ANALYTICAL METHOD FOR THE DETERMINATION OF CAPECITABINE AND ITS METABOLITES (5'-DFCR, 5'-DFUR AND 5-FU) IN HUMAN PLASMA (2 - 500 ng/mL) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY

Andrii Piatkivskyi[1], Toshiyuki Matsumoto[1], Manuel Acance[1],John Chen[2], Jenny Lin[1] and Noriaki Tatsuta[2]

[1] CMIC, Inc. 2860 Forbs Avenue, Hoffman Estates, IL 60192; [2] Synta Pharmaceuticals Corp. 45 Hartwell Avenue, Lexington, MA 02421‡

‡ Currently Madrigal Pharmaceuticals 500 Office Center Drive, Suite 400 Fort Washington, PA 19034.

Overview

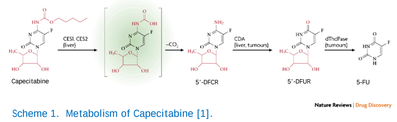
► The LC-MS/MS method for determination of Capecitabine and its metabolites (5'-DFCR, 5'-DFUR and 5-FU) in human plasma was developed and fully validated. The validation was carried out with respect to selectivity, matrix effects, calibration curve, intra- and inter-assay precision and accuracy, recovery, stability in plasma samples (freeze/thaw, short-term and long-term), post-preparative stability (batch length, re-injection and extract stability), stock solution stability, working solution stability, whole blood stability, dilution integrity, determination of lower limit of quantitation (LLOQ), and carryover.

► Each typical validation run included test samples and two sets of matrix blank samples (MB), a zero samples (Z, MB spiked with IS only), calibration standards, and QC samples. To evaluate the carryover MB was injected after the ULOQ sample.

► A variation of the high-throughput method for the analysis of Capecitabine, 5'-DFCR, 5'-DFUR, and 5-FU within a single run is presented for the discussion of future work and directions.

Introduction

5-Fluorouracil (5-FU) is well-known anti-cancer drug, widely used for decades against a variety of solid tumors. 5-Fluorouracil has a narrow therapeutic index, there is a very little difference between the theoretical minimum effective dose and the maximum tolerated dose, and therefore it needs to be administrated intravenously to control therapeutic dosage [2]. Capecitabine (Xeloda) is a prodrug with peroral administration; it is a novel tumor-activated and tumor-selective fluoropyrimidine carbamate derivative which has reduced gastrointestinal toxicity and high tumor selectivity [1]. It was designed to generate 5-Fluorouracil (5-FU) through the multiple metabolism steps (Scheme 1).



Scheme 1. Metabolism of Capecitabine [1].

The enzymatic bioconversion pathway initiates in the liver, where human carboxylesterases 1 and 2 (CES1 and CES2) cleave the ester bond of the carbamate. This is followed by a fast, spontaneous decarboxylation reaction resulting in 5'-DFCR formation. Generation of the parent drug (5-FU) continues in the liver, and to some extent in tumors, by cytidine deaminase (CDA), which converts 5'-DFUR. Finally, thymidine phosphorylase (ECGF1) releases the active drug 5-FU in the tumor [1].

In current study we had developed and validated a reliable and robust method for the analysis of Capecitabine and three of its metabolites.

There were multiple methods previously reported in the literature. A. Salvador *et al.* managed to separate all four analytes in a single run using single extraction procedure from human plasma. The simultaneous extraction of analytes was performed on an Atoll XWP solid phase support. They published an extensive overview of the different SPEs and evaluated their suitability. Separation and detection were performed by liquid chromatography (Waters Atlantis C18 reversed-phase column) and AB Sciex API 365 triple quadrupole mass spectrometer equipped with "Turbo spray" operated in negative ionization mode. The total run time for an LC-MS/MS analysis was 14 min per sample [3]. D. Montange *et al.* simultaneously detected all compounds using ionization mode polarity switching. Although this approach improved overall method sensitivity, the analytical run time was still 15 minutes. Compounds were extracted *via* a classic liquid–liquid extraction (LLE). Chromatographic analysis was performed on Thermo Accela HPLC using Polaris C18 reverse-phase column with detection by APCI LC-MS/MS. The method was fully validated following Food and Drug Administration (FDA) recommendations [4]. Hermes Licea-Perez *et al.* in their publication concentrated on the detection of only 5-FU and Capecitabine, as well as *α*-fluoro-*β*-alanine (FBAL) as a byproduct of metabolism, which induces cardiotoxicity and neurotoxicity. This approach excluded the most complex task of resolving 5'-DFUR and 5'-DFCR, which leaded to a higher throughput and great dynamic range. The analytes were extracted by LLE using a mixture of acetonitrile and ethyl acetate and modified with dansyl group. 5-FU and Capecitabine were further purified using LLE with methyl tertiary-butyl ether (MTBE) and analyzed using a reversed-phase column "Primesep D" with embedded basic ion-pairing groups and AB Sciex API 5000 mass spectrometer. The method was validated over the concentration ranges of 10–10,000, 5–5,000, and 1–1,000 ng/mL for FBAL, 5-FU, and Cape, respectively. Due to relatively small volume of plasma (50 µL) and relatively short LC run time (4.5 minutes for 5-FU/Capecitabine and 2.5 minutes for FBAL) the throughput of the method is fairly high [5]. Dhananjeyan *et al.* described a method for analysis of Capecitabine, 5-DFCR, 5-DFUR, and 5-FU in mouse plasma using protein precipitation and HPLC–UV detection, however the LLQ of the assay was 1,000 ng/mL and the run time was 10 minutes [6].

In this project we attempted to combine previous experience and expertise to create reliable, sensitive, high-throughput LC-MS/MS method. We highlighted three main challenges to overcome from the previous work. First challenge is a finding of compromised solution regarding detection mode, because Capecitabine and 5'-DFCR demonstrated higher sensitivity in positive ion mode, while 5'-DFUR and 5-FU had higher sensitivity in negative ion mode. Second challenge is a separation of 5'-DFCR and 5'-DFUR, which are very similar in structure, thus polarity. And last one is a separation of 5-FU from an endogenous plasma components with the same MRM transitions. Details of the method development are described in the discussion section.

Results

► **Sample Treatment Procedure:**
Analytical samples (plasma 100 µL) were spiked with 25 µL of isotopically-labeled IS, followed by protein precipitation with 500 µL of acetonitrile. The samples were vortexed and centrifuged at 2,000×g, 4°C, for 5 minutes. Approximately 500 µL of supernatant was transferred and evaporated at 40°C, for approximately 60 minutes, and the residue was reconstituted in 100 µL of water.

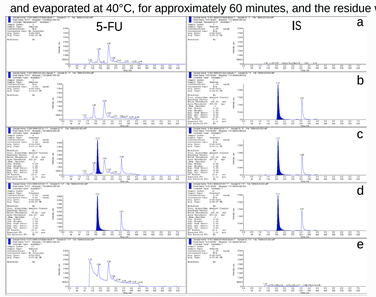


Figure 1. Representative chromatograms of 5-FU and IS: (a) MB, (b) Z, (c) S1 (2 ng/mL), (d) S9 (500 ng/mL), (e) MB after ULOQ to evaluate carryover.

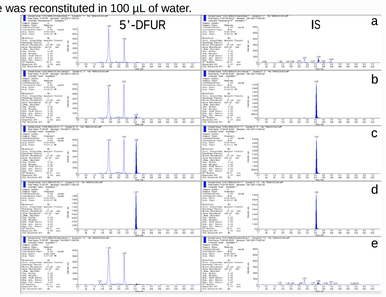


Figure 2. Representative chromatograms of 5'-DFUR and IS: (a) MB, (b) Z, (c) S1 (2 ng/mL), (d) S9 (500 ng/mL), (e) MB after ULOQ to evaluate carryover.

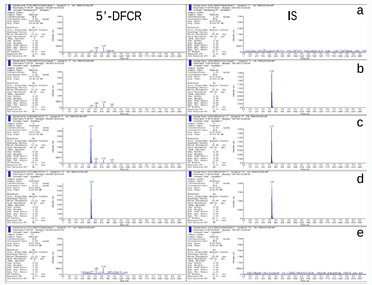


Figure 3. Representative chromatograms of 5'-DFCR and IS: (a) MB, (b) Z, (c) S1 (2 ng/mL), (d) S9 (500 ng/mL), (e) MB after ULOQ to evaluate carryover.

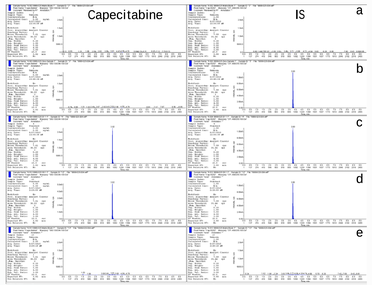


Figure 4. Representative chromatograms of Capecitabine and IS: (a) MB, (b) Z, (c) S1 (2 ng/mL), (d) S9 (500 ng/mL), (e) MB after ULOQ to evaluate carryover.

Analytes: 5-FU, 5'-DFUR

Time (min)	Gradient Conditions: Mobile Phase A (%)	Mobile Phase B (%)
Initial	100	0
0.50	100	0
2.50	85	15
4.00	85	15
4.10	5	95
6.00	5	95
6.10	100	0
7.50	100	0

Divert Valve Position	
Time (min)	Valve Position
0.0	Divert
1.3	MS
4.5	Divert

MS Conditions	
Ionization mode	APCI
Polarity/Scan type	Negative/(MRM)
Curtain gas (CUR)	Nitrogen, 20 psi
Nebulizer current (NC)	-1 µA
Temperature (TEM)	400°C
Nebulizer gas (GS1)	Dry air, 40 psi
Collision gas (CAD)	Nitrogen, 8

Monitoring ions:

Compound	Precursor Ion (m/z)		Product Ion (m/z)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
5-FU	128.9 [M-H]-	→	42.0	300	-80	-20	-9
5-FU-IS	131.8 [M-H]-	→	44.0	300	-90	-28	-9
5'-DFUR	244.8 [M-H]-	→	171.0	25	-100	-18	-7
5'-DFUR-IS	248.1 [M-H]-	→	173.9	25	-95	-20	-10

Analytes: 5'-DFCR and Capecitabine

Time (min)	Gradient Conditions: Mobile Phase A (%)	Mobile Phase B (%)
Initial	95	5
0.50	95	5
2.40	65	35
2.75	50	50
4.75	50	50
5.00	5	95
7.00	5	95
7.50	95	5
9.50	95	5

Divert Valve Position	
Time (min)	Valve Position
0.0	Divert
1.5	MS
4.8	Divert

MS Conditions	
Ionization mode	APCI
Polarity/Scan type	Positive/(MRM)
Curtain gas (CUR)	Nitrogen, 40 psi
Nebulizer current (NC)	3 µA
Temperature (TEM)	300°C
Nebulizer gas (GS1)	Dry air, 40 psi
Collision gas (CAD)	Nitrogen, 8

Monitoring ions:

Compound	Precursor Ion (m/z)		Product Ion (m/z)	Dwell (msec)	DP (V)	CE (V)	CXP (V)
5'-DFCR	246.1 [M+H]+	→	130.0	50	36	25	15
5'-DFCR-IS	249.1 [M+H]+	→	133.2	50	120	27	14
Capecitabine	360.1 [M+H]+	→	244.1	50	80	17	15
Cap-IS	371.4 [M+H]+	→	255.1	50	80	17	15

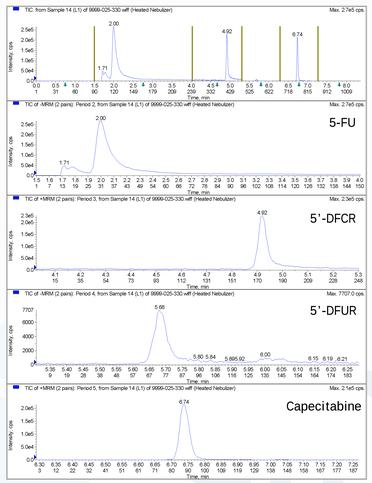


Figure 5. Combined method LQC (6ng/mL): (a) TIC and (b) XIC of 5-FU, (c) 5'-DFCR, (d) 5'-DFUR, and (e) Capecitabine.

Species	Human
Specificity	6 Lots Plasma from 3 Males and 3 Females (Un-pooled)
Matrix (volume)	Plasma (100 µL)
Analyte	Capecitabine 5'-Deoxy-5-fluoro Cytidine (5'-DFCR) 5'-Deoxy-5-fluorouridine (5'-DFUR) 5-Fluorouracil (5-FU)
Internal standard (IS)	Capecitabine-D_{11} (Cap-IS) 5'-Deoxy-5-fluorocytidine-^{13}C, $^{15}N_2$ (5'-DFCR-IS) 5'-Deoxyfluorouridine-^{13}C, $^{15}N_2$ (5'-DFUR-IS) 5-Fluorouracil-^{13}C, $^{15}N_2$ (5-FU-IS)
Analytical method	LC-MS/MS
Lower limit of quantification (LLOQ)	2 ng/mL
Quantitative working range	2 ng/mL to 500 ng/mL
Regression, Weighting	Linear $1/x^2$ (r >0.9900 [r^2 >0.9801])
Standard curve concentrations	2, 4, 10, 25, 50, 125, 250, 450 and 500 ng/mL
Quality control levels	2 ng/mL (LLQC), 6 ng/mL (LQC), 200 ng/mL (MQC), 400 ng/mL (HQC) and 2,500 ng/mL (DQC).

QC sample with 5'-DFUR only: 10,000 ng/mL (QC-DFUR)

Intra-assay precision and accuracy		Precision		Accuracy	
	QC Sample	Capecitabine	5'-DFCR	Capecitabine	5'-DFCR
	LLQC	3.2-4.9%	3.8-4.3%	97.5-99.0%	93.0-97.5%
	LQC	1.9-9.2%	1.1-9.3%	94.8-101.2%	98.3-103.8%
	MQC	1.1-5.4%	1.9-5.3%	94.0-101.0%	96.0-99.0%
	HQC	1.8-3.9%	2.5-3.2%	96.2-99.7%	93.7-96.7%
	QC Sample	Precision 5'-DFUR	5-FU	Accuracy 5'-DFUR	5-FU
	LLQC	6.1-10.8%	0.9-1.6%	92.0-95.5%	93.0-100.0%
	LQC	3.6-12.0%	0.7-9.8%	98.5-102.5%	96.2-101.2%
	MQC	3.2-5.1%	1.6-6.2%	100.0-105.0%	100.0-105.5%
	HQC	2.2-6.9%	1.2-1.7%	98.0-106.8%	98.0-102.8%

Inter-assay precision and accuracy	QC Sample	Precision Capecitabine	5'-DFCR	Accuracy Capecitabine	5'-DFCR
	LLQC	3.9%	4.6%	98.0%	96.0%
	LQC	6.0%	6.1%	98.0%	100.5%
	MQC	4.8%	3.9%	98.0%	98.0%
	HQC	3.5%	2.9%	97.5%	95.0%

Inter-assay precision and accuracy (Continued)	QC Sample	Precision 5'-DFUR	5-FU	Accuracy 5'-DFUR	5-FU
	LLQC	8.4%	3.4%	93.5%	97.0%
	LQC	7.6%	5.9%	101.2%	98.5%
	MQC	4.6%	4.6%	102.0%	102.5%
	HQC	6.5%	2.5%	101.3%	100.8%

Selectivity for individual matrix and pooled matrix spiked with concomitant drugs	No interfering peaks greater than acceptable limits observed at retention times of interest
Matrix effect	%CV of the IS normalized factor less than 15%
Recovery	%CV less than or equal to15%
Stability in plasma – Freeze/thaw	5 cycles thawed at refrigerated conditions (REF) and stored at -20°C and -80°C
Stability in plasma – Short-term	Capecitabine 26 hours at REF 5'-DFCR 25 hours at REF 5'-DFUR 26 hours at REF 5-FU 25 hours at REF **QC-DFUR 25 hours at REF**
Stability in plasma – Long-term	Capecitabine 3 months (90 days) at -20°C and -80°C 5'-DFUR 3 months (90 days) at -20°C and -80°C 5'-DFCR 3 months (90 days) at -20°C and -80°C 5-FU 3 months (90 days) at -20°C and -80°C **QC-DFUR 3 months (90 days) at -20°C and -80°C**
Post-preparative stability-Batch length	120 samples injection
Post-preparative stability -Re-injection	Capecitabine 87 hours at 5°C 5'-DFCR 87 hours at 5°C 5'-DFUR 90 hours at 5°C 5-FU 90 hours at 5°C **QC-DFUR 93 hours at 5°C**
Post-preparative stability-Extract	Capecitabine 85 hours at 5°C 5'-DFCR 85 hours at 5°C 5'-DFUR 74 hours at 5°C 5-FU 74 hours at 5°C **QC-DFUR 74 hours at 5°C** Capecitabine 24 hours at RmT and 3 months (91 days) at 5°C 5'-DFCR 24 hours at RmT and 3 months (91 days) at 5°C 5'-DFUR 24 hours at RmT and 3 months (91 days) at 5°C 5-FU 24 hours at RmT and 3 months (91 days) at 5°C Capecitabine 24 hours at RmT and 3 months (91 days) at 5°C 5'-DFCR 24 hours at RmT and 3 months (106 days) at 5°C 5'-DFUR 22 hours at RmT and 3 months (112 days) at 5°C 5-FU 24 hours at RmT and 3 months (91 days) at 5°C
Whole blood stability	2 hours at REF
Dilution integrity	2,500 ng/mL diluted 10-folds and 100-folds
Determination of LLOQ	2 ng/mL
Carryover	Met acceptance criteria

Discussion

In the current project we presented a novel method for determination of Capecitabine and its metabolites (5'-DFCR, 5'-DFUR and 5-FU) in human plasma. The analytical method was validated at CMIC, Inc. in compliance with Food and Drug Administration (FDA) recommendations. All of the validation parameters evaluated met acceptance criteria, as described in the approved validation plan and applicable CMIC SOPs. In conclusion, this analytical method is sufficiently reliable for the determination of Capecitabine and its metabolites (5'-DFCR, 5'-DFUR and 5-FU) in human plasma over the dynamic range of 2 ng/mL to 500 ng/mL.

Current method was designed to be robust and reliable, thus it was decided to conduct analysis in two separate runs to achieve the best sensitivity for each analyte and to eliminate potential cross-talk. Capecitabine and 5'-DFCR were analyzed in the positive ion mode, 5'-DFUR and 5-FU were analyzed in the negative ion mode, respectively. The APCI source has been chosen over ESI in order to reduce matrix effect. The overall sensitivity of the system was affected, but it was compensated with improved signal to noise ratio. Even though current method was fully validated the methodology can be improved and requires further work to eliminate potential problems in sample analysis.

Through the development process we obtained some promising results which would help to improve efficiency of the method. We are currently working on the method which could monitor all analytes within one 8.5 minute run (vs. 14 min A. Salvador *et al.*) **Fig. 5**. In order to obtain maximum sensitivity for all analytes we attempted to develop LC-MS/MS method which would utilize polarity switch, to detect analytes in the ionization modes they had demonstrated the best sensitivity, within a single run. Once working in polarity-switching mode, the AB Sciex Analyst 1.5.1 software generated broad 5-FU peak based on compound's TIC, which might potentially affect quantitation results during sample analysis. The newest version of the software has a feature "Scheduled MRM", which was designed specifically to handle analysis with polarity switching.

To address the separation challenge we designed a complex gradient. The elution gradient should start with 100% of mobile phase A (0.1% FA in water) in order to delay the elution of 5-FU and separate it from an endogenous plasma components, then slowly increase the content of organic solvent to resolve 5'-DFCR and 5'-DFUR. After their elution the content of organic solvent might be increased up to 90% to elute Capecitabine and returned to initial condition for re-equilibration. The Waters Select X reversed-phase column (3.0 × 100 mm, 3.5 µm) was chosen for the method development, because its stationary phase was specifically designed to handle extreme transitions in solvent polarity. The column length and temperature (35°C), and a flow rate (0.6 mL/min) were selected to achieve the best separation.

Based on the previous findings [2, 7] special care was taken to monitor formation of 5-FU out of 5'-DFUR by analyzing concentrated QC-5'-DFUR (10,000 ng/mL) sample for the content of 5-FU at the detectable concentration level. Usually the breakdown of prodrugs to the active drug within the sample should be inhibited or stopped prior to analysis, whether by cooling down the sample or chemically as a part of sample collection or sample treatment procedures. The results of our test satisfied all requirements, therefore it was concluded that sample handling on ice is sufficient enough to prevent decomposition of 5'-DFUR to 5-FU. This conclusion needs to be revised because QC-5'-DFUR samples were made in regular pooled plasma, but looking deeper into the Capecitabine metabolism process as opposed to the pooled plasma from healthy subjects, we might assume presence of corresponding enzymes in the sample which transforming a substrate after sample collection. In the case of 5-FU it was reported in [7] that addition of 0.3 nM of 5-Nitrouracil or 20 mM of Citric Acid to the sample might dramatically inhibit 5'-DFUR →5-FU conversion. It was determined that the collection procedure should include spiking of harvested plasma with citric acid prior to samples being frozen [7], so current findings should be adopted in the future work. Also, regarding the sample treatment, it is more beneficial to use SPE over protein precipitation only. This approach might be less cost-effective and more time-consuming, but it would prevent instrumental failures and analysis delays related to the frequent instrument service and maintenance.

References

1. Rautio, Jarkko, *et al.* "Prodrugs: design and clinical applications." *Nature Reviews Drug Discovery* 7.3 (2008): 255-270.

2. Gamelin, Erick, *et al.* "Dose monitoring of 5-Fluorouracil in patients with colorectal or head and neck cancer - status of the art." *Critical reviews in oncology/hematology* 30.1 (1999): 71-79.

3. Salvador, A, *et al.* "Simultaneous LC-MS-MS analysis of Capecitabine and its metabolites (5'-Deoxy-5-Fluorocytidine, 5'-Deoxy-5-Fluorouridine, 5-Fluorouracil) after off-line SPE from human plasma." *Chromatographia* 63.11-12 (2006): 609-615.

4. Montange, Damien, *et al.* "An APCI LC-MS/MS method for routine determination of Capecitabine and its metabolites in human plasma." *Journal of Mass Spectrometry* 45.6 (2010): 670-677.

5. Licea-Perez, Hermes, *et al.* "Development of a sensitive and selective LC-MS/MS method for the determination of α-Fluoro-β-Alanine, 5-Fluorouracil and Capecitabine in human plasma." *Journal of chromatography B* 877.11 (2009): 1040-1046.

6. Dhananjeyan, Mugunthu R., *et al.* "Rapid and simultaneous determination of Capecitabine and its metabolites in mouse plasma, mouse serum, and in rabbit bile by high-performance liquid chromatography." *Journal of Chromatography A* 1138.1 (2007): 101-108.

7. McKnight, Janine, *et al.*" ISR Failure for 5-Fluorouracil Analysis from Patients Dosed with Capecitabine." Presented at the 8th Workshop on Recent Issues in Bioanalysis (WRIB) Universal City, California 10-14 March 2014.