Published in final edited form as:

Biomed Chromatogr. 2015 May ; 29(5): 762–767. doi:10.1002/bmc.3354.

Effects of American ginseng on pharmacokinetics of 5-fluorouracil in rats

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Abstract

The pharmacokinetics of 5-fluorouracil (5-FU) in combination with or without American ginseng (seven-consecutive days oral dose) in rats were evaluated using liquid chromatography–electrospray ionization–mass spectrometry (LC-MS). Chromatographic separation was performed on a reverse LC column within a total run time of 6.5 min, which allowed for a relatively quick analysis. The limit of quantification for 5-FU was 15 ng/mL and this method was linear over 15–50,000 ng/mL. This method supported stabilizing determination of the plasma concentration of 5-FU over a period of 24 h. Precision both interday and intraday (coefficient of variation) was within 14% and accuracy (relative error) ranged from –5 to 14%. In view of the observed pharmacokinetic parameters, including maximum concentration, time to maximum concentration, area under the concentration–time curve (AUC), mean residence time, elimination half-life and clearance, our results showed no significant differences in all of the pharmacokinetic parameters between the ginseng co-treated group and 5-FU alone group. Some increase in AUC was observed in 5-FU plus ginseng group; however, the difference did not reach statistical significance compared with 5-FU alone. It appeared that American ginseng administration did not significantly alter the kinetics of 5-FU. More studies are still needed to confirm our results.

Keywords

5-fluorouracil; American ginseng; LC-MS; pharmacokinetics; herb–drug interaction

Introduction

5-Fluorouracil (5-FU) is a commonly used chemotherapeutic agent for the treatment of cancer, especially malignancies in the aerodigestive tract, breast, head and neck (Malet-Martino and Martino, 2002; Masuda *et al.*, 2001; Navolanic and McCubrey, 2005; Sakaeda *et al.*, 2009). Most of the cytotoxic mechanisms of 5-FU have been well explored. It was thought to produce its anticancer effects by thymidylate synthase through the incorporation

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of anabolic, inhibiting cytotoxic nucleotide metabolites of 5-FU, thereby blocking DNA replication (Boni *et al.*, 2010). Meanwhile, the pharmacokinetics of 5-FU have been reported extensively. It is worth noting that the pharmacokinetics of 5-FU could be altered by the use of other drugs (Chavez *et al.*, 2006), including herbal medicines (Fishbein *et al.*, 2009; Gu *et al.*, 2013; Qiao *et al.*, 2011a, b). To ensure the clinical efficacy and safety of 5-FU, it is necessary to study interactions between 5-FU and other compounds.

American ginseng is a very popular herb in the USA, and its main active components are ginsenosides, which have been reported to have a wide variety of biological activities, including immunomodulatory effects, hypoglycemic, anti-inflammatory and anti-tumor activity (Qi *et al.*, 2011a, b; Sen *et al.*, 2013; Yuan *et al.*, 2010; Yuan *et al.*, 1998). A clinical study showed that American ginseng may be helpful in cancer-related fatigue with good tolerance (Barton *et al.*, 2010). We previously reported that American ginseng increased the effects of cancer chemotherapy with 5-FU (Li *et al.*, 2009a, b), and this synergistic effect between American ginseng and 5-FU made it possible to reduce the dose of 5-FU in combination with the herb and thereby decrease 5-FU dose-related toxicity (Li *et al.*, 2009a, b). Therefore, 5-FU and American ginseng can be administered concomitantly. However, it is not certain whether the use of American ginseng affects the metabolism of 5-FU.

The aim of the present study was to investigate the effect of American ginseng on the pharmacokinetics of 5-FU. This data would be important to sufficiently recognize the potential combined effect of the co-administration of American ginseng and 5-FU.

Experimental

Chemicals, material and reagents

5-Fluorouracil and 5-bromouracil (5-BU) were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). Methanol and formic acid (HPLC grade) were obtained from ROE Scientific Inc. (Newcastle, PA, USA). Purified water prepared using the Millipore system (Millipore, Bedford, MA, USA) was employed for all the preparations. Other reagents were of analytical grade and purchased from Jiangsu Hanbon Sci. & Tech. Co. Ltd (Nanjing, Jiangsu, China).

Equipment and LC/MS condition

Chromatographic analysis was performed on an Agilent 1100 series LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a dual pump and a Rheodyne 7125i injection valve with a 20 μ L sample loop. Data were acquired and processed by a HP ChemStation. A Thermo Hypersil BDS C₁₈ column (4.6 $\times$ 255 mm i.d., 5 μ m) with an Agilent Zorbax ODS C₁₈ as the guard column (4.6 $\times$ 12.5 mm i.d., 5 μ m) was employed for the chromatographic separation. Columns were maintained at room temperature throughout the procedure. The mobile phase consisted of (A) 0.1% aqueous formic acid and (B) methanol. The isocratic elution condition was 15% B for 6.5 min. The flow rate was 1 mL/min and the injection volume was 10 μ L.

Detections were performed by an Agilent MSD SL-G2710BA with an electrospray ionization source (Agilent Corp, Santa Clara, CA, USA) by monitoring the deprotonated or

HCOO-adduct ions. The analysis parameters were as follows: fragmentor voltage, 100V; drying gas (N₂) flow rate, 8 L/min; gas temperature, 250°C; nebulizer gas (N₂) pressure, 40 psig; capillary voltage, 2500 V. Analysis for 5-FU and 5-BU was done in a negative ion mode with single ion monitoring m/z values restricted to specific retention time windows 0–4.5 min, m/z 129.00; 4.5–6.5 min, m/z 189.00.

Preparation and analysis of American ginseng extract

Four-year old American ginseng roots (*Panax quinquefolius* L.) were obtained from Roland Ginseng, LLC (Wausau, WI, USA). American ginseng samples were prepared with a slight modification from previous works. The air-dried roots of American ginseng were pulverized into powder and sieved through an 80 mesh screen. Eight grams of the powder were extracted twice by heat-reflux with 320 mL of 70% ethanol at 90°C for 4 h each. The combined extract was evaporated under vacuum and lyophilized. HPLC analysis data showed that each gram of American ginseng extract contained 389 mg of Rb1, 22 mg of Rc, 27 mg of Rd, 113 mg of Re and 4 mg of Rg1.

Preparation of standard solutions, calibration samples and quality control samples

Stock solutions of 5-FU were prepared in mobile phase solution at the concentration of 100 µg/mL. The stock solution of 5-BU was prepared in methanol at the concentration of 1 µg/mL. All solutions were kept in a brown volumetric flask at 4°C before use.

Calibration samples in plasma were prepared by spiking appropriate aliquots from the stock solutions of 5-FU into the blank rat plasma to obtain solutions at 15–50,000 ng/mL final concentrations. Quality control (QC) samples were also prepared in the same way to obtain solutions at 25 ng/mL (low), 1000 ng/mL (medium) and 30,000 ng/mL (high) final concentrations. All of the spiked plasma samples were then treated according to biosample preparation procedure.

Biosample preparation

Each 0.1 mL of plasma, 0.01 mL of internal standard (IS: 5-BU) solution and 1.0 mL of ethyl acetate/isopropyl alcohol (v/v, 9:1) were combined and vortex-mixed for 1 min and then centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was transferred to a test tube. The remaining sample was then extracted again with 0.5 mL of ethyl acetate–isopropyl alcohol in the same way. The supernatant was mixed in the test tube and evaporated to complete dryness by a pressured gas-blowing concentrator at 4°C. The residue was dissolved in 100 µL mobile phase with vortex-mixing for 1 min and then the centrifugation process was repeated. Ten microliters of the supernatant was injected into the LC–MS system to determine the concentration of 5-FU.

Method validation

Linearity, LOD and LOQ—To evaluate linearity, plasma calibration curves were developed and assayed. Plasma samples were quantified using the ratio of the peak area of analyte to that of IS. The peak area ratio (y) and concentration of each analyte (x) were subjected to a weighted ($1/x^2$) least squares linear regression analysis to calculate the calibration equation and correlation coefficients. The limit of detection (LOD) was

determined as the lowest concentration with a signal-to-noise (S/N) ratio of 3 and the limit of quantification (LOQ) was determined as the lowest concentration with a S/N ratio of 10.

Accuracy and precision—Intraday accuracy and precision were determined by analyzing five replicates of QC samples in the same day. Interday accuracy and precision were determined by analyzing five replicates at each concentration level on six consecutive days.

The accuracy was calculated as the relative error (RE) of the observed concentration from the spiked concentration. The precision was expressed by the coefficient of variation (CV). Each of them was defined as:

$$\text{Bias error (RE\%)} = [(\text{mean observed} - \text{theoretical value}) / \text{theoretical value}] \times 100$$

$$\text{Precision (CV\%)} = (\text{standard deviation} / \text{mean}) \times 100$$

Recovery and matrix effect—Recoveries of analyte at three QC levels ($n = 5$) were determined by comparing the responses of the analyte from QC samples with the responses of analyte spiked with postextracted matrix at equivalent concentrations. In set 1, blank plasma samples from five untreated rats were spiked with 5-FU plus IS prior to the sample extraction process. In set 2, blank plasma samples from five untreated rats were spiked with 5-FU plus IS post sample extraction. In set 3, 5-FU and IS concentrations were prepared in the chromatographic mobile phase solution. Matrix effects on the ionization of analyte were evaluated by comparing the above set 2 with set 3. Extraction efficiency (EE) and matrix effects (ME) were defined as:

$$\text{EE (\%)} = (\text{peak area ratio of 5-FU+IS in set 1} / \text{peak area ratio of 5-FU+IS in set 2}) \times 100$$

$$\text{ME (\%)} = 100 - [(\text{peak area ratio of 5-FU+IS in set 2} / \text{peak area ratio of 5-FU+IS in set 3}) \times 100]$$

Freeze-thaw stability—The stability of analytes was evaluated by analyzing QC samples at three concentrations stored at -80°C . Two freeze-thaw cycles of the QC samples were performed at 4 and 27 h ($n = 5$).

Animal experiment

Normal male Sprague-Dawley rats weighing 250–300 g were obtained from BiKai Co. Ltd. (Shanghai, China). Five rats were placed in one cage, and maintained at $25 \pm 2^{\circ}\text{C}$ and $65 \pm 5\%$ relative humidity on a 12 h light–12 h dark cycle. All animals had free access to food and water. All experimental procedures concerning the animals were in accordance with protocols that approved by the Review Committee of Animal care and Use of China Pharmaceutical University (approval date: 8 November 2013; no. 2008001632235).

After acclimatization for 7 days, all rats were randomly divided into group 1 (5-FU-treated group, $n = 5$) and group 2 (both 5-FU and American ginseng-treated group, $n = 5$). American ginseng extract (300 mg/kg) was administered orally to the group 2 animals for seven consecutive days while water was administered orally to the group 1 animals at the same time. After 7 days' administration, the rats were fasted for 12 h before the experiment. 5-FU (50 mg/kg) was administered by intraperitoneal injection to the rats of both groups. Under light ether anesthesia, blood samples (0.5 mL) were collected from the retro-orbital plexus of rats into heparinised tubes at time points 0, 5, 10, 20, 30, 60, 90, 120 and 240 min after dosing. Plasma samples were obtained by harvesting supernatant collected by centrifuging blood samples at 4500 rpm for 10 min at 4°C. Collected samples were stored at -80°C until further analysis.

The following pharmacokinetic parameters were obtained: maximum plasma concentration ($C_{\max}$), time to maximum plasma concentration ($T_{\max}$), mean residence time (MRT), volume of distribution (V_d), half-life ($T_{1/2}$), area under the concentration–time curve (AUC) and plasma clearance (CL). The data was obtained using a noncompartmental method using Drug and Statistics 2.0 (DAS 2.0) software package (Mathematical Pharmacology Professional Committee of China, Shanghai, China).

Statistical analysis

Results are expressed as the mean $\pm$ SD or SE where indicated. Statistical differences among groups were assessed by a two-tailed Student's *t*-test. Values of $p < 0.05$ were considered a significant difference, and values of $p < 0.01$ were considered remarkably significant difference.

Results and discussions

Method validation

Specificity—The selectivity of the method was verified by comparing the chromatograms of six different batches of blank rat plasma with the corresponding spiked plasma. Under optimized LC-ESI-MS conditions, the approximate retention times for 5-FU and the internal standard (IS) (Fig. 1) were 3.8 and 6.1 min (Fig.2), respectively. No significant interference from endogenous substances was observed, suggesting a good selectivity of the developed method.

Linearity, LOD and LOQ—The linear regression of 5-FU in the plasma of rats exhibited good linear relationships over the range from 15 to 50,000 ng/mL. The regression equation for 5-FU in the plasma of rats was: $y = 1.1965x + 0.588$ ($r^2 = 0.9913$).

Sensitivity was assessed by the LOD and LOQ. Pooled plasma samples were spiked to contain 10 ng/mL 5-FU and analyzed on five separate days. The peak area in chromatograms for the spiked plasma samples containing the above lowest concentrations was compared with the noise signal. The LOD had to have a precision of 10% and a S/N ratio 3. The same procedures were performed with contents of analytes in pooled plasma samples spiked with 15 ng/mL 5-FU to determine the LOQ. The LOQ had to have a precision of 15% and a S/N ratio 10, which were acceptable in the current assay.

Accuracy and precision

As shown in Table 1, for all of the samples evaluated, both interday and intraday precision (RSD) were within the acceptable range (<14%) and accuracy (% bias) ranged from –5 to 14%.

Recovery and matrix effect

Table 2 shows the extraction efficiencies as well as matrix effects for 5-FU. Extraction recoveries (~90%) at three concentration levels of the analyte were all within the acceptable range.

Owing to the ion suppression, the presence of the matrix effect can decrease or increase the response of the analyte. Thus, the evaluation of matrix effect should be conducted before the validation of any LC-MS method. The mechanisms for such matrix effects are not entirely understood. The results showed that the matrix effects of the QC samples were moderate (~23%). Although the matrix effect does interfere with the determination of 5-FU (Table 2), the analytical capability of this method was not affected significantly, as shown in method validation profile (Table 1).

Freeze–thaw stability

QC samples of 5-FU were tested for stability in plasma over two freeze–thaw cycles. Relative errors for 5-FU during freeze–thaw cycles were <6% after 4 h and 13% after 27h, indicating approved stability for subsequent study conditions. The results are presented in Table 3.

Pharmacokinetics analysis

The peak plasma concentrations ($C_{\max}$) for the control group and the AGS-treated group were 40.41 ± 5.97 (mean $\pm$ S.E.) and 39.31 ± 3.44 $\mu\text{g/mL}$, respectively ($p > 0.1$). The values of AUC for 5-FU were 1326.49 ± 19.27 mg min/L for the control group and 1526.84 ± 88.26 mg min/L for the AGS-treated group ($p > 0.05$). Likewise, no significant alteration was observed in the MRT, $T_{1/2}$ and CL between the two groups. The plasma concentration–time curves after a single intraperitoneal injection of 5-FU of the two groups are compared in Fig. 3. The corresponding pharmacokinetic parameters reflecting the bioavailability of experimental animals are summarized in Table 4. The plasma concentration of 5-FU appeared to manifest no significant difference when the rats were orally administered an amount of American ginseng that was equivalent to the daily dose for a human.

Discussion

5-FU has been used as clinical chemotherapy to treat solid tumors for over 50 years (Meyerhardt and Mayer, 2005). The key enzyme responsible for the catabolism of 5-FU is dihydropyrimidine dehydrogenase (DPD). Either delivered orally or by intraperitoneal injection, most 5-FU is rapidly catabolized in the liver by DPD, which converts 5-FU to 5-fluoro-5, 6-dihydrouracil (5-FUH₂) (Buchel *et al.*, 2013; Peer *et al.*, 2012).

Deglycosylation reactions in ginseng saponins by intestinal bacteria via the stepwise cleavage of the sugar moieties construct the main metabolic pathways of ginsenosides *in vivo*. Hepatic cytochrome P450 enzymes contribute to another metabolic pathway. Liver and bile are responsible for systemic clearance of ginseng saponins from the circulation. Among the described hepatic cytochrome P450 concerned with catalyzing ginsenoside metabolism, cytochrome P3A4-catalyzed oxygenation metabolism is essential in the hepatic disposition of ginsenosides (Qi *et al.*, 2011a, b). As a feedback effect, catalytic activities of hepatic cytochrome P2C9 and P3A4 in human liver microsomes could be inhibited by ginsenosides Rb1, Rb2, Rc and Rd (Deb *et al.*, 2014; He and Edeki, 2004). Therefore, the enzyme-inhibiting effect during the hepatic drug metabolizing process caused by ginsenosides should be taken into account. When anti-tumor agents are prescribed to tumor patients simultaneously consuming herbs containing these ginsenosides, therapies with cytochrome P2C9 substrates such as warfarin should be closely monitored (Haefeli and Carls, 2014).

Ginseng could be considered as an independent antiproliferative agent for many types of cancer: ovarian, breast, lung, melanoma, etc. The possible mechanism could be a variety of effects relating to anti-angiogenesis, anti-proliferation and apoptosis (Sagar *et al.*, 2006a, b). Furthermore, a synergistic inhibitory effect was observed in human gastric cancer cells (BGC823) when pretreated with ginseng extraction, while there was little impact on normal human gastric cells after the same administration. On the level of rats' body constitution, increased weight was observed after 10 days' *Panax ginseng* pretreatment (Gu *et al.*, 2013). Consequently, it could be inferred that ginsenosides present potential clinical relevance in improving the quality of life of cancer patients. In the present study, no manifest difference could be determined when comparing the pharmacokinetics of American ginseng co-administration with 5-FU and the administration of 5-FU alone.

Panax ginseng was found to change the pharmacokinetics of 5-FU when rats were pretreated with *Panax ginseng* and *Salvia miltiorrhiza*. The increase in $T_{1/2}$ (k_e) caused by *Panax ginseng* indicated that the rate of the drug elimination from the body was reduced and 5-FU remained at low concentration for a longer period of time, which might lead to a longer drug effect time and longer-lasting efficacy of 5-FU (Gu *et al.*, 2013). Compared with the previous study, our results showed that, apart from a slight increase in AUC, other pharmacokinetic parameters, including $T_{1/2}$ and CL_z/F in the group co-treated with ginseng and 5-FU, were not significantly different from those in the group treated with 5-FU alone.

Conclusions

In this project, we studied the effect of American ginseng on the pharmacokinetics of 5-FU in rats. A sensitive, accurate, convenient and reproducible LC–MS method was developed and validated for the quantification of 5-FU. Our data suggested that, in our experimental paradigm, American ginseng did not significantly affect the metabolism of 5-FU. More studies, especially controlled clinical trials, are needed to definitely elucidate the American ginseng and 5-FU interactions.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (no. 81373957, 81102763 and 81373919) and the Natural Science Foundation of Jiangsu Province (no. SBK201322119).

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Abbreviations used

5-FU	5-fluorouracil
5-BU	5-bromouracil
AGS	American ginseng
AUC	area under curve
CL	clearance
C_{max}	area maximum concentration
MRT	mean residence time
T_{max}	time to maximum concentration
T_{1/2}	elimination half-life

Figure 1. Chemical structures of (a) 5-fluorouracil (5-FU), molecular weight 130.08 Da; and (b) 5-bromouracil (5-BU), molecular weight 190.99 Da.

Figure 2.

Representative chromatograms for 5-FU and internal standard 5-BU in: (a) standard solution; (b) blank plasma without exogenous compounds; (c) blank plasma with standard solution; (d) plasma sample after single administration of 5-FU for 90 min; and (e) plasma sample after combination administration of 5-FU in combination with ginseng for 90 min.

Figure 3. Mean concentration–time profiles for 5-FU in rat plasma. All values are represented as means $\pm$ SD, with $n = 5$.

Table 1
Method validation for 5-FU in rat plasma (accuracy and precision)

	Theoretical (ng/mL)	Observed (ng/mL)	CV (100%) ^a	RE (100%) ^b
<i>Inter-day</i>				
	15	15.6 ± 1.9	11.97	6.13
	25	25.6 ± 3.3	12.88	2.24
	1,000	1,139 ± 128	3.43	13.91
	30,000	33,362 ± 2832	8.49	11.21
<i>Intra-day</i>				
	15	17.1 ± 1.72	10.09	13.87
	25	27.5 ± 3.56	12.98	9.84
	1,000	1,134 ± 148	13.09	13.39
	30,000	28,369 ± 2771	9.77	-5.44

^aPrecision (coefficient of variation, CV, %) = (standard deviation/mean) × 100.

^bBias error (relative error, RE, %) = [(mean observed – theoretical value)/theoretical value] × 100.

Concentrations measured are reported as means ± SD; *n* = 5 for both interday and intraday determination.

Table 2
Extraction efficiency and matrix effect for 5-FU in rat plasma

Added (ng/mL)	Extraction efficiency ^a (% mean, <i>n</i> = 5)	Matrix efficiency ^b (% mean, <i>n</i> = 5)
25	62.36 ± 11.5	23.63 ± 2.01
1,000	83.76 ± 6.62	23.72 ± 3.20
30,000	94.64 ± 6.58	18.47 ± 1.81

^aExtraction efficiency (%) = (peak area ratio of 5-FU + IS in set 1/peak area ratio of 5-FU + IS in set 2) × 100.

^bMatrix efficiency (%) = 100 – [(peak area ratio of 5-FU + IS in set 2/peak area ratio of 5-FU + IS in set 3) × 100].

Concentrations measured are reported as means ± SD; *n* = 5 for both extraction efficiency and matrix efficiency determination.

Table 3
Freeze–thaw stability for 5-FU in rat plasma

Theoretical (ng/mL)	RE ^a (%; 4 h)	RE (%; 27 h)
25	−5.22	−12.59
1000	−6.80	−12.62
30,000	−2.29	−13.47

^aPercentage relative error (RE, %) = [(mean observed – theoretical value)/theoretical value] × 100.

Table 4
Pharmacokinetic parameters for the single administration of 5-FU (50 mg/kg) and combination administration of 5-FU (50mg/kg) plus American ginseng (300mg/kg)

Pharmacokinetic parameters	5-FU	5-FU + ginseng
AUC (mg min/L)	1326.49 ± 119.27	1526.84 ± 88.26 [*]
MRT (min)	27.71 ± 0.28	29.93 ± 2.26 ^{**}
$T_{1/2}$ (min)	17.44 ± 2.18	15.93 ± 2.00 ^{**}
CL (L/min/kg)	0.038 ± 0.0036	0.033 ± 0.0022 ^{**}
$C_{\max}$ (mg/L)	40.41 ± 5.97	39.31 ± 3.44 ^{**}

Data expressed as means ± SE ($n = 5$). AUC, area under the concentration vs time curve; MRT, mean residence time; $T_{1/2}$, elimination half-life; CL, clearance; $C_{\max}$, maximum concentration.

^{*} $p > 0.05$ compared with single 50 mg/kg 5-FU administration group.

^{**} $p > 0.10$ compared with single 50 mg/kg 5-FU administration group.