Determination of 5-Fluorouracil in Serum Microsamples by a High-Performance Liquid Chromatographic Method and its Application to Pharmacokinetic Studies in Rats

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Abstract

A high-performance liquid chromatographic method is described for the quantitative determination of 5-fluorouracil (5FU) in only 30 µL of serum. A reversed-phase C_{18} column is used for the separation and analysis of 5FU. The mobile phase consists of methanol–20mmol/L phosphate buffer, pH 6.80 (5:95, v/v). The calibration curve for 5FU is linear over the range of 0.05–10 µg/mL. The extraction recoveries of 5FU and *p*-aminobenzoic acid (as an internal standard) are > 91.28% and 81.98%, respectively. The intra-day and inter-day coefficients of variation of 5FU are less than 6.29% and 9.57% at four different concentrations, respectively. The method is simple, sensitive, and reliable. It is superior to previous methods in that the sample volume needed is relatively small (just 30 µL of serum). Therefore, this method can be utilized to determine 5FU in rats for study in various fields, especially in pharmacokinetics, controlled-release, and combination therapy with 5FU, etc. In this study, it is successfully applied to pharmacokinetic studies of 5FU after administrations of an intravenous bolus dose (25 mg/kg) and two oral doses (25 and 75 mg/kg) in rats.

Introduction

5-Fluorouracil (5FU) is one of the most widely used anticancer drugs for the treatment of malignant tumors, including colorectal, breast, head and neck, and pancreatic cancers (1–4). To improve therapeutic effects and reduce toxicity, many new dosage forms have been designed and developed, such as a controlled-release preparation of 5FU. In the research for the development of the new dosage forms, pharmacokinetic studies of 5FU in laboratory animals are indispensable, especially in small laboratory animals. Therefore, it is essential to develop a sensitive and precise analytical method to determine the concentration of 5FU in biological matrices.

Although numerous high-performance liquid chromatography (HPLC) methods for the determination of 5FU have been previously reported (5–8), large volumes (100–500 µL) of serum or plasma were required. The sample size was too much to be feasible for studying the pharmacokinetics of 5FU in small laboratory animals (e.g., rats), because blood samples had to be drawn repeatedly to trace temporal changes in blood levels of the drug in individual animals. Therefore, we developed an HPLC method for analyzing 5FU in serum (9), and then improved its HPLC conditions by means of adjusting the pH of the mobile phase and optimizing the extraction solvent of 5FU to make it more sensitive and reliable.

Here we report an optimized method of the determination of 5FU in serum by HPLC. The new method just needs as small as 30 µL of serum. Compared with previous methods, the volume of serum for determination is greatly reduced to 30 µL. Because a microvolume (30 µL) of serum is enough to assay 5FU, blood can be drawn repeatedly from small laboratory animals after administration so that it is possible to study the pharmacokinetics of 5FU in rats. As a result, the method can be applied to rats for further research and development of 5FU, such as studies on the pharmacokinetics of new dosage forms of 5FU, release of 5FU from its controlled-release preparation, combination therapy with 5FU, etc. Therefore, it is necessary to develop a simple and sensitive method using small volumes of serum for the quantitative determination of 5FU.

Experimental

Reagents and materials

5-Fluorouracil and *p*-aminobenzoic acid (PABA) were purchased from Fluka Chemie AG (CH-9470 Buchs, Switzerland). Methanol was of HPLC grade and other chemicals used were of analytical grade.

Instrumentation and chromatographic conditions

The HPLC system (Agilent 1100 series, Palo Alto, CA) consisted of a quaternary pump, a mobile phase vacuum degassing unit, an autosampler, diode array, and multiple-wavelength detector. A C_{18} column (250 mm × 4.6mm, 5 µm,

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Hypersil, Dalian Elite Scientific Instrument Co., Dalian, China) was used for chromatographic separations. The wavelength of the detector was set at 270 nm. The composition of mobile phase was 5% methanol in 20 mmol/L phosphate buffer (pH6.80). The flow-rate was 0.7 mL/min. All the analyses were performed at 20ºC.

Preparation of standard solutions and samples

5FU and PABA as an internal standard were individually dissolved in methanol to make the standard stock solutions with concentrations of 1 mg/mL. The stock solutions were kept at 0–4ºC before use. Six standard solutions of 5FU were prepared for the calibration curve at the following concentration levels: 0.05, 0.1, 0.5, 1, 5, and 10 µg/mL, and they were made by further dilution of the stock solution with methanol. The internal standard (8 µg/mL) of PABA was also prepared from its stock solution.

Samples, as quality control standards (QC), were extracted with the following procedure: 15 µL of internal standard (PABA, 8 µg/mL) and 30 µL of standard were added to a 10-mL centrifuge tube, and the solvent was evaporated to dryness in an evaporator (Pierce, IL) at 60°C under nitrogen gas. Thirty microliters of serum were added and mixed well, then 1 mL of ethyl acetate–ethanol solution (ethyl acetate–ethanol, 75:25, v/v) was added. The mixture was rigorously vortexed for 1 min and centrifuged for 5 min at 10000 g to separate the different phases. The organic phase was carefully transferred into a fresh tube and evaporated under an N_2 flow to dry at 60°C. The residue was redissolved in 100 µL of mobile phase and mixed well. Then 60 µL of the reconstituted sample were injected onto the HPLC column.

The blood samples for serum drug analysis after administration were prepared identically except that 5FU standards were not added.

Method validation

Six standard solutions for the calibration curve were at the concentrations of 0.05, 0.1, 0.5, 1, 5, and 10 µg/mL. Standards at each concentration were extracted and analyzed as mentioned previously. The calibration curve, which was based on peak height ratio of 5FU to PABA, was constructed by least-square linear regression.

QCs to determine the recovery, accuracy, and precision were at four concentration levels (0.05, 0.5, 5, and 10 µg/mL) in the linear range for 5FU. They were extracted following the extraction procedure, and four unextracted standards at the same concentration levels were directly injected onto the column without extraction. The recoveries were calculated by the following equation:

Recovery (%) = (peak height of extracted standard) / (mean peak height of unextracted standards) × 100

The intra-day assay variability was assessed with replicates (n = 6) and the inter-day assay was done in five different days in the same concentration levels. The precisions were expressed as coefficient of variation (C.V. %) and accuracies were expressed as bias calculated by the following equation:

$$\text{Bias (\%)} = [(C_{obs} - C_{nom}) / C_{nom}] \times 100$$

Where C_{obs} and C_{nom} are the observed and the nominal concentrations, respectively.

The limit of detection (LOD) of 5FU was determined at a signal-to-noise ratio of 3.

Application to pharmacokinetics in rats

Animals and blood sampling

Four male, virus-free waster rats with a mean body mass of 304 g (± 8 g), were obtained from Capital University of Medical Sciences (Beijing, China). The right jugular vein was cannulated with a catheter. The animals were allowed to recover for four days from the catheterization prior to the drug administration series.

5FU was dissolved in 0.9% NaCl, and the drug solutions were given intravenously via the jugular vein catheter and orally by gavage, respectively, to four rats. All injections were given in a volume of 1 mL/kg body mass. Animals first received an i.v. 25 mg/kg dose via the jugular vein catheter. 5FU solution was delivered in 15 s. To ensure that the drug solution was completely administrated, 0.3 mL of sterile saline were delivered in 15 s to wash the drug from the catheter. After four days, animals also received an escalating oral 5FU dosing series (25 and 75 mg/kg), with each dose separated by four-day intervals. Before injecting drug solutions, a blood sample was taken as a blank. Serial blood samples (70 µL) were individually collected through jugular vein catheter at 2, 5, 10, 15, 20, 30, 60, 90, 120, and 180 min post-administration. In addition, two more blood samples were taken at 240 and 360 min after p.o. 75 mg/kg. An equal volume of sterile saline was injected to maintain a constant blood volume after each sampling. Blood samples were centrifuged at 10000 g for 10 min to separate serum. The serum samples were stored at –20°C until analysis by HPLC.

Pharmacokinetic analysis

Pharmacokinetic analysis was performed using WinNonlin 4.2 software system (Pharsight, CA). The data was described by an open two-compartment model for 5FU and fitted to the following equation:

$$Cp = Ae^{-\alpha t} + Be^{-\beta t}$$

Where Cp is the total serum drug concentration at time t; A and B are the extrapolated zero intercepts; α and β represent the apparent first-order distribution and elimination rate constants, respectively. The bioavaibility (F) of 5FU was estimated by the following equation:

$$F = \frac{(\text{AUC/Dose})_{po}}{(\text{AUC/Dose})_{iv}}$$

In addition, the pharmacokinetic parameters were calculated from serum concentration-time data by WinNonlin. These parameters were: apparent volume of distribution (V_1), elimination rate constant from central compartment (K_{10}), clearance (CL), distribution half-life ($t_{1/2}\alpha$), elimination half-life ($t_{1/2}\beta$), and area under serum concentration–time curve (AUC). Maximum

serum concentration (C_{max}) and time to reach a C_{max} (T_{max}) were reported as observed. All parameters are shown in Table I.

Results and Discussion

Chromatograms

The typical chromatograms of 5FU are shown in Figure 1. Interferences to 5FU were not observed in the blank serum (Figure 1A). 5FU and PABA were separated well and did not interfere with each other (Figure 1B). A representative chromatogram of a blood sample, obtained from a rat at 10 min following intravenous administration of 5FU (25 mg/kg), is shown in Figure 1C.

Linearity and extraction recovery

The relation between 5FU concentration and its peak height ratio of 5FU to PABA was linear from 0.05 to 10 µg/mL (y = 0.2137x + 0.0046, R^2 = 0.9986). The limit of detection of 5FU, at a signal-to-noise ratio of 3, was 20 ng/mL.

Table I. Pharmacokinetic Parameters for 5FU i.v. and p.o. Route Administrations in Rats (n = 4)

Dose	Parameter	Mean ± SD	Unit
i.v. 25 mg/kg	C_{max}	21.00 ± 3.26	µg/mL
	$t_{1/2}\alpha$	4.57 ± 1.29	min
	$t_{1/2}\beta$	22.65 ± 5.88	min
	K_{10}	0.116 ± 0.021	min
	V1	1208 ± 174	mL/kg
	CL	137.2 ± 12.3	mL/min/kg
	AUC	183.2 ± 16.5	min/mg/mL
p.o. 25 mg/kg	C_{max}	0.697 ± 0.044	µg/mL
	T_{max}	30.0 ± 0.0	min
	AUC	33.04 ± 5.06	min/mg/mL
	F	18.03 ± 2.76	%
p.o. 75 mg/kg	C_{max}	1.699 ± 0.503	µg/mL
	T_{max}	30.0 ± 0.0	min
	AUC	122.1 ± 40.9	min/mg/mL
	F	22.22 ± 7.45	%

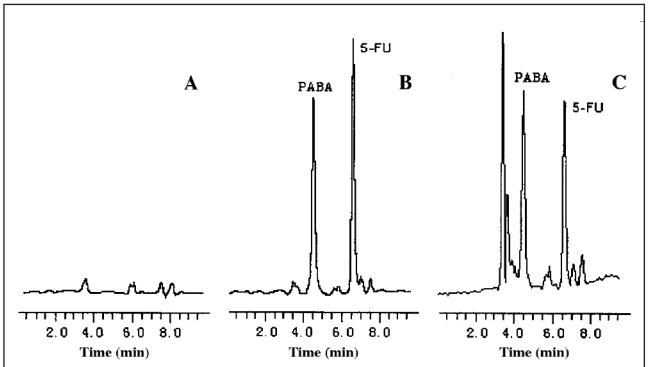

Figure 1. Chromatograms of a blank serum (A), a QC sample containing 5 µg/mL 5FU and 4 µg/mL PABA (B), and a rat serum sample obtained from a subject at 10 min after administration of i.v. 25 mg/kg 5FU (C).

The recoveries of 5FU and PABA from rat serum are summarized in Table II. The recoveries were consistent and less variable over the concentration range as shown. The average recovery of 5FU was 92.40% over the four concentrations. The average recovery of PABA was 82.82%.

Precision and accuracy

The internal standard method was used in the calibration and evaluation of unknown samples because the ratio of 5FU to PABA for each concentration is invariable in analysis process. PABA had been added prior to performing the extraction in order to correct irreproducibility imparted in the extraction process. The intra- and inter-day precisions of 5FU were evaluated at four different concentrations (0.05, 0.5, 5, and 10 µg/mL) by adding 5FU to blank serum. Bias values varied from –1.86% to 2.68%. Both intra- and inter-day data of 5FU are listed in Table III. The precision and accuracy of the assay are acceptable according to *Guidance for Industry* issued by US FDA (10), and they are sufficiently precise and accurate for application to pharmacokinetic studies.

Application to pharmacokinetic studies in rats

The validated method was successfully applied to the quantitation of pharmacokinetic studies. The serum 5FU concentration-time profiles were shown in Figure 2. The mean concentrations (symbols) of 5FU were determined after administrating bolus i.v.

Table II. Extraction Recoveries of 5FU and PABA in Rat Serum (n = 6)

Compound	Concentration (µg/mL)	Recovery (%) (Mean ± SD)	C.V. (%)
5FU	0.05	91.28 ± 6.91	7.57
	0.5	94.86 ± 5.75	6.06
	5	91.37 ± 4.33	4.74
	10	92.09 ± 2.13	2.31
PABA	4	83.67 ± 2.34	2.80
	8	81.98 ± 3.17	3.87

Table III. Precision and Accuracy of the HPLC Method for the Determination of 5FU in Rat Serum

Nominal concentration (µg/mL)	Observed concentration (Mean ± SD) (µg/mL)	Precision C.V. (%)	Accuracy Bias (%)
Intra-day (n = 6)			
0.05	0.0509 ± 0.0032	6.29	1.80
0.5	0.4991 ± 0.0085	1.70	–0.18
5	5.0058 ± 0.2806	5.61	0.12
10	10.007 ± 0.3016	3.01	0.07
Inter-day (n = 5)			
0.05	0.0512 ± 0.0049	9.57	2.40
0.5	0.4907 ± 0.0211	4.30	–1.86
5	5.0134 ± 0.2837	5.66	2.68
10	9.9987 ± 0.3580	3.58	–0.01

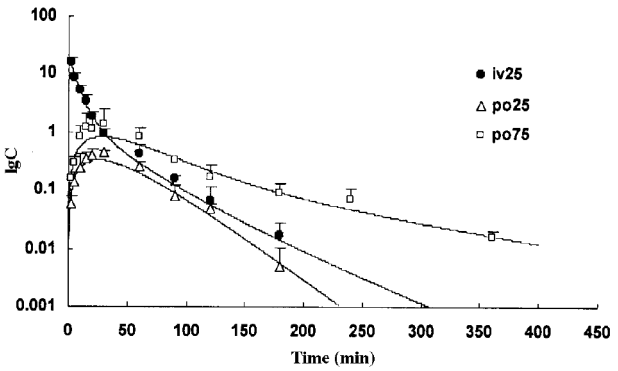

Figure 2. Concentration-time profiles of 5FU in serum following administrations of bolus doses of i.v. (25 mg/kg) and p.o. (25 and 75 mg/kg) in rats (n = 4).

(25 mg/kg) and p.o. (25 and 75 mg/kg), respectively, to four rats. The solid lines in Figure 2 were predicted to indicate serum concentration-time profiles. The pharmacokinetic parameters of 5FU are listed in Table I.

Conclusion

In the present study, we validated an HPLC method for the quantitative determination of 5FU in 30 µL of rat serum. The method has sufficient sensitivity with good precision and accuracy. It is simple, sensitive, and reliable. It is superior to previous methods in that the sample volume for the analysis of 5FU is relatively small (just 30 µL of serum). Therefore, it is significant that this method will be utilized to determine 5FU in rats for study in various fields, especially on the pharmacokinetics of new dosage forms of 5FU, release of 5FU from its controlled-release preparation, and combination therapy with 5FU, etc. In this study, it has been successfully applied to pharmacokinetic studies of 5FU after administrations of an intravenous bolus dose (25 mg/kg) and two oral doses (25 and 75 mg/kg) in rats.

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