administers 5-FU along with the irreversible DPD inhibitor eniluracil [8] in order to decrease 5-FU metabolism and clearance. This results in increased 5-FU bioavailability and decreased formation of the DPD-mediated 5-FU inactive metabolite α-fluoro-β-alanine (FBAL), which has been shown to be potentially neurotoxic but possess no significant anticancer properties [9].

In an alternative approach to enhance 5-FU bioavailability, the oral 5-FU prodrug tegafur (1-[2-tetrahydrofuranyl]-5FU, ftorafur; FT) is combined with the endogenous and reversible DPD competitive inhibitor uracil in a 4:1, uracil:tegafur molar ratio (UFT®) [10]. Fig. 1 depicts the chemical structures of uracil, 5-FU, and

Abbreviations: 5-FU, 5-fluorouracil; UFT®, uracil and tegafur; LV, leucovorin (folinic acid); PK, pharmacokinetic; AUC, area under the curve; RT, radiation therapy; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; UV, ultraviolet spectroscopy.

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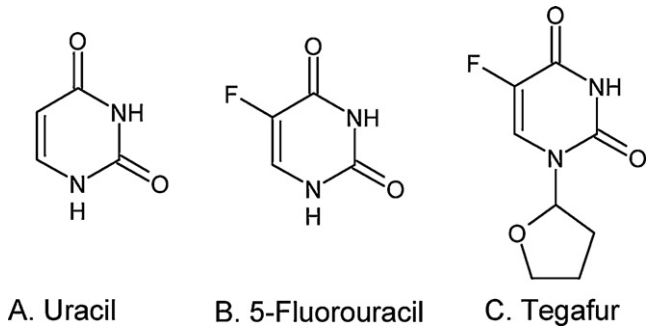

Fig. 1. Structures of (A) uracil, (B) 5-fluorouracil, and (C) tegafur.

tegafur. Tegafur undergoes cleavage of the tetrahydrofuran moiety, catalyzed mainly by cytochrome P450 isoform 2A6, as well as thymidine phosphorylase in the liver [10,11], into 5-FU. UFT® is often formulated with leucovorin (UFT®/LV), which augments 5-FU cytotoxicity. Daily oral administration of UFT®/LV results in a greater 5-FU maximum plasma concentration (C_{MAX}), average steady-state plasma concentration ($C_{SS,AVG}$), and prolonged 5-FU half-life compared to continuous intravenous infusion 5-FU [12,13]. However, exposure to 5-FU as well as efficacy is comparable between oral UFT®/LV and intravenous 5-FU and LV [12,13]. Another oral combination involving tegafur is S-1 (TS-1, Taiho Pharmaceutical), which is a combination of tegafur, gimeracil (an inhibitor of DPD), and oteracil (which inhibits the phosphorylation of fluorouracil in the gastrointestinal tract, thereby reducing the gastrointestinal toxic effects of 5-FU) in a molar ratio of 1:0.4:1 [14].

As of late 2011, only a few groups [15–18] have simultaneously measured both 5-FU and tegafur in human (and in one case dog) plasma. In 2003, Zufia et al. [15] simultaneously quantified 5-FU and tegafur using a gradient elution HPLC/UV method. Chu et al. [16] used a similar HPLC/UV method but with isocratic elution to measure both drugs in beagle dog plasma. Remaud et al. [17] in 2005 simultaneously quantified tegafur, 5-FU, and 5-fluoro-5,6-dihydrouracil (5-FUH_2) in human plasma. In order to quantify tegafur resulting from oral UFT® or S1 administration, the authors constructed two separate calibration curves for tegafur, the first in a range of 25–2500 ng/ml, the second in a higher range of 2500–25,000 ng/ml. Liu et al. [18] validated their LC–MS/MS method for simultaneous detection of low concentration 5-FU (2–500 ng/ml) and high concentration tegafur (12–3000 ng/ml) for use in oral administration of S1.

Presented here is a sensitive, selective, and robust combined LC–MS/MS and LC–UV method for the simultaneous detection of low plasma concentrations of 5-FU (8–200 ng/ml) and high plasma concentrations of tegafur (800–20,000 ng/ml) typically found after oral administration of UFT®/LV. This validated method was also subsequently applied for the first time to human plasma samples collected as part of a phase I clinical trial of UFT®/LV for patients with head and neck cancers [19]. Noncompartmental pharmacokinetic analysis was performed to further demonstrate the successful applicability of this novel bioanalytical method.

2. Experimental

2.1. Materials

Tegafur (1-[2-tetrahydrofuranyl]-5-FU) and 5-flurouracil were purchased from Sigma–Aldrich (St. Louis, MO, USA). The isotopically labeled 5-fluorouracil (iso-5-FU), the internal standard, was purchased from Medical Isotopes, Inc. (Pelham, NH, USA). Ethyl acetate, methanol, ammonium acetate were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Plasma extraction procedure

Tegafur and 5-FU were extracted from 200 μl of plasma using a liquid–liquid extraction procedure. Initially, 100 μl of internal standard spiking solution (1.00 μg/ml of 5-fluorouracil-^{13}C, $^{15}N_2$ in phosphate buffered saline solution) was added to the plasma contained in a 10 ml glass test tube. Next, 200 μl of saturated ammonium sulfate solution was added to precipitate plasma proteins with subsequent vortex mixing for 30 s followed by the addition of 5 ml of a 15:85 isopropanol:ethyl acetate mixture followed by another 30 s of vortex mixing. The sample was then centrifuged at 1000 × *g* for 15 min at 23 °C. Four milliliters of the supernatant was subsequently removed from each tube and transfered to an evaporation tube. Tubes were placed in a 45 °C water bath and evaporate under a stream of nitrogen. After evaporation was complete, 100 μl of 20:80 methanol:deionized H_2O was added to each tube and vortex mixed for 30 s to reconstitute the compounds. These mixtures were then transferred to HPLC autosampler vials for analysis.

2.3. Instrument conditions

Chromatographic separation was achieved using a Supelco® Discovery® RP Amide C16, 150 mm × 4.6 mm, 5 μm column with an isocratic flow of (3/97, v/v) methanol/water at 1 ml/min using an Agilent 1100 series HPLC system (Agilent Technologies, Inc, Santa Clara, CA, USA). Total run time was 17 min. Concentrations of tegafur were determined by absorbance detection at 272 nm. Approximately 30% flow (0.3 ml/min) was directed into a Waters Quattro Micro triple quadrupole mass spectrometer (Waters Micromass, Milford, MA) via splitter. Multiple reaction monitoring (MRM) was performed on 5-FU (*m/z* 128.7 > 41.40) and on the internal standard, iso-5-FU, (*m/z* 131.7 > 43.40) in the negative ion API mode with dwell times of 200 ms. Mass spectroscopic conditions were as follows: capillary voltage 3.50 kV, cone voltage 25.00 V, source temp 110 °C, desolvation temp 400 °C, collision gas pressure 3×10^{-3} mbar, cone gas flow 70 l/h and desolvation gas flow 460 l/h. Collision energy was set at 13.00 eV for the 5-FU transition, while 15.00 eV was used for the iso-5-FU transition.

2.4. Validation

Validation was achieved according to FDA regulatory guidelines for bioanalytical methods, with the primary requirement being that the intra- and inter-day accuracies and precisions below 15% variation [20].

2.4.1. Linearity

Five calibration standard solutions were prepared at 5-FU/tegafur concentrations of 8/800, 20/2000, 40/4000, 100/10,000, and 200/20,000 ng/ml, respectively, in cryopreserved human plasma. Calibration curves were calculated using a 1/*x* weighting, where *x* represented the ratio of analyte peak area to internal standard peak area for 5-FU and where x represents analyte peak area for tegafur. The lower limit of quantification (LLOQ) and detection (LLOD) for both tegafur and 5-FU were determined by signal-to-noise ratios of 5 and 3, respectively.

2.4.2. Accuracy and precision

Three quality control (QC) standards were also prepared in human plasma with 5-FU/tegafur concentrations of 14/1400, 60/6000, and 140/14,000 ng/ml, respectively. Intra-day accuracy (percent average deviation from the mean) and precision (percent

Table 1a
5-Fluorouracil validation results.

	Prepared concentration (ng/ml)	*n*	Average measured concentration (ng/ml)	Accuracy as average deviation (%)	Precision as standard deviation (%)	Recovery (%)	Stability as change in 24 h (%)
Intra-assay	14.0	3	14.4	3.05	2.65	86.4	1.23
	60.0	3	65.0	8.39	0.494	89.0	
	140	3	145	3.44	0.782	91.2	
Inter-assay	14.0	9	15.1	7.68	3.78		
	60.0	9	63.6	5.93	1.82		
	140	9	142	1.69	1.86		

standard deviation) were determined by the average of measured concentrations for the three QC standards. Inter-day accuracy and precision were determined in a similar manner over three consecutive days ($n = 9$).

2.4.3. Recovery

Absolute recoveries were calculated by comparing measured concentrations of standards prepared in plasma to the corresponding concentrations prepared in mobile phase solutions. Internal standards were not used in these measurements.

2.4.4. Autosampler stability

Autosampler stability of 5-FU and tegafur was assessed by analyzing standards immediately after preparation and again 24 h after the standards had remained in the autosampler compartment at 4 °C.

2.5. Clinical study design

Demonstration of the applicability of this bioanlaytical method was conducted by evaluation of blood samples from a clinical research protocol approved by the Duke University Institutional Review Board and performed according to the Declaration of Helsinki as amended in Somerset West (1996). All patients signed written informed consent before trial entry.

Adult patients with histologically proven adenocarcinoma or squamous cell carcinoma of the thoracic esophagus or gastroesophageal (GE) junction were included, as previously described [19]. Minimum laboratory requirements included the following: absolute neutrophil count of 1500/μl or greater, platelets of 100,000/μl or greater, total bilirubin level of 1.5 mg/dl or less, serum creatinine level no greater than 1.5 times the upper normal limit, and aspartate/alanine aminotransferase levels no more than 2.5 times upper normal limit.

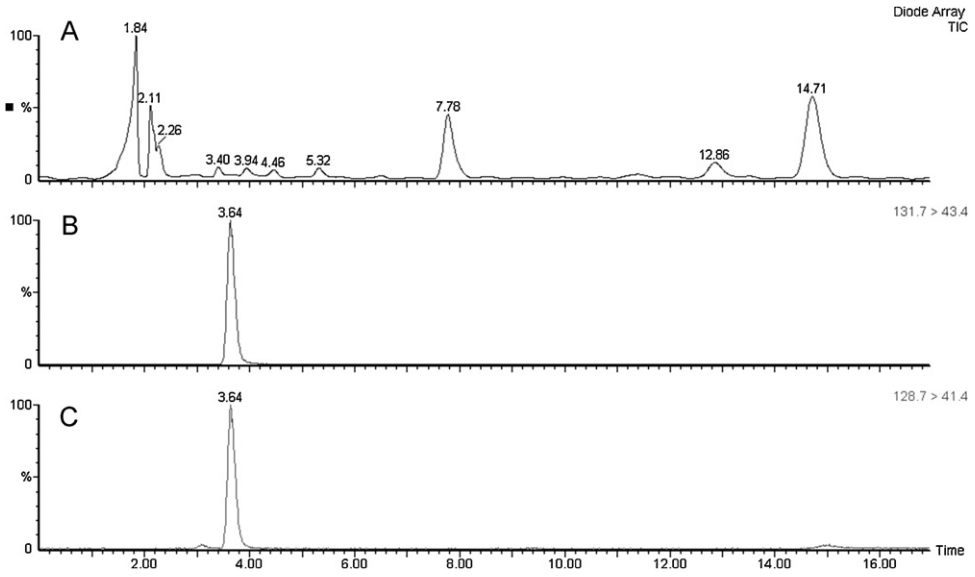

Fig. 2. UV tracing (A) of tegafur and tandem mass spectrometric chromatograms of ^{13}C, $^{15}N_2$-5-fluorouracil (B) and 5-fluorouracil (C).

Table 1b
Tegafur validation results.

	Prepared concentration (ng/ml)	*n*	Average measured concentration (ng/ml)	Accuracy as average deviation (%)	Precision as standard deviation (%)	Recovery (%)	Stability as change in 24 h (%)
Intra-assay	1400	3	1393	1.82	2.70	97.5	14.2
	6000	3	6557	9.28	0.711	110	
	14,000	3	14,187	1.33	0.292	105	
Inter-assay	1400	9	1468	5.62	4.17		
	6000	9	6240	5.59	4.97		
	14,000	9	13,871	4.79	5.95		

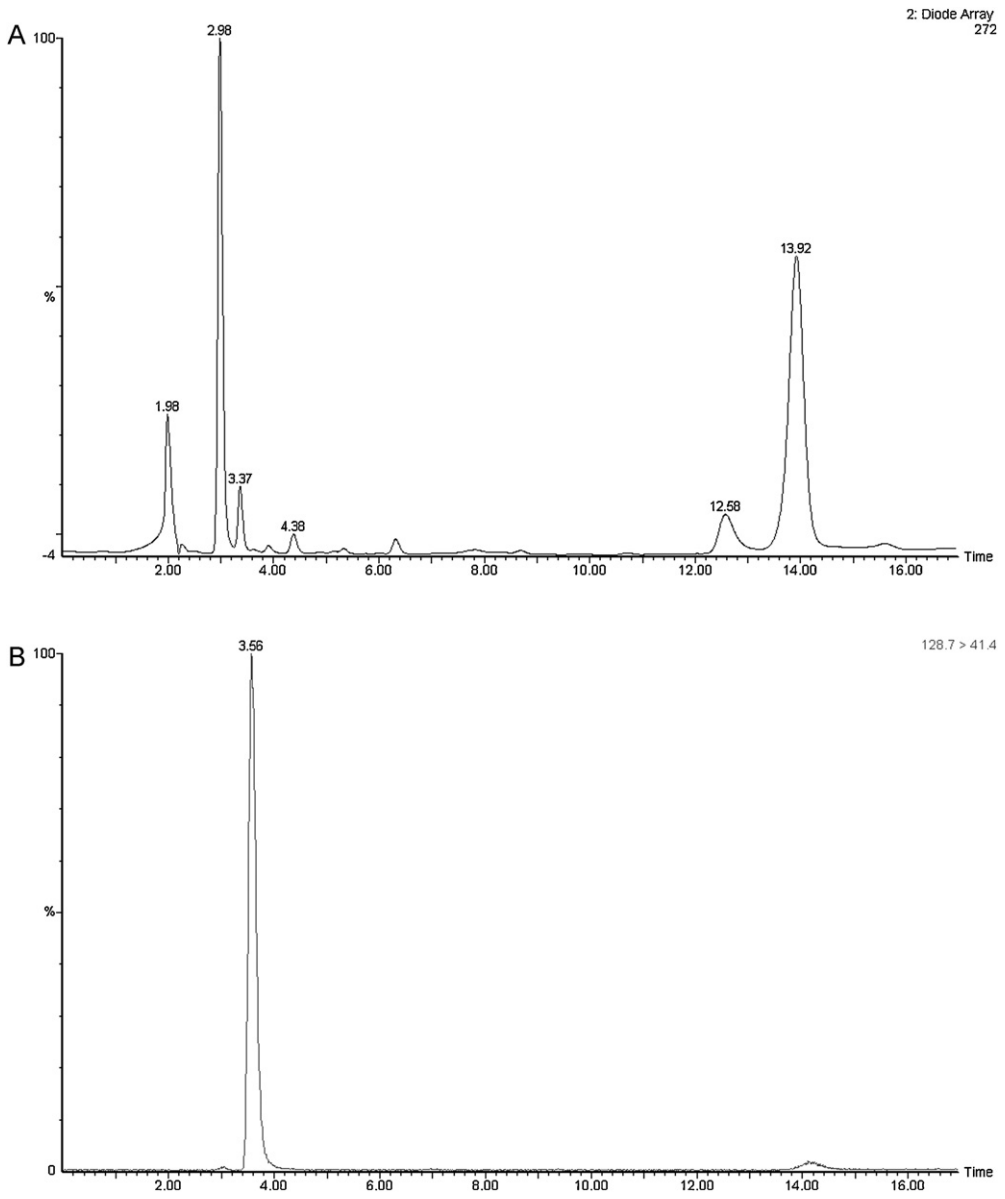

Fig. 3. Example of a tegafur UV chromatogram (A) and 5-FU MS/MS chromatogram (B) obtained from a clinical pharmacokinetic specimen.

Applicability of the bioanalytical method to clinical samples was conducted using twelve patients (median age 55 years; range, 19–70) with either squamous cell carcinoma or adenocarcinoma who participated in the clinical study. UFT®/LV doses were administered 5 days a week (M–F), by mouth, on an every 12 h schedule. Dose level 1 (UFT®/LV at 200/30 mg, twice daily) and dose level 2 (UFT®/LV at 300/30 mg in morning, 200/30 mg in evening) were administered on radiation days only, beginning on day 1 and finishing on the evening of the final radiation treatment day. Of the twelve patients in this study, six were dosed at dose level 1 (Patients 1–6) and six at dose level 2 (Patients 7–12).

2.5.1. Pharmacokinetic studies

Blood samples (3 ml) were drawn at pre-dose, 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 10 h on the first day of UFT®/LV administration to determine systemic disposition of the prodrug, tegafur, and its active product, 5-FU. Concentration versus time data were analyzed by a standard, two-stage approach using non-compartmental techniques (WinNonlin v1.1, Pharsight, Mountain View, CA, USA) in the Clinical Pharmacology Core Lab at the Mary Babb Randolph/WVU Cancer Center where the analytic development was also conducted.

3. Results

3.1. Analytic validation

The combined LC–MS/MS and LC–UV assay was validated over three days. Both intra- and inter-day accuracy and precision for 5-FU and tegafur were less than 15% as shown in Tables 1a and 1b. Fig. 2 (panel A) shows a tracing that represents a summation of UV spectra at 2 nm intervals from 190 nm to 600 nm. The tegafur peak at 14.7 min is shown to be well separated from the earlier eluted plasma peaks. Fig. 2 also depicts chromatograms demonstrating the selectivity of the two MRM transitions occurring simultaneously for 5-FU and iso-5-FU (panels B and C). Sufficient linearity was achieved for each corresponding calibration curve, with mean ($n = 3$) correlation coefficients of 0.9996 for 5-FU and 0.9990 for tegafur. The 24-h autosampler stability results

Table 2
First dose plasma pharmacokinetic parameter estimates for 5-FU and tegafur in subjects.

	5-FU		Tegafur	
	Dose level 1[a] ($n=6$)	Dose level 2[b] ($n=6$)	Dose level 1 ($n=6$)	Dose level 2 ($n=6$)
t_{MAX} (h)	1.42 ± 0.58	1.25 ± 0.93	1.83 ± 0.81	2.08 ± 1.11
C_{MAX} (ng/ml)	86.9 ± 88.5	149 ± 138	6065 ± 1773	9175 ± 2749
$AUC_{0-10\,h}$ (ng h/ml)	77.8 ± 67.0	232 ± 195	41,290 ± 22,575	45,589 ± 14,526

Data shown as mean ± SD.
[a] UFT®/LV at 200/30 mg, twice daily.
[b] UFT®/LV at 300/30 mg in morning; 200/30 mg in evening.

are also shown in Tables 1a and 1b. 5-Fluorouracil demonstrated excellent stability at 4 °C temperature with only a 1% change in concentrations. Tegafur proved somewhat less stable, with a 14% difference, presumably through ester hydrolysis of the tetrahydrofurane moiety. The LLOQ and LLOD for 5-FU were 8.0 and 5 ng/ml, respectively. The LLOQ and LLOD for tegafur were 800 and 250 ng/ml, respectively. Absolute recoveries were calculated for both tegafur and 5-FU and ranged from 97% to 110% and 86% to 91%, respectively.

3.2. Application to patient samples

Six patients received a 200 mg oral UFT dose (with 30 mg leucovorin), whereas the remaining six received a 300 mg oral UFT dose (with 30 mg leucovorin). Fig. 3A is a representative LC chromatogram depicting the tegafur peak with a retention time of 13.9 min for the 0.5 h post dose time point sample for a single subject, whereas Fig. 3B is the corresponding 5-FU MRM with a retention time of 3.56 min from the same sample. Fig. 4 depicts the mean concentration–time curves for all 12 subjects at both the high concentration tegafur (A) and low concentration 5-FU (B) during the first dose. The resulting clinical concentration–time data for tegafur suggests that the high concentration range for tegafur produces more applicable data for analysis of plasma samples in subjects on this therapy. Noncompartmental analysis was performed on day 1 to assess the first-dose pharmacokinetic parameters t_{MAX}, C_{MAX}, and $AUC_{0-10\,h}$, as shown in Table 2. Relatively high inter-subject variability was observed for each pharmacokinetic parameter, as anticipated based on previous clinical trials involving 5-FU [8,21]. Such is evident by the high standard deviations from the mean values shown in Table 2, as well as the error bars in the plasma concentration versus time curve for both 5-FU and tegafur shown in Fig. 4.

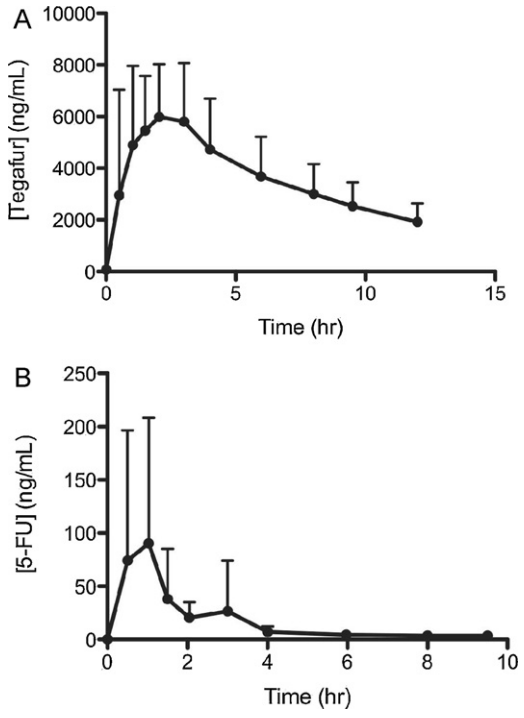

Fig. 4. Mean plasma concentration time curves for (A) tegafur, and (B) 5-FU, in 12 patients receiving first-dose UFT®/LV.

4. Discussion

This newly developed assay was designed to simultaneously measure low concentrations of 5-FU and high concentrations of tegafur in plasma resulting from the oral administration of UFT®/LV. The combined HPLC/UV and HPLC/MS/MS method contains analytical improvements over the two HPLC/UV assays and the two HPLC/MS/MS assays cited in the literature [15–18] while being specifically designed for UFT®/LV. An advantage of this assay over the HPLC/UV methods of Zufia et al. [15] and Chu et al. [16] is the short analysis time (17 min compared to 24–30 min). This improvement was achieved most likely by the use of a relatively polar column (Supelco® Discovery RP-Amide C16) and a very polar mobile phase (3/97, v/v) methanol/water delivered at a fast flow rate (1 ml/min), allowing for faster passage of analytes through the column. Another advantage of this method was that our extraction recoveries for 5-FU were better than those of Chu et al. with a range of 86–91% compared to 51–55%, respectively. High recoveries may produce more consistent results in cases where the extraction matrix (plasma) varies unexpectedly.

Compared to the method of Remaud et al. [17], the current approach is quicker, demonstrates comparable selectivity, requires significantly less sample volume (200 μl vs. 500 μl of plasma) and achieves sufficient sensitivity required for 5-FU measurement in human plasma after oral administration of UFT. Efficiency is also improved compared to that of Remaud et al. [17] at the higher concentration level for tegafur examined (800–20,000 ng/ml) since the use of only one calibration curve is required. An important difference in this method compared to others [17,18] entails use of UV absorption for quantitation. This approach has two major benefits compared to tegafur quantification by MRM in the mass spectrometer. First, inadvertent fragmentation of tegafur to 5-FU is avoided, which is sometimes noted during desolvation in the negative ion mode for API. Secondly, saturation of the mass spectrometer detector is avoided, which commonly occurs with analytes in concentrations as high as tegafur (20,000 ng/ml) in this assay, which is higher than the method of Liu (3000 ng/ml) [18].

This analytical approach allows for the first time the simultaneous detection of low concentrations of 5-FU and high concentrations of tegafur anticipated following administration of the UFT®/LV formulation in humans. Data generated from human samples were sufficient for estimation of pharmacokinetic parameters.

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References

[1] M. Minowa, Y. Kinoshita, S. Hasebe, T. Ito, M. Ogawa, K. Takebayashi, K. Ehara, M. Ueno, H. Udagawa, M. Hayashi, Gan To. Kagaku Ryoho 38 (2011) 393.
[2] Y. Osaka, M. Shinohara, S. Hoshino, T. Ogata, Y. Takagi, A. Tsuchida, T. Aoki, Anticancer Res. 31 (2011) 633.
[3] T. Hara, K. Nishikawa, M. Sakatoku, K. Oba, J. Sakamoto, K. Omura, Gastric Cancer (2011).
[4] L. Fischer von Weikersthal, A. Schalhorn, M. Stauch, D. Quietzsch, P.A. Maubach, H. Lambertz, D. Oruzio, R. Schlag, K. Weigang-Kohler, U. Vehling-Kaiser, M. Schulze, J. Truckenbrodt, M. Goebeler, J. Mittermuller, D. Bosse, B. Szukics, M. Grundeis, T. Zwingers, C. Giessen, V. Heinemann, Eur. J. Cancer 47 (2011) 206.
[5] B. Ullman, J. Kirsch, Mol. Pharmacol. 15 (1979) 357.
[6] P.V. Danenberg, A. Lockshin, Mol. Cell. Biochem. 43 (1982) 49.
[7] A.B. van Kuilenburg, J. Haasjes, D.J. Richel, L. Zoetekouw, H. Van Lenthe, R.A. De Abreu, J.G. Maring, P. Vreken, A.H. van Gennip, Clin. Cancer Res. 6 (2000) 4705.
[8] L. Ochoa, H.I. Hurwitz, G. Wilding, D. Cohen, J.P. Thomas, G. Schwartz, P. Monroe, W.P. Petros, V.P. Ertel, A. Hsieh, C. Hoffman, R. Drengler, S. Magnum, E.K. Rowinsky, Ann. Oncol. 11 (2000) 1313.
[9] T. Akiba, R. Okeda, T. Tajima, Acta Neuropathol. 92 (1996) 8.
[10] K. Ikeda, K. Yoshisue, E. Matsushima, S. Nagayama, K. Kobayashi, C.A. Tyson, K. Chiba, Y. Kawaguchi, Clin. Cancer Res. 6 (2000) 4409.
[11] T. Komatsu, H. Yamazaki, N. Shimada, S. Nagayama, Y. Kawaguchi, M. Nakajima, T. Yokoi, Clin. Cancer Res. 7 (2001) 675.
[12] D.H. Ho, R. Pazdur, W. Covington, N. Brown, Y.Y. Huo, Y. Lassere, J. Kuritani, Clin. Cancer Res. 4 (1998) 2085.
[13] P.M. Hoff, R. Pazdur, S.E. Benner, R. Canetta, Anticancer Drugs 9 (1998) 479.
[14] P. Schoffski, Anticancer Drugs 15 (2004) 85.
[15] L. Zufia, A. Aldaz, C. Castellanos, J. Giraldez, Ther. Drug Monit. 25 (2003) 221.
[16] D. Chu, J. Gu, W. Liu, J. Paul Fawcett, Q. Dong, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 795 (2003) 377.
[17] G. Remaud, M. Boisdron-Celle, A. Morel, A. Gamelin, J. Chromatogr. B: Analyt. Technol. Biomed. Life Sci. 824 (2005) 153.
[18] K. Liu, D. Zhong, H. Zou, X. Chen, J. Pharm. Biomed. Anal. 52 (2010) 550.
[19] B.G. Czito, D.P. Cohen, C.R. Kelsey, A.C. Lockhart, J.C. Bendell, C.G. Willett, W.P. Petros, T.A. D'Amico, R. Truax, H.I. Hurwitz, Int. J. Radiat. Oncol. Biol. Phys. 70 (2008) 1066.
[20] Food and Drug Administration. Guidelines for Industry: Bioanalytical Methods Validation, 2001.
[21] A. Berglund, G. Carlsson, B. Gustavsson, J.E. Frodin, P. Ragnhammar, B. Glimelius, Anticancer Res. 23 (2003) 1789.