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Liquid chromatography–tandem mass spectrometric assay for the analysis of uracil, 5,6-dihydrouracil and β-ureidopropionic acid in urine for the measurement of the activities of the pyrimidine catabolic enzymes☆

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Abstract

A liquid chromatography–tandem mass spectrometric assay for the determination of uracil, 5,6-dihydrouracil and β-ureidopropionic acid in urine was developed to measure the activities of enzymes involved in pyrimidine breakdown. The assay was required to investigate the relation between the uracil–dihydrouracil ratio and toxicities observed after treatment with fluoropyrimidines drugs. After addition of stable isotopically labelled internal standards, the analytes were isolated from a 100-μl urine sample using liquid–liquid extraction with ethyl acetate–2-propanol. Compounds were separated on an Atlantis dC18 column, using ammonium acetate–formic acid in water as the eluent. The eluate was totally led into an electrospray interface with positive ionisation and the analytes were quantified using triple quadrupole mass spectrometry. The assay was validated in the range 1.6–1600 μM, using both, artificial urine and pooled urine as matrices. Intra-day precisions were ≤8% and inter-day precisions were ≤10%. Accuracies between 91 and 108% were found. The analytes were chemically stable under all relevant conditions and the assay was successfully applied in two clinical studies of cancer patients treated with 5-fluorouracil or capecitabine.

Keywords: Uracil; 5,6-Dihydrouracil; β-Ureidopropionic acid; Pyrimidines; Liquid–liquid extraction; LC–MSMS

1. Introduction

The pharmacokinetics and pharmacodynamics of pyrimidine analogue drugs are strongly influenced by the activity of the enzymes responsible for pyrimidine breakdown. This catabolic route has been depicted for uracil in Fig. 1. The activity of the enzymes dihydropyrimidine dehydrogenase (DPD), dihydropyrimidinase (DHP) and β-ureidopropionase (UP), and the aminotransferases leading to total breakdown of uracil show high inter-individual variations. Especially genetic variations in genes coding for these enzymes can result in the total deficiency of one of these enzymes, predominantly resulting in neurological disorders [1] or in partial deficiencies. Inter-individual variability in pyrimidine metabolism can have a significant impact on the efficacy of pyrimidine analogues, such as the anticancer agents 5-fluorouracil (5-FU) and its pro-drug capecitabine. Because DPD catalyses the rate determining reaction of 5-FU catabolism, several studies have been undertaken to investigate the relation between DPD activity and the efficacy and/or toxicity of 5-FU [2–9]. The roles of DHP and UP are less important. The activity of DPD can be determined directly [2,5,8] or indirectly by measuring variations in the pyrimidine levels in plasma [3,4,7,9], but both show a high variation. In addition, the DPD expression can be evaluated [5,6] and the DPD variability can also be assessed genetically. Especially the splice-site mutation IVS14 + 1G > A has the most frequently observed impact on DPD phenotype but does not cover all deficient mutations; only 43% in a Dutch population [8] and even only one out of 28 deficient patients in a French population [10]. Further, the first death-case due

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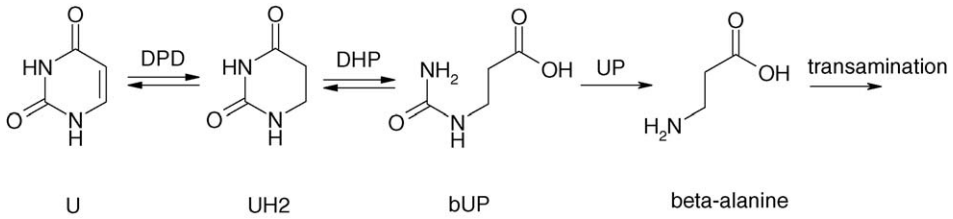

Fig. 1. Reaction pathway for uracil breakdown. DPD: dihydropyrimidine dehydrogenase; DHP: dihydropyrimidinase; UP: β-ureidopropionase; U: uracil; UH2: 5,6-dihydrouracil; bUP: β-ureidopropionic acid.

to capecitabine use by a DPD deficient patient showed the 1896C > T mutation [11]. Therefore, phenotyping may probably be preferred above genotyping methods. All studies report a positive, but often poor, correlation of the parameters studied with the clinical outcome of 5-FU treatment [2–9,12] and, if investigated, with toxicity [2,4,8,12]. The ultimate goal is dose individualization of fluoropyrimidines, hopefully resulting in less side effects and optimal efficacy. Although dose individualization charts based on plasma dihydrouracil–uracil ratio have already been proposed [3,4], clinical usefulness of all analytical approaches has not (yet) been proven is therefore still under investigation.

The activity of DPD and the other pyrimidine catabolic enzymes can be determined indirectly with radio-assays using liver [13–15] or lymphocyte samples [16], immunohistochemical assays using liver samples [5,6] or HPLC using peripheral blood mononuclear cells [17]. However, direct assays of pyrimidines and their catabolites in urine or plasma, reflecting the in vivo activity of enzymes responsible for pyrimidine breakdown, are faster, easier to use and do not require radio-labelled compounds or antibodies. Several direct methods for the determination of DPD activity using urine [18–25] or plasma [9,20,26–29] samples have been reported, using different probe compounds (uracil [9,18–29]), thymine [19,21,24,25] and 5-FU [9,26]) and different analytical techniques (LC–MSMS [18,20,24,25], LC-UV [9,22,23,26,27,29], GC–MS [19,21,28]. Some methods can simultaneously determine the activity of DHP [19,21–23,25] and UP [25,30]. Uracil seems to be the most preferred probe compound and urine the favorite sample matrix for the analysis of uracil and its catabolites. The higher levels in urine compared to plasma are probably advantageous. Typically, as far as we know, urinary assays were not yet used in clinical studies of pyrimidine analogue drugs. For the assays combining the determination of uracil and 5,6-dihydrouracil (UH2), two validated assays using LC-UV for plasma samples [26,27] and one LC–MSMS assay for both, urine and plasma samples [20] were reported. We developed and validated a chromatographic assay for the simultaneous determination of U, UH2 and bUP using LC–MSMS because of the superior selectivity of the detection method. A similar assay was reported previously [25] but with insufficient sensitivity for UH2. Further, we evaluated two pools of patient samples using the new assay to explore the possibilities of dose individualization of capecitabine. Urinary levels of uracil and its catabolites were measured as part of different clinical studies in order to relate their ratios with observed 5-FU toxicities [31].

2. Experimental

2.1. Chemicals

Uracil (98%) and 5,6-dihydrouracil (98%) were supplied by Aldrich (Milwaukee, WI, USA), β-ureidopropionic acid and uracil-1,3-$^{15}N_2$ (98 atom% ^{15}N) (U′) by Sigma (St. Louis, MO, USA) and 5,6-dihydrouracil-$^{13}C_4$-$^{15}N_2$ (98%+ $^{13}C_4$-$^{15}N_2$) (UH2′) by Cambridge Isotope Laboratories (Andover, MA, USA). Pooled urine was obtained from equal portions of urine from six healthy volunteers). Water of LC–MS quality was obtained from Biosolve (Valkenswaard, The Netherlands) and other water used was home-purified by reversed osmosis on a multi-laboratory scale. All other chemicals were of analytical grade and originated from Merck (Darmstadt, Germany).

β-Ureidopropionic acid-$^{13}C_4$-$^{15}N_2$ (bUP′) was obtained by hydrolysis of UH2′ [14]. Sodium hydroxide (400 μl, 0.2 M) was added to 200 μl of 33.3 mM UH2′ in water. After heating at 37 °C for 15 min, the mixture was neutralized with 600 μl of 0.1333 M hydrochloric acid. A 100% conversion of this reaction would result in a yield of 5.6 mM bUP′. Remaining UH2′ was not detected in the product using a GC–MS assay according to Kuhara et al. [19,30,32]. One liter of artificial urine comprised 1.3 g sodium dihydrogen phosphate monohydrate, 0.51 g disodium hydrogen phosphate dihydrate, 0.58 g trisodium citrate dihydrate, 6.8 g sodium chloride, 12 g ureum, 0.90 g calcium chloride dehydrate, 2.5 g disodium sulfate, 0.75 g magnesium sulfate, 2.3 g ammonium chloride and 6.1 g potassium chloride.

2.2. Equipment

The LC–MSMS equipment consisted of a DGU-14A degasser, a Sil-HTc autosampler and two LC10-ADvp-μ pumps (all from Shimadzu, Kyoto, Japan) and a Finnigan TSQ Quantum Discovery Max triple quadrupole mass spectrometer with electrospray ionization (Thermo Electron, Waltham, MA, USA). Data were recorded on and the system was controlled by a Dell Optiplex GX270 personal computer, equipped with the Finnigan Xcalibur software (version 1.4, Thermo Electron).

2.3. LC–MSMS conditions

Partial-loop injections (5 μl) were made on an Atlantis dC18 column (150 mm × 2.1 mm, d_p = 3 μm, average pore diameter = 10 nm, Waters Chromatography, Milford, MA, USA) with an Atlantis dC18 pre-column (10 mm × 2.1 mm, d_p = 3 μm,

Table 1
Tuned ESI and MS conditions, found by optimization with UH2 and bUP standards

Parameter	UH2	bUP
ESI spray voltage (V)	5000	5000
Ion transfer capillary temperature (°C)	216	300[a]
Argon pressure Q2 (mTorr)	1.0	1.0

[a] 216 °C was used instead of the optimum.

Waters). The column temperature was maintained at 0 °C using melting ice and the autosampler was maintained at 15 °C. The eluent comprised 0.075 g/l ammonium acetate and 0.0025% (w/v) formic acid in water.

The eluent flow rate was 0.2 ml/min and de eluate was led into the electrospray probe, oriented at "1.0" in the X-direction, at "−1" in the Y-direction and at "C" in the Z-direction. The calibration of the quadrupoles was performed using Tyr and the $(Tyr)_3$ and $(Tyr)_5$ peptides. The ion spray was tuned for UH2, the conditions found (Table 1) were also used for U, and for bUP, only the optimum temperature of the ion transfer tube was not used in the final method for this compound because cooling down the tube from 300 to 216 °C was too slow for the assay. Collision energies and used off set values of the tube lens for all parent and daughter ions of the three compounds and their labelled internal standards (ISs) are reported in Table 2. For uracil and UH2, the signals of the two most abundant daughter ions of each analyte (Table 2; Fig. 2) were added up. The mass resolution was set at 0.7 full with at half height (unit resolution) for all compounds. A 13-min run time was used.

2.4. Sample pre-treatment

To a 100-μl urine sample, pipetted into a glass tube, 50 μl of the IS-mixture (66 μM U′, 67 μM UH2′ and 56 μM bUP′ in water), 100 μl of saturated ammonium sulfate–formic acid (10:1, v/v) and 4 ml of ethyl acetate–2-propanol (10:1, v/v) were added. The sample was shaken on a rotary mixer for 10 min at 40 rpm and after centrifugation at 2.3×10^3 *g* for 5 min the organic phase was poured into a conical glass tube and evaporated under a stream of nitrogen at 25 °C. The residue was reconstituted in 100 μl water by vortex-mixing. After centrifugation at 2.3×10^3 *g* for 5 min, the clear solution was transferred into a 250-μl glass insert placed in an injection vial.

Table 2
MS parameters of individual compounds

Parameter	Uracil	U′	UH2	UH2′	bUP	bUP′
Parent mass	113.0	115.0	115.0	121.0	133.0	139.0
Daughter mass	70.0 96.0	71.0	55.1 73.1	58.0	115.0	121.0
Tube lens offset	65	65	76	76	89	89
Collision energy (V)	20 20	20	24 15	24	10	10

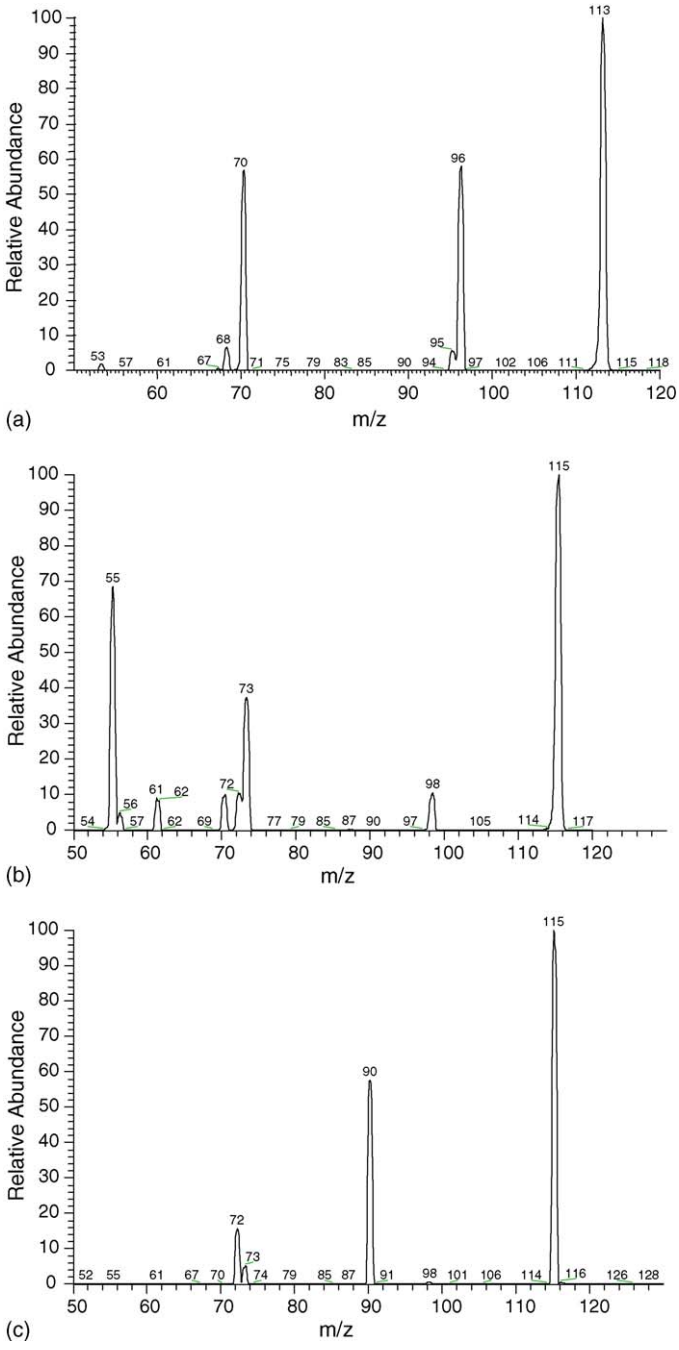

Fig. 2. Fragmentation spectra of (A) uracil, (B) dihydrouracil and (C) β-ureidopropionic acid. $[M+H]^+$ ions were fragmented with argon at the optimum collision energies of the most prominent daughter ions (Table 1).

2.5. Validation

2.5.1. Calibration

Stock solutions were prepared in water and were stored at −30 °C. Two stock solutions were prepared for each analyte and one for each IS. Three stock solutions were combined and diluted to a working solution containing 1665 μM uracil, 1550 μM UH2 and 1574 μM bUP. This solution, stored at −30 °C, was used daily to prepare calibration samples in water yielding ca. 1600, 400, 160, 40, 16, 4 and 1.6 μM for each compound. The

two samples at the lowest levels were analyzed duplicately for each analytical run, the others only once. Least-squares linear regression using the individual samples and weighted by X^{-2} (reciprocal for the squared concentration) was employed to define the calibration curve using the ratios of the peak heights of each compound and its stable isotopically labelled IS in each calibration sample. For U' a correction of 0.2% of the uracil response was used because of isotopic interference from uracil.

2.5.2. Precision and accuracy

The second stock solution of each analyte was used to obtain validation (quality control (QC)) samples in artificial urine at ca. 1.6 and 4 μM of each analyte. The pooled "blank" urine, representing an average individual or patient, as well as three spiked samples of the same urine were also used for the validation. QC-A was spiked with 1824 μM uracil, representing total DPD deficiency [19,21]; QC-B with 912 μM uracil and 1774 μM UH2, representing total DHP deficiency [19,21,22,33] and QC-C with 365 μM uracil, 709 μM UH2 and 1535 μM bUP, representing total UP deficiency [13,30]. Precisions and accuracies were determined by sextuple analysis of each validation sample in three analytical runs on three separate days for all QCs (total: $n = 18$). Relative standard deviations were calculated for both, the intra-day precision (repeatability) and the inter-day precision (reproducibility). Measured endogenous amounts of the analytes were added to the spiked amounts of QC-A, -B and -C, to obtain the expected values.

2.5.3. Selectivity

Six individual urine samples from different healthy donors were processed to test the selectivity of the assay. These samples were additionally processed without IS, after spiking with ca. 8 μM and after spiking with ca. 32 μM of each compound, respectively.

2.5.4. Recovery

The extraction yield was determined triplicately by comparing processed samples with samples obtained by reconstitution of blank residues (dried extracts) of the same matrix in water containing the analytes or ISs at the same concentrations. The ion-suppression was calculated by comparing these spiked extracts (triplicately) with "academic" solutions in water.

The analytes were tested at ca. 1600 and 160 μM in urine, 1600 and 4 μM in water and at ca. 16 μM in artificial urine. The internal standards were tested at the concentrations used in the assay in artificial urine, pooled urine, water and water containing ca. 1600 μM of each analyte (highest calibration sample).

2.5.5. Stability

The stability of the analytes was investigated in QC-C and in the pooled "blank" urine. Quadruple analysis of these samples was performed after the following additional storage conditions: 24 h at ambient temperature, three freeze–thaw cycles, 7 days at 4 °C and 13 months at −30 °C.

Furthermore, samples from complete validation runs were reinjected after additional storage of the reconstituted extracts for 4 days at 15 °C and for 7 days at 4 °C, respectively.

The stability of the analytes in the stock and working solutions was tested by diluting the samples to approximately 80 μM, sampling 100 μl duplicately and adding 50 μl of IS solution. The mixture was then analyzed, using the LC–MSMS analysis with 10-μl injections. Freshly weighted and diluted standards were used as a reference.

2.5.6. Patient samples

Patient samples were obtained from two clinical studies.

(A) Fifty-three cancer patients (32 female, 21 male; 92% Caucasian) were treated with 5-FU or capecitabine previously. Urine was obtained in the fasted state. Of these patients, 25 developed 5-FU-related toxicity in the past.
(B) Seventy-six cancer patients received combination therapy with capecitabine. Patients with advanced colorectal cancer additionally received oxaliplatin, patients with advanced gastroesophageal cancer epirubicin and cisplatin. Spot urine was taken from these patients, mostly in the fasting state shortly before receiving chemotherapy.

All study protocols were approved by the Medical Ethical Committee of the Antoni van Leeuwenhoek Hospital/The Netherlands Cancer Institute, Amsterdam, The Netherlands. They were performed according to the Good Clinical Practice Guidelines and written informed consent was obtained from each patient prior to the start of the study.

3. Results and discussion

3.1. Method development

Developing a chromatographic assay for urine was initially started at our laboratory using GC–MS according to the method of Kuhara et al. [19,30,32] using our labelled ISs. Unfortunately, the validation of this assay was not successful due to batch-to-batch variation of the N,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)/trimethylchlorosilane (TMCS) reagent, resulting in a poor yield of the derivatization for several batches. We, therefore, switched to LC–MSMS for development of the assay for uracil, HU2 and bUP.

Most previously published LC–MSMS assays used negative ionization for uracil and UH2 [18,20,24], with exception of the study of van Lenthe et al. [25] who used positive ionization for U, UH2 and bUP. All methods used long (15–25 cm) reversed-phase columns, effluent splitting before the electrospray and, with one isocratic exception [20], gradient elution. Several additions to the water–methanol eluents were used: ammonium formate (pH 5) [24], acetic acid/ammonium acetate (pH 4) [25], formic acid (pH 2.6) [18] or none [20]. Initially we tried to inject non-treated urine samples to avoid performing an extraction procedure; however, serious ion-suppression was observed, especially for UH2.

For the assignments of the daughter ions, the work of Kamel and Munson [34] was a helpful tool. The product ions of $[\text{uracil} + \text{H}]^+$ (*m*/*z* 113) (spectrum shown in Fig. 2A), could be explained by the loss of NH_3 (*m*/*z* 96) and the loss of HNCO

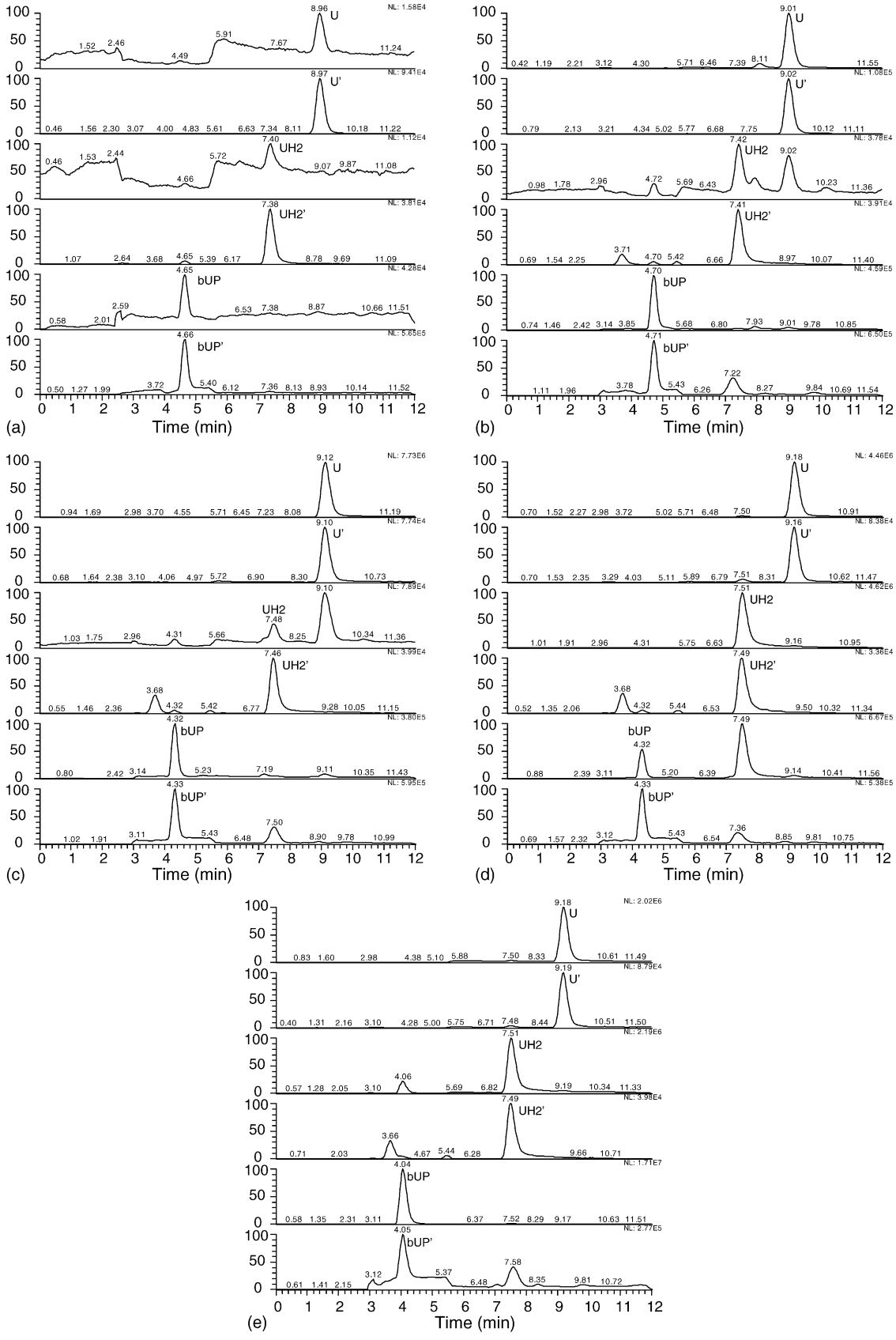

Fig. 3. MSMS chromatograms of (A) artificial urine spiked with analytes at level of LLQ, (B) pooled blank urine, (C) QC-A (1860 μM uracil, 9.6 μM UH2 and 16.8 μM bUP), representing DPD deficiency, (D) QC-B (912 μM uracil, 1774 μM UH2 and 16.8 μM bUP), representing DHP deficiency and (E) QC-C (365 μM uracil, 710 μM UH2 and 1535 μM bUP), representing UP deficiency. Monitored ions are reported in Table 2.

(*m/z* 70). The product ions of $[UH2+H]^+$ (*m/z* 115) (Fig. 2B) could be explained by the loss of H_2CCO (*m/z* 73) and the loss of NH_2CONH_2 (*m/z* 55). The main fragment (*m/z* 115) of $[bUP+H]^+$ (*m/z* 133) was caused by the loss of water, other ions could be explained by the loss of HNCO (*m/z* 90), HNCO and water (*m/z* 72) and the loss of NH_2CONH_2 (*m/z* 73) (Fig. 2C).

Using the chosen most prominent transitions, uracil and UH2 both showed a minor response (ca. 1% for both) in the transition of the other compound (cross-interference). Further, significant response from bUP at the UH2 transitions (*m/z* 115 → 96 and 70), probably due to the loss of H_2O during ionization, was observed. Therefore, chromatographic separation of all compounds was required. To achieve separation of uracil and UH2, the column had to be long enough (150 mm), this length was also necessary to prevent ion-suppression in the electrospray by the urine matrix. Other measures to avoid this suppression were a limited injection volume (5 μl) and the use of liquid–liquid extraction. The retention of bUP was increased by lowering the pH of the eluent with formic acid. While the concentration of ammonium acetate in the eluent resulting in the highest MSMS response for both uracil and UH2 was 0.1 g/l, a quarter of this amount was replaced by formic acid to obtain sufficient retention of bUP. Analogously, to facilitate liquid–liquid extraction of bUP, formic acid was added to the samples prior to extraction. Finally, the column was cooled to 0 °C in order to separate dihydrouracil from an interfering endogenous compound, possibly 5,6-dihydrouridine [25]. The ice bath, kept in an isolating box, remained at 0 °C overnight (>16 h).

3.2. Validation

Chromatograms of the lower limit of quantification (LLQ) in artificial urine, pooled urine, QC-A, QC-B and QC-C samples are shown in Fig. 3. The figures show complete separation of the analytes at all concentrations and an excellent discrimination between the different phenotypes.

3.2.1. Calibration

The assay was linear for all compounds in the whole concentration range (1.6–1600 μM) (Table 3). For eight calibration curves, the concentrations were back-calculated from the ratio of the peak heights and no deviations higher than 8% were observed (Table 3).

3.2.2. Precision and accuracy

Assay performance data are reported in Table 4. No intra-day variations higher than 10.3% and no deviations of the accuracy higher than 9.1% were observed. Therefore, upper and lower limits of the calibration can be assigned to the upper and lower limits of quantification [35–37], being 17 μM for U and 16 μM for both, UH2 and bUP.

3.2.3. Selectivity

No interference from the urine matrix was observed for both, analytes and ISs. The spiked amounts of the analytes were back-calculated and are reported in Table 5.

Table 3
Back-calculated concentrations ($n=8$) of the analytes from the calibration samples

Nominal concentration (μM)	Concentration found (μM)	Precision (%)	Accuracy (%)
Uracil			
1.67	1.67	5.2	100.4
4.16	4.14	3.8	99.4
16.65	16.39	1.6	98.4
41.62	40.80	0.7	98.1
166.5	162.4	1.7	97.6
416.2	144.2	2.7	99.0
1665	1794	3.3	107.7
5,6-Dihydrouracil			
1.55	1.54	10.3	99.6
3.87	3.89	7.5	100.5
15.50	16.05	3.1	103.6
38.74	38.78	3.7	100.1
155.0	157.1	2.4	101.4
387.4	381.7	1.8	98.5
1550	1493	1.9	96.3
β-Ureidopropionic acid			
1.57	1.58	4.0	100.3
3.94	3.92	5.0	99.6
15.74	15.60	4.1	99.1
39.36	31.14	2.6	98.3
157.4	157.4	3.0	99.9
393.6	392.9	2.9	99.8
1574	1624	5.0	103.1

Table 4
Assay performance data ($n=18$ on 3 days) for the determination of U, UH2 and bUP

Nominal concentration (μM)	Concentration found (μM)	Intra-day precision (%)	Inter-day precision (%)	Accuracy (%)
Uracil				
1.82 (art. Urine)	1.78	5.3	5.6	97.4
4.56 (art. Urine)	4.27	3.9	5.3	93.6
Pooled urine	36.1	2.9	3.1	
1824 (+36.1)	2005	2.2	2.6	107.7
912 (+36.1)	944.8	3.7	6.4	99.6
365 (+36.1)	391.6	2.9	2.9	97.6
5,6-Dihydrouracil				
1.77 (art. Urine)	1.72	6.9	8.0	96.9
4.44 (art. Urine)	4.24	5.3	5.5	95.7
Pooled urine	9.58	5.6	6.2	
0.00 (+9.6)	8.82	8.2	10.3	92.0
1774 (+9.6)	1706	4.7	8.9	95.7
710 (+9.6)	694	3.6	3.9	96.6
β-Ureidopropionic acid				
1.53 (art. urine)	1.54	1.8	4.9	99.8
3.84 (art. urine)	3.59	2.5	3.4	93.5
Pooled urine	16.8	2.9	5.4	
0 (+16.8, QC-A)	15.4	3.0	5.6	92.0
0 (+16.8, QC-B)	15.2	4.4	5.8	90.9
1535 (+16.8)	1529	2.4	4.7	98.5

Between brackets: endogenous concentrations.

Table 5
Back-calculated concentrations ($n = 6$) of the amounts of analyte spiked to individual urine samples; concentrations in blank samples were subtracted from concentrations measured in spiked samples

Nominal concentration (μM)	Concentration found (μM)	Precision (%)	Accuracy (%)	Range of endogenous concentrations (μM)
Uracil				11–102
36.5	37.2	4.7	102.0	
9.12	8.9	18	97.4	
5,6-Dihydrouracil				<1.6–30
35.5	38.3	3.5	108.0	
8.87	9.6	12	108.9	
β-Ureidopropionic acid				2.6–40
30.7	28.7	7.3	93.6	
7.67	6.6	14	85.5	

3.2.4. Recovery

Recoveries for extraction and ion-suppression, as well as the overall recovery, are reported in Table 6 for the analytes and in Table 7 for the ISs. Typically, the recovery of the extraction is in the range 70–90% under all tested conditions for all analytes. Ion-suppression by matrix constituents is also variable and increases in the order bUP < U < UH2 for the analytes as well as for the ISs. Auto-suppression at the highest concentration of the analytes is also variable and shows a marked increase in the order UH2 < U < bUP (Table 7).

3.2.5. Stability

Recoveries of the analytes under different storage conditions in urine are shown in Table 8. Injection of the reconstituted extracts of two complete validation runs after additional storage, 4 days at 15 °C and 7 days at 4 °C, respectively, resulted again

Table 6
Recoveries (±S.D.; $n = 3$), corrected for endogenous concentrations if required, of the analytes at different concentrations in different matrices

Concentration (μM)	Matrix	Extraction (%)	Ion-suppression (%)	Overall (%)
Uracil				
1824 (+36.1)	Urine	90 ± 9	80 ± 11	72 ± 7
182.4 (+36.1)	Urine	80 ± 8	80 ± 8	64 ± 6
1824	Water	77 ± 5	104 ± 9	80 ± 8
4.65	Water	82 ± 8	108 ± 8	88 ± 7
1.82	Art. urine	79 ± 10	86 ± 11	68 ± 8
5,6-Dihydrouracil				
1774 (+9.6)	Urine	90 ± 14	74 ± 10	67 ± 9
177.4 (+9.6)	Urine	80 ± 9	71 ± 7	56 ± 6
1774	Water	77 ± 5	102 ± 7	79 ± 7
4.44	Water	74 ± 8	125 ± 15	92 ± 14
1.77	Art. urine	81 ± 15	74 ± 11	60 ± 11
β-Ureidopropionic acid				
1535 (+16.8)	Urine	86 ± 13	96 ± 6	82 ± 12
153.5 (+16.8)	Urine	76 ± 4	77 ± 3	59 ± 3
1535	Water	75 ± 1	102 ± 2	77 ± 2
3.84	Water	73 ± 5	94 ± 5	69 ± 4
1.53	Art. urine	69 ± 2	85 ± 2	59 ± 2

Between brackets: endogenous concentrations.

Table 7
Recoveries (±S.D.; $n = 3$), of the ISs at their used concentrations (ca. 60 μM for each IS) in different matrices

Matrix	Ion-suppression (%)
U′	
QC 0.2 (Art. urine)	84 ± 9
Urine	68 ± 6
Water	94 ± 17
Highest calibration sample (water)	70 ± 3
UH2′	
QC 0.2 (Art. urine)	64 ± 9
Urine	49 ± 6
Water	93 ± 9
Highest calibration sample (water)	100 ± 6
bUP′	
QC 0.2 (Art. urine)	88 ± 11
Urine	71 ± 5
Water	94 ± 8
Highest calibration sample (water)	34 ± 1

Table 8
Stability data (±S.D.; $n = 4$), of the three analytes under different storage conditions

Conditions/sample	Pooled urine	QC-C[a]
Uracil		
24 h at ambient temperature	99.7 ± 3.6	97.3 ± 7.3[b,c]
Three freeze–thaw cycles	96.2 ± 1.5[b]	96.9 ± 2.5[b]
7 d at 4 °C	109.8 ± 3.3[b]	113.0 ± 0.9[b,c]
13 months at -30 °C	93.8 ± 1.9	89.3 ± 3.2
5,6-Dihydrouracil		
24 h at ambient temperature	100.3 ± 3.9	95.6 ± 5.3[b,c]
Three freeze–thaw cycles	99.1 ± 6.2[b]	96.7 ± 0.5[b]
7 d at 4 °C	111.5 ± 8.0[b]	106.6 ± 1.4[b,c]
13 months at -30 °C	101.7 ± 6.4	98.4 ± 4.9
β-Ureidopropionic acid		
24 h at ambient temperature	98.2 ± 3.6	95.9 ± 6.2[b,c]
Three freeze–thaw cycles	109.9 ± 3.5[b]	94.7 ± 2.2[b]
7 d at 4 °C	100.6 ± 4.1[b]	109.2 ± 1.3[b,c]
13 months at -30 °C	102.5 ± 2.9	98.7 ± 1.5

[a] QC-C: pooled urine spiked with 365 μM uracil, 710 μM UH2 and 1535 μM bUP.
[b] Samples were measured using a GC–MS assay according to Kuhara et al. [19,30,32].
[c] $n = 3$.

Table 9
Concentrations measured (±S.D.) in clinical samples

Population	C_U (μg/ml)	C_{UH2} (μg/ml)[a]	C_{bUP} (μg/ml)	n
Healthy subjects	30 ± 28	12 ± 10 (1)	20 ± 15	12
A[b]	33 ± 22	11 ± 7 (1)	29 ± 45	53
B[c]	17 ± 13	7.4 ± 5.7 (4)	11 ± 7	76

[a] Between brackets: number of measurements below LLQ (1.6 μM).
[b] A: Cancer patients previously treated with capecitabine or 5-FU.
[c] B: Patients with advanced colorectal cancer or with advanced gastroesophageal cancer.

in successful performances (data not shown). Recoveries of the analytes from individual stock and mixed working solutions after storage for 26 months at −30 °C ranged from 96 to 106%.

3.2.6. Patient samples

Twelve healthy subjects and 129 cancer patients were investigated. The concentrations of U, UH2 and bUP were assessed for the cancer patients as part of a study protocol. Measured concentrations are reported in Table 9, for UH2 the level was below the LLQ in a few occasions. The average molar ratios are reported in Table 10 and are compared with previously reported populations. The variation of the U/UH2 ratio between all studies is high and difficult to explain. Such a variation can also be observed for plasma ratios [2,4,9,29]. Different ratios for U/UH2 have been reported by different research groups, even for healthy volunteers. These discrepancies may be caused by circadian rhythm and sampling timing. In addition, these groups origin from different countries: Japan, China, France and The Netherlands. Therefore, ethnicity (unfortunately, often not reported) may also play a role. Our results for urine samples, however, do correspond with the Japanese studies [21,22,33]. The only bUP/UH2 ratio previously reported does not correspond with our results, but possibly the analytical interference of 5,6-dihydrouridine with UH2 in that study could have played a role [38].

Table 10
New and previously reported molar ratios of U, UH2 and bUP

U/UH2	bUP/UH2	n	Matrix	Population	Technique	Source
2.7 ± 0.7	1.6 ± 0.5	12	Urine	Healthy subjects	LC–MSMS+	New
3.6 ± 2.4	2.6 ± 1.8	53	Urine	A[a]	LC–MSMS+	New
2.3 ± 1.0	1.8 ± 0.9	76	Urine	B[b]	LC–MSMS+	New
1.24		1000	Urine	Newborn	LC-UV	[33]
3.9		18	Urine	3–8 y	LC-UV	[33]
0.54		48	Urine	Newborn	GC–MS	[19]
4.8		27	Urine	>4 y	GC–MS	[19]
2.9		1000	Urine	Infants	LC-UV	[22]
0.42	0.32	Unknown	Urine	Reference	bUP: LC-MSMS + U, UH2: unclear	[13]
Ca. 0.2		55	Urine	Healthy subjects	LC–MSMS-	[20]

[a] A: Cancer patients previously treated with capecitabine or 5-FU.
[b] B: Patients with advanced colorectal cancer or with advanced gastroesophageal cancer.

4. Conclusions

The reported assay can be used retrospectively and prospectively for phenotyping and activity measurements of pyrimidine catabolic enzymes as it allows good discrimination between normal activities and deficiencies of DPD, UHP and UP. Furthermore, this is the fist assay with sufficient sensitivity, at least for UH2, for simultaneous determination of uracil, UH2 and bUP in urine to be used for dose evaluation of fluoropyrimidines drugs and possibly also for dose adjustment. The assay meets common standards for precision, accuracy and linearity and is sufficiently sensitive; ion-suppression effects are all corrected by the labelled ISs. Variations between uracil/UH2 ratios from existing publications cannot be explained so far. Thus, cross-validation of the assay is warranted. Finally, the first data of urinary levels of U, UH2 and bUP in cancer patients have been reported now.

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