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Journal of Chromatography B, 774 (2002) 223–230

JOURNAL OF CHROMATOGRAPHY B

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Simple liquid chromatographic method for the determination of uracil and dihydrouracil plasma levels: a potential pretreatment predictor of 5-fluorouracil toxicity

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Received 28 December 2001; received in revised form 12 April 2002; accepted 12 April 2002

Abstract

5-Fluorouracil (5-FU) is a commonly used anti-cancer drug with notable activity in clinical practice, yet it causes significant unpredictable and often serious toxicity. Both 5-FU and uracil (U) are catabolised by dihydropyrimidine dehydrogenase (DPD) to form dihydrofluorouracil (FUH_2) and dihydrouracil (UH_2), respectively. A means of predicting toxicity before treatment would be more valuable. Variations in dihydropyrimidine dehydrogenase (DPD) activity between patients are at least partly responsible for variable toxicity. Measurement of the UH_2 to U ratio may be a measure of pyrimidine catabolism and thus be utilised to predict subsequent toxicity. We have developed an efficient extraction and detection method using HPLC for the simultaneous measurement of UH_2 and U in plasma. A single C_{18} Spherisorb ODS2 (25 cm) column using isocratic elution was utilised. U, UH_2 and the internal standard 4-chlorouracil were detected at wavelengths of 257, 220, and 268 nm, respectively. The chromatographic run time was 45 min which is half that of other methods. The detection limit was 0.02 μM for U and 0.1 μM for UH_2 using only 0.5 ml of plasma for both compounds. The basal plasma concentrations of U and UH_2 in 23 individuals ranged from 0.025 to 0.27 μM and 0.4–1.7 μM, respectively. This simple method may permit the assessment of pyrimidine catabolism, and therefore allow prediction of the toxicities associated with the use of fluorinated pyrimidines.

Keywords: Uracil; Dihydrouracil; 5-Fluorouracil

1. Introduction

5-Fluorouracil (5-FU) is one of the most commonly used anticancer drugs in the treatment of gastrointestinal, breast, and head and neck malignancies, using various schedules. 5-FU can produce severe haematological, mucosal and gastrointestinal toxicity, which is more often encountered with dose intensification strategies, but also occurs with moderate doses in adjuvant therapy treatments [1]. The metabolic pathways of 5-FU have been extensively studied, with particular focus on the catabolic pathway and its contribution to toxicity. There is a high degree of individual variability of 5-FU plasma pharmacokinetics, and several studies have demonstrated a close link between toxicity or response and individual pharmacokinetic parameters [1,2].

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1570-0232/02/$ – see front matter
PII: S1570-0232(02)00239-8

Variation in 5-FU pharmacokinetic parameters partially accounts for a proportion of the variance in toxicity [1,3,4]. Goldberg et al. showed that 5-FU area under the curve (AUC) correlated with subsequent neutropenia, but only accounted for 49% of the variance [4]. Attempts have been made to reduce the incidence of toxicity by individually adjusting dose to achieve a desired plasma level or AUC [1,5,6]. Individual dose adjustment during 5-FU treatment can only be effectively applied with infusional regimens and even in this setting has only met with partial success. A simple method whereby 5-FU catabolism or anabolism could be described prior to therapeutic dosing may allow a more precise prediction of 5-FU effects and therefore permit widespread individualised dosing in a variety of treatment schedules.

Dihydropyrimidine dehydrogenase (DPD) is the rate-controlling enzyme of pyrimidine and fluoropyrimdine catabolism and accounts for 70–80% of 5-FU clearance [7]. A wide range of variation in DPD activity is observed in otherwise normal individuals due to complete familial deficiencies and genetic polymorphisms [8,9]. Attempts have been made to measure DPD activity in patients prior to treatment in an effort to identify those at high risk of 5-FU toxicity. However, measurement of DPD activity involves a time consuming radioenzymatic assay that requires moderate volumes of blood and radiolabelled materials, and is unsuitable for routine application [10].

Since 5-FU and uracil (U) are catabolised by the same pathway (Fig. 1), measurement of plasma U and its dihydrogenated metabolite dihydrouracil (UH_2) may allow estimation of DPD activity and thereby 5-FU clearance. These parameters have previously been measured in plasma by Gamelin et al. using high-performance liquid chromatography (HPLC) [11], with the UH_2 to U ratio used as an indicator of 5-FU catabolic efficiency [12]. U, the natural substrate for DPD, accumulates in plasma when systemic DPD is inhibited [13]. Patients with low DPD activity produce less UH_2 and thus have a low UH_2 to U ratio. Gamelin et al. [12] showed that patients with a low UH_2 to U ratio have higher 5-FU plasma levels and greater toxicity. Thus, plasma UH_2 to U ratio can be used as a surrogate marker to monitor the dynamic status of systemic DPD activity. Uracil excretion in urine has been proposed as another method of screening for pyrimidine catabolic defects, but the absolute urine concentration can vary, and must be corrected for creatinine excretion in order to be accurately quantitated [14]. Defects in pyrimidine degradation have also been assessed by measuring urinary concentrations of uracil, thymine and their degradation products by using HPLC–electrospray tandem mass spectrometry [15], which is not practical as a routine laboratory assay.

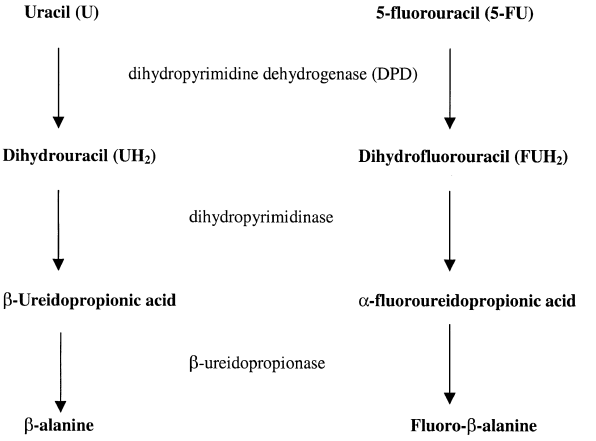

Fig. 1. Catabolic pathways of U and 5-FU.

We have developed a simple and comparatively rapid HPLC method to simultaneously measure U and UH_2 in plasma samples. This methodology utilises a simple extraction protocol and a single C_{18} Spherisorb ODS2 (25 cm) HPLC column using isocratic elution. U, UH_2 and the internal standard 4-chlorouracil (4-CU) were detected at wavelengths of 257, 220, and 268 nm, respectively. The chromatographic run time was 45 min which is half that of other methods.

2. Experimental

2.1. Chemicals and reagents

Standards: U and UH_2 were purchased from Sigma (NSW, Australia). 4-Chlorouracil was purchased from ICN Biomedicals (Seven Hills, Australia). Other reagents: ammonium sulfate

$(NH_4)_2SO_4$; potassium hydrogen phosphate (KH_2PO_4) were of analytical grade. Isopropanol and ethyl acetate were of HPLC grade (EM Science). Purified water was obtained by passage through a Nanopure II system (Sybron/Barnstead, Boston, MA, USA) and was further filtered through a 0.45 μm membrane filter (Millipore Australia, Rydalmere, Australia). Aqueous standard stock solutions (1 m*M*) were prepared by adding U and UH_2 powder to purified water. Plasma standards in the concentration range of 0.02–5.0 μ*M*, were prepared by adding aqueous stock standards to double-dialysed pooled human plasma. Double-dialysed plasma was utilised to remove endogenous U and UH_2 (dialysis tubing, to separate compounds with a molecular mass of ≤1200 from compounds of molecular mass >2000 (Sigma–Aldrich, Castle Hill, Australia)).

2.2. Plasma samples

Blood for generation of plasma standards was kindly provided by healthy volunteers. Clinical samples were obtained from patients prior to undergoing treatment with 5-FU. The institutional ethics committee approved the study and all patients gave written informed consent. Patient blood was collected before the infusion in 10-ml polypropylene tubes containing lithium heparin and kept on ice until centrifuged at 3000 *g* for 10 min. Plasma was transferred to 5-ml polypropylene tubes and kept at −80 °C until analysis.

2.3. Sample extraction

Thawed plasma samples (0.5 ml) in microcentrifuge tubes had 4-CU (20 μl, 100 μ*M*) added as an internal standard, then 200 μl of mobile phase (0.01 *M* KH_2PO_4, pH 3.0) and 100 μl of chloroform were added and vortex mixed. Saturated ammonium sulfate solution (200 μl, ~20 *M*) was added to precipitate plasma proteins. Samples were vortex mixed and then centrifuged in a microcentrifuge (15 850 *g*, 5 min) (Beckman Instruments, Gladesville, Australia). The supernatant was transferred to 100× mm borosilicate glass tubes (Australian Scientific, Kotara, Australia). Isopropranol–ethyl acetate (6 ml, 15:85, v/v) was added to the supernatant and vortex mixed thoroughly. Samples were then centrifuged (3000 *g*, 4 °C, 10 min) (Beckman Instruments) and the supernatant collected into a clean glass tube using a glass pipette. The organic extraction step was repeated collecting organic supernatant into the same glass tube. Samples were evaporated to dryness under a stream of nitrogen at 56 °C and reconstituted in 200 μl of mobile phase, vortex mixed and 50 μl of chloroform was added. The solution was centrifuged (3000 *g*, 4 °C, 3–5 min) and the supernatant was recovered. If needed, supernatant was filtered using a 0.20-μm pore nylon 66-membrane microspin centrifuge filter (Alltech Associates, Baulkham Hills, Australia). The supernatant or filtered samples were transferred to crimp-top polypropylene autosampler vials and a 50-μl volume was injected onto the HPLC system.

2.4. HPLC apparatus and conditions

The HPLC equipment consisted of a Shimadzu Model LC-10AD dual-piston pump, a Shimadzu autosampler Model SIL-10A, a Shimadzu Model SPD-10A variable-wavelength UV detector governed by a microcomputer running Shimadzu LC10 version 1.63 software, and a Shimadzu Model CT0-10AS_{VP} column oven (Shimadzu Oceania, Rydalmere, Australia). Separation was achieved by isocratic elution with a mobile phase (0.01 *M* KH_2PO_4, pH 3.0) at a flow-rate of 0.6 ml/min through a Waters Spherisorb ODS-2 analytical column (250×4.6 mm, 5 μm particle size) (Waters Australia, Rydalmere, Australia) maintained at 8 °C, preceded by a guard column (10×4.6 mm) of the same material.

The chromatographic run began at a wavelength of 268 nm, and was changed to 220 nm from 11.0 to 12.6 min in order to detect UH_2. This wavelength is slightly higher than the λ_{max} of UH_2 (208 nm) but was chosen to eliminate interference by co-eluting compounds. The wavelength was then changed to 257 nm at 12.6 min for a further about 5 min in order to detect U. This was followed by a switch to 268 nm for detection of the internal standard, 4-CU, which eluted at about 43 min. Integration of detector output was performed using Shimadzu LC-10 software to determine peak areas.

2.5. Assay validation

Various procedures were performed to validate the assay.

2.5.1. Extraction efficiency

Extraction efficiency was determined by spiking 0.5-ml aliquots of plasma and mobile phase with 0.05–2.0 μ*M* UH_2 and U in triplicate (Table 1). The peak area of extracted plasma was compared to the peak area recorded from an equivalent injection volume of mobile phase samples. Extraction efficiency for U ranged from 93 to 100% and for UH_2 ranged from 95 to 99%. Internal standard recovery at 4 μ*M* was 96±5.5% (n=8).

2.5.2. Minimum quantifiable concentration (MQC)

The minimum quantifiable concentration of this assay was calculated as the minimum concentration, which could be detected and quantified with ≤10% deviation from the actual concentration. Using these criteria, the minimum limit of quantitation was 0.1 μ*M* for UH_2 and 0.02 μ*M* for U, in 0.5 ml plasma standards. The basal plasma concentration of UH_2 and U in 23 individuals ranged from 0.4 to 1.7 μ*M* and 0.025–0.27 μ*M*, respectively. Thus, the minimum quantifiable concentration for each component using this methodology was below that encountered in patient samples.

2.5.3. Linearity

Plasma standards were prepared with double dialysed plasma obtained from healthy volunteers and spiked with U and UH_2 stock solutions. These samples were then subjected to the extraction and HPLC separation. The peak area ratios of UH_2 and U to that of the internal standard (UH_2/4-CU, U/4-CU) were calculated. Standard curves were drawn by plotting the peak area ratio versus the plasma concentration of each compound. Linearity of the relationship between injected plasma concentrations and detector response was assessed for extracted plasma standards over the range 0.02–5 μ*M*. The line of best fit was determined by using unweighted linear least-squares regression using MS Excel software. Data were added serially from low to high concentrations. The limit of linearity was taken as the highest concentration included which still gave an r^2>0.998. For 0.5 ml plasma standards linearity was satisfactory between 0.02 and 1 μ*M* for U, and 0.1–2 μ*M* for UH_2. U and UH_2 concentrations of patient samples were calculated by interpolation from the standard curves using HPLC software. Control concentrations of 2 and 5 μ*M* were also accurately measured by extrapolation from these calibration curves, respectively. Higher plasma concentrations can also be measured using a higher concentration range linear calibration curve between 5 and 100 μ*M*. In routine practice, plasma standards were analysed concurrently with each set of unknown samples.

2.5.4. Precision and accuracy

Data for the validation of within-day and between-day assay precision and accuracy are presented in Table 2. Assay precision refers to the degree of reproducibility of the assay, and was assessed at all standard concentrations in plasma (0.02–5 μ*M*). Accuracy refers to the ratio of measured compound concentration to the known concentration. For with-

Table 1
Extraction efficiency

Concentration (μ*M*)	Extraction efficiency (%, mean±SD)		
	Uracil (n=3)	Dihydrouracil (n=3)	4-Chlorouracil (n=8)
0.05	95±3.0	nd	
0.1	93±1.1	99±2.9	
0.2	93±3.8	95±4.6	
0.5	99±10.4	95±6.2	
1	97±3.4	99±3.6	
2	100±6.4	96±10.4	
4			96±5.5

nd=Not detected.

Table 2
Precision and accuracy of the method

	Actual concentration (μM)	Within-day			Between-day		
		Measured concentration (μM) mean±SD (n=3)	RSD (%)	Accuracy (%)	Measured concentration (μM) mean±SD (n=3)	RSD (%)	Accuracy (%)
Uracil	0.02	0.019±0.003	3.6	96	0.021±0.002	6.7	107
	0.05	0.046±0.004	7.2	93	0.047±0.001	7.0	93
	0.1	0.095±0.012	5.3	95	0.099±0.005	0.7	99
	0.2	0.208±0.007	4.1	104	0.201±0.007	0.4	100
	0.5	0.496±0.030	0.8	99	0.499±0.008	0.2	100
	1	1.060±0.073	1.6	102	1.006±0.010	0.6	101
	2	2.074±0.087	3.7	104	1.915±0.173	4.2	96
Dihydrouracil	0.02	nd			nd		
	0.05	nd			nd		
	0.1	0.110±0.002	9.7	110	0.110±0.004	9.9	110
	0.2	0.182±0.003	9.2	91	0.194±0.011	3.1	97
	0.5	0.477±0.020	4.6	95	0.489±0.012	2.2	98
	1	0.957±0.082	4.3	96	0.971±0.019	2.9	97
	2	2.051±0.08	2.6	103	2.025±0.024	1.2	101
	5	5.216±0.275	4.3	104	5.218±0.276	4.4	104

nd=Not detected.

in-day precision and accuracy, three control samples of each concentration were extracted and injected on the same day. For between-day assessment all standard concentrations were extracted and injected on each of 3 days. The within-day precision (RSD) of U varied between 0.8 and 7% and between-day precision varied between 0.2 and 7%, while for UH_2 the within-day precision varied between 2.6 and 9.7% and between-day precision varied between 1.2 and 9.9%. The accuracy at the tested concentrations ranged from 93 to 107% for U and 91–110% for UH_2 (Table 2).

3. Results and discussion

This plasma extraction and HPLC method is an adaptation of that described by Gamelin et al. [11], and is designed to be more applicable for routine clinical laboratory practice. Initially we proposed to use Gamelin et al.'s method for measurement of U and UH_2. The intricacies of this assay led us to explore improvements to the method without compromising sensitivity and precision, and render the method adaptable for routine clinical practice. To optimise the sensitivity and precision of the method while maintaining its simplicity we explored alternatives to the components of Gamelin et al.'s procedure: extraction (protein precipitation and solvent extraction); internal standard; mobile phase; column type; flow-rate; column temperature and choice of wavelength. The final result is an easier, shorter and more economical method that can be used routinely.

A number of modifications to the described extraction method [11] were explored. Microcentrifugation after adding $(NH_4)_2SO_4$ for protein precipitation produced better quality chromatograms. Vortexing the solvent extraction mix followed by centrifugation for 10 min at 3000 g was more efficient than slow mixing for longer periods of time. Furthermore, two rounds of liquid–liquid solvent extraction with isopropranol–ethyl acetate (6 ml, 15:85, v/v) resulted in better sample recovery than a single round.

Several internal standards (5-fluorocytosine, 5-chlorouracil, 4-chlorouracil, 5-iodouracil, and 5-iodoorotic acid) were tested to find the most appropriate one for this method. Other methods have utilised 5-bromouracil (5-BU). However, in our hands 5-BU co-eluted with endogenous plasma peaks in the extracted plasma samples. 4-Chlorouracil was finally selected as the internal standard for this assay,

as it appeared isolated on the chromatogram with a retention time of 43 min.

A variety of chromatographic mobile phases and the HPLC columns were tested. The mobile phase 0.01 *M* KH_2PO_4, pH 3.0 produced better chromatograms than others with different isotonic strengths of phosphates, different pH values, and phosphate solutions containing 10% methanol. Mobile phase pH is critical for stability of retention times of U and UH_2, with fresh mobile phase prepared daily allowing consistent retention times to be achieved. Gamelin et al. [11] used ODS1 (100 mm) and ODS2 (250 mm) columns in series to effectively separate the compounds of interest, but with a long run time of 90 min. We tested various combinations of these two columns, with varying lengths in an effort to reduce the run time and to effectively separate the compounds of interest. A single ODS2 (250 mm) column preceded by an ODS-2 guard column produced the best separation of U and UH_2 and reduced the run time significantly. Increasing the mobile phase flow-rate from 0.6 to 1.0 ml/min, resulted in poorer separation between U and UH_2. Cooling of column from room temperature to 8 °C facilitated an increase in resolution factor.

Various alternative wavelengths to detect U and UH_2 were tested. The rapid wavelength switching function of the Shimadzu detector allows each compound to be detected at or near its maximum absorption. The chromatographic run began at a wavelength of 268 nm and was then changed to 220 nm at 11.0 min for 1.6 min to detect UH_2. This wavelength is slightly higher than the λ_{max} of UH_2 (208 nm), but was chosen to eliminate interference by co-eluting compounds. The wavelength was then changed to 257 nm (λ_{max} for U) from 12.6 min to 17.5 min in order to detect U. This was followed by a switch to 268 nm for the remainder of the run to detect the internal standard 4-CU. Gamelin et al. [11] chose to use 205 nm to simultaneously detect all compounds of interest with no switching throughout the run. However, at 205 nm, we found interference from unknown plasma matrix compounds, which was significantly reduced by using longer wavelengths.

The extraction and HPLC assay developed produced a symmetrical peak shape and good baseline resolution of U, UH_2 and 4-CU (Fig. 2). Plasma matrix components did not interfere with the analysis. Using this system, the retention times for UH_2, U and 4-CU are approximately 11.6, 13.4 and 43 min, respectively. Wavelength switching times (11.0 and 12.6 min) are occasionally critical for accurate analysis of UH_2. The total analysis time for each run was 45 min. Plasma for standards was dialysed twice to remove endogenous U and UH_2 and to obtain a ''true'' blank plasma sample. This process also removed various other plasma matrix peaks from the chromatogram compared to non-dialyzed samples but peaks of interest were not affected. The presence of extra plasma matrix peaks in patient samples did not produce any sample carryover.

We hypothesised that U and UH_2 would have similar stability to 5-FU and FUH_2, which was previously assessed under various conditions to determine the optimal requirements for storage and processing during analysis [16]. At 4 °C, 2 μ*M* aqueous standards of U and UH_2 were stored for 5 months, with no evidence of apparent degradation. U and UH_2 were also found to be stable for up to 5 days at 24 °C.

Plasma standards stored for 6 months at −20 °C compared with fresh standards revealed that U did not degrade under storage, whereas UH_2 was found to undergo almost 50% degradation under these conditions. Alternatively plasma standards and controls can be prepared at the same time as patient samples and stored together for future analysis. In contrast, no degradation of U or UH_2 occurred in plasma samples that had been extracted and reconstituted before storage at 4 °C for up to 2–3 months. Thus, extracted plasma samples can be stored at 4 °C along with extracted plasma standards and controls for subsequent HPLC analysis. Therefore, we recommend storage of water standards at 4 °C or at −20 °C for up to 4–5 months, extracted plasma samples and standards for up to 2 months but unextracted plasma samples and standards should not be stored for extended periods of time.

4. Conclusion

The HPLC assay developed by Gamelin et al. [11] was until now, the only method described that could measure pyrimidines and their dihydrogenated me-

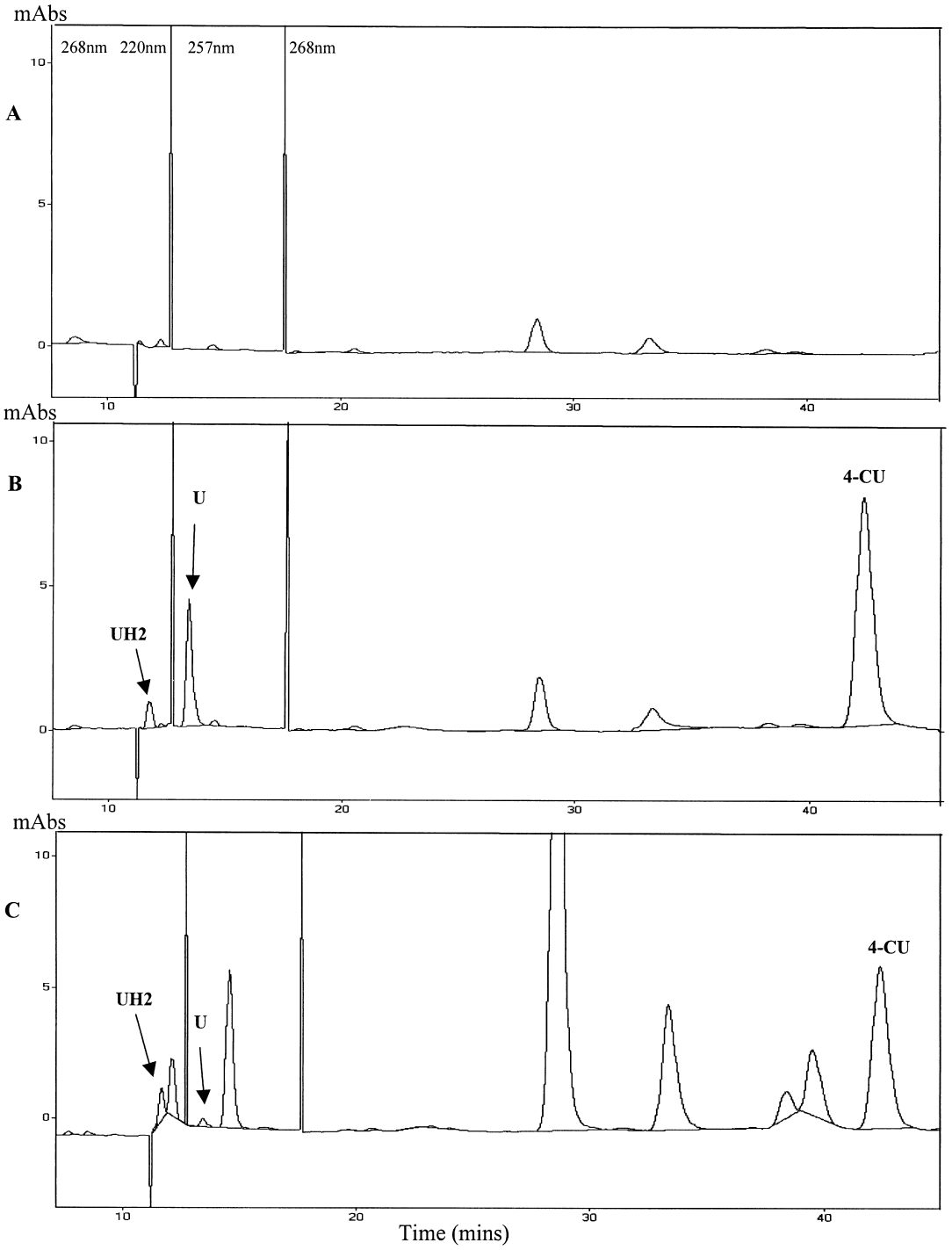

Fig. 2. HPLC chromatograms of (A) a blank dialysed plasma sample with minimal U and UH2 levels, (B) a standard plasma sample spiked with 2.5 μM U, 2.5 μM UH2 and 4 μM 4-CU, (C) a patient plasma sample containing 0.15 μM U and 1.77 μM UH2 and added internal standard 4-CU. Spikes at 10.5 min, 12.0 min and 16.0 min represent wavelength changes.

tabolites together in plasma. This assay is quite complex, requiring two columns set up in series maintained at a low temperature, with a long run time of 90 min per sample. We have developed a relatively rapid and simple HPLC method adapted from Gamelin et al.'s method to simultaneously measure the DPD substrate (U) and its dihydrogenated metabolite (UH_2) in plasma. Our method involves a simple liquid–liquid extraction procedure and uses only one HPLC column with a run time of 45 min. The method requires column cooling facilities and a variable wavelength uv/vis detector whose wavelength can be changed automatically during a run. This HPLC assay is as sensitive as other methods using gas chromatography–mass spectrometry [17] but it is more amenable for routine laboratory use.

We have used this method to assess the baseline value of UH_2 to U ratio in pretreatment plasma samples from 23 cancer patients receiving 5-FU. If required, 5-FU and FUH_2 can be measured simultaneously in plasma using our previously published method [16]. We are in the process of correlating pretreatment UH_2 to U ratio with 5-FU toxicity variables in patient samples.

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