

# Bioanalytical Method Development and Validation of Capecitabine by RP-HPLC Method

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**Abstract:** A rapid high-performance liquid chromatographic bioanalytical method has been developed and validated for capecitabine in human plasma. Capecitabine a prodrug of 5-fluorouracil (5-FU), is an oral tumor – selective fluoro pyrimidine carbamate approved in the treatment of colorectal and breast cancer. Capecitabine was found with symmetrical peak shapes on an analytical column, Phenomenex Luna C<sub>18</sub> using 70% Ammonium acetate with pH 5.0 and 30% acetonitrile as the mobile phase. The retention times of capecitabine and 5-bromouracil the internal standard were 5.7 and 7.8 min respectively. Linear calibration curves were obtained for each compound across a range of 623.69-8400.59 ng/ml. The limit of detection and limit of Quantification were found to be 300ng/ml and 600ng/ml respectively for capecitabine. Greater than 90% recoveries were obtained for capecitabine. The intra and interday relative standard deviation (%RSD) were found to be <5%.

**Keywords:** Capecitabine; 5-bromouracil.

## 1. Introduction

Capecitabine (5-deoxy-5-fluoro-N-[(pentyloxy) carbonyl]-cytidine, 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 [1]. Carboxylesterases 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 both in liver and tumour to 5'-deoxy-5-fluorouridine (DFUR). The conversion of 5'-DFUR to 5-FU by TP, an important enzyme which is up-regulated in solid tumors, is associated with tumor angiogenesis and has shown anti-apoptotic properties. 5-FU is enzymatically cleared from plasma, and the initial, rate-limiting step is catalyzed by dihydropyrimidine dehydrogenase (DPD) to produce dihydro-5-fluorouracil (FUH2); two subsequent steps result in the formation of fluorou Reidopropionic acid (FUPA) and  $\alpha$ -fluoro- $\beta$ -alanine (FBAL), respectively, with release of CO<sub>2</sub> and NH<sub>3</sub>. It has been shown that

high expression of TP is associated with resistance to conventional 5-FU treatment in various gastrointestinal tract tumors, particularly colon cancer (Fig. 1).

Many methods have reported for the analysis of capecitabine including HPLC with UV detection [2], HPLC method for simultaneous determination along with its metabolite [3], LC/MS/MS method for determination of capecitabine with its metabolites [4-6], LC/MS and GC/MS for anticancer drug monitoring [7]

Our objective in this investigation was to develop and validate an HPLC method for the determination of capecitabine in plasma. This report describes optimization and validation of a simple HPLC assay method with ultraviolet detection which permits the bioanalytical determination of capecitabine. Sample handling and chromatographic run times were minimized to provide fast quantitative results while maintaining the specificity, accuracy and precision required for evaluation of capecitabine.

## 2. Experimental

### 2.1. Reagents

Capecitabine and 5-bromo uracil (IS) were gently supplied by cipla pharmaceuticals Pvt Ltd., Bangalore. HPLC Grade solvents (Acetonitrile, water) were obtained from S. D. Fine Chemicals Ltd., India, Ranbaxy India Ltd. HPLC Grade Ammonium acetate and phosphoric acid were purchased from Qualigens Fine Chemicals Ltd., Mumbai.

Stock standard solutions of capecitabine and the internal standard were prepared by dissolving appropriate amounts of compounds in a known volume of acetonitrile and ammonium acetate and stored at 4°C.

### 2.2. Sample preparation

Blood samples were collected in heparinized tubes and immediately placed on ice and taken to the lab where they were centrifuged at 5000rpm for 5 min at room temperature. The resulting plasma samples were stored at -30°C until analysis.

#### 2.2.1. Protein Precipitation

The blank plasma sample was prepared by adding 1ml of plasma and 1ml of methanol and vortex for 30sec and then centrifuged the solution at 4°C, 5000 RPM for 5min. The supernatant liquid was taken and transferred to HPLC vials.

The blank plasma sample was prepared by adding 1ml of spiked plasma, 1ml of methanol and 0.2ml of IS (250µg/ml) and vortex for 30sec, then centrifuged the solution at 4°C, with a speed of 5000 RPM for 5min. The supernatant liquid was transferred to HPLC vials.

### 2.3. Equipment:

The chromatographic system used in the study was a Shimadzu LC-20 HPLC system. The separation of compounds was achieved using a Phenomenex Luna C18 column (5µm, 25cmx4.6mm id). The mobile phase used was 70% 0.01 Ammonium acetate at pH 5.0 adjusted with phosphoric acid and 30% Acetonitrile. The eluent was monitored at a wavelength of 300nm for capecitabine with a run time of 10min.

### 2.4. Quantification of capecitabine in plasma:

A Standard curve was prepared by injecting various concentrations of capecitabine in plasma. The concentrations of the plasma and quality control samples were calculated by using the regressed equation of the straight line  $y = ax + b$ . The individual chromatograms of blank plasma and the plasma containing capecitabine along with the IS are presented in Fig. 2 and 3.

The limit of detection (LOD) and limit of quantification (LOQ) were determined by using 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 and the concentration was calculated. There were 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" were calculated and multiplied by either 3 (LOD) or 5 (LOQ). LOQ values were subsequently confirmed using six replicates spiked at the target concentration within an acceptable variance of 20%.

### 2.5. Determination of recovery, accuracy and precision

The absolute recovery of capecitabine was determined by comparison of the peak areas from non extracted and extracted samples of QC-3 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 (RSD).

### 2.6. Stability:

#### 2.6.1. Short term stock stability:

A stock solution of capecitabine and IS was kept at room temperature for 8 hours and checked for its stability.

#### 2.6.2. Long term stock stability

A stock solution of capecitabine and IS was kept at room temperature for 45 days and checked for its stability.

#### 2.6.3. Bench top stability

The three replicate concentration of low and high quality concentration samples were determined by comparing the mean area ratio of freshly thawed samples which have been kept at room temperature for about 6 hours.

#### 2.6.4. Coolant stability

The three replicate concentration of low and high quality concentration samples were determined by comparing the mean area ratio of freshly thawed samples which have been kept at room temperature for about 24 hours.

#### 2.6.5. Freeze thaw stability

The stability of low and high quality concentration samples was determined after three freeze thaw cycles. The percent degradation was determined by comparing the mean ratio of area of Capecitabine.

#### 2.6.6. Long term plasma stability

Three aliquots of each of low and high concentrations at same conditions were analyzed on three separate occasions. Storage time not exceeds the time between the date of first sample analysis and the date of last sample analysis.

### 2.7. Ruggedness

The ruggedness of the method was studied by changing the experimental condition such as,

- Changing to another column of similar type (Phenomenex Gemini C18)
- Different operation in the same laboratory.

## 3. Results and Discussion

The method was validated in terms of limit of quantification, Recovery, Selectivity, Precision, accuracy and stability (Table 1).

### 3.1. Linearity

The method was validated over the range of 623.69-8400.59ng/ml. The slope, intercept, correlation co-efficient were found to be 0.000111, 0.050543, 0.9987 respectively.

### 3.2. Sensitivity

In the plasma, the calculated limit of quantification was 600ng/ml and limit of detection was 300ng/ml for capecitabine.

### 3.3. Recovery

The recovery was determined by comparing the aqueous solution and the spiked drug. The percentage recovery of the drug and the internal

standard was calculated and it was found to be 97.22% and 99.57% respectively.

### 3.4. Precision and Accuracy

The accuracy, precision and intraday precision were carried out by preparing 6 individual samples of HQC, MQC and LQC and the percentage CV and percentage nominal was calculated .

### 3.5. Stability

Stability of the method was carried out by performing short term and long term stock stability. The percentage mean ratio of the drug and internal standard were calculated.

Stability of the plasma samples was carried out by performing coolant, bench top and freeze thaw stability studies. The percentage mean ratio of the HQC and LQC were calculated.

The ruggedness of the method was carried out by changing the column and the analyst in the same lab. The percentage CV of the HQC and LQC were calculated.

The long term plasma stability was carried out by performing from the initial sample to the date of last sample. The percentage CV of the HQC and LQC were calculated.

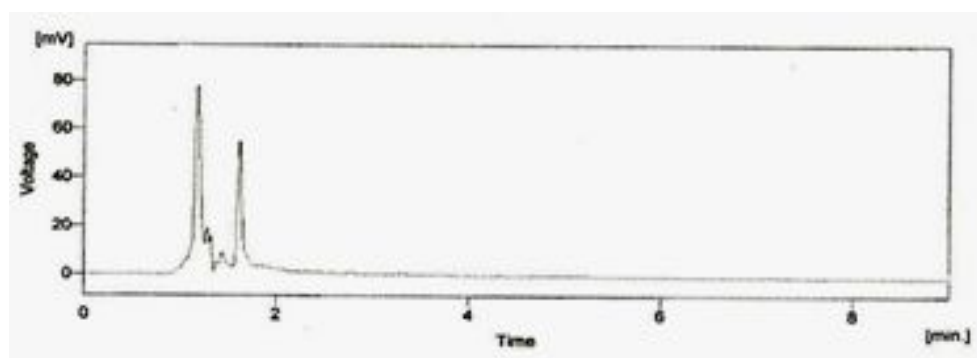


Fig.1. Chromatogram of blank plasma

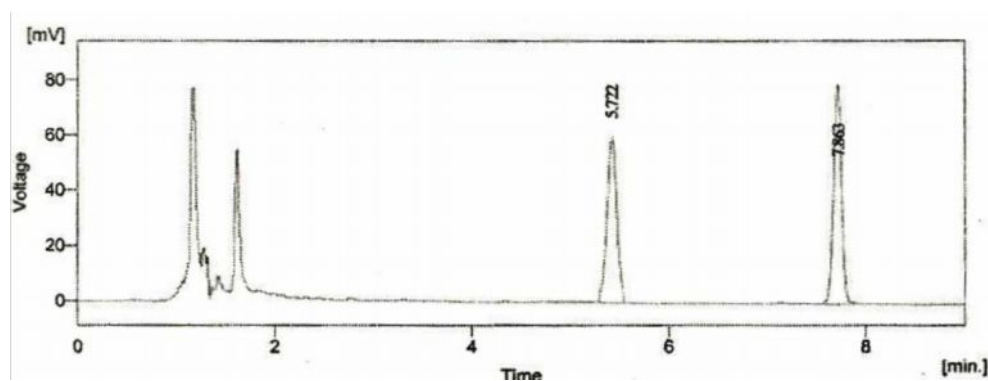


Fig.2. Chromatogram of Capecitabine and Internal standard

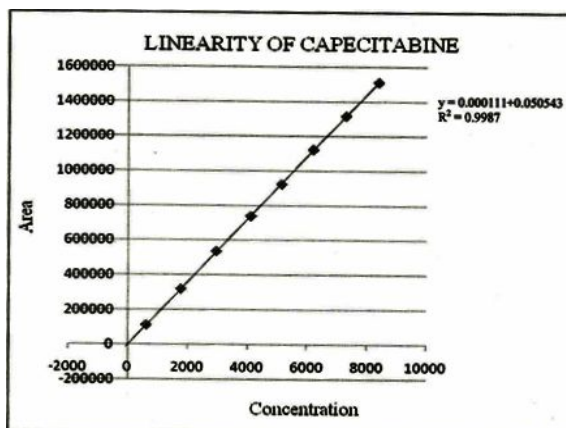


Fig.3. Calibration Curve

Table.1. Results of the method

S.NO	PARAMETERS	RESULT
01.	Selectivity	Pass
02.	System suitability	Pass
03.	LOD	300 ng
04.	LOQ	600 ng
05.	Accuracy and precision	Pass
06.	Intra day precision and accuracy	Pass
07.	Linearity	$R^2 = 0.9987$
08.	Short term stock stability	8.0 hrs
09.	Long term stock stability	45 days
10.	Long term plasma stability	60 days
11..	Bench top stability	6 hrs
12.	Coolant stability	24 hrs
13.	Freeze thaw stability	Pass
14.	Ruggedness	Pass
15.	Recovery	Pass

#### 4. Conclusion

The bioanalytical method developed is simple and shows good accuracy, specificity and reproducibility. It can be used for the estimation of Capecitabine in biofluids. The separation method

developed produce acceptable values of recovery. The chromatogram developed has well resolved peak of Capecitabine without any interference. The developed method could be applied in bioequivalence, Pharmacokinetic and toxicokinetic studies.

## 5. References

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