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Short Communication

Analysis of 5-fluorouracil in plasma and urine by high-performance liquid chromatography

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ABSTRACT

A relatively simple, sensitive and rapid high-performance liquid chromatographic method is described for measuring the anticancer drug 5-fluorouracil (5-FU) in human plasma and urine. The procedure includes liquid–liquid extraction using ethyl acetate–methanol (95:5) and preparative column chromatography to separate 5-FU from constituents normally occurring in these biological samples. The columns contained a specially modified form of diatomaceous earth, which requires no pre-conditioning washes. Reversed-phase high-performance liquid chromatography was performed on a C_{18} column (70 mm × 4.6 mm I.D.) with a mobile phase of water–methanol (95:5) and ultraviolet detection (268 nm). The overall recovery from plasma and urine was 91 and 94%, respectively, at the concentration of 50 ng/ml. The determination limit of the assay for 5-FU was 10 ng/ml of plasma and urine. Concentrations of 5-FU between 10 and 500 ng/ml were measured in plasma and urine with a relative standard deviation of 6.8%. In order to evaluate the procedure, plasma and urine samples from three patients treated with 5-FU by continuous intravenous perfusion, were investigated.

INTRODUCTION

5-Fluorouracil (5-FU) is an antimetabolite frequently used in the treatment of carcinomas of the gastrointestinal tract and breast [1,2]. This compound is used both as a single agent and in combination therapy.

Reversed-phase (RP) [3–5], reversed-phase ion-pair (RP-IP) [6–10] and normal-phase (NP) high-performance liquid chromatographic (HPLC) systems [11] were described for the analysis of 5-FU in the presence of structurally related compounds. The RP-HPLC system is the most widely applied. The disadvantage of the majority of these procedures is the sensitivity, which is not always sufficient for pharmacokinetic experiments. In most cases of HPLC, UV detection at 254–280 nm was associated with limits of determination in the range 50–100 ng/ml of plasma. The disadvantage of the NP system is the necessity for a derivatization procedure.

The RP-IP system consists of polymeric PRP-1 material [9] as the stationary phase and cetrimonium bromide as the ion-pairing reagent. In this system 5-FU has a relatively high k' value and in order to achieve an acceptable k' value, valve-switching or gradient-elution techniques can be

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applied. The use of cetrimonium bromide as the ion-pairing agent in gradient elution results in relatively unstable systems [10].

Schaaf *et al.* [4] used a relatively simple and sensitive HPLC method for the simultaneous determination of 5-FU and its prodrug FdUrd (5-fluoro-2′-deoxyuridine) in biological fluids. Sample pretreatment consisted of the addition of 3% phosphoric acid and liquid–liquid extraction with ethyl acetate–2-propanol. Recoveries of 66 and 61% for 5-FU and FdUrd, respectively, were obtained, but application of the method to urine analysis was not reported. In another paper [6], we have described the investigation of several parameters using an RP-IP system with a microbore column in order to obtain optimal separation of 5-FU and its main metabolites FUrd (5-fluoro-2′-uridine), FdUrd and FdUMP (5-fluoro-2′-deoxyuridine monophosphate). The detection limit was 2 and 10 ng/ml for 5-FU and the two nucleosides, respectively. However, this method was unable to quantify 5-FU in urine samples and, moreover, the complexity of the extraction procedure resulted in a method with a limited use in rapid routine analysis. Setson *et al.* [12] were able to monitor 5-FU concentrations in peritoneal fluid at 25 ng/ml. Sample preparation involved deproteinization with ammonium sulphate followed by liquid–liquid extraction with ethyl acetate. In addition, 5-FU concentrations were determined routinely only in the concentration range 50–2000 ng/ml, in plasma and peritoneal fluid.

According to previous studies [3–5,12], values lower than 50 ng/ml could not be determined with liquid chromatography in plasma and urine samples; valve-switching brought these limits down to 5 ng/ml [10]. The comprehensive pharmacokinetic data available on 5-FU in humans have been reviewed [13,14]. In contrast, only limited information has been published on a very rapid and sensitive method for monitoring 5-FU in blood and urine during treatment. This paper describes a rapid (<60 min, including sample pretreatment and work-up), sensitive (10 ng/ml) and selective method for the determination of 5-FU in urine and plasma using an RP column and spectrophotometric detection. The method involves simple liquid–liquid extraction, including a preparative column that requires no pre-conditioning.

EXPERIMENTAL

Apparatus

The HPLC system was a computer-monitored GOLD PC apparatus (Model 126 pumps, Model 166 detector), an Ultrasphere XL ODS 3-μm column (70 mm × 4.6 mm I.D.) (Beckman, Gagny, France) and a WISP 512 autosampler (Waters, Millipore, Molsheim, France).

Reagents

5-FU was purchased from Roche (Neuilly, France) and iodouracil (IU) from Sigma (La Verpillère, France). All other reagents were obtained from Prolabo (Paris, France) and were of the highest purity available. Chem-Elut columns (Analytichem International, Harbor City, CA, USA) were used for plasma and urine sample preparation. Throughout the study deionized water (Milli Q water purification system, Millipore) was used.

HPLC conditions

Room temperature and a flow-rate of 1 ml/min were maintained throughout the analyses. The absorbance spectrum of a solution of 5-FU in water showed a maximum at 268 nm, and this wavelength was selected for UV detection. A pressure of 100 bar was used. A volume of 30 μl of the samples in water was injected into the column. The mobile phase was water–methanol (95:5, v/v).

Sample pretreatment

Blood samples were collected in EDTA tubes and immediately centrifuged at 3000 *g*. The separated plasma was rapidly frozen in polypropylene tubes at −20°C until analysis. A 500-μl plasma sample was mixed for 10 s using a rotating mixer, and 50 ng of IU were added as the internal standard.

The aqueous samples were added to the dry

column for 3–5 min, so they were absorbed and distributed in a thin film over the hydrophilic packing material. The ethyl acetate–methanol mixture (95:5, v/v) was used to elute the first aliquot (2 ml). As the solvent trickled through the column, it extracted the 5-FU from the aqueous layer. A 4-ml volume of ethyl acetate–methanol (95:5, v/v) was used to end the extraction. The extract was concentrated by evaporating the 6 ml of eluents under a gentle stream of nitrogen gas at ambient temperature (15 min). The residue was reconstituted with 200 μl of water by placing the tube in an ultrasonic water-bath for 10 min. The reconstituted residue was then vortex-mixed and transferred to an autosampler microvial.

Chemotherapy protocol

Three patients (women) agreed to participate in this study after having been informed of its aims and nature. All had a recidiving breast cancer and received our standard chemotherapy protocol for this disease. Patients received CDDP on day 1 (Cisplatyl; Roger Bellon Labs., Neuilly, France) at a dose of 150 mg/m^2. CDDP was diluted in 250 ml of 0.9% NaCl solution and infused intravenously at a rate of 1 mg/min, starting at 5 p.m. On days 2, 3, 4, 5 and 6, 5-FU (Fluorouracile, Roche Labs., Paris, France) was infused intravenously at a constant rate with a volumetric pump (VMM; Vial Medical, St. Etienne-les-deux-Saints-Jeoirs, France). 5-FU infusion was initiated at 8 p.m. on day 1. The arm vein on which the infusion was performed was shielded from light.

Blood samples (5 ml) were taken during the infusion, at 8 a.m. and 5 p.m. on days 2, 3, 4 and 5 and at 6 p.m. on day 1 before 5-FU treatment and at 8 a.m. on day 6. They were collected into EDTA-containing tubes. The tubes were centrifuged immediately at 4°C at 3000 *g* for 10 min, and stored at −20°C until analysis. Urine samples (10 ml) were taken during the infusion, at 8 a.m. on days 2, 3, 4, 5 and 6, and collected into plastic tubes.

RESULTS AND DISCUSSION

Chromatograms from a plasma and a urine sample from a cancer patient spiked with 600 ng/ml IU (internal standard) are shown in Figs. 1 and 2, respectively. At a signal-to-noise ratio of 3, the minimal detectable concentration after the extraction procedure was 10 ng/ml, by injecting 30 μl of the 200 μl of reconstituted plasma and urine extract at a sensitivity setting of 0.0002 a.u.f.s. 5-FU could be determined with an acceptable precision ($\leq$ 6.8%) (Table I) at a concentration of 10 ng/ml (Fig. 1B).

Linearity and precision

In order to investigate the linearity of the procedure, blank plasma and urine samples were spiked with different amounts of 5-FU: 0.01–0.5 μg/ml and 0.01–1 μg/ml, respectively. The cali-

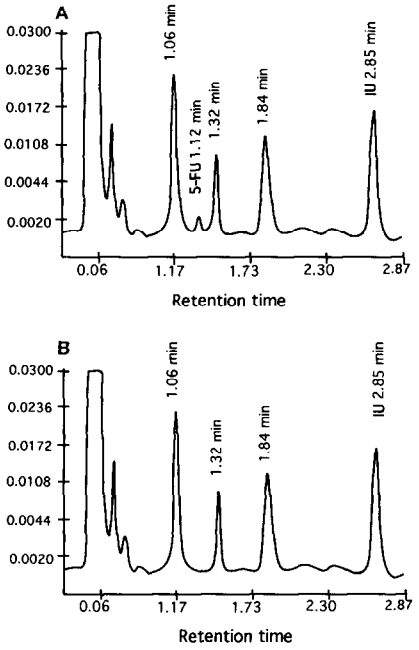

Fig. 1. (A) Chromatogram of a patient plasma sample containing 5-FU at 66 ng/ml and IU at 583 ng/ml. (B) Chromatogram of a blank plasma sample with 471 ng/ml IU (internal standard). For chromatographic conditions and extraction procedure, see text.

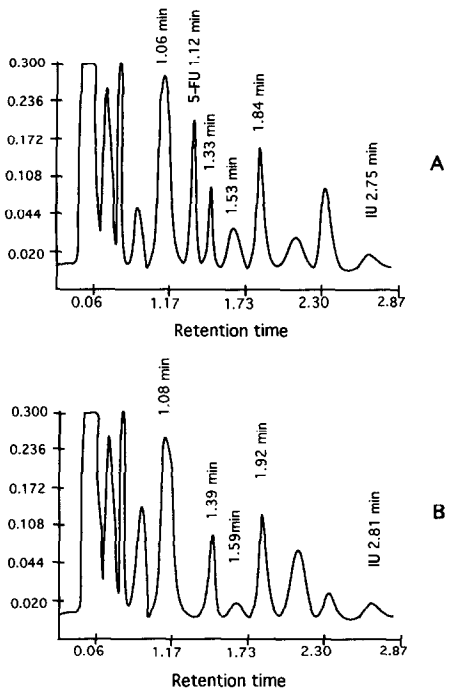

Fig. 2. (A) Chromatogram of a patient urine sample containing 5-FU at 15 323 ng/ml and IU at 586 ng/ml. (B) Chromatogram of a blank urine sample with 498 ng/ml IU (internal standard). For chromatographic conditions and extraction procedure, see text.

bration curves showed good linearity: plasma, $y = 0.565 \pm 0.001\ x - 0.037 \pm 0.001$ ($r \geq 0.998$); urine, $y = 0.758 \pm 0.004\ x - 0.082 \pm 0.004$ ($r \geq 0.998$). Both calibration curves were obtained from six samples, and x and y are the concentrations of 5-FU (μg/ml) and the peak area, respectively, and r is the correlation coefficient.

The within-day relative standard deviation (R.S.D.) based on these determinations was less than 6.8% for 5-FU at all concentrations (Table I). The between-day R.S.D. was calculated by performing six analyses of plasma and urine samples containing 5-FU at four concentrations on six different days. The between-day R.S.D. were all less than 16% (4.7–15.6%). A summary of the analyses is presented in Tables II and III.

The mean recoveries of 5-FU from plasma and urine samples in the concentration ranges 10–500 and 10–1000 ng/ml, respectively, are shown in the Table II and III. The recovery rate was above 90%, except in plasma at 10 ng/ml 5-FU (84%). Overloading of the column should be strictly avoided because, if more aqueous sample is added than the column was designed for, the sample could break through the bottom of the column into the collection tubes. The large surface area between the aqueous and organic layers led to

TABLE I

WITHIN-DAY VARIABILITY OF PLASMA ASSAY ($n = 6$)

Plasma		Urine	
5-FU (ng/ml)	R.S.D. (%)	5-FU (ng/ml)	R.S.D. (%)
10	6.8	10	5.7
50	4.2	100	4.3
100	3.8	500	3.8
500	5.2	1000	4.1

TABLE III

BETWEEN-DAY VARIABILITY OF URINE ASSAY ($n = 6$)

5-FU (ng/ml)	R.S.D. (%)	Recovery (%)
10	5.6	94
100	4.7	95
500	5.8	94
1000	7.2	93

TABLE II

BETWEEN-DAY VARIABILITY OF PLASMA ASSAY ($n = 6$)

5-FU (ng/ml)	R.S.D. (%)	Recovery (%)
10	15.6	84
50	8.6	91
100	7.8	92
500	7.3	93

excellent recoveries (Tables II and III) and eliminated emulsion problems.

These extraction columns contained a specially modified form of diatomaceous earth, which requires no pre-conditioning washes, but they are not reusable. Thus, one of the main advantages of HPLC over other chromatographic procedures was the possibility of a very simple and rapid extraction, which gave excellent recoveries from both plasma and urine.

This extraction procedure gave the same determination limit as those previously reported, but was less complex [9]. The solvent (water–methanol) was suitable for routine bioanalysis, as it was not necessary to rinse the column very often. We have analysed 300 samples successively without rinsing the column. This prolonged the column life, permitting the analysis of about 1800 samples with one column.

The variation of k' with pH has been previously studied [6]. The retention time of 5-FU cannot be altered by solvent pH variations.

Pharmacokinetic results

In order to evaluate the procedure described for the analysis of 5-FU, plasma and urine samples from three patients were investigated. Plasma and urine concentration–time curves are given in Figs. 3 and 4, respectively.

First described by Kish *et al.* [15], the chemotherapy protocol associating 5-FU with CDDP for the treatment of head and neck cancer seemed promising, since it gave a response rate of 88%.

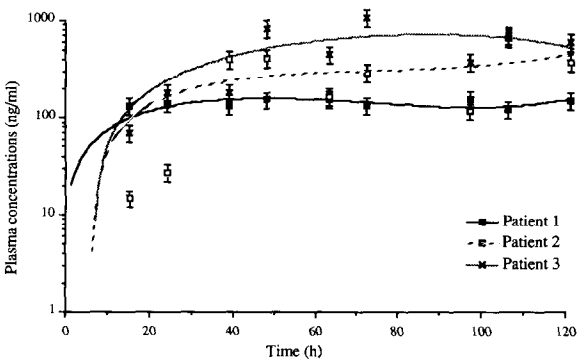

Fig. 3. Plasma concentration–time curves of 5-FU for three patients after continuous intravenous perfusion for 120 h.

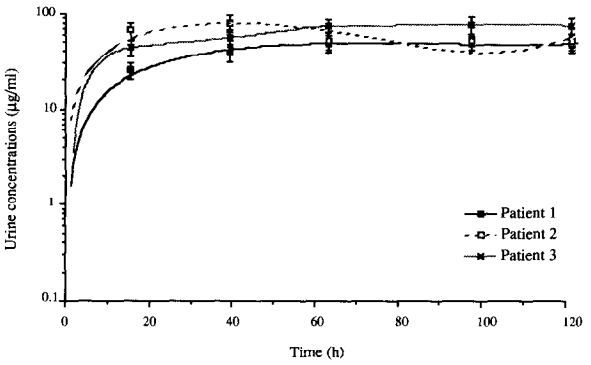

Fig. 4. Urinary excretion curves of 5-FU for three patients after continuous intravenous perfusion for 120 h.

Moreover, this response was often associated with myelosuppression and/or gastrointestinal toxicity (mucositis and diarrhea). Milano *et al.* [16] found a positive correlation between toxicity and the steady-state level of 5-FU in plasma. Their observations of a C (concentration) $\times$ T (time) threshold value (30 000 ng/ml h) that was highly predictive of toxicity was of practical use, because it enabled early recognition of high-risk patients. These investigators [15,16] identified a threshold day 1–3 area under the curve (AUC_{1-3}) of 15 000 ng/ml h that identified patients at greatest risk of toxicity. So AUC_{1-3} values were measured, and if they were greater than 15 000 ng/ml h, doses of 5-FU were reduced for the remainder of the infusion. Actually, the increase observed in individual $C \times T$ values from cycle to cycle is the single 5-FU dose adaptation. In order to make the fastest 5-FU dose adaptation, the results should be given immediately, so the choice of this HPLC dosage method is very appropriate.

In conclusion, a very rapid and accurate method for the analysis of 5-FU has been described that can be used for therapeutic monitoring of pharmacokinetic studies, as well as for testing the content uniformity of its products [15–18]. Owing to the highly efficient liquid–liquid extraction (Tables II and III), and because of its sensitivity (10 ng/ml) and the selectivity, this HPLC method is very suitable for routine analysis in bioavailability studies.

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