Abstract

Capecitabine is an orally-administered chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers. Capecitabine is a prodrug, that is enzymatically converted to 5-fluorouracil in the tumor, where it inhibits DNA synthesis and slows the growth of the tumor tissue. The present work involves the hydrolytic degradation of Capecitabine at 600°C followed by isolation of degradant 5'-DFCR through preparative HPLC. The enriched impurity was characterized by FTIR and NMR techniques and a sensitive fast UPLC method was developed. The method was developed on Acquity UPLC system, using Inertsil ODS 3V Column (50mm x 2.1mm x 1.8µm) in gradient elution mode with mobile phase consisting of 0.1% Trifluoro acetic acid as the Solution A and Acetonitrile and Methanol in the ratio 50: 50 was taken as the Solution B and the column was maintained at 35°C. The detection wavelength of Capecitabine was selected as 272nm and the flow rate was maintained at 0.5ml/min. The method was validated as per International Conference of Harmonization (ICH) Guidelines in terms of System suitability, Specificity, Limit of Detection (LOD), Limit of Quantification (LOQ), Linearity, Accuracy and Precision. The LOD and LOQ values were found to be 0.1µg/ml and 0.2µg/ml, respectively. The method is linear within the range of 0.2µg/ml - 0.75µg/mL for 5'-DFCR.

Keywords: Capecitabine, 5'-DFCR, Metastatic breast cancer, Hydrolytic degradation, Ultra Performance Liquid Chromatography (UPLC).

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INTRODUCTION

Capecitabine, Pentyl[1-(3,4-dihydroxy-5-methyltetrahydrofuran-2-yl)-5-fluoro-2-oxo-1H-pyrimidin-4-yl]carbamate, is an orally-administered chemotherapeutic agent used in the treatment of metastatic breast and colorectal cancers. Capecitabine is a prodrug, that is enzymatically converted to 5-fluorouracil in the tumor, where it inhibits DNA synthesis and slows down the growth of tumor tissue. The activation of Capecitabine follows a pathway with three enzymatic steps and two intermediary metabolites, 5'-deoxy-5-fluorocytidine (5'-DFCR) and 5'-deoxy-5-fluorouridine (5'-DFUR), to form 5-fluorouracil [1-4].

Fig.1: Structure of Capecitabine

ORIGIN OF DEGRADATION PRODUCT/DEGRADATION RELATED IMPURITIES

The main cause of appearance of impurities in drug substances or products is due to its degradation. The chemical instability of the drug substance under the conditions of heat, humidity, solvent, pH, and light encountered during manufacture, isolation, purification, drying, storage, transportation, and/or formulation is main cause of its degradation. It is governed by inherent chemical stability of the drug substance. The major routes of degradation of any drug substance include hydrolysis, oxidation, heat and photolysis. The stress testing helps in the generation of all possible degradation products that may form under different conditions. During the manufacture of bulk drugs, the degradation of end products results in the formation of impurities. Degradation products arise from synthetic processes, storage, formulation of dosage form and aging [5-8].

LITERATURE SURVEY

Literature survey reveals the conventional reverse phase HPLC method for the determination of 5'-deoxy-5-fluorocytidine in Capecitabine. However, the major problem encountered in the previous methods is a long run time [9].
No methods have so far been reported for determining the content of 5'-DFCR in Capecitabine by Ultra Performance Liquid Chromatography (UPLC) whereby the analysis could be performed in less than 5 minutes per injection [10-12].

**AIM OF PRESENT WORK**

The present work involves the hydrolytic degradation of Capecitabine followed by isolation of the degradant 5'-DFCR through preparative HPLC. The enriched impurity was characterized by LCMS, FTIR and NMR techniques and a sensitive fast UPLC method was developed and validated to determine its trace level in the Capecitabine drug substance.

However, the present work is an extensive approach for the method development of a Short Run-time Reverse Phase Method for the quantification of 5'-DFCR in Capecitabine by UPLC.

**Preparation of Capecitabine 5'-DFCR**

About 10g of Capecitabine API sample was taken in a 100ml conical flask containing about 50 ml of water. The solution was kept for stirring at 60°C. The aliquots were collected at an interval of 20 minutes and analyzed by the UPLC method for conversion of Capecitabine to 5'-DFCR. After 2 hours it was observed that Capecitabine was converted to 5'-DFCR (60%). No further degradation was observed even when the API was heated at a higher temperature and for a longer period.

Isolation of the degradant was carried out using the Waters 2000 Prep HPLC equipped with a UV detector monitored at 272 nm and a YMC-ODS-A C18 (250 × 50 mm x 10 μm) preparative column. The mobile phase comprised of Water: Acetonitrile: Methanol in the volume ratio 50: 25: 25. The impurity fractions were collected from several injections and then pooled. These pooled fractions were concentrated separately by using the Rotavapor (Heidolph Laboratory 4002 control) under high vacuum. The aqueous solutions were subjected to lyophilization to obtain the enriched impurity in solid state. The relative retention time of the isolated impurity was further
confirmed by using HPLC spiking studies, and the molecular mass was confirmed by LC-MS. The purity of the isolated impurities was about 90%.

Fig.3: Degradation of Capecitabine

CHARACTERIZATION DATA OF 5'-DFCR BY IR AND NMR OF 5'-DFCR

IR (cm⁻¹): 3482, 3324 (OH/NH Stretch), 2978, 2930 (Aliphatic C-H Stretch), 1685 C=O stretch, 1610, 1539, 1510 Aromatic ring stretch, 1244, 1223 C-O-C stretch, 1116, 1093 C-N stretch.

¹H NMR (DMSO): 3.80 (t,1H), 3.94 (t,1H), 4.12 (s,1H), 5.39 (bs,1H), 5.81 (bs,1H), 5.82 d,1H), 7.91 (s,2H).

MATERIALS AND METHODS

EXPERIMENTAL DETAILS

Several methods are also reported for the analysis of Capecitabine by HPLC. However, it was found that there were no methods were reported for the quantification of 5'-DFCR, a Degradation Impurity in Capecitabine using Ultra performance liquid chromatography (UPLC). Therefore, a sensitive, rugged and time-efficient method was developed and validated on UPLC (Ultra High Pressure Liquid Chromatography) for the quantification of the degradation impurity, 5'-deoxy-5-fluorocytidine. The chromatographic separation was achieved with Inertsil ODS 3V, 50 mm x 2.1 mm, 1.8 µm using gradient elution. The developed method was validated for parameters like
specificity and system suitability, limit of detection, limit of quantification, Linearity, precision and accuracy.

CHEMICALS AND REAGENTS
Capecitabine was obtained from Hope International. The analytical reagents required for the quantification of 5’-DFCR by UPLC i.e., Trifluoroacetic Acid and Acetonitrile were purchased from Sigma-Aldrich. HPLC grade water was obtained from Milli-Q water purification system (Millipore, Milford, USA).

INSTRUMENTATION AND SOFTWARE
UPLC analysis was performed using a Waters Acquity system equipped with binary solvent delivery pump and an auto sampler, connected to Waters Empower 2 software.

CHROMATOGRAPHIC CONDITIONS
A mobile phase consisting of Methanol: Acetonitrile: 0.1 % Acetic acid (7:1:12) as solvent A and Methanol: Acetonitrile: 0.1 % Acetic acid (16:1: 3) as solvent B using Inertsil ODS 3V Column (50 mm x 4.6 mm x 3 µm) with a Gradient [(Time-Solution B): 0-100; 5-100; 20-49; 30-49; 31-100; 40-100 as listed in the Revision Bulletin was performed. However, the baseline was a bit noisy and resolution was not achieved between 5’-DFCR unknown impurity. Further, the mobile phase was changed to 0.1% Trifluoro acetic acid as the Solution A and Acetonitrile and Methanol in the ratio 50: 50 was taken as the Solution B. The Column was also replaced by Inertsil ODS 3V Column (50mm x 2.1mm x 1.8µm) with a flow rate of 0.5 ml/minute and a column oven temperature of 35ºC. The wavelength was changed to 272 nm and the injection volume was set at 5µl. The diluent used was Solution A: Solution B (50:50).

The system suitability mixture containing 5’-DFCR and Capecitabine prepared and injected in an isocratic mode of solvent A: solvent B (80:20) followed by the Capecitabine sample in the diluents at a concentration of 500 ppm, wherein a good peak shape was observed for 5’-DFCR, but Capecitabine sample was not eluted.
Therefore, a gradient [(Time-Solution B): 0-10; 2-10; 3.5-80; 5.0-80; 5.01-10; 7-10] was introduced and the chromatogram obtained was clear and appreciable with the sample eluting at 3.4 minutes, whereas the impurities i.e., 5’-DFCR eluting at 0.5 minutes and a total run time of 7 minutes.

PREPARATION OF SAMPLE AND STANDARD SOLUTIONS
An Impurity Stock solution was prepared by accurately weighing 0.01 g of 5’-DFCR in a 100 ml volumetric flask and diluted up to the mark with the diluent methanol.

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A Capecitabine Standard Stock solution was also prepared by accurately weighing 0.01 g of Capecitabine USP Standard in a 100 ml volumetric flask and diluted up to the mark with the diluent.

Further, 0.5 ml of each of the Impurity Stock solution as well as the Capecitabine Standard Stock solution was transferred to a 100 ml volumetric flask and was diluted up to the mark with the diluent. This solution was the standard solution.

Capecitabine sample solution for the determination of 5’-DFCR content was prepared by accurately weighing and dissolving 0.025 g of the Capecitabine sample in a 50 ml volumetric flask and diluting it up to the mark with the diluent.

RESULTS AND DISCUSSION

Fig.6: Sample Chromatogram

METHOD VALIDATION

SYSTEM SUITABILITY

A system suitability test should be carried out to determine if the operating system is performing properly. System suitability tests are used to verify that the reproducibility of the equipment is adequate for the analysis to be carried out. These tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system, which can be evaluated as such.

System suitability tests were performed in accordance with the ICH guidelines to confirm whether the reproducibility of the equipment was adequate for the analysis to be performed. System suitability was performed by injecting system suitability solution and determining % RSD for peak area was calculated (less than 5 %).
Table 1: Results for System suitability

<table>
<thead>
<tr>
<th>Area</th>
<th>5’-DFCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9750</td>
</tr>
<tr>
<td></td>
<td>10077</td>
</tr>
<tr>
<td></td>
<td>10127</td>
</tr>
<tr>
<td></td>
<td>10208</td>
</tr>
<tr>
<td></td>
<td>9686</td>
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<td></td>
<td>9695</td>
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<tr>
<td>Mean</td>
<td>9924</td>
</tr>
<tr>
<td>SD</td>
<td>238</td>
</tr>
<tr>
<td>%RSD</td>
<td>2.4</td>
</tr>
</tbody>
</table>

SPECIFICITY
Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities and degradation products. In the present work, since only one impurity was monitored, the impurity standard (5’-DFCR) solution at the Limit Level and the sample solution with the concentration specified above (0.5 mg/ml) as well as a spiked standard solution with the above mentioned concentrations were prepared and injected. However, the Capecitabine Standard solution was prepared along with 5’-DFCR and Capecitabine so as to obtain the resolution between 5’-DFCR and Capecitabine.

LIMIT OF DETECTION
The sensitivity for detection can be demonstrated by determining the limit of detection (LOD). A signal to noise (S/N) ratio between 3 to 10 is generally considered to be acceptable for estimating the detection limit. S/N ratios of individual peaks were determined at different concentrations at the estimated LOD and respective % RSD was calculated for replicate injections (n=3). The LOD was found to be 4 for 0.1µg/ml. The results are shown in the Table 2.
LIMIT OF QUANTIFICATION

The quantification limit is the lowest concentration of a substance that can be quantified with acceptable precision and accuracy. A typical S/N ratio of 10-30 is generally considered to be acceptable for estimating the limit of quantification. S/N rations of individual peaks were determined at different concentrations to estimate the limit of quantification (LOQ) and respective %RSD was calculated for replicate injections (n=6). The LOQ was determined to be 0.2µg/ml. The results are shown in Table 2.

LINEARITY

Linearity of the method was checked by preparing solutions at four concentration levels of 0.2 µg/ml (Level 1), 0.25 µg/ml (Level 2), 0.50 µg/ml (Level 3) and 0.75 µg/ml (Level 4) for impurity A. Level 1 and Level 4 were injected in triplicate whereas Level 2 and Level 3 were injected in duplicate. The mean area responses recorded for 5’-DFCR were plotted against

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concentration. The correlation coefficient for 5’-DFCR was found to be 0.9997, which indicates good linearity. %Y Intercept for 5’-DFCR: 0.91 Linearity of 5’-DFCR.

**Table-3: Concentration of Linearity level**

<table>
<thead>
<tr>
<th>Level</th>
<th>Concentration</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.04%</td>
<td>3971</td>
</tr>
<tr>
<td>2</td>
<td>0.05%</td>
<td>5171</td>
</tr>
<tr>
<td>3</td>
<td>0.10%</td>
<td>10144</td>
</tr>
<tr>
<td>4</td>
<td>0.15%</td>
<td>15037</td>
</tr>
</tbody>
</table>

**Fig.10: Linearity graph 5’-DFCR**

**ACCURACY**

Impurity Stock solution containing the 5’-DFCR was spiked in Capecitabine at different concentrations of 0.20µg/ml, 0.25µg/ml, 0.50µg/ml and 0.75µg/ml. Each spiked solution was prepared in duplicate and injected. The recovery percentage and % RSD were calculated for each level impurity. Recovery of 5’-DFCR is shown in Table 3. The acceptance criteria for recovery of 5’-DFCR at concentration level of LOQ are between 85 and 115% and Limit level is between 90-110%.

**Fig.11: Impurity spiked in Capecitabine sample**
Table 4: Accuracy of 5′-DFCR

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Results obtained</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.20 µg/ml</td>
<td>93.00% - 99.49%</td>
</tr>
<tr>
<td>0.25 µg/ml</td>
<td>97.01% - 102.12%</td>
</tr>
<tr>
<td>0.50 µg/ml</td>
<td>97.92% - 99.00%</td>
</tr>
<tr>
<td>0.75 µg/ml</td>
<td>98.42% - 97.69%</td>
</tr>
</tbody>
</table>

SYSTEM AND METHOD PRECISION

The system for 5′-DFCR was checked for repeatability. The sample was prepared by spiking Capecitabine with the 5′-DFCR solution with a concentration of 0.5µg/ml of target analyte concentration and injected six times. The % RSD was found to be less than 5.0 % for system precision.

To determine the method precision six independent solutions were prepared by spiking Capecitabine with the 5′-DFCR at a concentration of 0.5µg/ml with respect to the target analyte concentration. Each solution was injected once. The variation in the results for the 5′-DFCR was expressed in terms of % RSD. The values calculated were found to be below 5.0 % RSD for impurities, indicating satisfactory method precision.

SAMPLE PREPARATION OF CAPECITABINE FOR ROUTINE ANALYSIS

Accurately weighed 25 mg of Capecitabine sample was transferred to a 50 ml volumetric flask, dissolved in the diluent and the volume was made up to the mark with the diluent. This solution was injected into the UPLC system to determine the amount of 5′-DFCR present in the sample. Three different batches of Capecitabine were analyzed under the developed conditions. The results are given in Table 5. The chromatogram obtained after the analysis was shown in (fig. 10).

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**Fig. 12: Sample Capecitabine**
CONCLUSION

Capecitabine sample was degraded by water hydrolysis at 60ºC and the impurity was isolated using preparative HPLC. The aliquots were collected at an interval of 20 minutes and analyzed by using the UPLC method for conversion of Capecitabine to 5’-DFCR. After 2 hours it was observed that Capecitabine converted in 5’-DFCR (60%). The analytical method developed and validated for 5’-DFCR in Capecitabine complies with the acceptance criteria of the analytical parameters, such as specificity, system suitability, and limit of detection, limit of quantification, Linearity, range, Precision and accuracy. Hence the method stands validated. The method can be used for routine quality control and stability analysis.

ACKNOWLEDGEMENT

I am grateful to the PAHER University Udaipur Rajasthan for providing the necessary facilities to carry out the research work.

REFERENCES


Table-5: Results obtained from three batches of Capecitabine

<table>
<thead>
<tr>
<th>Compound name</th>
<th>5’-DFCR Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>B.No. A</td>
<td>Below LOQ</td>
</tr>
<tr>
<td>B.No. B</td>
<td>Below LOQ</td>
</tr>
<tr>
<td>B.No. C</td>
<td>Below LOQ</td>
</tr>
</tbody>
</table>