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Simultaneous determination of 5-fluorouracil and its active metabolites in serum and tissue by high-performance liquid chromatography

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Abstract

In order to observe the biodistribution of 5-fluorouracil (5-FU) and its main metabolites in different kinds of tissue (tumor, liver, kidney, spleen, mucosa, lungs, heart, peritoneum, pancreas) and serum according to various novel application forms, a simple and rapid method for the simultaneous determination of 5-FU and its active metabolites 5-fluorouridine (5-FUrd) and 5-fluoro-2′-deoxyuridine (5-FdUrd) has been established. Proteins in serum and tissue samples were precipitated by perchloric acid after addition of the internal standard 5-bromouracil. The compounds were separated using an ODS Hypersil (5 μm) column and detected by UV absorbance (254 nm). Specificity, linearity, reproducibility, intermediate precision and accuracy of the method were established. The lower limit of quantitation (LOQ) for the compounds in serum and various tissue samples was determined. Data on the recovery of the compounds and the internal standard are provided. © 1997 Elsevier Science B.V.

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

1. Introduction

5-Fluorouracil (5-FU) has been used for the chemotherapeutic treatment of various solid tumors (liver, breast, colon) for more than twenty years [1–3]. This compound is used both as a single agent and in combination therapy. The antineoplastic effects of 5-FU are not produced by 5-FU alone but also by its metabolites [1–3]. 5-Fluoro-2′-deoxyuridine (5-FdUrd) monophosphate inhibits the thymidylate synthase and thus the synthesis of DNA.

5-Fluorouridine (5-FUrd) triphosphate on the other hand forms fraudulent RNA [4,5] (Fig. 1). It is therefore of interest to measure not only the 5-FU levels but also those of its metabolites and the residual amounts in various body parts. These can be determined by measuring the non-phosphorylated form.

Reversed-phase (RP), reversed-phase ion-pair (RP-IP) and normal-phase (NP) high-performance liquid chromatography (HPLC) was described for the analysis of 5-FU in the presence of structurally related compounds [4–16]. The RP-HPLC is the system most widely applied. The disadvantage of the

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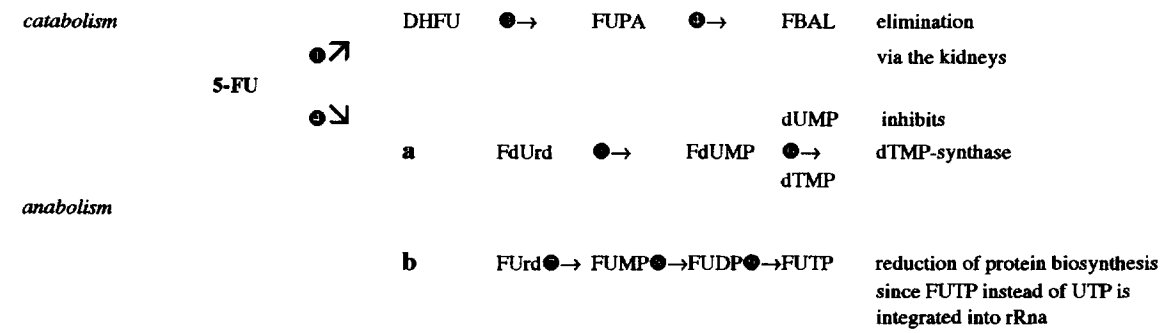

Fig. 1. 5-Fluorouracil metabolism. Abbreviations: 5-FU = 5-fluorouracil, 5-DHFU = 5-fluoro-5,6-dihydrouracil, FUPA = α-fluoro-β-ureidopropionase acid, FBAL = α-fluoro-β-alanine, 5-FdUrd = 5-fluoro-2′-deoxyuridine, 5-FdUMP = 5-fluoro-2′-deoxyuridine-5′-monophosphate, 5-FUrd = 5-fluorouridine, 5-FUMP = 5-fluorouridine-monophosphate, 5-FUDP = 5-fluorouridine-diphosphate, 5-FUTP = 5-fluorouridine-triphposphate, dTMP = thymidine-monophosphate, dUMP = uridine-monophosphate, UTP = uridinetriphosphate, (1) = DHU-dehydrogenase ($NAPH \rightarrow NADP^+$), (2) = dihydropyrimidase, (3) = ureidopropionase (4) (a) = thimidine phosphorylase (+rib-1-P), (b) = uridine phosphorylase (+deoxyrib-1-P), (5) = thymidine kinase (+ATP), (6) = thymidylate synthase and (7) = uridine kinase (+ATP).

majority of these procedures is the recovery, since simultaneous isolation and separation of 5-FU and its metabolites from tissue samples is problematic for several reasons: firstly because of the different chromatographic properties of 5-FU and its metabolites and secondly because of the interfering matrix components extracted during separation. Most of the commonly used extraction procedures are inefficient because of the unfavorable separation of the compounds in the organic layer. A large amount of organic solvent is required which lengthens the time needed for evaporation, so that the metabolites in the sample may be destroyed. Therefore, a method was developed that would enable simultaneous isolation of 5-FU and its most important metabolites (5-FUrd, 5-FdUrd), both from serum and tissue samples. In addition, a chromatographic separation principle was developed in order to separate the metabolites from each other and from the interfering matrix components.

2. Experimental

2.1. Chemicals and reagents

5-FU, 5-bromouracil (5-BU) (used as internal standard), 5-FUrd, 5-FdUrd were purchased from Sigma (Deideshofen, Germany). HPLC-grade methanol and analytical-grade acetic acid and perchloric acid were purchased from Merck (Darmstadt, Germany).

2.1.1. Remark

5-BU was chosen as an internal standard because of its similarity with 5-FU regarding structure, maximum absorption wavelength and percentage recovery. 5-BU is not used as a therapeutic agent and is not a metabolite of either 5-FU or 5-FUrd or 5-FdUrd.

2.2. Equipment

The chromatographic system consisted of an HPLC pump Gynkotek (high-precision pump, Model 300 C), an LKB (Bromma) 2157 autosampler and a programmable UV–VIS spectrophotometric detector (Shimadzu, SPD-6AV). The separation was performed by an ODS hypersil, 5 μm particle size, 250 mm×4.6 mm I.D. (VDS Optilab) analytical column protected by a pre-column packed with the same material (VDS Optilab, 4.5 cm×4.6 mm I.D.) Data were recorded by a D 2500 chromato-integrator (Merck–Hitachi).

2.3. Preparation of calibration standards and quality control samples

10 μl of an aqueous solution containing 5-FU, 5-FUrd and 5-FdUrd was added to 100 μl blank serum and 100 mg of blank tissue samples in order

to obtain concentrations of 1.0, 2.5, 5.0, 10.0, 20.0 and 50.0 μg/ml or μg/g. Then 20 μl of the internal standard (200 μg/ml) was added.

2.4. Preparation of serum and tissue samples of animals

Between 200 mg and 1000 mg were homogenized depending on the kind of tissue sample (liver, tumor, kidneys, spleen, peritoneum, gastric mucosa, lungs, heart and pancreas of male WAG-rats). Here the samples are directly homogenized for 1 min without puffer in an ice-cooled beaker. For the homogenization we used a dispersing device (Ultra-Turrax T 25 with UT dispersing tools S25 KR-18G, IKA-Labortechnik, Staufen; no-load speed=20 500 U/min). After homogenization the samples were weighed. The serum (200–600 μl) was applied directly.

2.5. Precipitation of protein

After adding 100 μl of 10% perchloric acid and 20 μl internal standard per 100 μl serum or 100 μg tissue, the samples were prepared as described in Section 2.4, shaken for 2 min and then centrifuged (2000 *g*) for 10 min. The supernatant was filtered (Millipore 45 μm), pipetted in 1.5 ml vials, and 10 μl were injected into the HPLC system.

2.6. Chromatographic conditions

The mobile phase was methanol–acetic acid (99%)–water (3:0.05:96.95, v/v/v), and was degassed with helium. The flow-rate was 1 ml/min and the temperature was 30°C. The eluate was monitored by UV absorbance at 254 nm.

2.7. Data evaluation and calculations

All calculations were done on an IBM-type personal computer using Excel 5.0 software (Microsoft Corporation, USA). The ratio of the peak area of 5-FU and its metabolites to that of the internal standard was used as assay parameter. Peak-area ratios were plotted against theoretical concentrations.

2.8. Defining assay characteristics

2.8.1. Specificity

To demonstrate the specificity of the method, blank serum and tissue samples of ten different rats were analyzed. Then five pooled serum and tissue samples ($n=5$) were spiked with standard solution (10 μg/ml of each compound for serum samples and 2 μg/g for tissue samples) and also analyzed. For the retention time of each compound data for the coefficients of variation are provided.

2.8.2. Standard curve and linearity

In order to investigate the linearity of the procedure, blank plasma and tissue samples were spiked with different amounts of 5-FU, 5-FUrd and 5-FdUrd (1–50 μg/ml).

The standard curves were obtained from unweighted least-squares linear regression analysis of the data and determined on each day of a three-day validation ($n=6$ at each concentration). Peak area ratios were used to calculate the standard curves. A plot of concentration versus signal and the relative error (R.E.) of the interpolated concentrations of the standards were applied to evaluate the linearity. Also the comparisons of the intercept with zero, the correlation coefficients and the coefficients of variation (C.V.s) were determined.

$$\text{R.E.} = \frac{\text{interpolated conc. of standard} - \text{nominal conc. of stand.} \times 100\%}{\text{nominal conc. of stand.}}$$

$$\text{C.V.} = \frac{100 \times s_n}{m}$$

where m=mean of each concentration and s_n= standard deviation.

2.8.3. Precision and accuracy

The reproducibility (intra-day assay precision), intermediate (inter-day) precision and accuracy were calculated from data obtained during the three-day validation. The concentrations were within the range of the standard curve. Serum and tissue samples spiked with these concentrations were analyzed on each day of the three-day validation ($n=6$ at each concentration). The precision was expressed as the coefficient of variation (C.V.) of the interpolated concentrations. All standard deviations of results obtained from standard curves were calculated ac-

cording to the rules of error propagation. Accuracy was expressed as the mean relative error (R.E.) of the interpolated concentration of the samples. According to international conference reports, a precision (C.V.) $\leq 15\%$ and an accuracy (R.E.) $\leq 15\%$ are acceptable [17,18].

2.8.4. Limit of quantitation (LOQ)

The LOQ is the lowest concentration of analyte that can be determined with acceptable precision and accuracy under the stated experimental conditions. It was determined from the peak and the standard deviation of the noise level (s_N). The LOQ was defined as the sample concentration resulting in a peak area of 10-times s_N. s_N was estimated by extrapolation to zero. The determination was done by repeated analysis of spiked serum and tissue samples ($n=10$). Precision and accuracy of quantification were calculated as described in Section 2.8.3. Following international recommendations a precision (C.V.) $\leq 20\%$ and accuracy $\leq 20\%$ are acceptable at the LOQ [17,18].

2.8.5. Recovery

For 5-FU, 5-FUrd, 5-FdUrd and the internal standard 5-BU, recovery was determined at a concentration of 10 μg/ml. Therefore, the absolute recovery (A.R.) was calculated using peak areas of extracted serum and tissue samples ($n=6$) and directly injected aqueous solutions of the same concentration:

$$\text{A.R.} = \frac{\text{peak areas (extracted analyte)} \times 100\%}{\text{peak area (solution of analyte)}}$$

2.9. Stability studies

For the stability studies, serum and various tissue samples were spiked with different amounts of 5-FU, 5-FUrd, 5-FdURd and 5-BU. The short-term stability in serum and tissue was assessed at 0.5, 1, 2, 4, 6 and 12 h both under normal laboratory conditions (20°C at daylight exposure) and at 4°C. The long-term stability in frozen samples (−20°C) was determined by periodic analyses over six months. Samples were analyzed after preparation and storage. Prior to analysis, the samples were brought up to room temperature and vortex-mixed.

3. Results

3.1. Specificity

The chromatograms of blank samples of serum, tumor and liver samples spiked with the compounds (10 μg/ml for serum samples and 2 μg/g for tissue samples) are shown in Fig. 2. The chromatograms demonstrate that the compounds in question could be detected separately from endogenous compounds. No interfering peaks were detected at the retention times of the compounds. The chromatograms of the other tissue samples showed the same results. For 5-FU the coefficients of variation of the retention time ranged from 1.66% for inter-day reproducibility to 2.64% for inter-day reproducibility. For 5-FUrd it ranged from 1.32% to 2.25%, for 5-BU from 1.11% to 2.33% and for 5-FdUrd from 1.26% to 2.67%.

3.2. Standard curve and linearity

In serum and in tissue samples the peak area ratios of 5-FU, its metabolites and the internal standard varied linearly with concentrations above the 1.0 to 50 μg/ml range. Intra-assay reproducibility was determined for the calibration curves prepared on the same day in replicate ($n=6$) using the same stock solutions. Inter-assay reproducibility was determined for the calibration curves prepared on three different days ($n=18$). The average results for intra- and inter-day assays showed a good linearity for the standard curves. Correlation coefficients of the calibration curves ranged from 0.9912 to 0.9977. Coefficients of variation (C.V.s) of 5-FU, 5-FUrd and 5-FdUrd ranged from −7.16% to 9.99%.

3.3. Precision and accuracy

The values obtained during the three-day validation for reproducibility, intermediate precision and accuracy are summarized in Table 1. The intermediate precision ranged from 3.6% to 10.2% for 5-FU, from 2.1% to 10.0% for 5-FUrd and from 2.1% to 10.0% for 5-FdUrd (concentration 1.0–50 μg/ml, $n=18$ for each compound). The accuracy was determined as 0.2% to 12.0% for 5-FU, 0.2% to 13.6% for 5-FUrd and 0.2% to 14.8% for 5-FdUrd (concentration 1.0–50 μg/ml, $n=6$ for each compound).

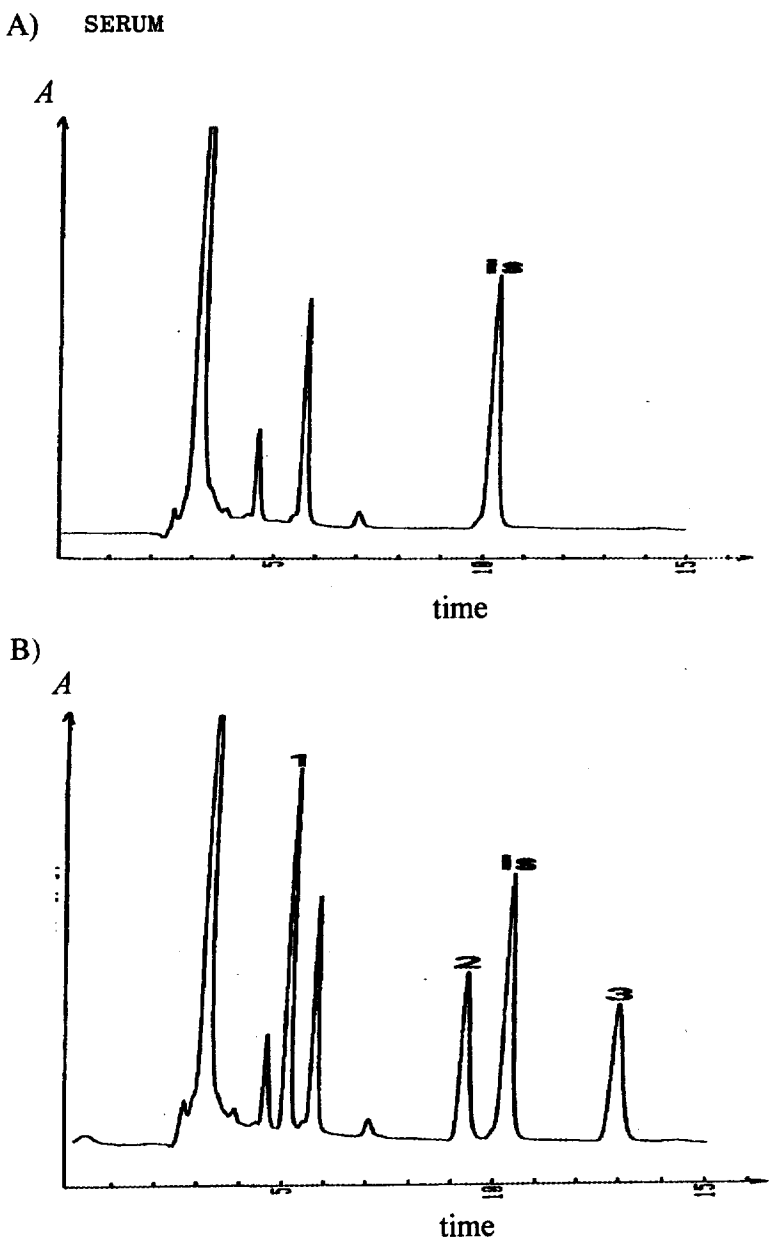

Fig. 2. (I) Representative chromatograms from serum samples (A) blank + internal standard (I.S.) and (B) spiked with 5-FU (1) $t_R = 4.94$, 5-FUrd (2) $t_R = 9.28$, 5-FdUrd (3) $t_R = 12.78$ + I.S. $t_R = 10.34$. (II) Representative chromatograms from tumor samples (A) blank + I.S. and (B) spiked with 5-FU (1) $t_R = 4.94$, 5-FUrd (2) $t_R = 9.32$, 5-FdUrd (3) $t_R = 12.75$ + I.S. $t_R = 10.38$. (III) Representative chromatograms from liver samples (A) blank and (B) spiked with 5-FU (1) $t_R = 4.96$, 5-FUrd (2) $t_R = 9.36$, 5-FdUrd (3) $t_R = 12.79$ + I.S. $t_R = 10.36$.

3.4. LOQ

The LOQs within the C.V. and the mean R.E. determined by repeated analysis for 5-FU, 5-FUrd and 5-FdUrd are shown in Table 2. LOQ of 5-FU was determined as 0.10 μg/ml in serum and 0.30–0.50 μg/g in various tissue samples. For 5-FUrd and 5-FdUrd, it was 0.20 μg/ml in serum and 0.6 μg/g

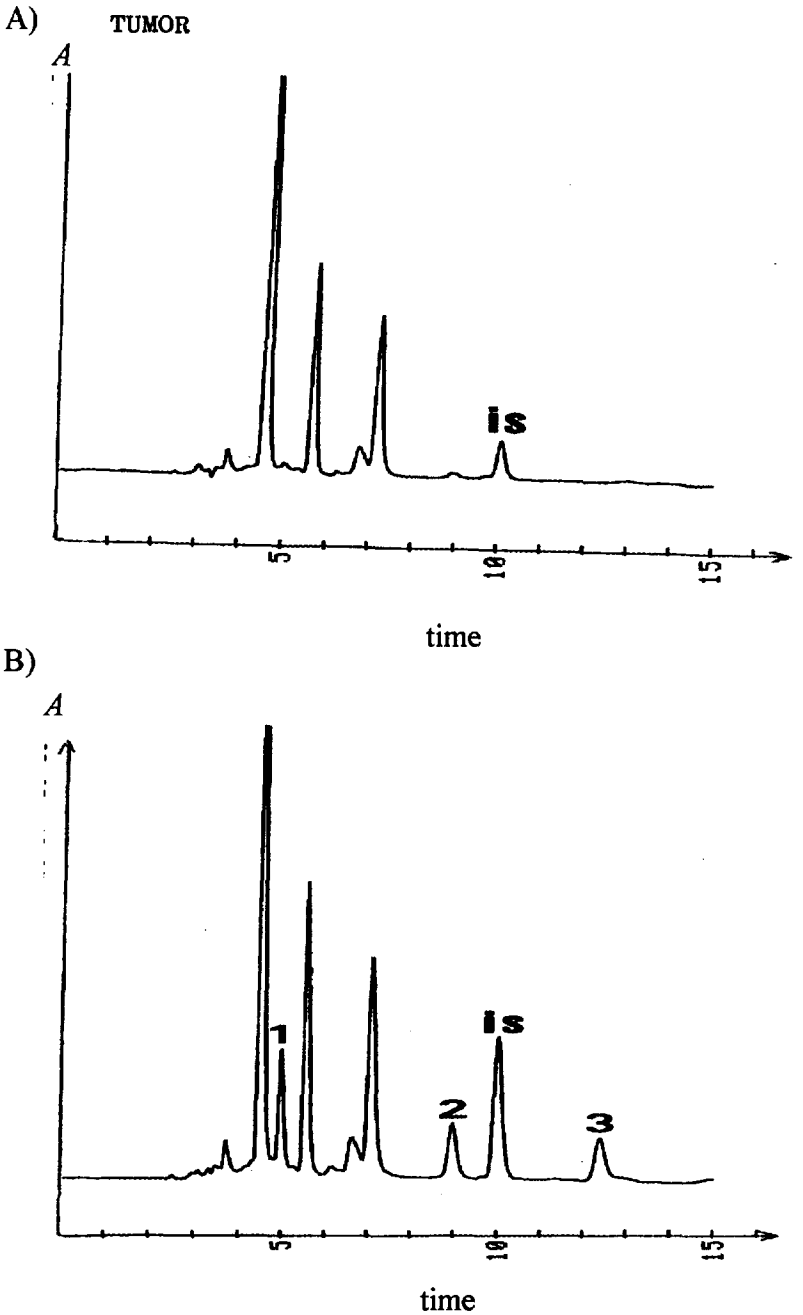

Fig. 2. (*continued*)

in tissue samples. Intermediate precision of the LOQ ranged from 8.9% to 16.2% for 5-FU, from 8.1% to 15.2% for 5-FUrd, and from 8.6% to 14.8% for 5-FdUrd. The accuracy of the LOQ ranged from 6.0% to 20.0% for 5-FU, from 3.0% to 15.0% for 5-FUrd, and from 2.1% to 15.0% for 5-FdUrd.

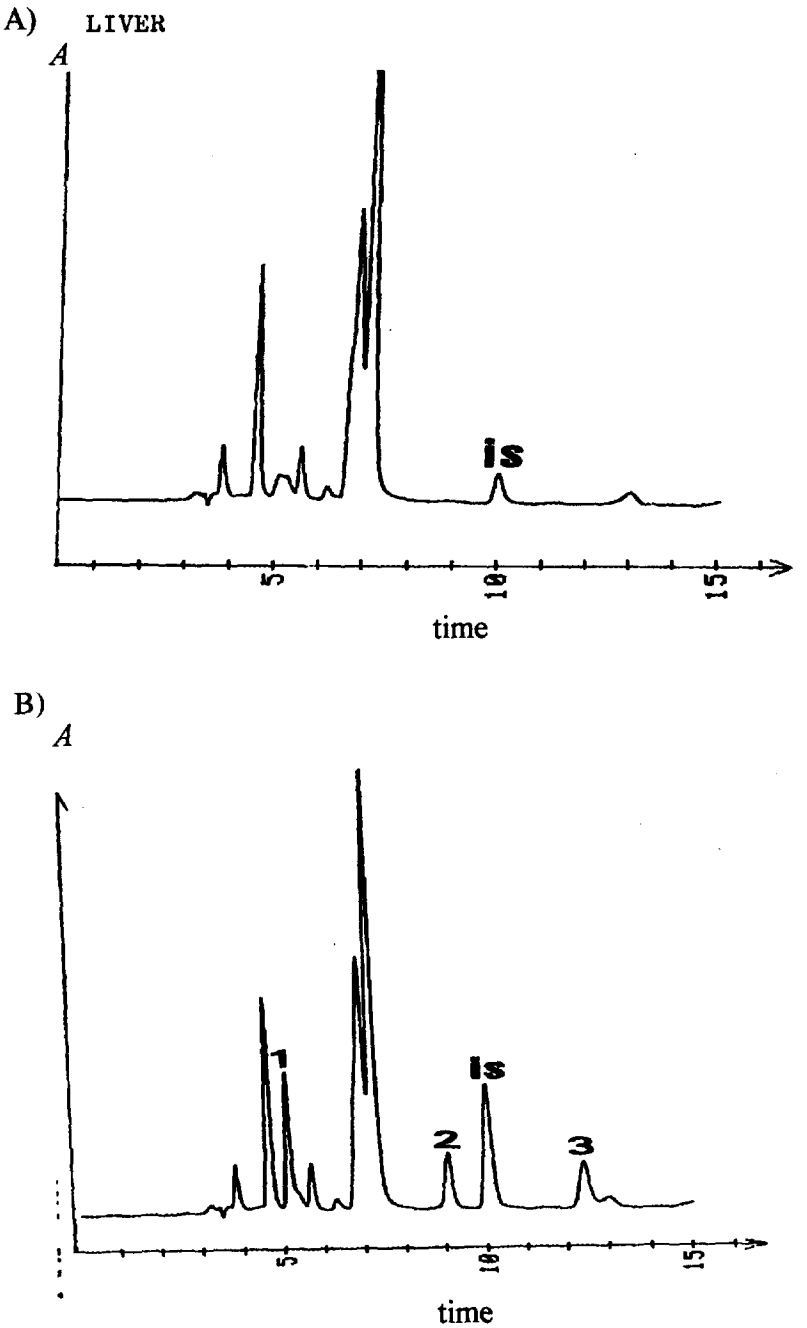

Fig. 2. (*continued*)

Table 1
Results of the three-day validation

Theoretical concentration (μg/ml or g)	5-FU			5-FUrd			5-FdUrd		
	Conc. int. (μg/ml or g) (mean±S.D.)	R.E. (%)	C.V. (%)	Conc. int. (μg/ml or g) (mean±S.D.)	R.E. (%)	C.V. (%)	Conc. int. (μg/ml or g) (mean±S.D.)	R.E. (%)	C.V. (%)
Serum									
Intra-day reproducibility ($n=6$)									
50.0	48.5±2.43	−3.6	5.01	49.9±3.17	−0.2	6.35	47.1±2.87	−5.8	6.09
20.0	20.4±1.44	2.0	7.06	19.8±1.63	−1.0	8.23	20.4±1.28	2.0	6.27
10.0	9.42±0.81	−5.8	8.60	9.84±0.68	−1.6	6.91	9.92±0.72	−0.8	7.26
5.0	4.84±0.38	−3.2	7.85	4.88±0.29	−2.4	5.94	4.87±0.26	−2.6	5.34
2.5	2.31±0.19	−7.6	8.23	2.41±0.17	−3.6	7.05	2.43±0.10	−2.8	4.00
1.0	0.97±0.07	−3.0	7.63	0.97±0.08	−3.0	8.45	1.09±0.08	9.0	7.43
Inter-day reproducibility ($n=18$)									
50	48.3±3.64	−3.4	7.54	47.6±3.54	−5.8	9.15	47.1±4.31	−5.8	9.15
20	19.8±1.53	−1.0	7.73	19.3±1.62	−2.5	7.23	19.5±1.41	−2.5	7.23
10	9.83±0.88	−1.7	8.95	10.2±0.92	−0.5	7.44	9.95±0.74	−0.5	7.44
5.0	5.04±0.29	1.0	5.75	4.88±0.26	−4.2	2.09	4.79±0.32	−4.2	2.09
2.5	2.41±0.17	−3.6	7.05	2.52±0.10	−2.0	6.12	2.45±0.15	−2.0	6.12
1.0	1.02±0.08	2.0	7.55	0.97±0.07	−6.0	8.72	0.94±0.08	−6.0	8.72
Liver									
Intra-day reproducibility ($n=6$)									
50.0	52.0±3.07	4.0	5.90	44.0±2.85	−12.0	6.48	47.0±2.94	−6.0	6.26
20.0	20.8±2.12	4.0	10.2	18.4±1.71	−8.0	9.29	18.8±1.52	−6.0	8.09
10.0	9.98±0.84	−0.2	9.42	9.37±0.72	−6.3	7.68	9.89±0.80	−1.1	8.09
5.0	5.01±0.41	0.2	8.18	4.81±0.32	−3.8	6.65	4.74±0.30	−13.8	6.33
2.5	2.31±0.21	−7.6	9.09	2.37±0.22	−5.2	9.29	2.47±0.10	−1.2	4.00
1.0	1.02±0.06	2.0	8.24	1.02±0.09	2.0	8.82	0.97±0.06	−3.0	6.29
Inter-day reproducibility ($n=18$)									
50	52.4±4.37	4.8	8.34	44.4±3.59	−11.2	8.09	51.4±5.07	2.8	9.86
20	20.7±2.03	3.5	9.81	21.8±2.11	9.0	9.68	21.2±1.82	6.0	8.58
10	10.8±0.93	8.0	8.61	11.0±0.97	10.0	8.82	11.2±0.82	12.0	7.32
5.0	5.14±0.34	2.8	6.61	4.78±0.31	−4.4	6.48	5.18±0.41	3.6	7.92
2.5	2.61±0.19	4.4	7.28	2.40±0.12	−4.0	5.00	2.65±0.13	14.8	4.91
1.0	1.04±0.09	4.0	8.85	0.88±0.06	−12.0	6.70	0.94±0.08	−6.0	8.19
Tumor									
Intra-day reproducibility ($n=6$)									
50.0	49.5±3.33	−1.0	6.73	46.5±3.75	−7.0	5.60	44.5±3.90	−5.8	8.76
20.0	19.8±1.92	−1.0	4.64	18.6±1.51	−7.0	8.12	17.7±1.19	−10.5	6.65
10.0	9.61±0.88	−3.8	9.16	10.1±0.80	1.0	8.00	9.72±0.6ß	−2.8	7.72
5.0	4.91±0.32	−1.8	6.52	4.78±0.26	−4.4	5.44	4.77±0.36	−4.6	7.55
2.5	2.54±0.09	1.6	3.58	2.61±0.16	4.4	6.13	2.53±0.09	−1.2	3.64
1.0	1.09±0.05	9.0	4.95	0.96±0.08	−4.0	8.23	1.07±0.08	7.0	7.85
Inter-day reproducibility ($n=18$)									
50	49.3±2.83	−1.4	5.74	46.2±3.75	−7.6	8.12	44.2±4.06	−11.6	9.19
20	19.7±1.49	−1.5	7.56	19.1±1.50	−4.5	7.85	17.7±1.60	−11.4	9.04
10	9.72±0.79	−2.8	8.13	10.4±0.94	4.0	9.04	9.85±0.84	−1.5	8.53
5.0	4.90±0.28	−2.0	5.71	4.78±0.27	−4.4	5.65	4.72±0.34	−5.6	7.20
2.5	2.58±0.21	3.6	8.11	2.62±0.10	4.8	3.59	2.47±0.20	−1.2	8.10
1.0	1.04±0.06	4.0	6.15	0.94±0.06	−6.0	6.06	0.95±0.09	−5.0	9.68

Concentrations of the standard curves and interpolated concentrations (conc. int) as well as relative error (R.E.) to document accuracy, and coefficient of variation (C.V.) to document reproducibility and intermediate precision of interpolated concentrations for 5-FU. 5-FUrd and 5-FdUrd (intra- and inter-assay reproducibilities).

Table 2
Limits of quantification (LOQs) of 5-FU, 5-FUrd and 5-FdUrd ($n=6$)

Serum or tissue type	5-FU			5-FUrd			5-FdUrd		
	LOQ (mean±S.D.) (μg/g)	C.V. (%)	R.E. (%)	LOQ (mean±S.D.) (μg/g)	C.V (%)	R.E. (%)	LOQ (mean±S.D.) (μg/g)	C.V. (%)	R.E. (%)
Serum (μg/ml)	0.12±0.02	16.2	20.0	0.21±0.03	15.2	5.0	0.23±0.03	14.8	15.0
Liver	0.33±0.04	12.7	10.0	0.62±0.05	8.1	3.3	0.61±0.07	11.1	1.7
Tumor	0.36±0.04	20.0	20.0	0.63±0.05	8.4	5.0	0.66±0.06	8.6	10.0
Kidneys	0.45±0.05	10.2	12.5	0.66±0.06	9.2	5.8	0.61±0.06	10.3	1.7
Spleen	0.43±0.05	11.6	7.5	0.68±0.07	10.9	13.3	0.64±0.09	14.1	6.7
Gastric-mucosa	0.53±0.06	11.5	6.0	0.68±0.07	10.5	13.3	0.69±0.08	12.0	15.0
Peritoneum	0.57±0.06	10.4	14.0	0.67±0.08	11.6	11.7	0.63±0.08	12.5	5.0
Heart	0.48±0.05	10.0	20.0	0.67±0.08	12.1	11.7	0.63±0.09	14.0	5.0
Lungs	0.47±0.04	8.9	17.5	0.69±0.07	10.4	15.0	0.66±0.09	13.9	10.0
Pancreas	0.59±0.06	10.2	18.0	0.69±0.08	10.9	15.0	0.68±0.08	11.3	13.3

3.5. Recovery

The absolute recoveries of 5-FU, 5-FUrd and 5-FdUrd are listed in Table 3. The C.V. ranged from 3.0% to 10.3%.

3.6. Stability studies

Stock solutions of 5-FU, 5-FUrd, 5-FdUrd and 5-BU (1–50 μg/ml or 1–50 μg/g) were stable for at least 5–6 months. In serum and tissue samples, the concentration of 5-FUrd and 5-FdUrd decreased slowly after 1 h at 20°C. After 6 h, only 80% of the original concentration was still present. A slower decrease of the concentration at room temperature was observed in 5-FU and 5-BU. After 48 h, 90% of the original concentration was still present. At 4°C, all samples were stable for up to five days, subsequently a minor decrease of the concentration was observed in 5-FUrd and 5-FdUrd, whereas 5-FU and 5-BU remained stable for 2–3 weeks and decreased then. Aqueous 5-FU and 5-BU solutions can be maintained longer at 4°C.

4. Discussion

In order to observe the biodistribution of 5-FU and its main metabolites 5-FUrd and 5-FdUrd in different kind of tissues (tumor, liver, kidney, spleen, mucosa, lungs, heart, peritoneum, pancreas) and serum according to various novel application forms an effi-

Table 3
Absolute recoveries of 5-FU, 5-FUrd, 5-FdUrd and 5-BU (I.S.) (10 μg/ml or g) ($n=6$)

Serum or tissue type	5-FU		5-FUrd		5-FdUrd		5-BU	
	A.R. (%) (mean±S.D.)	C.V. (%)	A.R. (%) (mean±S.D.)	C.V. (%)	A.R. (%) (mean±S.D.)	C.V. (%)	A.R. (%) (mean±S.D.)	C.V. (%)
Serum	97±4.1	4.2	99±3.8	3.8	94±4.4	4.9	98±2.9	3.0
Liver	104±7.3	7.0	88±7.4	8.4	98±6.4	6.5	102±6.1	6.0
Tumor	99±4.6	4.6	93±6.3	6.8	89±3.6	4.0	97±4.5	4.6
Kidneys	106±8.9	8.3	91±6.8	7.5	86±8.1	9.9	94±8.4	8.9
Spleen	101±7.6	7.5	91±5.9	6.5	98±6.9	7.0	89±9.2	10.3
Gastric-mucosa	97±6.4	6.6	87±7.8	9.0	102±9.6	9.4	103±8.6	8.3
Peritoneum	109±9.8	9.0	99±6.9	7.0	105±7.7	7.3	105±6.7	6.4
Heart	97±7.1	7.3	87±4.6	5.3	97±4.2	4.3	97±4.6	4.7
Lungs	95±6.1	6.3	91±5.2	5.7	99±3.8	3.8	98±5.2	5.3
Pancreas	108±9.7	9.0	103±9.4	9.1	105±8.7	8.3	108±9.4	8.7

cient, simple and rapid method for the simultaneous quantitative determination of these compounds is described. Extensive extraction and washing steps can be avoided by a sample pretreatment procedure involving direct deproteinization with perchloric acid. Assay performance was assessed both on the basis of the statistical characteristics of individual calibration lines and the results of quality control samples. The method validated for concentrations ranging from 1.0 to 50 μg/ml or μg/g has a good reproducibility and accuracy. The specificity of the method is satisfactory with respect to endogenous substances and drugs that may be co-administered. This method with its rapid and simple extraction steps and 15 min HPLC determination is useful for observation of the pharmacokinetic behaviour of various novel therapy models with 5-FU, 5-FUrd, 5-FdUrd and their liposome-encapsulated application forms. With this method we can determinate simultaneous 5-FU-, 5-FUrd- and 5-FdUrd concentrations over a long time in many animal trials. The stability studies carried out directly in serum and tissue samples were stable for at least three months when stored at −20°C.

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