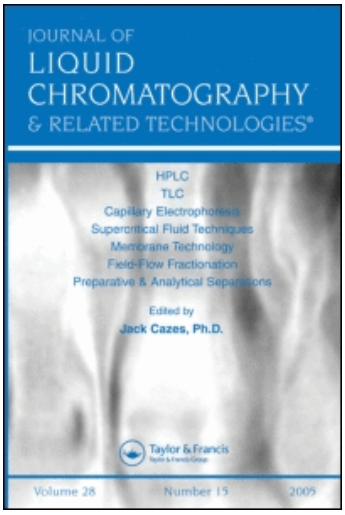

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A LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR THE SIMULTANEOUS DETERMINATION OF CAPECITABINE, 5'-DEOXY-5-FLUOROCYTIDINE, 5'-DEOXY-5-FLUOROURIDINE, 5-FLUOROURACIL, AND 5-FLUORODIHYDROURACIL IN HUMAN PLASMA

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A LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR THE SIMULTANEOUS DETERMINATION OF CAPECITABINE, 5′-DEOXY-5-FLUOROCYTIDINE, 5′-DEOXY-5-FLUOROURIDINE, 5-FLUOROURACIL, AND 5-FLUORODIHYDROURACIL IN HUMAN PLASMA

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□ *The objective of the present study was to develop a selective and sensitive liquid chromatography-mass spectrometry (LC-MS) method for the simultaneous quantitation of capecitabine, 5′-deoxy-5-fluorocytidine (5′-DFCR), 5′-deoxy-5-fluorouridine (5′-DFUR), 5-fluorouracil (5-FU), and 5-fluorodihydrouracil (5-FUH$_2$) in human plasma. Chromatography was performed on an Atlantis dC18 column with a mobile phase consisting of 1% formic acid in acetonitrile and 1% formic acid in water gradient elution. 5-fluorocytosine (5-FC) was used as internal standard. LC-MS data were acquired in SIM mode at m/z 130 for 5-FC, m/z 131 for 5-FU, m/z 133 for 5-FUH$_2$, m/z 246 for 5′-DFCR, m/z 247 for 5′-DFUR, and m/z 360 for capecitabine. The drug/internal standard peak area ratios were linked via quadratic relationships to concentrations (100–10000 μg/L for 5-FU; 50–10000 μg/L for 5-FUH$_2$; and 25–10000 μg/L for 5′-DFCR, 5′-DFUR, and capecitabine). The analysis of blank matrices from different donors showed the absence of interfering endogenous components at the retention times of the analytes. No evidence of matrix effect was observed. The method was precise (precision, 0.2–8.3%) and accurate (recovery, 99–104%). Mean extraction efficiencies >89% for each analyte were obtained. The lower limits of quantitation were 25 μg/L for capecitabine, 5′-DFCR, and 5′-DFUR; 50 μg/L for 5-FUH$_2$, and 100 μg/L for 5-FU. This method was successfully used to investigate plasma concentrations of capecitabine and its metabolites in a pharmacokinetic study carried out in patients with metastatic solid tumors receiving oral administration of capecitabine (1600 to 3420 mg according to the patient) twice a day.*

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Keywords 5′-deoxy-5-fluorocytidine, 5′-deoxy-5-fluorouridine, 5-fluorodihydrouracil, 5-fluorouracil, capecitabine, LC-ESI-MS quantitation

INTRODUCTION

Capecitabine (Xeloda®) is an orally-administered tumor-selective fluoropyrimidine used in the treatment of metastatic breast and colorectal cancers.[1] Capecitabine is a prodrug, that is enzymatically converted to 5-fluorouracil (5-FU) in the tumor, where it inhibits DNA synthesis and slows growth of tumor tissue.[2] Capecitabine is metabolized primarily in the liver by a carboxylesterase to 5′-deoxy-5-fluorocytidine (5′-DFCR). The latter is then converted to 5′-deoxy-5-fluorouridine (5′-DFUR) by cytidine deaminase. Finally, 5′-DFUR is converted into 5-FU by thymidine phosphorilase, which is present at high concentrations in tumor tissues.[3] 5-FU is enzymatically cleared from plasma to produce 5-fluorodihydrouracil (5-FUH_2), which is the rate-limiting step catalyzed by dihydropyrimidine dehydrogenase (DPD).[4]

Even though capecitabine is generally well tolerated, some patients can develop severe diarrhea due to the release of 5′-DFUR within the small intestine.[5] Moreover, hand-foot syndrome has proven to be a chronic dose-limiting toxicity of capecitabine, leading to significant morbidity in patients receiving this drug.[6,7] Thus, a sensitive and accurate bioanalytical method to analyze capecitabine and its metabolites is required for monitoring chemotherapy treatment in cancer patients.

Several liquid chromatography (LC) methods with ultra-violet (UV),[8] mass spectrometry,[9,10] or tandem mass spectrometry[11–13] detection have been developed over the last years for the analysis of capecitabine and its metabolites in human plasma. In most of them, both capecitabine and 5-FU were quantified. Other methods have been developed to quantify capecitabine and its metabolites in animal matrices.[10,14] Zufía et al.[8] reported an LC method with UV detection allowing the simultaneous quantitation of capecitabine, 5′-DFUR, 5-FU, and 5-FUH_2. However, this method required tedious extraction and purification steps, and long analysis times. Likewise, Licea-Perez et al.[12] validated an LC-MS/MS method for the determination of capecitabine, 5-FU, and α-fluoro-β-alanine in human plasma. Different liquid-liquid extraction steps followed by derivatization of 5-FU and capecitabine were performed. Xu et al.[9] described an on-line sample clean-up procedure method to simultaneously quantify capecitabine, 5′-DFCR, and 5′-DFUR in human plasma. But, using this method, it was not possible to quantify 5-FU. Siethoff et al.[11] published an LC-MS/MS assay to only quantify capecitabine and 5-FU. The sample pretreatment procedure involved

column switching techniques. Recently, Vainchtein et al.[13] developed a LC-MS/MS method for the simultaneous determination of capecitabine and the following four metabolites, 5′-DFCR, 5′-DFUR, 5-FU, and 5-FUH_2 in human plasma. These authors used two internal standards: fludarabine and 5-chloro uracil; the sample pretreatment procedure consisted in a simple single-step protein precipitation using 10% trifluoroacetic acid in water. However, it was not possible to replicate their method in LC-MS due to the presence of interfering peaks at the retention times of the analytes.

The objective of our work was to develop a selective and sensitive LC-MS method for the simultaneous quantitation of capecitabine, 5′-DFCR, 5′-DFUR, 5-FU, and 5-FUH_2 in human plasma. Sample handling and chromatographic conditions were optimized to provide fast quantitative results while maintaining the specificity, sensitivity, accuracy, and precision required for pharmacokinetic studies and therapeutic drug monitoring. The sample pretreatment procedure involved liquid-liquid extraction; 5-fluorocytosine (5-FC) was used as internal standard. The method was fully validated according to the FDA guidelines on bioanalytical validation;[15] it was used to quantify these drugs in plasma samples from patients receiving capecitabine.

EXPERIMENTAL

Materials and Reagents

Capecitabine and 5′-DFCR reference standards were generously provided by Hoffmann-La Roche (Basel, Suisse). 5-FUH_2 was obtained from Toronto Research Chemicals (North York, Canada). 5′-DFUR, 5-FC (IS) and formic acid were purchased from Sigma-Aldrich (Lyon, France). 5-FU and high purity acetonitrile were from Merck (Darmstadt, Germany); trifluoracetic acid, ethyl acetate and methanol were obtained from Prolabo (Fontenay sous Bois, France). All reagents were of HPLC grade or equivalent purity.

Ultra-high quality water was obtained from a Milli-Q water-purifying system (Millipore, Bedford, MA, USA).

For the validation of the method, drug-free Caucasian human plasma samples (6 different batches from 6 different pools of donors) were obtained from the Etablissement Français du Sang (Montpellier, France). These plasma pools were obtained from blood collected on lithium heparinate to prevent coagulation. Blank matrices were aliquoted and stored at −20°C until used. The same batch was used during the study for the preparation of calibrators and quality control (QC) samples. The other batches

were used to study the selectivity/specificity of the method and to assess the matrix effect.

Stock solutions of 5-FU, 5′-DFCR, 5′-DFUR, capecitabine, and 5-FC were individually prepared in purified water, stock solution of 5-FUH_2 was prepared in methanol. All stock solutions at a target concentration of 1 g/L were stored at -20°C. They were stable under conditions of daily use for at least 1 month (data not shown). Stock solutions were further diluted daily with purified water (methanol for 5-FUH_2) to obtain working solutions (from 1 to 150 mg/L). They were used to prepare calibration curves and quality control (QC) samples. Two different stock solutions were used to prepare calibrators and QC samples.

Reference solutions containing 5-FU, 5-FUH_2, 5′-DFCR, 5′-DFUR, capecitabine (250, 750, 7500 μg/L), and 5-FC (7500 μg/L) were prepared daily in purified water to check the performance of the LC-MS system.

Instrumentation and Chromatography

LC-MS experiments were carried on a Hewlett Packard Agilent 1100 quadrupole mass spectrometer (Agilent Technologies, Les Ulis, France) with an electrospray ionization source (ESI) operated in the positive ionization mode. Optimization of various experimental parameters including nature of the stationary phase, composition of the eluent, nature of the organic modifier, capillary voltage, nebulizer pressure, and sampling cone voltage was carried out (data not shown).

An LC system from Agilent Technologies, consisting of a 1100 series quaternary pumping unit, a degasser and an autosampler was used. Data were acquired and processed with HPChem software (version 08.04) from Agilent Technologies. The analytical column was an Atlantis dC18 column (5 μm, 4.6 × 100 mm, Waters, Milford, MA, USA). The column temperature was maintained at 20°C and the sample compartment was maintained at 4°C. Mobile phase A consisted of 1% (v/v) of formic acid in water and mobile phase B was 1% (v/v) formic acid in acetonitrile. The mobile phases were deaerated ultrasonically before use and with a stream of helium during use. Table 1 shows the variations in proportions of solvents A and B. The total run time was 15 min and the flow rate was maintained constant at 0.6 mL/min throughout the run. An injection volume of 10 μL in a 100 μL loop was used.

The mass spectrometer was tuned with the procedures provided by Agilent Technologies to give a maximum response for m/z 118, 230, 508, and 997. The instrument was optimized by infusing a 10 mg/L solution of all analytes in the mobile phase (95%water-5%acetonitrile containing 1% formic acid) at 600 μL/min through the Agilent pump directly connected

TABLE 1 Gradient Elution Profile

Time (min)	Solvent A (%)	Solvent B (%)
0.0	95	5
2.0	95	5
8.0	0	100
9.0	0	100
9.1	95	5
15.0	95	5

to the mass spectrometer. Voltages were set at +3.0 kV for the capillary and +0.5 kV for the skimmer lens. Main spectrometer parameters were as follows: source temperature, 100°C; quadrupole temperature, 99°C; nebulizing gas (nitrogen), 0.25 MPa; drying gas (nitrogen), 720 L/h. During all experiments, mass spectra were obtained by scanning from m/z 100 to m/z 500. Single-ion-monitoring (SIM) mode was used for sample quantitation by sequentially monitoring the protonated molecules $(M+H)^+$, of each analyte: m/z 130 for 5-FC, m/z 131 for 5-FU, m/z 133 for 5-FUH_2, m/z 246 for 5′-DFCR, m/z 247 for 5′-DFUR, and m/z 360 for capecitabine (Figure 1). Data acquisition was carried out with a dwell time of 95 ms. The sampling cone voltages were 180, 80, 140, 35, 80, and 60 V, respectively.

Preparation of Standards and QC Samples

Calibration standards containing capecitabine and metabolites were prepared freshly by adding appropriate volumes of working solutions into 0.5 mL of plasma at the concentrations of 100, 500, 1000, 5000, and 10000 μg/L for 5-FU; 50, 100, 500, 1000, 5000, and 10000 μg/L for 5-FUH_2; and 25, 50, 100, 500, 1000, 5000, and 10000 μg/L for 5′-DFCR, 5′-DFUR and capecitabine. The calibration standards were vortex-mixed for approximately 30 s before processing.

QC samples were independently prepared as aforementioned to yield concentrations of 250, 750, and 7500 μg/L for all analytes. For 5′-DFCR, 5′-DFUR, and capecitabine an additional QC sample was prepared at 75 μg/L.

The percentage of non-matrix solvent used to spike calibration standards and QC samples were less than 5% (v/v).

Sample Pretreatment Procedure

A 25 μL aliquot of IS working solution (150 mg/L) was added to 0.5 mL sample aliquots and vortex mixed for 10 s. Plasma proteins were then precipitated with 0.5 mL 1% (v/v) TFA in water; after vortex mixing for a

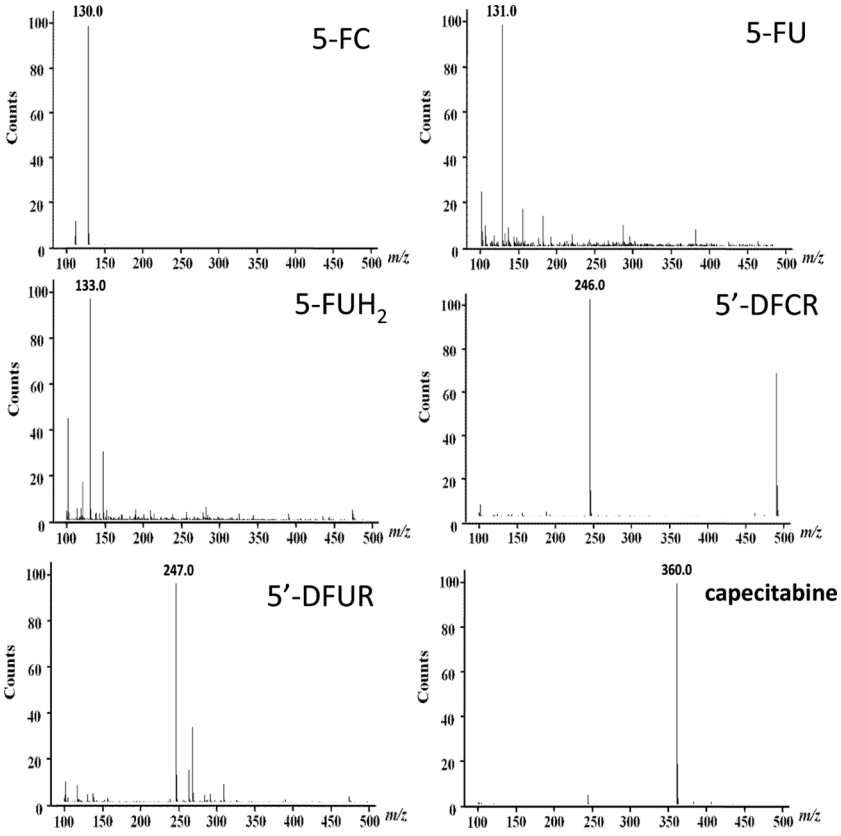

FIGURE 1 Mass spectra (scan mode), 5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil, 5-FUH_2, 5-fluorodihydrouracil.

few seconds, the mixture was centrifuged at 16000 × *g* for 10 min (+4°C). The supernatant was transferred to a glass tube and 5 mL of ethyl acetate/methanol (95:5, v/v) were added as extraction solvent. The sample was vortex mixed again for 5 s, shaken for 5 min and then centrifuged at 16000 × g for 10 min at +4°C to separate aqueous and organic layers. The organic phase was transferred to an empty glass tube and evaporated to dryness at 45°C under nitrogen stream for 30 min. The dried extract was re-dissolved in 100 µL of water containing 1% formic acid (v/v) and transferred to an injection vial.

Data Analysis

Quantitation utilized an analyte to internal standard area ratio. To link peak area ratios and theoretical concentrations of each analyte, different

models were tested: 1) unweighted or weighted least-squares linear regression analysis ($Y = aX + b$) and 2) quadratic relationship ($Y = aX^2 + bX + c$) with Y = peak area ratio and X = nominal concentration. The regression curve was not forced through zero. The resulting equation parameters were used to back-calculate concentrations for the calibrators that were statistically evaluated.

Matrix Effect

For the determination of the matrix effect (i.e., suppression or enhancement of ionization of analytes by the presence of matrix components),[16] six different pools of control drug-free human plasma samples were processed according the procedure described previously. The dried extract was re-dissolved in 100 µL of 1% formic acid in water spiked with solutions of the capecitabine and its metabolites to yield the same concentrations as in the processed QC samples. The unprocessed samples were prepared in water containing 1% formic acid at the same nominal concentrations. Matrix effect was defined as a ratio of the analyte peak response in the presence of matrix ions to the analyte peak response in the absence of matrix ions. Each determination was replicated 3-fold. Matrix factor values between 0.85–1.15 were judged acceptable.

Validation

Specificity

For the determination of the specificity of the analytical method, chromatograms from human drug-free plasma samples (six different pools) were examined for visible evidence of interference by endogenous compounds with the compounds of interest.

Plasma samples from patients receiving other drugs (irinotecan, ondansetron, metoclopramide, racecadotril, paracetamol, nonsteroidal anti-inflammatory drugs, and corticosteroids) were analyzed for interference.

Precision and Accuracy

The intra-assay and inter-assay precision and accuracy of the method were validated by analyzing QC samples against a calibration curve. Determinations were performed with six replicates per QC on the same day as well as each day for 7 separate days. The precision of the method at each concentration was calculated as the relative standard deviation of the mean (R.S.D.).

The accuracy of the procedure was determined as the relative mean error (RME) using the following equation: RME = [mean found concentration/theoretical spiked concentration] × 100.

Extraction Efficiency

The absolute recoveries of capecitabine, 5′-DFCR, 5′-DFUR, 5-FU, and 5-FUH_2 from plasma were evaluated based on the comparison of the peak areas of extracted QC samples with those obtained by direct injections of the same amount of compounds (n = 3 per concentration) dissolved in water containing 1% formic acid. The extraction efficiency of the internal standard was also calculated.

Determination of the Lower Limit of Quantitation (LLOQ)

The LLOQ estimated on QC samples was defined as the lowest drug concentration that could be determined with a precision ≤20% and an accuracy of 80–120%.

Application to Clinical Pharmacokinetic Study

This overall analytical procedure has been used to determine concentrations of capecitabine and its metabolites in plasma samples from patients aged 18–75 years old with metastatic solid tumor. The study protocol was reviewed and approved by the institutional review board. Capecitabine was administered orally at a dose of 1600 to 3420 mg, twice a day combined with irinotecan, 180 mg/m^2, and for 10 patients combined with oxaliplatin, 85 mg/m^2. Pharmacokinetic analysis was carried out using the Pk-fit software.[17,18] Pharmacokinetic parameters were determined from the plasma concentration-time data using a compartmental approach and included the total area under the plasma concentration-time curve (AUC), elimination half-life ($t_{1/2}$), lag-time (t_{lag}), total clearance (CL/F), and volume of distribution (V/F). CL and V were uncorrected for bioavailability (F).

RESULTS

Method Development

The objective of the present study was to develop a rugged, specific, and sensitive method allowing simultaneous determination of capecitabine, 5′-DFCR, 5′-DFUR, 5-FU, and 5-FUH_2 in human plasma. The major problem encountered during method development was the significant differences in polarity between the analytes which lead to sample extraction difficulties. Chromatographic conditions were optimized to obtain sharp

peak shape with adequate response. This included column type (C8, C18, phenyl, Shield RP18 or Atlantis dC18), composition of the mobile phase, pH of buffer solution, and flow rate. The presence of organic acid (formic, acetic or trifluoroacetic acid) or buffer (formate or acetate) in the mobile phase was evaluated. Best separations of the analytes and relative short analysis time were obtained on a Atlantis dC18 using a mobile phase consisting of eluent A, 1% of formic acid in water and eluent B, 1% formic acid in acetonitrile. The sample pretreatment was also optimized, both liquid-liquid and solid phase extractions were tested. The best results were obtained using liquid-liquid extraction by ethyl acetate containing 5% methanol after protein precipitation by 1% TFA in water.

Retention Times and Specificity

The representative chromatograms in Figure 2 show drug-free human plasma, drug-free human plasma spiked with the five analytes at the LLOQ

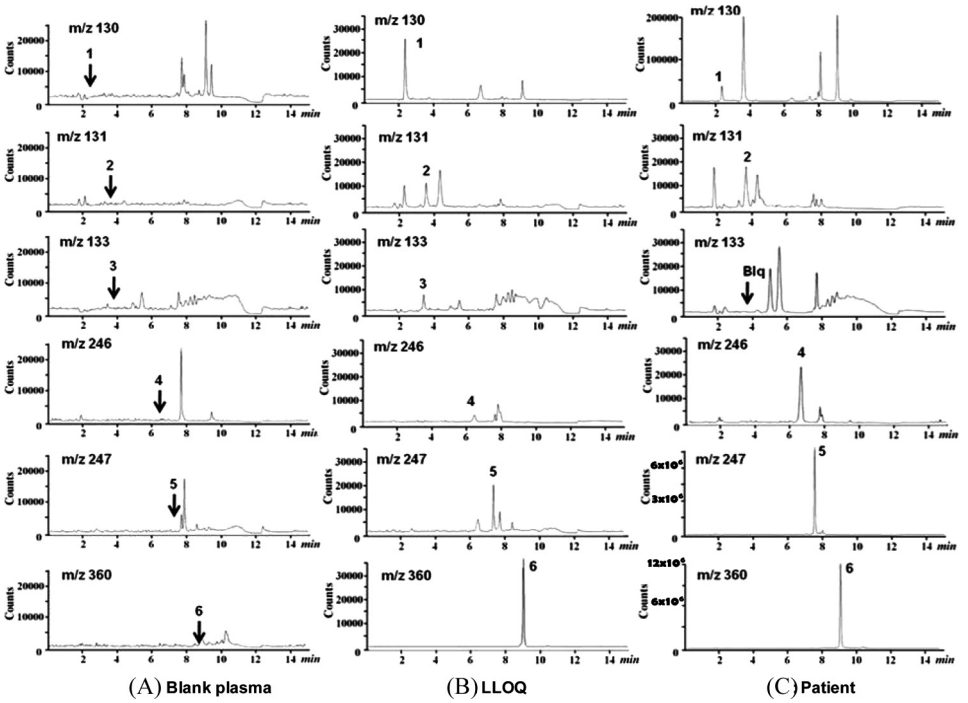

FIGURE 2 Typical chromatograms of blank plasma (A); plasma spiked with the five analytes at the LLOQ (B); and plasma drawn 1 h after capecitabine administration (2500 mg) (C): 5-FU 150 μg/L, 5′-DFCR 293 μg/L, 5′-DFUR μg/L, and capecitabine 8904 μg/L, Peak 1: internal standard; Peak 2: 5-fluorouracil (5-FU); Peak 3: 5-fluorodihydrouracil (5-FUH_2); Peak 4: 5′-deoxy-5-fluorocytidine (5′-DFCR); Peak 5: 5′-deoxy-5-fluorouridine (5′-DFUR); Peak 6: capecitabine.

and human plasma obtained 1 h after oral administration of capecitabine (2500 mg). The retention times of capecitabine, 5′-DFCR, 5′-DFUR, 5-FU, 5-FUH_2, and internal standard were 9.0, 6.5, 7.5, 3.5, 3.3, and 2.3 min, respectively (CVs, 0.6–2.2%, n = 20). The calibration curves consisted of 5–7 calibration points.

The six different lots of human plasma, commercially procured, were chromatographically screened for interfering substances. No interferences at the retention times of the analytes were observed (Figure 2A). The tested drugs do not interfere with any of the analytes in the described assay.

Drug/Detector Response Relationship

For all analytes, quadratic calibration curves gave the best fit based on the statistical analysis results[19] and precision and recovery of QC samples. CV values on the slope b were <14% (Table 2). Mean back-calculated concentrations are summarized in Table 3. For each analyte, the goodness of fit between back-calculated concentrations and nominal concentrations was statistically verified (Student's *t*-test). The mean relative predictor error calculated from the difference between back-calculated and nominal concentrations was not statistically different from zero.

Matrix Effect, Precision, Accuracy, Extraction Efficiency, and LLOQ

No evidence of matrix effect was observed at the retention times of the analytes (Table 4); the matrix factor ranged from 85 to 115%. The

TABLE 2 Mean Values of Quadratic Regression Parameters[a]

Reproducibility	r^2 (Mean ± S.D.)	a (Mean ± S.D.)	b (Mean ± S.D.)	c (Mean ± S.D.)
Intra-assay (n = 6)				
5-FU	0.992 ± 0.004	−0.104 ± 0.078	0.175 ± 0.024	0.002 ± 0.001
5-FUH_2	0.997 ± 0.003	−0.074 ± 0.080	0.345 ± 0.024	0.012 ± 0.019
5′-DFCR	0.999 ± 0.001	−0.549 ± 0.161	1.76 ± 0.224	−0.001 ± 0.002
5′-DFUR	0.999 ± 0.001	−0.067 ± 0.049	0.612 ± 0.048	0.015 ± 0.028
Capecitabine	0.998 ± 0.003	0.260 ± 0.040	11.0 ± 0.831	0.226 ± 0.404
Inter-assay (n = 7)				
5-FU	0.992 ± 0.011	−0.143 ± 0.064	0.233 ± 0.016	0.002 ± 0.002
5-FUH_2	0.997 ± 0.002	−0.095 ± 0.034	0.386 ± 0.031	0.002 ± 0.001
5′-DFCR	0.996 ± 0.002	−0.357 ± 0.220	1.65 ± 0.114	−0.001 ± 0.003
5′-DFUR	0.998 ± 0.001	−0.060 ± 0.069	0.652 ± 0.053	0.042 ± 0.053
Capecitabine	0.998 ± 0.003	0.220 ± 0.084	11.4 ± 1.27	0.131 ± 0.309

[a]Quadratic unweighted regression, formula: $y = ax^2 + bx + c$.

r^2: determination coefficient; n: number of replicates; S.D., standard deviation.

5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil, 5-FUH_2, 5-fluorodihydrouracil.

TABLE 3 Relative Standard Deviation (R.S.D.) and Recoveries Calculated from Mean Back-Calculated Concentrations (Calibration Curves)

	25 µg/L		50 µg/L		100 µg/L		500 µg/L		1000 µg/L		5000 µg/L		10000 µg/L	
	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)	R.S.D. (%)	Recovery (%)
Intra-assay (n = 6)														
5-FU	–	–	–	–	3.8	101.0	4.0	93.0	3.6	104.0	5.40	96.0	7.70	97.2
5-FUH_2	–	–	6.5	99.9	7.0	103.0	8.1	102.0	5.3	94.9	2.50	98.3	1.80	99.1
5′-DFCR	5.1	100.0	5.5	105.0	6.7	103.0	8.0	102.0	6.5	101.0	3.00	98.8	1.80	101.0
5′-DFUR	3.8	102.0	8.0	101.0	2.3	104.0	6.2	104.0	4.8	99.0	0.67	99.6	1.10	101.0
Capecitabine	6.3	95.7	6.7	101.0	7.8	97.9	2.2	104.0	1.5	102.0	0.95	99.4	0.83	102.0
Inter-assay (n = 7)														
5-FU	–	–	–	–	6.0	102.0	3.9	103.0	3.2	104.0	6.00	95.5	10.0	99.8
5-FUH_2	–	–	11.1	97.8	5.9	99.8	11.2	103.0	7.0	100.0	6.00	103.0	2.50	98.8
5′-DFCR	3.1	102.0	8.9	102.0	8.0	98.4	11.5	103.0	7.3	97.6	5.70	102.0	2.30	100.0
5′-DFUR	9.9	92.9	8.8	105.0	9.0	98.4	7.2	99.3	5.8	98.4	1.50	101.0	0.48	99.9
Capecitabine	9.4	103.0	8.4	104.0	9.7	103.0	7.4	100.0	8.3	102.0	5.00	96.8	5.50	100.0

n: number of replicates; 5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil, 5-FUH_2, 5-fluorodihydrouracil.

TABLE 4 Matrix Effect

	250 μg/L		750 μg/L		7500 μg/L	
n = 6	Mean ± S.D.	Range	Mean ± S.D.	Range	Mean ± S.D.	Range
5-FU	0.99 ± 0.03	0.97–1.05	0.90 ± 0.03	0.87–0.94	0.91 ± 0.07	0.87–1.03
5-FUH_2	0.93 ± 0.05	0.87–1.01	1.02 ± 0.10	0.88–1.15	0.89 ± 0.04	0.88–0.94
5′-DFCR	1.12 ± 0.03	1.08–1.15	1.10 ± 0.05	1.05–1.15	1.05 ± 0.05	0.98–1.11
5′-DFUR	1.02 ± 0.04	0.97–1.07	1.11 ± 0.04	1.04–1.15	0.94 ± 0.04	0.87–0.98
Capecitabine	1.02 ± 0.07	0.91–1.07	1.00 ± 0.09	0.89–1.14	0.93 ± 0.08	0.85–1.03

n: number of different matrices studied; S.D., standard deviation; 5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil, 5-FUH_2, 5-fluorodihydrouracil.

Each value is the mean from 3 replicates in each batch of matrix.

intra-assay and inter-assay precision and accuracy results are presented in Table 5; precision ranged from 0.2 to 8.3% and accuracy was 99–104%. Values were within acceptable limits.[15,16,19]

Mean extraction recoveries were 98.4% (R.S.D., 4.8%) for capecitabine, 94.9% (R.S.D., 5.5%) for 5′-DFCR, 94.2% (R.S.D., 6.7%) for 5′-DFUR, 89.7% (R.S.D., 7.9%) for 5-FU, 91.8% (R.S.D., 8.1%) for 5-FUH_2, and 93.5% (R.S.D., 5.4%) for the internal standard. They were not statistically different over the range of concentrations studied.

The LLOQs were 25 μg/L for capecitabine, 5′-DFCR, and 5′-DFUR; 50 μg/L for 5-FUH_2; and 100 μg/L for 5-FU.

TABLE 5 Intra-Assay and Inter-Assay Precision and Accuracy of the Method

	75 μg/L		250 μg/L		750 μg/L		7500 μg/L	
	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)	Precision (%)	Accuracy (%)
Intra-assay (n = 6)								
5-FU	–	–	0.6	104	0.7	103.0	0.8	99.3
5-FUH_2	–	–	1.4	102	2.7	100.0	0.8	101.0
5′-DFCR	1.0	104.0	0.6	103	2.4	101.0	0.4	101.0
5′-DFUR	3.7	100.0	0.8	103	3.1	99.5	0.8	102.0
Capecitabine	4.2	99.8	1.1	103	2.4	100.0	0.6	102.0
Inter-assay (n = 7)								
5-FU	–	–	2.8	104	8.0	101.0	0.6	100.0
5-FUH_2	–	–	2.9	104	2.7	102.0	0.2	101.0
5′-DFCR	7.5	101.0	4.9	101	2.0	102.0	0.8	99.8
5′-DFUR	7.0	102.0	5.6	102	2.3	103.0	0.6	101.0
Capecitabine	6.0	102.0	8.3	102	5.3	101.0	0.5	101.0

n: number of replicates; 5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxy-5-fluorouridine; 5-FU, 5-fluorouracil, 5-FUH_2, 5-fluorodihydrouracil.

TABLE 6 Mean ± (S.D.) Pharmacokinetic Parameters of Capecitabine and its Main Metabolites

	Capecitabine	5′-DFCR	5′-DFUR	5-FUH_2
V/F, L	443 ± 888	–	–	–
CL/F, L/h	268 ± 424	–	–	–
$t_{1/2}$, h	1.03 ± 0.66	0.89 ± 0.76	1.12 ± 1.37	0.99 ± 0.38
AUC, mgxh/L	13.0 ± 8.85	4.83 ± 3.84	39.6 ± 28.4	2.43 ± 0.81

S.D., standard deviation; 5′-DFCR, 5′-deoxy-5-fluorocytidine; 5′-DFUR, 5′-deoxy-5-fluorouridine, 5-FUH_2, 5-fluorodihydrouracil.

V, volume of distribution; CL/F total clearance uncorrected for bioavailability (F); $t_{1/2}$, terminal elimination half-life; AUC, area under curve normalized to a 2500 mg administered dose.

Clinical Study

For capecitabine and its main metabolites mean pharmacokinetic parameters are given in Table 6. Mean concentration versus time profiles are presented in Figure 3. The absorption process was rapid ($\sim 4\,h^{-1}$) with a lag time ($\sim$0.4 h). For some patients with deficiency in DPD, 5-FUH_2 concentrations were rapidly below the LLOQ of the method. 5-FU concentrations were only measurable in some patients 1 and 2 h after capecitabine administration; they were near to the LLOQ (i.e., 100 μg/L). At the other sampling times, 5-FU concentrations were below the LLOQ.

DISCUSSION AND CONCLUSION

A LC method with MS detection for the simultaneous determination of capecitabine, 5′-DFCR, 5′-DFUR, 5-FU, and 5-FUH_2 in human plasma was

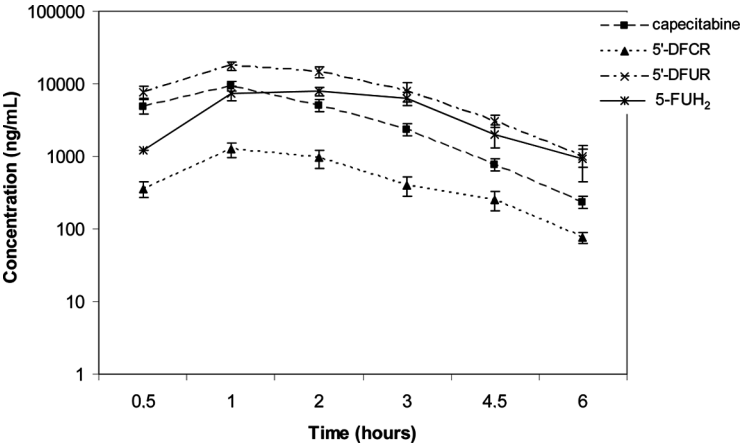

FIGURE 3 Mean (standard deviation) concentration vs time profiles of capecitabine, 5′-DFCR, 5′-DFUR and 5-FUH_2 in patients with metastatic solid tumors.

developed. The assay is accurate and precise. Good recoveries are achieved in a single solvent mixture extraction step. This method was used to analyze plasma samples drawn during a pharmacokinetic study carried out in patients with metastatic solid tumors receiving capecitabine. Compared to the method published by Vainchtein et al.[13] by LC-MS/MS, in the present study, the LLOQs of 25 μg/L for capecitabine, 5′-DFCR, and 5′-DFUR are about two times higher. However, this sensitivity is sufficient to follow concentrations of these three compounds in patients 12 h following capecitabine intake. For 5-FU, it was not possible to decrease the LLOQ below 100 μg/L. Since 5-FU is eluted in the first minutes, the high aqueous content of the mobile phase would impair the desolvation and, hence, the ionization process.

The capecitabine clearance uncorrected for bioavailability, 268 L/h, was lower but in the order of previously reported estimates from non-compartmental analyses, 347 L/h/1.8 m^2,[20] 311 to 378 L/h/1.8 m^2[21], and 315 L/h/1.8 m^2[22] but similar to that reported by Urien et al., 231 L/h/m^2.[23] The capecitabine $t_{1/2}$, 1 h, was also in the range of previous estimates.[21] The elimination of metabolites is clearly rate-limited, that is, apparent half-lives of the three metabolites were close to the capecitabine half-life.

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