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Measurement of endogenous uracil and dihydrouracil in plasma and urine of normal subjects by liquid chromatography–tandem mass spectrometry

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Abstract

A sensitive and specific HPLC–MS–MS method was developed for the determination of endogenous uracil (Ura) and its metabolite dihydrouracil (UH2) in human plasma and urine samples. Plasma samples were extracted with ethyl acetate–isopropanol (85:15, v/v) following added ammonium sulfate, and then separated on a Discovery Amide C_{16} column with 3% methanol solution as the mobile phase; urine samples were just centrifuged at 2500 *g* for detection. Quantitation was carried out by LC–MS–MS in the multiple reaction monitoring (MRM) mode. The limits of quantitation of the method for Ura and UH2 were 0.5 and 5 ng ml^{-1} (for plasma), and 50 and 100 ng ml^{-1} (for urine), respectively. This method can be useful to evaluate the activity of dihydropyrimidine dehydrogenase (DPD), a rate-limiting enzyme of the chemotherapy drug fluoropyrimidine, which will be helpful in investigating subject variation of DPD and adjusting clinical dosage in pyrimidine chemotherapy.

Keywords: Uracil; Dihydrouracil

1. Introduction

Dihydropyrimidine dehydrogenase (DPD) is a key rate-limiting enzyme responsible for endogenous pyrimidine base metabolism. For example, it can catabolize uracil (Ura), one of its endogenous substrates, to dihydrouracil (UH2). Significant elevations of Ura concentration in plasma and urine will occur in subjects with DPD deficiency [1]. 5-Fluorouracil (5-FU), a first line antimetabolic chemotherapy drug commonly used to treat digestive, breast, and head and neck cancer, has a chemical structure similar to DPD substrates. The level of DPD becomes the major determinant of 5-FU clearance in patients administered high dose 5-FU [2–4]. Pharmacokinetic research data on 5-FU showed that, in vivo, more than 80% of an administered 5-FU dose was catabolized by DPD, and the remaining unchanged drug was excreted in urine. Thus, potentially lethal drug interactions may appear when coadministered with other pyrimidine drugs inhibiting DPD [5].

As DPD influences both pharmacokinetics and the effects of fluoropyrimidine drugs, much research has

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been done on the measurement of DPD activity [6,7]. DPD enzyme is distributed in many tissues, especially in liver, and also in peripheral blood mononuclear cells (PBMC), bone marrow, intestinal mucosa and spleen. Detection of DPD activity in PBMC may be the most convenient method to determine total DPD level. But there is a weak correlation between the level of DPD in PBMC and 5-FU clearance as well as the concentration of 5-FU in plasma [1]. This indicates that DPD recovery in PBMC cannot reflect the full systemic recovery of DPD in other important tissues, such as the liver or gastrointestinal tract. Another result has shown that 5-FU systemic clearance significantly exceeds liver blood flow, which indicates that DPD activity in other tissues also contributes greatly to 5-FU metabolism [4]. Therefore, the level of DPD in certain tissues cannot accurately reflect those in the whole body.

A theoretically more sensitive index for total DPD level is the UH2–Ura concentration ratio in plasma and urine. Although concentration of Ura in plasma [4] or urine [8,9] may reflect the ability of DPD to metabolize Ura, many other factors, such as diet and beverages containing a large amount of Ura, will interfere with the Ura concentration in biological fluids. Besides, the concentration of Ura in urine must be revised by urinary creatinine when evaluating DPD activity [10]. In this paper, we introduce UH2–Ura concentration ratio to eliminate such interference in order to reflect the actual level of DPD. So the concentrations of Ura and UH2 in the plasma and urine of healthy subjects were simultaneously detected in our test with liquid chromatography–tandem mass spectrometry, a highly sensitive and selective technique. This method is very suitable for the quantification of Ura and UH2 considering their high polarity and endogenous trace substances. Other methods of detecting Ura or UH2 are not only complex and time-consuming but also less sensitive [4,11–14].

2. Experimental

2.1. Chemicals and HPLC reagents

Ura, UH2, 5-bromouracil (5-BU), 5-fluorouracil (5-FU) and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Ammonium sulfate, isopropanol and ethyl acetate were of HPLC grade from Beijing Chemical Plant (Beijing, China). Methanol was supplied by Fisher (Fair Lawn, NJ, USA). The water used was of Milli-Q grade and degassed with helium. Saline was supplied by Shijiazhuang drug plant (Beijing, China).

2.2. LC–MS–MS

The LC–MS–MS system consists of a PE-series-200 autosampler, two Waters-510 pumps, an automated Waters gradient controller, a Harvard syringe pump, API-3000 triple-quadrupole mass spectrometer, and a workstation. Mobile phase was 3% methanol solution, and flow rate was 1 ml min^{-1}. Column was Amide C_{16} (150×4.6 mm I.D.) and 10% of mobile phase flowing from the end of the column was infused into the MS–MS system by a build-in splitter. The temperature of the analytical column was 20 °C. Mass spectrometry parameters were optimized with the syringe filled with standard solutions, and some ion source parameters (OR, RNG) as well as triple-quadrupole parameters (RO1, RO2, ST3, etc.) were recorded automatically. Nebulizer temperature, voltage and nitrogen pressure were optimized manually. Main spectrometer parameters were as follows: nebulizer temperature, 400 °C; nebulizer voltage, 3500 V; nebulizer gas (N_2), 13 l min^{-1}; curtain gas (N_2), 9 l min^{-1}; CAD gas (N_2), 8 l min^{-1}; auxiliary gas (N_2), 7 l min^{-1}. Multiple reaction monitoring (MRM) was used for detecting Ura and UH2 as well as their internal standards (I.S.), 5-BU and 5-FU. OR, RNG, RO1, RO2, ST3 (mass dependent parameters) for Ura (m/z 110.8→42.1) and UH2 (m/z 112.8→42.1) were −26, −70, 38, 40, 42 V and −61, −140, 32, 34, 36 V, respectively.

2.3. Sample collection

2.3.1. Plasma

Plasma samples from 123 healthy volunteers (56 males, 29.09±7.98 years old; 67 females, 30.22±8.04 years old) were collected between 08:00 and 09:00 h (in the fasted state) to minimize the influence of DPD circadian variation. Serological tests for HCV and HIV, and clinical chemistry and

hematology results were all normal in these subjects. A 5-ml sample of whole blood was collected in a heparinized tube and ~3 ml plasma was allocated to two Eppendorf tubes and then stored at −30 °C until analysis.

2.3.2. Urine

Urine samples from 55 volunteers (24 males, 26.6±4.6 years old; 31 females, 53.6±4.4 years old) were collected between 07:00 and 08:00 h. Serological tests for HCV and HIV, and clinical chemistry and hematology results for all volunteers were normal. The urine (10 ml) was centrifuged for 10 min (1000 *g*), and the supernatant was transferred into Eppendorf tubes and stored at −30 °C.

2.4. Sample preparation

2.4.1. Plasma

First 100 μl internal standard (5-BU, 400 ng ml^{-1}) was added to 200 μl plasma sample and vortex-mixed. Ammonium sulfate (150 mg) was then added and vortex-mixed for 20 s. Then 5 ml isopropanol–ethyl acetate (15:85, v/v) was added. The tube was vortex-mixed for 1 min and centrifuged for 15 min (1000 *g*). Next 4 ml supernatant was separated and transferred to a glass tube and evaporated at 45 °C for 15 min under a stream of nitrogen. The dry extract was reconstituted with 100 μl 10% methanol solution and an aliquot of 20 μl was injected onto HPLC column for LC–MS–MS analysis.

2.4.2. Urine

Urine sample was centrifuged at 2500 *g*. Then 200 μl supernatant and 50 μl internal standard (5-FU, 1 μg ml^{-1}) were vortex-mixed and 20 μl of the mixture was injected into the LC–MS–MS system for detection.

2.5. Method validation

2.5.1. Stock solutions, calibration samples and validation samples

Ura, UH2 and I.S. (5-BU and 5-FU) were all dissolved in methanol–water (1:1) at a concentration of 200 μg ml^{-1} and stored at −80 °C. First 1.25 ml Ura (200 μg ml^{-1}) and 12.5 ml UH2 (200 μg ml^{-1}) solutions were mixed and diluted with water to 50 ml in a volumetric flask. This was then diluted to working solutions at concentrations of 0.5/5, 1/10, 5/50, 10/100, 50/500, and 100/1000 ng ml^{-1} Ura/UH2 with 3% BAS. Working solutions for quantitation in urine were prepared with saline in the same way containing the following concentrations of Ura/UH2: 0.05/0.1, 0.1/0.2, 0.5/1, 1/2, 5/10, and 10/20 μg ml^{-1}. Validation samples were also prepared according to the method described above. The concentrations were 1/10, 10/100, and 100/1000 ng ml^{-1} (Ura/UH2) in 3% BAS and 100/200, 1000/2000, 10000/2000 ng ml^{-1} (Ura/UH2) in saline.

2.5.2. Calibration line, precision, accuracy and extraction recovery

After the sample was prepared, a 20-μl sample solution was injected into the LC–MS–MS system and peak areas of Ura, UH2 and internal standard (I.S.) were recorded. Peak-area ratios between the analyte and its internal standard were used to construct the least-squares regression calibration lines.

Precision of method was derived from the results of validation samples. Validation samples were low, medium and high concentration ($n=6$) in each run ($n=3$). Within-run and between-run coefficients of variation (or precision) were calculated by RSD% (relative standard deviation). The accuracy was expressed as the ratio of the compound added to that measured.

Extraction recoveries of two analytes in biologic samples were investigated. Mean extraction recovery was calculated by the ratio of linear slopes derived from extracted and un-extracted plasma samples (low, medium, high concentrations, $n=6$) [15]. Otherwise, extraction recovery of Ura/UH2 in urine was regarded as 100%, owing to no extraction procedure before detection.

2.5.3. Influence of substitute plasma (3% BSA) on extraction recovery

Standard samples (1/10, 10/100, and 100/1000 ng ml^{-1}, Ura/UH2) spiked with 3% BSA and plasma ($n=6$) were prepared according to Section 2.5.1. After these samples were analyzed, the linear slopes of peak-area ratio versus concentration in two matrixes could be compared.

3. Results

3.1. Method selectivity

Product ion mass spectra of Ura, UH2, 5-FU and 5-BU (Fig. 1) show precursor ion and their corresponding product ions, which were used as paired ions for measuring analytes in MRM mode. Impurities in 3% BSA/plasma or saline/urine did not interfere with the detection of Ura and UH2 or I.S. (5-BU and 5-FU). Chromatograms of plasma and urine obtained from the analysis of the lowest quantitated Ura and UH2 are shown in Fig. 2A,B, respectively. When testing for the influence of substitute plasma (3% BSA) on extraction recovery, there was no statistical difference in linear slopes in two matrixes ($P>0.05$, $n=6$), which showed that there was no discrepancy in extraction recovery (Table 1).

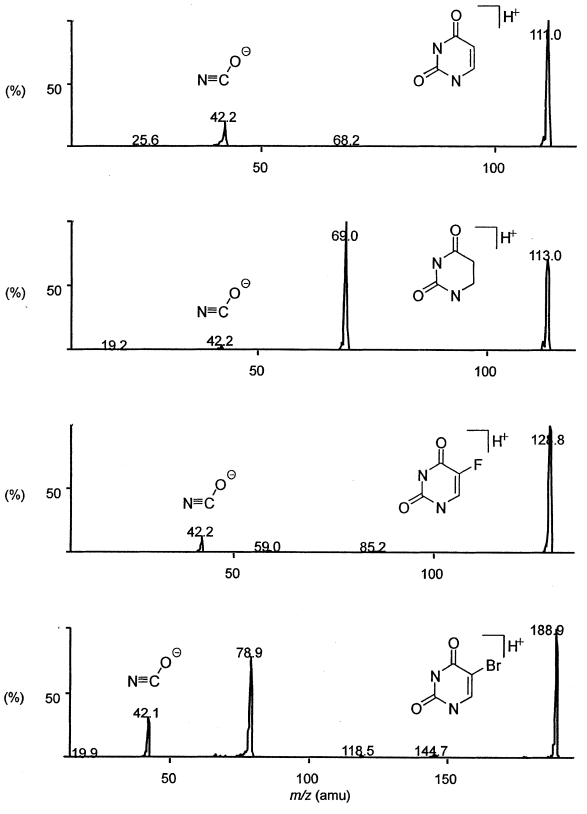

Fig. 1. Product ion spectra of Ura (m/z 111.0), UH2 (m/z 113.0), FU (m/z 128.8) and BU (m/z 188.9) produced by triple-tandem quadrupole mass spectrometry.

Thus substitute plasma (3% BSA) is suitable for preparing standard curve samples for quantitation.

3.2. Detection limit

The limits of quantitation (LOQ) for Ura/UH2 were 0.5/5 ng ml^{-1} (in plasma) and 0.05/0.1 μg ml^{-1} (in urine), respectively. Signal/noise ratio was not less than 5.0 at LOQ.

3.3. Linearity

There were good linear relationships over the concentration ranges 0.5–100 ng ml^{-1} (Ura) and 5–1000 ng ml^{-1} (UH2) in 3% BSA as well as 0.05–10 μg ml^{-1} (Ura) and 0.1–20 μg ml^{-1} (UH2) in saline. The correlation coefficients for the calibration curves were all >0.9900.

3.4. Accuracy and precision

The data for the validation of within-run and between-run precision are presented in Table 2. The results show very low coefficients of variation, even for low plasma levels of analytes. The accuracy of the method is also presented in Table 2.

3.5. Mean extraction recovery of plasma samples

The mean extraction recoveries for Ura and UH2 in plasma samples were 83.6 and 87.2%, respectively.

3.6. Stability of samples in preparation and detection

Analytes were stable in 3% BSA/plasma and saline/urine during storage at −30 °C for 6 months. No obvious variation in sample preparation and freeze–thaw cycle (data not shown) was observed.

3.7. Application for quantitating the concentrations of Ura and UH2

Concentrations of Ura and UH2 in plasma and urine samples of healthy subjects were measured and their concentration ratios calculated. These results (Figs. 3 and 4, Table 3) indicate that there are

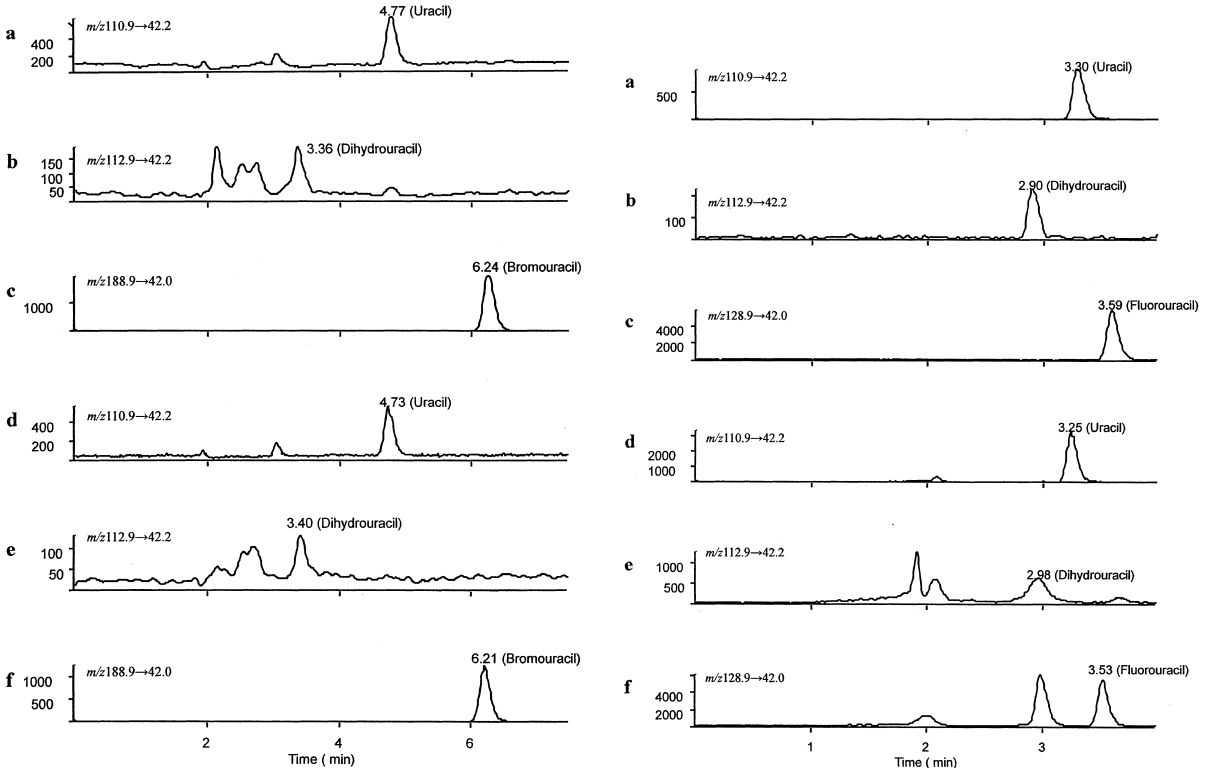

Fig. 2. (A) Multiple reaction monitoring chromatograms of plasma samples: (a–c) standard spiking sample containing the lowest concentration of uracil and dihydrouracil; (d–f) plasma sample of normal subject. I.S.: bromouracil. (B) Multiple reaction monitoring chromatograms of urine samples: (a–c) standard spiking sample containing the lowest concentration of uracil and dihydrouracil; (d–f) urine sample of normal subject. I.S.: bromouracil.

Table 1
Linear slopes of the area ratios (analyte to I.S.) to the corresponding concentrations ($n=6$) in the test for influence of 3% BSA on extraction recovery

	Ura		UH2	
	Plasma ($\times10^{-3}$)	3% BSA ($\times10^{-3}$)	Plasma ($\times10^{-3}$)	Saline ($\times10^{-3}$)
	4.905	4.433	4.978	5.527
	5.060	5.783	5.287	5.132
	5.228	5.665	5.537	5.315
	5.314	5.801	5.109	5.339
	4.876	5.366	5.070	4.520
	5.315	5.242	5.418	5.855
Mean ($\times10^{-3}$)	5.116	5.382*	5.233	5.281*
SD ($\times10^{-3}$)	0.198	0.517	0.217	0.446
RSD (%)	3.9	9.6	4.2	8.4

*$P>0.05$.

Table 2
Accuracy and precision for the detection of Ura and UH2 in plasma and urine (in method validation, $n=24$)

	Analyte	Concentration (ng ml^{-1})		Recovery (%)	RSD (%)	
		Added	Found		Within-run	Between-run
Plasma	Ura	1	1.015	101.5	6.3	6.7
		10	9.942	99.4	6.7	6.6
		100	93.11	93.1	5.1	5.9
	UH2	10	10.19	101.9	6.0	6.2
		100	102.2	102.2	7.7	8.8
		1000	974.2	97.4	6.8	8.7
Urine	Ura	100	1.003	100.3	6.7	4.1
		1000	9.560	95.6	6.1	13.9
		10000	113.5	113.5	7.0	6.0
	UH2	200	10.26	102.6	6.8	2.1
		2000	96.70	96.7	9.0	11.5
		20000	1083	108.3	6.8	4.0

obvious differences in the concentrations of Ura and UH2 and the UH2/Ura ratio among subjects, and these were not significantly normal distributions. Correlation of UH2 to Ura plasma concentration was significant but weak ($r=0.289$, $P=0.001$, $n=123$); however, concentrations of UH2 and Ura were strongly correlated in urine ($r=0.683$, $P=0.000$, $n=55$). Lines for 5 and 95% percentiles of UH2/Ura ratio are shown in Figs. 3 and 4, indicating that the subjects with points outside the range have abnormal levels of DPD. Correlation between UH2/Ura ratio and age, gender or blood type was analyzed and was not significant ($P>0.05$ for all).

4. Discussion

The sensitivity of other reported methods for detecting Ura or UH2 could be improved further

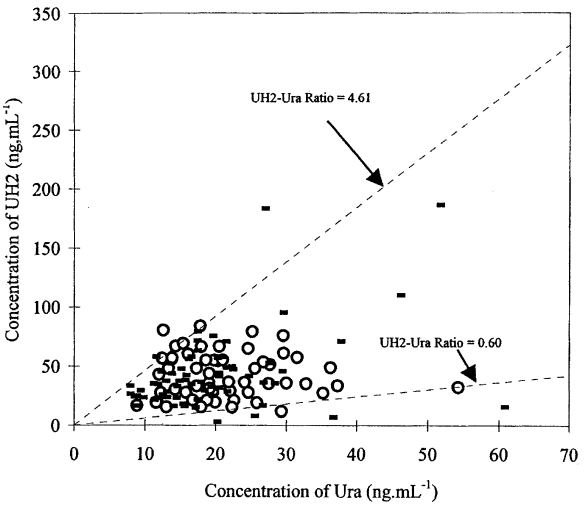

Fig. 3. Distribution of Ura and UH2 concentrations in plasma of healthy subjects; $r=0.289$, $P=0.001$, n (male, ''o'')=56, n (female, ''–'')=67; 5 and 95% percentiles of UH2–Ura ratio were 0.60 and 4.61, respectively.

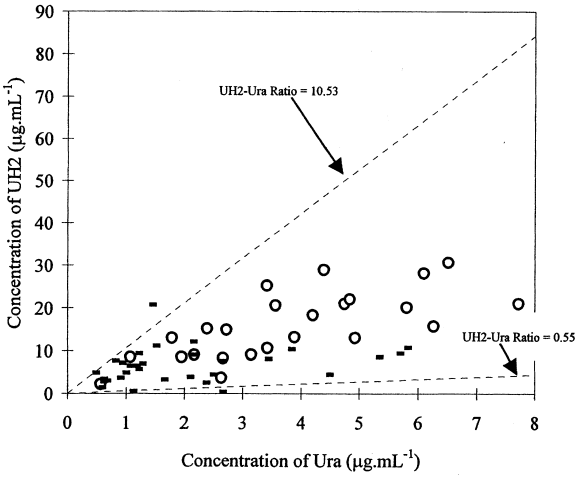

Fig. 4. Distribution of Ura and UH2 concentrations in urine of healthy subjects; $r=0.683$, $P=0.000$, n (male, ''o'')=24, n (female, ''–'')=31; 5 and 95% percentiles of UH2–Ura ratio were 0.55 and 10.53, respectively.

Table 3
Statistic parameters of Ura (ng ml^{-1}), UH2 (ng ml^{-1}) and UH2/Ura ratio in plasma and urine of healthy subjects

Samples	Analyte	Group	*n*	Medium	Minimum	Maximum	5% Percentile	95% Percentile	Normality test (*P*)	*t*-test (*P*)
Plasma	Ura	Male	56	19.76	8.90	54.30	11.77	36.48	0.063*	0.142
		Female	67	17.06	7.61	60.47	8.21	42.48	0.000	
		Total	123	18.65	7.61	60.47	8.55	37.14	0.000	
	UH2	Male	56	36.41	11.82	84.22	15.57	79.56	0.011	0.872
		Female	67	35.47	2.85	186.82	10.77	104.55	0.000	
		Total	123	35.91	2.85	186.82	15.43	80.33	0.000	
	UH2/Ura ratio	Male	56	1.87	0.40	6.38	0.68	4.67	0.007	0.314
		Female	67	2.30	0.14	6.88	0.28	4.54	0.200*	
		Total	123	2.13	0.14	6.88	0.60	4.61	0.022	
Urine	UH2/Ura ratio	Male	24	4.39	2.73	7.38	2.73	7.35	0.011	0.943
		Female	31	4.19	0.13	14.57	0.32	12.26	0.143*	
		Total	55	4.36	0.13	14.57	0.55	10.53	0.054*	

*$P>0.05$, significant normality distribution.

owing to their weak UV absorbance. The GC–MS method with high sensitivity but a time-consuming derivatization procedure [4] is also unfeasible. However, the high sensitivity offered by LC–MS–MS can easily measure concentrations of Ura and UH2 in plasma and urine with a broad linear range and a simple preparatory procedure.

Ura and its metabolite UH2 are endogenous high polarity substances, which results in difficult liquid–liquid extraction and ultimately low detection. Solid phase extraction (SPE) was not very suitable for the separation of Ura and UH2 from plasma because they can co-elute with other endogenous substances in plasma. Precipitating plasma protein by organic solvents (methanol, acetonitrile) or trichloroacetate (TCA) is accompanied by interference of large amounts of endogenous substances during detection. Moreover, samples were also diluted by this method. Therefore, liquid–liquid extraction may be the only way to prepare samples. In our method, we optimized the condition of extracting plasma samples after studying many methods [4,11,16] in order to achieve a higher detection limit. A constitution ratio of 15:85 (v/v) for isopropanol to ethyl acetate was better than 10:90 (v/v) or 20:80 (v/v). Since pH in plasma is also a key factor for extraction, pH 2, 5, 6.8, 11 in plasma were tested during extraction procedure, and results indicated that the highest extraction coefficient was achieved at neutral pH of plasma. We also found that addition of neutral salt can cause water-soluble substances to salt-out and enter organic phase effectively. The amount of ammonium sulfate added before extraction must be less than half the volume of plasma, which greatly increases the extraction coefficient from ~15% (without ammonium sulfate) to >80%.

With respect to detecting Ura and UH2 concentrations by MS, the following three points are important:

(a) A fraction of 0.1 ml min^{-1} was infused into LC–MS–MS for easily atomization in ion source because detection sensitivity was just proportional to the concentration of analyte.

(b) Any salt or acid added to the mobile phase will decrease detection sensitivity of analyte, probably due to competition of ionization in ion source.

(c) The addition of ammonium sulfate during plasma extraction may cause impurity to interfere with the detection of Ura and UH2. Therefore, ammonium sulfate must be purified by isopropanol–ethyl acetate before use (15:85, v/v).

Pyrimidine antimetabolic drugs and Ura have the same metabolic pathway via DPD in vivo due to their similar chemical structures. This can also be demonstrated by the marked increase of Ura in plasma or urine when in the presence of an inhibitor of DPD. So analyzing endogenous Ura and UH2 as well as their concentration ratio in biologic fluids

before administering drugs will help to prospectively evaluate toxic or therapeutic effects on patients, especially for patients with DPD deficiency. Determination of DPD level in peripheral blood mononuclear cells (PBMC) or tumor tissues reported in many papers is regarded as a global method to estimate the total DPD level or the sensitivity of tumor to pyrimidine drugs. But this method is laborious and expensive and cannot actually reflect total DPD level compared with our method of using analog as a probe compound to estimate the metabolic degree of drugs in vivo. Our method is also more accurate than simply detecting the concentration of uracil in biologic fluids [4,9]. Besides, concentrations of Ura and UH2 in urine need not be revised by creatinine [9] when estimating subject DPD levels.

Our assay has successfully measured baseline Ura and UH2 concentrations in plasma and urine of healthy subjects. Plasma Ura concentration appears to be narrowly distributed with a mean value of 0.179 ± 0.082 μM ($n=123$) which is very close to that reported by Bi et al. [4], 0.17 ± 0.05 μM ($n=11$). UH2–Ura concentration ratios in plasma and urine are 2.301 ± 1.236 ($n=123$) and 4.462 ± 2.696 ($n=55$), respectively. If this accurate and simple method were used for estimating DPD levels of patients before administration of pyrimidine drugs, its clinical importance would be shown clearly. Further research in this field will identify the practicality of measuring plasma/urine UH2–Ura concentration ratio for dosage guidance of clinical pyrimidine chemotherapy.

In conclusion, we have developed a simple, sensitive, and selective analytic method for measuring endogenous Ura and its DPD metabolite in plasma and urine. UH2–Ura concentration ratio as an index to indicate the total DPD level is theoretically better than others [4,14]. Additional studies are ongoing to better define the clinical utility of this index.

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