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Simultaneous determination of capecitabine and its three nucleoside metabolites in human plasma by high performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Capecitabine (Cape) is a prodrug that is metabolized into 5′-deoxy-5-fluorocytidine (DFCR), 5′-deoxy-5-fluorouridine (DFUR), and 5-fluorouracil (5-FU) after oral administration. A liquid chromatography–tandem mass spectrometry method for the simultaneous determination of capecitabine and its three metabolites in human plasma was developed and validated. The *ex vivo* conversion of DFCR to DFUR in human blood was investigated and an appropriate blood sample handling condition was recommended. Capecitabine and its metabolites were extracted from 100 μL of plasma by protein precipitation. Adequate chromatographic retention and efficient separation were achieved on an Atlantis dC_{18} column under gradient elution. Interferences from endogenous matrix and the naturally occurring heavy isotopic species were avoided. Detection was performed in electrospray ionization mode using a polarity-switching strategy. The method was linear in the range of 10.0–5000 ng/mL for Cape, DFCR, and DFUR, and 2.00–200 ng/mL for 5-FU. The LLOQ was established at 10.0 ng/mL for Cape, DFCR, and DFUR, and 2.00 ng/mL for 5-FU. The inter- and intra-day precisions were less than 13.5%, 11.1%, 9.7%, and 11.4%, and the accuracy was in the range of −13.2% to 1.6%, −2.4% to 2.5%, −7.1% to 8.2%, and −2.0% to 3.8% for Cape, DFCR, DFUR, and 5-FU, respectively. The matrix effect was negligible under the current conditions. The mean extraction recoveries were within 105–115%, 92.6–101%, 94.0–100%, and 85.1–99.9% for Cape, DFCR, DFUR, and 5-FU, respectively. Stability testing showed that the four analytes remained stable under all relevant analytical conditions. This method has been applied to a clinical bioequivalence study.

1. Introduction

Capecitabine (Cape) is a fluoropyrimidine carbamate used in the treatment of metastatic breast and colorectal cancers. As a prodrug, Cape is converted into the active agent 5-fluorouracil (5-FU) through a three-step enzymatic process after oral drug administration [1,2]. First, hydrolysis by carboxylesterases leads to the formation of 5′-deoxy-5-fluorocytidine (DFCR). Second, cytidine deaminase catalyzes the conversion of DFCR to 5′-deoxy-5-fluorouridine (DFUR). Finally, further catabolism by thymidine phosphorylase produces 5-FU [1,2].

The pharmacokinetic parameters of Cape and its metabolites exhibited marked inter-individual variability [1]. Therefore, to completely evaluate the safety and efficacy of the Cape formulation, the determination of the plasma concentrations of the parent drug, active metabolite, and metabolites closely related to the generation of the active metabolite is necessary. The simultaneous determination of Cape and its metabolites in human plasma remains challenging. Among these three metabolites, DFCR and DFUR are major ones with plasma concentrations near the parent drug level [1,3–7], whereas 5-FU is present in plasma at a significantly lower level [1,3,5–7]. Therefore, linearity should be obtained at markedly different analyte concentration ranges. The differences

Abbreviations: 5-FU, 5-fluorouracil; Cape, capecitabine; DFCR, 5′-deoxy-5-fluorocytidine; DFUR, 5′-deoxy-5-fluorouridine; EMA, European Medicines Agency; FDA, Food and Drug Administration; HQC, high quality control; IS, internal standard; LLOQ, lower limit of quantification; LQC, low quality control; ME, matrix effect; MF, matrix factor; MQC, middle quality control; MRM, multiple reaction monitoring; SIL, stable isotope labeled; $T_{1/2}$, half life; TIS, Turbolonspray; ULOQ, upper limit of quantification.

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in hydrophobicity between Cape and its metabolites also pose a challenge for the simultaneous analysis of these compounds using LC. In addition, the interference between DFCR and DFUR should be considered because the molecular weights of these compounds differ by only one unit. Moreover, *ex vivo* transformation of DFCR to DFUR in blood by cytidine deaminase should be considered during blood collection and processing. To date, several LC–MS/MS methods have been reported for the determination of Cape and its metabolites in human plasma [3,8–12]. In some studies, tetrahydrouridine, a cytidine deaminase inhibitor, was added into blood to prevent *ex vivo* conversion of DFCR to DFUR [8,13]. In another study, it was suggested that obtained blood samples should be immediately cooled on ice-water, and plasma should be stabilized using tetrahydrouridine for an extended period of storage [9].

In the present study, a sensitive and selective LC–MS/MS method with a polarity-switching strategy was developed for the simultaneous quantification of Cape, DFCR, DFUR, and 5-FU in human plasma. LC conditions were optimized to avoid isotopic interferences among DFCR, DFUR, and their isotope-labeled internal standard (IS) $^{13}C,^{15}N_2$-DFCR. In addition, *ex vivo* conversion of DFCR to DFUR was quantitatively investigated using fresh human blood. This method was fully validated according to the US Food and Drug Administration (FDA) [14] and European Medicines Agency (EMA) guidelines [15], and successfully applied to characterize the pharmacokinetic profiles of Cape and its three main metabolites in cancer patients after a single oral administration of 2000 mg of Cape.

2. Materials and methods

2.1. Chemical and reagents

Cape was kindly provided by Qilu Tianhe Pharmaceutical Co., Ltd. (Shandong, China). DFCR, DFUR, d_{11}-Cape, $^{13}C,^{15}N_2$-DFCR, and $^{13}C,^{15}N_2$-5-FU were purchased from TLC Pharmachem., Inc. (Toronto, Ontario, Canada). 5-FU was supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC-grade methanol, acetonitrile, and ammonium hydroxide were obtained from Sigma–Aldrich (St. Louis, MO, USA). Other regents were of analytical grade and were supplied by Tedia (Fairfield, OH, USA). Deionized water was generated by a Millipore Milli-Q Gradient Water Purification System (Molsheim, France).

2.2. Instrumentation

An HPLC system consisting of a DGU-20A3 vacuum degasser, a LC-20AD pump, a CTO-20A column oven, and a SIL-20AC autosampler (Shimadzu, Kyoto, Japan) was used for solvent and sample delivery. Mass spectrometry detection was conducted on an AB Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) equipped with a TurboIonspray (TIS) interface. Analyst™ Version 1.5.2 (Applied Biosystems, Concord, Ontario, Canada) was used for data acquisition.

2.3. Liquid chromatography–tandem mass spectrometry conditions

The analytes were separated on an Atlantis dC_{18} column (100 mm × 4.6 mm, 3 μm; Waters, Milford, MA, USA) maintained at 40 °C with a C_{18} guard column (4.0 mm × 3.0 mm, 5 μm; Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) a mixture of 0.025% acetic acid and 0.0025% ammonium hydroxide solution (pH 3.8), and (B) methanol. A gradient elution method was used (Table 1).

Table 1
MS parameters and gradient elution conditions for the analysis of Cape, DFCR, DFUR, and 5-FU.

MS parameters

Compound	Precursor ion	Product ion	Collision energy (eV)	Declustering potential (V)
Cape	360.2	244.2	5	90
d_{11}-Cape	371.2	255.1	20	60
DFCR	244.0	107.0	−20	−50
DFUR	245.0	108.0	−24	−50
$^{13}C,^{15}N_2$-DFCR	247.0	131.0	−20	−50
5-FU	129.2	42.2	−33	−50
$^{13}C,^{15}N_2$-5-FU	132.2	44.2	−33	−50

Gradient elution conditions

Time (min)	Flow rate (mL/min)	Solvent A* (%)	Solvent B* (%)
0.0	0.60	100	0
2.0	0.60	100	0
2.5	0.60	60	40
3.8	0.60	60	40
4.5	0.60	10	90
7.5	0.60	10	90
7.6	0.60	100	0
9.3	1.50	100	0
10.5	1.50	100	0

* Solvent A is a mixture of 0.025% acetic acid and 0.0025% ammonium hydroxide solution, and solvent B is methanol.

The electrospray ionization (ESI) source was operated with polarity switching during two periods in a single run. The first period, in negative ionization (ESI−) mode, was applied between 0 min and 7.5 min. This mode allowed the detection of DFCR, DFUR, $^{13}C,^{15}N_2$-DFCR, 5-FU, and $^{13}C,^{15}N_2$-5-FU. The second period, in positive ionization (ESI+) mode, was used for the detection of Cape and its IS d_{11}-Cape between 7.5 min and 10.5 min. Quantification was performed using multiple reaction monitoring (MRM). In the ESI− mode, the MS/MS setting parameters were as follows: 25 psi curtain gas; 50 psi nebulizer gas (GS1); 50 psi turbo gas (GS2); −4500 V ion spray voltage; 550 °C source temperature; and 200 ms dwell time. In the ESI+ mode, the MS/MS setting parameters were as follows: 25 psi curtain gas; 50 psi GS1; 50 psi GS2; +4000 V ion spray voltage; 550 °C source temperature; and 200 ms dwell time. The MRM transitions and the optimized MS parameters for each analyte are summarized in Table 1.

2.4. Preparation of standards and quality control (QC) samples

Stock solutions of Cape, DFCR, DFUR, and 5-FU at concentrations of approximately 1.00 mg/mL were prepared in methanol. The solutions were serially diluted with 50% aqueous methanol to obtain Cape/DFCR/DFUR/5-FU working solutions of 1.00/1.00/1.00/0.20, 3.00/3.00/3.00/0.50, 10.0/10.0/10.0/1.00, 30.0/30.0/30.0/2.00, 80.0/80.0/80.0/5.00, 200/200/200/10.0, and 500/500/500/20.0 μg/mL. Calibration standard samples were prepared by spiking 10.0 μL of working solutions into 1000 μL of blank plasma to obtain the final concentrations of 10.0/10.0/10.0/2.00, 30.0/30.0/30.0/5.00, 100/100/100/10.0, 300/300/300/20.0, 800/800/800/50.0, 2000/2000/2000/100, and 5000/5000/5000/200 ng/mL for Cape/DFCR/DFUR/5-FU. QC solutions were prepared through the separate weighing of standard references. QC samples were independently prepared in blank plasma at four concentrations: 10.0/10.0/10.0/2.00 ng/mL (LLOQ), 25.0/25.0/25.0/5.00 ng/mL (low quality control, LQC), 200/200/200/20.0 ng/mL (middle quality control, MQC), and 4000/4000/4000/160 ng/mL (high quality control, HQC) for Cape/DFCR/DFUR/5-FU. Stock solutions of d_{11}-Cape, $^{13}C,^{15}N_2$-DFCR, and $^{13}C,^{15}N_2$-5-FU were prepared in methanol and diluted