# Validated High-Performance Liquid Chromatographic Technique for Determination of 5-Fluorouracil: Applications to Stability Studies and Simulated Colonic Media

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## Abstract

**A simple isocratic stability-indicating high-performance liquid chromatographic method with UV detection using thymine as an internal standard is developed. The method is validated and the degradation products are determined. The method is applied for the assessment of the stability of 5-fluorouracil in rat caecal content as a simulated colon medium under anaerobic conditions. The drug decomposes under acidic, alkaline, thermal, and oxidative stress. The drug is highly susceptible to acidic, alkaline, and oxidative hydrolysis as compared to alkaline conditions. Separation of the drug from major and minor degradation products is successfully achieved on a $C_{18}$ analytical, µ-bondapak column. The detection wavelength is 260 nm. The method is validated, and the response is found to be linear in the drug concentration range of 0.1–2.0 µg/mL. The high linearity of the standard calibration curve of 5-fluorouracil in the rat content is found to be $R^2$ = 0.998 in the concentration range from 0.5 to 5 µg/mL. No degradation occurred after incubation of 5-fluorouracil in the rat caecal contents. The standard deviation and coefficient of variation values for intra- and inter-day precision study exhibit acceptable accuracy and precision data throughout the concentration range investigated.**

## Introduction

5-Fluorouracil (5-FU; Figure 1A) is an anticancer agent and the most widely used drug in the treatment of malignancies arising from breast, gastrointestinal tract, head, and neck regions of the body (1). This antimetabolite antineoplastic agent is widely used for the treatment of colorectal cancer (2,3). For several decades, 5-FU was the only chemotherapeutic agent with clinical activity against colorectal cancer (4). 5-FU can produce severe haematological, mucosal, and gastrointestinal toxicities, which are more often encountered with dose intensification strategies but also occur with moderate doses in adjuvant therapy treatments (5).

Due to erratic oral bioavailability, intravenous administration of this drug is currently in clinical use (6). Oral site-specific rate-controlled 5-FU delivery is expected to reduce systemic side-effects and to also provide an effective and safe therapy for colon cancer with reduced dose and duration of therapy. A great deal of research work has concerned site-specific drug delivery to the colon, as documented by several reviews on this subject (7–11). The stability of 5-FU in the colonic medium is a critical issue that needs to be well-defined for the success of such an approach.

Several analytical methods have been previously reported for the determination of 5-FU, including assays based on gas liquid chromatography (12), gas chromatography combined with mass spectroscopy (13), and reversed-phase high-performance liquid chromatography (HPLC) (14). Most of the earlier analytical methods were not properly validated according to current requirements (15–16) and/or made use of large sample volumes (500–1000 µL).

The parent drug stability test guidelines issued by International Conference on Harmonization (ICH) (17) suggest that

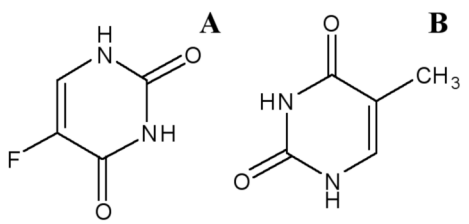


**Figure 1.** Chemical structures of: 5-FU (A) and thymine (B).

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stress studies should be carried out on a drug to establish its inherent stability characteristics, leading to identification of degradation products and hence supporting the suitability of the proposed analytical procedures. It also requires that analytical test procedures for stability samples should be stability-indicating and that they should be fully validated.

This study provides more dimensions to the work because it represents an important application for the stability-indicating assay. A large volume of literature research work has been concerned with site-specific drug delivery of 5-FU to the colon (18–21). Such a delivery system is expected to reduce systemic side effects and provide safe and effective therapy for colon cancer, involving reduced drug dosage levels along with duration of therapy. However, the stability of 5-FU in the colonic medium was never addressed in the literature. Such a study is essential for any drug intended to be localized in the colon, which is rich with a variety of degrading enzymes.

Therefore, the aim of this present study was to develop a simple, rapid, and properly validated HPLC method coupled with UV detection for quantitative determination of 5-FU utilizing a single column and isocratic elution, taking into considerations a variety of ICH-recommended test conditions. The developed method will be utilized for studying the stability of 5-FU. The method will be applied for the assessment of stability in the rat caecal content media under anaerobic conditions as a simulated colonic medium, which can be used in subsequent studies to determine the suitability and effectiveness of a potential carrier system for colon cancer.

## Experimental

### Materials

5-FU (lot # C5666A, purity > 99%) was generously donated by Alfa Aesar, A Johnson Matthey Company (Ward Hill, MA). The internal standard, thymine (Figure 1B) (lot # 4226), was purchased from Riedel Dehaën (Darmstadt, Germany). Potassium dihydrogen phosphate and potassium hydroxide were obtained from BDH Laboratory Supplies (BDH Chemicals Ltd., Poole, U.K.). Distilled and deionized water was obtained by passage through ELGA (a trade name of Vivendi Water Systems Ltd., Wycombe, Bucks, U.K.), and was further filtered through a 0.22-µm membrane filter (Millipore, Bedford, MA). Stock solutions were prepared by dissolving the compounds in water. The standard solutions were prepared daily, stored in the dark, and refrigerated.

### Apparatus

*HPLC apparatus and conditions*. Chromatographic separation was optimized with respect to the stationary and mobile phase compositions, flow-rate, sample volume, detection wavelength, and internal standard. The HPLC system consisted of a Waters Model 1515 HPLC pump, a Waters autosampler Model 717 plus, a Waters 2487 dual λ absorbance UV detector (Waters Inc., Bedford, MA) governed by a microcomputer running Empower build version 1154 software, and vortex mixer (Scientific Industries, Inc., Stony Brook, NY). The detector wavelength was set at 260 nm. Separation was achieved by isocratic elution with a mobile phase of 40 mM phosphate buffer adjusted to pH 7.0 (10% w/v potassium hydroxide), delivered at a flow-rate of 1.0 mL/min at ambient temperature through a $C_{18}$ analytical, µ-bondapak column (150 mm length × 4.6 mm i.d., 10 µm particle size).

*Stock solutions and standards*. Stock solutions of 5-FU were prepared in triplicate by dissolving 10.0 mg 5-FU in 100 mL water, resulting in a solutions containing 100 µg/mL. This solution was diluted 10-fold by water to give a working standard solution concentration of 10 µg/mL. A stock solution of the internal standard at a concentration of 100 µg/mL was prepared by dissolving 10.0 mg of thymine in 100 mL water. Working solutions of 5-FU (10 µg/mL) and 100 µg/mL of thymine were prepared by dilution of the stock solutions in water. Standards were prepared with the following concentrations of 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 µg/mL for 5-FU.

### Assay validation

Validation of this assay was in compliance with the current Food and Drug Administration (FDA) guidelines for method validation (22). A validation study included a set of calibration and lower limit of quantification (LLOQ) samples employing quality control portions at three levels performed on six separate occasions. The precision values obtained were calculated by one-way analysis of variance (ANOVA) for each test concentration, using the run-day as the classification variable.

*Precision, accuracy, and limit of quantitation*. Multiple injections ($n$ = 6) were performed on a single day to establish the intra-day (within-day) coefficient of variation (CV; precision). The inter-day CV was determined by the same way. Carry-over between injections was minimal. Before each sample run, the syringe was rinsed and the injector loop was backflushed with mobile phase at a flow-rate of 1.0 mL/min. Standards were assayed in order of increasing concentration. The accuracy was expressed as the ratio of the compound added to that measured (mean value/nominal value) × 100. The limit of detection (LOD) was obtained as the concentration that gives the smallest measurable peak with a signal-to-noise ratio at least equal 3. LLOQ was taken as the lowest concentration in the standard calibration curve. The LLOQ was 100 ng/mL and the LOD of 5-FU was 10 ng/mL.

*Specificity*. Specificity of the method with regard to 5-FU was studied by determining the purity of the drug peak response from a mixture of stressed samples. A resolution factor study was also performed involving the 5-FU peak separation from the nearest resolved degradation product peak (23).

### Accelerated stability studies

In order to determine whether the analytical method and assay were stability-indicating, the stability of 5-FU was determined by subjecting it to alkaline, acidic, oxidative, and thermal conditions in order to accelerate degradation. Regulatory guidances ICH Q2A, Q2B, and Q3B, and FDA 21 CFR section 2111 require the development and validation of stability-indicating potency assays. All stress decomposition studies were carried out at a drug concentration of 1 µg/mL in

water except for oxidative degradation, where an amount of 2 µg/mL was used and found to be sufficient to clearly demonstrate the degradation behavior. HPLC studies on 5-FU under different stress conditions exhibit varying degrees of degradation behavior. It was not the intention of this study to identify degradation products but merely to show that they wouldn't interfere if and when present.

*Degradation in alkali*. An appropriate volume of 5-FU stock solution was taken and diluted with water to prepare a 1.0 µg/mL solution. An aliquot of this solution (1.0 mL) was exposed to hydrolysis by adding 4.0 mL of 0.1 N NaOH. This mixture was protected from light, incubated in a water bath at 60ºC for 60 min, neutralized by addition of 4.0 mL of 0.1 N HCL, and assayed for 5-FU content.

*Acidic conditions*. An appropriate volume of 5-FU stock solution was taken and diluted with water to prepare a 1.0 µg/mL solution. An aliquot of this solution (1.0 mL) was exposed to hydrolysis by adding 4.0 mL of 0.1 N HCl. This mixture was protected from light and incubated in a water bath at 60ºC for 60 min, neutralized by addition of 4.0 mL of 0.1 N NaOH, and assayed for 5-FU content.

*Oxidative conditions*. An appropriate volume of 5-FU stock solution was taken and diluted with water to prepare a 2.0 µg/mL solution. An aliquot of this solution (2.0 mL) was exposed to oxidative degradation by adding 2.0 mL of 30% hydrogen peroxide. This mixture was protected from light and incubated in a shaking water bath at 30ºC for 30 min before analysis.

*Thermal conditions*. An appropriate volume of 5-FU stock solution was taken and diluted with water to prepare a 1.0 µg/mL solution. An aliquot of this solution was protected from light and incubated at 60ºC. After seven days, samples were withdrawn at the appropriate time and subjected to HPLC analysis after suitable dilution.

### Simulated colonic media

*Construction of standard curve in rat caecal content*. Male Wistar rats weighing 200–300 g were used throughout this part. The rats will be sacrificed while under ether anesthesia and the caeci exteriorized, legated at two ends, and cut loose. The contents of the formed caecal bags were individually weighed, pooled, and suspended in chilled phosphate buffer (pH 7) to give a final dilution of 3% (w/v). A 100 mL volume was centrifuged at 4000 rpm for 10 min, and the clear supernatant was taken. Standard solutions of 5-FU were prepared in rat caecal extract supernatant with the following concentrations: 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 µg/mL. Each solution was spiked with 10 µL prior to injection into the HPLC column. The same HPLC conditions used previously were applied here.

*Stability of 5-FU in the colon medium*. The stability of 5-FU in the colon was assessed by incubation of known weights of the drug in rat caecal contents. After sacrificing the rats, the contents of the formed caecal bags were individually weighed, pooled, and suspended in chilled phosphate buffer saline (pH 7) to give a final dilution of 3% (w/v). Ten milligrams of 5-FU were incubated separately in 100 mL of the suspension at 37ºC ± 0.5 and shaken at 80 rpm using a thermostatic shaker. The experiments were performed under nitrogen atmosphere to simulate anaerobic conditions. Half-milliliter samples were withdrawn after 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 12.0, and 24 h post-incubation. The samples were centrifuged at 4000 rpm for 10 min and the clear supernatant was diluted to 25 mL with water. Fifty microliters were injected in the column for analysis using the same previously mentioned HPLC method.

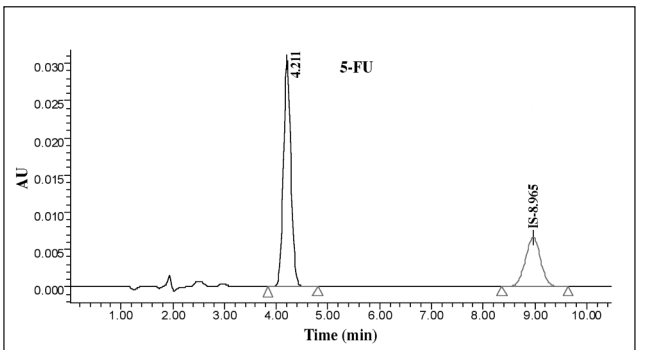


**Figure 2.** A typical HPLC chromatogram of 5-FU and the internal standard, thymine.

**Table I. Intra-Day and Inter-Day Accuracy and Precision of 5-FU (mean ± SD, *n* = 6)***

| $C_{nominal}$ (µg/mL) | Intra-day | | | Inter-day† | | |
|---|---|---|---|---|---|---|
| | $C_{est.}$ (µg/mL) | Precision as CV% | Accuracy % | $C_{est.}$ (µg/mL) | Precision as CV% | Accuracy % |
| 0.1 | 0.099 ± 0.01 | 6.83 | 99.67 | 0.099 ± 0.03 | 2.89 | 99.5 |
| 0.25 | 0.248 ± 0.004 | 1.42 | 99.06 | 0.249 ± 0.014 | 4.15 | 99.6 |
| 0.5 | 0.505 ± 0.023 | 4.59 | 101.07 | 0.503 ± 0.022 | 4.33 | 100.63 |
| 0.75 | 0. 75 ± 0.036 | 4.75 | 100.04 | 0.752 ± 0.023 | 3.08 | 100.22 |
| 1.0 | 0.999 ± 0.013 | 1.27 | 99.93 | 0.996 ± 0.013 | 1.34 | 99.57 |
| 1.5 | 1.496 ± 0.006 | 0.401 | 99.73 | 1.501 ± 0.01 | 0.69 | 100.09 |
| 2.0 | 2.003 ± 0.012 | 0.596 | 100.13 | 2.001 ± 0.009 | 0.43 | 100.03 |

* $C_{nominal}$ = nominal concentration; $C_{est.}$ = estimated concentration.
† Inter-day reproducibility was determined from 6 different runs over a 8-week period at seven concentrations. The concentration of each run was determined from a single calibration run on the first day of the study.

## Results and Discussion

### HPLC

HPLC with UV detection was chosen as a simple, fast, and effective separation method for the determination of 5-FU and its degradation products (24–26). The mobile phase consisting of 40 mmol/L potassium dihydrogen phosphate buffer (pH 7.0) was found to be an appropriate mobile phase, allowing adequate separation of the drug and the internal standard using a $C_{18}$ µ-bondapak column at a flow-rate of 1.0 mL/min. The selected chromatographic conditions provided optimum resolution of 5-FU and the internal standard. Retention times (RT) in minutes for 5-FU and thymine were 4.1 and 8.7 min, respectively.

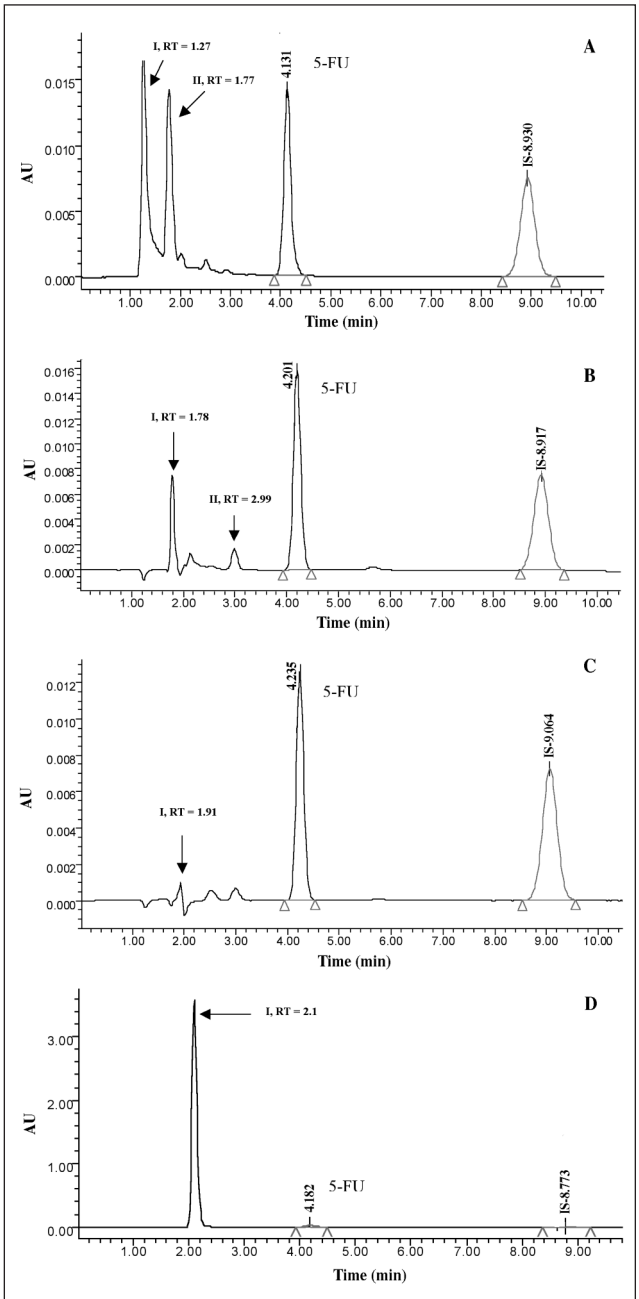


**Figure 3.** Representative HPLC chromatograms of 5-FU samples: sample degraded in 0.1 N HCl (A), sample degraded in 0.1 NaOH (B), sample subjected to thermal analysis (C), and sample subjected to oxidative degradation (D).

### Method validation

*Quantification and linearity*. The calibration curves were linear from 0.1 to 2.0 µg/mL for 5-FU. The mean (± standard deviation; SD) regression equation for six replicated calibration curves constructed on different days was (10.5 ± 2.1461) C + (–0.436 ± 0.3471), $r^2$ = 0.9998 ± 0.0001. Standard curves were constructed over an 8-week period to determine the variability of the slopes and intercepts. The results show little day-to-day variability in the slopes and intercepts. ANOVA of the slopes, intercepts, and correlation coefficients of the six standard plots indicated non-significant differences ($p > 0.05$). The results confirm the linearity of the standard curves and the excellent reproducibility of the assay method. The LOQ for this method was attained with samples containing 10 ng/mL of 5-FU. Figure 2 shows a typical HPLC chromatogram of 5-FU and the internal standard. The chromatogram shown in Figure 2 demonstrates that there is no interference and no noticeable degradation products in the analysis of the original samples.

*Sensitivity*. The LOD, which is defined as the lowest concentration of the analyte which can be detected but not necessarily quantitated, was found to be 10.0 ng/mL.

*Precision and accuracy*. The intra- and inter-day precision and accuracy of the method are presented in Table I. The developed method was found to be precise, as the intra-day SD values of six replicate determinations for one day at the usual working concentrations of 0.1 to 2.0 µg/mL ranged from 0.004 to 0.036 µg/mL with the CV (precision) ranging from 0.4% to 6.83%. In addition, the developed method was found to be precise, as the inter-day (Table I) SD values of six replicate determinations in six consecutive days at the same working concentrations mentioned previously were between 0.003 and 0.023 µg/mL, with CV being in the range of 0.43% to 4.33%. These low values of SD and CV indicate the precision of this stability-indicating method (27–28).

The accuracy was calculated as the % of the drug recovered after analysis relative to the corresponding nominal concentrations. The intra-day (Table I) accuracy was between 99.06% and 101.13%, and the inter-day (Table I) accuracy was between 99.5% and 100.63%. These high values of the % drug recovered reflect the accuracy of the assay method (29). Clearly, the assay method is reliable and applicable for stability assessment of 5-FU under various degradation conditions. Both accuracy and precision values throughout the concentration range (0.1–2.0 µg/mL) were acceptable (22). The specificity of the method with regard to 5-FU was ascertained by the absence of any co-eluted or chromatographic interference peaks of degradation and/or metabolite products (Figures 3A–C).

*Stress studies*. HPLC studies on 5-FU under different stress conditions using phosphate buffer as the solvent system suggested the following degradation behaviors. The results of the degradation studies under various stress conditions (acidic, alkali, oxidative, and thermal) are presented in Table II.

*Acidic conditions*. The drug decreased with time on heating

at 60ºC in 0.1 N HCL, forming two major degradation products at RT (minutes) of 1.27 and 1.77 min, respectively. The rate of hydrolysis in acid was faster as compared to that in alkali (Figure 3A).

*Degradation in alkali*. The drug was found to be less liable to alkaline hydrolysis than acidic hydrolysis. Decomposition of 5-FU results in a major degradation product at an RT of 1.78 min and a minor degradation product at 2.99 min (Figure 3B). The method developed was compared to previously related works (30–31). The previous reports described much less sensitive methods with LOQ ≥ 1250 ng/mL. Our results were in agreement with regard to the degradation of 5-FU in both strong acids and alkali solutions.

*Thermal degradation*. Similar to acidic conditions, the drug was found to be liable to degradation. The percentage of drug degraded was 87.8% ($p > 0.05$) (Table II). This resulted in the appearance of an extraneous peak in the chromatogram at an RT of approximately 1.91 min, which could be due to a degradation product as shown in Figure 3C.

*Oxidative conditions*. The drug was found to be highly labile to oxidative hydrolysis. The reaction in 2.0 mL of 30% hydrogen peroxide was so rapid that under these experimental conditions, the concentration of the parent drug fell by 90% with a corresponding concentration increase for the degradation product (Table II). The major degradation product was detected at an RT of 2.1 min, as illustrated in Figure 3D.

*Stability of 5-FU in the colon medium*. The standard calibration curve of 5-FU in the rat caecal content was $R^2 = 0.9988$ in the concentration range from 0.5 to 5 µg/mL, indicating excellent linearity over the experimental range. Figure 4 shows the chromatograms of 5-FU over various incubation times in the rat caecal contents. It is clear that no degradation occurred after incubation of 5-FU in the rat caecal contents over the 24 h time period. The ratio of the drug area to the internal standard area remained the same.

**Table II. Analysis of 5-FU Under Various Stress Conditions (mean ± SD, *n* = 3)**

| Stress condition | Initial drug concentration | Amount of drug remaining | CV (%) | Drug remaining (%) | Drug degraded (%) |
|---|---|---|---|---|---|
| Acidic | 1.0 µg/mL | 0.133 ± 0.015 | 11.45 | 13.33 | 86.67 |
| Alakine | 1.0 µg/mL | 0.168 ± 0.044 | 26.58 | 16.83 | 83.17 |
| Thermal | 1.0 µg/mL | 0.122 ± 0.009 | 8.02 | 12.22 | 87.78 |
| Oxidative | 2.0 µg/mL | 0.199 ± 0.008 | 4.380 | 9.98 | 90.07 |

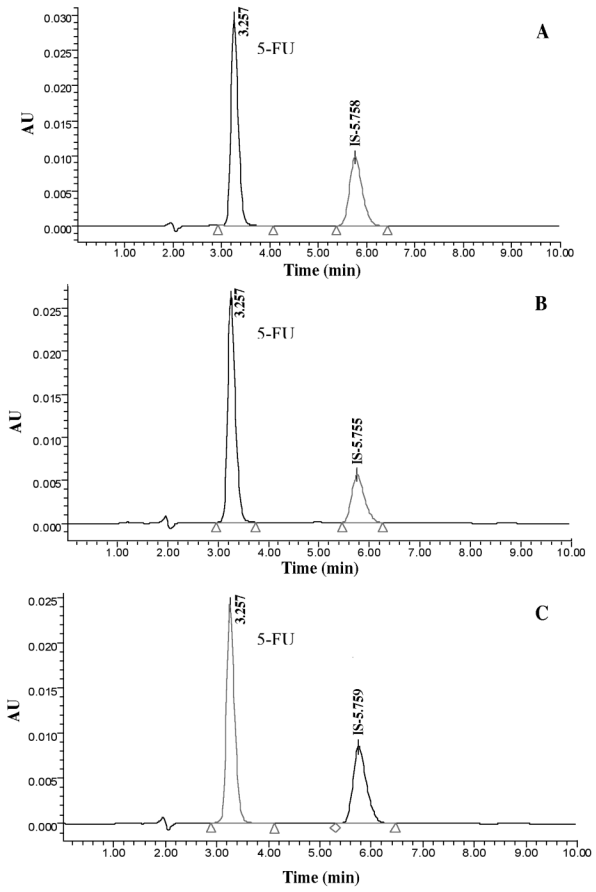


**Figure 4.** HPLC chromatograms of 5-FU incubated in 3% suspension of rat caecal content under anaerobic conditions: after 1 h (A), after 6 h (B), and after 24 h (C).

## Conclusion

The HPLC method developed in this study is rapid, sensitive, and specific. The precision and accuracy of the method are within acceptable range. The simplicity of technique, the minimal volume requirements, and the high sensitivity make this technique particularly attractive for the quantification of 5-FU in pharmaceuticals. The results of the stress testing, undertaken according to the ICH, reveal that the method is selective and stability-indicating. It is clear that no degradation occurred after incubation of 5-FU in the rat caecal contents for 24 h. The method can also be readily adapted to routine quality control analysis.

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