Abstract. A simple and precise analytical method for the determination of 5’-deoxy-5-fluorocytidine (DFCR) and 5’-deoxy-5-fluorouridine (DFUR), the enzymatically formed metabolites of capecitabine in plasma, was developed using a reversed-phase high performance liquid chromatography gradient method with external standard method. Blood samples were analyzed after separation of DFCR/DFUR by solid-phase extraction from matrix compounds using a C16 amide reversed-phase column operated at a flow rate of 0.8 ml/min in gradient elution mode with a mobile phase composed of water-methanol (10 mM ammonium acetate in water; m/v). Excellent recoveries in plasma ranging from 77.5-99.12% for DFCR and 84.70-99.15% for DFUR, respectively, were obtained. For both compounds the calibration curves were linear over the range from 0.156 to 5.0 μg/ml. The present assay is robust, selective and sensitive, and is being applied in our laboratories to monitor plasma concentrations of DFCR and DFUR in clinical phase I and phase II studies.

Capecitabine, a fluoropyrimidine carbamate, is an orally administered pro-drug of 5’-deoxy-5-fluorouridine (5’-DFUR), which is converted into 5-fluorouracil (5-FU) an anti-metabolite with activity against numerous types of neoplasms, including those of the breast, esophagus, larynx, gastrointestinal and genitourinary tracts (1-3). Capecitabine is rapidly and extensively absorbed through the gastrointestinal wall as an intact molecule, and rapidly metabolized to 5-FU by a three-step enzymatic cascade (4, 5). Firstly, capecitabine is metabolized into 5’-deoxy-5-fluorocytidine (DFCR) by the human carboxyesterase isoenzyme 2, primarily in the liver (Figure 1). Subsequently, 5’-DFCR is converted to 5’-DFUR by cytidine deaminase in the liver and in tumor cells. Finally, 5’-DFUR is metabolized into the cytotoxic 5-FU by thymidine phosphorylase, which is significantly more active in tumor than in the adjacent healthy tissue (6).

The antitumor activity of 5-FU is attributed to inhibition of the enzyme thymidylate synthase that is essential for DNA synthesis.

In our pharmacokinetic and drug monitoring studies, capecitabine is used for the treatment of patients suffering from metastatic pancreatic cancer in a triple combination together with the human epidermal growth factor receptor type-1/epidermal growth factor receptor tyrosine kinase inhibitor erlotinib and vascular endothelial growth factor inhibitor bevacizumab.

The aim of this investigation was to establish a simple, accurate, selective and sensitive method to quantitate 5’-DFCR and 5’-DFUR in plasma samples of patients receiving chemotherapeutic treatment, especially combination regimens with low doses of capecitabine (500 mg/m²-950 mg/m²). Furthermore this method should be suitable for outpatient drug monitoring purposes.

Materials and Methods

Materials. Pure chemical standards (purity ≥98%) of 5’-DFCR and 5’-DFUR were purchased from AK Scientific, Inc. (Union City, CA, USA) and TCI Europe (Eschborn, Germany), respectively. Water and methanol of high-performance liquid chromatography grade were obtained from Merck Chemicals (Darmstadt, Germany). Oasis
HLB® (1 cm³, 30 mg packing volume) solid-phase extraction (SPE) cartridges were obtained from Waters Corporation (Vienna, Austria). Ammonium acetate was purchased from Aldrich-Chemie (Steinheim, Germany).

Apparatus and analytical conditions. HPLC analysis was performed using a La Chrom System (Merck-Hitachi Ltd., Tokyo, Japan) consisting of a D-7200 Autosampler including Peltier cooling unit, a L-7120 Pump, a column oven (Shimadzu CTO-10A) and a La Chrom L-7400 UV Detector. HPLC components were connected by a D-7000 Interface. Model D-7000 chromatography software (Merck-Hitachi Ltd.) was used for controlling, data acquisition and peak integration.

Separation of compounds was performed on Discovery® reversed-phase C16 amide column (15 cm × 4.6 mm, 5 μm; Supelco, Bellefonte, PA, USA) protected by a guard column (LiChrospher® 100 RP-18 5 μm) and maintained at 36±1˚C. Gradient elution mode was performed using an online de-gasser (Model VWR 2003) and a mobile phase consisting of water-methanol (10 mM ammonium acetate in water; m/v) for solvent A at a ratio of 90:10 and for solvent B at a ratio of 10:90, respectively. The flow rate was 0.8 ml/min. Gradient method was achieved by mixing solvent A and B together as follows: From 0 to 7 min 100% of solvent A was used, from 7 to 15 min solvent B was increased from 0 to 100% (solvent A decreases from 100% to 0%). From 15 to 20 min HPLC was performed by 100% eluent B. Then solvent A was increased from 0% at 20 min up to 100% at 22 min and kept at 100% until the ending of the chromatogram. By this method, gradient elution finished within 30 min. The detection wavelength was 254 nm for both metabolites.

Standard solutions. Stock solutions of both metabolites were prepared by dissolving 1.0 mg/ml of 5'-DFCR and 5'-DFUR in water-methanol (50:50; v/v). Subsequently, these solutions were serially-diluted with pooled human plasma from the Red Cross Austria to obtain concentrations of 5.0, 2.5, 1.25, 0.625, 0.312, 0.165 μg/ml.

Sample preparation. CCB metabolites were separated from matrix by solid-phase extraction (SPE) using Oasis HLB® cartridges (1 cm³; 30 mg packing volume; Waters, Austria). The SPE unit was operated under vacuum (~5 bar) at a flow rate of 1 ml/min. Firstly, the cartridge was activated with 1.0 ml methanol and then preconditioned with 1.0 ml water. Then 1.0 ml of plasma sample was applied on the cartridge ad metabolites were eluted from the SPE column with 1.0 ml of water-methanol (95:5; v/v). Finally this eluate was pipetted into the autosampler vial and chromatographed.

Calibration curve. Calibration standards at concentrations of 0.156, 0.312, 0.625, 1.25, 2.5, 5.0 μg were prepared and assayed as described above.

The calibration curve was obtained by plotting the peak area of each metabolite versus the metabolite concentration in the standard solutions using linear least-square regression method.

For linear regression analysis and descriptive statistics, the Graph Pad Prism Version 5.04 was used (GraphPad Software, Inc.; La Jolla, CA, USA).

Results

Assay validation. The analytical procedure was validated in terms of selectivity, recovery, linearity of assay, linearity of volume of injection, limit of detection, limit of quantification, stability, robustness, precision and accuracy to reveal that this HPLC method is applicable to pharmacokinetic studies. It is now being used for determination of capecitabine metabolite concentrations in plasma from patients with pancreatic and advanced colorectal cancer undergoing various new treatments with capecitabine, monoclonal antibodies and small molecules.

Selectivity. Three different blank plasma samples were extracted and analyzed for potential peaks from endogenous substances which could interfere with peaks resulting from 5'-DFCR and 5'-DFUR.

As can be seen in Figure 2, 5'-DFCR and 5'-DFUR are well-separated from each other and there is no overlap with peaks resulting from matrix components. In buffer samples, the metabolites are base-line separated within seven minutes. Separation is very similar to that for patients’ plasma samples, but resolution is slightly lower due to small peaks resulting
from compounds of the pre- and co-medication. Some patients received up to fifteen other drugs at the same time.

The baseline drift starting after twelve minutes results from changes in the eluent density caused by gradient solvent mixing.

**Linearity of assay: Calibration curve.** Each calibration curve, prepared by plotting a peak area against sample concentration, was linear over the range of 0.156-5.0 μg with a correlation coefficient better than 0.9996 for 5’-DFCR and 0.9992 for 5’-DFUR. The detailed parameters of the calibration curve obtained are listed in Table I, the goodness of the assay data can be seen by negligible residuals for 5’-DFCR and 5’-DFUR.

**Volume of injection.** The linearity of the volume of injection for 5’-DFCR and 5’-DFUR was assessed (n=3 for each injection volume) by analyzing samples with a known concentration of 1 μg/ml in buffer. Injection volume was linear over the range of 20 to 60 μl for both metabolites (r=0.9994).

**Accuracy, precision and recovery.** Daily accuracy and precision were evaluated by analysis of samples at concentrations of 0.156, 1.25, 5.0 μg on the same day. To assess the day-to-day accuracy and precision, samples at the same concentration were re-analyzed on day 1 and on day 7. Table II lists the detailed information. Generally, recovery was better for 5’-DFCR than for 5’-DFUR.

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**Table I. Data used to draw the Calibration curve obtained from determination of 5’-DFCR and 5’-DFUR on different days.**

<table>
<thead>
<tr>
<th>Concentration (μg)</th>
<th>Mean area</th>
<th>Residual area</th>
<th>Slope</th>
<th>Y-intercept</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-DFCR</td>
<td>0.156</td>
<td>8.05</td>
<td>0.200</td>
<td>Slope=19.32±0.2710</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.312</td>
<td>11.48</td>
<td>0.616</td>
<td>Y-intercept=4.835±0.6387</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>17.56</td>
<td>0.648</td>
<td>R=0.9996</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>28.36</td>
<td>-0.629</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>51.41</td>
<td>-1.733</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>102.35</td>
<td>0.898</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5’-DFUR</td>
<td>0.156</td>
<td>1.37</td>
<td>1.778</td>
<td>Slope=25.96±0.5215</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.312</td>
<td>5.02</td>
<td>1.378</td>
<td>Y-intercept=–4.458±1.229</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>11.82</td>
<td>0.053</td>
<td>R=0.9992</td>
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</tr>
<tr>
<td></td>
<td>1.25</td>
<td>24.98</td>
<td>-3.013</td>
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<td></td>
<td>2.5</td>
<td>58.84</td>
<td>-1.603</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>5.0</td>
<td>126.75</td>
<td>1.406</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R (Correlation coefficient); residual area (difference between interpolated and measured value).

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Figure 2. HPLC analysis of A: Blank plasma sample; B: 5 μg of 5’-DFCR and 5’-DFUR in buffer; C: blank plasma of healthy volunteer spiked with 5 μg of (5’-DFCR) and (5’-DFUR) the two metabolites appear at around 5.8 minutes for 5’-DFCR and 6.4 minutes for 5’-DFUR; D: plasma sample obtained from a patient collected 90 minutes after administration of capecitabine.
LOD and LOQ. Blank plasma samples were spiked with pure chemical standards of both metabolites containing 0.0019, 0.039 μg/ml and 0.078, 0.156 μg/ml, respectively to determine the LOD and the LOQ. The LOD was found to be 39 ng referring to a signal-to-noise ratio of at least 1:5. Referring to Table II LOQ was chosen as 156 ng due to a recovery of 90.36% (5'-DFCR) and 84.68% (5'-DFUR), respectively.

Stability of analytes. In previous studies, we observed a loss of compound during frozen storage, therefore we investigated the stability of 5'-DFCR and 5'-DFUR under various conditions. The effect of freeze-thaw cycles on the stability of the metabolites in human plasma as well as the stability on the machine was assessed at same concentration levels as given in Table III. Recovery was determined on day one and day seven when the samples were left on the machine and when stored in the freezer at –80˚C. As can be seen from Table III recoveries of both metabolites were significantly lower when plasma samples were left on the machine especially in a low concentration range (recovery below 80%). The stability of the samples, when stored at –80˚C showed high recoveries up to 100%.

Robustness. The change of the mobile phase composition from 90:10 to 95:5 lead to an extension of the retention time of ±2.28 min (±0.064) for 5'-DFCR and ±2.73 min (±0.015) for 5'-DFUR, respectively.

Column characteristics. Column characteristics were as follows, the capacity factor (k') was 1.41 (±0.011), the number of theoretical plates N/m (according the guidelines of the European Pharmacopoeia; EUP) was 16,828 (±1258.3) and the asymmetric factor was 1.15 (±0.122) for 5'-DFCR. For 5'-DFUR k’ was 1.69 (±0.008), N/m (EUP) was 26,491 (±13562.8) and the asymmetric factor was 1.26 (±0.045). The resolution (EUP) was 1.51 (±0.222) and the selectivity (α) was 1.20 (±0.009). This system suitability test was performed directly on the machine. These column parameters meet the requirements set according to the Good Laboratory Practice (GLP) Guidelines of the European Medical Agency.

Freeze and thaw stability of stock solutions. Blank plasma was spiked for 5'-DFCR and 5'-DFUR to investigate whether freezing and thawing has a negative impact on the stability of these two metabolites. Therefore, three samples with a concentration of 1 μg/ml were prepared and analyzed immediately after preparation and ten days after storage at –80˚C. It was found that the concentration of 5'-DFCR decreased by 34.99% and in the case of 5'-DFUR by 13.73%.

Long-term stability of stock solutions. Both chemical standards were dissolved in water-methanol (50:50; v/v) and samples at three different concentration levels were prepared to assess the long-term stability when stored at –80˚C. We found that the prepared stock solutions are not stable for a time period of three months, especially when samples with low concentrations are frozen. Loss of substance for 5'-DFCR was: at 0.156 μg=68.63%, at 1.25 μg=67.50%, and at 5.0 μg=65.61%; and for DFUR at 0.156 μg=33.07%, at 1.25 μg=18.73%, and at 5.0 μg=77.22%. Therefore, it is necessary to analyze plasma samples of metabolites within a few weeks. Interestingly, the parent compound capecitabine is stable under frozen conditions for many months. Generally, plasma samples should be analyzed as soon as possible to prevent from inaccurate pharmacokinetic results.
Discussion

As capecitabine is quite common in use for anticancer treatment, not only as a single-agent (6) but also in combination with other cytotoxic drugs (7), it is of great interest to provide an accurate sensitive robust and reproducible method for the analysis of this compound. Thus, treatment with a regimen containing capecitabine should be accompanied by therapeutic drug monitoring to optimize treatment conditions, while limiting cytotoxic side-effects.

Two assays have already been described in different species and published for the quantitation of CCB and its metabolites (8-10). These assays either use mass spectrometric detection (10) that is rather insensitive, or have an LOQ of 1 μg/ml (9), which is not suitable for quantifying small amounts of 5'-DFCR and 5'-DFUR in investigations with long-term plasma sampling.

Figure 3 depicts the mean concentration-time curves of 5'-DFCR and 5'-DFUR after two different doses of capecitabine. These graphs present data from an ongoing study concerning the triple combination of capecitabine, bevacizumab and erlotinib, with six patients per dose level. Data obtained from blood samples collected at 300 and 360 min after capecitabine administration give evidence of the suitability of our HPLC method to quantitate small amounts of capecitabine metabolites. Only such a sensitive method permits for precise pharmacokinetic parameters, such as terminal elimination half-life or AUC_{inf} values, to be obtained.

Our actual work is a consequence of a previous analytical article of Farkouh et al. (11) describing a method for the determination of capecitabine itself, and reveals a simple, precise, robust and reliable method for the determination of 5'-DFCR and 5'-DFUR, the direct precursor of 5-FU, which is predominately formed inside the tumor.

The described method is already being applied successfully for the analysis of plasma samples collected from patients who are part of a pharmacokinetic phase Ib study. It makes the quantitation of metabolite plasma concentrations possible for up to 12 hours.

We also assessed another method which allows for the simultaneous determination of capecitabine, 5'-DFCR, 5'-DFUR and 5-FU, but it is far less sensitive than the current one and there are overlaps between the 5-FU peak and peaks of the pre-medication.

The presented method is being used in an ongoing clinical trial to quantitate the metabolites 5'-DFCR and 5'-DFUR, especially after administration of low doses of capecitabine when given concomitantly with erlotinib and bevacizumab (12). The described assay is suitable for detecting even minor alterations in the plasma concentrations with good accuracy,
precision and recovery. Additionally no peak interferences with pre- or co-administered drugs have been observed.

Due to its sensitivity and selectivity it meets the GLP requirements to be applied in further clinical studies where low doses of capecitabine are to be administered.

References


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