Journal of Chromatography B, 913–914 (2013) 30–40

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantitative determination of capecitabine and its six metabolites in human plasma using liquid chromatography coupled to electrospray tandem mass spectrometry

Maarten J. Deenen[a,b], Hilde Rosing[c], Michel J. Hillebrand[c], Jan H.M. Schellens[a,b,d], Jos H. Beijnen[c,d,*]

[a] Division of Clinical Pharmacology, Department of Medical Oncology, The Netherlands Cancer Institute, PO Box 90203, 1006 BE Amsterdam, The Netherlands
[b] Department of Experimental Therapy, The Netherlands Cancer Institute, PO Box 90203, 1006 BE Amsterdam, The Netherlands
[c] Department of Pharmacy & Pharmacology, Slotervaart Hospital, PO Box 90440, 1006 BK Amsterdam, The Netherlands
[d] Faculty of Science, Department of Pharmaceutical Sciences, Division of Pharmaco-Epidemiology and Clinical Pharmacology, Utrecht University, Utrecht, The Netherlands

ARTICLE INFO

Article history:
Received 8 June 2012
Accepted 27 November 2012
Available online 7 December 2012

Keywords:
Capecitabine
HPLC–MS/MS
Bioanalysis
5-Fluorouracil
Chromatography
Cancer

ABSTRACT

Capecitabine is the oral prodrug of the anticancer drug 5-fluorouracil (5-FU). The purpose of this study was to quantify capecitabine and its metabolites including 5′-deoxy-5-fluorocytidine (5′-dFCR), 5′-deoxy-5-fluorouridine (5′-dFUR), 5-FU, dihydro-5-fluorouracil (FUH_2), α-fluoro-ureidopropionic acid (FUPA) and fluoro-β-alanine (FBAL) in human plasma using liquid chromatography coupled to electrospray tandem mass spectrometry. To this end two individual assays were developed: one for the simultaneous quantification of capecitabine, 5′-dFCR and 5′-dFUR using reversed phase chromatography and gradient elution, and one assay for 5-FU, FUH_2, FUPA and FBAL using hydrophilic interaction chromatography and isocratic elution. Both assays were fully validated according to current FDA guidelines. Total run time for the capecitabine assay was 9.0 min, and of the 5-FU assay 5.0 min. Analyte extraction was performed by protein precipitation. Stable labeled isotopes for each of the analytes were used as internal standards. The linear ranges of the analytes were 50–6000 ng/mL for the capecitabine assay and 50–5000 ng/mL for the 5-FU assay. Validation results demonstrate that capecitabine and its metabolites can be rapidly, accurately, precisely and robustly quantified in human plasma with the presented methods. Both assays are currently in extensive use in support of pharmacokinetic studies in patients treated with capecitabine or 5-FU.

1. Introduction

5-Fluorouracil (5-FU) and its oral prodrug capecitabine (Xeloda®) belong to the group of fluoropyrimidines, and are among the most commonly prescribed anticancer drugs for the adjuvant and palliative treatment of various types of solid tumors. While 5-FU is administered intravenously, capecitabine is applied orally, usually in bi-daily doses of 825, 1000 or 1250 mg/m^2 of body surface area [1]. Upon ingestion, capecitabine is rapidly and almost completely absorbed in the gastro-intestinal tract as unchanged drug. Subsequently, it is converted by carboxylesterase to 5′-deoxy-5-fluorocytidine (5′-dFCR), then via cytidine deaminase to 5′-deoxy-5-fluorouridine (5′-dFUR) and thereafter by thymidine phosphorylase to 5-FU. 5-FU is intracellularly phosphorylated into its active moieties that primarily interfere with DNA and RNA synthesis. However, the majority of 5-FU is inactivated by dihydropyrimidine dehydrogenase mainly in the liver to dihydro-5-fluorouracil (FUH_2). Subsequent and last catabolites in the fluoropyrimidine degradation cascade are α-fluoro-ureidopropionic acid (FUPA) formed by dihydropyrimidinase, and α-fluoro-β-alanine (FBAL) that is formed by β-ureidopropionase with the co-release of CO_2 and NH_3 (Fig. 1) [2].

The main and dose-limiting side effects of fluoropyrimidines are diarrhea, mucositis, stomatitis, nausea and vomiting, bone marrow suppression, and especially in the case of capecitabine, hand-foot syndrome [3,4]. Interestingly, a randomized phase III study showed that with pharmacokinetically guided dose adjustments of 5-FU, the incidence and severity of adverse events were significantly reduced, while the response rate improved, with a trend toward increased overall survival [5]. Whether individual dose adjustments based on pharmacokinetic monitoring also improves clinical outcome of patients treated with capecitabine remains to be established.

Obviously, bio-analytical assays for the quantitative determination of capecitabine and its metabolites are essential in support for clinical pharmacological studies with fluoropyrimidines. Several

* Corresponding author at: Department of Pharmacy & Pharmacology, Slotervaart Hospital, PO Box 90440, 1006 BK Amsterdam, The Netherlands.
Tel.: +31 020 512 4481; fax: +31 020 512 4753.
E-mail address: jos.beijnen@slz.nl (J.H. Beijnen).

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http://dx.doi.org/10.1016/j.jchromb.2012.11.033

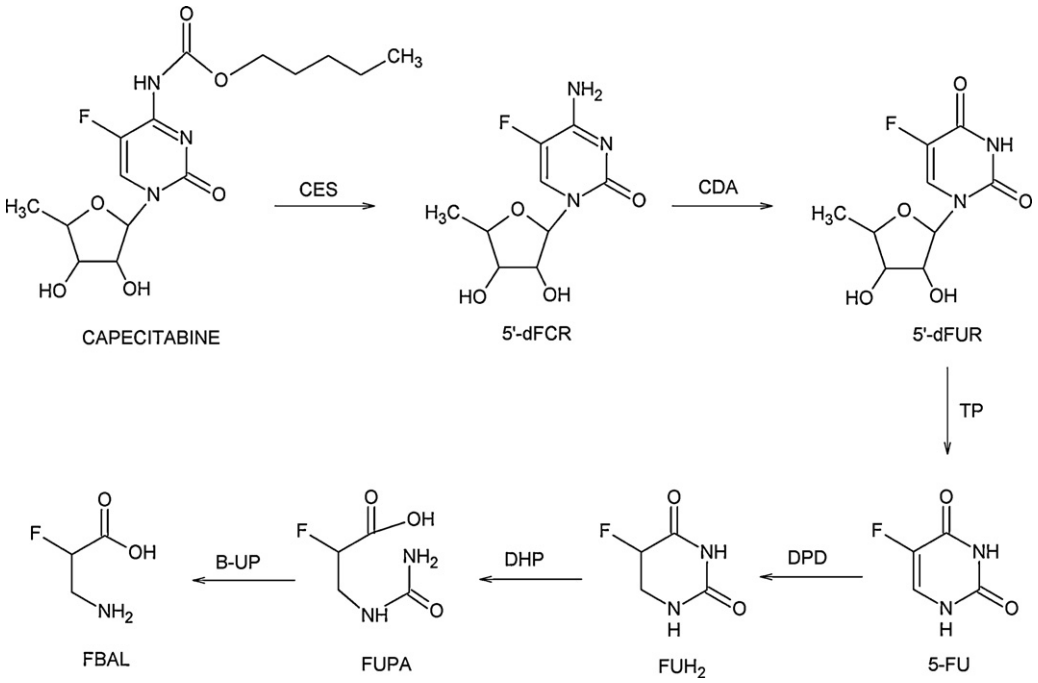

Fig. 1. Biotransformation of capecitabine and 5-FU. Capecitabine is metabolized by carboxylesterase (CES) to 5′-dFCR, which is subsequently metabolized by cytidine deaminase (CDA) to 5′-dFUR, followed by conversion to 5-FU by thymidine phosphorylase (TP). 5-FU is inactivated by dihydropyrimidine dehydrogenase (DPD) to dihydrofluorouracil (FUH_2), which is thereafter converted by dihydropyrimidinase (DHP) to α-fluoro-β-ureidopropionic acid (FUPA). α-Fluoro-β-alanine (FBAL) is the final catabolite in this cascade and is formed by β-ureidopropionase (B-UP).

high performance liquid chromatography (HPLC) assays for the quantification of capecitabine and various of its metabolites have been described with either mass spectrometric (MS) [6–11] or ultraviolet [12,13] detection. However, none of these assays is complete in the sense that quantification of all metabolites is described. Other limitations are extensive sample pretreatments, long run times, necessity for column switching, insufficient lower limit of quantification, or lack of details for method replication. We previously described a HPLC coupled to tandem mass spectrometry (HPLC–MS/MS) assay for the simultaneous quantification of capecitabine, 5′-dFCR, 5′-dFUR, 5-FU and FUH_2 using fludarabine and 5-chlorouracil as internal standards [10]. In an attempt to gain additional sensitivity and accuracy, we firstly aimed at replacing fludarabine and 5-chlorouracil by stable isotopes for each of the analytes of interest. Secondary aims were shorter run-time and increased robustness. In addition, we included the two remaining and final metabolites FUPA and FBAL. Herein, we describe the development and validation of the quantitative determination of capecitabine, 5′-dFCR, 5′-dFUR, 5-FU, FUH_2, FUPA and FBAL in human plasma using HPLC–MS/MS. Since 5-FU and subsequent metabolites show substantially different physicochemical properties compared to capecitabine, 5′-dFCR and 5′-dFUR, the quantification was split into two independent assays, respectively. Both assays are currently extensively applied in support of clinical pharmacological studies with capecitabine and 5-FU.

2. Materials and methods

2.1. Chemicals and materials

Capecitabine ($C_{15}H_{22}N_3O_6F$), 5′-dFCR ($C_9H_{12}N_3O_4F$), 5′-dFUR ($C_9H_{11}N_2O_5F$), 5-FU ($C_4H_3FN_2O_2$), FUH_2 ($C_4H_5FN_2O_2$), FUPA ($C_4H_7FN_2O_3$), FBAL ($C_3H_6FNO_2$), capecitabine-d11, 5′-dFCR-$^{13}C^{15}N_2$, 5′-dFUR-$^{13}C^{15}N_2$, 5-FU-$^{13}C^{15}N_2$, FUH_2-$^{13}C^{15}N_2$, FUPA-$^{13}C_3$, and FBAL-$^{13}C_3$ were purchased from Toronto Research Chemicals Inc. (North York, Toronto, Canada). Acetonitrile and methanol (supra-gradient grade) were from Biosolve Ltd. (Valkenswaard, The Netherlands). Formic acid 98% and water (LiChrosolve) originated from Merck (Darmstadt, Germany) and distilled water was obtained from Aqua B. Braun (Melsungen, Germany).

2.2. Mass spectrometry

Detection of the analytes was performed on a triple quadrupole mass spectrometer equipped with turbo ionspray interface (API4000, AB Sciex, Foster City, CA, USA). An Agilent 1100 series liquid chromatography system was used consisting of a binary pump, in-line degasser, column oven and autosampler (Agilent Technologies, Palo Alto, CA, USA). Capecitabine and 5′-dFCR were detected in the positive mode, whereas the other analytes in the negative ion mode. MS/MS experiments were performed to determine the most abundant product ions, for which multiple reaction monitoring (MRM) parameters were optimized. Optimized mass transitions and operating procedures are provided in Table 1. Analyst™ software v1.5 (AB Sciex) was used for data processing.

2.3. Chromatography for capecitabine, 5′-dFCR and 5′-dFUR

Capecitabine, 5′-dFCR and 5′-dFUR were separated using a XBridge C18 column (50 mm × 2.1 mm ID, particle size 5 μm; Waters Corp., Mildford, MA, USA) protected with a 0.5 μm filter (Upchurch Scientific, Oak Harbor, WA, USA), and thermostatted at 30 °C. Chromatography was performed using a gradient system consisting of mobile phase solution A (0.05% formic acid in water) and solution B (0.05% formic acid in methanol). The gradient started for the first 3 min with solution A:B 95:5 (v/v) at a constant flow rate of 0.3 mL/min. From 3.01 to 6.00 min the flow rate was set at 0.4 mL/min and a linear gradient was applied to A:B 5:95 (v/v). At t = 6.01 min, the ratio returned to A:B 95:5 (v/v) and the flow rate was increased to 0.5 mL/min for the following 2 min. At t = 8.01 min, the flow rate was set to its starting value of 0.3 mL/min for

Table 1
Mass spectrometer settings.

	Method 1	Method 2
Analytes	Capecitabine, 5′-dFCR and 5′-dFUR	5-FU, FUH_2, FUPA and FBAL
Interface	Turbo Ion Spray (TIS)	Turbo Ion Spray (TIS)
Scan type	MRM	MRM
Resolution Q1 and Q3	Unit	Unit
Pause time	5 ms	5 ms
Interface heater	On	On
Nebulizer gas (GS1)	30	50
Turbo gas (GS2)	60	50
Collision gas (CAD)	6	8
Curtain gas (CUR)	50	50
Temperature TIS	600 °C	700 °C

Analyte	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Period/experiment	Ion spray voltage (V)	Dwell (ms)	DP	EP	CE	CXP
Method 1									
Capecitabine	360	130	3/1	5500	150	46	10	45	8
Capecitabine-d11	371	131	3/1	5500	150	46	10	29	12
5′-dFCR	246	130	1/1	4500	300	41	10	53	8
5′-DFCR-$^{13}C^{15}N_2$	249	133	1/1	4500	300	41	10	53	8
5′-dFUR	245	108	2/1	−4500	150	−65	−10	−28	−7
5′-dFUR-$^{13}C^{15}N_2$	248	109	2/1	−4500	150	−65	−10	−20	−13
Method 2									
5-FU	129	42	1/1	−4500	50	−65	−10	−30	−7
5-FU-$^{13}C^{15}N_2$	132	44	1/1	−4500	50	−65	−10	−30	−7
FUH_2	131	83	1/1	−4500	50	−55	−6	−17	−12
FUH_2-$^{13}C^{15}N_2$	134	85	1/1	−4500	50	−55	−6	−17	−12
FUPA	149	106	1/1	−4500	50	−35	−10	−12	−7
FUPA-$^{13}C_3$	152	109	1/1	−4500	50	−35	−10	−12	−7
FBAL	106	86	1/1	−4500	50	−55	−6	−14	−5
FBAL-$^{13}C_3$	109	89	1/1	−4500	50	−55	−6	−14	−5

equilibration. Total run time of the assay was 9.0 min. The eluent was directed to the mass spectrometer in the period between 1.8 and 7.0 min. Outside this time period, the eluent was directed to waste.

2.4. Chromatography for 5-FU, FUH_2, FUPA and FBAL

The chromatography for 5-FU, FUH_2, FUPA and FBAL was performed on a Luna HILIC column (150 mm × 2.1 mm ID, particle size 3 μm; Phenomenex, Torrance, CA, USA) protected with an inline filter of 0.5 μm (Upchurch Scientific) with the column oven maintained at ambient temperature. 5-FU, FUH_2, FUPA and FBAL were chromatographically separated using isocratic elution consisting of 20% solution A (10 mM formic acid in water at pH 4.0) mixed with 80% solution B (100% acetonitrile). The flow rate was maintained at 0.3 mL/min and total run time was 5.0 min. During the first 1.2 min and after 4.5 min the flow was directed to waste.

2.5. Preparation of stock and working solutions

For both assays, independent stock and working solutions were prepared. For each of the analytes, two stock solutions of 1.0 mg/mL in water were prepared from two independent weightings: one for the calibration standards and one for the validation samples. Stock solutions were further diluted in water to obtain seven working solutions containing capecitabine, 5′-dFCR and 5′-dFUR in concentrations of 120,000, 70,000, 20,000, 10,000, 5000, 2000 and 1000 ng/mL, respectively. Eight working solutions were prepared for 5-FU, FUH_2, FUPA and FBAL in concentrations of 100,000, 75,000, 50,000, 20,000, 10,000, 5000, 2000 and 1000 ng/mL. The quality control working solutions for both assays contained analyte concentrations of 90,000, 10,000, 3000 and 1000 ng/mL. Stock solutions of the internal standards were prepared by dissolving approximately 1 mg of compound in 1.0 mL water, except for FUH_2-$^{13}C^{15}N_2$, FUPA-$^{13}C_3$ and FBAL-$^{13}C_3$, which were dissolved in 1.0 mL of DMSO. For both assays, internal standard working solutions were prepared in 10.0 mL of water at concentrations of the stable isotopes of 10,000 ng/mL. All stock and working solutions were stored at −20 °C.

2.6. Preparation of calibration and quality control samples in plasma

For both assays, independent calibration standards and quality control samples were prepared in control drug-free human heparinized plasma that was centrifuged for 5 min at 2000 × *g* before use. Calibration standards were freshly prepared at concentrations of capecitabine, 5′-dFCR and 5′-dFUR of 50, 100, 250, 500, 1000, 3500 and 6000 ng/mL, and at 50, 100, 250, 500, 1000, 2500, 3750 and 5000 ng/mL for 5-FU, FUH_2, FUPA and FBAL, each by adding 20 μL of the appropriate working solution to 380 μL of plasma, followed by short vortex mixing.

Quality control samples were prepared for both assays in analyte concentrations of 50 (lower limit of quantification [LLOQ]), 150 (low), 500 (mid), 4500 (high) and 25,000 (>upper limit of quantification [ULOQ]) ng/mL by diluting the quality control working solutions 20 times in control human heparinized plasma. Quality control samples were stored in replicates of 200 μL at −20 °C.

2.7. Sample pre-treatment for the analysis of capecitabine, 5′-dFCR and 5′-dFUR

An amount of 10 μL of the internal standard working solution (10,000 ng/mL) was added to 100 μL human lithium heparinized plasma. Proteins were precipitated by adding 200 μL of methanol:acetonitrile 1:1 (v/v), followed by short vortex mixing and automated shaking at 1250 rpm for 10 min. Then, samples were centrifuged for 10 min at approximately 23,100 × *g*, and 50 μL of the supernatant was transferred into a glass autosampler vial with insert containing 150 μL water. Injection volume of the processed sample solution was 5 μL.

2.8. Sample pre-treatment for the analysis of 5-FU, FUH_2, FUPA and FBAL

To 100 μL of human heparinized plasma, 10 μL of the internal standard working solution (10,000 ng/mL) was added. Protein precipitation was performed by adding 400 μL acetonitrile, followed by short vortex mixing and automated shaking at 1250 rpm for 10 min. Samples were then centrifuged for 10 min at approximately 23,100 × *g*. A volume of 100 μL of the supernatant was transferred into a glass autosampler vial with insert for the analysis of 5-FU, FUPA and FBAL. The remainder of the supernatant was filtered through a HybridSPE-PPT cartridge (Sigma–Aldrich, Zwijndrecht, The Netherlands). The filtrate was transferred into a glass autosampler vial with insert for the analysis of FUH_2. Aliquots of 5 μL of the processed sample solutions were injected onto the column.

2.9. Validation procedures

Both assays were validated in accordance with the current FDA guideline on bioanalytical method validation [14].

2.9.1. Linearity

Calibration standards, including standards containing no analyte or internal standard (double blank) and samples containing only internal standard (blank), were analyzed in duplicate in three separate analytical runs. The linear regression of the peak area versus the concentration was weighted by $1/x^2$ for determination of the concentration of the analytes. To assess linearity, deviations of the back-calculated concentrations from the nominal concentrations should be within 85–115%. At the lower limit of quantification (LLOQ) level a deviation of ±20% was allowed.

2.9.2. Accuracy and precision

Five replicates of each of the quality control samples at LLOQ, low, mid, and high concentrations were analyzed in three separate analytical runs. Five replicates above the upper limit of quantification (ULOQ) at 25,000 ng/mL were diluted ten times with tetrahydrouridine stabilized control lithium heparinized plasma and analyzed in one analytical run. The inter- and intra-assay accuracies were calculated as the difference between the nominal and measured concentrations. The coefficient of variation (CV) was calculated to assess the precision. Accuracy should be within ±20% for the LLOQ and within ±15% at the other concentrations. The CVs should be less than 20% at the LLOQ, and less than 15% at the other concentration levels.

2.9.3. Specificity and selectivity

Six individual batches of control drug-free lithium heparinized human plasma were used to investigate whether endogenous matrix constituents interfere with the assay. Blank, double blank, and samples spiked at the LLOQ were freshly prepared, and analyzed in one analytical run. Peak areas of compounds co-eluting with the analyte or internal standard should not exceed 20% of the analyte peak area at the LLOQ, or 5% of the internal standard area. Deviations at the LLOQ level from the nominal concentrations should be within ±20%.

To determine the cross analyte and internal standard interferences, all analytes were separately spiked at their ULOQ concentration to control human plasma. The internal standards were separately spiked at their nominal concentration. The interference at the retention times of the analytes and internal standards should be less than 20% and 5% of the peak areas detected in the LLOQ sample, respectively.

2.9.4. Matrix factor

The matrix effect (ion suppression) was determined in triplicate by analyzing the analytes at low, mid and high concentrations in water, and measuring the analytes spiked in blank plasma extract in the same concentrations as in processed QC low, mid and high samples, respectively. The absolute matrix factor was defined as the ratio of each analyte peak response in the presence and absence of matrix ions. The relative matrix factor was determined by correcting with the internal standard.

2.9.5. Carry-over

Carry-over was determined in one analytical run by injecting a double blank matrix sample after the highest calibration standard. Responses in the double blank matrix at the retention times of the analytes and internal standards should be less than 20% and 5% of the mean response detected in the LLOQ sample, respectively.

2.9.6. Stability

Stability of the stock solutions in water was determined after 6 h of storage at room temperature, and after 4 months of storage at −20 °C. The stability of the analytes in plasma was investigated during three freeze (−20 °C)/thaw cycles. Various stability experiments were performed of the analytes in plasma when stored at temperatures of −70 °C, −20 °C, on ice-water and at ambient temperature, and also when stabilized with 0.1 mg/mL tetrahydrouridine (THU). The stability of the processed samples was determined after storage for two weeks at 2–8 °C. The re-injection reproducibility in the autosampler was determined 24 h after start of the original run, during which period samples were stored at 2–8 °C. Plasma stability experiments were performed in triplicate at concentration levels of QC low, mid and high, or at QC mid. The analytes were considered stable if the determined concentrations did not deviate more than ±15% from the initial concentrations, and the precisions should be less than 15%.

3. Results and discussion

3.1. Chromatography

In our previous report for the determination of capecitabine, 5′-dFCR, 5′-dFUR, 5-FU and FUH_2 in plasma, a Hypercarb column (30 mm × 2.1 mm ID, particle size 5 μm) was used, by which all analytes could be successfully separated in one assay within 15 min [10]. Upon frequent application of the assay however, we noticed that the signal intensity decreased in time, thereby significantly loosing sensitivity. In addition, the number of runs that could be maximally performed using this column was limited, despite extensive washing and reconditioning procedures. Therefore, we decided to develop a new assay using a different type of chromatography. Given the extensive differences in physicochemical properties of capecitabine, 5′-dFCR and 5′-dFUR compared to 5-FU and subsequent metabolites, it was decided to develop two independent assays. Hereby, analytes in the capecitabine assay could be separated within 9.0 min and for the 5-FU assay within 5.0 min. Both assays proved to be very stable and robust, also when extensively used. In addition, the capecitabine assay could be successfully applied using 96-wells plates. Fig. 2 depicts the representative chromatograms of both assays.

3.2. Sample pre-treatment

Sample pre-treatment was initially started using 10% (w/v) trichloroacetic acid (TCA) for protein precipitation [10]. Pre-validation results revealed however that the stable isotope capecitabine-d11 rapidly converted into 5′-dFUR in acid environment. Storage of the TCA-processed samples at 2–8 °C for only a

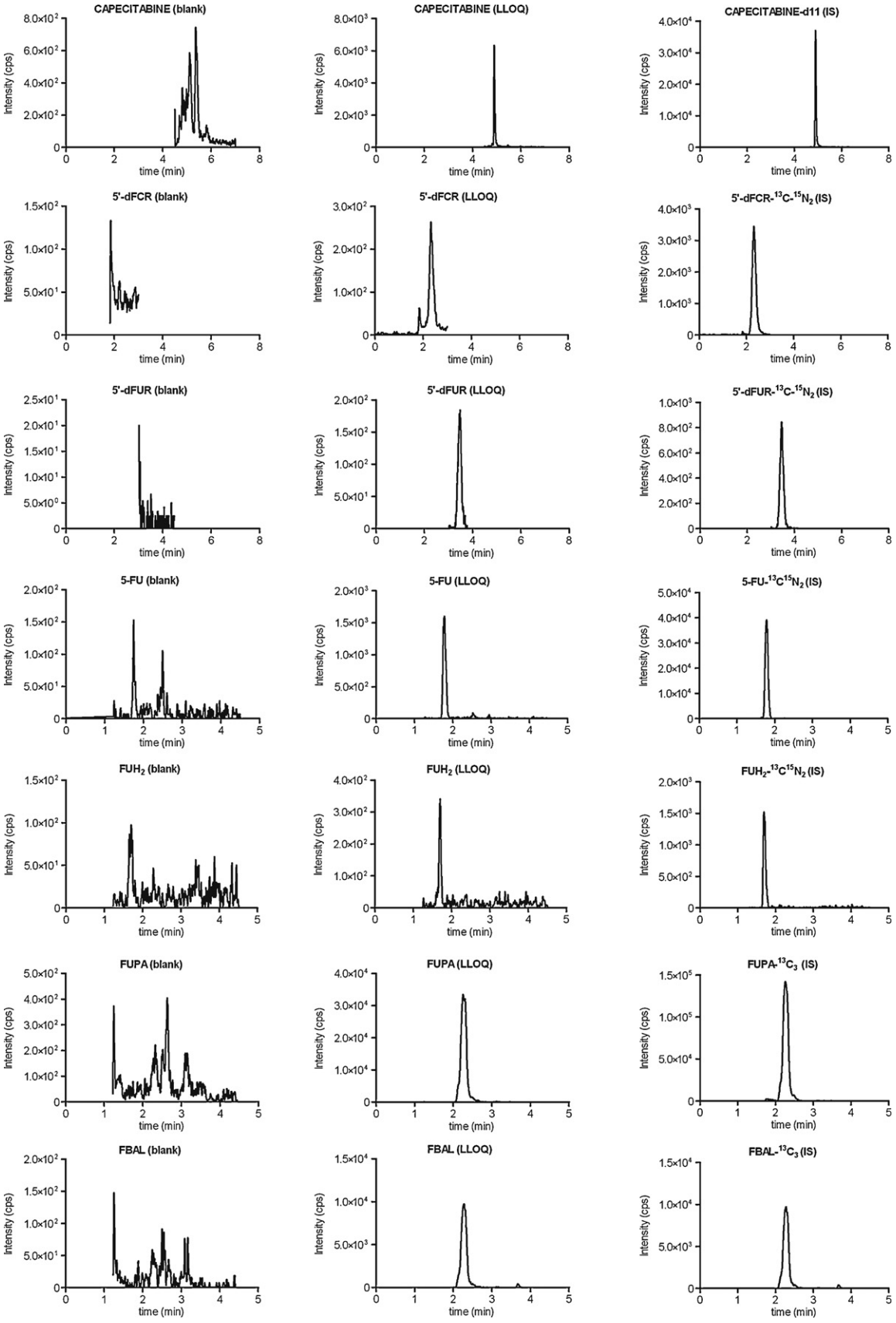

Fig. 2. Representative HPLC–MS/MS chromatograms from blank human plasma, spiked plasma samples at the LLOQ of capecitabine, 5′-dFCR, 5′-dFUR, 5-FU, FUH_2, FUPA and FBAL, and spiked plasma with their internal standards (IS), respectively.

couple of hours already resulted in an unacceptable increase in the concentration of 5′-dFUR of more than 25%, whereas the area of capecitabine-d11 decreased with a similar amount. Therefore, 10% TCA appeared not suitable for protein precipitation in this assay. Using a pH-neutral solution of methanol:acetonitrile (1:1, v/v), protein precipitation was successful, and guaranteed sufficient stability of capecitabine, 5′-dFCR, 5′-dFUR and their stable isotopes, respectively. For the 5-FU assay, 100% acetonitrile was used for protein precipitation, which was added in a 4:1 (v/v) ratio to plasma. Herewith, the same percentage of acetonitrile as is used in the mobile phase of the isocratic elution is reached. Thereby, when injecting a processed sample, potentially disturbing effects on the chromatography caused by differences in polarity are minimized. During pre-validation experiments, it was noted that the sensitivity of FUH_2 significantly increased using a HybridSPE-PPT cartridge filter, which removes among others phospholipids from the extract. The filtration resulted in a reduction of the noise and an increase of the FUH_2 signal, resulting in a sensitivity gain of a factor of 50. Therefore, FUH_2 was quantified after filtration, whereas 5-FU, FUPA and FBAL were quantified unfiltered.

3.3. Mass spectrometry

Fig. 3 depicts the MS/MS product ion scans and proposed fragmentation pattern of all analytes. Clear responses were observed at m/z of 360, 246, 245, 129, 131, 149, and 106, which correspond to the protonated molecular ions of capecitabine and 5′-dFCR, and deprotonated molecular ions of 5′-dFUR, 5-FU, FUH_2, FUPA and FBAL, respectively. The most abundant fragments of capecitabine and 5′-dFCR were product ions with m/z of 130, corresponding to loss of the sugar moiety and for capecitabine additionally the pentanoic acid side chain. For 5′-dFUR the m/z of the product ion was 108, corresponding to the loss of the sugar moiety and the fluorine atom. The product ion of 5-FU had a m/z of 42 and represents the formamide moiety; the product ion of FUH_2 with m/z 83 represents loss of the fluoro-ethane group; the product ion of FUPA had a m/z 106 corresponding to the loss of the formamide moiety; and the m/z for the product ion of FBAL was 86, representing the loss of the fluorine atom.

3.4. Validation

3.4.1. Linearity

The assays were linear over the tested concentration range of 50–6000 ng/mL for capecitabine, 5′-dFCR and 5′-dFUR, and 50–5000 ng/mL for 5-FU, FUH_2, FUPA and FBAL in human plasma. The mean accuracies did not deviate more than −5.8% and 3.2% from the nominal concentrations for all compounds at all concentration levels, with a maximum CV for the precision of 9.6% above LLOQ concentrations, and a maximum CV of 16.3% at the LLOQ. Correlation coefficients for all compounds were 0.995 or higher.

3.4.2. Accuracy and precision

Table 2 lists the assay performance data (inter-assay accuracies and precisions) for capecitabine and its metabolites. The intra-assay accuracies at the LLOQ were within −10.2% and 2.5%, and at the higher levels within −6.4% and 7.2%. The maximum intra-assay precisions at the LLOQ and at the other levels were 9.9% and 8.7%, respectively. In addition, 10-fold dilution of the samples above the ULOQ resulted in acceptable deviations from nominal concentrations with intra-assay accuracies within −7.9% and 11.0% and intra-assay precisions were maximally CV = 12.6% (data not shown). In summary, all accuracies and precisions for all compounds were within the predefined acceptance criteria.

3.4.3. Specificity and selectivity

The endogenous, cross analyte and internal standard interference tests showed no co-eluting peaks in the blanks with areas exceeding 20% of the area at the LLOQ level of the analytes in the blanks, and no co-eluting peaks exceeding 5% of the area of the internal standards. The deviations from the nominal concentrations were within ±20%, therefore, the specificity and selectivity of the assay were considered acceptable.

3.4.4. Matrix factor

Table 3 lists the results of the matrix factor analysis. While no absolute matrix effect was observed for capecitabine, 5′-dFCR, 5′-dFUR and FUPA across the validated range, 5-FU, FUH_2 and FBAL did show a matrix effect (ion suppression). The isotopically labeled internal standards, however, proved to be most effective at minimizing the influence of matrix ions: the values of the relative matrix effect of all analytes ranged between 0.94 and 1.05, with a maximum variability of 5.8%.

3.4.5. Carry-over

The responses in the first double blank sample after injection of the highest calibration standard were 0.00% of the areas of a LLOQ sample for all analytes and internal standards, except for capecitabine, for which the carry-over was 11.6% of the area at LLOQ. The carry-over, therefore, proved to be satisfactory.

3.4.6. Stability

Table 4 shows the results of the stability experiments. All analytes were stable in stock solution at ambient temperature for at least 6 h. The deviation of the freeze/thaw stability experiments also remained within 15% of the nominal concentrations. In the final extract, all analytes were stable for at least 15 days when stored at 2–8 °C, and also, the re-injection reproducibility was satisfactory. All analytes were stable in plasma within the margins of acceptance at ambient temperature for 6 h, except for FUH_2, which concentration decreased by more than 40% during this storage period. However, when kept on ice-water for 2 h, no degradation occurred. Furthermore, storage of plasma at ambient temperature showed a decrease in the concentration of 5′-dFCR, whereas the concentration of 5′-dFUR increased. Upon storage for 24 h at ambient temperature, a definitively unacceptable conversion of 5′-dFCR into 5′-dFUR was noted. The enzyme that mediates this conversion is cytidine deaminase, and has high activity in plasma, but can be competitively inhibited using the substrate tetrahydrouridine [15,16]. Therefore, additional stability experiments were performed with tetrahydrouridine-stabilized plasma samples, but also with unstabilized plasma stored on ice-water. Under both conditions, the CDA-mediated conversion of 5′-dFCR to 5′-dFUR was successfully inhibited for at least 48 h. Long-term stability in plasma at −20 °C was acceptable when stored for 3 months, however, also under this condition, 5′-dFCR slowly metabolized to 5′-dFUR. Importantly, at 6 months, the decrease in the concentration of 5′-dFCR exceeded the predefined level of acceptance. Therefore, long-term stability experiments at −70 °C were performed, and showed no significant deviations from the nominal concentrations when stored for 6 months under this condition.

In summary, to obtain the most reliable pharmacokinetic data of patients treated with capecitabine, obtained blood samples should be immediately cooled on ice-water and centrifuged at 2–8 °C. Plasma should be stored at −70 °C, and in case of storage for more than 6 months, should be stabilized using tetrahydrouridine. Ideally, plasma samples are kept on ice-water before processing. Final extracts are stable under the tested conditions.

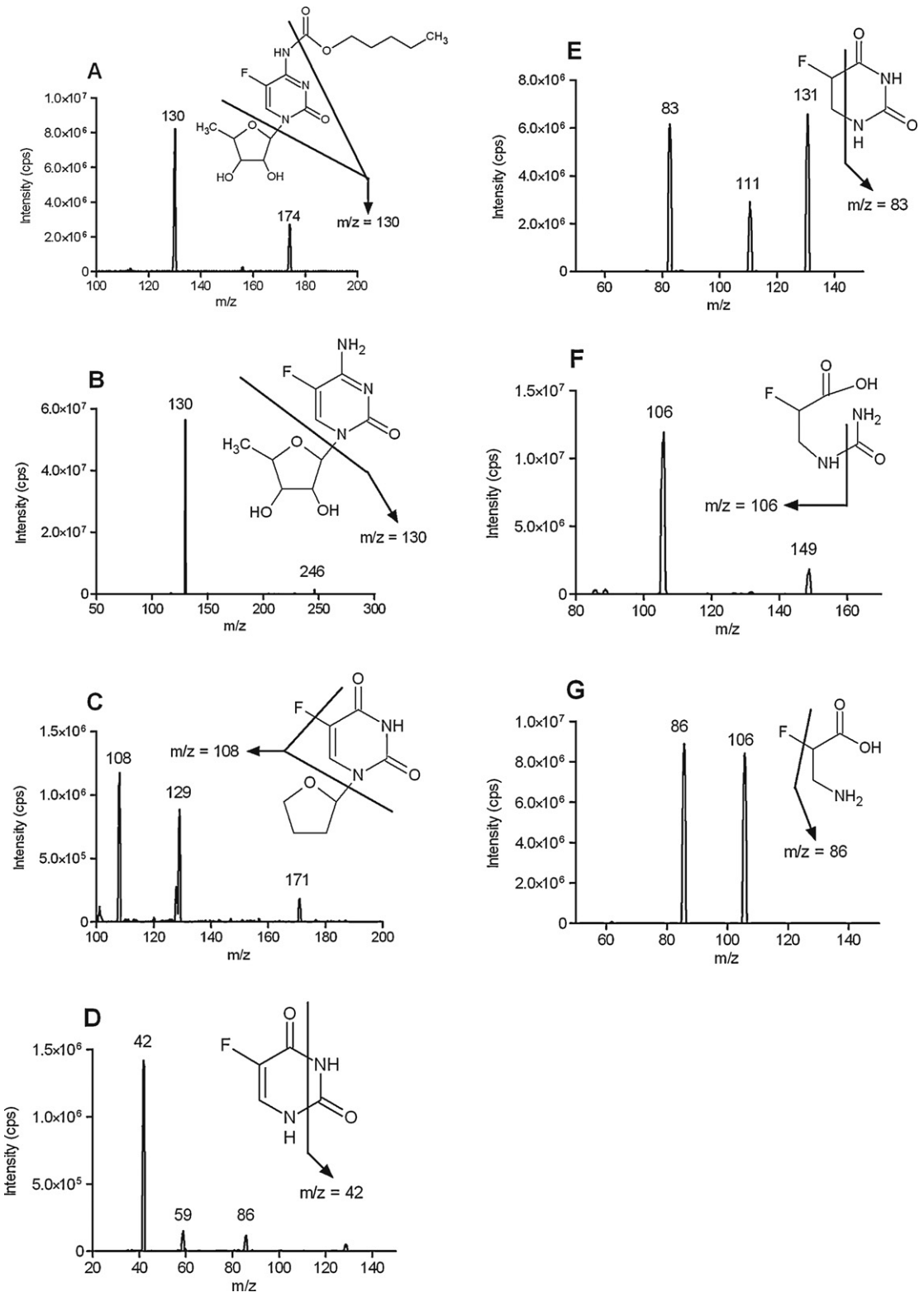

Fig. 3. Product ion scans of capecitabine (A), 5′-dFCR (B), 5′-dFUR (C), 5-FU (D), FUH_2 (E), FUPA (F) and FBAL (G).

4. Application of the assays

The described assays are currently successfully applied in support of pharmacokinetic studies in patients treated with capecitabine or 5-FU. Fig. 4 shows the measured concentrations of capecitabine plus its metabolites in a patient with gastric cancer after oral administration of 1650 mg capecitabine. The calculated pharmacokinetic parameters including area under the concentration–time curve (AUC), maximum concentration (C_{max}), time to reach C_{max} (T_{max}), and the half-lives ($t_{1/2}$) are reported in Table 5. Samples were taken and analyzed after written informed consent from the patient had been obtained. The selected linear ranges cover typically observed plasma concentrations for all analytes after administration of capecitabine [17]. The plasma levels of

Table 2
Assay performance data for capecitabine and metabolites in human plasma.

Compound	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Inter-assay accuracy (%)	Inter-assay precision (%)	Number of replicates
Capecitabine	48.6	46.6	−4.1	9.6	15
	146	143	−2.3	4.3	15
	486	489	0.5	2.2	15
	4370	4480	2.5	2.0	15
5′-dFCR	50.4	45.3	−10.2	9.2	15
	151	143	−5.5	6.7	15
	504	472	−6.4	4.7	15
	4530	4400	−2.9	4.3	15
5′-dFUR	49.5	47.5	−4.0	9.7	15
	149	152	2.3	6.4	15
	495	527	6.5	3.9	15
	4460	4780	7.2	3.3	15
5-FU	49.8	50.2	0.8	7.0	15
	149	148	−0.6	5.1	15
	498	495	−0.5	3.0	15
	4480	4490	0.3	3.7	15
FUH_2	49.0	49.3	0.6	9.9	15
	147	148	0.6	8.1	15
	490	482	−1.6	5.7	15
	4410	4480	1.6	6.5	15
FUPA	51.5	51.7	0.0	2.3	15
	155	157	1.4	3.0	15
	515	532	3.3	2.1	15
	4610	4320	−6.3	3.2	15
FBAL	58.0	59.5	2.5	8.5	15
	174	171	−1.6	8.7	15
	580	563	−3.0	6.3	15
	5220	5310	1.7	6.4	15

capecitabine and its metabolites are characterized by a wide inter-patient variability. In addition, half-lives of all analytes, except for the final metabolite FBAL, are all relatively short, with values of approximately 1 h. The half of FBAL is 3–10 h [17]. For these reasons it is desirable to be able to measure capecitabine plus its metabolites in a wide range of concentrations. The linear range of the here described assays are 50–6000 ng/mL for capecitabine, 5′-dFCR and 5′-dFUR, and 50–5000 ng/mL for 5-FU, FUH_2, FUPA and FBAL in human plasma. Thereby, the typically observed plasma concentrations of (metabolites of) capecitabine are covered.

Pharmacokinetic data of capecitabine and its metabolites may potentially be useful for monitoring, or potentially even guiding treatment with capecitabine or 5-fluorouracil. A large pharmacokinetic study in 481 patients showed that the area under the concentration–time curve of FBAL was significantly associated with a higher incidence of severe capecitabine-induced diarrhea [18].

Table 3
Matrix factor for capecitabine and its metabolites.

Analyte	Concentration (ng/mL)	Absolute matrix factor		Relative matrix factor	
		Mean	CV (%)	Mean	CV (%)
Capecitabine	147	0.96	0.7	0.99	2.1
	489	0.95	1.3	0.98	1.5
	4400	0.96	2.1	0.99	0.4
5′-dFCR	148	0.95	3.1	1.02	3.6
	493	0.95	0.6	0.99	2.7
	4440	0.96	2.3	1.01	1.1
5′-dFUR	151	0.97	6.2	1.03	4.7
	502	0.93	1.7	0.97	2.9
	4520	0.96	2.5	1.00	2.0
5-FU	160	0.49	7.9	1.05	5.8
	534	0.49	2.2	1.02	2.9
	4810	0.56	2.0	0.97	1.3
FUH_2	154	0.23	16	1.04	2.6
	513	0.15	22	1.00	5.2
	4620	0.14	2.3	1.04	13
FUPA	155	1.12	5.5	1.05	2.2
	515	1.02	2.0	1.01	1.8
	4640	1.01	1.2	1.03	1.2
FBAL	174	0.37	6.6	1.01	4.5
	580	0.32	7.2	0.94	3.8
	5220	0.32	1.0	1.04	1.8

Table 4
Stability data for capecitabine and its metabolites.

Matrix	Condition	Compound	Initial concentration (ng/mL)	Measured concentration (ng/mL)	Deviation (%)	CV (%)
Stock solution	Ambient, 6 h	Capecitabine	1.00×10^6	1.01×10^6	1.4	1.8
		5′-dFCR	1.00×10^6	1.02×10^6	1.5	5.8
		5′-dFUR	1.00×10^6	0.9×10^6	−1.7	1.0
		5-FU	1.00×10^6	0.97×10^6	−3.0	1.9
		FUH_2	1.00×10^6	1.03×10^6	2.6	6.8
		FUPA	1.00×10^6	1.05×10^6	4.6	4.3
		FBAL	1.00×10^6	1.01×10^6	0.8	1.7
Stock solution	−20 °C, 4 months	Capecitabine	1.00×10^6	0.98×10^6	−2.4	4.5
		5′-dFCR	1.00×10^6	1.03×10^6	3.4	0.9
		5′-dFUR	1.00×10^6	1.03×10^6	2.5	0.8
		5-FU	1.00×10^6	0.99×10^6	−0.8	1.1
		FUH_2	1.00×10^6	0.98×10^6	−2.0	6.0
		FUPA	1.00×10^6	1.03×10^6	−3.4	1.4
		FBAL	1.00×10^6	1.03×10^6	3.0	0.5
Plasma	3 freeze (−20 °C)/thaw cycles	Capecitabine	146	144	−1.1	5.8
			4370	4440	1.7	0.9
		5′-dFCR	151	134	−11.0	3.0
			4530	4460	−1.5	1.1
		5′-dFUR	149	165	10.5	2.8
			4460	4790	7.4	2.8
		5-FU	149	142	−4.9	7.9
			4480	4373	−2.4	1.4
		FUH_2	147	154	4.8	12.2
			4410	4353	−1.3	6.8
		FUPA	155	154	−0.9	6.3
			4640	4510	−2.8	1.2
		FBAL	156	154	−1.5	2.0
			4670	4580	−1.9	0.0
Plasma	3 months, −70 °C	Capecitabine	145	142	−1.8	1.5
			4340	4487	3.4	0.7
		5′-dFCR	148	144	−2.7	0.7
			4450	4537	1.9	1.1
		5′-dFUR	146	139	−4.6	1.7
			4390	4177	−4.9	1.7
Plasma	6 months, −70 °C	Capecitabine	145	144	−0.7	2.1
			4340	4443	2.4	4.2
		5′-dFCR	148	142	−4.3	1.5
			4450	4543	2.1	5.7
		5′-dFUR	146	143	−1.8	1.1
			4390	4273	−2.7	3.9
Plasma	3 months, −20 °C	Capecitabine	146	145	−0.7	0.7
			4370	4490	2.7	2.2
		5′-dFCR	151	139	−7.9	4.5
			4530	4143	−8.5	2.8
		5′-dFUR	149	167	12.1	3.3
			4460	5073	13.8	4.0
		5-FU	149	152	1.8	5.3
			4480	4470	−0.2	2.1
		FUH_2	147	131	−11.1	9.2
			4410	4100	−7.0	12.0
		FUPA	155	154	−0.9	1.4
			4640	4720	1.8	11.8
		FBAL	174	183	5.0	10.2
			5220	5087	−2.6	2.8
Plasma	6 months, −20 °C	Capecitabine	146	144	−1.6	2.6
			4370	4357	−0.3	2.4
		5′-dFCR	151	129	−14.3	15.1
			4530	3753	−17.1	5.6
		5′-dFUR	149	154	3.4	9.4
			4460	5013	12.4	4.9
Plasma	Ambient, 6 h	Capecitabine	146	145	−0.9	3.1
			482	467	−3.1	3.5
			4370	4330	−0.9	1.6
		5′-dFCR	151	135	−10.6	2.2
			494	444	−10.2	3.3
			4530	3923	−13.4	0.4
		5′-dFUR	149	171	14.5	5.0
			488	501	2.6	4.4
			4460	4903	9.9	5.4

Table 4 (*Continued*)

Matrix	Condition	Compound	Initial concentration (ng/mL)	Measured concentration (ng/mL)	Deviation (%)	CV (%)
		5-FU	149	141	−5.6	4.7
			4480	4393	−1.9	0.3
		FUH_2	147	85	−42.3	8.7
			4410	2573	−41.6	5.2
		FUPA	155	151	−2.6	6.1
			4640	4463	−3.8	0.7
		FBAL	174	159	−8.6	8.6
			5220	5253	0.6	5.5
		Capecitabine	146	134	−8.2	0.8
Plasma	Ambient, 24 h		482	441	−8.4	5.3
			4370	4137	−5.3	1.2
		5′-dFCR	151	107	−29.2	9.2
			494	373	−24.6	6.5
			4530	3303	−27.1	2.3
		5′-dFUR	149	186	24.8	3.8
			488	582	19.3	3.2
			4460	5740	28.7	2.7
		Capecitabine	482	407	−15.6	0.9
Plasma	Ambient, 48 h	5′-dFCR	494	320	−35.2	6.9
		5′-dFUR	488	642	31.6	6.9
		Capecitabine	482	452	−6.2	1.4
Plasma	Ice-water, 6 h	5′-dFCR	494	478	−3.2	4.5
		5′-dFUR	488	469	−4.0	4.4
		Capecitabine	482	443	−8.1	3.2
Plasma	Ice-water, 24 h	5′-dFCR	494	452	−8.6	6.6
		5′-dFUR	488	504	3.3	8.4
		Capecitabine	482	450	−6.7	2.7
Plasma	Ice-water, 48 h	5′-dFCR	494	448	−9.3	4.5
		5′-dFUR	488	496	1.5	4.4
Plasma	Ice-water, 2 h	FUH_2	147	128	−13.2	9.2
			4410	4380	−0.7	8.3
		Capecitabine	482	482	−0.1	3.4
Plasma	Ambient, stabilized with THU, 6 h	5′-dFCR	494	515	4.1	7.3
		5′-dFUR	488	473	−3.1	3.2
		Capecitabine	482	450	−6.6	3.3
Plasma	Ambient, stabilized with THU, 24 h	5′-dFCR	494	517	4.6	5.8
		5′-dFUR	488	481	−1.5	5.6
		Capecitabine	482	418	−13.2	2.6
Plasma	Ambient, stabilized with THU, 48 h	5′-dFCR	494	523	5.9	3.2
		5′-dFUR	488	472	−3.2	2.4
		Capecitabine	146	149	2.3	3.7
Final extract	2–8 °C, 15 days		4370	4330	−1.0	1.3
		5′-dFCR	151	163	8.2	3.5
			4530	4400	−2.8	0.7
		5′-dFUR	149	165	11.0	4.5
			4460	4770	7.0	2.2
		5-FU	149	149	0.2	5.5
Final extract	2–8 °C, 17 days		4480	4570	2.0	2.2
		FUH_2	147	149	1.4	11.1
			4410	4610	4.5	5.1
		FUPA	155	171	10.1	12.0
			4640	4710	1.5	5.9
		FBAL	174	164	−5.6	5.2
			5220	4893	−6.3	4.4
		Capecitabine	146	139	−4.6	6.1
			486	483	−0.5	4.5
			4370	4320	−1.1	2.9
Final extract	Re-injection reproducibility, 2–8 °C, 24 h	5′-dFCR	151	141	−6.4	7.6
			504	490	−2.8	5.8
			4530	4220	−6.8	2.0
		5′-dFUR	149	137	−8.1	7.6
			495	511	3.3	5.8
			4460	4400	−1.4	3.3
		5-FU	149	143	−3.8	1.5
			498	493	−1.1	1.2
			4480	4313	−3.7	4.8

Table 4 (*Continued*)

Matrix	Condition	Compound	Initial concentration (ng/mL)	Measured concentration (ng/mL)	Deviation (%)	CV (%)
		FUH_2	147	135	−8.2	13.7
			490	462	−5.7	2.0
			4410	4590	4.1	5.0
		FUPA	155	153	−1.3	2.6
			515	514	−0.3	2.9
			4640	4427	−4.6	2.3
		FBAL	156	146	−6.2	1.7
			519	491	−5.4	1.9
			4670	4410	−5.6	1.3

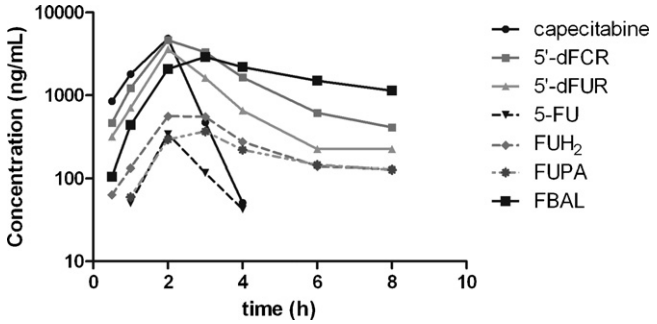

Fig. 4. Plasma concentration–time curves of capecitabine, 5′-dFCR, 5′-dFUR, 5-FU, FUH_2, FUPA and FBAL in a patient with gastric cancer following administration of 1650 mg capecitabine.

Table 5
Calculated pharmacokinetic parameters for capecitabine and its metabolites in a patient with gastric cancer given 1650 mg of capecitabine.

Analyte	AUC (h ng/mL)	T_{max} (h)	C_{max} (ng/mL)	$t_{1/2}$ (h^{-1})
Capecitabine	7330	2	4860	0.4
5′-dFCR	14,330	2	4640	1.4
5′-dFUR	8140	2	3590	1.3
5-FU	625	2	339	0.8
FUH_2	2220	2	559	1.6
FUPA	1750	3	366	2.4
FBAL	16,500	3	2910	3.1

Another phase III study in patients with metastatic colorectal cancer showed that pharmacokinetically guided 5-FU dosing resulted in a significantly increased response rate, and fewer severe toxicities [5]. Recently, Saif et al. extensively reviewed the potential of pharmacokinetically guided dose adjustments of 5-FU-based chemotherapy [19]. All these data support the value of pharmacokinetically guided treatment with capecitabine or 5-fluorouracil. Thereby, the need for analytical methods for the quantitative determination of capecitabine and its metabolites is also demonstrated.

5. Conclusion

We report the development and validation of the quantitative determination of the frequently applied anticancer drug capecitabine and its six metabolites 5′-dFCR, 5′-dFUR, 5-FU, FUH_2, FUPA and FBAL in human plasma, using HPLC–MS/MS. Due to significant differences in physicochemical properties of capecitabine, 5′-dFCR and 5′-dFUR compared to 5-FU, FUH_2, FUPA and FBAL, two different assays were developed and validated. Thereby, a highly robust, accurate, sensitive and specific quantification could be achieved, maintaining short run-times. All analytes were extracted using protein precipitation methods, and stable isotopes for each of the analytes were used as internal standard. Reversed-phase chromatography was used for the capecitabine assay; separation in the 5-FU assay was conducted using hydrophilic interaction chromatography. The tested linear range of the analytes was 50–6000 ng/mL for the capecitabine assay, and 50–5000 ng/mL for the 5-FU assay. These concentrations cover the ranges of typically observed plasma concentrations after administration of capecitabine or 5-FU. Both assays appeared highly robust and well-suitable for support of pharmacokinetic studies with capecitabine and 5-FU.

Acknowledgements

Luc Lucas and Abadi Gebretensae are greatly acknowledged for their excellent technical support.

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