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Determination of the concentrations of 5-fluorouracil and its metabolites in rabbit plasma and tissues by high-performance liquid chromatography☆

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Abstract

The concentrations of 5-fluorouracil, 5-fluoro-5,6-dihydrouracil, 5-fluorouridine and 5-fluoro-2′-deoxyuridine in plasma, liver, kidney, lung and heart of rabbits were determined by high-performance liquid chromatography (HPLC) after drug administration by two different routes. HPLC was carried out by using a Spherisorb 5 ODS 2 column and 0.05 *M* phosphate buffer as the mobile phase with UV detection at 200 nm. The pH of the mobile phase, organic modifier content and column temperature were found to have a profound influence on the results, hence it was necessary to optimize a procedure for each matrix. A comparison of the efficiency of intravenous and peritoneal administration revealed that the latter provides higher drug concentrations in the liver and minimal contents in plasma and all other tissues studied.

1. Introduction

5-Fluorouracil (5-Fu) and its metabolites are widely used for treating liver cancer [1,2]. Their high toxicity has promoted testing of various administration routes [1,3–7] in order to determine which provides the maximum possible concentrations in liver tissue and minimum levels in plasma so as to ensure maximum efficiency and minimum toxicity.

The drug is rapidly metabolized on administration, particularly by the liver, where it gives rise to various metabolites with well known antineoplastic properties [8]. It is therefore of interest not only to measure the drug levels, but also those of their metabolites and the residual amounts remaining in various body parts in order to develop an optimum procedure for administration of the drug. Among the various techniques available for the determination of 5-Fu [9–16], we chose high-performance liquid chromatography (HPLC), which previously provided good results in the determination of 5-Fu in vitreous gel and liquid samples [17]. The original procedure was modified in order to ensure optimum conditions for each type of matrix by using *n*-propanol and diethyl ether in different proportions and under various conditions as extractants [18].

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2. Experimental

2.1. Reagents

5-Fluorouracil (5-Fu), 5-fluoro-5,6-dihydrouracil (FuH_2), 5-fluorouridine (Furd), 5-fluoro-2′-deoxyuridine (Fdurd), 4-chlorouracil (4-Clu) and 5-fluorocytosine (5-Fc) were purchased from Sigma (St. Louis, MO, USA). Ammonium dihydrogenphosphate and all other chemicals used to prepare the buffers were of analytical-reagent grade and supplied by Merck (Darmstadt, Germany). The water used was purified by passage through a Nanopure II system from Barnstead (Newton, MA, USA) and is referred to as Nanopure water. Diethyl ether and *n*-propanol were obtained from SDS (Peypin, France).

2.2. Apparatus and chromatographic conditions

The chromatographic set-up used consisted of a CD4000 multiple-solvent partitioning pump and an SM4000 variable-wavelength UV–Vis detector, both from LDC Analytical (Riviera Beach, FL, USA), in addition to a JCL6000 chromatography data system from Jones Chromatography (Littleton, CO, USA).

The column used was 25 cm × 0.46 cm I.D. and packed with 5-μm particles of Spherisorb 5 ODS 2 purchased from Phenomenex (Torrance, CA, USA). It was thermostated as required by using a CAL 9900 advanced air oven from CAL Controls (Libertyville, IL, USA). The mobile phase was 0.05 *M* phosphate buffer (pH 2–8) containing 0.5% of acetonitrile, pumped at a flow-rate of 1.0 ml/min. Samples were injected by means of a Marathon autosampler from Spark Holland (Emmen, Netherlands) furnished with a fixed-volume (20-μl) loop.

2.3. Administration procedure

Intravenous route

Rabbits were given 30 mg of 5-Fu per kg of body mass through the right femoral vein of the rabbit. Blood samples were then withdrawn from the ears at various times. The samples were collected in tubes containing sodium heparin, centrifuged for 10 min at 3000 *g* and frozen for later analysis. The animals were killed at different times after administration of the drug in order to obtain tissue specimens that were frozen until analysis.

Peritoneal route

Rabbits were anaesthetized with scandicain for abdominal implanting of a catheter that was connected to an absorption bed consisting of peritofundin, glucose and 30 mg 5-Fu/kg mass. The solution was propelled into the abdomen by means of a piston pump in such a way that 5-Fu was continuously diffused to the blood and tissues. Blood and tissue specimens were obtained as in the previous case.

2.4. Internal standard calibration

Stock standard solutions of 5-Fu, FuH_2, Furd, Fdurd, 5-Fc and 4-Clu each at a concentration of 100 μg/ml were prepared in Nanopure water. They were stable for at least 3 months if stored at 4°C. These solutions were used to prepare different working standard solutions by sequential dilution with Nanopure water.

Drug-free samples spiked with known amounts of 5-Fu, its metabolites and an appropriate internal standard (5-Fc for plasma and 4-Clu for tissue samples) were analysed concurrently with each set of unknown samples. At least seven different concentrations of 5-Fu and its metabolites across the working range were measured in quintuplicate. Calibration graphs were obtained by using the least-squares method. Peak-area ratios between each analyte and its corresponding internal standard were used to construct the least-squares regression lines. The concentrations of 5-Fu and its metabolites in plasma and tissues were determined by interpolation from the graphs using the peak-area ratios obtained from unknown samples. Plasma and tissue blanks were used to monitor potential interfering substances.

2.5. Sample extraction

Prior to HPLC analysis, samples were subjected to an extraction and clean-up procedure in order to remove matrix compounds that might overlap with the compounds of interest.

In previous work [18], a clean-up procedure for the determination of 5-Fu in biological matrices was optimized for each type of fluid examined. Because the other analytes to be determined in this work were 5-Fu metabolites, we tried the same procedures in the belief that they would be equally effective. Each of the matrices was subjected to two experiments involving the same amount of tissue and plasma; in one of them, the sample was spiked with the compounds studied. Application of the optimized extraction procedure provided extraction yields between 80% and 99% (Table 1); also, interfering peaks, although still present to some extent, had no adverse effect on the determination of the compounds of interest.

2.6. HPLC analysis

In view of the results obtained in previous work [17], we chose to use the same column (25 cm × 0.46 cm I.D., packed with 5-μm particles of Spherisorb 5 ODS 2) and 0.05 *M* phosphate buffer as the mobile phase. In order to maximize the resolution, the different experimental parameters influencing retention (pH of the mobile phase, percentage of organic modifier and column temperature) were optimized, and the most suitable wavelength to detect the compounds studied was selected.

3. Results and discussion

The absorption spectra of both the analytes and of 5-Fc and 4-Cl used as internal standards exhibited two absorbance maxima at *ca.* 200 and 270 nm, except for FuH_2, which showed a single maximum at 190 nm followed by a sharp absorbance decrease. We therefore selected a wavelength of 200 nm for the simultaneous determination of 5-Fu and its metabolites, which also allowed FuH_2 to be detected and the sensitivity to the other compounds to be increased.

3.1. Influence of separation parameters

As noted earlier, we studied the effect of the pH of the mobile phase, the organic modifier content in the phase and the column temperature on the determination in order to optimize the resolution.

pH

The influence of the pH of the mobile phase was studied by using 0.05 *M* solutions of ammonium dihydrogenphosphate at various pH values that were adjusted to 2–8 with 1 *M* HCl or 1 *M* NaOH. These solutions were used as the mobile phase and a mixture of the analytes and both internal standards was injected concurrently with each mobile phase. Fig. 1 shows the retention times for the compounds at different pH values of the mobile phase. As can be seen, the pH had a marked effect mainly on retention of 4-Clu. In fact, the column selectivity for 4-Clu was reversed at pH 4.7 in relation to Furd. On the other hand, a pH above 7.2 was counter-

Table 1
Extraction recoveries of 5-Fu and its metabolites in plasma and tissues

Sample	Recovery (mean ± S.D., $n = 3$) (%)			
	FuH_2	5-Fu	Furd	Fdurd
Plasma	89.54 ± 0.85	89.00 ± 0.89	81.89 ± 0.93	88.67 ± 0.98
Liver	80.16 ± 0.92	81.20 ± 0.95	80.15 ± 0.95	85.92 ± 0.95
Kidney	82.48 ± 0.88	84.75 ± 0.93	80.06 ± 0.89	88.26 ± 0.92
Lung	90.01 ± 0.93	95.04 ± 0.92	85.30 ± 0.93	98.15 ± 0.91
Heart	88.20 ± 0.85	93.89 ± 0.94	82.17 ± 0.94	97.00 ± 0.95

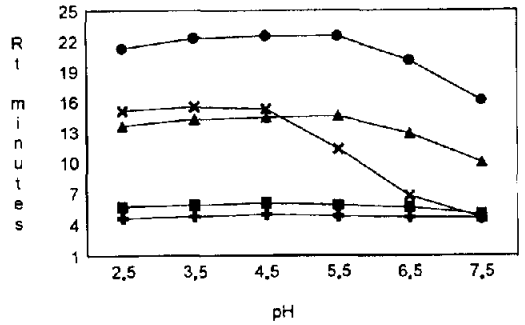

Fig. 1. Variations in the retention time of the compounds as a function of the pH of the mobile phase. (+) FuH_2; (■) 5-Fu; (▲) Furd; (●) Fdurd; (×) 4-Clu.

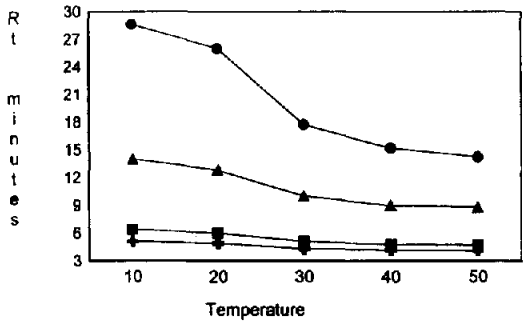

Fig. 3. Influence of the column temperature on the retention times of the compounds. Symbols as in Fig. 1.

productive as it resulted in 4-Clu overlapping with 5-Fu.

Organic modifier content

We used acetonitrile at concentrations between 0.1% and 2% as an organic modifier; the use of higher concentrations resulted in overlapped peaks of 5-Fu and FuH_2. The mobile phase used was 0.05 *M* phosphate buffer (pH 3.0) containing various proportions of acetonitrile. The mixture of standards was thus injected with mobile phases of different composition. Fig. 2 shows the retention times obtained for the different compounds as a function of the acetonitrile content in the mobile phase. As can be seen, 0.5% acetonitrile provided maximum resolution, which was similar to that achieved in its absence but with the added advantage of increased speed, the first compound being eluted within 20 min.

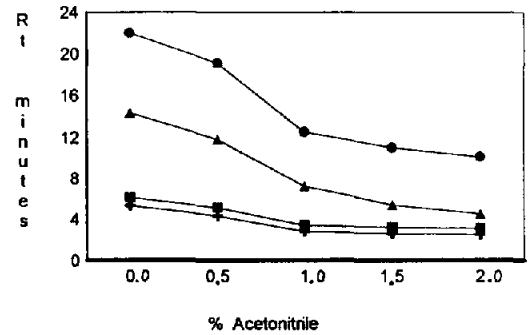

Fig. 2. Variations in the retention times of the compounds as a function of the percentage of acetonitrile in the mobile phase. Symbols as in Fig. 1.

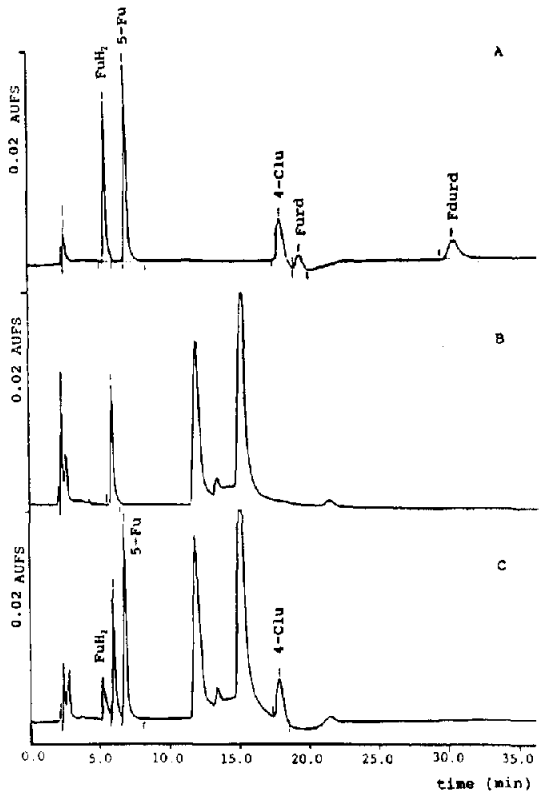

Fig. 4. Chromatogram showing the separation of (A) a standard mixture, (B) a blank of a liver sample and (C) a liver sample after the extraction procedure.

Column temperature

In order to determine the effect of temperature, the column was thermostated from 10 to 50°C at 10°C intervals, and a mobile phase consisting of 0.05 *M* phosphate buffer (pH 4) containing 0.5% of acetonitrile was used. Fig. 3 shows the retention times obtained as a function of the column temperature. As can be seen, increasing temperature resulted in decreased retention times, particularly for the more strongly retained compounds, the t_R values for which were more than halved in some instances.

After the influence of the above parameters on resolution had been determined, the optimum conditions for plasma and each type of tissue studied were established. The mobile phase used was the same for all matrices: 0.05 *M* phosphate buffer containing 0.5% acetonitrile. On the other hand, the pH and column temperature were adjusted to different values in order to achieve the best possible resolution between the analytes to interest and interfering compounds. The optimum conditions for each type of sample were as follows: liver, pH 3, 15°C; kidney, pH 6, 10°C; lung, pH 5, 35°C; heart, pH 5, 20°C; and plasma, pH 2.5, 25°C.

Figs. 4–8 show the chromatograms obtained under the optimum HPLC for a standard mixture, plasma and tissue blanks and plasma and tissue samples after the extraction procedure.

3.2. Calibration graphs

The calibration graphs for 5-Fu and metabolites obtained from the extraction of spiked samples were linear from the limit of quantifica-

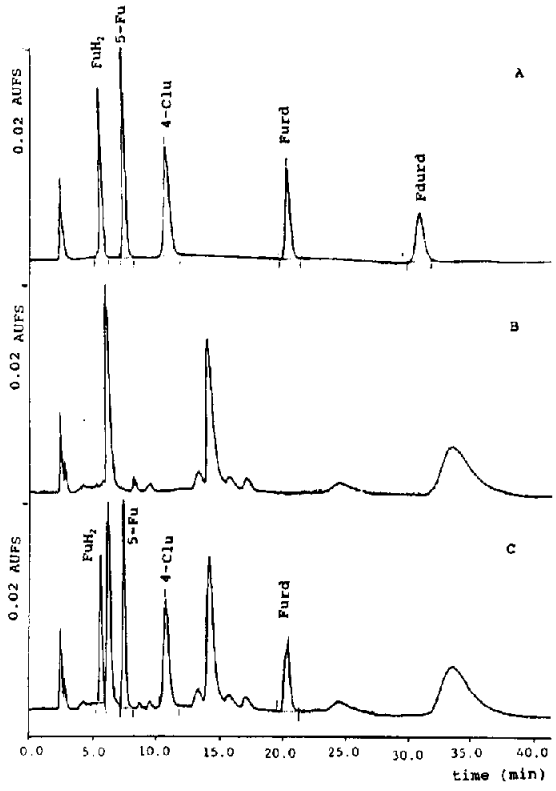

Fig. 5. Chromatogram showing the separation of (A) a standard mixture, (B) a blank of a kidney sample and (C) a kidney sample after the extraction procedure.

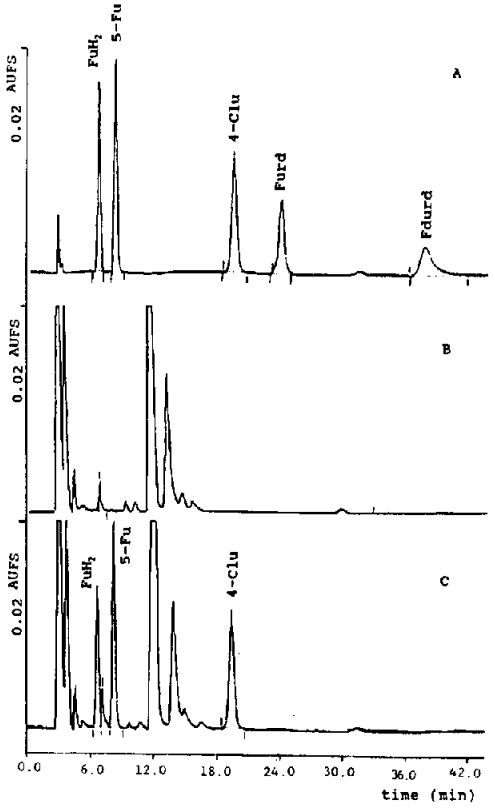

Fig. 6. Chromatogram showing the separation of (A) a standard mixture, (B) a blank of a heart sample and (C) a heart sample after the extraction procedure.

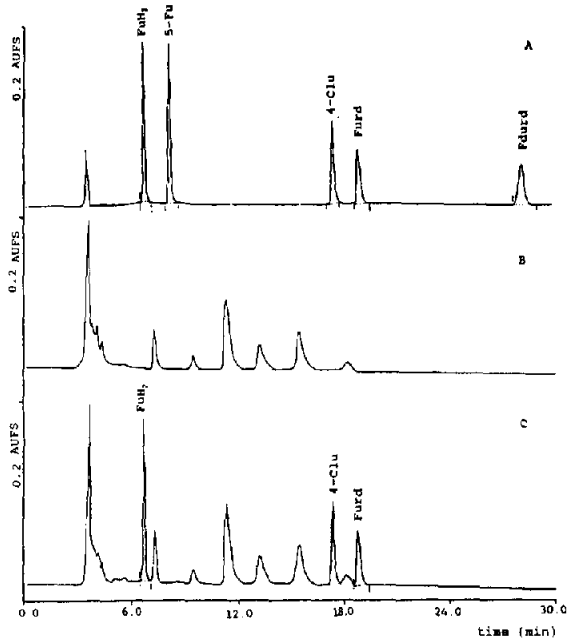

Fig. 7. Chromatogram showing the separation of (A) a standard mixture, (B) a blank of a lung sample and (C) a lung sample after the extraction procedure.

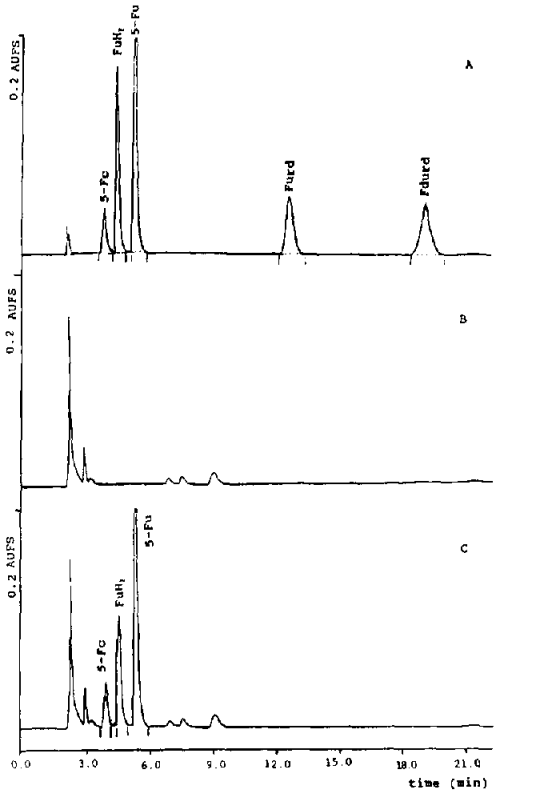

Fig. 8. Chromatogram showing the separation of (A) a standard mixture, (B) a blank of a plasma sample and (C) a plasma sample after the extraction procedure.

tion to at least 100 μg/ml or μg/g. The limits of quantification obtained ($s_b + 10\sigma$, s_b being the average signal of the blank and σ the standard deviation) for plasma and tissues are given in Table 2.

3.3. Animal experiments

Plasma, liver, kidney, lung and heart samples from the rabbits were analysed after intravenous and peritoneal administration of the drug. The results obtained are given in Tables 3–5.

Intravenous administration

The 5-Fu concentration in rabbit plasma was maximum 1 min after injection and decreased sharply afterwards up to 30 min and more gradually thereafter (Fig. 9). On the other hand, the drug concentration in tissues peaked at 15 min, after which it decreased sharply and became virtually undetectable at 2 h. Also, the drug was not detected in the lung. FuH_2 became detectable in plasma at 1 min. Its concentration subsequently increased to a maximum of 6.40 μg/ml at 60 min, after which it started to decrease.

Fdurd was not detected in any of the tissues. On the other hand, FuH_2 was found to be present in all of them within 15 min. Its maxi-

Table 2
Limits of quantification of 5-Fu and its metabolites ($s_b + 10\sigma$)

Compound	Limit of quantification				
	Liver (μg/g)	Kidney (μg/g)	Lung (μg/g)	Heart (μg/g)	Plasma (μg/ml)
5-Fu	0.57	0.24	0.10	0.80	0.30
FuH_2	0.53	0.39	0.25	0.48	0.33
Furd	0.20	0.24	0.43	0.30	0.50
Fdurd	0.50	0.21	0.09	0.11	0.67

Table 3
Concentrations of 5-Fu and its metabolites in tissues after intravenous administration of 5-Fu

Time after administration (h)	Concentration (mean ± S.D., $n = 3$) (μg/g)								
	Liver		Kidney			Lung		Heart	
	5-Fu	FuH_2	5-Fu	FuH_2	Furd	FuH_2	Furd	5-Fu	FuH_2
0.25	9.19 ± 0.01	1.19 ± 0.01	31.14 ± 0.08	3.24 ± 0.03	8.03 ± 0.01	20.15 ± 1.75	63.30 ± 4.04	7.95 ± 0.40	2.15 ± 0.48
0.5	3.06 ± 0.08	4.30 ± 1.09	11.00 ± 0.01	5.78 ± 1.32	8.18 ± 0.09	23.47 ± 2.71	32.59 ± 1.99	7.47 ± 0.01	4.48 ± 0.72
1	1.45 ± 0.07	5.20 ± 1.03	0.50 ± 0.15	6.32 ± 0.01	8.61 ± 0.08	25.64 ± 0.26	–	0.96 ± 0.33	6.77 ± 0.60
2	–[a]	0.67 ± 0.02	–	7.14 ± 0.04	–	26.78 ± 2.06	–	–	0.81 ± 0.26
4	–	–	–	9.20 ± 2.13	–	33.01 ± 2.09	–	–	–
8	–	–	–	6.96 ± 0.51	–	24.45 ± 0.05	–	–	–
16	–	–	–	6.42 ± 0.03	–	21.87 ± 0.03	–	–	–
32	–	–	–	5.37 ± 0.51	–	20.20 ± 0.03	–	–	–

[a] Dashes indicate below the limit of quantification.

mum concentration in the liver and heart was detected at 1 h, whereas those in the kidney and lung were detected 4 h later. The highest and most persistent concentrations were found in the lung. Furd was present in the kidney and lung at 15 min, after which it decreased to undetectable levels at 2 h. It was not detected in the liver or heart.

Peritoneal administration

The 5-Fu concentration in plasma was low (0.38–1.63 μg/ml) at all intervals. On the other hand, those in the liver and kidney increased to a maximum at 1 h, after which they decreased gradually to undetectable levels at 24 h in the liver and 4 h in the kidney. The drug was undetectable in the lung during the first 15 min after administration and at any time in the heart.

No 5-Fu metabolites were detected in plasma, which can be attributed to the very low concentration of the drug. Any metabolites produced would have occurred at levels below the detection limit of the technique.

None of the tissues was found to contain Fdurd. On the other hand, FuH_2 was primarily found in the kidney and lung, at initially decreas-

Table 4
Concentrations of 5-Fu and its metabolites in tissues after peritoneal administration of 5-Fu

Time after administration (h)	Concentration (mean ± S.D., $n = 3$) (μg/g)								
	Liver			Kidney			Lung	Heart	
	5-Fu	FuH_2	Furd	5-Fu	FuH_2	Furd	FuH_2	5-Fu	FuH_2
0.25	–[a]	–	–	–	22.20 ± 5.12	12.22 ± 0.02	7.67 ± 0.54	12.84 ± 2.17	–
0.5	11.13 ± 0.06	3.71 ± 0.55	–	1.64 ± 0.10	18.23 ± 4.43	9.90 ± 0.54	20.83 ± 0.56	–	8.52 ± 0.58
1	27.12 ± 2.50	–	–	10.61 ± 1.54	9.19 ± 1.71	8.14 ± 0.01	18.05 ± 2.20	–	–
2	5.63 ± 0.69	–	–	6.99 ± 1.67	7.30 ± 0.11	7.42 ± 0.01	4.78 ± 0.28	–	–
4	5.25 ± 0.05	–	26.70 ± 3.60	–	6.15 ± 0.30	5.08 ± 0.04	–	–	–
8	0.63 ± 0.03	–	39.19 ± 0.04	–	3.87 ± 0.12	–	–	–	–
24	–	–	61.67 ± 0.04	–	3.42 ± 0.15	–	–	–	–
24.25	–	–	74.85 ± 0.56	–	7.96 ± 0.86	–	12.69 ± 1.65	–	–
25	–	–	84.71 ± 0.04	–	6.85 ± 1.17	–	9.02 ± 1.60	–	–
32	–	–	–	–	5.85 ± 0.02	–	5.13 ± 0.32	–	–
48	–	–	–	–	4.49 ± 0.96	–	–	–	–

[a] Dashes indicate below the limit of quantification.

Table 5
Concentrations of 5-Fu and its metabolites in plasma after intravenous and peritoneal administration of 5-Fu

Time after administration	Concentration (mean ± S.D., $n = 10$) (μg/ml)		
	Intravenous administration		Peritoneal administration
	5-Fu	FuH_2	5-Fu
1 min	92.65 ± 5.36	2.01 ± 0.58	–
5 min	41.40 ± 4.56	3.49 ± 0.96	0.48 ± 0.14
10 min	29.57 ± 3.69	2.78 ± 0.98	0.57 ± 0.08
15 min	27.35 ± 3.25	3.12 ± 1.15	0.71 ± 0.09
30 min	16.04 ± 3.10	3.97 ± 0.95	0.71 ± 0.10
45 min	13.15 ± 2.64	4.74 ± 1.08	0.87 ± 0.13
60 min	9.66 ± 2.21	6.40 ± 1.56	0.92 ± 0.21
90 min	3.98 ± 1.02	2.15 ± 0.81	1.63 ± 0.36
2 h	2.27 ± 0.69	2.70 ± 0.47	1.04 ± 0.28
4 h	–[a]	3.85 ± 1.21	0.57 ± 0.07
8 h	–	–	0.55 ± 0.12
16 h	–	–	0.51 ± 0.15
24 h	–	–	0.38 ± 0.11

[a] Dashes indicate below the limit of quantification.

ing concentrations that rose sharply at 24 h and continued to fall afterwards. Such an abrupt rise in the FuH_2 concentration was concurrent with the end of 5-Fu administration (removal of catheters from the rabbits' abdomens). Therefore, the animals might have reacted by metabolizing the drug. Furd was not detected in the lung or heart. On the other hand, its concentration in the kidney decreased gradually during the first 8 h, at which time it was undetectable, whereas that in the liver became detectable at 4 h and increased subsequently for another 25 h, after which it started to decrease sharply.

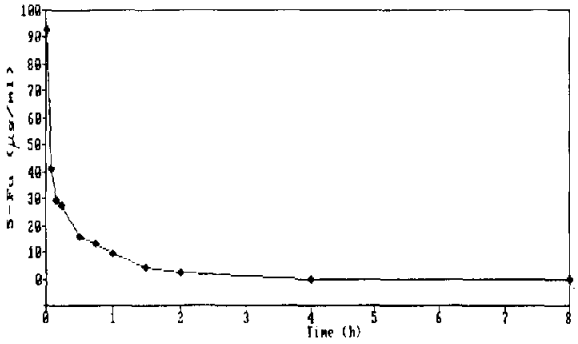

Fig. 9. Plasma concentration–time profile of 5-Fu after intravenous administration.

4. Conclusions

5-Fluorouracil and its metabolites can readily be determined by HPLC in plasma and biological tissues by using phosphate buffer as the mobile phase, the pH of which, in addition to the proportion of acetonitrile that it contains and the column temperature used, have a critical effect, so they must be optimized for each type of sample. Peritoneal administration of the drug is seemingly more effective than intravenous injection for treatment of liver cancer as it provides higher drug concentrations in the liver and much lower concentrations in plasma and other types of tissue.

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