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Note

Rapid and sensitive method for the determination of 5-fluorocytosine in human plasma by ion-pair high-performance liquid chromatography

HIDEO HOSOTSUBO

Central Laboratory for Clinical Investigation, Osaka University Hospital, 1-1-50, Fukushima-ku, Osaka 553 (Japan)

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5-Fluorocytosine (5-FC) is a fluorinated pyrimidine and an oral antifungal agent and is currently the drug of choice for the treatment of severe systemic fungal infections. The major limitation to the use of 5-FC is its toxicity as some patients develop adverse reactions, dose-related hepatotoxicity [1] and depression of bone marrow function [2]. There is some evidence that 5-FC is metabolized to 5-fluorouracil to a small extent in man [3], but about 80–90% of a given dose is excreted in the urine. However, it is accumulated in patients with impaired renal function [4]. 5-FC therapy therefore involves a balancing of toxic effects against antifungal activity [5].

Many of the problems associated with pharmacokinetic studies of 5-FC arise from the difficulty in obtaining rapid, accurate and reproducible measurements of drug levels in various biological fluids. Monitoring of the concentrations of this drug in serum during treatment may assist clinical management. Only a few procedures exist for the measurement of 5-FC, including microbiological [6], fluorimetric [7], gas chromatographic [8,9] and high-performance liquid chromatographic (HPLC) utilizing ion-exchange [10,11] or reversed-phase [12–14] column methods. The microbiological method is time-consuming, insensitive, imprecise, difficult to standardize and shows considerable variability. The fluorimetric method is slow and the recovery is variable. Gas chromatographic methods have many advantages over the more commonly used microbiological method but have the disadvantage of low recovery (30–40%) and the requirement for derivatization of 5-FC.

Several HPLC methods [10–14] have been reported that offer faster and more accurate and reproducible alternatives for both pharmacokinetic studies [4,5]

0378-4347/89/$03 50

and routine clinical use. HPLC also offers improved sensitivity and specificity and is easier to standardize than bioassay. This paper describes a simple, accurate, selective and sensitive method for the determination of 5-FC by ion-pair reversed-phase HPLC that does not require extraction of the 5-FC or the use of internal standards.

EXPERIMENTAL

Chemicals

Organic solvents of HPLC grade were purchased from Katayama Chemical (Osaka, Japan). Purified water was obtained using a Milli-Q system equipped with ion-exchange, organic and carbon filters (Japan Millipore, Tokyo, Japan). The ion-pairing agent, sodium octanesulphonate, was purchased from Kanto Chemical (Tokyo, Japan). 5-FC was a gift from Nippon Roche (Tokyo, Japan).

HPLC instrumentation

The HPLC instrumentation consisted of a Model LC-6A system (Shimadzu, Kyoto, Japan) and a Model 7125 sample injection valve (Rheodyne, Cotati, CA, U.S.A.) with a 5-μl injection loop. Ion-pair reversed-phase chromatographic analysis was performed on a Shimadzu Shim-pack CLC-C8 (5 μm) column (150 mm$\times$6.0 mm I.D.). A Shimadzu SPD-6A variable-wavelength UV detector set at 280 nm was used. Peak height was integrated by a Shimadzu C-R4A computing integrator.

Chromatographic conditions

The mobile phase was 5 m*M* sodium octanesulphonate–methanol–acetic acid (70:30:1, v/v) (pH 3.0) at a flow-rate of 1.0 ml/min. The column temperature was maintained at 22–25 °C. The column effluent was monitored at 280 nm with a sensitivity setting of 0.10 a.u.f.s. and the integrator attenuation was set at 5.

Sample preparation

In a 1.5-ml microcentrifuge tube were placed 0.1 ml of plasma standard or unknown plasma sample and 0.1 ml of acetonitrile. After mixing on a vortex mixer for 5 s and then allowing to stand for 5 min, the sample was centrifuged for 2 min at 12 000 *g* and a 5-μl aliquot of the clear supernatant was injected into the chromatographic system. All preparations were performed at 22–25 °C.

Calibration procedure

A stock solution of 5-FC was prepared in water at a concentration of 4.0 g/l. Eleven reference samples containing 10, 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 μg/ml 5-FC were prepared by diluting this stock solution with drug-free pooled plasma. These samples were used to assess the calibration, accuracy and precision of the assay. Quantification was effected by comparing the peak height of an unknown with a calibration graph.

Application to clinical study

A 2-g dose of 5-FC was administered orally to two patients (A, 38 years old, male; B, 52 years old, male). Blood samples were taken at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 h following the dosing. Blood samples were collected in K_2EDTA-coated tubes, centrifuged for 10 min at 3000 *g* (4°C) and the plasma was stored at −70°C until analysis.

RESULTS

Chromatographic separation

The chromatogram of drug-free plasma is shown in Fig. 1A. Any endogenous compounds absorbing at 280 nm appear to elute with the solvent front as no later peaks are seen. 5-FC is well separated from the front peak; it has a retention time of 5.8 min (Fig. 1B and 1C). The total time required (from sample preparation until elution of 5-FC) was less than 15 min. Good resolution of the 5-FC peak allowed its quantification by measurement of the peak height. It was not necessary to use an internal standard with this method as suitable coefficients of variation (C.V.) were obtained for intra- and inter-assay reproducibility (Table I).

Precision

To the define intra- and inter-assay precision for plasma specimens with the HPLC method, each of the five reference assays were analysed (Table I). The coefficients of variation ranged from 1.90 to 3.20% for the intra-assay precision and from 2.13 to 5.93% for the inter-assay precision.

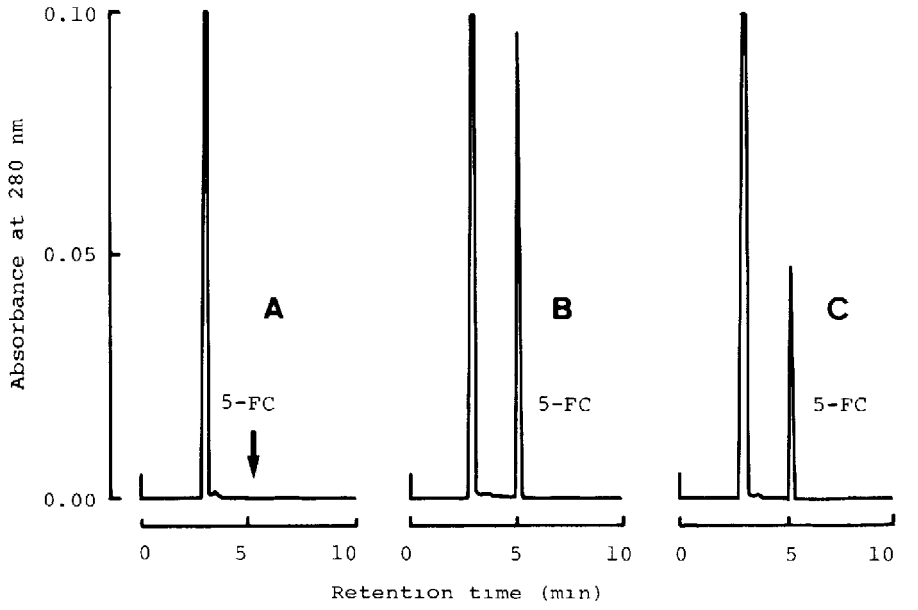

Fig. 1. High-performance liquid chromatograms of plasma samples. (A) Drug-free plasma extract; (B) spiked plasma containing 100 μg/ml 5-FC; (C) plasma collected from a patient 4 h after an oral dose of 2 g of 5-FC (calculated concentration of 5-FC, 45 6 μg/ml).

TABLE I

PRECISION OF THE METHOD FOR 5-FC IN PLASMA

5-FC concentration (μg/ml)	Observed concentration (μg/ml)			
	Intra-assay ($n=10$)		Inter-assay ($n=5$)	
	Mean ± S.D.	C.V. (%)	Mean ± S.D.	C.V. (%)
20	19.7 ± 0.63	3.20	20.4 ± 1.21	5.93
40	40.2 ± 1.15	2.86	40.7 ± 1.74	4.28
60	59.1 ± 1.28	2.17	60.2 ± 2.18	3.62
80	80.8 ± 1.73	2.14	79.8 ± 2.27	2.84
120	121.4 ± 2.31	1.90	118.7 ± 2.53	2.13

TABLE II

RECOVERY OF 5-FC FROM PLASMA USING THE HPLC METHOD

5-FC added (μg/ml)	5-FC found (mean ± S.D., $n=6$) (μg/ml)	Recovery (mean ± S.D.) (%)
10	9.8 ± 0.36	98.0 ± 3.6
20	19.6 ± 0.58	98.0 ± 2.9
40	40.4 ± 1.23	101.0 ± 3.1
60	60.1 ± 1.32	100.2 ± 2.2
80	80.6 ± 1.82	100.8 ± 2.3
120	119.8 ± 2.41	99.8 ± 2.0
	Mean	99.6 ± 2.7

Recovery and calibration graph

The analytical recovery of 5-FC added to plasma is shown in Table II.

A calibration graph was generated by least-squares linear regression analysis of the peak height of 5-FC and was linear over the range of drug concentration used (10–200 μg/ml, $n=6$). The calculated equation was $y=0.027x-0.02$ ($n=11$) with a correlation coefficient of 0.999. The detection limit was 0.1 μg/ml (signal-to-noise ratio 2:1).

Interference studies

Interference of different drugs with the 5-FC peak was also studied. About twenty commonly prescribed drugs were tested and shown not to interfere. These included seven antifungal drugs (amphotericin, clotrimazole, griseofulvin, ketoconazole, miconazole, natamycin and nystatin), six antibiotics (amikacin, ampicillin, chloramphenicol, gentamicin, tobramycin and vancomycin), aspirin, ethenzamide, ibuprofen, paracetamol, salicylamide and salicylate. They all eluted much earlier (<4 min) than the 5-FC peak under the same chromatographic conditions.

TABLE III

PLASMA CONCENTRATIONS OF 5-FC AFTER AN ORAL DOSE OF 2 g TO TWO HUMAN PATIENTS

Time (h)	Plasma concentration (μg/ml)	
	Patient A	Patient B
0	0	0
0.5	20.5	15.8
1	44.6	22.4
2	59.7	29.6
4	44.3	58.2
6	29.6	46.3
8	25.4	37 1
12	15.3	24.7
24	4.2	12.9

Clinical application studies

The method was applied to plasma samples from two human subjects after a single oral dose of 2.0 g of 5-FC. The results in Table III show that the plasma levels reached a maximum of about 60 μg/ml 2 and 4 h after dosing for patients A and B, respectively. The plasma concentrations then declined with apparent half-lives of 4 h (patient A) and 7 h (patient B) for the terminal slope.

DISCUSSION

The method for the HPLC assay of 5-FC described here is a significant improvement over those previously reported [6–14] in terms of sensitivity, ease of sample preparation and simple extraction procedure. For conventional reversed-phase columns [12–14] 5-FC was poorly retained and only new, high-efficiency columns had sufficient resolution to allow quantitation. Ion-exchange columns [10,11] could resolve 5-FC, but a poor column efficiency and band broadening effects limited this method to samples containing high concentrations of 5-FC. However, ion-pair reversed-phase HPLC eliminates these problems.

The use of sodium octanesulphonate as the counter ion provided sufficient column capacity for resolving 5-FC from unretained sample components, as the 5-FC was converted into a cation for chromatography by using an acidic mobile phase (pH 3.0). The octanesulphonyl ion was added to the mobile phase to pair with the 5-FC cation and increase its k' value. One could selectively retain 5-FC on the column by increasing the concentration of the sodium octanesulphonate. No interference from endogenous substances or selected drugs occurred in this region of the chromatogram.

The proposed method quantified the results by using an external standard. The theoretical considerations of and limitations to the use of an internal standard and its effect on assay precision have been previously discussed [15]. For this

assay, an internal standard would not improve the precision because sample injection, which otherwise is the principal source of error in the external standard calibration procedure, included the entire contents of a fitted sample injection loop. In addition, the use of an internal standard would not correct for potential errors in a sample preparation. The advantage of this method is its simplicity, the absence of a need for extraction, internal standard or derivatization and its usefulness for the range of drug concentrations normal for 5-FC in plasma. This assay should prove useful in pharmacokinetic studies and has the additional advantages of speed (7–8 min per injection) for routine use in the clinical laboratory.

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