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Journal of Chromatography B, 677 (1996) 199–203

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

High-performance liquid chromatographic determination of 5-fluorouracil and its prodrugs, tegafur and 4-deoxy-5-fluorouracil, in rat plasma

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Received 21 July 1995; revised 12 September 1995; accepted 2 October 1995

Abstract

A rapid, sensitive and reproducible ion-pair, reversed-phase high-performance liquid chromatographic (HPLC) method was developed to simultaneously quantitate 5-fluorouracil (5FU) and its prodrugs, tegafur (TF) and 4-deoxy-5-fluorouracil (DFU), in rat plasma. 5FU, TF, and internal standard, 2′,3′-didehydro-2′-deoxythymidine (D4T, stavudine), were detected by UV absorption at 254 nm, and DFU was detected at 313 nm. Extraction recoveries for all compounds ranged between 80% and 92%. Retention times of 5FU, D4T, DFU and TF were 4.6, 5.5, 6.8 and 11.5 min, respectively. Calibration plots were linear over the range of 0.1 μg/ml to 50 μg/ml for 5FU, and 0.25 μg/ml to 50 μg/ml for TF and DFU. The limit of quantitation was 0.1 μg/ml for 5FU and 0.25 μg/ml for TF and DFU. The intra- and inter-day variations were less than 10% and accuracy was greater than 90%. This method was applied to plasma samples collected from rats that were administered 5FU, TF and DFU intravenously.

Keywords: 5-Fluorouracil; Tegafur; 4-Deoxy-5-fluorouracil

1. Introduction

5-Fluorouracil (5FU) is used in the treatment of a variety of human solid tumors such as breast cancer, cancer of gastrointestinal tract, cancer of head and neck, and ovarian cancer [1]. However, administration of the compound often causes severe gastrointestinal toxicity and myelosuppression [2]. To overcome these toxicities, many derivatives and related compounds have been synthesized. Tegafur (TF), a tetrahydro-2-furanyl derivative of 5FU, in clinical studies has shown antineoplastic activity comparable to that of 5FU against several tumor types including breast cancer [3]. This prodrug acts as a depot form of 5FU and produces little myelosuppression relative to 5FU [4]. Another prodrug of 5FU, 4-deoxy 5-fluorouracil (DFU), has been synthesized [5] and is being investigated as an anticancer agent. The chemical structures of 5FU, TF, and DFU are depicted in Fig. 1.

In order to study the preclinical in vivo distribution and biotransformation of these prodrugs, a method of simultaneous analysis of the prodrugs and

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0378-4347/96/$15.00
SSDI 0378-4347(95)00438-6

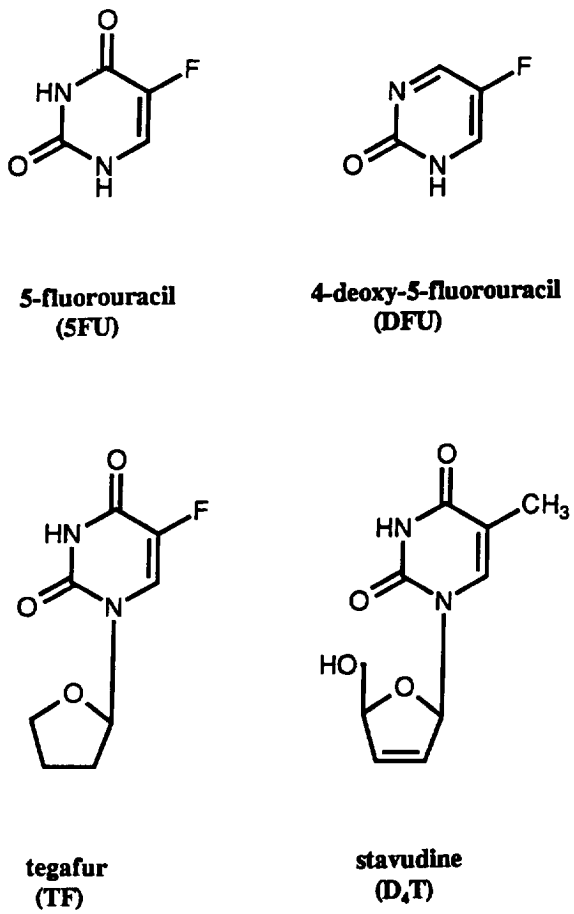

Fig. 1. Chemical structures of 5FU, DFU, TF, and D4T.

the parent compound is essential. Several methods of high-performance liquid chromatographic (HPLC) analysis have been reported for 5FU [6–11]. Although there are a few HPLC methods to simultaneously detect TF and 5FU in human plasma [12,13], there is no analytical method available for the determination of DFU. The objective of this study was to develop a single HPLC analytical method, based on earlier procedures developed by Rustum and Hoffman [10], for the determination of 5FU and its prodrugs, TF and DFU, in rat plasma. This new method was applied to plasma samples collected from rats that were administered 5FU, TF, and DFU intravenously.

2. Experimental

2.1. Chemicals and reagents

5FU, TF, and tetrabutyl ammonium hydroxide were purchased from Sigma (St. Louis, MO, USA). DFU was kindly provided by Dr. C.K. Chu of the Department of Medicinal Chemistry, University of Georgia College of Pharmacy. 2′,3′-Didehydro-2′-deoxythymidine (D4T, stavudine), used as an internal standard (I.S.), was provided by the Developmental Therapeutic Branch, AIDS Program, National Institutes of Health (Rockville, MD, USA). The chemical purity of the compounds determined by spectral and HPLC analysis was greater than 98.5%. HPLC grade acetonitrile and all other chemicals (reagent grade) were purchased from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Preparation of standards

Standard solutions of 100, 10 and 1.0 μg/ml of the compounds were prepared in distilled, deionized water. Standard solutions were added to rat plasma to provide calibration concentrations of 0.1, 0.25, 0.5, 1, 2.5, 5, 12.5, 25 and 50 μg/ml.

2.3. Extraction procedure

Plasma samples (200 μl) were placed in 17 × 100 mm polypropylene centrifuge tubes followed by 50 μl of internal standard, D4T (50 μg/ml). Ice-cold acetonitrile (1 ml) was added to precipitate plasma proteins. The tubes were vigorously mixed for 30 s and centrifuged for 7 min at 9000 g. The supernatant was transferred to a clean polypropylene centrifuge tube, and excess crystalline magnesium sulfate was added. After mixing for 2 min, the tubes were centrifuged for 10 min at 9000 g. The clear supernatant was transferred to another polypropylene tube and evaporated to dryness under a stream of nitrogen gas at ambient temperature. The extraction residue was reconstituted with 200 μl of mobile phase and transferred to a disposable 300-μl polypropylene injection tube. Injection volumes, based on expected drug and prodrug concentrations, ranged from 15 μl to 150 μl.

2.4. Chromatography

The HPLC system consisted of a Waters Model 501 solvent delivery system, a Model 710B WISP autosampler and a Waters Model 440 UV absorbance detector. The two channel UV detector was equipped with a 254 nm filter for the detection of 5FU, TF, and internal standard and a 313 nm filter for the detection of DFU. The detector was set up such that eluant flowed in sequence from one detection cell to the other. Data analysis was performed simultaneously by a Hewlett-Packard Model 3390A integrator for peaks detected at 254 nm and by a LDL Analytical D-2500 computing integrator for peaks detected at 313 nm. A 250 × 4.1 mm I.D., 10-μm particle size Hamilton PRP-1 column (Alltech Assoc, Deerfield, IL, USA) protected by a 25 × 2.3 mm I.D. PRP-1 guard column was used for the separation of the compounds. The mobile phase was 5 m*M* tetrabutyl ammonium hydroxide (pH 11.1):acetonitrile (84:16). The compounds were eluted at a flow-rate of 1.5 ml/min. The chromatography was performed at ambient temperature.

2.5. Quantitation

Concentrations of 5FU, TF and DFU in unknown samples were determined from the slopes of calibration curves of the peak-area ratio (drug/internal standard) versus standard drug concentrations. Slopes were determined using a linear regression analysis with a weighting factor of $1/x^2$ [14]. Use of this weighting factor generated a normal distribution of weighted residuals around the standard curve over the entire range of drug concentrations.

2.6. Assay specifications

Extraction recoveries of 5FU, TF, and DFU were assessed at plasma concentrations of 0.25, 2.5 and 25 μg/ml. The peak areas from six extracted plasma samples and from six direct injections of the same amount of drug in water were compared. The percentage extraction recovery was computed from (peak $\text{area}_{\text{extract}}$/mean peak $\text{area}_{\text{direct injection}}$) × 100%.

The intra- and inter-day accuracy and precision of the analytical method were determined by analysis of six plasma samples containing 25 μg/ml, 2.5 μg/ml, and 0.25 μg/ml concentrations for the three compounds. To determine inter-day precision, the quality control samples were divided into 200 μl aliqouts and frozen at −20°C. Over a period of three days, samples were thawed and assayed. Assay

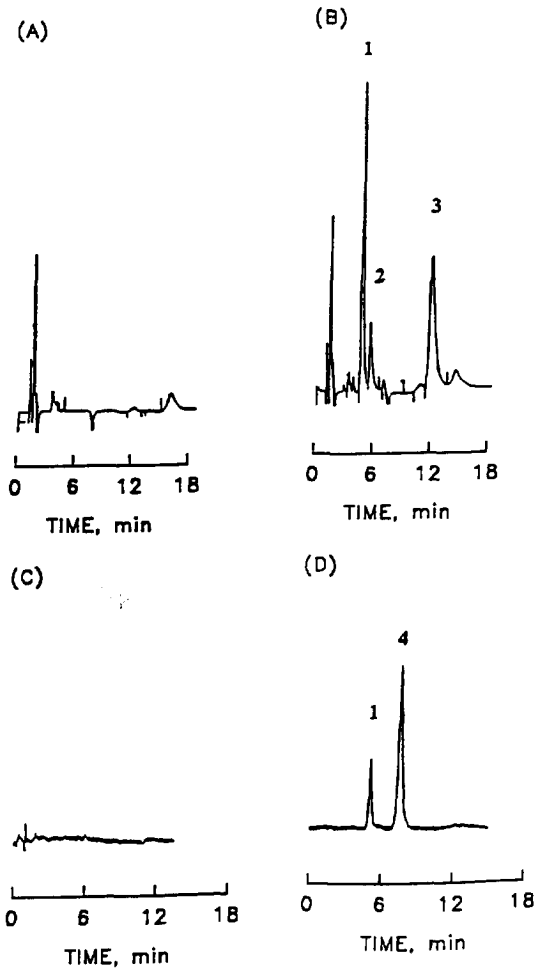

Fig. 2. Representative chromatograms for (1) 5FU, (2) D4T, (3) TF, and (4) DFU in (A) blank plasma using a UV detection wavelength of 254 nm, (B) plasma with 5FU, D4T, TF, and DFU added at 254 nm, (C) blank plasma using a UV detection wavelength of 313 nm, and (D) plasma with 5FU, D4T, TF, and DFU added at 313 nm. The concentrations of 5FU, TF and DFU were 25 μg/ml.

precision was determined by calculating relative standard deviations for each drug concentration. Accuracy was calculated by comparing measured concentrations to the known values.

2.7. Animal studies

Rats were administered 50 mg/kg (385 μmol/kg) of 5FU or an equimolar dose of TF (77 mg/kg) or DFU (44 mg/kg) intravenously. Blood samples were collected at selected times after compound administration into heparinized microcentrifuge tubes. Plasma was obtained by centrifugation and stored at −20°C until analysis.

3. Results and discussion

The objective of this study was to develop a single HPLC analytical method for the determination of 5FU and its prodrugs, TF and DFU, in rat plasma. The newly developed method was based on earlier procedures developed by Rustum and Hoffman for the determination of 5FU [10]. In addition to measuring prodrug concentrations, as well as 5FU in plasma, the present method employs a simplified extraction procedure and an internal standard. The method presented here also uses two different wavelengths to detect 5FU and its prodrugs simultaneously.

Chromatograms corresponding to extracts of (a) blank rat plasma using a UV detection wavelength of 254 nm, (b) a plasma sample spiked with 5FU (25 μg/ml), TF (25 μg/ml) and I.S. (2.5 μg/ml) using 254 nm, (c) blank rat plasma using a UV detection wavelength of 313 nm, and (d) a plasma sample containing DFU (25 μg/ml) using 313 nm are illustrated in Fig. 2. Each compound eluted with a sharp peak and distinct separation at baseline. The retention times of 5FU, D4T, DFU, and TF were 4.6, 5.5, 6.8, and 11.5 min, respectively. Drug free plasma samples were consistently free of interferences at the retention times corresponding to the compounds of interest.

The limit of quantitation of the assay was 0.1 μg/ml for 5FU and 0.25 μg/ml for both prodrugs at a signal-to-noise ratio of 3. Calibration plots were linear over the range 0.1–50 μg/ml (0.77–385 μM) for 5FU, 0.25–50 μg/ml (1.25–250 μM) for TF, and 0.25–50 μg/ml (2.20–440 μM) for DFU. The assay specifications including, extraction recovery, intra- and inter-day precision and accuracy for 5FU, TF, and DFU at 0.25, 2.5, and 25 μg/ml concentrations, are presented in Table 1. The extraction recovery of 5FU, TF, and DFU was similar for the three compounds at all concentrations studied, averaging approximately 85%. The intra- and inter-day precision of the assay was satisfactory. As shown in Table 1, relative standard deviations were less than 10%. The accuracy of the analytical method was greater than 90% for all three compounds at low, medium, and high concentrations.

Plasma concentrations as a function of time for (a) 5FU after intravenous administration of 5FU, (b) TF and 5FU after intravenous administration of TF, and (c) DFU and 5FU after intravenous administration of DFU are shown in Fig. 3. Both prodrugs yielded parent drug, however higher plasma concentrations

Table 1
Assay specifications

Compound	Concentration (μg/ml)	Recovery (%)	Precision (%)		Accuracy (%)	
			Intra-day	Inter-day	Intra-day	Inter-day
5FU	0.25	83.3±8.4	9.34	5.60	95.60	94.34
	2.50	83.8±9.5	5.28	7.49	98.62	93.24
	25.0	91.9±9.0	8.47	2.61	99.80	96.26
TF	0.25	81.2±8.9	7.50	8.93	92.94	98.42
	2.50	82.7±6.3	4.58	3.98	96.34	98.12
	25.0	92.3±7.1	9.29	3.11	98.04	98.31
DFU	0.25	80.4±6.7	6.73	9.33	97.60	90.80
	2.50	81.9±8.2	5.47	4.78	94.34	96.52
	25.0	85.0±8.9	4.69	5.30	94.79	99.15

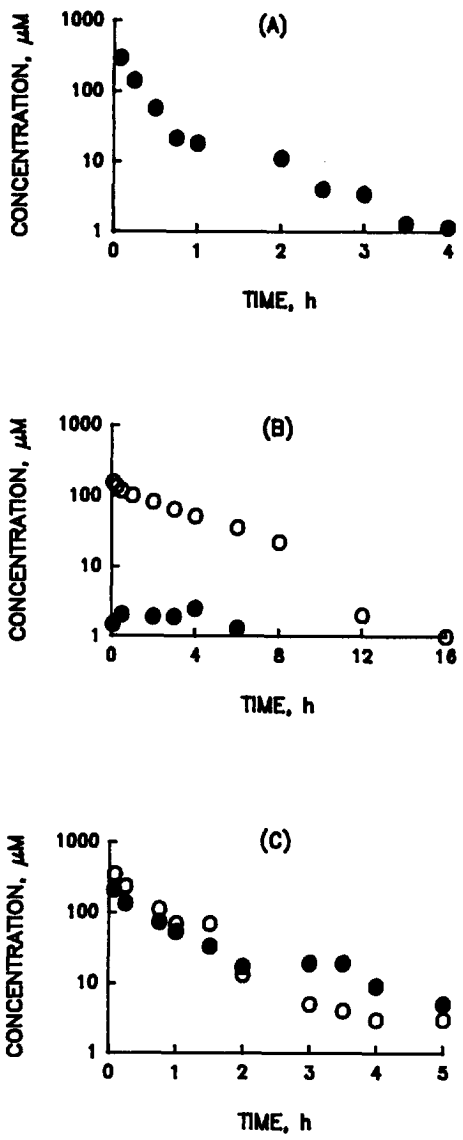

Fig. 3. Plasma concentration versus time profiles of (A) 5FU (●) after intravenous administration of 5FU, (B) TF (○) and 5FU (●) after intravenous administration of TF, and (C) DFU (○) and 5FU (●) after intravenous administration of DFU.

of 5FU were observed after administration of DFU than after TF.

The determination of 5FU and its prodrugs, TF and DFU, in rat plasma by this HPLC method is rapid, sensitive and reproducible. The limit of quantitation of this method was sufficient to characterize the disposition of the prodrugs and their bioconversion to 5FU. This method can be applied to studies on the pharmacokinetics of 5FU, TF, and DFU in rats.

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