

# Simple Quantitation of Flucytosine in Low Volume Serum Samples and Evaluation of Common Endogenous Interferences by HPLC-UV

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## Abstract

5-Fluorocytosine (5-FC) is used for the treatment of several infections. It is extremely important to monitor blood level concentration for maximum activity to avoid its side effects. A simple, faster, and more accurate analytical method is developed and validated using high-performance liquid chromatography with UV detection in a very low-volume serum sample. Exactly 50  $\mu\text{L}$  of serum was precipitated with 5% trichloroacetic acid. After mixing and centrifugation, 20  $\mu\text{L}$  of supernatant was injected into the HPLC column. Detection was performed at 280 nm. The method is very specific and free from interfering substances due to different drugs and their different circulating metabolites. The limit of detection (LOD) and limit of quantification (LOQ) were found to be 0.50  $\mu\text{g/L}$  and 1.0  $\mu\text{g/L}$ , respectively. The method was linear in the range of 5 - 150  $\mu\text{g/L}$  in the serum sample. In method comparison, the correlation coefficient  $r^2$  was 0.999 and the percentage recovery was 90% - 105% on four levels of the quality control samples. Within run and between run precision was found to be less than 2.2% at four different concentrations (5, 25, 50, and 100  $\mu\text{g/L}$ ). A simple, faster, and more accurate HPLC-UV method is developed which is very useful for monitoring 5-FC concentration in low volume serum samples without evaporation step and ion exchange chromatography within minutes.

## Keywords

5-Fluorocytosine, Serum Sample, HPLC-UV Method, Quantification,

## 1. Introduction

5-Fluorocytosine (5-FC) is a fluorinated synthetic analog of cytosine. Its antimycotic action comes from its fast conversion into another molecule called 5-fluorouracil (5-FU) which is an antimetabolite that produces RNA coding errors and prevents DNA synthesis [1]. 5-Fluorocytosine is clinically used along with amphotericin B for the treatment of several systemic mycotic infections such as visceral *Candida albicans*, *Cryptococcal meningitis*, chromomycosis, and torulopsis [2] [3] [4].

It has been reported that flucytosine converted into its active metabolites especially 5-FU which caused severe side effects including bone marrow depression and hepatotoxicity [5] [6] [7]. About 80% - 90% of 5-FC is excreted into urine samples in the unchanged form [8]. Therefore, it is very important to monitor the concentration of 5-FC to maintain its adequate blood level concentration, avoid drug-related side effects, and modify the dosage in a patient with renal dysfunction.

Several analytical methods for the determination of 5-FC have been reported. Microbiological and enzymatic methods were the oldest methods used for routine analysis but they had limitations due to longer analysis time especially when the rapid determination of 5-FC was needed to adjust the dose for the patients [9] [10]. HPLC methods are the most frequently used for the quantitative determination of 5-FC in biological samples with solid phase extraction [11], ultrafiltration technique [12], and liquid-liquid extraction with different organic solvents [13] [14] [15]. Some methods used perchloric acid [16], trichloroacetic acid [17], acetonitrile [18], and ethanol [19] to precipitate proteins for faster sample preparation before HPLC analysis. We found several disadvantages in all these reported HPLC methods and most of them were likely not suitable for routine analysis of 5-FC in high-volume clinical labs. The most common drawbacks are lack of internal standard, use of a longer HPLC column (250 mm × 4.6 mm) with a guard column (50 mm × 4.6 mm) [20], long chromatographic run time [21], large sample volume [22], laborious sample extraction, lack of interference study due other drug and absence of carryover evaluation due to the auto-samplers of HPLC system. An ultrafiltration HPLC method required 1 mL sample volume with double centrifugation without an internal standard and produced low recovery. A cation exchange HPLC column was used which is not available in most clinical laboratories [12]. In another report, both 5-FC and 5-FU were determined together with two different internal standards in a C8 column, however, it also required a higher sample volume (0.5 mL of plasma) and injection volume (50 µL) without any information about the removal of other plasma constituents from the column after the elution of both molecules.

An extra dilution step with phosphate buffer is required due to the utilization of a strong cation ion exchange HPLC column for chromatographic separation [23]. No HPLC method has studied the effect of the most common endogenous interference due to lipemia, hemolysis, and icterus on flucytosine concentration in serum or plasma samples.

Therefore, a different procedure was required that would be free from the aforementioned shortcomings and which would be suitable for high-volume therapeutic drug monitoring labs. Herein, we modified one reported HPLC method [23] from another researcher in which an extra dilution step with phosphate buffer was removed, the ion exchange column was replaced with C18 columns, and the 2-minute washing step with 80% methanol was added before the next injection. In addition, six points calibration standards were prepared in serum samples instead of single-point calibration in saline. These changes reduced back pressure, provided increased column life by removing serum constituents, good resolution, higher theoretical plate count, sharp, stable peak shape for more than 1500 samples, and better assessment of linearity in routine analysis. We also evaluated the effect of the most common cause of pre-analytical error due to endogenous substances like lipemia, hemolysis, and icterus. The method is fully validated according to FDA bioanalytical method guidelines as described below.

## 2. Experimental

### 2.1. Reagents and Chemicals

5-Flucytosine (Catalogue # F7129), 5-iodocytosine (Catalogue # I6875), trichloroacetic acid (Catalogue # T6399) and potassium phosphate monobasic (Catalogue # P0662) were obtained from Sigma-Aldrich, USA. HPLC grade methanol was purchased from Fisher Scientific. HPLC column 150 mm × 3 mm with 5 μm particle size was obtained from Chromsystems Instruments & Chemicals GmbH Germany (Part 38130). HPLC grade water was obtained from the Millipore unit.

### 2.2. HPLC System

A high-performance liquid chromatographic system was obtained from Waters, USA. Alliance HPLC 2790 separation module was composed of the binary pump and an auto-sampler. An absorbance detector 2487 UV detector at 280 nm was used for monitoring 5-FC and its internal standard. Patient data were processed by using Waters empower 2 software. A volume of 20 μL from calibration, quality controls, and patient samples was loaded into the HPLC system.

### 2.3. Reagent Preparation

Mobile phase A was prepared by dissolving 1.36 g of potassium phosphate monobasic into 1000 mL of HPLC grade water and pH was adjusted to 5.5 with 2M sodium hydroxide solution. Mobile phase B was prepared by mixing 800 mL of methanol with 200 mL of HPLC grade water. Both mobile phases were filtered

with a 0.45  $\mu\text{m}$  filter by vacuum and sonicated for 10 minutes before use. A 50% mixture of methanol and HPLC water was used for needle wash and seal wash. Precipitation reagent was made by dissolving 5.0 g of trichloroacetic acid into 100 mL of HPLC grade water. All these reagents were stored at room temperature. The internal standard was prepared by dissolving about 0.025 g of 5-iodocytosine in 50 mL of HPLC grade water in a volumetric flask. Prepared internal was stored at 2°C - 4°C in an amber color glass bottle.

#### **2.4. Calibration and Quality Controls**

Accurately weighted 200 mg of 5-flucytosine was dissolved into 100 mL of HPLC grade water. This stock standard was diluted to prepare 50, 100, 250, 500, 1000, and 1500  $\mu\text{g/L}$  secondary standard solutions. Six points calibration standards (5, 10, 25, 50, 100, and 150  $\mu\text{g/L}$ ) were made by spiking 1mL of these secondary standard solutions into 9 mL of drug-free serum samples separately. Similarly, the corresponding secondary stock solution was used to prepare four levels of quality controls (5, 25, 50, and 100  $\mu\text{g/mL}$ ) in drug-free serum samples. These calibration standards and quality controls were aliquoted (100  $\mu\text{L}$  each) and stored at -20°C until consumed.

#### **2.5. Sample Preparation**

50  $\mu\text{L}$  of serum calibration standards, quality controls, and patient samples were transferred into separate 1.5 mL Eppendorf centrifuge tubes. A volume of 50  $\mu\text{L}$  of internal standard and 100  $\mu\text{L}$  of 5% trichloroacetic acid were added to each tube. All the tubes were vortexed for 30 seconds and centrifuged for 5 minutes at 15,000 RPM. The supernatant was transferred into V-shaped HPLC vials and 20  $\mu\text{L}$  was injected into the HPLC column.

#### **2.6. HPLC Parameters**

HPLC column was equilibrated with a 50% mixture of mobile phase A & B. Gradient elution was started with 0.5 mL/min with 100% mobile phase A which changed to 50% of mobile A and B for the next 6 minutes. At 6.1 minutes, the flow rate was changed to 0.75 mL/min with 100% mobile phase B for the next two minutes. The gradient and flow rate were brought to the initial condition with 100% mobile phase A at 0.5 mL/min for the next two minutes before the next injection. 20  $\mu\text{L}$  of blank, calibrations, quality controls, and patient samples were injected into the HPLC column. The tunable UV detector was operated at 280 nm for both compounds. Six points calibration curve was prepared by empowering software using the internal standard mode of quantitation against the concentration of six calibration standards.

### **3. Method Validation**

#### **3.1. Specificity and Selectivity**

Ten different serum samples were collected from healthy individuals and in-

jected into the HPLC system after the extraction procedure. The chromatograms were compared with reference standard solution containing 5-flucytosine and internal standard in water.

Serum samples from patients taking commonly used medication such as acetaminophen, amoxicillin, amikacin, carbamazepine, gentamicin, phenobarbital, phenytoin, and theophylline were collected and analyzed by the assay procedure.

The effect of common interferences was also evaluated. Different serum samples were prepared and supplemented with three increasing concentrations of hemolysate, bilirubin, and triglyceride. These samples were tested by the developed HPLC method.

### 3.2. Linearity and Range

A six-point calibration curve at a concentration of 5, 10, 25, 50, 100, and 150 µg/L in serum samples was created by the area ratio of 5-flucytosine to its internal standard.

### 3.3. Accuracy and Precision

Four levels of quality control serum samples (5, 25, 50, and 100 µg/L) were prepared by spiking a pure standard solution of 5-flucytosine into the drug-free serum. Five replicates from each control sample were assayed to determine within-run accuracy and precision. Between run accuracy and precision were determined from the results of five replicate ( $n = 5$ ) analyses of four quality controls (5, 25, 50, and 100 µg/mL) for three consecutive days. Precision was calculated by percent coefficient of variance (% CV) and accuracy was measured as percent recovery of the spiked and recovered amount.

The accuracy of the internal standard was assessed by comparing its peak area in pure calibration standards and serum quality control samples.

### 3.4. Comparison Study

About 20 patient samples of varying concentrations tested in our lab were sent to Bioscientia for method comparison. The correlation coefficient was found to be 0.999 with no significant bias between the two methods.

### 3.5. Limit of Detection and Quantitation (LOD & LOQ)

Low concentration serum samples of 0.5 µg/L and 1.0 µg/L were prepared by adding a pure standard of 5-FC into the drug-free serum. Each sample was analyzed ten times to determine precision, accuracy, and single-to-noise ratio.

### 3.6. Carry over

A low concentration control sample was tested five times repeatedly and the mean value was calculated. The same quality control sample was injected after the high-quality control sample. The experiment was repeated five times and percent carryover was measured from two mean values of low QC sample.

### 3.7. Stability Study

Two levels of quality controls (10 and 50 µg/mL) were prepared by spiking a pure standard of 5-FC into pool serum. After measuring the initial results, the controls were aliquoted and stored at 2°C - 4°C for 10 days. The aliquots of control samples were retested every 2 days and the results were compared with the initial values. The stability of extracted samples was also assessed by storing and analyzing the samples in an auto-sampler after 0, 1, 2, and 3 days at 10°C.

### 3.8. System Suitability

Water empowers software automatically calculated system suitability parameters for the peaks of 5-FC and internal standards such as theoretical plate, tailing factor, and resolution factor.

## 4. Results

Chromatograms of a blank serum, calibration standard, and patient sample are shown in **Figures 1-3**, respectively. After the extraction procedure, ten healthy individual serum samples were injected into the HPLC system. There is no peak found at the typical retention time of 5-FC and internal standard.

Most commonly used medications such as acetaminophen, amoxicillin, amikacin, carbamazepine, gentamicin, phenobarbital, phenytoin, and theophylline were spiked in serum samples and injected into the HPLC system after protein precipitation. No interfering peaks were detected at the retention of 5-FC and internal standard.

A pool serum from normal patients was prepared and supplemented with four increasing concentrations of hemolysate, bilirubin, and triglyceride. This sample was tested by the assay procedure. The results are shown in **Figure 4**.

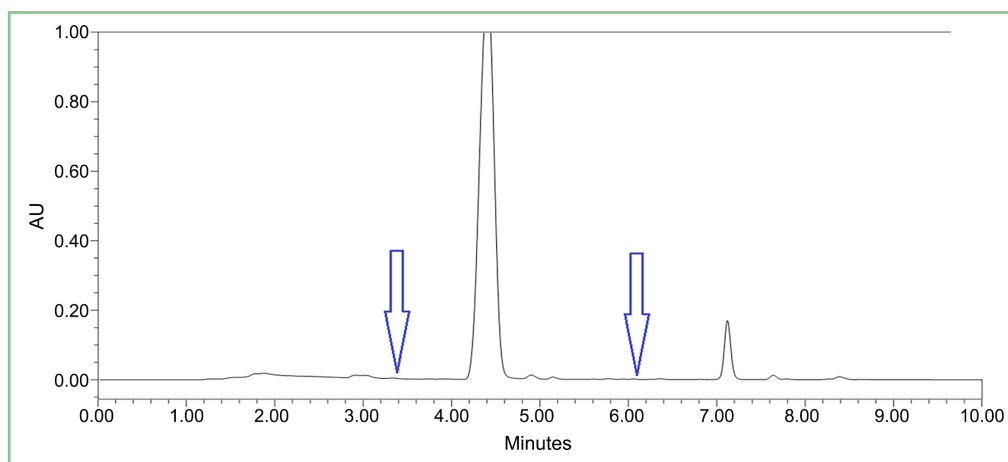
Six calibration standards were prepared by spiking a pure standard of 5-FC in drug-free serum at a concentration of 5, 10, 25, 50, 100, and 150 µg/L. These samples were precipitated and the supernatant was analyzed in duplicate. A linear calibration curve was obtained by plotting the peak area ratio of 5-FC to the peak area of internal standard against the corresponding concentrations. Linear regression analysis was performed and results were calculated (**Table 1**).

Within run accuracy and precision were determined by analyzing five replicates of four different levels of quality control samples. Between run accuracy and precision were tested by analyzing five replicates of four-level quality control for three consecutive days. Mean recovery and percent coefficient of variance (%CV) was calculated (**Table 1**).

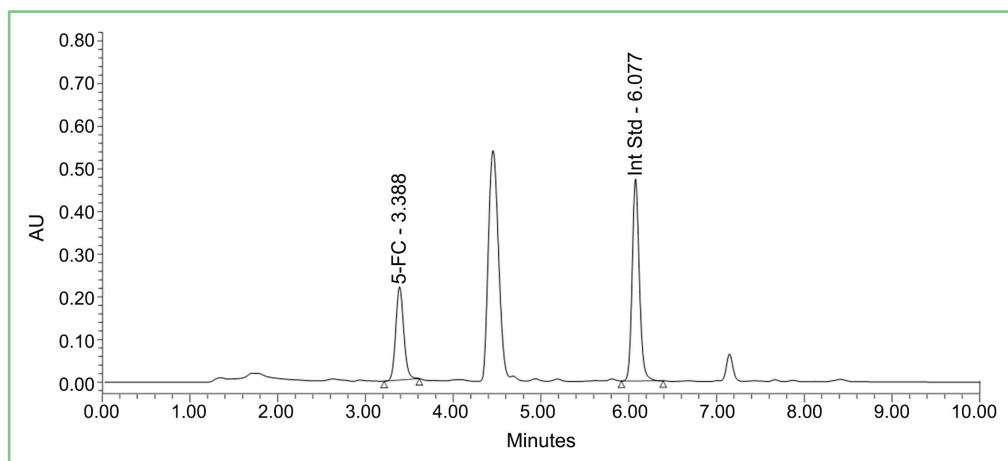
About twenty patient samples of varying concentrations were analyzed in our lab and Biosciencia Lab, Germany for a method comparison study. The slope, intercept, bias, and correlation coefficient were measured (**Table 1**).

Results obtained for a limit of detection (LOD), the limit of quantitation

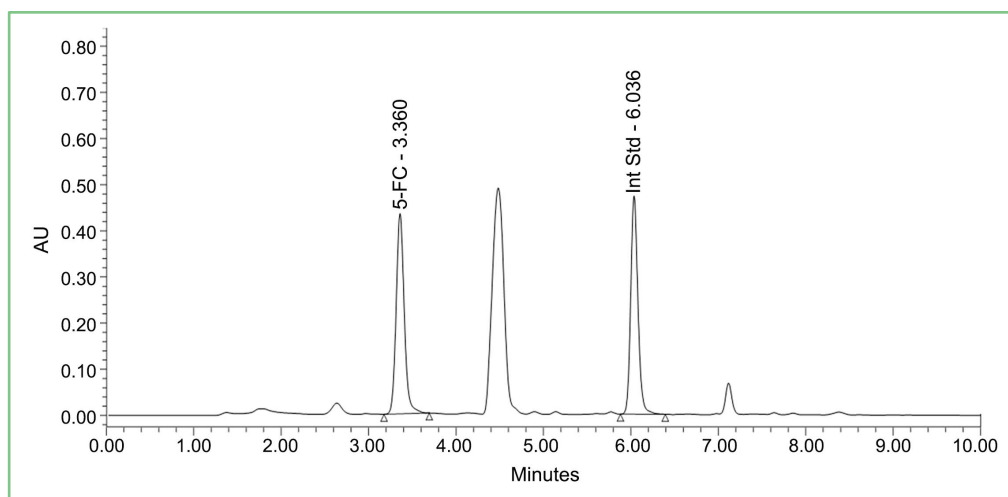
(LOQ), carry-over studies, stability studies, and system suitability parameters were summarized in **Table 1**.



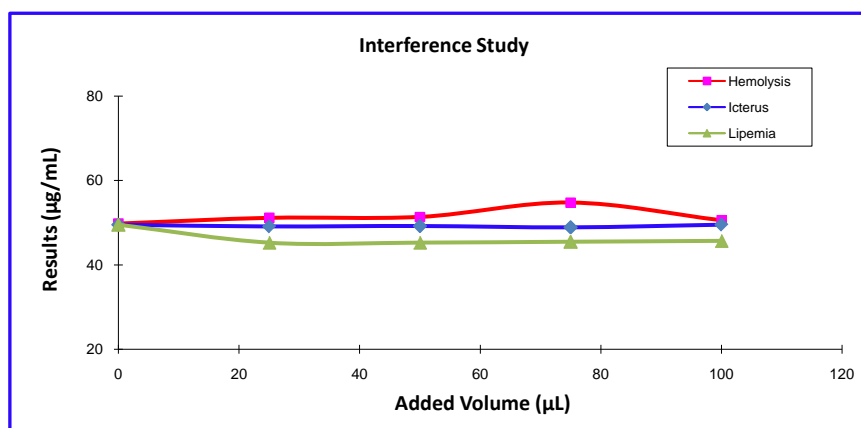
**Figure 1.** Chromatogram of blank serum sample (Arrows show the absence of peak).



**Figure 2.** Chromatogram of serum calibration standard (25 µg/L).



**Figure 3.** Chromatogram of patient sample (42 µg/L).



**Figure 4.** Effect on 5-FC concentration due to hemolysis, icterus, and lipemic samples.

**Table 1.** Validation data of linearity, range, precision, accuracy, comparison, and extraction efficiency.

Linearity (N = 2)	Range (µg/L)	Slope	Intercept	R <sup>2</sup>
	5 - 150	0.9983	0.0459	0.9997
Precision	%CV at 5 µg/L	%CV at 25 µg/L	%CV at 50 µg/L	%CV at 100 µg/L
Within Run (N = 5)	1.3	0.5	0.4	0.8
Between Run (N = 15)	2.1	1.7	1.6	1.0
Accuracy	% Recovery at 5 µg/L	% Recovery at 25 µg/L	% Recovery at 50 µg/L	% Recovery at 100 µg/L
Within Run (N = 5)	93.3	102.9	100.1	102.0
Between Run (N = 15)	94.8	100.9	99.0	101.0
Comparison (N = 20)	Slope	Intercept	Bias (µg/L)	R <sup>2</sup>
	0.94	1.2	4.32	0.999
Sensitivity	LOD (µg/L)	% Recovery/% CV	LOQ (µg/L)	%Recovery/% CV
	0.5	121/22	1.0	102/2.5
Carry Over	% Carry Over		0.02%	
System Suitability Parameters				
Compound	Retention Time (min)	Theoretical Plate	Tailing Factor	Resolution Factor
5-FC	3.37	7189	1.13	26
Internal Standard	6.05	11,174	1.14	

## 5. Discussion

The reverse phase HPLC method with UV detection for the determination of 5-FC in serum is preferred instead of ion exchange chromatography. The method involves simple protein precipitation from a very low sample volume (50 µL) with 5% trichloroacetic acid after the addition of an internal standard. 5-iodocytosine is used as an internal standard due to its structural similarity with 5-FC.

Three different solvents (acetonitrile, ethanol, and 5% trichloroacetic acid) were tested for protein precipitation. It was found that the peak of 5-FC resolved



from the solvent front with mobile phases A and B with stated gradient while it was not resolved when protein was precipitated with acetonitrile and ethanol due to other components of the serum sample. Both 5-FC and internal standard peaks were separated on the C<sub>18</sub>-HPLC column in 5% trichloroacetic acid supernatant without any adverse effect on column life and its chromatographic performance.

Six points calibration standards were prepared both in water and drug-free serum samples. Both calibration curves were identical in slope and  $y$ -intercept which showed that aqueous-based calibration standards can be used for the quantitation of patient samples. Since the retention time of 5-FC and internal standard are highly sensitive to the small variation in the mobile phase composition, we recommended using the same lot of mobile phase during the analysis of a batch of patient samples. A huge peak at 4.4 min was present in all serum samples but well resolved and did not interfere with both compounds even at very low concentrations. The calibration curve was prepared from 5 to 150  $\mu\text{g/L}$  since the blood levels of flucytosine in patients with normal renal function ranged between 50 and 100  $\mu\text{g/L}$  when normally recommended doses were taken by the patient. Both, 5-FC and internal standard were well resolved and separated from other components of serum sample at  $p\text{H}$  5 of mobile phase A with selected gradient used in this method. Mobile phase B was used to wash the column and to remove other components of the serum samples before the next injection for 2 - 3 minutes. The intensity at 280 nm was much higher than 254 nm, therefore, we used 280 nm in our lab for the routine analysis of patient samples.

Effects of the most common endogenous interference due to lipemia, hemolysis, and icterus on flucytosine concentration in serum have been evaluated and found no significant effect both on measured concentration and chromatographic separation. A large number of drugs and their circulating metabolites were also tested and no interfering peaks were found at the retention time of 5-FC and its internal standard.

The method is faster and analysis of calibration standard, three controls samples, and 20 patient samples can be performed in 3 - 4 h including sample preparation time. This method has significant advantages over other published methods in terms of low sample volume, simplicity, rapidity, specificity, reproducibility, and cost-effectiveness and is suitable for therapeutic drug monitoring laboratories.

### Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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