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Note

Separation of 5-fluorouracil and uracil by ion-pair reversed-phase high-performance liquid chromatography on a column with porous polymeric packing

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The pyrimidine antimetabolite 5-fluorouracil (5-FU) is widely used in clinical practice for the treatment of various forms of cancers. However, its applicability is limited by myeloid and gastrointestinal toxicity. Recently, delayed uridine administration was shown to rescue mice from high-dose 5-FU toxicity and to enhance the antitumour activity of 5-FU [1—3]. To evaluate this combination in a clinical phase I study, pharmacokinetics of 5-FU and uridine were determined [4].

Uridine and its catabolite uracil could be determined in plasma and urine by high-performance liquid chromatography (HPLC) using an Aminex A-29 column as described previously [5]. In addition, 5-FU was well resolved from uridine and uracil. However, low concentrations of 5-FU in the micromolar range could not be determined due to the presence of uric acid. HPLC separation of 5-FU and uracil is difficult with commonly used reversed-phase columns. Miller et al. [6] tested several of such columns for their ability to separate 5-FU and uracil and found that with every column tested these compounds were poorly resolved. Since 5-FU and uracil have relatively high pK_a values of 7.98 and 9.50, respectively [7], their separation could theoretically be achieved at high pH values. With most reversed-phase columns HPLC is restricted to the use of eluents at pH < 7. However, PRP-1 columns packed with poly(styrene-divinylbenzene) can be used over a wide pH range

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from 1 to 13 [8]. With this column, different separations of nucleosides and bases are possible at various pH values. In the present study, we compared the separation of uridine, uracil and 5-FU with a LiChrosorb 10-RP-18 column and a PRP-1 column at high pH with the ion-pairing agent cetrimide.

MATERIALS AND METHODS

HPLC was carried out using instrumentation from Perkin-Elmer (Norwalk, CT, U.S.A.) consisting of a Series 2/2 solvent delivery system connected to two LC75 variable-wavelength detectors set at 254 and 280 nm. Kontron (Zürich, Switzerland) apparatus was also used, consisting of an LC410 pump connected to two Uvikon 740 LC fixed-wavelength detectors (254 and 280 nm). At 280 nm, the absorbance by interfering peaks was lower. The 280/254 ratio enabled identification of the compounds. A LiChrosorb 10-RP-18 column (150 × 4.6 mm) was obtained from Chrompack (Middelburg, The Netherlands). The PRP-1 material (particle size between 8 and 12 μm) was obtained from Hamilton (Reno, NV, U.S.A.) and was packed in a 150 × 4.1 mm column as described by Zygmunt et al. [9]. Uracil, uridine and 5-FU were purchased from Sigma (St. Louis, MO, U.S.A.), and cetyltrimethylammonium bromide (cetrimide) was from BDH (Poole, U.K.). Other chemicals were of the highest quality commercially available. Eluents were prepared with de-ionized water which was further purified with a Millipore Milli Q system, filtered through a 0.22-μm filter and sonicated. The PRP-1 column was routinely run at 0.5—1 ml/min (pressure about 100 bars). The column was run overnight at 0.1 ml/min and stored in methanol—water (1:1) between use.

Plasma obtained from heparinized blood was deproteinized for 20 min on ice using trichloroacetic acid (8% final concentration) or perchloric acid (0.4 *M* final concentration) The extracts were clarified by centrifugation and neutralized by thoroughly mixing 1 vol. of supernatant with 2 vols. of trioctylamine—Freon (1:4, v/v) as described previously [10, 11]. After centrifugation, the aqueous phase was pipetted off and stored at −20°C until analysis. No difference between the two extraction procedures was found.

The following equation was used to calculate the capacity factor (k'): $k' = (V_p - V_0)/V_0$, where V_0 is the void volume and V_p the volume required to elute the compound. The relative capacity factors for uracil and 5-FU were expressed as the selectivity factor r, defined as $k'_{5\text{-FU}}/k'_{\text{uracil}}$.

RESULTS AND DISCUSSION

5-FU concentrations in plasma could be determined with a LiChrosorb 10-RP-18 column (Fig. 1A). This system could also be employed to study the pharmacokinetics of uridine, since uridine and uracil could be separated. The compound which was coeluted with uracil did not interfere with the determination of uracil at concentrations of uracil higher than 10 μM. 5-FU and uracil were also resolved. However, 5-FU could not be detected when the uracil concentration (about 100 μM) exceeded that of 5-FU as in the treatment of patients in whom 5-FU administration is followed by uridine. Such conditions resulted in the detection of the 5-FU peak as a shoulder on the uracil peak

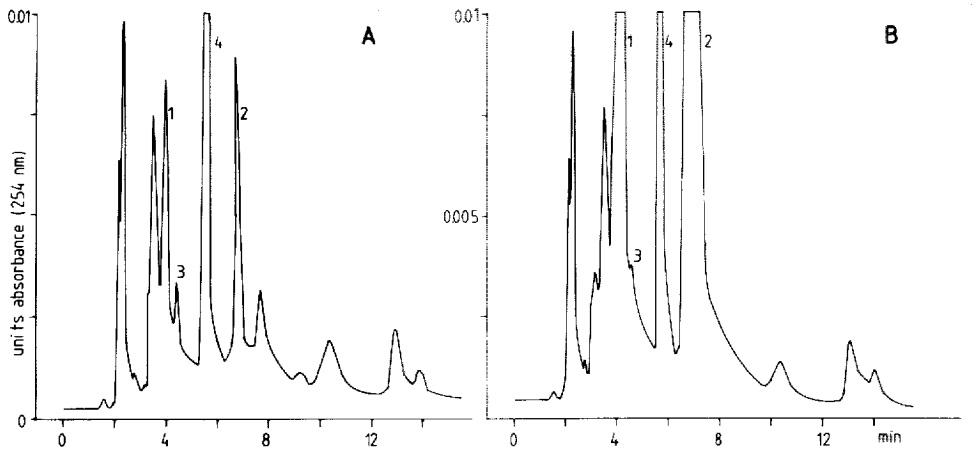

Fig. 1. Chromatograms of plasma of patients, using a LiChrosorb 10-RP-18 column with isocratic conditions. The elution solvent was 0.01 *M* $NH_4H_2PO_4$, pH 3.8, containing 2.5% methanol. The flow-rate was 1 ml/min. Retention times were: uracil (1) 4.0 min; 5-FU (3) 4.4 min; uric acid (4) 5.6 min; and uridine (2) 6.7 min. Uracil coeluted with an unknown plasma compound. The equivalent of 40 µl of undiluted deproteinized plasma (total injection volume 50 µl) was injected. (A) plasma of a 5-FU-treated patient (700 mg/m², bolus injection), 2 h after injection; (B) plasma of the same patient at the end of a 1-h uridine infusion (5 g/m², intravenous, 2 h after 5-FU).

(Fig. 1B). Phosphorolysis of uridine led to concentrations of uracil 100 times higher than those of 5-FU. With other reversed-phase HPLC columns improvement of separation was not expected [6]. Since the PRP-1 column could be used with buffers over a wide pH range, we attempted to improve the separation of 5-FU and uracil by varying the pH of the elution solvent. Ammonium phosphate buffers between pH 7 and 9 were tested. Although 5-FU is ionized to a greater extent than uracil at pH 8 [7], the resolution of 5-FU and uracil at this pH was unsatisfactory. Also, no separation was achieved at the other pH values. Subsequently, ion-pair HPLC was performed to increase the retention of ionized compounds. With cetrimide as the ion-pairing agent, 5-FU and uracil could be separated although coelution of uridine and uracil occurred (Fig. 2A).

In drug-free plasma, no interfering peaks were present at the retention time of 5-FU (Fig. 2B). Uric acid, the main interfering peak in plasma, was separated from 5-FU. An anion-exchange system using an Aminex A-29 column [5] at pH 9.1 (0.01 *M* disodium hydrogen phosphate, 0.005 *M* citric acid, 19% ethanol; at 0.3 ml/min and 70°C) was also capable of separating 5-FU from uracil; however, 5-FU was poorly resolved from uric acid (data not shown). The concentration of 5-FU determined in plasma of 5-FU-treated patients with the PRP-1 column was similar to the concentration determined with the LiChrosorb 10-RP-18 column. The presence of high concentrations of uridine and uracil in plasma of patients treated with 5-FU—uridine did not interfere with the determination of 5-FU (Fig. 2C). The detection limit of the method was 20 pmol (in 20 µl of plasma), and the peak area was linear with concentration to at least 500 pmol. Since the 5-FU dose may be higher in the 5-FU—uridine regimen than in the conventional 5-FU schemes, the period during which plasma drug concentrations can be measured may be extended.

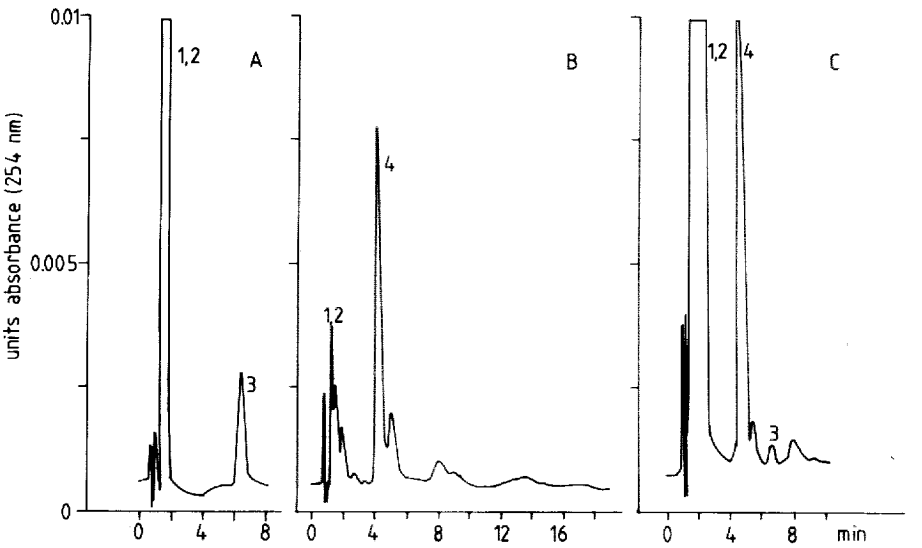

Fig. 2. HPLC separation of uracil (1), uridine (2), 5-FU (3) and uric acid (4) on a PRP-1 column under isocratic conditions. The elution solvent was 0.05 *M* Tris—HCl, 0.025 *M* cetrimide (pH 8.0), and the flow-rate was 1 ml/min. Retention times were: uracil and uridine, 1.8 min; uric acid, 4.5 min; and 5-FU, 6.8 min. (A) 20 μl of a mixture of standards (10 μ*M* uridine, 10 μ*M* uracil and 25 μ*M* 5-FU); (B) 20 μl diluted deproteinized patient plasma (1 part plasma, 2 parts water) before treatment; (C) 20 μl diluted deproteinized plasma (1 part plasma, 1 part water) of the same patient described in Fig. 1B at the end of the 1-h uridine infusion.

TABLE I

CAPACITY AND SELECTIVITY FACTORS FOR 5-FU, URIDINE, URACIL AND URIC ACID

Capacity (k') and selectivity (r) factors were calculated for separations with the eluents described in Figs. 1 and 2.

	LiChrosorb 10-RP-18	PRP-1
k'		
5-FU	2.9	12.1
Uracil	2.5	3.1
Uridine	4.4	3.1
Uric acid	3.7	7.9
r		
5-FU—uracil	1.2	3.9
5-FU—uridine	1.5	3.9
5-FU—uric acid	1.3	1.5
Uridine—uracil	1.8	1
Uridine—uric acid	1.2	2.5
Uracil—uric acid	1.5	2.5

The column performance decreased during prolonged use, but could be regained by careful refilling the top of the column with the packing slurry. Table I shows a comparison of the separation characteristics of the LiChrosorb

10-RP-18 and the PRP-1 columns. It is apparent that the PRP-1 column gives a good separation of 5-FU and uracil.

This paper shows that the use of the PRP-1 column at high pH allows an enhanced separation of 5-FU and uracil which has not been possible with other reversed-phase columns. Furthermore, this separation which takes advantage of modest differences in ionization is only possible with ion-pairing. Resolution of 5-FU and uracil is applicable in clinical studies of the combination of 5-FU and uridine. Also, this HPLC method may serve as an alternative in the separation of 5-FU from other plasma components.

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