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Short communication

Simultaneous determination of 5-fluorouracil and uracil by high-performance liquid chromatography using four serial columns

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Abstract

A sensitive assay was developed for the quantitation of 5-fluorouracil (5-FU) and uracil using liquid–liquid extraction (LLE) and HPLC with UV detection. Analyses were performed with four μBondapak C_{18} columns connected in series using 20 m*M* acetic acid with 1% ACN as mobile phase. The calibration curves were linear across the range of 26–1000 ng ml^{-1} (0.21–7.8 μ*M*) for 5-FU and 1.0–14.0 μg ml^{-1} (0.01–110 μ*M*) for uracil. This assay has been implemented to determine the plasma concentrations for pharmacokinetic studies for 5-FU and uracil in conjunction with clinical trials.

Keywords: Uracil; 5-Fluorouracil

1. Introduction

5-Fluorouracil (5-FU) has been one of the most widely used chemotherapeutic agents used in the treatment of common malignancies. In the liver, the catabolic clearance of 5-FU is mediated by a series of enzymes that are normally responsible for the breakdown of pyrimidines like uracil and thymine. Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in this series [1]. DPD ultimately converts 5-FU into inactive metabolites that are excreted in the urine and bile [2]. Patients with congenital deficiencies of DPD are at risk for unusually severe adverse drug reactions after exposure to 5-FU [2]. Neurotoxicity is a severe reaction to increased levels of uracil and 5-FU in the brain [3]. Diasio et al. have demonstrated that DPD activity is correlated with 5-FU clearance [4]. A drug that has been coadministered with 5-FU, 5-ethynyluracil (GW 776C85, 5-EU), is an irreversible inactivator of DPD. Unusually higher levels of uracil in plasma occur in patients who are given this drug.

5-FU is most conveniently assayed by HPLC with UV detection [5–11]. However, the retention times of 5-FU and uracil are very close together, making quantitation of 5-FU difficult in the presence of high concentrations of uracil. We have developed a new and sensitive assay to simultaneously measure 5-FU and uracil in plasma using a modified HPLC method

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[3]. In this method, a series of four columns was employed to obtain optimal resolution of 5-FU and uracil.

2. Conditions

2.1. Chemicals

Samples of 5-FU, 5-chlorouracil (5-Clu), and uracil were supplied by Sigma–Aldrich (St. Louis, MO, USA). Ethyl acetate, acetonitrile (both HPLC grade), hydrochloric acid and glacial acetic acid were supplied by Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was filtered using a Milli-Q water purification system supplied by Millipore (Bedford, MA, USA). Normal human plasma from healthy volunteers was obtained from the blood bank at the University of Chicago Hospitals (Chicago, IL, USA). For stock solutions, 5-FU was dissolved in 0.1 *M* HCl and stored at 4°C in darkness.

2.2. Equipment

The HPLC system was manufactured by Hitachi Instruments (Tokyo, Japan). It consisted of an interface (L-7000), pump (L-7100), autosampler (L-7200) and UV detector (L-4000H). UV detection was at 275 nm.

2.3. Sample preparation

Standards were prepared with the following concentrations of 25, 75, 100, 200, 400, 500, 750, 1000 ng ml^{-1} for 5-FU and 1, 2, 4, 5, 8, 9, 11, 12, 14 μg ml^{-1} for uracil. Plasma samples (1.0 ml) were supplemented with internal standard (50 μl of 2 μg ml^{-1} 5-Clu) and 8 ml ethyl acetate.

After shaking vigorously for 5 min, the samples were centrifuged at 2250×*g* (15 min, RT). The aqueous layers were dried under nitrogen (37°C) and reconstituted in distilled water. After centrifuging at 7200×*g* (10 min, RT) and removing any precipitate from the top layer, 100 μl was injected into the HPLC system.

2.4. High-performance liquid chromatography

A Nova–Pak C_{18} guard column and four C_{18} μBondapak columns (10 μm, 125 Å, 300×3.9 mm) were provided by Waters (Milford, MA, USA) (Fig. 1). The flow-rate was 0.9 ml min^{-1} at room temperature.

2.5. Mobile phase

The mobile phase consisted of 1% ACN in 20 m*M* acetic acid filtered through a nylon filter membrane, 0.45 μm (Micron Separations, Westboro, MA, USA) and degassed under vacuum.

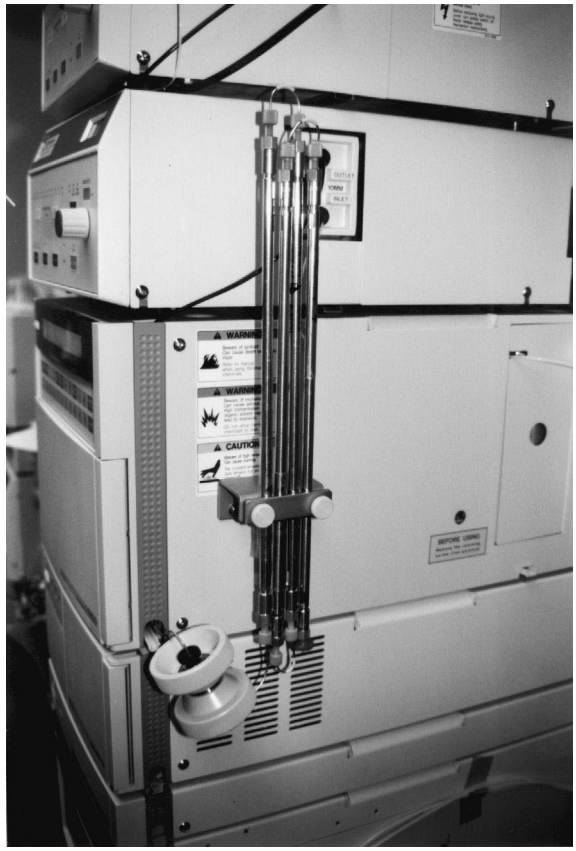

Fig. 1. Four columns were connected in series in order to obtain best peak separation. Due to the length of the connected columns, the solvent front eluted at 16 min producing a run time of 50 min. In order to keep the columns clean, a wash solvent of 10% ACN in d-H_2O was added from 35 min to 40 min. The pressure through the three day validation period averaged 3500 psi.

3. Results

Representative chromatograms from human plasma are shown in Fig. 2.

This assay was validated in terms of linearity, accuracy, precision, lower limit of quantitation, determination of unknown concentrations (blind samples) and recovery. Linear relationships were observed from 25 to 1000 ng ml^{-1} for 5-FU (r= 0.9939) and 1 to 14 μg ml^{-1} for uracil (r=0.9953). Linearity was determined by plotting concentration to the ratio of peak areas between 5-FU (or uracil) and 5-Clu. Each point was determined from nine assays over three days.

Accuracy is determined by dividing the mean measured concentration by the spiked concentration and multiplying it by 100. Precision (C.V.) is determined by dividing the standard deviation by the mean measured concentration and multiplying it by 100. Accuracy ranged from 92.4 to 105.7% for 5-FU and 96.5 to 101.5% for uracil (Tables 1 and 2). The precision ranged from 1.7 to 8.0% for 5-FU and 0.3 to 7.3% for uracil (Tables 1 and 2).

The lower limit of quantitation (LLQ) is the

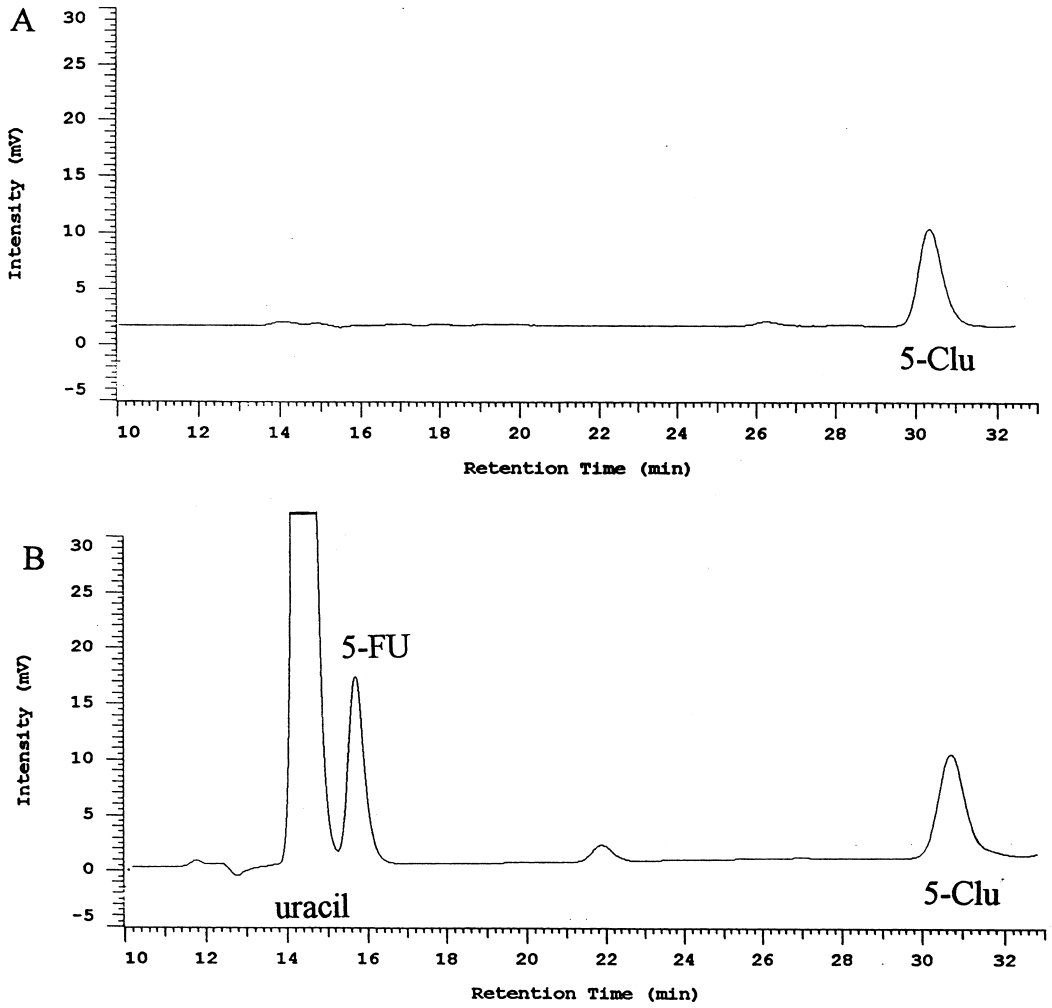

Fig. 2. (A) Chromatogram of a blank plasma sample with 1 μg ml^{-1} 5-Clu (internal standard). (B) Chromatogram of a spiked plasma sample containing uracil at 14.32 μg ml^{-1}, 5-FU at 1010 ng ml^{-1}, and 5-Clu at 1 μg ml^{-1}.

Table 1
Intra-day and Inter-day accuracy and precision for the determination of 5-FU in human plasma by HPLC

Theoretical concentration (ng ml^{-1})	Mean found concentration (ng ml^{-1})	Accuracy (% found/ theoretical)	Precision (C.V., %)
Intra-day (n=3)			
26.4	24.4±1.2	92.4	4.9
73.4	71.6±1.3	97.5	1.8
98.9	95.3±3.9	96.4	4.1
208	204±2.1	98.3	1.0
414	438±22.7	105.7	5.2
514	503±20.9	97.9	4.2
767	798±13.3	104.1	1.7
1015	1067±18.6	105.2	1.7
Inter-day (n=9)			
26.4	24.9±2	94.3	8
73.4	72.2±3.8	98.4	5.3
98.9	96.4±5	97.4	5.2
208	204±12	97.9	5.9
414	427±32	103.1	7.5
514	511±29	99.4	5.7
767	796±47	103.7	5.9
1010	1058±63	104.3	6

Table 2
Intra-day and Inter-day accuracy and precision for the determination of uracil in human plasma by HPLC

Theoretical concentration (μg ml^{-1})	Mean found concentration (μg ml^{-1})	Accuracy (% found/ theoretical)	Precision (C.V., %)
Intra-day (n=3)			
1.04	1.04±0.02	100.7	1.9
2.29	2.26±0.02	98.7	0.9
3.98	3.98±0.08	100	2
5.65	5.52±0.05	97.7	0.9
7.90	8.02±0.05	101.5	0.6
8.99	9.17±0.06	102	0.7
11.16	11.11±0.03	99.6	0.3
12.21	12.26±0.08	100.4	0.7
14.32	14.25±0.08	99.5	0.6
Inter-day (n=9)			
1.04	1.05±0.05	101	4.8
2.29	2.25±0.07	98.3	3.1
3.98	3.87±0.23	97.2	5.9
5.65	5.45±0.29	96.5	5.3
7.90	8.00±0.30	101.3	3.8
8.99	9.29±0.40	103.3	4.3
11.16	11.13±0.81	99.7	7.3
12.21	12.38±0.50	101.4	4
14.32	14.43±0.50	100.8	3.5

lowest concentration of analyte that can be measured with accuracy within 85–115%, and precision within 15%. It defines the reporting limit and the concentration of the lowest calibration standard. The limit of quantitation for 5-FU was determined as 26.4 ng ml^{-1}. The limit of quantitation was not studied for uracil because of high expected concentrations in patients.

As part of quality control, samples of unknown concentrations (blind samples) were analyzed. The accuracy was within 15%.

The recovery of 5-FU and uracil was determined from the comparison of peak areas obtained after injection of stock solutions and extracted plasma of equivalent concentrations. The studied concentrations for 5-FU were 500 and 1000 ng ml^{-1}. The studied concentrations for uracil were 1, 11 and 14 μg ml^{-1}. The recoveries for 5-FU were 43% and 49% respectively. The uracil recoveries were 78%, 83%, and 65% respectively.

Uracil and 5-FU are stable in human plasma at −80°C for at least three months. Representative chromatograms of a patient treated with 5-FU and a patient given 5-EU prior to treatment with 5-FU are shown in Fig. 3.

4. Discussion

The alternatives to using this method include GC–MS (the most popular), and column switching. Marunaka et al. discussed the extraction of ftorafur (an effective anti-tumor agent), 5-FU, and uracil utilizing GC–MS [12]. The advantages of their method include high sensitivity (1 ng ml^{-1}) and a short run time of about 10 min. The disadvantages of their method include limited accessibility to a GC–MS system, complex extracting procedures, and silylation [12].

A column switching protocol has been utilized to separate 5-FU from uracil [13,14]. Although this protocol has been set up to determine drug levels in tissue samples, the authors state that it can be used for serum and plasma samples as well [13]. Since a microbore column is used, the assay is highly sensitive (3 ng g^{-1}) and solvent usage is reduced. However, this method involves complex sample and

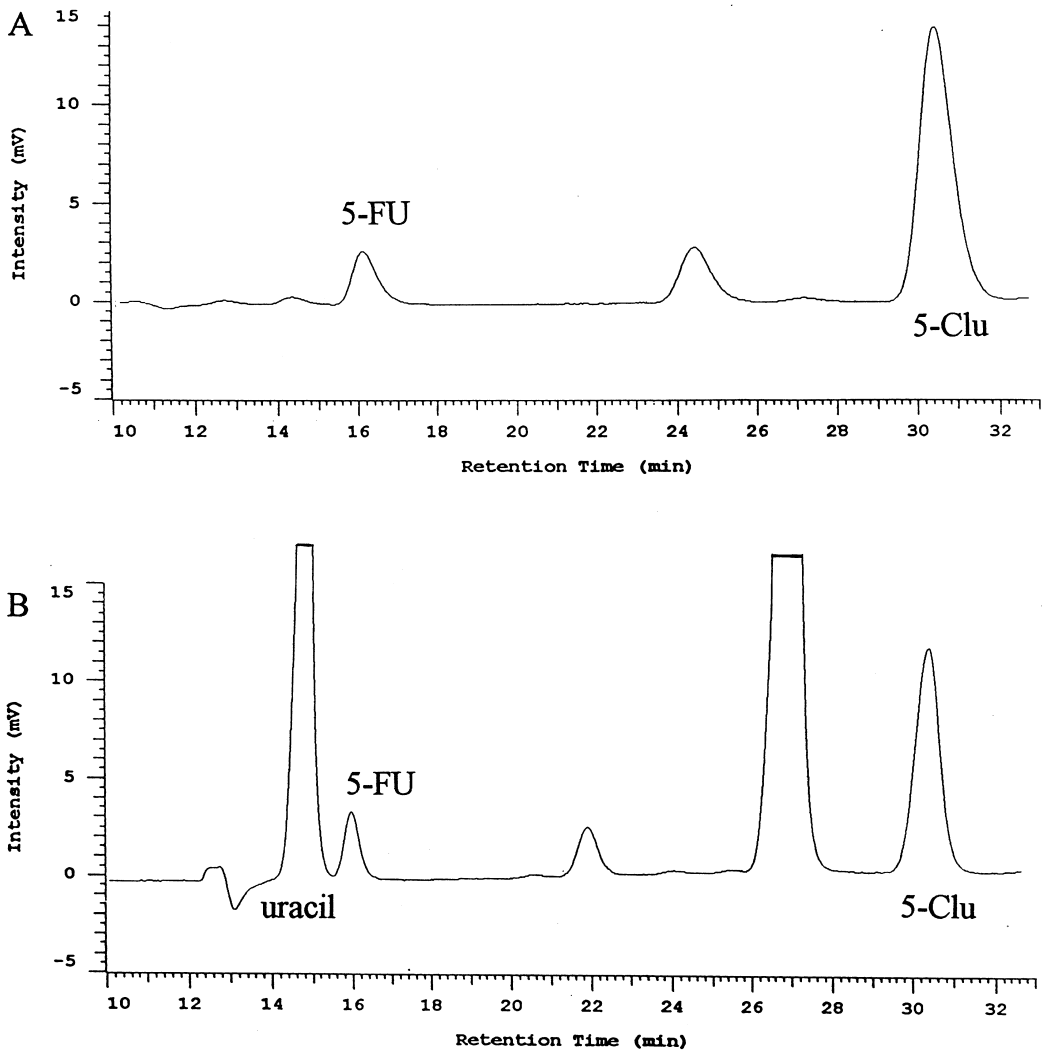

Fig. 3. (A) Chromatogram of a typical patient not given the DPD blocking drug, 5-EU; where uracil was below quantitation limits, 5-FU was 217 ng ml^{-1}, and 5-Clu was 1 μg ml^{-1}. (B) Chromatogram of a typical patient pretreated with 5-EU; where uracil was 4.89 μg ml^{-1}, 5-FU was 110 ng ml^{-1} and 5-Clu was 1 μg ml^{-1}.

pre-column derivatization with 4-bromomethyl-7-methoxycoumarin [13].

We have tried Waters Nova-Pak C_{18} columns (4-μm particle size, 3.9×300 mm). Unfortunately, the smaller particle size did not improve resolution and led to higher pressures. μBondapak columns are widely used for separating many compounds. Using four μBondapak columns in series resulted in the separation of all compounds of interest, and an acceptable backpressure of 3500 psi for the four columns in series. We did not evaluate microbore systems, which may also be a feasible approach.

In summary, 5-FU and uracil can be separated via four μBondapak C_{18} columns connected in series using a modified HPLC method. This method has been applied to determine the plasma concentrations of 5-FU and uracil for pharmacokinetic study as shown in Fig. 3. This assay is quick, simple, and rugged; thereby, lessening the possibility of error due to complicated extraction procedures.

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