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Determination of 5-fluorouracil and its main metabolites in plasma by high-performance liquid chromatography: Application to a pharmacokinetic study

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Abstract

This paper describes a relatively simple and sensitive high-performance liquid chromatographic assay (HPLC) with ultraviolet absorbance detection for 5-fluorouracil (5-FUra) and its two main metabolites, 5-fluorouridine (5-FUrd) and 5-fluoro-2′-deoxyuridine (5-FdUrd), in plasma. In this study, two plasma clean-up procedures involving addition of internal standard, solid-phase and liquid–liquid extractions have been developed. A reversed-phase Kromasil C_{18} column was used. The detection was performed at 268 nm for 5-FUra and at 275 nm for the two metabolites. Linear detection responses were obtained for concentrations ranging from 25 to 1000 ng/ml. The average recovery from plasma was 35, 42 and 48% for 5-FUra, 5-FUrd and 5-FdUrd, respectively. Precision, expressed as C.V., ranged from 2.7 to 13% and the mean recovery from 94 to 105%. The limits of quantitation and detection of the three analytes were 20 and 10 ng/ml, respectively. The method was used to monitor the pharmacokinetic profile of 5-FUra and its two metabolites in patients with metastatic colorectal cancer.

Keywords: 5-Fluorouracil; 5-Fluorouridine; 5-Fluoro-2′-deoxyuridine

1. Introduction

5-Fluorouracil (5-FUra) is an antineoplasic agent that is widely used alone or in combination chemotherapy regimens for the treatment of advanced gastrointestinal cancer, breast cancer and several other types of cancer [1]. In the body, 5-FUra is rapidly metabolized, particularly by the liver, to give various metabolites with well known antineoplastic properties [2]; among them, 5-fluorouridine (5-FUrd) and 5-fluoro-2′-deoxyuridine (5-FdUrd) nucleoside analogues (Fig. 1).

Several analytical methods, which include gas chromatography [3], mass spectrometry [4], isotachophoresis [5] and high-performance liquid chromatography (HPLC) [9–18] have been developed to detect 5-FUra in plasma of patients undergoing cancer therapy. Some methods involved both the quantitation of 5-FUra and its nucleosides (5-FUrd, 5-FdUrd) in serum [8,14,16,18].

This paper describes rapid, specific, reliable and sensitive analytical methods based on reversed-phase liquid chromatography that require separate assays

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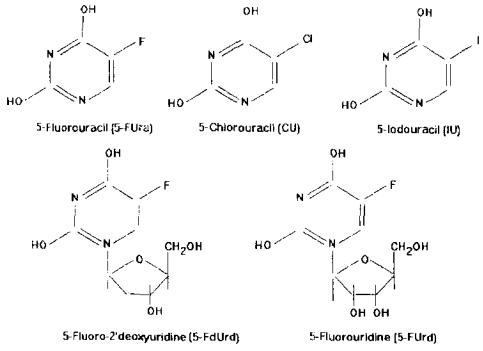

Fig. 1. Structural formulae of 5-FUra, 5-FUrd, 5-FdUrd and internal standards used for the analytical method.

for the quantitation of 5-FUra and its main metabolites in plasma. To quantify 5-FUra, the procedure involved a sample clean-up by solid-phase extraction while for the two nucleosides the sample pretreatment included a liquid–liquid extraction followed by a solid-phase extraction. These methods are more accurate due to the use of internal standards. They were validated according validation procedures, parameters and acceptance criteria based on USP XXIII guidelines [19] and recommendations of Shah et al. [20]; moreover, stability tests under different conditions have been performed.

2. Experimental

2.1. Materials and reagents

5-FUra (Roche, Neuilly, France) was purchased as an injectable form (1 g/20 ml). The metabolites, 5-FUrd and 5-FdUrd, as well as 5-iodouracil (IU) and 5-chlorouracil (CU), for use as internal standards, were obtained from Sigma (St. Quentin-Fallavier, France). Methanol, ethyl acetate, acetonitrile and chloroform were all of analytical-reagent grade and were purchased from Prolabo (Paris, France). C_{18} Chem Elut extraction cartridges were purchased from Varian (Les Ulis, France) and used without activation and washing prior to sample loading.

The 5-FUra stock solution was diluted 1:49 with purified water (Laboratoires Fandre, Ludres, France) to yield the 5-FUra standard solution containing 1 mg/ml. The standard solutions of metabolites and internal standards were prepared in purified water at concentrations of 1 mg/ml. Just before use, these solutions were used to prepare different working solutions by sequential dilutions (1:9, 1:99) with purified water. These solutions were used to spike the plasma samples prior to extraction. An unextracted working standard solution (400 ng/ml) in purified water was prepared daily to check the resolution of the chromatographic system.

Pooled free plasma samples from healthy volunteers were used for the validation of the method.

2.2. Instrumentation

The chromatographic system consisted of a Shimadzu model LC9A pump (Kyoto, Japan), a Shimadzu model SPD10AV variable-wavelength UV–Vis detector, a Rheodyne loading valve (model 7010) fitted with a 20 μl sample loop (Touzart and Matignon, Paris, France) and a Shimadzu integrator model C-R5A (chart speed, 3 mm/min). HPLC separation was performed on a stainless-steel Kromasil C_{18} analytical column (150×4.6 mm I.D.) packed with 5 μm diameter particles (Touzart and Matignon, Paris, France).

2.3. Chromatographic conditions

The eluent mixture was composed of methanol and purified water (3:97, v/v), and was deaerated ultrasonically prior to use. The flow-rate was set at 0.6 ml/min, which corresponds to a back pressure of about 14 MPa. The volume injected was 20 μl. Chromatography was performed at ambient temperature (20°C). The eluent was monitored at 268 and 275 nm for 5-FUra and the two metabolites (5-FUrd and 5-FdUrd), respectively.

2.4. Extraction procedure

2.4.1. 5-FUra

A 0.5 ml volume of plasma was mixed with 50 μl of internal standard solution (CU, 5 μg/ml) in a 5 ml glass tube and then loaded on a C_{18} Chem Elut cartridge. Washing of this cartridge was not necessary prior to the elution. The analytes were eluted with 4×2 ml of an ethyl acetate–methanol (95:5, v/v) mixture, and the solid-phase extraction cartridge

was flushed with nitrogen at a flow-rate of 1 ml/min. The eluate fraction was collected in a 10 ml glass tube and evaporated to dryness under a stream of nitrogen for 30 min at 40°C. The dried residue was reconstituted in 200 μl of water, placed in an ultrasonic bath for 10 min and then filtered through a 0.45 μm HV4 membrane (Millipore, Molsheim, France). A 20 μl volume was injected onto the column.

2.4.2. 5-FUrd and 5-FdUrd

In a 5 ml screw-capped glass tube, an aliquot of plasma (0.5 ml) was mixed with 50 μl of internal standard (IU, 5 μg/ml). Protein precipitation was performed by addition of 2 ml of acetonitrile. The tubes were gently shaken by rotation for 10 min (20 rpm). The resulting mixture was centrifuged (3000 *g*) for 10 min at 4°C to separate the two phases. The supernatant was removed and transferred to a screw-capped tube, and 3 ml of chloroform were added. After Vortex-mixing (2630 rpm) for 2 min, samples were frozen at −80°C for 30 min in order to improve separation of both phases, thawed then centrifuged at 3000 *g* for 10 min at 4°C. A 500 μl volume of the upper aqueous layer was pipetted and applied to a C_{18} Chem Elut Cartridge. The cartridge was eluted with 4×2 ml of an ethyl acetate–methanol (80:20, v/v) mixture. The following procedure was identical to the one described above.

2.5. Instrument calibration

For all components, calibration samples were prepared by spiking 0.5 ml of blank plasma with appropriate volumes of working solutions in order to obtain concentrations of 25, 50, 100, 200, 350, 500 and 1000 ng/ml. The volume added was always smaller than or equal to 2% of total volume of the samples, so that the integrity of the plasma was maintained.

2.6. Data analysis

Quantitation was based on peak areas measured by the integrator. The ratio of the peak area of 5-FUra, 5-FUrd and 5-FdUrd to that of internal standard was used as the assay parameter. Peak area ratios were plotted against theoretical concentrations.

Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data. The quality of fit was evaluated by comparing back-calculated concentrations to the nominal ones. The resulting slopes and intercepts were used to obtain concentration values for that day's quality control samples and unknown samples.

The linearity of the method was confirmed using the classical statistical tests; i.e., comparison of intercept with zero and correlation coefficients. Moreover, the Kolmogorov–Smirnov test was used to compare the distribution of the residuals (difference between nominal and back-calculated concentrations) to the expected one (N(0,1), i.e., normal distribution and centered around zero).

2.7. Specificity

Several human plasma samples from different healthy subjects were tested for the absence of interfering compounds. The retention times of endogenous compounds in plasma were compared with those of 5-FUra, its metabolites and the internal standards.

Plasma samples from patients receiving other drugs commonly used in combination with 5-FUra were analyzed for interference. The following drugs were checked: doxorubicin, cyclophosphamide, *l*-folinic acid, dexamethasone, granisetron, *cis*-dichlorodiaminoplatinum, carboplatine.

2.8. Recovery

Five samples of plasma were spiked with 50, 500 and 1000 ng/ml of each drug. They were assayed and the recoveries of 5-FUra and its metabolites were determined by comparing the resulting peak areas with the peak areas from aqueous solutions at the same concentrations injected directly onto the analytical column.

The extraction efficiency was also determined for the internal standard.

In order to study the effect of co-extracted biological material, recoveries were also computed by comparison of extracts from spiked samples with blank extracts spiked after pre-treatment.

2.9. Precision and accuracy

Both intra- and inter-day precision and accuracy of the method were tested. Samples of plasma were spiked at three concentrations, low (75 ng/ml), middle (400 ng/ml) and high (800 ng/ml). Six aliquots of each sample were tested on the same day, and the resulting coefficient of variation (C.V.) indicated the intra-assay reproducibility. Aliquots of the same sample were tested once a day during six days, and the resulting C.V. indicated the inter-assay reproducibility.

Accuracy was evaluated by calculating the percent difference (bias %) between the measured mean concentrations and the corresponding nominal concentrations.

2.10. Determination of the limit of quantitation and of the limit of detection

The limit of quantitation (LOQ) was defined as the lowest drug concentration which can be determined with an accuracy and precision ≤20% on a day-to-day basis [20]. Its corresponds to sample concentrations of 5-FUra, 5-FUrd and 5-FdUrd giving a peak area of 10 times the noise level (S_N). The estimate of S_N was determined by extrapolation to zero. To determine the accuracy and precision on the LOQ, spiked plasma were used ($n=6$).

The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of 3 times S_N.

2.11. Stability studies

The working solutions (0.1 mg/ml, 1 μg/ml, 0.1 μg/ml) were repeatedly ($n=3$) injected into the chromatograph immediately after preparation (time 0) and at 1, 2, 3, 4 and 6 h after bench-top storage at room temperature.

For stability studies in plasma, quality control concentrations of 75, 400 and 800 ng/ml of 5-FUra, 5-FUrd, 5-FdUrd were analyzed in replicate ($n=5$). The short-term stability was assessed at the following times: 0.25, 0.5, 1, 2, 4 and 6 h after storage under ordinary laboratory conditions (20°C at daylight exposure) and at 4°C. The long-term stability in frozen human plasma (−30°C) was determined by periodic analysis over 2 months. Prior to their analyses, samples were brought to room temperature and Vortex-mixed well. Samples were analyzed immediately after preparation (reference values) and after storage.

The freeze–thaw stability was also determined. Spiked plasma were analysed immediately after preparation and on a daily basis after repeated freezing–thawing cycles at −30°C on three consecutive days.

A drug was considered stable if more than 90% of the intact drug was retained at the end of the study period.

3. Results

3.1. Retention times and specificity

Typical chromatograms of 5-FUra, 5-FUrd and 5-FdUrd in plasma are shown in Figs. 2 and 3. Each chromatographic run required approximately 13 min for the parent drug and 25 min for the metabolites.

The retention times were 5.8 for 5-FUra (intra- and inter-day C.V.s: 1.0 and 1.5%, respectively) and 10.7 min for CU (intra- and inter-day C.Vs.: 1.1 and 1.0%, respectively). They were 13.2 (intra- and inter-day C.V.s: 1.5 and 1.2%, respectively), 19.8 (intra- and inter-day C.V.s: 2.0 and 1.5%, respectively) and 22.1 min (intra- and inter-day C.V.s: 1.4%) for 5-FUrd, 5-FdUrd and IU, respectively.

About 600 injections could be carried out without any change of column performances.

Extraction and chromatographic analysis of ten separate blank plasma samples confirmed that there were no endogenous peaks that coeluted with the respective analytes (Fig. 2a Fig. 3a).

None of the drugs tested that could be co-administered with 5-FUra interfered.

3.2. Linearity

Linear relationship was observed by plotting drug concentrations (x) against peak area ratios (y) at concentrations ranging from 25 to 1000 ng/ml. The correlation coefficients for calibration curves were equal or better than 0.993.

Intra-assay reproducibility was determined for

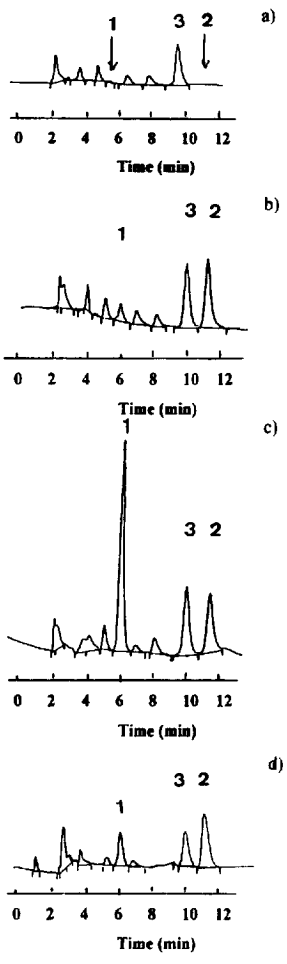

Fig. 2. Chromatograms of blank plasma (a), of plasma spiked with 75 (b), and 1000 ng/ml (c) of 5-FUra, and of plasma sample obtained from a cancer patient (d) treated with 5-FUra (concentration of 5-FUra: 251 ng/ml). Peak 1 is 5-FUra, peak 2 is internal standard (CU) and peak 3 is an unknown compound. For chromatographic conditions see Section 2.3.

calibration curves prepared on the same day in replicate ($n=6$) using the same stock solutions. Inter-assay reproducibility was determined for calibration curves prepared on different days ($n=7$). The results are given Table 1. For each point of calibration, the concentrations were back-calculated from the equation of the linear regression curves (experimental concentrations) and the coefficients of variation (C.V.%) were computed. The results are given in Table 2.

A linear regression of the back-calculated concentrations versus the nominal ones provided a unit slope and an intercept equal to 0 (Student t-test).

The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero. Moreover, the residuals (difference between nominal and back-calculated concentrations) were normally distributed and centered around zero (Kolmogorov–Smirnov test).

3.3. Recovery

The mean recovery of 5-FUra averaged $35.3\pm1.2\%$ ($n=15$). It was $41.7\pm2.9\%$ ($n=15$) for 5-FUrd and $48.0\pm3.6\%$ ($n=15$) for 5-FdUrd. It was not statistically different over the range of concentrations studied.

Recoveries were $45\pm1.3\%$ ($n=5$) for CU and $41\pm1.5\%$ ($n=5$) for IU.

No effect of the co-extracted biological material was detected.

3.4. Precision and accuracy

For concentrations of calibration standards ranging from 25 to 1000 ng/ml, the precision around the mean value did not exceed 15% coefficient of variation (Table 2).

Intra-day and inter-day precision and accuracy of the method, assessed by analysing quality control samples are presented in Table 3.

3.5. Limit of quantitation and limit of detection

The limit of quantitation was 20 ng/ml for 5-FUra and its metabolites. At this level, the precision of determination, expressed as coefficient of variation was less than 18%, with an adequate assay accuracy. The recovery averaged 97%.

The limit of detection was 10 ng/ml for 5-FUra and its metabolites.

3.6. Stability studies

Standard solutions of 5 FUra, 5-FUrd, 5-FdUrd and internal standards (1 mg/ml) were stable for at least 3 months if stored at 4°C.

In plasma samples spiked with 75, 400 and 800

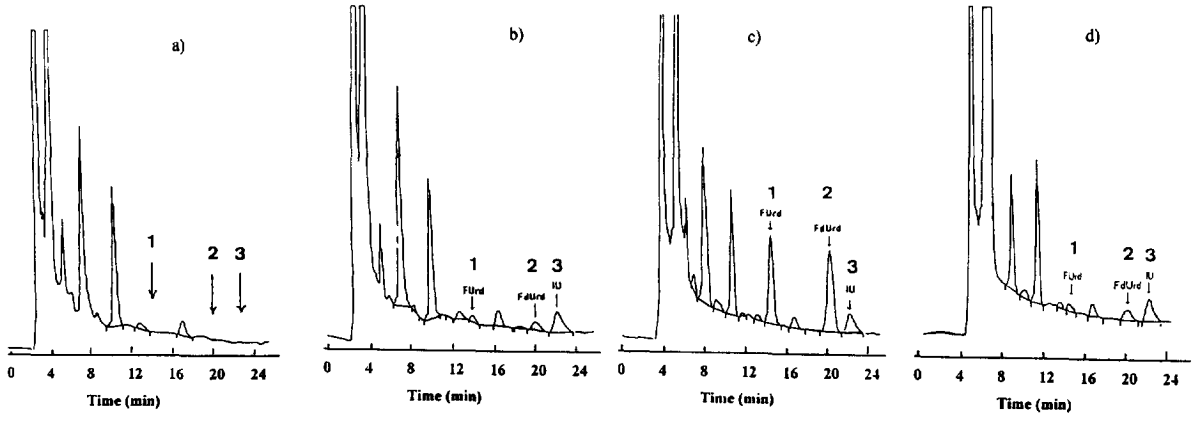

Fig. 3. Chromatograms of blank plasma (a), of plasma spiked with 75 (b) and 1000 ng/ml (c) of 5-FUrd and 5-FdUrd, and of plasma sample obtained from a cancer patient (d) treated with 5-FUra (concentrations of 5-FUrd and 5-FdUrd: 131 and 168 ng/ml, respectively). Peak 1 is 5-FUrd, peak 2 is 5-FdUrd and peak 3 is internal standard (IU). For chromatographic conditions see Section 2.3.

ng/ml, concentrations did not significantly decrease after 6 h at 4°C and 20°C (Table 4).

The long-term freezer stability indicated that 5-FUra and its two metabolites were stable during 2 months, the percent recovery ranged from 93 to 105%. Compared to the reference values, there was no statistical difference.

At least three freeze–thaw cycles can be tolerated without losses higher than 10%.

4. Discussion and conclusion

The developed methods proved to be useful and reliable for the determination of plasma concentrations of 5-FUra and its two main metabolites. In order to improve the specificity and the limit of quantitation of each analyte, two plasma clean-up procedures involving solid-phase and liquid–liquid extractions followed by reversed-phase chromatography with ultraviolet absorbance detection, have been developed, one to quantify 5-FUra and the other to quantify the metabolites. Assay performance was assessed both on the basis of the statistical characteristics of individual calibration curves and from the results of quality control samples. This method, validated for concentrations ranging from 25 to 1000 ng/ml, has a good reproducibility and accuracy, and low limits of quantitation and detection compared to the most published HPLC methods [6,7,9,10,12,13,16]. The limit of quantitation or of detection was of the same order of magnitude as that reported by some authors [8,11]. However, lower detection limits to quantify 5-FUra have been reported by Barberi-Heyob et al. [14] using ion-pair HPLC (2 ng/ml), by Jochheim et al. [17] using precolumn derivatization with a fluorescent reagent and a column switching method (3 ng/ml) and by Palmisano et al. [15] using a binary gradient (5 ng/ml). However, using the method described by Barberi-Heyob et al. [14], previous assays have shown that, according to the drug free plasma used, several extraneous peaks due to the matrix occurred at the retention time of 5-FUra. Nevertheless, the limits of quantitation and detection of the proposed analytical procedure can be improved two-fold by increasing the injected volume to 50 μl. The specificity of this method with respect to endogenous substances and drugs that may be co-administered is satisfactory. Stability studies carried out directly in plasma indicated that samples were stable for at least 2 months when stored at −30°C. The time for sample clean-up and chromatography required approximately 45 min for the 5-FUra; thus, this method allows therapeutic drug monitoring. Nowadays, in our laboratory a population pharmacokinetic program to optimize individual dosage regimens based on the determination of 5-FUra, 5-FUrd and 5-FdUrd is under development.

The overall analytical procedure has been used to

Table 1
Assay linearity

	5-FUra			5-FUrd			5-FdUrd		
	Correlation coefficient of the linear regression analysis[a] (r) (mean±S.D.)	Slope (b) (mean±S.D.)	Intercept (a) (mean±S.D.)	Correlation coefficient of the linear regression analysis[a] (r) (mean±S.D.)	Slope (b) (mean±S.D.)	Intercept (a) (mean±S.D.)	Correlation coefficient of the linear regression analysis[a] (r) (mean±S.D.)	Slope (b) (mean±S.D.)	Intercept (a) (mean±S.D.)
Intra-day reproducibility ($n=6$)	$0.996\pm2.5\times10^{-3}$ (C.V.=0.25%)	$0.0031\pm3\times10^{-4}$ (C.V.=5.5%)	0.0068±0.052	$0.996\pm2.9\times10^{-3}$ (C.V.=0.29%)	$0.00302\pm1.9\times10^{-4}$ (C.V.=6.3%)	−0.071±0.116	$0.997\pm1.4\times10^{-3}$ (C.V.=0.14%)	$0.00372\pm2.7\times10^{-4}$ (C.V.=7.2%)	−0.028±0.08
Inter-day reproducibility ($n=9$)	$0.998\pm1.7\times10^{-3}$ (C.V.=0.17%)	$0.0030\pm1.68\times10^{-4}$ (C.V.=5.6%)	0.027±0.057	$0.996\pm1.6\times10^{-3}$ (C.V.=0.16%)	$0.00294\pm1.9\times10^{-4}$ (C.V.=6.6%)	−0.029±0.058	$0.997\pm1.8\times10^{-3}$ (C.V.=0.18%)	$0.0035\pm1.0\times10^{-4}$ (C.V.=2.9%)	0.0063±0.048

r=Correlation coefficient.
[a] Linear unweighted regression, formula: $y=a+bx$.

Table 2
Inter- and intra-assay reproducibilities of the HPLC analysis

Theoretical concentration (ng/ml)	5-FUra				5-FUrd				5-FdUrd			
	Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=9$)		Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=9$)		Intra-assay reproducibility ($n=6$)		Inter-assay reproducibility ($n=9$)	
	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)
25	26.3±3.1	11.8	23.9±3.5	14.6	24.0±2.5	10.4	22.3±1.8	8.1	26.4±3.1	11.7	24.2±3.8	15.7
50	44.0±4.5	10.2	51.8±6.0	11.6	48.9±2.9	5.9	48.4±7.1	14.7	53.4±3.0	5.6	46.0±5.6	12.2
100	101±9.1	9.0	93.6±7.8	8.3	99.8±11.7	11.7	101±9.6	9.5	98.8±11.7	11.8	93.9±4.4	4.7
200	197±14.2	7.2	205±17.6	8.6	211±17.5	8.3	201±20.4	10.1	204±19.0	9.3	199±12.8	6.4
350	352±38.2	10.9	348±28.2	8.1	333±20.4	6.1	330±28.3	8.6	340±18.1	5.3	356±35.3	9.9
500	491±35.8	7.3	502±19.9	4.0	493±44.4	9.0	501±42.8	8.5	487±25.3	5.2	522±32.9	6.3
1000	1001±12.0	1.2	999±7.7	0.77	1007±17.1	1.7	1004±21.2	2.1	1009±14.6	1.4	989±16.2	1.6

Table 3
Intra-day and inter-day accuracy and precision of the HPLC method

Theoretical concentration (ng/ml)	n	5-FUra				5-FUrd				5-FdUrd			
		Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Mean recovery (%)	Relative error (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Mean recovery (%)	Relative error (%)	Experimental concentration (ng/ml) (mean±S.D.)	C.V. (%)	Mean recovery (%)	Relative error (%)
Intra-day precision													
75	6	79±9.9	12.5	105.3	5.3	71±4.5	6.3	94.5	5.5	74±8.9	12.0	98.7	1.3
400	6	394±48.8	12.4	98.5	1.5	400±36.5	9.1	100.0	0.0	399±10.6	2.7	99.8	0.2
800	6	786±70.3	8.9	98.3	1.7	797±66.3	8.3	99.6	0.4	795±49.8	6.3	99.4	0.6
Inter-day precision													
75	6	74±5.5	7.4	98.7	1.3	75±5.2	6.9	100.0	0.0	78±7.3	9.4	104.0	4.0
400	6	380±36.3	9.6	95.0	5.0	389±32.5	8.4	97.3	2.7	398±41.5	10.4	99.5	0.5
800	6	770±47.4	6.2	96.3	3.7	831±72.5	8.7	103.9	3.9	814±79.8	9.8	101.8	1.8

Table 4
Mean percent recoveries after 6 h of storage at 20°C and 4°C

Concentration added (n=5) (ng/ml)	Recovery (mean±S.D.) (%)					
	5-FUra		5-FUrd		5-FdUrd	
	20°C	4°C	20°C	4°C	20°C	4°C
75	95.1±3.1	93.9±2.6	98.7±7.4	101±9.3	96.0±6.3	103±8.0
400	95.5±5.5	99.4±6.3	103±10.4	106±8.9	98.0±7.9	107±13.8
800	101±7.3	96.2±5.3	97.1±7.8	104±8.7	99.9±8.7	106±13.4

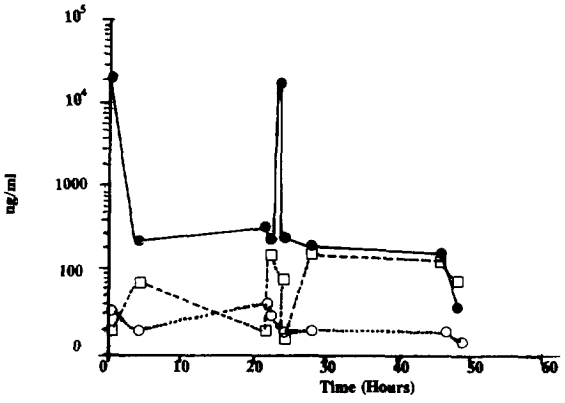

Fig. 4. Plasma concentration–time profile of (●) 5-FUra, (○) 5-FUrd and (□) 5-FdUrd after intravenous administration of 5-FUra to a patient with metastatic colorectal cancer.

determine plasma concentrations of 5-FUra and its two main metabolites in plasma samples from patients with metastatic colorectal cancer receiving folinic acid, followed by 5-FUra daily for two consecutive days (this study was reviewed and approved by the regional ethical committee) [21]. The treatment was given as folinic acid (200 mg/m^2) by intravenous (i.v.) infusion over 2 h in 0.9% (w/v) sodium chloride, then 5-FUra (400 mg/m^2) i.v. bolus, then 5-FUra 600 mg/m^2 by i.v. infusion over 22 h in 5% dextrose. This was repeated over the next 24 h. Venous blood samples were drawn into heparinized tubes before, just after the i.v. bolus, and at 4 and 21.75 h after the start of each 5-FUra infusion. Additional samples were taken 15 and 120 min after the end of treatment. Fig. 4 illustrates the plasma concentrations versus time plots for 5-FUra, 5-FUrd and 5-FdUrd obtained from a patient.

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