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Capecitabine is an anticancer agent and is the oral prodrug of 5-fluorouracil (5-FU). In this study, an ultra-high performance liquid chromatography coupled to turbo ion spray tandem mass spectrometry (UPLC-MS/MS) method was developed and validated to quantify capecitabine and its metabolites including 5'-deoxy-5-fluorocytidine (5'-dFCR), 5'-deoxy-5-fluorouridine (5'-dFUR), 5-FU, and fluoro-β-alanine (FBAL) in lithium heparinized human plasma. Analytes were extracted by protein precipitation, chromatographically separated by Acquity UPLC HSS T3 column with gradient elution, and analyzed with a tandem mass spectrometer equipped with an electrospray ionization source. Capecitabine and 5'-dFCR were quantified in positive ion mode and 5'-dFUR, 5-FU, and FBAL were quantified in negative ion mode. The total chromatographic run time was 9 min. Stable isotopically labeled internal standards were used for all analytes. The assay was validated over the range from 25.0 to 2,500 ng/mL for capecitabine, 10.0 to 1,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU and 50 to 5,000 ng/mL for FBAL in human plasma. Validation results have shown the developed assay allows for reliable quantitative analysis of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in plasma samples.

1. Introduction

5-Fluorouracil (5-FU) and capecitabine (Xeloda®) are fluoropyrimidine-based chemotherapeutic agents used in the treatment of several solid tumors (Knikman et al. 2021). 5-FU is administered intravenously, whereas capecitabine, as the pro-drug of 5-FU, can be administered orally. Capecitabine is converted through three enzymatic steps into 5-FU, which in its turn is taken up by the cell and metabolized into the active intracellular metabolites. Capecitabine is converted to 5'-deoxy-5-fluorocytidine (5'-dFCR) by carboxyl esterase, upon which it is converted to 5'-deoxy-5-fluorouridine (5'-dFUR) and 5-FU by cytidine deaminase and thymidine phosphorylase, respectively (Miwa et al. 1998; Schellens 2007). Approximately 80-90% of the administered dose of 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD) primarily in the liver (Xeloda [SmPC] 2017). DPD converts 5-FU into the inactive metabolite 5,6-dihydro-5-fluorouracil (5-FUH_2). Thereupon, 5-FUH_2 is converted into α-fluoro-β-ureidopropionic (FUPA) and α-fluoro-β-alanine (FBAL) by dihydropyrimidinase and β-ureidopropionase, respectively (See Fig. 1) (Malet-Martino 2004). A small fraction of 5-FU is phosphorylated intracellularly to active metabolites which are incorporated into RNA or DNA which interferes with normal RNA function and causes DNA damage, respectively (Álvarez et al. 2012; Grem 1997). The main side-effects attributed to fluoropyrimidines are mucositis, gastrointestinal side effects and, hand-foot syndrome (Hoff et al. 2017; Johnston and Kaye 2001). Bioanalytical assays for the quantitative determination of capecitabine and its metabolites are necessary in support of clinical pharmacological studies and could also be used for therapeutic drug monitoring. Over the years, multiple liquid chromatography (LC) methods have been developed for the analysis of capecitabine and its metabolites (Knikman et al. 2020). LC was combined with either ultraviolet (UV) (Buchner et al. 2013; Dhananjeyan et al. 2007; Farkouh et al. 2010; Piórkowska et al. 2014; Thorat et al. 2018; Zufía et al. 2004) or mass spectrometric (Deenen et al. 2013; Deng et al. 2015; Guichard et al. 2005; Licea-Perez et al. 2009; Montange et al. 2010; Salvador et al. 2006; Siethoff et al. 2004; Vainchtein et al. 2010; Xu and Grem, 2003) detection. However, none of these assays can quantify capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL simultaneously in a single assay due to the differences in physiochemical properties. Deenen et al. (2013) reported the quantification of these metabolites but this method consists of two separate assays, one for the quantification of capecitabine, 5'-dFCR and, 5'-dFUR and another assay to determine 5-FU, 5-FUH_2, FUPA and FBAL.

Herein we describe the development and validation of a selective and sensitive LC-MS/MS for the simultaneous quantification of

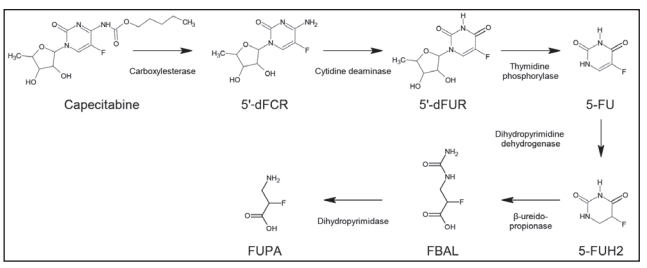

Fig. 1: Metabolism of capecitabine. Abbreviations: 5'-dFCR: 5'-deoxy-5-fluorocytidine, 5'-dFUR: 5'-deoxy-5-fluorouridine, 5-FU: 5-Fluoruracil, 5-FUH2: 5,6-dihydro-5-fluorouracil CAP: Capecitabine, FBAL: α-fluoro-β-alanine, FUPA: α-fluoro-β-ureidopropionic. Abbreviations: 5'-dFCR: 5'-deoxy-5-fluorocytidine, 5'-dFUR: 5'-deoxy-5-fluorouridine, 5-FU: 5-Fluoruracil, CAP: Capecitabine, FBAL: α-fluoro-β-alanine.

capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in human plasma. Assay validation has been based on the EMA and FDA guidelines on bioanalytical validation (European Medicines Agency 2011a; U.S. Department of Health and Human Services Food and Drug Administration 2018) and is applied in pharmacokinetic studies with capecitabine.

2. Investigations, results and discussion

2.1. Chromatography

In one of our previously described quantification methods capecitabine and its metabolites 5'-dFCR, 5'-dFUR, 5-FU, and 5-FUH_2 could be measured in plasma using a hypercarb (porous graphitic carbon) column. This column was not suited for measuring large batches of samples. The chromatography deteriorated and signal intensity decreased in time, which led to a significant loss of sensitivity. Furthermore, the batch-to-batch variability of the packed columns was considerable (Vainchtein et al. 2010). In another report, two separate assays were needed due to the physicochemical differences between capecitabine and its hydrophilic metabolites (Deenen et al. 2013). Capecitabine has a long carbon chain and shows lipophilicity. However, during biotransformation, the polarity of the metabolites gradually increases which become more hydrophilic (Knikman et al. 2020). Therefore, we decided to develop a new assay in which capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL can be separated and measured in a single run. Reversed-phase chromatography was applied using an Acquity UPLC HSS T3 column. Using this column in combination with gradient allowed for an adequate analyte retention (even for the most hydrophilic metabolite FBAL) and separation allowing for multiplexing of all analytes. The total run time was 9 min and typical retentions of capecitabine, 5'-dFUR, 5'-dFCR, 5-FU, and FBAL were 6.47, 5.14, 4.91, 3.10, and 1.12 min, respectively. The assay proved to be very stable and robust, also when extensively used for a period of 1.5 years.

2.2. Sample pre-treatment

Sample pre-treatment and analytical run time had to be efficient for rapid and high-throughput quantification of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in human plasma. Protein precipitation was chosen as the sample pre-treatment procedure to ensure high sample throughput. A mixture of methanol and acetonitrile (50:50, v/v) was successfully applied and added to the plasma in a ratio of 1:3. However, only protein precipitation was not sufficient to achieve acceptable sensitivity for FBAL. Therefore, an extra step was introduced in which after protein precipitation supernatants were evaporated under a gentle stream of nitrogen, reconstituted in mobile phase A and transferred to a 96-well plate. This step concentrates the sample by factor 3, which increases the signal intensity and allows for a lower LLOQ. The use of a 96-well plate allows for the analysis of large batches of samples. During validation, it was found that 5-FUH_2 converted to FUPA during evaporation. Therefore, this method is not suitable for the quantification of 5-FUH_2, and FUPA. This conversion did not affect the data integrity of the other analytes.

2.3. Mass spectrometry

To optimize the source settings and the analyte-specific conditions, flow injection analysis was applied. To obtain the largest signal-to-noise ratios, the mass spectrometer was utilized in the negative mode for quantification of 5'-dFUR, 5-FU, and FBAL and in the positive mode for 5'-dFCR and capecitabine. Optimized detector and analyte specific settings are presented in Table 1. Representative chromatograms of blank QC sample, QC LLOQ sample, and study sample are shown in Fig. 2.

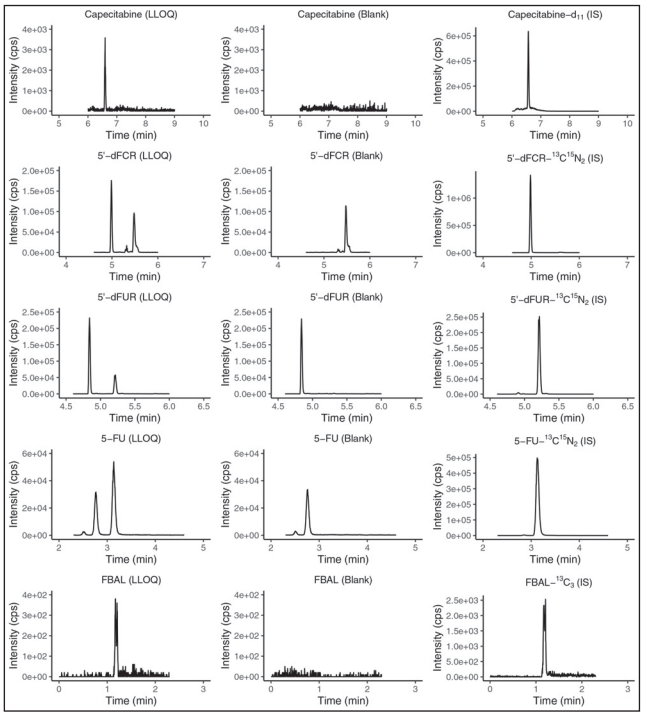

Fig. 2: Representative UPLC-MS/MS chromatogram from blank human samples, spiked plasma samples at the LLOQ of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, FBAL, and spiked plasma with their internal standards (IS), respectively.

Table 1: Mass spectrometer settings for the quantification of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU and FBAL in human plasma

Analyte	Precursor ion (m/z)	Product ion (m/z)	Ion spray Voltage (V)	Dwell (ms)	DP (V)	EP (V)	CE (V)	CXP (V)
Capecitabine	360.0	243.9	5000	50	106	10	40	12
Capecitabine-d11	371.1	254.8	5000	50	106	10	40	12
5'-dFCR	246.0	130.0	5000	25	71	10	44	18
5'-dFCR-$^{13}C^{15}N_2$	249.0	133.0	5000	25	71	10	44	18
5'-dFUR	244.9	128.8	-4500	25	-100	-10	-22	-13
5'-dFUR-$^{13}C^{15}N_2$	246.9	130.8	-4500	25	-100	-10	-22	-13
5-FU	128.9	42.1	-4000	20	-70	-10	-22	-7
5-FU-$^{13}C^{15}N_2$	131.8	43.8	-4000	20	-70	-10	-22	-7
FBAL	105.9	85.9	-4000	30	-25	-10	-12	-5
FBAL$^{13}C_3$	108.8	88.7	-4000	30	-25	-10	-12	-5

Abbreviation: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-Fluoruracil; CE, Collision energy; CXP, Collision exit potential; DP, Declustering potential; EP, Entrance potential; FBAL, α-fluoro-β-alanine; V, voltage.

Table 2: Assay performance data for the quantification of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU and FBAL in human lithium heparinized plasma

Compound	Nominal concentration (ng/mL)	Mean measured concentration (ng/mL)	Inter-assay accuracy (%)	Intra-assay accuracy (%)	Inter-assay precision (%)	Intra-assay precision (%)
Capecitabine	25.0	21.8	± 13.0	≤ 17.8	± 6.2	≤ 5.2
	90.0	91.6	± 1.8	≤ 3.4	± 0.9	≤ 3.0
	625	628	± 0.5	≤ 1.5	± 0.4	≤ 2.5
	1500	1473	± 1.8	≤ 2.7	± 1.2	≤ 1.9
5'-dFCR	10	10.34	± 3.5	≤ 16.9	± 11.2	≤ 2.0
	37.5	38.5	± 2.7	≤ 8.5	± 4.9	≤ 2.9
	250	257.0	± 2.8	≤ 5.0	± 2.7	≤ 3.6
	624	651	± 4.4	≤ 5.3	-*	≤ 2.7
5'-dFUR	10	9.1	± 8.5	≤ 13.1	± 4.3	≤ 5.5
	37.5	37.17	± 0.9	≤ 3.8	± 2.5	≤ 2.2
	250	259	± 3.6	≤ 4.2	-*	≤ 4.1
	625	657	± 5.1	≤ 5.8	-*	≤ 2.0
5-FU	10	9.5	± 4.9	≤ 6.3	± 2.3	≤ 2.6
	37.5	38.15	± 1.7	≤ 2.7	± 0.4	≤ 2.8
	250	258.9	± 3.5	≤ 4.3	± 0.5	≤ 2.4
	625	634.2	± 1.5	≤ 1.9	-*	≤ 2.9
FBAL	50.0	47.0	± 6.1	≤ 13.4	± 9.7	≤ 12.0
	187.5	188	± 0.1	≤ 1.4	-*	≤ 7.3
	1250	1291	± 3.3	≤ 7.5	± 2.9	≤ 7.3
	3125	3236	± 3.6	≤ 7.0	± 3.4	≤ 5.4

* Inter-run precision could not be calculated (mean square between group is less then mean square within group). Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-Fluoruracil; FBAL, α-fluoro-β-alanine. Accuracy and precision were established in 5 replicates of each validation sample concentration in 3 separate analytical runs.

2.4. Validation

Calibration standards, including standards without internal standard nor analytes (double blank), a standard only containing an internal standard (blank) were analyzed at the beginning and the end of 3 separate analytical runs. Linear regression was applied (area ratio of 5-FU and FBAL vs the concentration) with a weighting factor of $1/x^2$, where x is the concentration of the analyte. The calibration data of capecitabine, 5'-dFUR, and 5'-dFCR quadratic fits were applied with a weighting factor of $1/x$. The assay was validated over the range from 25.0 to 2,500 ng/mL for capecitabine, 10.0 to 1,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU and 50 to 5,000 ng/mL for FBAL in human plasma. For all compounds at all concentration levels, deviations of measured concentrations from nominal concentrations ranged from -13.4% and 5.8%, with a maximum coefficient of variation (CV) value of 9.7%, and 8.2% for concentrations above LLOQ and at LLOQ, respectively. Correlation coefficients were 0.9964 or better for all compounds.

Five replicates of each validation sample concentration (LLOQ, low, mid and high) in human lithium heparinized plasma in three separate analytical runs were used to analyze and validate the accuracy and precision of this method. The intra-run bias should be within ±20% for the LLOQ and within ±15% for the other concentrations. A one-way ANOVA was used to estimate the intra- and inter-assay precision. The assay performance data (inter-assay accuracies and precisions) for capecitabine and its metabolites are listed in Table 2. The intra-assay accuracies ranged from 17.8% to 16.9%, and from 3.8% to 8.5% at LLOQ and at higher concentrations, respectively. The maximum intra-assay precision values were 0.4% and 12.0% at LLOQ and at the higher levels. Ultimately, the accuracy and precision for all compounds were within the acceptance criteria.

Analytes were separately spiked in control lithium heparinized plasma at their ULOQ concentration to assess cross-analyte interference. To assess potential interferences from internal standards separate samples were prepared by spiking control lithium heparinized plasma at the assay concentration. The relative interference for all analytes was ≤20% except for the following ULOQ samples: capecitabine (342.6% of the LLOQ area of 5'-dFCR and 37.0% of 5'-dFUR) and 5'-dFCR (897.6% of the LLOQ of DFUR). The impact of the interferences has been investigated by calculating the maximum tolerable ratio's (interference ≤20%). The ratios for capecitabine/5'-dFCR, capecitabine/5'-dFUR, and 5'-dFCR/5'-dFUR were 14.6, 135, and 2.22, respectively. The ratios were calculated in clinical samples (n=2148) obtained in several clinical studies and for the interference of capecitabine-5'-dFCR, capecitabine-5'-dFUR, and 5'-dFCR-5'-dFUR 99.8%, 100%, and 98.0% of the samples were within the maximum tolerable ratios. Based on this we demonstrated that cross-analyte interference was acceptable for these analytes. The relative interference for the internal standards were all ≤5.0% and within the criteria.

To analyze for the presence of endogenous interferences six different batches of double blanks and samples were separately spiked at their LLOQ. The LLOQ samples of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL were all within ±20% of their nominal concentrations in at least 4 out of 6 bathes. No interferences from endogenous material at the retention time of the analytes were observed in the blanks. Therefore, the tests for endogenous interferences were considered acceptable.

To assess the matrix effect (ion suppression), six batches of individual blank matrices at low and high concentrations in singular were investigated. The matrix effect was calculated for each lot of matrix by calculating the ratio of the peak area in the presence of matrix (measured by analyzing blank matrix spiked after extraction with analyte), to the peak area in absence of matrix (working solution of the analyte). The absolute matrix effect ranged from 2 to 60% signal reduction at both tested levels. However, SIL-ISs were able to correct for this (n = 6, RSD ranging from 0.4 – 2.2%). From data it can be concluded that the matrix effect of different matrix batches does not influence on the accuracy and precision of the method.

To determine carry-over, two double blank samples were injected after an ULOQ sample in at least three validation runs. The

Table 3: Stability of capecitabine and its metabolites tested under various conditions

Matrix	Condition	Compound	Initial concentration ng/mL	Measured concentration ng/mL	DEV (%)	CV (%)
Plasma	-70°C, 5 months	Capecitabine	90.0	97.8	8.7	2.2
			1500	1500	-1.1	4.3
		5'-dFCR	37.5	38.6	2.9	2.9
			625	640	2.3	2.9
		5'-dFUR	37.5	37.9	1.0	2.9
			625	631	0.9	1.2
		5-FU	37.5	38.0	1.2	0.9
			625	644	3.1	1.3
		FBAL	187.5	197.0	5.1	6.6
			3125	3277	4.9	1.2
Final extracts	2-8°C, 10 days	Capecitabine	90.0	95.2	5.8	4.4
			1500	1537	2.4	4.4
		5'-dFCR	37.5	38.2	1.8	0.2
			625	636	1.8	2.2
		5'-dFUR	37.5	36.8	-1.8	1.8
			625	634	1.4	2.7
		5-FU	37.5	37.2	-0.9	1.3
			625	634	1.4	0.9
		FBAL	187.5	192.7	2.8	1.6
			3125	3193	2.2	1.2
Dry extracts	2-8°C, 7 days	Capecitabine	90.0	94.0	2.6	1.8
			1500	1573	4.9	3.2
	2-8°C, 20 days	5'-dFCR	30.0	30.8	2.6	2.4
			750	702	-6.4	1.7
	2-8°C, 7 days	5'-dFUR	37.5	38.7	3.2	0.7
			625	665	6.5	3.1
	2-8°C, 7 days	5'-FU	37.5	37.3	-0.5	1.7
			625	646	3.4	2.1
	2-8°C, 7 days	FBAL	187.5	184.0	-1.9	6.6
			3125	3327	6.5	5.1
Freeze (-70°C) / Thaw (RT)	3 cycles	Capecitabine	90.0	101.3	12.6	1.9
				1545	3.0	2.2
			1500			
		5'-dFCR	37.5	39.1	4.3	3.5
			625	660	5.6	3.0
		5'-dFUR	37.5	38.2	1.7	2.5
			625	627	0.3	3.0
		5'-FU	37.5	37.4	-0.2	1.6
			625	627	0.4	1.3
		FBAL	187.5	190.5	1.6	3.5
			3125	3265	4.5	1.6

Abbreviations: 5'-dFCR, 5'-deoxy-5-fluorocytidine; 5'-dFUR, 5'-deoxy-5-fluorouridine; 5-FU, 5-Fluoruracil; CV, Coeffience of variance; DEV, Deviation; FBAL, α-fluoro-β-alanine.

response in the first two blank matrices at the retention time of the analytes and the internal standards was less than 1.6% (0.3% for the IS) of the mean response at the LLOQ for tested analytes. In conclusion, the carry-over was satisfactory.

To assess the stability, several experiments were performed. Analytes were considered stable if the determined concentration did not deviate more than ±15% from the nominal concentrations. Previously, benchtop stability of capecitabine and its metabolites in plasma was shown at ambient temperature for at least 6 h (Deenen et al. 2013). Table 3 shows the results of the stability experiments. The deviation after three freeze (-70 °C) /thaw (room temperature) cycles was within ±15% of the nominal concentrations. The stability of the final extract was evaluated after 10 days at 2-8 °C in processed human lithium heparinized plasma samples. The deviation for low concentrations was less than or equal to 5.8% of the nominal concentration for all analytes and the CV was less than or equal to 4.4%. The stability of the dried extract was evaluated after 7 days at 2-8 °C and the deviation for CAP, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL at both the low and high concentrations were less than or equal to 6.5%. Therefore, it is concluded that samples are stable in dry extract plasma samples after at least 7 days at 2-8 °C for capecitabine, 5'-dFUR, 5-FU, and FBAL and at least 20 days at 2-8 °C for 5'-dFCR. The long-term stability was studied for up to 5 months at -70 °C and showed that the deviation was less or equal to 8.7% of the nominal concentration for all analytes, indicating that the samples are stable when stored for at least 5 months under these conditions.

2.5. Clinical application

The presented method was successfully used in support of a large clinical trial (NCT02324452)(Henricks et al. 2018), where patients were treated with fluoropyrimidine-based chemotherapy to determine plasma concentrations of capecitabine, 5'-dFUR, 5'-dFCR, 5-FU, and FBAL. Plasma lithium-heparin samples were collected after approval by the medical ethical committee and informed consent of

all patients. Plasma samples were taken on day 1 prior to treatment with fluoropyrimidines (pre-dose), and after 0.25, 0.5, 1, 2, 3, 4, 6, and 8 hours after administration. A representative plasma concentration-time curve from pharmacokinetic analyses of a colorectal cancer patient treated with 1500 mg capecitabine is presented in Fig. 3. Pharmacokinetic data of capecitabine and its metabolites has the potential the be used for the monitoring and even dose-individualization of fluoropyrimidine-based chemotherapy. It has been shown that 5-FU plasma concentrations are correlated to the onset of severe fluoropyrimidine-related toxicity (Gamelin et al. 2008; Milano et al. 1994). In addition, exposure to FBAL has shown to be associated with the incidence of severe (grade 3-4) capecitabine-induced diarrhea (Gieschke et al. 2003). These data show the value of pharmacokinetic follow-up of patients treated with fluoropyrimidine-based chemotherapy and the utility for the simultaneous quantification of capecitabine, 5'-dFUR, 5'-dFCR, 5-FU, and FBAL.

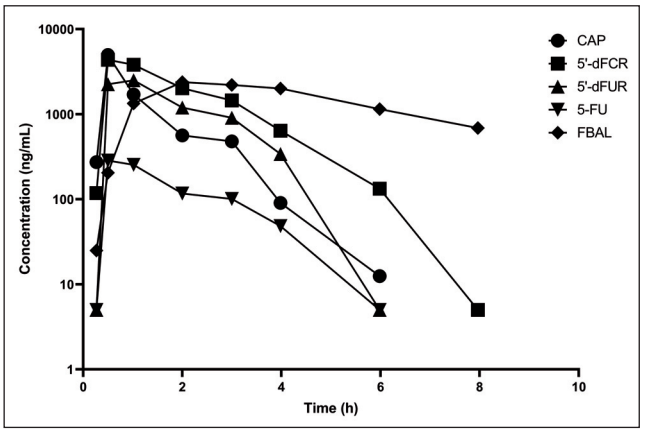

Fig. 3: Representative pharmacokinetic curves of capecitabine and its metabolites in a colorectal cancer patient treated with 1500 mg capecitabine. Abbreviations: 5'-dFCR: 5'-deoxy-5-fluorocytidine, 5'-dFUR: 5'-deoxy-5-fluorouridine, 5-FU: 5-Fluoruracil, CAP: Capecitabine, FBAL: α-fluoro-β-alanine.

2.6. Conclusion

An accurate, sensitive, and robust UPLC-MS/MS assay for the quantification of capecitabine, 5'-dFUR, 5'-dFCR, 5-FU and FBAL in human plasma was developed and validated. Sample pretreatment consists of protein precipitation in combination with evaporation and reconstitution. The validated concentrations were from 25 to 2,500 ng/mL for capecitabine, from 10 to 1,000 ng/mL for 5'-dFCR, 5'-dFUR and 5-FU and from 50 to 5,000 ng/mL for FBAL. Stable isotope labeled internal standards were used for all analytes. The stability of all analytes was adequate at 2-8 °C, at -70 °C, and after 3 free/thaw cycles. Examined assay validation parameters fulfilled the acceptance criteria of the US Food and Drug Administration (European Medicines Agency 2011b; U.S. Department of Health and Human Services Food and Drug Administration 2018). The developed assay allows for robust and reliable quantitative analysis of capecitabine, 5'-dFCR, 5'-dFUR, 5-FU, and FBAL in plasma samples and has shown its clinical applicability.

3. Experimental

3.1. Chemicals and materials

Capecitabine ($C_{15}H_{22}FN_3O_6$), 5'-dFCR ($C_9H_{12}FN_3O_4$), 5'-dFUR ($C_9H_{11}FN_2O_5$), 5-FU ($C_4H_3FN_2O_2$), FBAL ($C_3H_6FNO_2$), Capecitabine-d_{11} ($C_{15}H_{11}FN_3O_6$-$^2H_{11}$), 5'-DFCR-$^{13}C^{15}N_2$ ($C_8H_{12}FNO_4$-$^{13}C^{15}N_2$), 5'-DFUR-$^{13}C^{15}N_2$, ($C_8H_{11}FO_5$-$^{13}C^{15}N_2$), 5-FU-$^{13}C^{15}N_2$ ($C_3H_3FO_2$-$^{13}C^{15}N_2$), FBAL-$^{13}C_3$ (H_6FNO_2-$^{13}C_3$) were purchased from Toronto Research Chemicals Inc. (North York, TO, Canada). Acetonitrile (Supra-Gradient grade and ULC/MS grade), formic acid (ULC/MS grade), water (ULC/MS-grade), and methanol (Supra-Gradient grade) were purchased from Biosolve Ltd. (Valkenswaard, The Netherlands). Blank human lithium heparinized plasma was purchased from Bioreclamation (Hicksville, NY, USA).

3.2. Preparation of stock and working solutions

Two sets of stock solutions for capecitabine and its metabolites, which were used for the calibration standards and quality control samples, were prepared from two independent weightings. Both were prepared by weighing approximately 1.0 mg of each analyte and adding an appropriate amount of water to obtain a 1.0 mg/mL stock solution. The working solutions were prepared separately by further diluting the stock solutions in water. Eight working solutions were prepared containing all analytes. Working solutions were prepared with concentrations ranging from 500 ng/mL to 50,000 ng/mL for capecitabine, 200 ng/mL to 20,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU, and 1,000 ng/mL to 100,000 ng/mL for FBAL. The stock solutions for the quality control samples were diluted with water to obtain five working solutions. Working solutions were prepared with concentrations of 500, 1,500, 12,500, 37,500, and 50,000 ng/mL for capecitabine, 200, 600, 5,000, 15,000, and 20,000 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU, and 1,000, 3,000, 25,000, 75,000, and 100,000 ng/mL for FBAL. IS stock solutions were prepared by weighing approximately 1.0 mg of each IS. For capecitabine-d_{11} and FBAL-$^{13}C_3$ an appropriate amount of water is added to obtain stock solutions of 1.0 mg/mL. For 5'-DFCR-$^{13}C^{15}N_2$, 5'-DFUR-$^{13}C^{15}N_2$, and 5-FU-$^{13}C^{15}N_2$ an appropriate amount of dimethyl sulfoxide (DMSO) was added to obtain a 1.0 mg/mL separately prepared stock solutions. IS working solutions were made by diluting the IS stock solutions in water. A final concentration of 3,750 ng/mL was obtained for capecitabine--d_{11}, 1,500 ng/mL for 5'-DFCR-$^{13}C^{15}N_2$, 5'-DFUR-$^{13}C^{15}N_2$ and 5-FU-$^{13}C^{15}N_2$, and 7,500 ng/mL for FBAL-$^{13}C_3$. All stock and working solutions were stored at -20 °C.

3.3. Preparation of calibration standards and quality control samples

Both calibration standards and quality control samples were prepared by adding 200 µL of the appropriate working solution to 3800 µL in control lithium heparinized plasma, followed by short vortex mixing. Calibration standards were prepared at concentrations of 2,500, 1,800, 1,250, 625, 250, 125, 62.5, 25 ng/mL for capecitabine, 1000, 750, 500, 250, 100, 50, 25 and 10 ng/mL for 5'-dFCR, 5'-dFUR and 5-FU, 5000, 3750, 2500, 1250, 500, 250, 125 and 50 ng/mL for FBAL. For capecitabine, the quality control samples were prepared at concentrations of 1,875, 625, 75, and 25 ng/mL. The working solutions for the quality control samples were further diluted to concentrations of 750, 250, 30, and 10 ng/mL for 5'-dFCR, 5'-dFUR, and 5-FU. The quality control samples were prepared at concentrations of 3750, 1250, 150, and 50 ng/mL for FBAL by adding 200 µL of the appropriate working solution to 3800 µL in control lithium heparinized plasma. Both calibrations standards and quality control samples were stored in replicates of 300 µL in 2.0 mL tubes at -70°C until use.

3.4. Sample preparation

To 300 µL of sample aliquots, 20 µL of the internal standard working solution (Using stable isotope labeled (SIL) internal standards) for 5'-DFCR-$^{13}C^{15}N_2$, 5'-DFUR-$^{13}C^{15}N_2$, 5-FU-$^{13}C^{15}N_2$ and in H_2O for Capecitabine-d_{11} and FBAL-$^{13}C_3$) was added. Subsequently, proteins were precipitated with 900 µL of methanol:acetonitrile 1:1 (v/v), followed by short vortex mixing, 10 min of automatic shaking at 1,250 rpm, and centrifuging at 14,000 rpm for 10 min at room temperature. Thereupon, the supernatant was evaporated in the turbo evaporator. Afterward, the dry extract was dissolved in 100 µL of 0.1% formic acid in water. This was followed by short vortex mixing and centrifuging at 14,000 rpm at 4°C. The supernatant was then transferred to a vial with an insert or to a capped 96 wells plate.

3.5. Liquid chromatography – mass spectrometry

Capecitabine and its metabolites were separated using an Acquity UPLC HSS T3 column, 150 mm x 2.1 mm ID, particle size 1.8 µm (Waters Corp., Milford, MA, USA) protected with a 0.2 µm filter at a temperature of 30 °C. Chromatographic separation was achieved by using a gradient consisting of mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) at a constant flow rate of 300 µL/min. The gradient started for the first 2.50 min with 100% mobile phase A after which the proportion of mobile phase B increased linearly to 90% until 7.50 min. At t=7.5 min, the column was brought back to its original state of 100% mobile phase A. The total run time was 9.0 min. Detection of the analytes was performed on a quadruple trap mass spectrometer with a Turbo Ion Spray Interface (Q-trap 5500 triple quadrupole, Sciex, Framingham, M, USA). Detection was performed in the negative ion mode for FBAL, 5-FU, and 5'-DFUR and positive ion mode for 5'-DFCR and CAP. Nebulizing gas, turbo gas, collision gas, and curtain gas were set to 50, 50, 6, and 40 arbitrary units, respectively. The source temperature was 600 °C. Other mass spectrometric settings are shown in Table 1.

3.6. Validation

The validation of the assay was based on the current EMA and FDA guidelines on bioanalytical method validation (European Medicines Agency, 2011a; U.S. Department of Health and Human Services Food and Drug Administration 2018). The following validation parameters were assessed: calibration model, accuracy and precision, specificity and selectivity, matrix effect, carry-over, and stability. This assay has been used successfully in support of a large clinical trial (NCT02324452)(Henricks et al. 2018) thereby showing clinical applicability.

Conflicts of interest: The authors declare no conflict of interest.

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