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Simultaneous determination of capecitabine and its metabolites by HPLC and mass spectrometry for preclinical and clinical studies

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Abstract

A reverse-phase high-performance liquid chromatography method with electrospray ionization and detection by mass spectrometry is described for the simultaneous determination of capecitabine, its intermediate metabolites (DFCR, DFUR) and the active metabolite 5-fluorouracil in mouse plasma, liver and human xenograft tumours. The method was also cross-validated in human plasma and human tumour for clinical application. The method has greater sensitivity than previously published methods with an equivalent accuracy and precision. It uses less biological material (plasma, tissue) and should therefore be applicable to biopsies in patients treated with capecitabine.

Keywords: Capecitabine; 5-Fluorouracil; Quantification; Plasma; Tumour; Liver

1. Introduction

Capecitabine (N^4-pentoxycarbonyl-5′-deoxy-5-fluorocytidine, Xeloda®), is a fluoropyrimidine carbamate, which is converted in liver and tumour to the active agent 5-fluorouracil (5-FU). It is used in the chemotherapeutic treatment of patients with breast and colon cancer. Carboxylesterases (EC 3.1.1.1) located in the liver in human and in the plasma and liver in rodents convert capecitabine to 5′deoxy-5-fluorocytidine (DFCR). DFCR is then converted by cytidine deaminase (EC 3.5.4.5) both in liver and tumour to 5′deoxy-5-fluorouridine (DFUR). The formation of 5-FU from DFUR is catabolised by thymidine phosphorylase (EC 2.4.2.4), and preferential expression in tumours has been reported previously both in animal models [1] and in patients [2]. 5-FU is the active metabolite: its inhibition of thymidylate synthase (EC 2.1.1.45) and incorporation into nucleic acids are responsible for the cytotoxic activity. Extensive pharmacokinetic studies have been performed on capecitabine and its metabolites [3,4] based on phases II and III trials [4–6]. Marked inter-patient variability was observed during these studies, although pharmacokinetic parameters were not predictive of either toxicity or response to treatment [4]. Animal models have also been used to evaluate capecitabine efficacy in different xenograft models [1,7]. A physiologically-based pharmacokinetic model was developed and shown to predict accurately plasma concentrations of capecitabine and its metabolites [8].

As a single agent, capecitabine is generally used in the clinic using a twice daily administration schedule for 14 out of 21 days, but it is possible that other schedules of administration may be more beneficial for some patients. The duration of treatment may also be important. Finally, early markers of response/progression could be beneficial for patient management.

Performing preclinical studies in rodents allows the development of alternative schedules of administration linked to the determination of drug concentration in different organs. They may also be used to identify a better surrogate tissue to predict toxicity/response. However, one of their main pitfalls is the small quantities of biological material that can be recovered for pharmacokinetic studies, hence the interest in developing analytical methods using small volumes of blood and small quantities of tissue. This would also be beneficial in clinical studies when serial sampling is required. Finally, if an analytical method is validated for both rodent and human tissue, the comparison of preclinical and clinical data is facilitated.

Several HPLC methods have been developed over the recent years to study capecitabine and its metabolites. The difference in polarity between capecitabine and the active metabolite

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5-fluorouracil has so far prevented the simultaneous analysis of both compounds by HPLC: Reigner et al. in the original method analysed independently capecitabine, DFCR and DFUR by HPLC and UV detection and 5-FU using gas chromatography [9]. Subsequently, an MS–MS method was developed by the same team [10] but using a different sample extraction and chromatography conditions for capecitabine, DFCR, DFUR on one system and 5-FU and FBAL on another. More recently, a LC–MS technique has been developed for capecitabine, DFCR, DFUR but is not suitable for 5-FU determination [11]. Zufia et al. set up a method using UV detection that allows the simultaneous detection of capecitabine, DFUR, 5-FU and dihydro-5-FU in plasma [12]. Finally, Siethoff et al. were able to determine the plasma concentration of both capecitabine and its different metabolites but using a column switching and MS–MS detection [13].

We propose a new HPLC method using small volumes of biological material, validated in plasma and tissues (liver, tumour) from rodents and humans, which allows the simultaneous quantification of capecitabine and its metabolites with a single HPLC system coupled to mass spectrometry detection. The method is fully validated for both preclinical and clinical studies and can therefore be the basis for further preclinical and clinical studies with capecitabine.

2. Experimental

2.1. Chemicals and solutions

Capecitabine (batch # 26954-190A-MIL), 5′deoxy-5-fluorocytidine (DFCR) (batch # Ro 0218782-000-003), 5′deoxy-5-fluorouridine (DFUR) (batch # Ro 0219738-000-02), and 5-fluorouracil (5-FU) were provided by Hoffmann-La Roche, Basel, Switzerland. Ammonium acetate was from Sigma (Sigma, Gillingham, UK). Formic acid was from BDH (BDH, Poole, UK). HPLC grade acetonitrile was from Rathburn (Walkerburn, UK) or BDH.

2.2. Plasma and tissues from mouse and human

Human plasma was obtained from the Scottish National Blood Transfusion Service. Human colorectal tumour tissue was obtained, with patients' consent, from Prof. Dunlop (University of Edinburgh). Xenograft tissue from the human cancer cell line HCT-116 along with mouse liver and plasma were obtained from C57/Bl6 nude mice (Cancer Research UK, London). Tissues were collected in liquid nitrogen. Plasma and tissues were stored at −70 °C until analysis.

2.3. Plasma and tissue standard preparation

Solutions of capecitabine, DFCR, DFUR and 5-FU at a concentration of 1 mg ml^{-1} were prepared by dissolving the appropriate amounts of compounds in H_2O:acetonitrile (50:50, v/v) and stored at ca. +4 °C in the dark. Standard mixture working solutions of capecitabine, DFCR, DFUR and 5-FU were prepared by mixing and serial diluting the stock solutions. A separate stock for quality controls (QC) of each analyte was prepared.

Table 1
Dilutions used for the preparation of standard curves and quality controls

	Drug concentration (ng ml^{-1})				Fortification (for 50 μl or mg sample)
	Cap	DFCR	DFUR	5-FU	
0	0	0	0	0	–
Std-1	5	10	5	50	2.5 μl of S3
Std-2	10	20	10	100	5.0 μl of S3
Std-3	25	50	25	250	12.5 μl of S3
Std-4	50	100	50	500	2.5 μl of S2
Std-5	100	200	100	1000	5.0 μl of S2
Std-6	250	500	250	2500	12.5 μl of S2
Std-7	500	1000	500	5000	2.5 μl of S1
QC-1	10	20	10	100	5.0 μl of S3
QC-2	50	100	50	500	2.5 μl of S2
QC-3	500	1000	500	5000	2.5 μl of S1

S1 contained 10, 20, 10, 100 μg ml^{-1} of capecitabine, DFCR, DFUR and 5-FU. S2 and S3 are 1/10 and 1/100 dilutions of S1, respectively.

Various volumes of mixture solutions of capecitabine, DFCR, DFUR and 5-FU (Table 1) were added to 50 μl of plasma and 150 μl of acetonitrile into a 300 μl flat well 96-well microplate. For tumour and liver tissue, the mixture solutions of capecitabine, DFCR, DFUR and 5-FU were homogenised with 50 mg of tumour or liver tissue and 250 μl of 50 mM ammonium acetate: acetonitrile (1:3, v/v) into a 2.0 ml cryovial tube according to Table 1. After centrifugation at 3500 × *g* for 10 min at 4 °C, the supernatant was transferred into a 200 μl tapered-well 96-well microplate and evaporated to dryness using a centrifuge vacuum system at ∼30 °C. The dried extract was resuspended in 100 μl water and 10 μl were analysed by HPLC. Mouse plasma contains high levels of carboxylesterase [14]. QC and samples were therefore thawed on ice to avoid significant conversion of capecitabine to DFCR.

2.4. Instrumentation

The chromatographic system was a Thermo electron Surveyor 1.3 SP1 pump, Surveyor 1.3 SP1 HPLC Autosampler, and Finnigan TSQ Quantum Discovery mass spectrometer. Data were acquired and processed with Xcalibur SR1 and LC Quan 2.0 SP1 chromatography manager software.

Compounds were separated on a Develosil ODS-UG-3 column (4.6 mm × 150 mm, 3 μm) (Nomura Chemical) protected by a Waters Symmetry C18 (4.6 mm × 20 mm, 5 μm) guard cartridge. The method used gradient elution (Table 2) with a total run time of 12 min. The column was maintained at 30 °C.

Table 2
Gradient elution conditions

Time (min)	Formic acid	Water	Acetonitrile	Flow rate (ml min^{-1})
0	0.1	100	0	1
2	0.1	100	0	1
8	0.1	5	95	1
9	0.1	5	95	1
9.1	0.1	100	0	1
12	0.1	100	0	1

Table 3
Transitions and collision energies

Analyte	Capecitabine	DFCR	DFUR	5-FU
Transition	360.0 → 243.8	245.9 → 137.5 (245.9→130)	244.8 → 136.6 (244.8 → 129)	258.8 → 36.6 (129.1 → 42.4)
Collision energy (CE)	−10	−18	19	17

Confirmation transitions are mentioned in brackets.

The mass spectrometer was operated in the electrospray mode. The source temperature was 300 °C and the spray voltage 3 kV. The collision gas pressure was 0.2 Pa. All analytes were optimised using the software auto tune facility for SRM transitions (Table 3).

2.5. Quantification of capecitabine and metabolites

The peak areas of capecitabine, DFCR, DFUR and 5-FU were used to construct calibration curves using a regression analysis with $1/x$ weighting in accordance to Almeida et al. [15]. Quality control concentrations were calculated from the regressed equation.

The limit of detection (LOD) and limit of quantification (LOQ) were determined as follows: 6 blank samples from six separate subjects for each analyte and matrix were extracted and compared to a low standard of each analyte. Where an obvious peak existed at the same retention time as the analyte, a concentration was calculated for this peak. Where no discreet peak, or a series of small noise peaks existed at the same retention time as the analyte, the height of the noise was measured and compared to the height of the low standard. This provided a "concentration" for the noise. An average of the 6 "noise concentrations" was calculated and multiply by either 3 (LOD) or 5 (LOQ). LOQ values were subsequently confirmed using six replicates spiked at the target concentration as being within an acceptable variance of 20%.

2.6. Determination of recovery, accuracy and precision

The absolute recovery of capecitabine, DFCR, DFUR and 5-FU was determined by comparison of the peak areas from non-extracted and extracted samples of QC-3 (Table 1) in triplicate. The intra-day accuracy and precision were determined at three different concentrations from six replicate QC. The inter-day accuracy and precision were determined at three concentrations from six replicate QC on three independent occasions. The precision was calculated as the relative standard deviation of the mean (R.S.D.) with R.S.D. (%) = (standard deviation of the mean/mean) × 100. The accuracy was calculated as the relative mean error (RME) with RME (%) = [(mean-theoretical concentration)/theoretical concentration] × 100.

2.7. Stability

Short-term stability: three aliquots of each high, medium and low concentration QC were extracted and left at room temperature for 24 h before analysis.

Freezer stability: three aliquots at each of the low and high concentrations quality controls were stored at −70 °C for 6 months and analysed compared to fresh QC.

2.8. Cross-validation with human plasma and tumour tissues

The method was evaluated in terms of LOQ and intra-day accuracy and precision on human plasma and human tumour. Considering the difficulty in obtaining human tumour tissue, the chromatographic profile of three blank tumour tissues was analysed to determine the background signal and interfering peaks. Six human tumours were then pooled to evaluate the linearity, the accuracy and the precision. Six replicates set at the LOQ and at three concentrations were tested.

3. Results and discussion

The method was validated in terms of limits of quantification recovery, specificity, sensitivity, precision and accuracy, and stability.

3.1. Specificity

The specificity tested the ability of the method to differentiate and quantitate the analyte in the presence of other endogenous constituents in the sample and to detect potential interferences. The chromatographic separation of mouse plasma and human tumour xenograft tissue blank and spiked at the LOQ of capecitabine, DFCR, DFUR and 5-FU are presented Supplementary Fig. 1. No interfering peaks were observed and no significant peaks were found at the retention times of the analytes in plasma and tumour. However, small peaks eluting at the retention time of DFUR, DFCR and Capecitabine were detected in mouse liver and were taken into account in standard curves fitting.

3.2. Linearity

Standard curves were performed in triplicate for each analyte in plasma, tumour and liver. In all cases the regression coefficient was >0.99. Capecitabine and DFUR curves were linear over a range of 5–1000 ng ml^{-1} and DFCR over a range of 10–2000 ng ml^{-1} with a weighting on $1/x$. 5-FU response was quadratic with a weighting of $1/x$ from 50 to 10,000 ng ml^{-1}.

3.3. Sensitivity

In the plasma, the calculated limit of quantification was 4.0 ng ml^{-1} for capecitabine, 1.4 ng ml^{-1} for DFCR,

Table 4
Recovery of capecitabine, DFCR, DFUR and 5-FU in plasma, tumour and liver tissues

	Capecitabine, 500 ng ml^{-1}		DFCR, 1000 ng ml^{-1}		DFUR, 500 ng ml^{-1}		5-FU, 5000 ng ml^{-1}	
	% Recovery	% C.V.	% Recovery	% C.V.	% Recovery	% C.V.	% Recovery	% C.V.
Plasma	101.3	0.14	101.1	0.71	99.9	0.59	106.8	2.62
Tumour	78.2	1.68	90.2	4.05	88.1	5.90	97.3	2.42
Liver	94.1	4.69	103.7	4.18	103.8	2.83	116.1	2.60

3.3 ng ml^{-1} for DFUR and 45.8 ng ml^{-1} for 5-FU. In tumour tissue, the LOQ was 1.3 ng ml^{-1} for capecitabine, 1.7 ng ml^{-1} for DFCR, 0.5 ng ml^{-1} for DFUR and 50.0 ng ml^{-1} for 5-FU. In the liver tissue, the LOQ was 13.0 ng ml^{-1} for capecitabine, 3.0 ng ml^{-1} for DFCR and 92.0 ng ml^{-1} for 5-FU. Since no interference was observed for DFUR in this tissue, the LOQ could not be determined.

3.4. Recovery

The recovery was determined by calculating the concentration of the high QC processed in triplicate using a non-extracted calibration line (Table 4). The recovery in the plasma and the liver was excellent with values ranging from 99.9 ± 0.6% to 107 ± 2.6% in the plasma and 94.1 ± 4.8% to 116 ± 2.6% in the liver. No significant difference in recovery was observed among the four compounds in these two matrices. The recovery was lower in the tumour for capecitabine and DFUR: 78.2 ± 1.7% and 88.1 ± 5.9%, respectively. As recommended by Matuszewski et al. [16], evaluation of matrix effect by spiking matrix before and after extraction was performed for these two compounds in tumour tissue and concentrations calculated against non-extracted standards. Recovery from QC spiked after extraction was not significantly different from QC spiked before extraction suggesting a good extraction method. However, the recovery was lower than 100%, suggesting a limited matrix effect potentially due to an inhibition of ionization.

3.5. Intra-day accuracy and precision

The accuracy and precision were calculated from six QC at three concentrations for each compound. The results are summarised Table 5. The accuracy as determined by the relative mean error was comparable across matrices with a minimum of −12.1% for DFUR in the plasma and a maximum of 9% for 5-FU in plasma. The precision, evaluated by the relative standard deviation of the mean (R.S.D.), was similar in the plasma, tumour and the liver ranging from 2.8% for DFUR in plasma to 12.2% for 5-FU.

3.6. Inter-day accuracy and precision

The accuracy and precision were calculated from six QC at three concentrations for each compound on three independent

Table 5
Intra- and inter-day precision (R.S.D.) and accuracy (RME) for plasma, tumour and liver tissue

	Intra-day accuracy and precision						Inter-day accuracy and precision					
	Plasma		Tumour		Liver		Plasma		Tumour		Liver	
	R.S.D. (%)	RME (%)	R.S.D. (%)	RME (%)	R.S.D. (%)	RME (%)	R.S.D. (%)	RME (%)	R.S.D. (%)	RME (%)	R.S.D. (%)	RME (%)
Capecitabine												
10	3.55	2	6.77	−5.1	4.38	−6.1	6.5	2.7	9.4	−2	9.4	−3.4
50	9.51	−0.8	7.61	−8.2	9.3	−6.3	7.6	0.02	8.2	−3.1	8.5	−4.5
500	5.07	−3.6	8.24	−7.9	6.35	−0.01	7.2	0.03	7.1	−5.3	9.7	−3.6
DFCR												
20	6.51	1.1	7.4	−7.2	6.06	0.7	4.7	2.8	9.8	−0.5	7.9	5.9
100	3.22	0.9	8.02	−5.1	8.74	−8.6	3.7	4	7.5	−5.7	8	−4.5
1000	4.49	−10.3	8.21	−6.8	8.3	−4.5	7	−6.8	5.7	−7.1	9.2	−5.6
DFUR												
10	7.32	−10.3	5.05	−10.1	10.4	−3	10.1	0.5	11	0.5	8.4	4.9
50	3.67	−9.9	7.87	−8.5	6.81	−8.1	7.5	−1.2	6	−9.8	7.9	−6
500	2.81	−12.1	5.43	−0.8	6.93	−8.6	8.4	−9.2	6.6	−6.4	6.8	−8.1
5-FU												
100	3.6	9	6.21	−10.4	5.72	−7.5	5.1	8.6	8.7	−1.1	7.8	0.6
500	7.96	−0.4	8.51	−1.6	12.24	−4.8	9.3	−2.8	8.2	−3.8	10.6	2
5000	4.03	5.5	7.26	6.7	5.56	2.6	7.6	−1.7	10.7	−6	6.9	1.1

The intra-day accuracy and precision were determined at three different levels of concentrations from six replicate QC. The inter-day accuracy and precision were determined at three levels of concentrations from six replicate QC on three independent occasions.

Table 6
Stability of capecitabine, DFCR, DFUR and 5-FU in plasma, tumour and liver tissue at two to three concentrations

	Short-term stability						Long-term stability					
	Plasma		Tumour		Liver		Plasma		Tumour		Liver	
	% Recovery	CV (%)	% Recovery	CV (%)	% Recovery	CV (%)	% Recovery	CV (%)	% Recovery	CV (%)	% Recovery	CV (%)
Capecitabine												
10	112.8	2.9	91	4.4	100	8.2					n.d.	n.d.
50	103.3	4.9	93.8	4.5	103.5	7.5	111.1	2.2	112	1.8	n.d.	n.d.
500	99.5	3.9	98.7	5.2	83.7	2.9	96.8	5.7			n.d.	n.d.
1000									84.5	4.6	n.d.	n.d.
DFCR												
20	111.8	2.2	88.2	3.2	106	13					n.d.	n.d.
100	108.2	3.2	95.8	7.3	95.3	11.4	109	3.2	112	0.7	n.d.	n.d.
1000	97.6	7.8	108.6	4.8	86.4	2	95.2	5.8			n.d.	n.d.
2000									87	0.1	n.d.	n.d.
DFUR												
10	109.4	4.9	103.3	12.6	97.6	7.5					n.d.	n.d.
50	110.5	1.8	100.3	5.4	87.4	4	109.9	4.5	112	3.2	n.d.	n.d.
500	94.5	12.6	107.4	5	85.7	3.6	97.6	7.7			n.d.	n.d.
1000									83	2.3	n.d.	n.d.
5-FU												
100	107.3	4.8	93.5	14.8	95.7	15.5					n.d.	n.d.
500	88.5	5.6	95.6	11.3	109.4	10.6	82.1	11.4	92	0.4	n.d.	n.d.
5000	99.5	6.5	80.8	4.6	84.4	2.5	101.2	6.5			n.d.	n.d.
10,000									83	3.3	n.d.	n.d.

Short-term stability ($n = 6$) and long-term stability ($n = 3$) were evaluated. n.d.: not determined.

occasions. The accuracy was satisfactory in the three matrices tested with an overall value <10% for all compounds. The precision was also consistent across tissues ranging from −9.8% for DFUR in tumour to +8.6% for 5-FU in plasma.

3.7. Sample stability

Stability in a biological fluid is a function of the storage conditions, the chemical properties of the analyte, the matrix and the container system. In view of the high carboxylesterase activity in mouse plasma, QC and samples have to be thawed on ice to avoid significant conversion of capecitabine to DFCR. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling from being taken from the patient to final analysis. Therefore, we evaluated the short-term stability where samples were left in the autosampler overnight and the long-term stability when samples were stored at −70 °C for 6 months (Table 6). Capecitabine and its metabolites were stable in the conditions tested in all three matrices at the different concentrations ranging from 80.8% to 112.8%.

3.8. Cross-validation

Considering the paucity of human tumour tissue available, a cross-validation was performed based on the preclinical data, on human plasma and human colon tumour (Table 7). The limits of quantification tested were chosen as relevant for clinical purpose rather than based on a signal-to-noise ratio. The accuracy and precision were consistent between murine and human matrices.

Table 7
Cross-validation for the determination of capecitabine, DFCR, DFUR and 5-FU in human plasma and tumour tissue

	Plasma		Tumour	
	R.S.D. (%)	RME (%)	R.S.D. (%)	RME (%)
Intra-day accuracy and precision				
Capecitabine				
5 (LOQ)	7.1	−10.2	1.8	3.2
10	9.4	−2	4.7	−3.2
50	15	10.6	2.9	−2.7
500	4	3	5.7	−6.1
DFCR				
10 (LOQ)	3.4	2.5	5.2	−13.5
20	12.4	9.2	3.6	−6.5
100	3.4	1.2	2.5	0.06
1000	4.1	2.3	4.2	−8.2
DFUR				
5 (LOQ)	9.5	−1.2	7.7	8.3
10	7.5	7	1.6	−1
50	3.2	−0.05	2.8	−3.2
500	4.1	−2	5.5	−4.6
5-FU				
50 (LOQ)	6.5	6.3	7.0	10.3
100	10	−6	4.9	−9.9
500	7.2	−2	8.1	−1.8
5000	5.5	2.9	5.2	0.4

The accuracy and precision at the LOQ and at three levels of concentrations were evaluated on six replicates.

4. Conclusion

Several HPLC methods have been developed in the last few years for the quantification of capecitabine and/or its metabolites, but each has had associated limitations. In contrast to previous methods, the present method was validated in plasma, tumour and liver from both mouse and human origin. It can therefore be used both for preclinical and clinical studies. It uses small quantities of biological material (50 μl of plasma, 50 mg of tumour tissue) while previous methods used 250–500 μl of plasma and 500 mg of tissues. The amount of tissue required is compatible with quantities recovered from biopsies and is therefore potentially applicable to clinical pharmacokinetic studies investigating both plasma and tissue disposition. The parent drug and its metabolites are all analysed in the same run, reducing sample processing and variability of results in a total run time of only 12 min which allows the analysis of large series of samples and decreases the volume of solvent used.

The sensitivity of the method as expressed by the limit of quantification was superior or equivalent to previous published methods for capecitabine, DFCR and DFUR [9,13]. For 5-FU, the sensitivity was lower but is still relevant to concentrations observed in both plasma and tumour tissues in patients [17]. This may be due to the specifications of the mass spectrometer with regard to the molecular weight of the compound. Also, since 5-FU is eluted first, the high aqueous content of the mobile phase may impair the desolvation and hence the ionization process.

The reproducibility of the method was better than previously published work, which is particularly important when processing large sets of samples [11–13]. Finally, the recovery of capecitabine and its metabolites from tumour tissues was greatly improved as compared to the study performed by Schuller et al. [17].

Therefore, the present method is versatile enough to be used for both preclinical and clinical studies with capecitabine. The limited amount of biological material needed makes the method potentially usable for tissue biopsies in patients and could therefore allow the study of capecitabine and its metabolites in patients treated with capecitabine in the three main tissues where its disposition and metabolism occur: plasma, liver and tumour.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2005.09.010.

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