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ORIGINAL ARTICLE

Determination of 5-fluorouracil and dihydrofluorouracil levels by using a liquid chromatography–tandem mass spectrometry method for evaluation of dihydropyrimidine dehydrogenase enzyme activity

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Abstract
Purpose 5-Fluorouracil (5-FU), acting as a pyrimidine antagonist, is a major chemotherapy drug used for the treatment of tumors such as gastrointestinal, breast, ovary, and head and neck cancers. The key and rate-limiting enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DHPDH), whose partial or complete deficiency exposes to a severe 5-FU toxicity in patients. The determination of DHPDH activity in patients before the treatment and setting up a personalized therapy for each patient receiving the drug can help us to prevent the possible risk of toxicity.
Methods To isolate peripheral blood mononuclear cells (PBMCs), EDTA-anticoagulated blood samples were collected from randomly selected 47 patients and examined for 5-FU and its metabolite dihydrofluorouracil (FUH2) by using a liquid chromatography–tandem mass spectrometry (LC–MS/MS) to observe DHPDH activity at different intervals (0 and 4th hour) indirectly.
Results Intra-assay and interassay CV % values of samples from the measurements of the modified methods are found 1.3–11.9, 2.3–9.4 for 5-FU and 3.1–14.4, 3.3–12.6 for FUH2, respectively. The reference values derived from 45 patients treated with 5-FU are 1.84 ± 0.34 ug/gr protein for 5-FU, 40.15 ± 11.43 ng/gr protein for FUH2, respectively. FUH2/5-FU ratio is 21.9 ± 3.72. In addition, the results determined from two patients, in which the lack of DHPDH is considered, were 3.24 and 4.16 ug/gr protein for 5-FU, 4.1 and 6.7 ng/gr protein for FUH2. FUH2/5-FU ratio is 1.26 and 1.61.
Conclusion The measurements of 5-FU, FUH2, and especially their ratio (FUH2/5-FU) by the modified LC–MS/MS method could be used to determine DHPDH enzyme activity.

Keywords Dihydropyrimidine dehydrogenase · 5-Fluorouracil · Dihydrofluorouracil · LC–MS/MS

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Introduction

Fluorouracil (5-FU), an analogue of uracil acting as a pyrimidine antagonist, interferes with DNA synthesis by blocking the methylation of deoxyuridylic acid, inhibits thymidylate synthetase, or incorporates into RNA. 5-FU is a major chemotherapy drug used for the treatment of various solid tumors such as gastrointestinal, breast, ovary, and head and neck cancers, with reasonable results [1, 2]. The key enzyme in 5-FU catabolism is dihydropyrimidine dehydrogenase (DHPDH) is the initial and rate-limiting enzyme in the third-step pathway that converts uracil and thymine to B-alanine and B-aminoisobutyrate, respectively [3]. Seventy to eighty percentage of the administered 5-FU is degraded in vivo by DHPDH; the determination of DHPDH activity in patients is an index of 5-FU degradation [4–6]. 5-FU pharmacokinetics is characterized by a short half-life [7], mostly due to extensive liver catabolism by DHPDH [8]. In population studies of PBMCs, DHPDH has shown that enzyme activity is variable with a seven- to tenfold range observed [9–12]. These studies suggest that

although total deficiency is estimated to occur in about 0.1% of patients, relative DHPDH deficiency is projected to have an incidence of 3 to 5% [13] and thus be at increased risk of severe toxicity if treated with 5-FU [9, 14–17]. It was reported that the onset of toxicity of 5-FU appeared approximately twice as fast in patients with a partial DHPDH deficiency compared with patients with normal DHPDH activity [15, 16, 18].

An alternative indirect method has been proposed to assess DHPDH status in patients with cancer. Because this enzyme is responsible for the reduction of uracil (U) to dihydrouracil (FUH2), measurement of physiological 5-FU/FUH2 ratio could provide quick insight into DHPDH status. There are several methods for the measurement of both 5-FU and FUH2, either in plasma or urine [2, 4, 8, 19, 20].

The aim of this study was to develop a rapid and simple LC–MS/MS method for estimating 5-FU/FUH2 ratio in plasma, as an index of DHPDH status, and for assaying 5-FU as part of drug level monitoring. To demonstrate that intact peripheral blood mononuclear cells(PBMCs) can be an effective tool to study and evaluate the catabolism of 5-FU, reflecting the efficiency of the whole enzymatic machinery involved in the metabolism of the drug. Consequently, this procedure could be used to set up a personalized therapy for each patient receiving the drug [2].

Materials and methods

Study design and endpoints

The study was approved by the Kecioren Teaching and Research Hospital local ethics committee and all patients granted their written inform consent to participate.

5-FU, FUH2, and formic acid were purchased from Sigma–Aldrich (St. Louis, MO, USA). [15N2]-5-FU was obtained from Isotopes Inc. (Pointe-Claire, Quebec, Canada). All solvents were purchased from Fisher Chemicals (Fair Lawn, NJ, USA). All chemicals were of analytical grade.

Study design

The study involved 7 mL of EDTA-anticoagulated blood samples of randomly selected 47 patients which were collected between 8 and 9 AM to avoid changes in DHPDH activity due to its circadian variation [7, 21], and all of them were processed immediately to isolate peripheral blood mononuclear cell (PBMC).

Isolation of human PBMC

PBMCs were isolated from 7 mL of EDTA-anticoagulated blood using Lenfodex® (INNO-TRAIN Diagnostic GmbH, Kronberg, Taunus)). After 20-min centrifugation at 4,000 rpm (25°C), PBMC ring occured. Afterward, PBMC ring was collected, diluted with phosphate-buffered saline (PBS) to a final volume of 5 mL, and centrifuged at 5,000 rpm for 15 min at 25°C. Supernatant was removed, and the pellet was diluted with PBS to a final volume of 5 mL and centrifuged at 5,000 rpm for 15 min. This procedure repeated once more, and the pellet was finally resuspended in 250 μL of PBS. An aliquot (10 μL) was used for protein concentration measurement. Protein determinations were carried out using modified Lowry method [22]. The amount of protein in suspensions is set to be 100 mg/mL by using PBS.

Determination of the rate of 5-FU degradation [2]

The rate of 5-FU degradation was determined by measuring the decrease in a fixed concentration of 5-FU added to the solution of intact PBMC. The reaction mixture was obtained by mixing 64 μL of the cell suspension, freshly prepared PBMC, with 16 μL of 50 μg/mL 5-FU (10 μg/mL, final concentration). The reaction was carried out in an Eppendorf tube incubated in a thermomixer at 37°C for 0 and 4 h; the reaction was stopped by adding 150 μL of 1% formic acid containing internal standard ([15N2]-5-FU, 10 μg/mL, final concentration).

Samples were mixed for 5 min with an automatic pipette manually, and 20 μL of the supernatant was injected into the LC MS/MS. To calculate the rate of 5-FU degradation, the concentration of the drug after 4-h incubation was subtracted from the calculated concentration at 0 time. Calculations were done by measuring the rate of 5-FU reduction and FUH2 increase.

Precision, recovery study design

Linearity responses were observed in the 5-FU and FUH2 concentration 0.25–250 ng/mL 0.125–125 ng/mL, respectively.

The intra-assay precision and recovery were determined by using 9 amounts of 5-FU and FUH2 in 5 times within a day. Interassay precision was evaluated at the above amounts of 5-FU and FUH2 on 5 consecutive days.

Within the scope of this study, 150 μL of 0.1% formic acid–containing internal standard was added to 80 μL samples taken. In the recovery studies, 16 μL different standards are included after 150 μL of 0.1% formic acid–containing internal standard was added to prepared cell suspensions.

LC–MS/MS analysis

The LC–MS/MS system consisted of Shimadzu LC Prominence (Shimadzu Corporation) and Api 3200 triple

Table 1 Accuracy and precision

Concentration (ng/mL)		Recovery (%)		Intra-assay CV (%) $n = 5$		Interassay CV (%) $n = 5$	
5-FU	FUH2	5-FU	FUH2	5-FU	FUH2	5-FU	FUH2
0.25	0.125	145.6	133.5	25.2	26.8	33.6	42.4
0.5	0.25	105.8	128.5	11.9	21.4	14.4	29.7
1	0.5	101.2	99.3	8.9	9.4	13.2	12.6
5	1	102.3	98.2	8.4	7.5	10.2	8.8
10	2	101.2	99.3	9.2	3.2	11.2	7.4
25	5	98.4	102.4	5.3	4.4	6.2	5.6
50	10	97.6	101.5	3.3	5.9	4.2	6.4
100	50	99.4	97.2	3.2	3.2	5.2	4.3
250	100	100.8	102.1	1.3	2.3	3.1	3.3

quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with electrospray ionization (ESI) in negative-ion, multiple reaction monitoring (MRM) mode. The HPLC separation was performed using a 150 × 4.6 mm Luna 3 um C18 column (Phenomenex, Torrance, CA). Temperature was set at 400°C; dwell time was 0.3 s, DP -40, CE -28, EP -10 V. The MRM m/z transitions monitored were 128.0–41.6 for 5-FU, 131–43 for [15N2]-5-FU (internal standard), and 131.0–41.6 for FUH2. Solvent consisted of acetonitrile–water (50:50,v/v). Elution was performed at a flow rate of 200 µL/min for 8 min.

Table 2 5-FU, FUH2, and ratios results

	5-FU ug/gr protein	FUH2 ng/gr protein	FUH2/5-FU ratio
Normal DHPDH activity ($n = 45$)			
Mean	1.83	40.15	21.9
SD	0.34	11.43	3.72
Cutoff value	>2.5	<10	<10
DHPDH deficiency			
Patient 1	3.24	4.1	1.26
Patient 2	4.16	6.7	1.61

Results

Accuracy and precision of results after modification of the method are presented in Table 1.

Lower limit of quantitation (LLQ)

According to the FDA guidelines for bioanalytical method validation, the calibration curve describes the concentration versus response relationship adequately if the observed deviation and precision are <20% for the LLQ. LLQs were found to be 0.5 ng/mL for 5FU and FUH2.

The mean and SD values obtained from the patients who used 5-FU (45 patients) and 2 patients with DHPDH deficiency are presented in Table 2.

The outlook of LC MS/MS images belonging to the measurements of 5-FU and FUH2 from the modified method is presented in Fig. 1.

Discussion

The role of DHPDH in the degradation of 5-FU implies an inverse correlation between the DHPDH activity and the clearance of 5-FU [21, 23]. Various studies show that there are conflicting results as if a correlation exists between the DHPDH activity and the clearance of 5-FU in individuals with normal DHPDH activity [21, 24]. However, patients with a partial or profound DHPDH deficiency have a reduced capacity to degrade 5-FU, and therefore they are at risk of severe 5-FU toxicities [14, 15, 25, 26]. Patients with the peripheral blood mononuclear cell DHPDH activity less than 70% of the mean population were classified as having reduced DHPDH activity, and the frequency of profound deficiency is usually lower in population [10, 21, 27]. DHPDH activity also influences on the tumor response with the resistant tumors harboring higher level of DHPDH activity [16, 28].

Determination of the activity of DHPDH enzyme is a key method to identify individuals at risks of DHPDH deficiency. Various methods have been proposed to identify these patients at risk. Recently, screening the urinary uracil level and plasma fluoro-beta-alanine, the final metabolite of 5-FU [20, 29, 30] and C^{13}-uracil breath test, has been developed to identify DHPDH-deficient individuals [31, 32]. Also, several methods including HPLC, mass spectrometry, and denaturing high-performance liquid chromatography have been developed to identify DHPDH activity [25].

In this study, the measurement of 5-FU and FUH2 by the tandem mass method optimized by modifying provides convenience to determine both the 5-FU reduction and

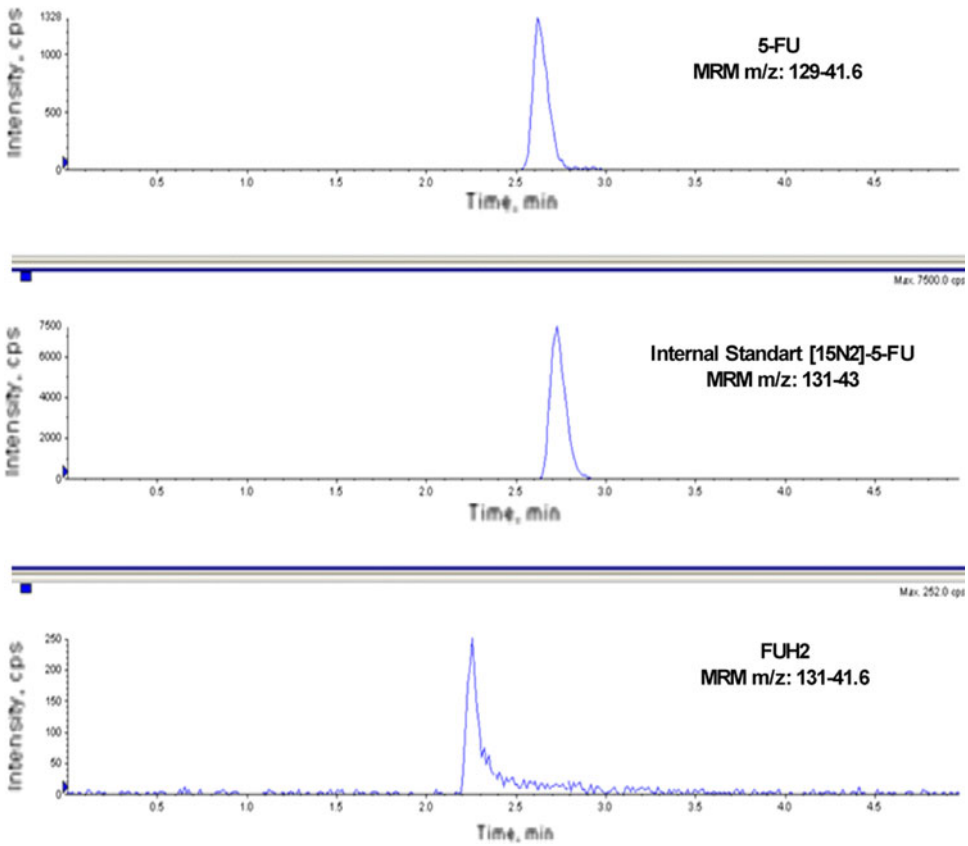

Fig. 1 The images of 5FU, FUH2, and internal standard in LC–MS/MS

FUH2 production. According to the results of the modified LC–MS/MS method, the measurement of 5-FU and FUH2 has appropriate analytical performance. Besides using 5-FU reduction to determine DHPDH activity, FUH2 measurement and especially FUH2/5-FU ratio may also be appropriate in the assessment of enzyme activity. Especially, in this process, in two patients applied because of toxicity, low enzyme activity detected, and therapeutic doses were revised accordingly. Eventually, analytical performance of modified 5-FU and FUH2 measurements were observed to be sufficient.

Because DHPDH activity displays both circadian regulations [23] and high variability according to the PBMC studied [21], single measurement of its activity or expression may not be adequate. To set up a reference range of 5-FU degradation rate in intact PBMC and a threshold to identify patients with an altered 5-FU metabolism, a larger clinical study would be required.

References

1. Hiroyuki B, Kenichi T, Toru K et al (2003) Dihydropyrimidine dehydrogenase and thymidylate synthase activities in hepatocellular carcinomas and in diseased livers. Cancer Chemother Pharmacol 52:469–476
2. Lostia AM, Lionetto L, Ialongo C, Gentile G, Viterbo A, Malaguti P, Paris I, Marchetti L, Marchetti P, De Blasi A, Simmaco M (2009) A liquid chromatography–tandem mass spectrometry method for the determination of 5-fluorouracil degradation rate by intact peripheral blood mononuclear cells. Ther Drug Monit 31:482–488
3. Miyazaki K, Shibahara T, Sato D et al (2006) Influence of chemotherapeutic agents and cytokines on the expression of 5-fluorouracil-associated enzymes in human colon cancer cell lines. J Gastroenterol 41:140–150
4. Deporte-Fety R, Picot M, Amiand M et al (2001) High-performance liquid chromatographic assay with ultraviolet detection for quantification of dihydrofluorouracil in human lymphocytes: application to measurement of dihydropyrimidine dehydrogenase activity. J Chromatogr B Biomed Sci Appl 762:203–209
5. Van Kuilenburg ABP, Klumpen HJ, Westermann AM et al (2007) Increased dihydropyrimidine dehydrogenase activity associated with mild toxicity in patients treated with 5-fluorouracil and leucovorin. Eur J Cancer 43:459–465
6. Guimbaud R, Guichard S, Dusseau C et al (2000) Dihydropyrimidine dehydrogenase activity in normal, inflammatory and tumour tissues of colon and liver in humans. Cancer Chemother Pharmacol 45:477–482
7. Milano G, Chamorey AL (2002) Clinical pharmacokinetics of 5-fluorouracil with consideration of chronopharmacokinetics. Chronobiol Int 19:177–189
8. Di Paolo A, Ibrahim T, Danesi R et al (2002) Relationship between plasma concentrations of 5-fluorouracil and 5-fluoro-5, 6-dihydrouracil and toxicity of 5-fluorouracil infusions in cancer patients. Ther Drug Monit 24:588–593
9. Ridge SA, Sludden J, Wei X et al (1998) Dihydropyrimidine dehydrogenase pharmacogenetics in patients with colorectal cancer. Br J Cancer 77:497–500

10. Lu ZH, Zhang RW, Diasio RB (1993) Dihydropyrimidine dehydrogenase activity in human peripheral blood mononuclear cells and liver: population characteristics, newly identified deficient patients, and clinical implication in 5-fluorouracil chemotherapy. Cancer Res 53:5433–5438
11. Etienne MC, Lagrange JL, Dassonville O, Fleming R, Thyss A, Renee N, Schneider M, Demard F, Milano G (1994) Population study of dihydropyrimidine dehydrogenase in cancer patients. J Clin Oncol 12:2248–2253
12. McMurrough J, McLeod HL (1996) Analysis of the dihydropyrimidine dehydrogenase polymorphism in a British population. Br J Clin Pharmacol 41:425–427
13. Meta-Analysis Group In Cancer (1998) Toxicity of fluorouracil in patients with advanced colorectal cancer: effect of administration schedule and prognostic factors. J Clin Oncol 16(11):3537–3541
14. Johnson MR, Hageboutros A, Wang K, High L, Smith JB, Diasio RB (1999) Life-threatening toxicity in a dihydropyrimidine dehydrogenase-deficient patient after treatment with topical 5-fluorouracil. Clin Cancer Res 5:2006–2011
15. Van Kuilenburg AB, Haasjes J, Richel DJ et al (2000) Clinical implications of dihydropyrimidine dehydrogenase (DHPDH) deficiency in patients with severe 5-fluorouracil-associated toxicity: identification of new mutations in the DHPDH gene. Clin Cancer Res 6:4705–4712
16. Omura K (2003) Clinical implications of dihydropyrimidine dehydrogenase (DHPDH) activity in 5-FU based chemotherapy: mutations in the DHPDH gene, and DHPDH inhibitory fluoropyrimidines. Int J Clin Oncolology 8:132–138
17. Raida M, Schwabe W, Hausler P, Van Kuilenburg AB, Van Gennip AH, Behnke D, Hoffken K (2001) Prevalence of a common point mutation in the dihydropyrimidine dehydrogenase (DHPDH) gene within the 5′-splice donor site of intron 14 in patients with severe 5-fluorouracil (5-FU)-related toxicity compared with controls. Clin Cancer Res 7:2832–2839
18. Etienne MC, Chatelut E, Pivot X et al (1998) Co-variables influencing 5-fluorouracil clearance during continuous venous infusion. A NONMEM analysis. Eur J Cancer 34:92–97
19. Van Kuilenburg AB, van Lenthe H, Blom MJ, Mul EP, Van Gennip AH (1999) Profound variation in dihydropyrimidine dehydrogenase activity in human blood cells: major implications for the detection of partly deficient patients. Br J Cancer 79:620–626
20. Furuhata T, Kawakami M, Okita K, Kimura Y, Kihara C, Tsuruma T, Ohnura T, Yamaguchi K, Hata F, Katsuramaki T, Sasaki K, Hirata K (2006) Plasma level of a 5-fluorouracil metabolite, fluoro-beta-alanine correlates with dihydropyrimidine dehydrogenase activity of peripheral blood mononuclear cells in 5-fluorouracil treated patients. J Exp Clin Cancer Res 25:79–82
21. Milano G, Etienne MC, Pierrefite V et al (1999) Dihydropyrimidine dehydrogenase deficiency and fluorouracil-related toxicity. Br J Cancer 79:627–630
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
23. Harris BE, Song R, Soong SJ, Diasio RB (1990) Relationship between dihydropyrimidine dehydrogenase activity and plasma 5-fluorouracil levels with evidence for circadian variation of enzyme activity and plasma drug levels in cancer patients receiving 5-fluorouracil by protracted continuous infusion. Cancer Res 50:197–201
24. Terashima M, Irinoda T, Kawamura H et al (2003) Intermittent FLDP: 24-h infusion of 5-FU on days 1, 3 and 5 combined with low-dose cisplatin on days 1–5 for gastric cancer, and its pharmacologic and kinetic rationale. Cancer Chemother Pharmacol 51:240–246
25. Van Kuilenburg ABP, Van Lenthe H, Tromp A et al (2000) Pitfalls in the diagnosis with a partial dihydropyrimidine dehydrogenase deficiency. Clin Chem 46:9–17
26. Johnson MR, Yan J, Shao L, Albin N, Diasio RB (1997) Semiautomated radioassay for determination of dihydropyrimidine dehydrogenase (DHPDH) activity. Screening cancer patients for DHPDH deficiency, a condition associated with 5-fluorouracil toxicity. J Chromatogr B Biomed Sci Appl 696:183–191
27. Ogura K, Ohnuma T, Minamide Y, Mizuno A, Nishiyama T, Nagashima S, Kanomaru M, Hiratsuka A, Watabe T, Uenmatsu T (2005) Dihydropyrimidine dehydrogenase activity in 150 healthy Japanese volunteers and identification of novel mutations. Clin Cancer Res 11:5104–5111
28. Jiang W, Lu Z, He Y, Diasio RB (1997) Dihydropyrimidine dehydrogenase activity in hepatocellular carcinoma: implication in 5-fluorouracil-based chemotherapy. Clin Cancer Res 3:395–399
29. Kouwaki M, Hamajima N, Sumi S, Nonaka M, Ssaki M, Dobashi K, Kidouchi K, Togari H, Wada Y (1998) Identification of novel mutations in the dihydropyrimidine dehydrogenase gene in a Japanese patient with 5-Fluorouracil toxicity. Clin Cancer Res 4:2999–3004
30. Sumi S, Kidouchi K, Hayashi K, Imaeda M, Asai M, Wada Y (1998) Urinary screening for pyrimidine metabolism disorders: reference ranges for dihydrouracil, uracil, and dihydroduracil/uracil ratio. Adv Exp Med Biol 431:191–195
31. Mattison LK, Ezzeldin H, Carpenter M, Modak A, Johnson MR, Diasio RB (2004) Rapid identification of dihydropyrimidine dehydrogenase deficiency by using a novel 2–13C-uracil breath test. Clin Cancer Res 10:2653–2658
32. Mattison LK, Fourie J, Hirao Y, Koga T, Desmond RA, King JR, Shimizu T, Diasio RB (2006) The uracil breath test in the assessment of dihydropyrimidine dehydrogenase activity: pharmacokinetic relationship between expired 13CO2 plasma [2–13C] dihydrouracil. Clin Cancer Res 15:549–555