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Automated determination of 5-fluorouracil and its metabolite in urine by high-performance liquid chromatography with column switching

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Abstract

We report a quantitative assay of 5-fluorouracil (FU) and its metabolite, 5-fluorodihydrouracil (FDHU) in human urine by used a column-switching high-performance liquid chromatographic method. The analyses were carried out using a molecular exclusion column for sample purification, and a cation-exchange column for separation. Each sample required only 40 min to analyze, and required no preparation other than filtration. Linearity was verified up to 1000 nmol/ml ($r>0.993$). The recovery of FU was 96–101%; recovery of FDHU was 96–105%. The imprecision (RSD) for FU (10–100 nmol/ml) was $<1.5\%$, same-day ($n=5$), and $<1.8\%$, day-to-day ($n=5$). The imprecision (RSD) for FDHU (10–100 nmol/ml) was $<3.2\%$, same-day ($n=5$), and $<4.0\%$, day-to-day ($n=5$). The detection limits were, respectively, 0.1 nmol/ml. We measured FU and FDHU in urine of seven cancer patients after oral administration of FU. The cumulative quantity ratio of the FDHU and FU (FDHU/FU) excreted in their urine within 120 min after FU administration was a constant value in all seven patients. Based on these results, we believe that our method provides a useful tool for evaluating FU metabolism.

Keywords: 5-Fluorouracil; 5-Fluorodihydrouracil

1. Introduction

Although 5-fluorouracil (hereafter referred to as FU) is one of the most widely used anti-cancer drugs; many details concerning its mode of action and its kinetics within the human body remain largely unknown.

FU is metabolized primarily in the liver, being metabolized in the pyrimidine metabolic system. In this process, FU is first metabolized to dihydropyrimidine dehydrogenase (DPD) by 5-fluorodihydrouracil (hereafter referred to as FDHU),

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and then metabolized to 2-fluoro-β-alanine by dihydropyrimidinase (DHPase) [1], with both of these metabolites being excreted in the urine (Fig. 1).

As approximately 80% of the administered FU is metabolized in this manner [2], the levels of FU in the blood of patients with pyrimidine metabolic disorders such as DPD deficiency, etc., tend to be abnormally high, and could possibly trigger serious adverse reactions [3]. Two such incidents were reported in Japan (Japan Cancer Society, Japan Cancer Treatment Society, 1997). In both cases, the patients showed severe myelosuppression; conditions believed to be caused by decreased DPD activity.

Although the importance of testing for DPD deficiency in order to prevent adverse reactions to FU has been recognized in Europe and the USA, the current method of using peripheral blood mononuclear cells to measure DPD activity [4–6] is both complicated and unsuited to screening which occurs before the FU is administered. With the efficacy of FU treatment established, a simple method of screening for pyrimidine metabolic disorders is essential in order to prevent adverse reactions to FU.

We report quick and easy measurement of the levels of FU and FDHU in urine with a high-performance liquid chromatography (HPLC) column-switching method.

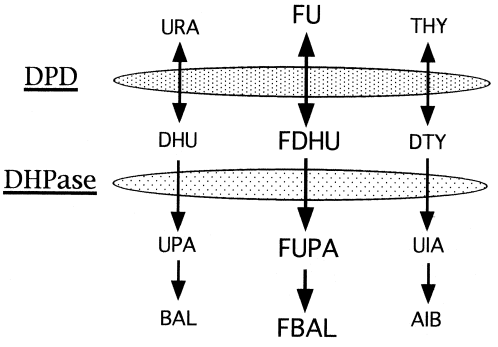

Fig. 1. Pyrimidine catabolic pathway. DPD, dihydropyrimidine dehydrogenase; DHPase, dihydropyrimidinase; FU, 5-fluorouracil; FDHU, 5-fluorodihydrouracil; FUPA, 2-fluoro-3-ureidopropionate; FBAL, α-fluoro-β-alanine; URA, uracil; DHU, dihydrouracil; UPA, β-ureidopropionate; BAL, β-alanine; THY, thymine; DTY, dihydrothymine; UIA, β-ureidoisobutyric acid; AIB, β-amino-isobutyric acid.

Moreover, we tested our method with seven cancer patients who were orally administered FU. The FU and FDHU levels in their urine were measured in order to evaluate the ability of FU metabolism and to screen for adverse reactions to FU caused by pyrimidine metabolic disorders such as DPD deficiency. The results of these tests are presented in this paper.

2. Experimental

2.1. Chemicals and solutions

The sulfuric acid (special test grade), acetonitrile (for HPLC), and FU (special test grade) used in the test were purchased from Wako (Tokyo, Japan). The FDHU sample used in the test was synthesized by Kankyo Kagaku Kenkyusho (Tokyo, Japan). The distilled water used in the test was subjected to ion exchange processing.

2.2. Columns and solvents

The following columns were used in the test.

Column 1 was a molecular exclusion type column manufactured by Asahi Seiko Kogyo, Tokyo, Japan (Asahipak GS-320H Q, 300 mm×7.6 mm I.D., 9 μm particle size).

Column 2 was a cation-exchange resin type column manufactured by Mitsubishi Kasei, Tokyo, Japan (MCIGEL CK08EH, H+ type, 300 mm×8 mm I.D., 9 μm particle size).

5 m*M* Sulfuric acid was used as the mobile phase and a mixture of acetonitrile–water (50:50) was used as the column 1 clean-up solvent.

2.3. HPLC apparatus

The following devices were used in the test.

(i) Two flow pumps, an L-6320 gradient system model (Hitachi, Tokyo, Japan) and a LP-1100 model (Tokyo Rika Kikai, Tokyo, Japan)

(ii) A GT-103 degasser, a LV-306R automatic high-pressure switching valve (six directions), and a CA-202 column oven (Lab-Quatec, Tokyo, Japan).

(iii) Two UV-8000 ultraviolet sensors (Tokyo Rika Kikai, Tokyo, Japan).

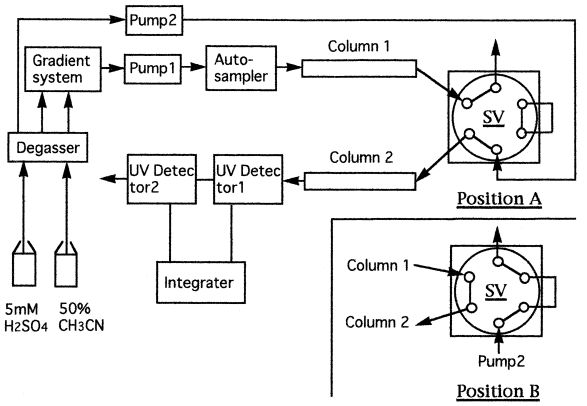

Fig. 2. HPLC configuration. In position A of the switching valve (SV), 5 m*M* sulfuric acid delivered by pump 1 (1.0 ml/min) flushed the sample to column 1. 14.5 min after injection, the SV switched to position B and the fraction containing FU and FDHU transferred to column 2. Columns 1 and 2 were coupled for 3 min, after which the SV switched back to position A and the separation on column 2 proceeded. 18 min after injection, 50% acetonitrile delivered by pump 1 (1.0 ml/min) cleaned-up column 1 for 5 min.

(iv) An AS-8020 autosampler (Toso, Tokyo, Japan).

The HPLC configuration used is shown in Fig. 2.

2.4. Sample preparation

The collected urine samples were immediately frozen (−20°C) for storage. The samples were prepared by filtering out the minute particles using an ULTRAFREE-MC (made by Millipore, Bedford, MA, USA) with hole diameters of 0.45 μm.

2.5. HPLC procedure

A precisely measured 20-μl aliquot of each sample was injected into column 1 when the valve was in position A (Fig. 2). 14.5 min after sample injection, the six-port valve rotated to position B, coupling column 1 to column 2. The fraction containing the FU and FDHU from column 1 was delivered to column 2. 17.5 min after sample injection, the valve switched back to position A, separation on the column 2 continued. Column 1 was eluted with 5 m*M* H_2SO_4 for 18 min after injection, and was then washed with acetonitrile–water (50:50) for 5 min. Finally, it was re-equilibrated with 5 m*M* H_2SO_4 for 17 min. Column 2 was eluted consecutively with 5 m*M* H_2SO_4. Both columns were maintained at 40°C in a column oven. The flow-rate of column 1 was 1.0 ml/min, and of column 2 was 0.8 ml/min. The two columns were connected for a period of 14.5 min (just before FDHU was eluted) to 17.5 min (just after FU was eluted) by use of column-switching techniques. The operating sequence is listed in Table 1. The eluent from column 2 was continuously monitored at 210 and 270 nm. Quantitation of FU and FDHU concentrations was performed by a weighted linear calibration curve of peak-height vs. concentration.

2.6. Urine samples

Urine samples were obtained from seven patients with breast cancer, who were orally administered 200 mg/day of FU after surgical chemotherapy. They were given informed consents to participate in this study. 100 mg of FU was administered orally, and a series of urine samples were collected over a period of 120 min. The FU and FDHU levels in the collected urine were measured.

Urine concentrations were corrected using creatinine, and the creatinine was measured with an autoanalyzer (TBA80FR, Toshiba, Tokyo, Japan).

Prior to this study, we measured urinary pyrimidine of the patients, and confirmed they were not homozygotes of DPD deficiency and DHPase deficiency.

Table 1
Operating sequence

Time (min)	Event	Valve position	50% Acetonitrile (ml/min)
0.0	Inject Columns 1 and 2	A	0.0
14.5	Connect	B	0.0
17.5		A	0.0
18.0	Wash	A	1.0
23.0	Re-equilibrate	A	0.0
40.0	End	A	0.0

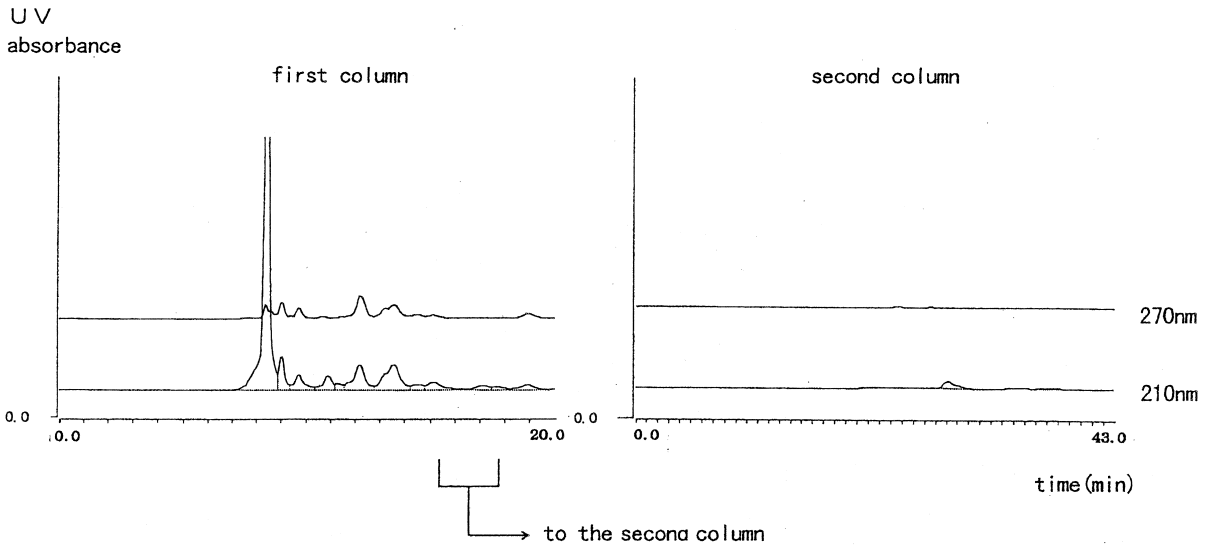

Fig. 3. Chromatogram of urine from a healthy person.

3. Results

3.1. Chromatograms

The urinary chromatograms from a healthy person and from a cancer patient who had been administered FU are shown in Figs. 3 and 4. The chromatographic profile of the urine sample from the cancer patient after administration of FU had two major peaks, corresponding to the standard mixture of FU and FDHU (retention times and absorption ratio at 250/270 nm for FU and 210/230 nm for FDHU). No interference from endogenous components was observed in urine from the healthy person. The re-

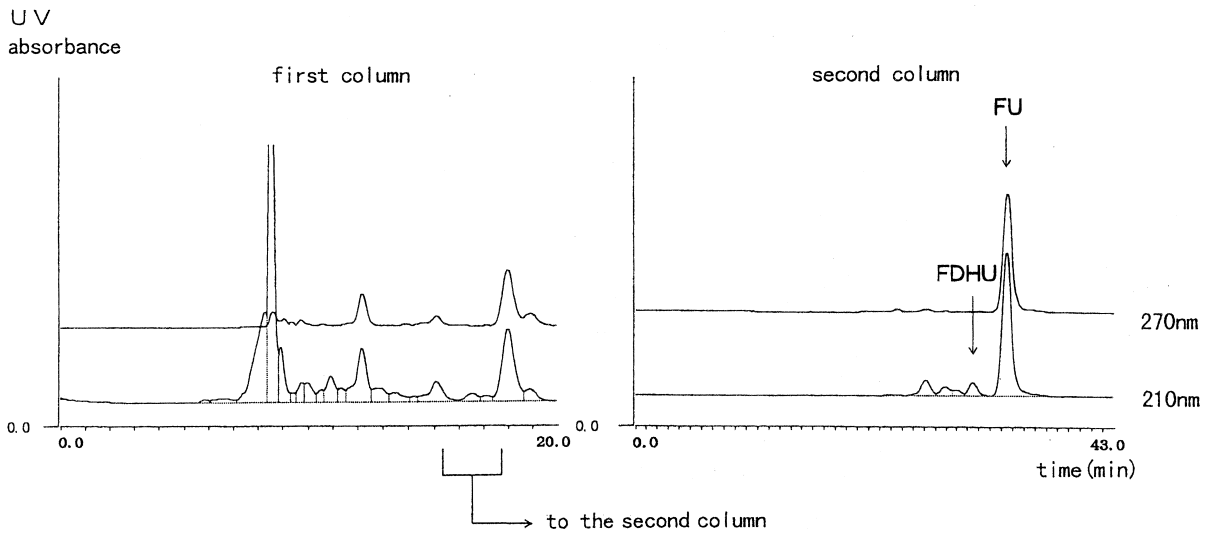

Fig. 4. Chromatogram of urine from a patient receiving 5-fluorouracil (FU) (during a 120 min period after oral administration of 100 mg FU).

tention times (mean±SD, $n=5$, in series) of FDHU and FU were 28.75±0.6 min and 31.56±0.4 min, respectively.

3.2. Linearity

The calibration curves for FU and FDHU was obtained using standard mixture solution at different concentrations (1, 10, 50, 100, 500, 1000 nmol/ml; sample diluted with distilled water). The relationships between standard concentrations and peak-heights were linear in the concentration range 1–1000 nmol/ml. The regression equation for FU is: $y=1.99\ (\pm 0.03)x+3.44(\pm 0.04)$, $r=0.9994\ (\pm 0.0002)$; and for FDHU is: $y=1.34\ (\pm 0.02)x+8.62(\pm 0.04)$, $r=0.9993\ (\pm 0.0003)$, (x in nmol/ml; y in peak-height; SD in parentheses, $n=5$, in series).

3.3. Analytical recovery

The analytical recovery of standard compounds added to urine is shown in Table 2. The analytical recovery of FU was in the range 96–101%; for FDHU, analytical recovery was in the range 96–105%.

3.4. Precision

The accuracy and precision were determined with five determinations per concentration. Same-day and day-to-day accuracy and precision values are shown in Table 3. The same-day RSDs for measurement of FU (10–100 nmol/ml) were in the range 0.61–1.52%, for measurement of FDHU (10–100 nmol/ml) were in the range 1.48–3.29% ($n=5$). The day-to-day RSDs for measurement of FU (10–100 nmol/ml) were in the range 0.51–1.84%, for measurement of FDHU (10–100 nmol/ml) were in the range 2.56–4.04% ($n=5$).

The detection limit both for FU and FDHU was 0.1 nmol/ml per 20 μl injected.

3.5. Results in urine from cancer patients

Concentrations of FU, FDHU and FDHU/FU ratio for seven cancer patients are shown in Table 4. The mean±SD of FU, FDHU and FDHU/FU ratio were 55.4±12.9 μmol/g Cre, 29.2±9.1 μmol/g Cre, and 0.52±0.07. The maximum urinary FU and FDHU concentration level varies widely from person-to-person. During the 120 min period after FU is administered, however, the FDHU/FU ratio in the urine of the seven cancer patients showed a nearly constant value. Both the FU and FDHU urinary levels fell below the detection threshold 180 min after being administered (data not shown).

4. Discussion

In Europe and the USA, DPD deficiency condition is diagnosed by measuring the DPD activity in peripheral blood mononuclear cells [4–6]. However, that method requires the use of a radioactive substance, and sample preparation is complicated [7]. It

Table 2
Analytical recovery of FU and FDHU added to urine sample[a]

Analyte	Added amount (nmol/ml)	Concentration found (nmol/ml)	Recovery rate (%)	Recovery RSD (%)
FU	10	9.62±0.16[b]	96.16	1.42
	25	24.75±0.21	98.99	0.69
	50	49.75±0.50	99.13	0.79
	100	101.24±0.47	101.24	0.38
FDHU	10	10.50±0.60	105.03	5.09
	25	24.81±0.76	99.23	2.60
	50	48.21±2.50	96.42	3.45
	100	99.58±2.72	99.58	2.37

[a] Analysis was done five times for each concentration.
[b] Mean±SD.

Table 3
Imprecision of FU and FDHU determination[a]

Analyte	Theoretical concentration (nmol/ml)	Same day		Day-to-day	
		Concentration, found (nmol/ml)	RSD (%)	Concentration found (nmol/ml)	RSD (%)
FU	10	9.84±0.21[b]	1.52	10.02±0.20	1.84
	25	25.01±0.35	0.84	24.81±0.21	0.79
	50	49.79±0.36	0.88	50.13±0.46	0.61
	100	100.91±0.64	0.61	100.14±0.48	0.51
FDHU	10	10.11±0.58	3.29	10.08±0.62	4.04
	25	24.96±0.61	2.10	25.12±0.71	3.19
	50	50.11±0.89	2.44	49.89±0.44	3.01
	100	100.24±1.08	1.48	99.98±1.04	2.56

[a] Analysis was done five times. Quantitation was performed by a weighted calibration curve of peak-height vs. concentration over the range of 10–100 nmol/ml.
[b] Mean±SD.

is also not generally used for screening patients to whom FU is to be administered.

In the previous uracil loading test which we performed [8], we found that it was possible to diagnose DHPase deficiency heterozygotes, as well as DPD heterozygotes conditions, by measuring the amount of uracil and dihydrouracil in the urine. However, it was impossible to diagnosis heterozygotes by measuring serum uracil and dihydrouracil.

We speculated that urinary pyrimidine is a better index than serum pyrimidine for detecting DPD or DHPase heterozygotes [9]. Because the measured substances (FU and FDHU) do not naturally exist in organisms, they are ideal for use in the former uracil loading test method in which the uracil had to labeled with fluorine. The FU and FDHU method is also less influenced by variations in individuals than the uracil method, as uracil is naturally present in organisms.

Table 4
Urinary concentrations of FU, FDHU and FU ratio for seven cancer patients

Patient	Age	FU (μmol/g Cre)	FDHU (μmol/g Cre)	FDHU/FU (ratio)
N.K.	53	65.6	35.8	0.55
K.I.	54	43.9	26.1	0.59
H.H.	67	80.1	46.3	0.58
M.K.	48	54.1	20.9	0.39
K.H.	64	46.4	21.2	0.46
A.T.	59	51.5	29.1	0.57
T.K.	45	48.1	25.2	0.52
Mean±SD		55.4±12.9	29.2±9.1	0.52±0.07

Moreover, our current method (determining the FDHU/FU ratio by measuring the amounts of FU and FDHU excreted in the urine during a 120 min period after FU is administered) allows us to estimate the amount of DPD and DHPase activity in organisms. Because the patients with pyrimidine metabolism disorder showed abnormal dihydrouracil/uracil ratio [10], we speculate that patients as well as show abnormal FDHU/FU ratio.

We suggest that the measurement of FDHU/FU ratio is a more accurate diagnosis of DPD or DHPase heterozygotes, and allows us to evaluate the risk of adverse reactions to FU caused by pyrimidine metabolic disorders before these reactions occur.

This method needs no sample preparation apart from filtration while the previous HPLC methods [11,12] and the gas chromatographic–mass spectrometric methods [13] need rather complicated procedure. Moreover, this method is able to simultaneously measure FDHU as well as FU, though previous methods can measure only FU.

Urine was chosen as the analysis subject for three reasons: (1) FU has an extremely large kidney clearance. (2) Urine reflects the pyrimidine metabolism of all internal organs, rather than just the blood and liver. (3) In the uracil loading test, urinary

concentration was better index than serum concentration for detecting DHPuria [9].

In conclusion, our current method requires only a small amount of urine, minimizing the burden on the patient. Moreover,this method needs simple procedure for measurement. We believe that this is ideal for routine examination in the FU chemotherapy.

We suggest that this method is useful for detection of DPD deficiency and evaluating the FU metabolism. But further data of many patients is necessary to prove our suggestion.

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