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Short communication

Sensitive liquid chromatographic assay for the simultaneous determination of 5-fluorouracil and its prodrug, tegafur, in beagle dog plasma

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Abstract

A high-performance liquid chromatographic (HPLC) method has been developed for the simultaneous determination of 5-fluorouracil (5-FU) and its prodrug, tegafur (TF), in dog plasma. 5-FU, the internal standard, 5-bromouracil, and TF were separated on a C_{18} Spherisorb ODS_2 column using isocratic elution with retention times of 4.4, 8.0 and 21.2 min, respectively. Detection by UV absorption at 260 nm gave a limit of quantitation of 4 μg/l for 5-FU in plasma. Calibration curves for 5-FU and TF were linear over the ranges of 4–160 μg/l and 0.48–19.2 mg/l, respectively. Intra- and inter-day precision over these concentration ranges were <10.9 and <13.6% for 5-FU and TF, respectively, with good accuracy for both compounds. The method was successfully applied to define plasma concentration–time curves of TF and 5-FU in dogs administered a single oral dose containing TF (100 mg) and uracil (224 mg).

Keywords: 5-Fluorouracil; Tegafur

1. Introduction

5-Fluorouracil (5-FU) has been widely used in the chemotherapy of a variety of human carcinomas including those of the head and neck, gastrointestinal tract and breast, using various schedules [1]. However, administration of the compound often causes severe gastrointestinal toxicity and myelosuppression [2]. To overcome the toxicity, many derivatives and related compounds have been synthesized. Tegafur (TF), a 2-tetrahydrofuranyl derivative of 5-FU, has been shown to have a broad spectrum of anti-tumor activity when administered intravenously or orally [3]. It acts as a prodrug of 5-FU and produces comparatively little myelosuppression [4]. The chemical structures of 5-FU and TF are depicted in Fig. 1.

Therapeutic doses of TF produce very low plasma concentrations of 5-FU [5] requiring sensitive analytical methods for pharmacokinetic studies. High-performance liquid chromatography (HPLC)

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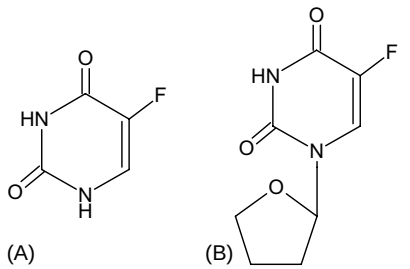

Fig. 1. Chemical structures of: (A) 5-fluorouracil and (B) tegafur.

and gas chromatography–mass spectrometry (GC–MS) methods have been developed [6]. HPLC methods with fluorescence or chemiluminescence detection possess adequate sensitivity but involve complex derivatization steps [7,8]. An HPLC method with UV detection has been reported for the simultaneous determination of 5-FU and TF in rat plasma [9], but was not suitable for detailed pharmacokinetic studies because of its poor specificity and inadequate sensitivity (0.1 mg/l).

This paper describes a simple and sensitive HPLC method with UV detection for the simultaneous determination of 5-FU and TF using 5-bromouracil (5-BU) as internal standard. The assay was developed to improve on an earlier method described by Gamelin et al. [10]. The method was validated and used successfully to determine pharmacokinetic profiles of 5-FU and TF in dogs after a single oral dose of a combined dosage form (UFT capsules) containing TF 100 mg and uracil 224 mg. Concurrent administration of uracil with TF has been reported to enhance anti-tumour activity of 5-FU [11].

2. Experimental

2.1. Materials

5-FU, TF and 5-BU were purchased from Sigma Chemical Co (St Louis, MO, USA). HPLC grade ammonium sulfate, isopropanol, ethyl acetate, acetic acid and methanol were supplied by Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was filtered using a Milli-Q water purification system supplied by Millipore (Bedford, MA, USA). UFT capsules containing TF 100 mg, uracil 224 mg were supplied by Taihe Pharmaceutical Co., Tianjin, China.

2.2. Preparation of standards

Six standard solutions containing binary mixtures of 5-FU and TF were prepared in methanol. Concentrations of 5-FU and TF were 20, 40, 100, 200, 400, 800 μg/l and 2.4, 4.8, 12, 24, 48, 96 mg/l, respectively. A solution of 5-BU (10 mg/l) was also prepared in methanol. Aliquots of the standard solutions (100 μl) were added to samples of dog plasma (500 μl) to provide six calibration standards containing the equivalent 5-FU and TF concentrations in plasma of 4, 8, 20, 40, 80, 160 μg/l and 0.48, 0.96, 2.4, 4.8, 9.6, 19.2 mg/l, respectively. Low, medium and high quality control (QC) samples were similarly prepared containing the equivalent 5-FU and TF concentrations in plasma of 4, 20, 80 μg/l and 0.48, 2.4, 9.6 mg/l, respectively.

2.3. Extraction procedure

Plasma samples (500 μl) and aliquots of methanol (100 μl), calibration standards (600 μl) or QC samples were placed in centrifuge tubes together with aliquots of 5-BU solution (60 μl). Tubes were vortex-mixed after which plasma proteins were precipitated by the addition of 100 mg of ammonium sulfate. Tubes were vigorously mixed for 1 min and centrifuged for 5 min at 6000 × *g*. Aliquots (2 ml) of isopropanol:ethyl acetate (15:85) were added and tubes were vortex-mixed for 3 min and centrifuged for 15 min at 6000 × *g*. Organic layers were transferred to clean tubes and evaporated to dryness at 40 °C under a stream of nitrogen. Residues were reconstituted in mobile phase (150 μl), briefly vortex-mixed and 25 μl injected into the HPLC system.

2.4. Chromatography

The HPLC system (Waters, USA) consisted of a 515 pump, a 7725I Rheodyne injector and a 2487 UV detector set at 260 nm. Samples were analyzed on a C_{18} Spherisorb ODS_2 column (250 mm × 4.6 mm i.d., 5 μm particle size, Jahnsson, China), protected by a guard column (15 mm × 4.6 mm i.d.) of the same material. The mobile phase (10 mM acetic acid:methanol, 90:10 (v/v)) was vacuum degassed and filtered through a 0.45 μm Millipore membrane filter before use. The flow-rate was 1.0 ml/min and chromatography was performed at ambient temperature.

2.5. Assay validation

2.5.1. Selectivity

HPLC peaks of 5-FU, TF and 5-BU were identified on the basis of their retention times and UV spectra obtained using a "scan spectra" method of stop–flow. Plasma samples from six, drug-free, healthy beagle dogs were tested for the presence of endogenous compounds co-eluting with 5-FU, TF and 5-BU.

2.5.2. Recovery, stability and limit of quantitation

Recoveries of 5-FU and TF were determined by comparison of peak heights obtained after injection of extracted QC samples and corresponding standard

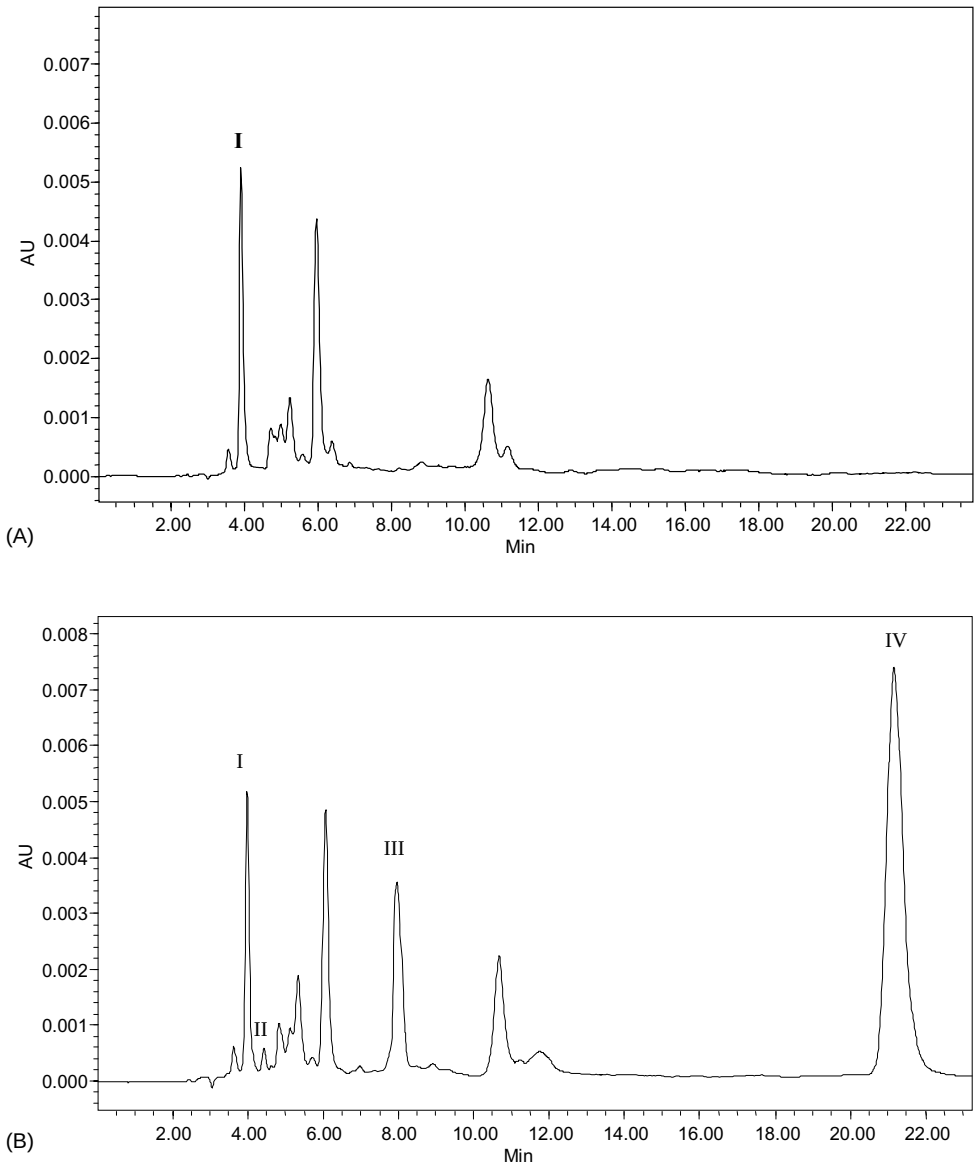

Fig. 2. HPLC chromatograms of: (A) blank plasma, (B) a calibration standard containing 20 μg/l 5-FU, 0.6 mg/l 5-BU and 2.4 mg/l TF, and (C) a dog plasma sample 0.75 h after a single oral dose of a UFT capsule (TF 100 mg, uracil 224 mg). Chromatographic peaks I, II, III and IV are uracil, 5-FU, 5-BU and TF, respectively.

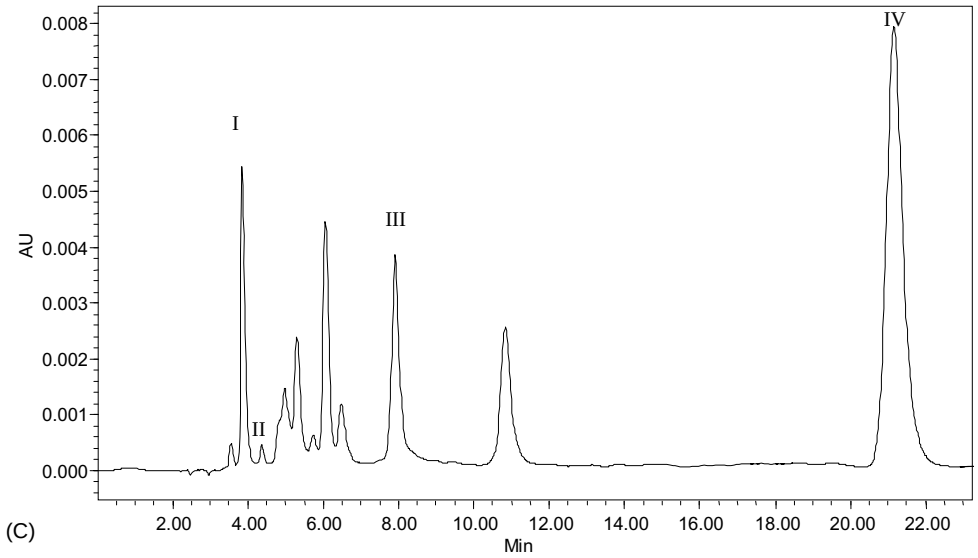

Fig. 2. (*Continued*).

solutions ($n = 6$) at the same concentration. The stability of 5-FU and TF in mobile phase was studied by comparing duplicate low, medium and high QC samples stored for 6 and 12 h at room temperature with freshly prepared QC samples. The limit of quantitation of 5-FU was calculated as the minimum concentration that could be quantified with no more than 15% relative standard deviation (R.S.D. (%)).

2.5.3. Linearity

Calibration standards were subjected to extraction and HPLC analysis in triplicate. Quantitation of 5-FU was based on calibration curves of peak height ratio (drug/internal standard) versus concentration. Linearity was assessed by linear regression analysis with a weighting factor of $1/\chi^2$.

2.5.4. Precision and accuracy

Intra- and inter-day precision and accuracy were based on analysis of six replicates of the low, medium and high QC samples on three different days. Values were calculated by one-way analysis of variance (ANOVA).

2.6. Animal study

Six beagle dogs (10 ± 1.0 kg) were administered a single oral dose of a UFT capsule. Blood samples (3 ml) were collected into heparinized tubes before and at 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 12 and 16 h after drug administration. Plasma was separated by centrifugation for 10 min at $1000 \times g$ and 4 °C. Plasma was stored at −20 °C pending analysis for TF and 5-FU as described above.

3. Results and discussion

The aim of this study was to develop an HPLC assay for the simultaneous determination of 5-FU and its prodrug TF in plasma superior to a previous HPLC assay described by Gamelin et al. [10]. The previous assay used 0.01 M KH_2PO_4 at pH 3.0 as mobile phase and 5-BU as internal standard. The run-time was about 60 min and the absence of an organic modifier in the mobile phase resulted in some endogenous compounds being retained on the column with consequent reduction in column life. We tested a number of mobile phases and found that 10 mM acetic acid with 10% methanol gave excellent separation of the compounds of interest in a run time of 25 min. Using acetonitrile instead of methanol or >10% methanol resulted in poor separation of 5-FU from endogenous compounds. Phosphate was found to be unnecessary for resolution.

Table 1
Recovery (mean ± S.D.), precision and accuracy for the simultaneous determination of 5-FU and TF in dog plasma by HPLC

Drug	Nominal concentration	Recovery (%)	Measured concentration (mean ± S.D.)	Accuracy (%)	Intra-day precision (R.S.D. (%))	Inter-day precision (R.S.D. (%))
5-FU	4.00 μg/l	50.8 ± 5.1	3.93 ± 0.22	98.2	5.7	5.1
	20.0 μg/l	54.6 ± 5.9	20.07 ± 0.83	100.4	1.9	10.9
	80.0 μg/l	51.4 ± 4.1	80.40 ± 2.53	100.5	1.2	6.6
TF	0.48 mg/l	78.9 ± 6.6	0.48 ± 0.03	100	4.4	13.6
	2.40 mg/l	84.7 ± 7.7	2.46 ± 0.11	102.5	4.2	5.7
	9.60 mg/l	78.9 ± 3.7	9.31 ± 0.32	97	2.7	6.6

Data are based on analysis of six replicates on three separate days.

Chromatograms of blank plasma and a representative calibration standard are shown in Fig. 2. The retention times of 5-FU, 5-BU and FT were 4.4, 8.0 and 21.2 min, respectively. There was no interference from endogenous compounds in dog plasma including uracil [12] (identified by LC/MS/MS), which eluted with a retention time of 4.0 min along with other endogenous compounds. The assay was robust despite the fact that the 5-FU peak is observed in the region of concentrated endogenous peaks.

Recovery, precision and accuracy of the assay are summarized in Table 1. The limit of quantitation of 5-FU was 4 μg/l. Linearity was satisfactory with correlation coefficients of >0.996 for 5-FU and >0.998 for TF. 5-FU and TF were found to be stable in mobile phase at ambient temperature for up to 12 h allowing a large number of samples to be processed in each analytical run.

Mean concentration versus time curves for 5-FU and TF in plasma after a single oral dose of a UFT capsule (TF 100 mg, uracil 224 mg) to six beagle dogs are presented in Fig. 3. As previously found in a human study [5], low plasma concentrations of 5-FU were observed after administration of TF. The maximum concentrations of 5-FU and TF were 17.4 ± 4.78 μg/l (range: 11.9–26.3 μg/l) and 14.5 ± 3.58 mg/l (range: 8.8–17.7 mg/l), respectively, with corresponding times of 90 min (range: 60–120 min) for both compounds. Concentrations of 5-FU were below the limit of quantitation 10 h after the administration of the drug. Detailed results of this pharmacokinetic study will be reported elsewhere.

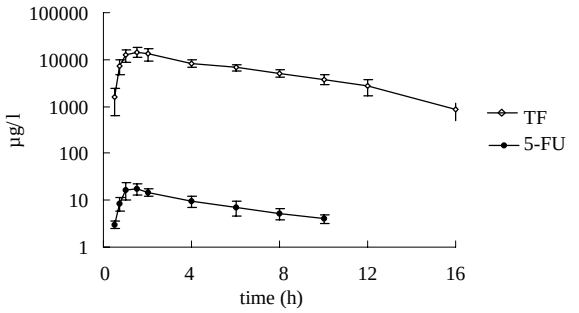

Fig. 3. Plasma concentration vs. time curves for 5-FU and TF after a single oral dose of a UFT capsule (TF 100 mg, uracil 224 mg) to beagle dogs. Each point represents mean ± S.D. ($n = 6$).

In conclusion, the assay reported here for the simultaneous determination of 5-FU and TF in dog plasma is rapid, simple and sensitive. The limit of quantitation of 5-FU is adequate to characterize the disposition of this active metabolite after an oral dose of TF. The method can be applied to studies of the pharmacokinetics of TF and 5-FU in dogs.

Acknowledgements

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