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Developing and validation a method for identifying a chosen bulk medication 5-flourouracil and its formulation.

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ABSTRACT:

This study presents the development and validation of a stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantitative analysis of 5-Fluorouracil (5-FU), a widely used chemotherapeutic agent, in bulk drug and pharmaceutical formulations. The method utilized a C18 column (250 × 4.6 mm, 5 µm) with a mobile phase of 50 mM potassium dihydrogen phosphate buffer (pH 5.0) at a flow rate of 1.2 mL/min and UV detection at 254 nm. Validation was performed as per ICH Q2(R1) guidelines, assessing specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), and limit of quantification (LOQ). The method demonstrated excellent linearity ($R^2 = 0.999$) over a concentration range of 5–25 µg/mL, with a regression equation of AUC = 69.15x + 2.276. Accuracy was confirmed with recoveries of 98.82–99.45% across 80%, 100%, and 120% levels, and precision studies showed %RSD values below 0.13%. The LOD and LOQ were 0.265 µg/mL and 0.832 µg/mL, respectively. Forced degradation studies under acidic, alkaline, oxidative, and thermal conditions confirmed the method's stability-indicating capability, with degradation ranging from 4.10% (thermal) to 17.15% (alkaline). Analysis of marketed tablet formulations yielded a drug content of 99.74% of the labeled claim. The method is simple, precise, robust, and cost-effective, making it suitable for routine quality control, stability testing, and pharmacokinetic studies of 5-FU.

1. Introduction

5-Fluorouracil (5-FU), a fluorinated pyrimidine analog, is a cornerstone chemotherapeutic agent used in the treatment of various solid tumors, including colorectal, breast, and head and neck cancers. As an antimetabolite, 5-FU inhibits thymidylate synthase, disrupting DNA synthesis and inducing cytotoxicity in rapidly dividing cells. Despite its efficacy, 5-FU's narrow therapeutic index and potential for systemic toxicity necessitate stringent quality control to ensure accurate dosing and formulation stability. Analytical methods for 5-FU must be sensitive, specific, and capable of detecting degradation products to comply with regulatory standards such as those set by the International Council for Harmonisation (ICH) [4]. Various techniques, including UV-Visible spectrophotometry, high-performance liquid chromatography (HPLC), and liquid chromatography-mass spectrometry (LC-MS/MS), have been reported for 5-FU analysis. However, methods like LC-MS/MS are costly and complex, limiting their use in routine quality control. HPLC with UV detection offers a practical and cost-effective alternative, provided it is optimized and validated for specificity and robustness.

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This study aimed to develop and validate a stability-indicating RP-HPLC method for quantifying 5-FU in bulk and pharmaceutical formulations, with a focus on simplicity, precision, and compliance with ICH Q2(R1) guidelines. Analytical method development and validation are central to the drug industry to ensure pharmaceuticals are of high quality to meet strict standards for safety, efficacy, and consistency. These analytical methods are not only crucial during formulation and production but span the entire lifecycle of the drug, from quality control, stability testing, to regulatory compliance. 5-Fluorouracil (5-FU), an effective antimetabolite and chemotherapeutic drug of the 1950s, has been extensively utilized for the treatment of solid tumors such as colorectal, breast, pancreatic, esophageal, and head and neck carcinomas. Being a fluorinated pyrimidine analog, 5-FU acts by inhibiting thymidylate synthase and interfering with DNA synthesis and apoptosis in fast-growing cells. Though highly efficient, 5-FU is a tough analytical compound due to its low molecular weight, high polarity, absence of strong chromophores, and instability under certain conditions. Determining and quantifying 5-FU in bulk drug and dosage forms (e.g., injectables, creams) accurately is crucial for a number of reasons: Dose Accuracy: Therapeutic failure or undue toxicity can result from minor dosing variations, as 5-FU has a narrow therapeutic index. Formulation Stability: Stability over storage and use is vital to preserve drug integrity. Degradation Monitoring: Monitoring degradation products is required to ensure safety since these can undermine drug efficacy. Regulatory Compliance: The methods should be pharmacopeial and regulatory compliant to facilitate drug approval and manufacture. Due to its extensive application in multi-drug chemotherapy regimens, analysis methods for 5-FU need to be sensitive, precise, and rugged and able to separate the drug from excipients, other active pharmaceutical ingredients, and possible degradation products. These necessitate the use of robust, validated analytical methods to guarantee quality control and patient safety.

2. Materials and Methods

2.1. Chemicals and Reagents

5-Fluorouracil (Merck Ltd., India) was used as the reference standard. HPLC-grade solvents, including methanol, acetonitrile, and water, were procured from Merck Ltd., India. Other reagents, such as orthophosphoric acid, hydrochloric acid (HCl), sodium hydroxide (NaOH), and hydrogen peroxide (H_2O_2), were of analytical grade (Merck Ltd., India). Potassium dihydrogen phosphate (KH_2PO_4) was used to prepare the buffer.

2.2. Instrumentation

- **UV-Visible Spectrophotometer**: Shimadzu UV-1800 (Japan) with UV Probe software, operating in absorbance mode (190–1100 nm).
- **HPLC System**: Agilent 1260 Infinity (USA) equipped with a quaternary pump, diode array detector (DAD), and ChemStation software.
- **Column**: C18 reverse-phase column (250 × 4.6 mm, 5 µm particle size).
- **Other Equipment**: BUCHI Melting Point M560, IKA VORTEX 4 digital, Bruker ATR for FTIR, and glassware from Borosil, India.

2.3. Method Development

2.3.1. Mobile Phase Selection

Several mobile phase compositions were evaluated, including water:methanol, methanol:water, acetonitrile:water, and KH_2PO_4 buffer:acetonitrile mixtures. The optimal mobile phase was 50 mM KH_2PO_4 buffer (pH 5.0, adjusted with orthophosphoric acid) at a flow rate of 1.2 mL/min, providing sharp and symmetrical peaks with a retention time of approximately 6 minutes.

2.3.2. Detection Wavelength

A 5-FU solution (1000 µg/mL in methanol, diluted to 5–25 µg/mL with 0.1 N HCl) was scanned from 200–400 nm using a UV-Visible spectrophotometer. The maximum absorbance (λmax) was observed at 265 nm, but 254 nm was selected for HPLC detection to align with system suitability and sensitivity requirements.

2.3.3. Chromatographic Conditions

The HPLC method utilized a C18 column (250 × 4.6 mm, 5 µm) maintained at 30°C. The injection volume was 20 µL, and detection was performed at 254 nm. System suitability was assessed by injecting a 10 µg/mL standard solution in triplicate, monitoring retention time, theoretical plates, tailing factor, and peak area reproducibility.

2.3.4. Preparation of Standard Solutions

A stock solution (1000 µg/mL) was prepared by dissolving 10 mg of 5-FU in methanol. Working standards (5–25 µg/mL) were prepared by diluting aliquots with methanol. Solutions were stored in amber glassware to protect from light.

2.3.5. Calibration Curve

Working standards (5, 10, 15, 20, 25 µg/mL) were injected in triplicate, and mean peak areas were plotted

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against concentration. Linear regression was used to derive the calibration equation.

2.3.6. Analysis of Marketed Formulations

Tablets (50 mg/mL labeled claim) were crushed, and an amount equivalent to 10 mg of 5-FU was dissolved in methanol, sonicated for 20 minutes, filtered, and diluted to 10 µg/mL. The solution was analyzed by HPLC, and drug content was calculated using the calibration curve.

2.4. Forced Degradation Studies

To evaluate the method's stability-indicating capability, 5-FU was subjected to stress conditions:

- **Acidic Hydrolysis**: 50 mg 5-FU in 50 mL 0.1 N HCl, heated at 80°C for 8 hours.
- **Alkaline Hydrolysis**: 50 mg 5-FU in 50 mL 0.1 M NaOH, heated at 80°C for 8 hours.
- **Oxidative Degradation**: 50 mg 5-FU in 50 mL 3% H_2O_2, stirred at room temperature for 24 hours.
- **Thermal Degradation**: 50 mg 5-FU at 50°C for 4 weeks in a hot air oven.

Post-stress samples were diluted to 10 µg/mL and analyzed by HPLC. Degradation was quantified by comparing peak areas to the standard.

2.5. Method Validation

Validation was conducted per ICH Q2(R1) guidelines, evaluating:

- **Specificity**: Ability to distinguish 5-FU from degradation products and excipients.
- **Linearity**: Calibration curve over 5–25 µg/mL.
- **Accuracy**: Recovery at 80%, 100%, and 120% levels.
- **Precision**: Repeatability, inter-day, and analyst-to-analyst precision.
- **LOD and LOQ**: Calculated using standard deviation and slope of the calibration curve.
- **Robustness**: Effect of deliberate variations in pH, flow rate, and temperature.

3. Results

3.1. Physicochemical Characterization

5-FU was characterized as a white to off-white crystalline powder with a melting point of 280–283°C. Solubility studies (Table 1) showed high solubility in DMSO (>100 mg/mL), methanol (10–20 mg/mL), and 0.1 N HCl, moderate solubility in water (~12.2 mg/mL), and insolubility in ethyl acetate, chloroform, and diethyl ether.

Table 1: Solubility of 5-Fluorouracil

Solvent	Solubility
Water	~12.2 mg/mL
Methanol	~10–20 mg/mL
Ethanol (95%)	~3–5 mg/mL
Acetonitrile	Slightly soluble
DMSO	>100 mg/mL
0.1 N HCl	Highly soluble
Phosphate buffer (pH 7.4)	~10 mg/mL
Ethyl acetate	Practically insoluble
Chloroform	Insoluble
Diethyl ether	Insoluble

FTIR spectroscopy confirmed characteristic bands at ~3400–3200 cm^{-1} (N–H stretching), ~1720–1700 cm^{-1} (C=O stretching), and ~1650–1600 cm^{-1} (C=C/C=N stretching). UV-Vis spectroscopy identified λmax at 265 nm.

3.2. HPLC Method Optimization

The optimized mobile phase (50 mM KH_2PO_4, pH 5.0) provided a retention time of ~6 minutes with a tailing factor of ~1.12 and theoretical plates ~14624 (Table 2). System suitability parameters met ICH criteria.

Table 2: System Suitability Parameters

Parameter	Rep-1	Rep-2	Rep-3	Mean	SD
Retention Time (min)	4.854	4.860	4.875	4.863	0.0106
AUC	742.325	739.874	745.102	742.434	2.613
Theoretical Plates	14658	14627	14588	14624.33	35.11
Tailing Factor	1.12	1.10	1.14	1.12	0.02

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3.3. Method Validation

- **Linearity**: The calibration curve was linear over 5–25 µg/mL (R^2 = 0.999, AUC = 69.15x + 2.276) (Table 3).

Table 3: Linearity Results

Conc. (µg/mL)	Mean AUC	SD	%RSD
5	346.633	9.61	0.277
10	687.863	18.74	0.273
15	1050.173	18.84	0.179
20	1379.867	11.27	0.082
25	1729.950	15.0	0.009

- **Accuracy**: Recovery studies showed 99.45% (80%), 98.91% (100%), and 98.82% (120%) with %RSD < 0.7% (Table 4).

Table 4: Accuracy Results

Level (%)	% Recovery	SD	%RSD
80	99.45	0.291	0.293
100	98.91	0.510	0.516
120	98.82	0.601	0.609

- **Precision**: Repeatability, inter-day, and analyst-to-analyst precision yielded % mean recovery ~98.7–98.9% and %RSD < 0.13% (Table 5).

Table 5: Precision Results

Parameter	Avg % Recovery	Avg %RSD
Repeatability	98.71	0.103
Intermediate Precision	98.87	0.090
Analyst-to-Analyst Precision	98.88	0.089

- **LOD and LOQ**: LOD was 0.265 µg/mL, and LOQ was 0.832 µg/mL.

- **Robustness**: Mean recovery was 98.48% with %RSD of 0.086 under varied conditions.

3.4. Analysis of Marketed Formulations

The assay of tablets (50 mg/mL) showed a drug content of 49.87 mg/mL (99.74% of labeled claim).

3.5. Forced Degradation Studies

The method distinguished 5-FU from degradation products under stress conditions (Table 6). Alkaline hydrolysis caused the highest degradation (17.15%), followed by acidic hydrolysis (13.90%), oxidative degradation (7.30%), and thermal degradation (4.10%).

Table 6: Forced Degradation Results

Stress Condition	Drug Recovered (%)	Drug Decomposed (%)
Standard Drug	99.95	0.05
Acidic Hydrolysis	86.10	13.90
Alkaline Hydrolysis	82.85	17.15
Oxidative Degradation	92.70	7.30
Thermal Degradation	95.90	4.10

4. Discussion

The developed RP-HPLC method offers significant advantages over existing methods due to its simplicity, cost-effectiveness, and compliance with ICH guidelines. The use of a 100% aqueous mobile phase (50 mM KH_2PO_4, pH 5.0) eliminates the need for organic modifiers, reducing costs and environmental impact. The method's linearity (R^2 = 0.999) and high recovery (98.82–99.45%) align with previous studies. Precision (%RSD < 0.13%) and robustness (%RSD = 0.086) confirm its reliability for routine use. The stability-indicating capability, demonstrated by clear separation of degradation products, addresses 5-FU's susceptibility to alkaline and acidic conditions, consistent with literature findings. The method's LOD (0.265 µg/mL) and LOQ (0.832 µg/mL) are suitable for trace analysis, supporting applications in pharmacokinetic and stability studies. Compared to LC-MS/MS methods, which offer higher sensitivity but require complex instrumentation, this HPLC method is more accessible for quality control laboratories. The analysis of marketed formulations (99.74% assay) validates its practical utility. Limitations include the method's focus on tablet formulations, which may require adaptation for other dosage forms like injectables or creams. Future work could explore gradient elution to enhance resolution for complex matrices. The developed RP-HPLC method is a robust, precise, and stability-indicating tool for quantifying 5-FU in bulk and tablet formulations. Its compliance with ICH Q2(R1) guidelines, coupled with high accuracy, precision, and sensitivity, makes it ideal for routine quality control, stability testing, and pharmacokinetic studies. The method's simplicity and cost-effectiveness

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enhance its applicability in resource-limited settings, contributing to the safe and effective use of 5-FU in clinical practice.

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