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Simple and sensitive determination of 5-fluorouracil in plasma by high-performance liquid chromatography Application to clinical pharmacokinetic studies

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Abstract

5-Fluorouracil (5-FU) is an antineoplastic agent widely employed in the treatment of many types of cancer. Recent studies have proved the need for individual adjustment of 5-FU dosage based on pharmacokinetics. A simple and sensitive high-performance liquid chromatographic method for the determination of 5-FU in plasma and their preliminary clinical pharmacokinetics is described. After sample acidification with 20 μl of orthophosphoric acid (5%), the drug is extracted from plasma using *n*-propanol–diethyl ether (16:84). The organic layer is evaporated to dryness, the residue dissolved in 100 μl of mobile phase and 20 μl of this mixture is injected into a LiChrospher 100RP-18 (5 μm, 250×4.0 mm) analytical column. Mobile phase consisted of potassium dihydrogenphosphate (0.05 *M*, adjusted to pH 3). The limit of quantitation was 2 ng/ml. The method showed good precision: the within-day relative standard deviation (RSD) for 5-FU (10–20 000 ng/ml) was 3.75% (2.57–5.93); the between-day RSD for 5-FU, in the previously described range, was 5.74% (4.35–7.20). The method presented here is accurate, precise and sensitive and it has been successfully applied for 5-FU pharmacokinetic investigation and therapeutic drug monitoring.

Keywords: 5-Fluorouracil

1. Introduction

5-Fluorouracil (5-FU) is widely used in the treatment of a large range of tumours and according to various schedules. Some studies have proved a relationship between 5-FU plasma concentrations and the toxic and therapeutic effects of the treatment in different types of tumours [1–7]. This finding was the basis for the determination of a therapeutic range for this drug [3–7], which is essential for individual adjustment of 5-FU dosage. By means of individual dosage based on 5-FU concentrations, Gamelin et al. [4] reached a percentage of objective responses of 56% while this value was approximately 15% for 5-FU in monotherapy [8].

Numerous high-performance liquid chromatography (HPLC) methods for 5-FU determination reported previously are relatively sensitive but also time-consuming. Sample pretreatment procedures vary a lot and most of them involve several phases such as deproteination of plasma either by an ion-exchange column [13,14] or a precipitating agent such as trichloroacetic acid [9,10], ammonium sulphate [11] or perchloric acid [12]. Many of the

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extraction procedures are long and tedious and some of them involve back extraction [13,15,18] or clean-up steps such as silica gel column chromatography [19]. Besides, limits of detection and quantitation of several of these methods are quite high, 25–300 ng/ml [9,10,12,16–19].

The present method is very sensitive, simple and rapid, avoiding back extraction and allowing full resolution of 5-FU from uracil by means of a commonly used reversed-phase column.

Using this assay methodology we have successfully determined plasma concentrations of 5-FU in colorectal and pancreas cancer patients receiving a 24-h intravenous infusion of a high dose of 5-FU (2.5 g/m^2).

2. Experimental

2.1. Reagents

Uracil (U), 5-FU, 5-bromouracil (5-BU) and 5-fluorocytosine (5-FC) were purchased from Sigma–Aldrich (Steinheim, Germany). HPLC-grade diethyl ether, *n*-propanol, methanol, acetonitrile, chloroform, ethyl acetate and isopropanol, and analytical grade orthophosphoric acid, trichloroacetic acid and ammonium sulphate were obtained from Panreac (Barcelona, Spain).

For stock solutions, all compounds were dissolved in distilled water and stored at 4°C in darkness.

2.2. Sample preparation

Blood samples were collected in heparinized tubes and immediately centrifuged at 3000 *g* for 5 min. The plasma was stored at −30°C until analysis. Each sample was allowed to thaw at room temperature, and 50 μl of a solution of 5-BU (30 μg/ml) as internal standard and 20 μl of orthophosphoric acid (5%) were added to 500 μl of plasma. The tube was vortex-mixed for 10 s and 6 ml of *n*-propanol–diethyl ether (16:84) was added as extraction solvent. After vortex-mixing for 2 min, the mixture was centrifuged at 3000 *g* for 15 min; the organic layer was separated and evaporated to dryness in a vortex-evaporator. The residue was dissolved in 100 μl of mobile phase and 20 μl was injected into the HPLC system.

Standards were prepared from normal human plasma spiked with different amounts of 5-FU and analysed as patient samples.

2.3. High-performance liquid chromatography

The HPLC equipment consisted of a HP 1100 Model with a diode array detector. Separation of compounds was achieved using a LiChrospher 100RP-18 (5 μm, 250×4.0 mm) analytical column protected by a LiChrospher 100RP-18 precolumn (5 μm, 4.0×4.0 mm). The mobile phase was potassium dihydrogenphosphate (0.05 *M*, adjusted to pH 3 with 85% orthophosphoric acid); flow was initially held at 1 ml/min over 7 min (just after the 5-FU peak appears) and then increased to 1.7 ml/min until the end of the chromatogram. The column temperature was first set at 25°C over 7 min; at that time, the column is cooled down to 17°C. The detector wavelength was set at 266 nm.

2.4. Determination of 5-FU in plasma

The concentrations of 5-FU were determined from area ratios of 5-FU to the internal standard by reference to the calibration graph obtained in the concentration range 10–20 000 ng/ml.

The limit of quantitation in plasma was calculated as three-times the standard deviation of the lowest concentration included in the calibration graph (10 ng/ml).

2.5. Determination of recovery, precision and accuracy

The recovery from plasma was determined by comparing the areas of pure standards with those of extracted plasma samples containing the same amount of standards. Five replicates with three different concentrations ranging from 10 to 20 000 ng/ml of 5-FU in human plasma were processed as described above to determine the within-day and between-day reproducibility. The precision of the method at each concentration was calculated as the relative standard deviation (RSD); the accuracy of the procedure was determined by expressing the

mean calculated concentration as a percentage of the spiked concentration.

2.6. Application to clinical pharmacokinetic studies

This assay was utilised to determine 5-FU concentrations in plasma following a 24-h infusion of the drug (2.5 g/m^2) to six patients. Blood samples were obtained at predose and at different times during and after the 5-FU infusion.

Pharmacokinetic parameters of 5-FU were calculated using standard equations [20]. The following parameters were determined: plasma concentration at the steady-state (C_{ss}) was determined for each patient as the mean of all the concentration values obtained, considering that steady-state was reached within the first 2 h of infusion; area under the concentration–time curve ($AUC_{0-24\ h}$) was estimated by the trapezoidal method (5-FU concentration at the end of infusion, $C_{24\ h}$, was estimated as C_{ss}); total plasma clearance (Cl) was calculated as $Dose/AUC_{0-24\ h}$; mean residence time (MRT) was determined as $AUC_{0-24\ h}/AUMC_{0-24\ h}$, where $AUMC_{0-24\ h}$ is the area under the moment curve.

3. Results and discussion

3.1. HPLC assay development

3.1.1. UV wavelength selection

The absorption spectra of 5-FU, U, 5-FC and 5-BU exhibited two absorbance maxima at approximately 200 and 260 nm. The wavelength of 266 nm, which is the second absorbance maxima for 5-FU, was selected for the simultaneous determination of the analytes in order to obtain cleaner chromatograms.

3.1.2. Column type

5-FU is not easily separated from structurally related compounds as other pyrimidines with commonly used reversed-phase columns. An ordinary chromatographic problem is the separation of 5-FU peak from U, usually poorly resolved.

We tested three C_{18} columns: (A) Hypersil ODS (5 μm, 200×4.6 mm); (B) Nucleosil RP 18 (5 μm, 250×4.0 mm) and (C) LiChrospher 100RP-18 (5 μm, 250×4.0 mm). Since column C enabled precise measurement of 5-FU and provided better resolution from U, it was selected to perform chromatographic analysis.

3.1.3. Column temperature

The optimum temperature for 5-FU and U resolution with the LiChrospher column was 25°C. However, at this temperature 5-FC could not be perfectly resolved from U; moreover, if 5-BU was used as internal standard, it could not be separated from an interfering peak and the optimum temperature was then 17°C.

These findings stress the need for temperature gradient during the analysis: column temperature is set at 25°C over the first 7 min, after the 5-FU peak appears, then column is cooled down to 17°C until 5-BU, internal standard, is detected (15 min). Finally, the column is heated again up to 25°C and maintained at this temperature until the end of the chromatogram in order to allow the column to reach its initial condition for immediate analysis.

3.1.4. Selection of the internal standard

Since the analytical column provided better resolution for 5-BU than for 5-FC, 5-BU was chosen as internal standard. Furthermore, the 5-BU peak appears in an area of the chromatogram almost free from interfering peaks while 5-FC is detected very close to uracil and other endogenous peaks.

3.1.5. Flow of the mobile phase

Initially, mobile phase flows at 1 ml/min. After the 5-FU peak appears (7 min) flow was elevated to 1.7 ml/min in order to decrease 5-BU retention time and hence reduce analysis time. Flow is maintained at 1.7 ml/min for 13 min in order to allow the appearance of an endogenous peak which could interfere in the immediate analysis. Finally, flow comes down to 1 ml/min 20 min after the injection and held at this flow until the end of the chromatogram (22 min) in order to allow the column to return to initial conditions for the next analysis.

3.1.6. Influence of different parameters on the extraction ratio

3.1.6.1. pH of plasma samples. Since the pK_a values of acidic compounds 5-FU and 5-BU are approximately 8, an extraction pH of approximately 6 has to be chosen to ensure that they would exist in the unionised form. Plasma acidification has been already applied in several methods [15,19]; in the present assay, it was achieved by adding 20 μl of orthophosphoric acid (5%). This step is very important considering that, although plasma obtained from healthy subjects has a mean value of 6.5–7, plasma from cancer patients often has pH values higher than 7. For example, a plasma pH of 7.5 means that only 70% of the drug exists in the unionised form, therefore extraction with an organic solvent would be less effective.

3.1.6.2. Precipitating agents. In numerous HPLC methods, deproteinisation improves the quality of the chromatogram. We tested several precipitating agents as trichloroacetic acid [9,10], methanol, acetonitrile and ammonium sulphate, as a powder [11] and in solution; despite the fact that these chemicals produced cleaner chromatograms, all of them had a great repercussion on recovery since 5-FU and 5-BU coprecipitate with them.

3.1.6.3. Extraction solvent. Different solvents were analysed for 5-FU extraction from plasma samples. The internal standard was added immediately before extraction; the recovery was calculated by reference to the non-extracted aqueous solution to which identical quantities of internal standard had been added.

Several solutions and volumes were tested: chloroform, 16% propanol in diethyl ether [15,18], ethyl acetate [9,13,21,22] and 15% isopropanol in ethyl acetate [11,19]. Best recovery was obtained using 16% *n*-propanol in ethyl ether. Chloroform provided poor recoveries, $<10\%$ for both 5-FU and 5-BU. Ethyl acetate and isopropanol–ethyl acetate gave nearly 40% recovery for 5-FU and 20% for 5-BU. The volume of the extraction had a great influence on 5-FU and 5-BU recoveries: 5.5–6 ml is the minimum volume to reach a 5-FU recovery $>70\%$ from 500 μl of plasma. Time of vortex mixing was also an essential step in the extraction: over 2 min the recovery gain was insignificant.

3.2. Assay validation

The analytical methodology was validated in terms of selectivity, recovery, linearity, limit of quantitation, precision and accuracy.

3.2.1. Selectivity

No interfering peaks were observed in any of the plasma pools used in our studies, or in plasma samples obtained from patients who had not received prior administration of 5-FU (Fig. 1A). In this figure, an endogenous peak of U can be seen at 5.77 min. A representative chromatogram of a human plasma extract after administration of 5-FU at 2.5 g/m^2 is shown in Fig. 1B. Resolution of the compounds of interest was optimum with a mobile phase composition of 0.05 *M* potassium dihydrogenphosphate (pH 3).

3.2.2. Recovery

The overall mean recovery of 5-FU was 73.9±4.87 (Table 1). The recovery of the internal standard, 5-BU, measured at the concentration used in the analysis (3 μg/ml) was (mean±SD)

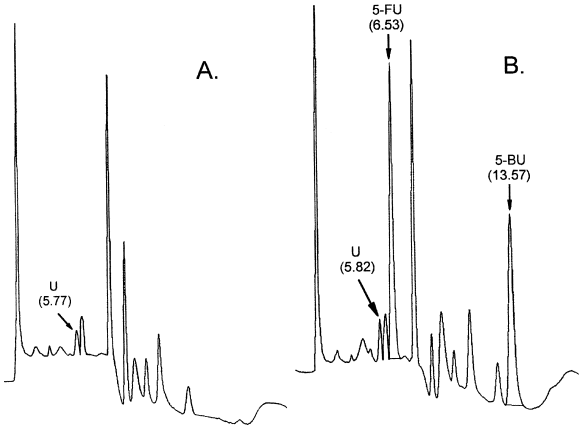

Fig. 1. HPLC of: (A) a predose plasma extract; notice the appearance of an endogenous peak of uracil (U) at 5.77 min; and (B) a human plasma extract after administration of 2.5 g/m^2 5-FU. Peaks: U (5.82 min), 5-FU (6.53 min) and internal standard 5-BU (13.57 min).

Table 1
Recovery of 5-FU from human plasma ($n=10$); results are expressed as mean±SD (standard deviation)

Concentration (ng/ml)	Recovery (%)
10	71.2±5.13
50	70.4±5.29
200	78.6±4.37
1000	72.2±4.43
5000	75.0±3.23
20 000	72.1±5.84

66.7±4.05 ($n=10$). As shown in Table 1, recoveries for 5-FU are similar for every concentration studied.

3.2.3. Linearity

The calibration graph was estimated as the mean of five graphs obtained on five different days and yielded the following equation: $y=1.076x-1.564$, where y is the area ratio of 5-FU to the internal standard (5-BU) and x is the concentration of 5-FU. The correlation coefficient (r) for each calibration graph was >0.999 and the RSDs of the response factors (RFs) ($RF=y/x$ for each concentration assayed) were below 10%.

3.2.4. Limit of quantitation

The limit of quantitation was 2.0 ng/ml for 5-FU; the signal-to-noise ratio for this concentration was approximately 3. As 5-FU is rapidly eliminated from the systemic circulation, elimination half-life is 9–22 min [23–25], increased sensitivity is an important factor for thorough pharmacokinetic analysis. Using this method, it is possible to determine plasma concentrations of 5-FU from samples obtained 1–2 h after the administration of a 24-h infusion of a 2.5 g/m^2 dose of this drug.

3.2.5. Precision and accuracy

The precision was good, with a mean within-day RSD of 3.75%, yielding a mean accuracy of 2.15%; the mean between-day RSD was 5.74%, with a mean accuracy of 5.43% (Table 2). As can be seen in this table, precision and accuracy of this method hardly depends on the concentration assayed or on the day of the assay.

3.3. Clinical pharmacokinetics of 5-FU

Plasma concentrations of 5-FU in six patients receiving 2.5 g/m^2 of 5-FU as a 24-h infusion are shown in Fig. 2. Plasma concentration at the steady state (C_{ss}) was (mean±SD) 895±198 ng/ml; mean values for $AUC_{0-24\ h}$, Cl and MRT were, respectively, 20.71±4.33 mg·h/l, 126±28 l/h·m^2 and 25.0±0.9 h.

4. Conclusions

The method we describe has several advantages as its simplicity, since it only requires a one-step extraction without a previous plasma deproteinisation, precision and specificity, but its main advantage is improved sensitivity that allows the quantification of 2 ng/ml of 5-FU in plasma samples.

This assay has been successfully applied for 5-FU pharmacokinetic investigation and therapeutic drug monitoring in cancer patients receiving 5-FU in different schedules.

Table 2
Within-day and between-day precision and accuracy of the HPLC determination of 5-FU in human plasma ($n=5$)

Concentration (ng/ml)	Within-day		Between-day	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
10	4.12	3.38	6.29	9.88
50	3.67	3.36	5.80	5.96
200	5.93	1.90	6.30	7.00
1000	2.57	1.74	7.20	3.55
5000	3.05	1.44	4.51	2.20
20 000	3.17	1.1	4.35	4.00

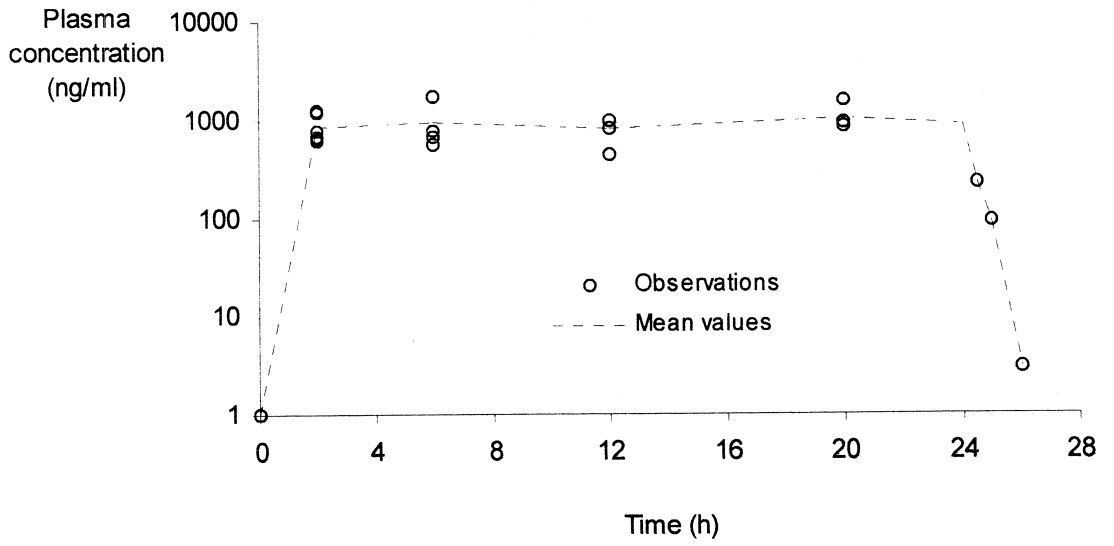

Fig. 2. Representative plasma concentrations of 5-FU in six patients after the administration of 5-FU at a dose level of 2.5 g/m^2 as a 24-h infusion. Mean plasma concentration at the end of infusion is considered to be C_{ss}. Plasma concentrations were determined by HPLC with UV detection.

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