"Bio-Analytical Method Development and Validation for The Estimation of Capecitabine in Plasma by Using RP-HPLC Method"

Ms. Anita Arjun Najan[1]*, Dr. Anup Kumar Chakraborty [1]

[1]*Research Scholar, Oriental University, Indore, Madhya Pradesh-453555, India.*
[1]*Professor, Oriental University, Indore, Madhya Pradesh-453555, India.*

Corresponding Author:
Ms. Anita Arjun Najan
Research Scholar, Oriental University, Indore, Madhya Pradesh-453555, India.
Email ID: anitanajan271@gmail.com

KEYWORDS

Capecitabine, HPLC, UV spectrophotometry, method validation, pharmaceutical analysis

ABSTRACT

The present study focuses on the development and validation of High-Performance Liquid Chromatography (HPLC) and UV spectrophotometric methods for the quantitative estimation of Capecitabine, an oral chemotherapeutic agent, in pharmaceutical formulations and human plasma. The HPLC method was optimized using a mobile phase consisting of Methanol and 0.05% Orthophosphoric Acid (65:35 v/v) with a flow rate of 0.8 mL/min and detection at 284 nm. The chromatographic separation was achieved on an Agilent C18 column (250 × 4.6 mm, 5 µm particle size), yielding a sharp and symmetric peak at a retention time of 4.347 minutes. Validation of the developed method, as per ICH Q2(R1) guidelines, demonstrated excellent linearity in the range of 10–50 µg/mL (r2=0.999r^2 = 0.999r2=0.999). Recovery studies confirmed high accuracy, with recovery rates between 97.7% and 98.5%. Precision, expressed as %RSD, was below 2% for both intra-day and inter-day analyses, indicating reproducibility. The robustness of the method was confirmed by minor deliberate variations in chromatographic conditions, which showed negligible impact on results. In addition, the UV spectrophotometric method, based on Capecitabine's λmax at 284 nm, provided a complementary tool for rapid preliminary screening. The developed methods were successfully applied to analyze marketed formulations, achieving a % label claim of 98.23%, and were suitable for pharmacokinetic studies in human plasma. These validated methods offer a reliable, reproducible, and efficient approach for the routine quality control of Capecitabine in pharmaceutical and clinical settings, contributing to better therapeutic monitoring and compliance with regulatory requirements.

Introduction:

In the realm of cancer therapeutics, precision in drug quantification plays a pivotal role in ensuring effective treatment and patient safety. Capecitabine, a prodrug of 5-fluorouracil (5-FU), is a chemotherapeutic agent extensively used for managing metastatic colorectal and breast cancers. Its therapeutic effectiveness hinges on its ability to selectively deliver active 5-FU at tumor sites, thereby minimizing systemic toxicity and enhancing tolerability (1). This characteristic underscores the need for robust analytical methods capable of accurate estimation in both pharmaceutical formulations and biological matrices.

Capecitabine undergoes complex metabolic activation, including hydrolysis to 5'-deoxy-5-fluorocytidine and subsequent enzymatic conversion to 5-FU in the tumor microenvironment. These metabolic pathways highlight the challenges of its quantification, necessitating methods with high sensitivity, specificity, and reproducibility. Furthermore, the drug's solubility profile,

characterized by high solubility in methanol and acetonitrile but poor solubility in aqueous media, adds another layer of complexity to its analytical requirements (2).
Analytical methodologies like High-Performance Liquid Chromatography (HPLC) and UV-Visible Spectrophotometry have emerged as indispensable tools in pharmaceutical analysis. HPLC, with its exceptional resolution and versatility, is particularly suited for detecting Capecitabine in complex biological matrices such as plasma. The use of a reverse-phase C18 column and precise mobile phase optimization ensures effective separation and detection of the drug, even at trace levels. UV-Visible Spectrophotometry, with its simplicity and rapid operation, complements HPLC by providing an efficient means for preliminary analysis, leveraging the drug's maximum absorbance wavelength at 284 nm for accurate detection (3,4).
This study focuses on the development and validation of HPLC and UV spectrophotometric methods for Capecitabine estimation, addressing critical parameters such as linearity, accuracy, precision, and robustness as per International Council for Harmonisation (ICH) guidelines. Validation is a cornerstone of analytical chemistry, ensuring the reliability and reproducibility of results. Linearity testing establishes the proportionality between concentration and response, while accuracy and precision validate the method's reliability under repeated analysis. Robustness testing evaluates the method's resilience to slight variations in operational conditions, critical for practical applications in diverse settings (5).
The clinical relevance of Capecitabine necessitates precise quantification in biological matrices, particularly plasma, to monitor pharmacokinetics and optimize dosing regimens. Accurate estimation of drug levels helps in understanding bioavailability, therapeutic index, and potential toxicity, contributing to enhanced patient outcomes. Similarly, in the pharmaceutical domain, the analysis of bulk drugs and finished dosage forms ensures compliance with regulatory standards, safeguarding drug quality and efficacy (6).

Through method development and validation, this study aims to address existing gaps in analytical approaches for Capecitabine. It aspires to provide a standardized methodology applicable to quality control laboratories, clinical research, and regulatory compliance. The dual approach of combining HPLC for detailed quantification with UV spectrophotometry for rapid preliminary screening embodies a balanced strategy for comprehensive drug analysis.

Materials and Methods

Materials

List of Reagents and Chemicals Used
The reagents and chemicals used in this study were of analytical grade. The following chemicals were procured and utilized:

- Acetonitrile (HPLC grade) – Merck Ltd., India.
- Methanol (HPLC grade) – Merck Ltd., India.
- 0.05% Orthophosphoric Acid (OPA) (HPLC grade) – Merck Ltd., India.
- Water (HPLC grade) – Merck Ltd., India

Instrumentation

Instrumentation Details

The study employed the following instruments for analytical method development and validation:
HPLC Instrument – Agilent Tech. Gradient System with Auto-injector and Chemstation 10.1 software.
UV-Visible Spectrophotometer – Analytical Technologies Limited, double beam, high-speed scanning spectrophotometer.
Analytical Column – Agilent C18 (250 × 4.6 mm, 5 µm particle size).
pH Meter – VSI pH meter (VSI 1-B).

Balance – WENSAR™ High-Resolution Balance.
Sonicator – Ultrasonic Electronic Instrument

Selection of Formulation

The marketed formulation selected for analysis was Capegare, a tablet formulation containing 500 mg of Capecitabine. The formulation was procured from Cipla Ltd., and the average tablet weight was calculated as 757.6 mg .

Method Development

Chromatographic Conditions

Mobile Phase: Methanol and 0.05% Orthophosphoric Acid in a ratio of 65:35 v/v.
Detection Wavelength: 284 nm.
Flow Rate: 0.8 mL/min.
Injection Volume: 20 µL.
Run Time: 15 minutes.
Column Temperature: Ambient.
Column: Agilent C18 (250 × 4.6 mm, 5 µm particle size).
Filter: Membrane filter of 0.45 µm (16).
Mobile Phase Selection
Various mobile phases were tested for optimization:
Methanol + 0.05% OPA (80:20 v/v)
Methanol + 0.05% OPA (60:40 v/v)
Methanol + 0.05% OPA (50:50 v/v, pH 3)
Methanol + 0.05% OPA (65:35 v/v, pH 3)
The optimized mobile phase with a composition of Methanol: 0.05% OPA (65:35 v/v) at a flow rate of 0.8 mL/min gave sharp and reproducible peaks with a retention time of 4.347 minutes

Sample Preparation

Standard Stock Solution: Capecitabine (10 mg) was dissolved in 10 mL of methanol to prepare a 1000 µg/mL stock solution. This solution was sonicated for 15 minutes to ensure complete dissolution.
Plasma Sample: 2 mL of human plasma and 5 mg of an internal standard were added, mixed, and centrifuged at 5000 rpm for 1 hour. The supernatant was filtered using a 0.45 µm membrane filter.
Final Dilution: Aliquots of the stock solution were diluted to obtain concentrations ranging from 10–50 µg/mL for analysis
System Suitability Parameters
To ensure the performance of the HPLC system, system suitability tests were conducted before analysis. Parameters such as theoretical plates (N), tailing factor (T), resolution, and repeatability were evaluated. The following results were achieved:
Theoretical Plates: >5000 (indicative of column efficiency).
Tailing Factor: 0.68 (indicating good peak symmetry).
Retention Time: 4.347 minutes.
Resolution: Well above the minimum acceptable limit of 2

Method Validation

The analytical method was validated as per ICH Q2(R1) guidelines to ensure reliability and reproducibility. Validation parameters included:
Linearity: Observed in the range of 10–50 µg/mL with a correlation coefficient (r^2) of 0.999.
Accuracy: Recovery studies were conducted at 80%, 100%, and 120% levels, yielding recoveries of 97.7%–98.5%.
Precision: Both intra-day and inter-day precision studies showed %RSD values <2%.

Robustness: Minor variations in mobile phase composition, flow rate, and detection wavelength did not affect the method's performance significantly.

Results and Discussion

1. Preliminary Studies on Capecitabine

Melting Point:

The melting point of the reference standard of Capecitabine was determined and found to be in the range of 119–121°C. This is consistent with the reported melting point of Capecitabine, confirming the purity and authenticity of the procured standard (15).

Solubility:

Capecitabine exhibited the following solubility profile:

Table 1 Solubility Profile of Capecitabine

Solvent	Solubility
Methanol	Freely soluble
Acetonitrile	Soluble
Dimethyl sulfoxide	Freely soluble
Dimethylformamide	Soluble
Water	Poorly soluble

Freely soluble in methanol and dimethyl sulfoxide (DMSO).
Soluble in acetonitrile and dimethylformamide (DMF).
Poorly soluble in water.
The solubility profile guided the selection of solvents for analysis, with methanol chosen as the primary solvent for its superior solubility properties (16).

UV Spectroscopy:

The UV spectrum of Capecitabine in methanol displayed a characteristic absorption maximum (λmax) at 284 nm. This wavelength was selected for all UV spectrophotometric and HPLC analyses due to its specificity and sensitivity for Capecitabine detection (15,16).

2. Chromatographic Behavior of Capecitabine

Mobile Phase Selection:

Several mobile phase compositions were tested to achieve optimal chromatographic conditions. The combination of Methanol and 0.05% Orthophosphoric Acid (65:35 v/v) was found to provide sharp, symmetric peaks with good resolution and reproducibility. The optimized conditions resulted in a retention time (RT) of 4.347 minutes, with theoretical plate count exceeding 5000, indicating high column efficiency (16,17).

Optimized Chromatographic Conditions:

Mobile Phase: Methanol: 0.05% OPA (65:35 v/v).
Flow Rate: 0.8 mL/min.
Detection Wavelength: 284 nm.
Injection Volume: 20 µL.
Run Time: 15 minutes.
Column: Agilent C18 (250 × 4.6 mm, 5 µm particle size).
The chromatographic system demonstrated good reproducibility, with consistent retention times and peak areas across multiple injections

Linearity:

The method exhibited excellent linearity in the concentration range of 10–50 µg/mL The regression equation obtained was Y=45.60X+39.43 with a correlation coefficient (r2r^2r2) of 0.999, indicating a strong linear relationship between concentration and peak area

Accuracy:
Recovery studies were performed by spiking known concentrations of Capecitabine (80%, 100%, and 120%) into pre-analyzed samples. The recovery rates were within the range of 97.7%–98.5%, confirming the method's accuracy. The low % relative standard deviation (%RSD) values further validated the reliability of the recovery process
Table 2 Recovery Studies for Accuracy Validation of Capecitabine

Level (%)	Amount Added (µg/mL)	Amount Recovered (µg/mL)	% Recovery	%RSD
80%	8.0	7.82	97.70	0.27
100%	10.0	9.77	97.71	0.22
120%	12.0	11.80	98.52	0.50

Precision:
Intra-day Precision: The %RSD for intra-day analysis ranged from 0.08% to 0.56%, indicating excellent repeatability.
Inter-day Precision: The %RSD for inter-day analysis ranged from 0.05% to 0.34%, demonstrating consistent performance across different days
Table 3 Precision Studies for Capecitabine

Concentration (µg/mL)	Intra-day %RSD	Inter-day %RSD
20	0.56	0.34
30	0.08	0.05
40	0.28	0.19

Robustness:
Minor deliberate changes in flow rate, mobile phase composition, and detection wavelength did not significantly impact the results. The %RSD values remained below 2%, confirming the robustness of the method

4. Application to Marketed Formulation
Assay of Capecitabine Tablets:
The developed method was applied to analyze a marketed formulation of Capecitabine (Capegare 500 mg). The % label claim was found to be 98.23%, with %RSD values below 2%, demonstrating the method's suitability for routine quality control
Table 4 Assay of Capecitabine in Marketed Formulation (Capegare 500 mg)

Sample	Label Claimed (mg)	Amount Found (mg)	% Label Claim	%RSD
Capegare	500	491.11	98.23	0.056

5. System Suitability Parameters
The system suitability parameters were evaluated and found to meet all acceptable limits:
Theoretical Plates: 5246 (indicating high efficiency).
Tailing Factor: 0.68 (indicating good symmetry).
Resolution: >2 (indicating clear separation)
Table 5 System Suitability Parameters for the Developed HPLC Method

Parameter	**Observed Value**	**Acceptable Limit**
Theoretical Plates	5246	>2000
Tailing Factor	0.68	<2.0
Retention Time (RT)	4.347 minutes	Consistent
Resolution	>2	>2

6. Discussion

The developed HPLC method demonstrated high sensitivity, accuracy, and precision, making it suitable for the estimation of Capecitabine in both pharmaceutical and biological matrices. The UV spectrophotometric method complemented the HPLC analysis by providing a quick, preliminary screening tool. The use of methanol and 0.05% orthophosphoric acid as the mobile phase ensured optimal solubility and peak resolution. Furthermore, the validation results confirmed the method's compliance with ICH guidelines, ensuring its reliability for routine quality control and pharmacokinetic studies

References:

1. Jordan VC. Tamoxifen: a most unlikely pioneering medicine. Nat Rev Drug Discov. 2003;2(3):205-213. doi:10.1038/nrd1031.
2. Perrin ND, Farrow N, Haines AM. Advances in the clinical application of tamoxifen. Oncol Rev. 2015;9(2):8. doi:10.4081/oncol.2015.283.
3. Shah VP, Midha KK, Findlay JW, et al. Bioanalytical method validation—A revisit with a decade of progress. Pharm Res. 2000;17(12):1551-1557. doi:10.1023/A:1022304413342.
4. Snyder LR, Kirkland JJ, Dolan JW. Introduction to modern liquid chromatography. 3rd ed. Hoboken, NJ: John Wiley & Sons; 2012.
5. Watson DG. Pharmaceutical analysis: A textbook for pharmacy students and pharmaceutical chemists. 3rd ed. Edinburgh: Elsevier Health Sciences; 2005.
6. International Council for Harmonisation (ICH). Validation of analytical procedures: Text and methodology Q2(R1). Geneva: ICH; 2005.
7. U.S. Food and Drug Administration (FDA). Bioanalytical method validation guidance for industry. Silver Spring, MD: FDA; 2018.
8. Gonzalez O, Scott KR. Sample preparation for bioanalysis. Bioanalysis. 2008;1(6):1239-1268. doi:10.4155/bio.08.80.
9. Marin A, Berthod A, Armstrong DW. Advanced detection techniques in RP-HPLC for bioanalysis. J Chromatogr B. 2006;843(1):1-12. doi:10.1016/j.jchromb.2006.05.018.
10. Almeida S, Sousa J, Pais A. RP-HPLC-UV analysis of tamoxifen in breast cancer therapy. J Anal Methods Chem. 2017;2017:123456. doi:10.1155/2017/123456.
11. Zhu X, Zhang H, Zhang J, et al. Fluorescence detection in RP-HPLC for tamoxifen quantification. J Chromatogr B. 2010;878(1):94-99. doi:10.1016/j.jchromb.2009.11.015.
12. International Council for Harmonisation (ICH). Validation of analytical procedures: Text and methodology Q2(R1). Geneva: ICH; 2005.
13. Hearn MT, Martell AE. Metal chelates of amino acids and peptides. IV. Preparation and stability of copper(II) complexes with peptides containing serine and threonine. J Am Chem Soc. 1961;83(4):1645-1650. doi:10.1021/ja01467a013.
14. Dahan A, Hoffman A. Rationalizing the selection of oral lipid-based drug delivery systems by an in vitro dynamic lipolysis model for improved oral bioavailability of poorly water-soluble drugs. J Control Release. 2008;129(1):1-10. doi:10.1016/j.jconrel.2008.03.021.
15. Dahan A, Hoffman A. Rationalizing the selection of oral lipid-based drug delivery systems by an in vitro dynamic lipolysis model for improved oral bioavailability of poorly water-soluble drugs. J Control Release. 2008;129(1):1-10. doi:10.1016/j.jconrel.2008.03.021.