Determination of S-1 (combined drug of tegafur, 5-chloro-2,4-dihydroxypyridine and potassium oxonate) and 5-fluorouracil in human plasma and urine using high-performance liquid chromatography and gas chromatography–negative ion chemical ionization mass spectrometry

Eiji Matsushima*, Kozo Yoshida, Ryuichi Kitamura, Ken-ichiro Yoshida

Pharmacokinetics Research Laboratory, Taiho Pharmaceutical Co. Ltd., 224-2 Ebisuno, Hiraishi, Kawauchi-cho, Tokushima 771-01, Japan

Abstract

A high-performance liquid chromatography (HPLC) and gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICI-MS) method was developed for the analysis of the combined antitumor drug S-1 (tegafur, 5-chloro-2,4-dihydroxypyridine and potassium oxonate) and active metabolite 5-fluorouracil in human plasma and urine. Tegafur was fractionated from biological fluids by extraction with dichloromethane and analyzed by HPLC. 5-Fluorouracil and 5-chloro-2,4-dihydroxypyridine were extracted with ethyl acetate from the residual layer after extraction of tegafur, and converted to pentafluorobenzyl (PFB) derivatives. Potassium oxonate was cleaned up with an anion-exchange column (Bond Elut NH$_2$). The extracted potassium oxonate was degraded to 5-azauracil and converted to PFB derivatives. The PFB derivatives were analyzed by GC–NICI-MS. A stable isotope was employed as the internal standard in the GC–NICI-MS analysis. The limits of quantitation of tegafur, 5-fluorouracil, 5-chloro-2,4-dihydroxypyridine and potassium oxonate in plasma were 10, 1, 2 and 1 ng/ml, respectively. The reproducibility of the analytical method according to the statistical coefficients is ~10%. The accuracy of the method is good; that is, the relative error is <10%. The methods were applied to pharmacokinetic studies of S-1 in patients.

Keywords: S-1; Tegafur; 5-Chloro-2,4-dihydroxypyridine; Potassium oxonate; 5-Fluorouracil

1. Introduction

S-1, a new antitumor agent, was developed based on biochemical modulation of 5-fluorouracil (5-FU), consisting of tegafur (FT), 5-chloro-2,4-dihydroxypyridine (CDHP) and potassium oxonate (Oxo) in a

molar ratio of 1:0.4:1 (Fig. 1). FT, which is a prodrug of 5-FU, plays a role as an effector. Both CDHP and Oxo, which do not have antitumor activity themselves, act as modulators. CDHP competitively inhibits dihydropyrimidine dehydrogenase, which degrades 5-FU, about 180 times more effectively than uracil does in vitro [1], leading to the prolonged retention of a concentration of 5-FU in
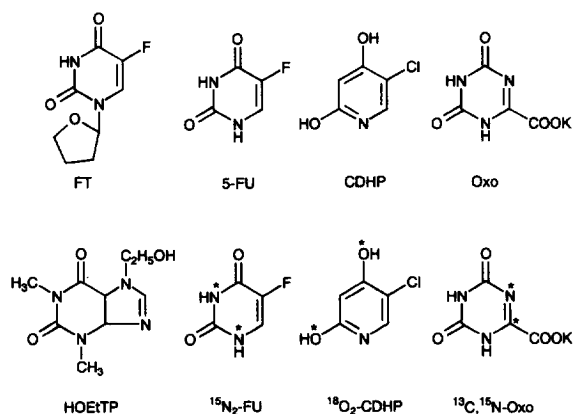
*Corresponding author.

Fig. 1. Structures of FT, 5-FU, CDHP and Oxo and the internal standards. * symbols indicate the position of stable isotopic labels.

blood [2]. Oxo competitively inhibits pyrimidine phosphoribosyltransferase, which converts 5-FU to fluorouridine monophosphate (FUMP), in vitro [3]. Oxo is mainly distributed in the gastrointestinal (GI) tract after oral administration to rats, leading to relief of GI toxicity induced by 5-FU [3]. S-1 showed a better therapeutic effect on various rat tumors and human xenografts than other oral fluoropyrimidines [2].

In pharmacokinetic studies, sensitive, specific and accurate detection methods are required. Furthermore, simple and rapid preparation methods are necessary for the determination of the four compounds, FT, CDHP, Oxo and 5-FU, which have different chemical and physical properties. The high-performance liquid chromatography (HPLC) determination of FT, and the sensitive gas chromatography–mass spectrometry (GC–MS) determination method for 5-FU are the most widely used instrumental techniques. Many derivatizing reagents for 5-FU have been reported. In these reports, the sensitivity was insufficient in both the electron impact (EI) ionization method with the silylation [4–7] and the methylation [8–10]. The pentafluorobenzyl derivative [11,12] and the ditrifluoromethylbenzyl derivative [13] were suitable for gas chromatography–negative ion chemical ionization mass spectrometry (GC–NICI-MS), which enabled more sensitive determination than EI-MS. The enzyme immunoassay (EIA) method for the determination of Oxo in plasma [14] and the HPLC method

for the determination of CDHP in vitro sample [1] have been reported. However, these methods were not suitable for detailed pharmacokinetic studies in humans, because of poor specificity or inadequate sensitivity.

In this paper, we report the extraction from biological fluids and pentafluorobenzyl derivatives of CDHP and Oxo for GC–NICI-MS analysis and a combination FT and 5-FU determination method. In addition, these methods were validated and used successfully to determine the pharmacokinetic profiles of FT, CDHP, Oxo and 5-FU in humans after a single oral administration of S-1.

2. Experimental

2.1. Materials and reagents

FT, CDHP and Oxo were synthesized in Taiho Pharmaceuticals (Tokyo, Japan), β-hydroxyethyltheophylline (HOEtTP) and 5-FU were purchased from Sigma (St. Louis, MO, USA), [$^{15}N_2$]-FU from PCR (Gainesville, FL, USA), [$^{18}O_2$]-CDHP and [$^{13}C,^{15}N$]-Oxo were synthesized in our laboratory (Fig. 1). Bond Elut NH_2 column (1 cc/100 mg) was from Varian (Harbor City, CA, USA). Pentafluorobenzylbromide was purchased from GL Sciences (Tokyo, Japan). Silica-gel for column chromatography was purchased from Wako Pure Chemical (Osaka, Japan). Water was purified with a Milli-QII water system (Nihon Millipore Kogyo, Yonezawa, Japan). All solvents were HPLC grade, and other reagents were analytical grade.

Pooled drug-free plasma and urine samples from healthy volunteers were used for validation of the methods.

2.2. Instruments

HPLC was performed with an LC-6 dual pump system with an SIL-6B auto sample injector and SPD-6A variable-wavelength UV detector (Shimadzu, Kyoto, Japan) operated at 270 nm. HPLC separation was achieved at ambient temperature with a reversed-phase Inertsil ODS-2 column (15 cm×4.6 mm I.D., 5 μm particle size; GL

Sciences) and a 1.0 ml/min of flow-rate of 15% methanol in 10 mM phosphate buffer (pH 5.5).

GC–NICI-MS was carried out using a Hewlett-Packard 5890 gas chromatograph interfaced with TRIO-1000 quadrupole mass spectrometry (VG Masslab, Manchester, UK). Negative ion chemical ionization (NICI) was performed with isobutane as the reagent gas. The source pressure was adjusted and optimized to obtain maximum sensitivity. Ionization was initiated with 70 eV with an emission current of 150 µA. Electron impact (EI) mass spectra were measured with 70 eV ionization energy, 100 µA ionization current. The source temperature was 210°C. The GC column was interfaced directly to the ion source and the interface temperature was maintained at 250°C. Gas chromatographic separation was performed on a DB-5 chemical bonded capillary column (15 m×0.32 mm I.D., film thickness 0.25 µm) (J&W Scientific, Folsom, CA, USA). The column temperature was programmed with a two-ramp temperature program; the oven temperature was held at 100°C for 1 min, then increased at 40°C/min to 200°C for the first ramp and increased at 20°C/min to 300°C for the second ramp. The injection was made using a Hewlett-Packard 7673 automatic sampler. For instrument control and data acquisition, an Intel 386 personal computer with the VG Lab-Base data system version Rel 2.00 was utilized.

^{1}H and ^{13}C NMR spectra were recorded in chloroform-d$_1$ solution using a JEOL GSX-400 NMR spectrometer (Tokyo, Japan) operating at 399.8 and 100.5 MHz, respectively. Temperature was controlled at 30°C, and tetramethylsilane (TMS) was used as the internal reference.

2.3. Preparation and isolation of pentafuruorobenzyl derivative of CDHP

A 10 mg amount of CDHP was dissolved in 0.5 ml of acetonitrile and 0.1 ml of triethylamine, and 0.1 ml of pentafluorobenzylbromide was added. The mixture was shaken for 1 h at room temperature, then extracted with 5 ml of n-hexane–ethanol mixture (10:1, v/v). The extract was concentrated to dryness under nitrogen gas and the residue was dissolved in 0.25 ml of chloroform and applied to silica-gel column (Wakogel C-200, 100–200 mesh,

10 cm×6 mm I.D.). The pentafuruorobenzyl derivative of CDHP was eluted with chloroform and two isomers were fractionated.

2.4. Preparation of standard solutions

Stock standard solutions of FT and HOEtTP were prepared in methanol, and 5-FU, CDHP, Oxo and the stable isotope-labeled compounds ([^{15}N$_2$]-FU, [^{18}O$_2$]-CDHP, [^{13}C,^{15}N]-Oxo) were prepared in water and stored below 5°C. Aliquots of the stock standard solution were diluted with water to provide working standard solutions.

2.5. Sample preparation

Plasma and urine samples were stored in polypropylene tube at −80°C until analysis.

2.5.1. Ft, 5-FU and CDHP

To 0.25 ml of plasma or urine diluted (1:10, v/v) with water, 0.1 ml of 0.2 M phosphate buffer (pH 7.0) and 0.1 ml of internal standard solution (HOEtTP; 10 µg/ml, [^{15}N$_2$]-FU; 250 ng/ml and [^{18}O$_2$]-CDHP; 500 ng/ml) were added. After adding 4 ml of dichloromethane, the mixture was shaken for 10 min, then centrifuged at 2000 g for 5 min. The organic layer was transferred to another test tube, and this extraction was repeated once. The combined organic layer was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was dissolved in 0.2 ml of HPLC mobile phase, and an aliquot (40 µl) was injected into an HPLC column for the chromatographic separation of FT.

To the residual aqueous layer, 0.4 ml of 1 M potassium dihydrogenphosphate and 4 ml of ethyl acetate were added. The mixture was shaken for 10 min, then centrifuged at 2000 g for 5 min. The organic layer was transferred to another test tube and this extraction was repeated once. The combined ethyl acetate layer was evaporated to dryness at 40°C under a stream of nitrogen and the residue was dissolved in acetonitrile (30 µl), and this was followed by the addition of pentafluorobenzylbromide (10 µl) and triethylamine (10 µl). The solution was allowed to stand at room temperature (30 min) and then ethyl acetate (0.05 ml) followed by n-hexane (0.5 ml) were added, and the solution was

vortex-mixed and then centrifuged. The separated upper layer was evaporated to dryness under a stream of nitrogen and the residue was dissolved in 0.2 ml of ethyl acetate and aliquot (1 μl) was injected into the GC–MS system.

2.5.2. Oxo

To 0.5 ml of plasma or urine diluted (1:10, v/v) with water, 0.5 ml of 10 mM Tris buffer (pH 7.4) and 0.05 ml of internal standard solution ([^{13}C,^{15}N]-Oxo; 250 ng/ml) were added. The mixture was applied to a Bond Elut NH$_2$ column conditioned by successively washing with methanol (1 ml), water (1 ml) and 10 mM Tris buffer (pH 7.4, 1 ml). The loaded column was washed with 4 ml of 10 mM tris buffer (pH 7.4) and eluted with 1 ml of 0.2 M carbonate buffer (pH 10). Then, 0.25 ml of 1 M hydrochloride was added to the eluate and heated at 60°C for 1 h. The mixture was evaporated to dryness under nitrogen gas, and the residue was converted into PFB derivative similar to 5-FU and CDHP.

2.6. Calibration and calculations

Calibration standards for control plasma or diluted urine (1:10) were prepared using FT concentrations of 4000, 1000, 250, 50 and 10 ng/ml; 5-FU concentrations of 400, 100, 25, 5 and 1 ng/ml; CDHP concentrations of 800, 200, 50, 10 and 2 ng/ml and Oxo concentrations of 200, 100, 25, 5 and 1 ng/ml. These standards were treated concurrently and in the same manner as the samples to be analyzed.

The peak area of FT, 5-FU, CDHP and Oxo to that of internal standard was used as the assay parameter. Peak-area ratios were plotted against theoretical concentrations. Calibration curves were obtained from a weighted ($1/x^2$) least-squares linear regression analysis of the data.

3. Results and discussion

3.1. Pentafluorobenzyl derivative of CDHP and Oxo

The products of CDHP and decalboxylated Oxo (5-azauracil) with pentafluorobenzylbromide were

subjected to the GC–MS at the EI or the NICI mode. Reaction of 5-azauracil provided a single peak on the total ion chromatogram, and its EI mass spectrum showed the structure as bis-PFB derivative of 5-azauracil. In the NICI mode, it was similar to that reported for the bis-PFB derivative of 5-FU [11], only one major fragment [M−C$_7$H$_2$F$_5$]$^-$ at m/z 292 and a weak fragment [M−C$_{14}$H$_3$F$_{10}$]$^-$ at m/z 112. The reaction of CDHP afforded two peaks (peak 1: 5.63 min, peak 2: 6.55 min) on the total ion chromatogram (Fig. 2), and these peaks showed identical EI and CINI mass spectra corresponding to bis-PFB derivative (Fig. 3). We isolated these peaks by silica-gel column chromatography to clarify the differences in structure. By comparing the ^{1}H and ^{13}C NMR spectra, peak 1 and peak 2 were assigned to (2-O,4-O)-bis-PFB derivative and (1-N,4-O)-bis-PFB derivative, respectively.

^{1}H and ^{13}C NMR spectra exhibited the following signals for peak 1: ^{1}H NMR (CDCl$_3$, 30°C) δ 8.04 (1H, s, H-6), 6.39 (1H, s, H-3), 5.44 (2H, m, OCH$_2$-2), 5.16 (2H, m, OCH$_2$-4); ^{13}C NMR (CDCl$_3$, 30°C) δ 162.75(s), 161.37(s), 147.04(s), 146.15(d), 144.54(s), 115.48(s), 110.3(s), 108.44(s), 94.45(d), 58.01(t), 55.39(t), and for peak 2: ^{1}H NMR (CDCl$_3$, 30°C) δ 7.39 (1H, s, H-6), 6.04 (1H, s, H-3), 5.09 (4H, m, NCH$_2$, OCH$_2$-4); ^{13}C NMR (CDCl$_3$, 30°C) δ 162.15(s), 161.73(s), 146.9(s), 144.5(s), 138.9(s), 136.4(s), 135.27(d), 134.53(s), 106.94(s), 98.48(d), 58.25(t), 40.77(t).

Quantitative determinations were performed by monitoring the peak 1 (short retention time) and ions at m/z 324 for CDHP and at m/z 292 for Oxo.
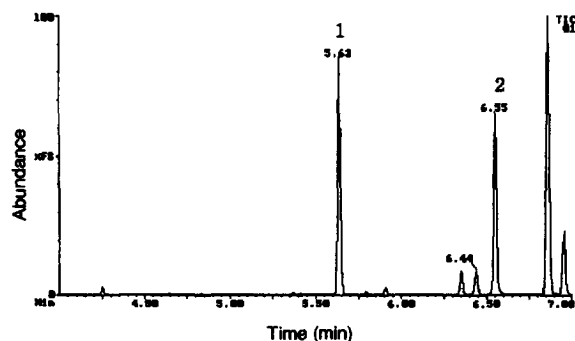

Fig. 2. GC–MS total ion chromatogram of pentafluorobenzyl derivative of CDHP (peak 1: 5.63 min, peak 2: 6.55 min).
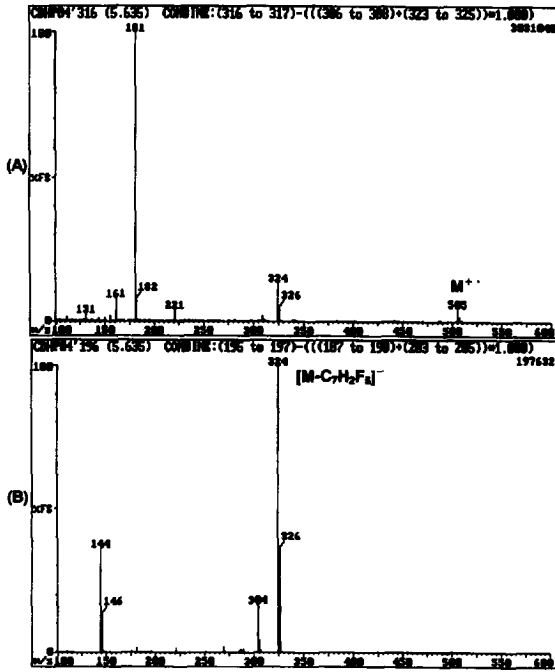
Fig. 3. GC mass spectrum of peak 1 (shown in Fig. 2) on (A) EI mode and (B) NICI mode.

3.2. Sample preparation

The liquid–liquid extraction technique was chosen for the determination of FT, 5-FU and CDHP, and the solid-phase extraction for Oxo. Under the given set of operating conditions, the recovery rates of FT, 5-FU and CDHP from plasma at all concentration ranges were 89.8±3.9%, 76.8±3.6% and 62.6±2.2%, respectively.

The extraction of Oxo from biological fluid was carried out under neutral conditions, because it was easily decarboxylated and resulted in 5-azauracil under acidic conditions. Oxo was retained to anion-exchange resin (Bond Elut NH_2 column) under neutral conditions. The endogenous interference and 5-azauracil, a minor metabolite of Oxo, were washed with tris buffer, and then Oxo was eluted with alkaline carbonate buffer (pH 10). The decarbonation condition of Oxo was carefully inspected, because the resulting 5-azauracil decomposed subsequently at low pH conditions. The eluate (1 ml of 0.2 M carbonate buffer, pH 10.0) was added to 0.25 ml of 1

M hydrochloride, and became pH 3–4; it was then heated at 60°C for 1 h without decomposition of the decarboxylated product. Under the given set of conditions of column extraction, the recovery of Oxo from plasma at all concentration ranges was 94.9±3.3%.

3.3. Chromatographic separation

To evaluate the specificity of the method, drug-free plasma and diluted (1:10) drug-free urine were analyzed by the assay procedure, and the retention times of endogenous compounds in plasma and urine were compared with those of FT, 5-FU, CDHP and Oxo.

In HPLC analysis, the observed retention times were 6.95 and 10.53 min for FT and HOEtTP (I.S.), respectively (Fig. 4). No peaks interfered at the retention times of FT or internal standard in plasma or urine. The repeat time required for HPLC injection was 20 min for plasma analysis and 30 min for urine analysis.

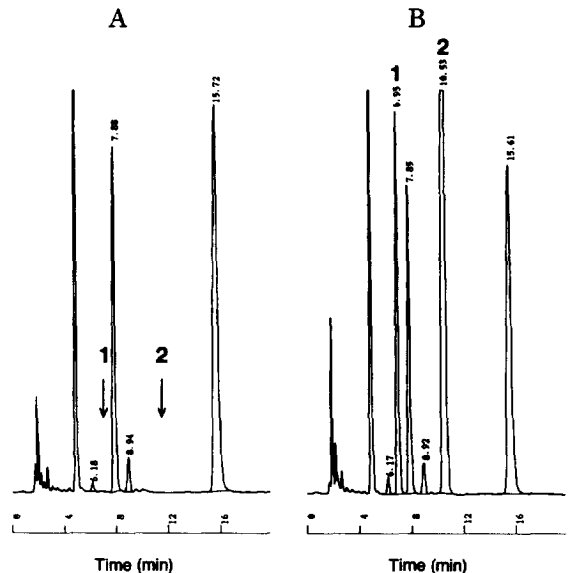The PFB derivatives of 5-FU, CDHP and 5-azauracil (decarboxylated Oxo) were analyzed under

Fig. 4. HPLC chromatograms of (A) blank plasma and of (B) plasma spiked with 1 μg/ml FT. Peaks: 1=FT, 2=internal standard (HOEtTP).
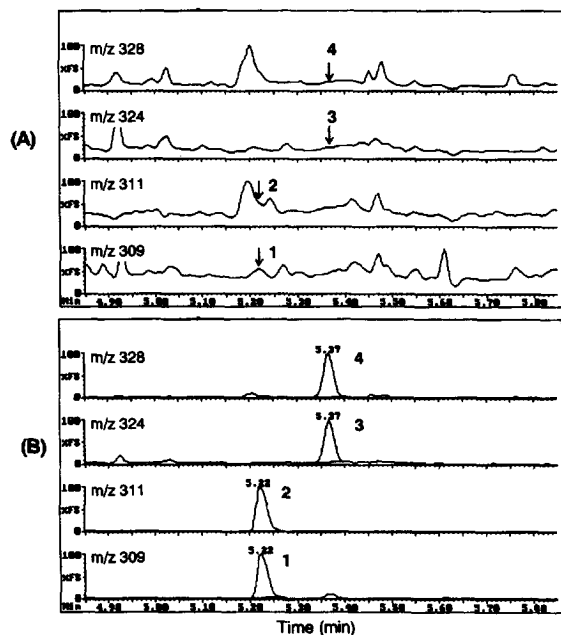
Fig. 5. GC–NICI-SIM chromatograms of (A) blank plasma and of (B) plasma spiked with 25 ng/ml 5-FU and 50 ng/ml CDHP. Peaks: 1=5-FU, 2=internal standard ([^{15}N$_2$]-FU), 3=CDHP, 4= internal standard ([^{18}O$_2$]-CDHP).

Fig. 6. GC–NICI-SIM chromatograms of (A) blank plasma and of (B) plasma spiked with 25 ng/ml Oxo. Peaks: 1=Oxo, 2=internal standard ([^{13}C,^{15}N]-Oxo).

the same GC–NICI-MS conditions, and each major fragment ion [M−C$_7$H$_2$F$_5$]$^-$ was monitored (Fig. 5 Fig. 6). Observed retention times were 5.22, 5.37 and 5.77 min for 5-FU, CDHP and 5-azauracil, respectively. No peaks interfered at the retention times, or with each monitored ion of these compounds and the internal standard in plasma or urine.

3.4. Linearity and LOQ

The peak-area ratio of FT, 5-FU, CDHP and Oxo over the internal standard in plasma and urine varied linearly with concentration over the ranges used; that is, 10–4000 ng/ml for FT, 1–400 ng/ml for 5-FU, 2–800 ng/ml for CDHP, and 1–200 ng/ml for Oxo in plasma and diluted urine. The between-day (n=5) average slope of the fitted straight lines, the mean correlation coefficient and the mean intercept are presented in Table 1. The linearity of the method was confirmed.
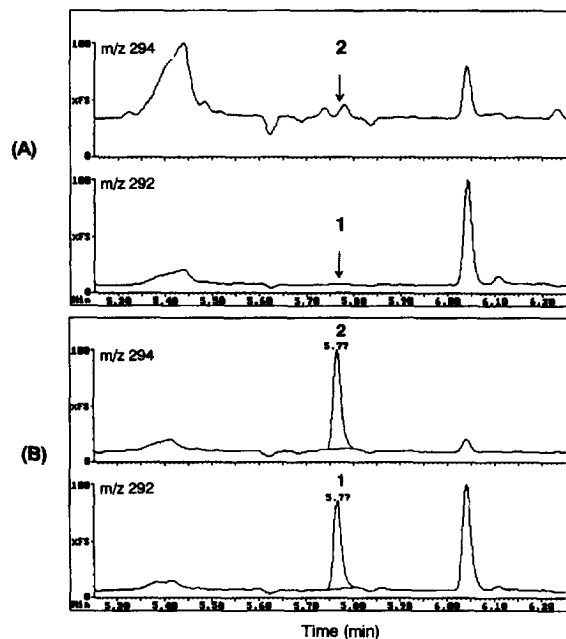
In plasma, the limit of quantitation (LOQ) was 10 ng/ml for FT, 1 ng/ml for 5-FU, 2 ng/ml for CDHP, and 1 ng/ml for Oxo. In urine, the LOQ was ten times the concentration of plasma.

3.5. Precision and accuracy

Between-day repeatability in human plasma and diluted urine was assessed by performing replicate analyses of spiked samples against a calibration curve. The procedure was repeated for different days on the same spiked samples at high, middle and low concentrations (3000, 300 and 30 ng/ml for FT in plasma; 300, 30 and 3 ng/ml for 5-FU in plasma; 600, 60 and 6 ng/ml for CDHP in plasma; 150, 30 and 3 ng/ml for Oxo in plasma; 30 000, 3000 and 300 ng/ml for FT in urine; 3000, 300 and 30 ng/ml for 5-FU in urine; 6000, 600 and 60 ng/ml for CDHP in urine; 1500, 300 and 30 ng/ml for Oxo in urine). Within-day repeatability was determined by treating spiked plasma samples and spiked urine

Table 1
Inter-assay linearity[a]

Sample	Compound	Concentration range (ng/ml)	Coefficient of the linear regression analysis (mean±S.D.)	Slope (mean±S.D.) (ml/ng×10⁻³)	Intercept (mean±S.D.) (×10⁻³)
Human plasma	FT	10–4000	0.9998±0.00024	0.3040±0.0038	0.2733±0.1698
	5-FU	1–400	0.9992±0.00011	13.53±0.16	4.46±2.20
	CDHP	2–800	0.9997±0.00009	7.04±0.04	0.75±1.53
	Oxo	1–200	0.9983±0.00074	40.56±0.61	11.93±7.76
Human urine[b]	FT	10–4000	0.9998±0.00012	0.2992±0.0025	0.3472±0.3908
	5-FU	1–400	0.9991±0.00029	13.61±0.50	3.50±0.67
	CDHP	2–800	0.9997±0.00012	6.88±0.09	4.40±3.75
	Oxo	1–200	0.9988±0.00043	39.10±1.31	24.65±9.88

[a] Calibration standards were prepared for five days.
[b] Human urine was diluted with water (1:10).

samples in replicate the same day. The accuracy, expressed as percent deviation of the observed concentration from the theoretical concentration, with the relative error, was evaluated.

The results for accuracy, within-day and between-day repeatability are presented in Table 2 Table 3. The relative error was less than ±10% for plasma and urine samples except at the LOQ, where it did not exceed ±20%. The precision around the mean value was less than 10% of the coefficient of variation (C.V.) for plasma and urine samples except at the LOQ, where it did not exceed 15%. The dilution method was confirmed to determine the concentration over the calibration range. Plasma spiked analyte above the highest calibration range was diluted with water to twenty-fold of volumes, then determined. The relative error was less than ±10% and the C.V. was less than 10% (Table 2).

3.6. Stability studies

The stability of FT, 5-FU, CDHP and Oxo in plasma and urine were studied. Spiked plasma and urine samples were placed in polypropylene tubes in freezer storage at −20°C and −80°C.

In plasma and urine at −80°C, FT, 5-FU, CDHP and Oxo were stable during a three month period. The concentration of storage sample compared with the initial concentration showed a relative error of <15%. However, CDHP was not stable over a period of two months at −20°C; the concentration in

plasma and urine decreased to 71% and 76%, respectively, compared with the initial concentration. The concentration of each QC sample after freeze-thaw was not changed. The influence of FT, presented in the clinical sample after the administration of S-1, on the concentration of 5-FU was evaluated. FT was not converted into 5-FU during all preparation steps or during storage at 5°C for an 8-h period.

All stock solutions of standards and internal standards were stable at 5°C during the three-month period.
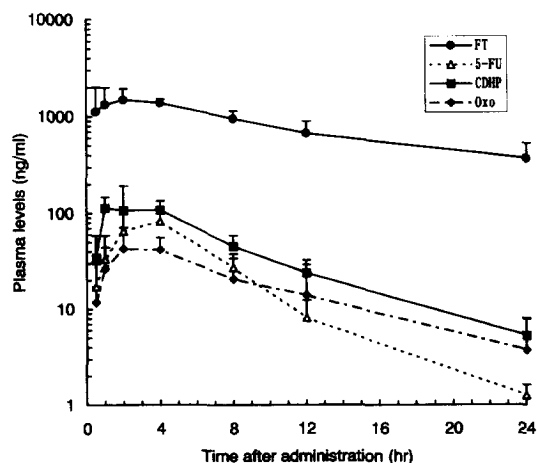

Fig. 7. Plasma levels of FT, 5-FU, CDHP and Oxo after single administration of S-1 (50 mg/body, as FT) to four patients. Each point represents the mean±S.D.

Table 2
Intra-assay precision and accuracy

Compound	Theoretical concentraion (ng/ml)	Experimental concentration (mean ± S.D.)[a] (ng/ml)	C.V. (%)	Mean relative error (%)
Human plasma				
FT	30 000[b]	31 915 ± 1502	4.7	6.4
	3000	2825 ± 58	2.0	−5.8
	300	282.6 ± 6.5	2.3	−5.8
	30	27.6 ± 2.5	8.9	−7.9
	10 (LOQ)	10.6 ± 1.4	12.8	6.5
5-FU	3000[b]	3213 ± 136	4.2	7.1
	300	275.8 ± 4.4	1.6	−8.1
	30	29.18 ± 1.02	3.5	−2.7
	3	2.93 ± 0.08	2.8	−2.2
	1 (LOQ)	0.97 ± 0.06	6.0	−3.5
CDHP	6000[b]	6483 ± 309	4.8	8.1
	600	567.7 ± 7.7	1.4	−5.4
	60	58.83 ± 2.62	4.5	−2.0
	6	5.71 ± 0.13	2.3	−4.8
	2 (LOQ)	2.22 ± 0.26	11.9	11.1
Oxo	1500[b]	1515 ± 31	2.1	1.0
	150	137.8 ± 1.0	0.7	−8.1
	30	29.89 ± 0.44	1.5	−0.4
	3	3.12 ± 0.04	1.3	3.9
	1 (LOQ)	1.05 ± 0.08	7.5	5.1
Human urine				
FT	30 000	30 651 ± 1412	4.6	2.2
	3000	3245 ± 80	2.5	8.2
	300	276.0 ± 8.5	3.1	−8.0
	100 (LOQ)	95.8 ± 11.1	11.6	−4.2
5-FU	3000	2940 ± 85	2.9	−2.0
	300	321.4 ± 9.9	3.1	7.1
	30	29.3 ± 2.1	7.3	−2.4
	10 (LOQ)	11.7 ± 0.7	5.9	16.9
CDHP	6000	6420 ± 414	6.5	7.0
	600	643.0 ± 11.6	1.8	7.2
	60	60.0 ± 3.2	5.4	0.1
	20 (LOQ)	20.4 ± 1.9	9.2	2.1
Oxo	1500	1355 ± 46	3.4	−9.7
	300	298.6 ± 7.8	2.6	−0.5
	30	29.3 ± 0.5	1.6	−2.4
	10 (LOQ)	10.9 ± 0.9	8.6	8.8

[a] Mean ± S.D. of five determinations.
[b] Plasma samples were diluted with water to twenty-fold of volumes.

3.7. Application in pharmacokinetics study

The mean concentration versus time curves for FT, 5-FU,CDHP and Oxo in plasma after a single oral administration of S-1 (50 mg/body, as FT) to four patients with solid tumor are presented in Fig. 7. The maximum concentrations of FT, 5-FU, CDHP and Oxo were 1.693 ± 0.455 µg/ml, 83.1 ± 35.1 ng/ml, 129.3 ± 60.1 ng/ml and 48.0 ± 20.4 ng/ml, respectively. The detailed results of this phar-

Table 3
Inter-assay precision and accuracy

Compound	Theoretical concentration (ng/ml)	Experimental concentration (mean±S.D.)[a] (ng/ml)	C.V. (%)	Mean relative error (%)
Human plasma				
FT	3000	2892±65	2.3	−3.6
	300	290.0±9.6	3.3	−3.4
	30	29.6±3.1	10.5	−1.4
5-FU	300	284.1±6.9	2.4	−5.3
	30	30.37±0.81	2.7	1.2
	3	3.10±0.25	8.1	3.3
CDHP	600	577.1±8.3	1.4	−3.8
	60	60.97±1.54	2.5	1.6
	6	5.95±0.24	4.1	−0.9
Oxo	150	142.5±3.7	2.6	−5.0
	30	30.96±0.39	1.2	3.2
	3	3.09±0.06	2.0	2.9
Human urine				
FT	30 000	30 092±1470	4.9	0.3
	3000	3041±174	5.7	1.4
	300	306.0±24.2	7.9	2.0
5-FU	3000	2865±108	3.8	4.5
	300	312.1±18.3	5.9	4.1
	30	31.0±1.9	6.1	3.4
CDHP	6000	5907±330	5.6	−1.6
	600	619.6±41.0	6.6	3.3
	60	61.9±3.8	6.1	3.1
Oxo	1500	1405±27	1.9	−6.4
	300	305.6±3.7	1.2	1.9
	30	32.4±2.4	7.3	7.9

[a] Mean±S.D. of five determinations.

macokinetic study of S-1 will be reported elsewhere.

By the fractionation of FT from 5-FU and CDHP with liquid–liquid extraction, these substances were analyzed by using the most suitable methods, which were simple HPLC and highly sensitive GC–NICI-MS. Furthermore, the fractionation method required only 0.75 ml of plasma for the determination of all analytes. The performance of these assays proved to be excellent during the course of the phar-macokinetic study in humans.

References

[1] K. Tatsumi, M. Fukushima, T. Shirasaka and S. Fujii, Jpn. J. Cancer Res., 78 (1987) 748.

[2] J. Uchida, T. Takechi, K. Nakano, S. Takeda, M. Fukushima, N. Unemi and T. Shirasaka, Proceedings of 86th Annual Meeting of American Association for Cancer Research, Vol. 36, Toronto, March 1995, p. 406.

[3] T. Shirasaka, Y. Shimamoto and M. Fukushima, Cancer Res., 53 (1993) 4004.

[4] D.B. Lakings and R.H. Adamson, J. Chromatogr., 146 (1978) 512.

[5] T. Marunaka and Y. Umeno, J. Chromatogr., 221 (1980) 382.

[6] T. Marunaka and Y. Umeno, K. Yoshida, M. Nagamachi, Y. Minami and S. Fujii, J. Pharm. Sci., 69 (1980) 1296.

[7] M. Kubo, H. Sasabe and T. Shimizu, J. Chromatogr., 564 (1991) 137.

[8] C. Aubert, C. Luccioni, P. Coassolo, J.P. Sommadossi and J.P. Cano, Arzneim. Forsch., 31 (1981) 2048.

[9] H. Isomura, S. Higuchi and S. Kawamura, J. Chromatogr., 224 (1981) 423.

[10] H. Odagiri, S. Ichihara, E. Semura, M. Utoh, M. Tateishi and I. Kuruma, J. Pharmacobio-Dyn., 11 (1988) 234.

[11] R.M. Kok, A.P.J.M. De Jong, C.J. Van Groeningen, G.J. Peters and J. Lankelma, J. Chromatogr., 343 (1985) 59.

[12] L.W. Anderson, R.J. Parker, J.M. Collins, J.D. Ahlgren, D. Wilkinson and J.M. Strong, J. Chromatogr., 581 (1992) 195.

[13] C.D. Bates, D.G. Watson, N. Willmott, H. Logan and J. Golberg, J. Pharm. Biomed. Anal., 9 (1991) 19.

[14] R. Kitamura, T. Satoh, M. Maeda and A. Tsuji, Yakugaku Zasshi, 114 (1994) 171.