

Development and Validation of an HPLC-DAD Method for the Determination of 5-Fluorouracil in Plasma

Bui Van Hoi^{1*}, Nguyen Phuong Thao¹, Chu Dinh Binh²

¹University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, Ha Noi, Vietnam

²School of Chemistry and Life Science, Hanoi University of Science and Technology, Ha Noi, Vietnam

*Corresponding author email: bui-van.hoi@usth.edu.vn

Abstract

Cancer remains one of the most significant health challenges worldwide, characterized by uncontrolled cell growth. However, it can be cured by using anticancer drugs. 5-fluorouracil is a drug used to treat various types of cancer with the inhibitory mechanism of the enzyme thymidylate synthase, which is crucial for DNA replication in cancer cells, leading to errors in their growth. Although effective, the short half-life of 5-FU and individual metabolic differences among patients limit its therapeutic potential. Therefore, it is necessary to monitor the 5-FU concentration during treatment to ensure the treatment efficiency. However, monitoring this compound in plasma is challenging due to the coexistence of complicated matrices. The study optimized and validated a sensitive method to quantify 5-fluorouracil in mice plasma using liquid-liquid extraction combined with a high-performance liquid chromatography-photodiode array detector (HPLC-PAD). The technique achieved excellent linearity, precision, accuracy, detection limits (LOD = 1.12 µg/L), and quantification (LOQ = 3.74 µg/L). The optimal extraction solvent was ethyl acetate in two replicates (2×3mL), PSA/C18 was the most effective cleaning agent with 97.9 ± 0.2% recovery and the mass of PSA/C18 did not affect the extraction recovery. However, high plasma volume reduced the recovery of 5-FU in plasma. Finally, in vivo studies in mice were used to confirm this method. The findings of this research underscored the importance of personalized dosing strategies based on 5-FU concentration in blood.

Keywords: 5-Fluorouracil (5-FU), HPLC-DAD, plasma, PSA, C18, pharmacokinetics.

1. Introduction

Cancer remains a leading cause of death worldwide, claiming millions of lives annually. According to GLOBOCAN statistics, which provide insights into the cancer situation based on reports from 185 out of 204 countries, predicts that in 2040 the number of cancer cases will increase to 28.4 million [1]. Developed in the 1950s, 5-fluorouracil (5-fluoropyrimidine-2,4(1H,3H)-dione) is an antimetabolite and antineoplastic agent targeting uracil metabolism in uncontrolled dividing cancer cells. Uracil is a key nucleotide base, and its metabolism is essential for synthesizing and repairing DNA and RNA. 5-FU's mechanism involves replacing the normal nucleotide uracil in RNA/DNA, consequently breaking the integrity of their genetic material, and inhibiting thymidylate synthase (TS), an enzyme involved in DNA replication and repair [2]. By inhibiting TS, 5-FU depletes the cell's supply of thymidine, leading to DNA damage and cell death, particularly during the DNA-synthesis phase of the cell cycle (S-phase), leading effectively against various types of cancer, especially the colorectal [3]. Moreover, metabolites of 5-FU, such as 5-fluorouridine triphosphate (FUTP) and 5-fluoro-2'-deoxyuridine

triphosphate (FdUTP), can be also integrated into RNA and DNA. This integration disrupts normal RNA processing and functioning, and causes DNA damage, further impairing cell division and leading to cell death.

However, 5-FU has a short half-life (the time it takes for the body to eliminate half the drug) and limited activity, which is most effective during the DNA synthesis phase (S-phase). Moreover, patients have different changes in metabolism and clearance rates in the blood. These differences lead to reduced treatment efficiency (not enough drug to kill cancer cells) or side effects (too much drug). To address this, personalized treatment regimens and approaches are important. By monitoring the 5-FU concentration in a patient's plasma, doctors can regulate the suitable dosage for each individual, allowing for a targeted and safer treatment strategy [4]. Analytical chemistry includes many analytical methods for quantifying anticancer drugs in biological samples, such as high-performance liquid chromatography (HPLC), gas chromatography (GC), and capillary electrophoresis (CE) [5]. However, the quantification of anticancer drugs like 5-fluorouracil (5-FU) in biological samples is a challenge for analytical chemists because the sample matrices such as protein,

fat, and other organic compounds can affect the analytical signals. Furthermore, GC requires volatile samples, but 5-FU is not volatile in its natural state and needs derivatization (chemical modification) before analysis [6]. Besides, CE only introduces a low amount of sample volume into the capillary (nanoliters) and a low absorption path length if UV detection is used, which leads to poor concentration sensitivity. Therefore, HPLC is often chosen over other analytical chemistry methods thanks to its speed, accuracy, and reproducibility. This technique can analyze a large number of compounds, including drugs and their metabolites, allowing for the identification and quantification of individual compounds in a mixture [7].

Previously, different studies have examined 5-FU's clinical pharmacokinetics in the body cells or plasma. These studies have explored various HPLC methods to accurately measure 5-FU levels. However, the methods had a limit of quantitation (LOQ) and a limit of detection (LOD) that were slightly high (20 µg/L and 10 µg/L, respectively) and low recovery (35-75%) [8-10]. These researches have only focused on the use of strong solvents to precipitate protein before analysis. The presence of other organic substances (non-precipitated protein, lipid,...) can lead to an increase in background noise. Therefore, an extra clean-up step is needed to remove these impurities. In addition, low recovery rates can lead to the inability to determine the exact dose, causing underdosing or unnecessary dose adjustments. This presents a gap in our understanding and warrants dedicated investigation to explore the analytical chemistry of 5-FU in plasma. Therefore, analytical methods for 5-FU measurement should target a lower LOD, a lower LOQ, and a recovery rate closer to 100%.

To address this gap, this research introduces an analytical method combining liquid-liquid extraction and HPLC with a Diode Array Detector (HPLC-DAD) that offers better sensitivity for detection and higher recovery of 5-FU from blood samples. This study aims to optimize a sensitive quantitative method of 5-FU. The validated method was used to analyze 5-FU in plasma samples using liquid-liquid extraction (LLE) combined with an HPLC-DAD. This method was also applied to monitor the kinetics of 5-FU in mice.

2. Materials and Methods

2.1. Chemicals and Reagents

The 5-FU standard (>98%, purity) was obtained from the National Institute of Drug Quality Control (NIDQC). Ethyl acetate (EA), acetonitrile (ACN), phosphoric acid (H₃PO₄), and sodium dihydrogen phosphate (NaH₂PO₄) were purchased from Fisher Scientific. The clean-up phases originated from different suppliers: PSA (1200 mg MgSO₄/400 mg PSA, DisQuE, Ireland), PSA/C18 (1200mg MgSO₄/400mg PSA/400mg C18), C18 (Agilent Technologies, USA), and HLB (Waters, USA). The ultrapure water (UPW) was produced from the GENPURE UV/UF system

(Thermo Scientific, England) and was used throughout this study. Since the extraction optimization requires a large volume of plasma and each mouse can provide only 1.2 - 1.5 mL of blood. Therefore, the pooled human plasma sample was used for this experiment. this research used human plasma collected from Bach Mai hospital from volunteers to optimize the extraction process. The pooled sample was prepared by mixing all donated human plasma samples in the same portions.

2.2. Stock and Standard Solutions

The stock solution of 1000 mg/L 5-FU was prepared by dissolving pre-weighted amount of 5-FU in methanol (LC-MS grade, Sigma Aldrich, Singapore). After that, a working solution at 50 mg/L was prepared monthly by dilution of stock solution using methanol. Both stock solution and working solution were stored at -20 °C. The calibration series (5, 10, 20, 50, 100, 200, 500, 1000, and 2000 µg/L) were daily prepared by diluting the working solution in the mobile phase.

2.3. Kinetic of 5-FU in studies

Twenty-five BALB/c mice (7-8 weeks old, approximately 25 g) were housed at 25 °C and administered a single oral dose of 5-FU at 50 mg/kg body weight. Blood samples were collected from the mice at predetermined times (30, 90, 150, 210, and 270 minutes after the drug administration, denoted as *t*₀, *t*₁, *t*₂, *t*₃, and *t*₄, each sampling, five mice were used to collect blood, and put in the tube containing antifreeze EDTA (1.2 mg to 2 mg/mL blood). The blood samples were then centrifuged at 4200 rpm for 30 minutes at 15 °C to isolate the plasma. The collected plasmas were stored in a fridge at 4 °C, then extracted and analyzed within 48h after the experiment.

2.4. Extraction and Clean-Up Procedure

All experiments were performed in triplicates. A plasma volume ranging from 50-500 µL was pipetted into a 15 mL centrifuge tube. Then, the sample was spiked with a 5-FU standard solution (targeted 50 to 1000 µg/L via analysis). To optimize the extraction process, different volumes of solvents (Ethyl acetate or acetonitrile) were added and vortexed for 1 minute followed by an ultrasonic for 5 minutes. Samples were then centrifuged at 4200 rpm for 10 minutes. After centrifugation, the upper organic layer was carefully transferred to another 15 mL falcon for the clean-up step. During the next phase, 50-200 mg of the cleaning sorbent was added to the extract. The mixture was vortexed for 1 minute and was centrifuged at 4200 rpm for 10 minutes to separate the extract and clean-up phase. The extract was collected and evaporated under a gentle stream of nitrogen at 50 °C until dryness. The residue was reconstituted in 500 µL of a water/methanol (H₂O/MeOH, 90/10, v/v), filtered through a syringe filter (13 mm i.d., 0.2 µm pore size), and transferred to the vial before subjecting to the HPLC system.

2.5. Method Development and Validation According to IUPAC Guidelines

2.5.1. Chromatographic optimization

The HPLC (Vanquish, Thermofisher Scientific, UK) equipped with a C18 reversed-phase column (250 mm × 4.6 mm, 5 μm, ODS-H, UK) was used. The instrumental setup and data treatment were monitored by Chromeleon software (version 7, ThermoScientific, USA). Different compositions of mobile phases were investigated and a mixture of methanol: water (containing 40 mM phosphate buffer, pH 3) at a ratio of 10:90 (v/v) in isocratic mode was chosen. The flow rate was constantly set at 1.2 mL/minute with an injection volume of 10 μL. The column was stabilized at 40 °C to reduce the viscosity of the mobile phase.

2.5.2. Precision and accuracy

Intra- and inter-day precisions were measured at low (50 μg/L), medium (200 μg/L), and high (1000 μg/L) concentrations within a single day and three consecutive days, respectively. The accuracy as well as precision were evaluated by comparing the measured concentrations of 5-FU to the theoretical values (recovery). The recovery values close to 100% indicate that the method accurately measures the 5-FU concentrations [11].

2.5.3. Sensitivity

The analytical method's sensitivity was assessed through the determination of the limit of detection (LOD) and limit of quantification (LOQ). The *LOD* is the minimum amount of analyte that can be detected which differentiates the signal from background interference. However, the *LOQ* defines the minimum concentration at which the analyte can be quantified with acceptable precision and accuracy [11]. *LOD* and *LOQ* were calculated following equations (1) and (2) respectively.

$$LOD = 3 \times \frac{S}{N} \quad (1)$$

$$LOQ = 10 \times \frac{S}{N} \quad (2)$$

where *S* is the signal (peak height) and *N* is the noise (baseline noise or baseline fluctuation nearby 5FU peak) that were determined on the chromatogram of spiking plasma sample at low concentration [11].

3. Results and Discussion

3.1. HPLC Performance and Method Validation

Validation of this study was done in compliance with IUPAC guidelines [12]. The following parameters were assayed: linearity range, limit of detection (LOD) and quantification (LOQ), precision, and recovery.

3.1.1. Chromatographic optimization

In general, a C18 reversed phase column (BDS Hypersil™ C18, 250 mm × 4.6 mm, 5 μm) and a

mobile phase consisting 90% of 40mM phosphate buffer at pH equal 3 and 10% of MeOH (volume/volume) at a flow rate of 1.2 mL/minute. The UV spectrum of 5-FU was recorded by 3D scan mode to obtain the optimal absorbance wavelength for quantification.

For quantification, 265 nm was selected for 5-FU in all analyses. The typical peak separation and response using this analytical method are shown in Fig. 1. The retention times obtained were 2.8 minutes for 5-fluorouracil with a total time for the chromatographic determination of 7 minutes. Fig. 1 shows the chromatogram of 1 mg/L 5-FU standard at 265 nm. 5-FU has different absorbance wavelengths at 195 nm [13] and 265 nm [14]. However, methanol used as a mobile phase has a UV cut-off at 205 nm, and this has affected the absorbance of 5-FU [15]. Chromatographic conditions were chosen according to results obtained from previous experiments.

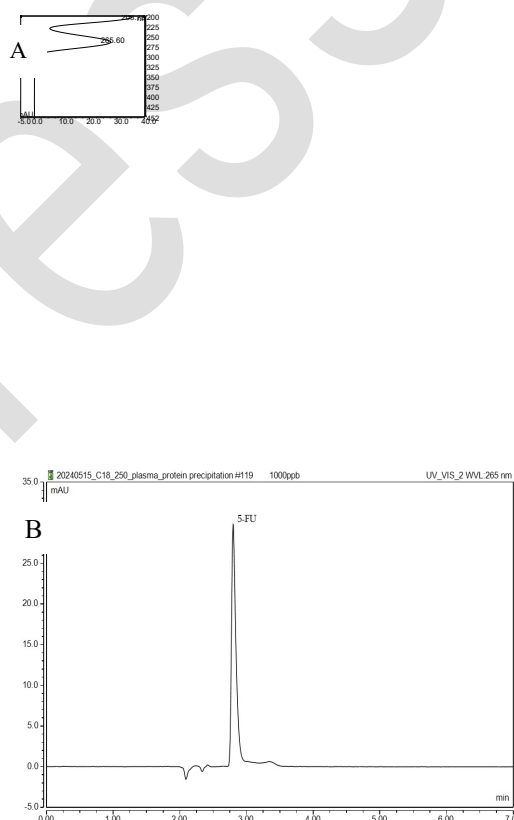


Fig. 1. A) UV spectrum of 5-FU and B) Chromatogram of 5-FU at 1000 μg/L standard at 265 nm.

3.1.2. Precision and accuracy

The precision of the method used to measure 5-fluorouracil (5-FU) concentration in plasma samples using HPLC-DAD by inter- and intra-day precision, measured at three different 5-FU concentrations (low: 50 μg/L, medium: 200 μg/L, high: 1000 μg/L). The results were evaluated in Table 1. The observed Relative Standard Deviation (*RSD*) values in both intra- and inter-day measurements are relatively low,

suggesting that the method is reliable for measuring 5-FU concentration.

Table 1. The recovery of 1000 µg/L 5-FU in plasma depending on the mass of the cleaning agent.

Mass of PSA/C18 (mg)	Recovery (± SD)	RSD (%)
50	92.9 ± 10.2	11.0
100	97.9 ± 0.2	0.2
200	104.4 ± 1.2	1.1
500	94.5 ± 3.7	3.9

3.1.3 Linearity, limit of detection, and limit of quantitation

A series of 5-FU standards was prepared at concentrations ranging from 20 to 2000 µg/L. The linearity was statistically confirmed the regression coefficient was not different from 1 ($R^2 = 0.9969$) which signifies how well the data points fit a straight line (Fig. 2). The LOD and LOQ of this method were determined as mentioned above and LOD and LOQ were 1.12 µg/L and 3.74 µg/L, respectively. LOD and LOQ were high enough for accurate and reliable analysis of 5-FU in the plasma sample [16].

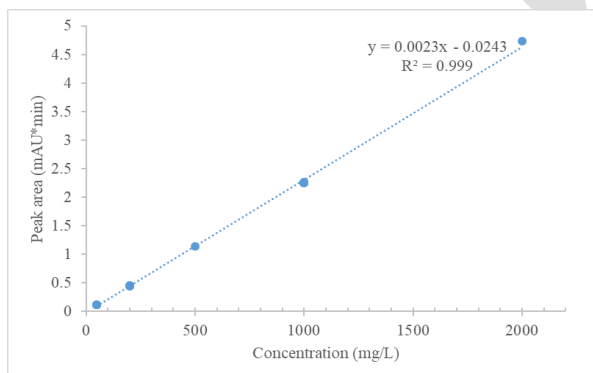


Fig. 2. Linearity range of 5-FU

3.2. Optimization of Extraction Solvent

3.2.1. Choosing extract solvent

This experiment aimed to identify the optimal solvent for extracting 5-FU from plasma samples. Two common extraction solvents EA and ACN were evaluated. Fig. 3A shows the chromatogram of plasma samples extracted by ACN, EA, and in standard at the same concentration (1000 µg/L). The baseline of the sample extracted by ACN (black) was significantly higher than that of the standard solution (pink). However, the baseline from the EA solvent (blue) closely matched the baseline of the standard solution. A higher baseline in the chromatogram indicates the presence of interfering substances that elute alongside the analyte (5-FU) and can potentially mask its signal. It suggests that the lower the baseline, the less impurities were eluted, which led to higher recovery [17]. Therefore, EA was selected for extracting 5-FU from plasma samples.

3.2.2. Optimization of solvent volume

Following the selection of EA, this experiment aimed to optimize the volume of EA required for efficient extraction. Plasma samples were spiked with 5-FU (1000 µg/L) and extracted with varying volumes of EA: 1 mL, 2 mL, 3 mL, 4 mL, 2×2.5mL, and 2×3mL (Fig. 3B). The results show increasing recoveries with increasing solvent volume, reaching a maximum of around 92.4% with 2×3mL. This suggests that with higher volumes, 5-FU is efficiently extracted from the plasma matrix. RSD values are low (below 0.5%) across all volumes, indicating good precision (reproducibility) within each tested volume [18]. Increasing the volume over 6mL is not necessary and is less desirable due to factors like increased processing time and solvent usage or waste of solvent.

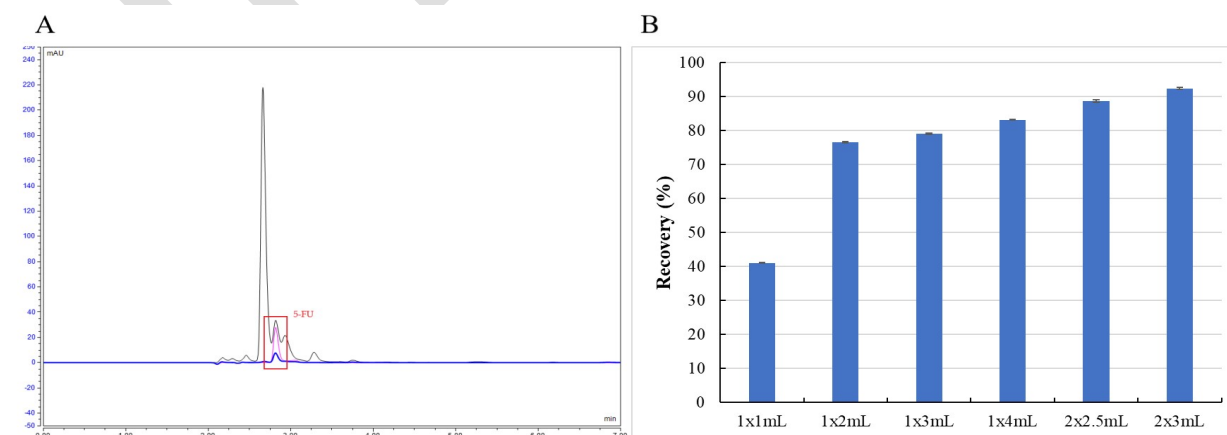


Fig. 3. The influence of solvent extraction on the 5-FU signal in the plasma sample. (A) HPLC chromatogram of plasma sample spiked with 1000 µg/L 5-FU extracted with EA (blue), extracted with ACN (black), and in standard solution (pink); (B) The recoveries of 5-FU at different extraction volumes

3.3. Optimization of Clean-up Phases

3.3.1. The type of cleaning sorbents

The cleaning sorbents were investigated with a weight of 100mg including Primary Secondary Amines (PSA), C18 stationary phase (C18), and the mixture of PSA with C18 (PSA/C18), and Hydrophilic-Lyophilic Balance (HLB). PSA/C18 showed the highest recovery (around 97.85%) with a low variation ($SD = 0.15$). However, PSA (combined with $MgSO_4$) and C18 have significantly lower recoveries (around 72% and 71% respectively) with higher variation (SD around 5 and 7). Moreover, Fig. 4 suggests that using PSA/C18 results in a chromatogram with the lowest baseline and least interference from other peaks in the sample. Besides, PSA (with $MgSO_4$) or C18 does not effectively remove interfering substances in the plasma samples compared to the PSA/C18 combination. The combination of PSA and C18 (PSA/C18) offers a synergistic effect [19], removing effectively interferences while minimizing 5-FU loss. HLB has a stronger affinity for 5-FU leading to its retention during cleanup and a lower recovery in the analysis, leading HLB to have the lowest recovery (around 68.5%).

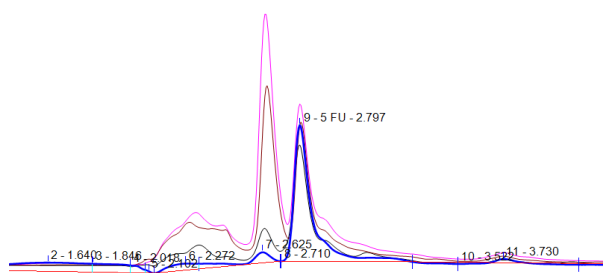


Fig. 4. The influence of background noise when analyzing 5-FU in different cleaning-agents. (Blue: PSA/C18, black: PSA, brown: C18, pink: HLB)

3.3.2 Effect of the clean-up weight

In principle, increasing the mass of the clean-up phase could remove the higher amount of impurities. However, using a higher mass of the clean-up phase might lead to a higher analysis cost. This experiment investigated the impact of PSA/C18 mass on recovery efficiency. Variable masses of PSA/C18 were used at 50 mg, 100 mg, 200 mg, and 500 mg. As results from Table 1, the lowest dose of 50 mg, the recovery was relatively high at 92.9%, but the relative standard deviation (RSD) was quite large at 11%. This high variability was due to the low removal of the matrix, leading to an increase in background noise. Using 100 mg, 200 mg, and 500 mg of PSA/C18 also resulted in high recoveries with extremely small RSD s, indicating consistent and efficient cleanup. However, to ensure cost-effectiveness in method optimization, 100 mg of PSA/C18 was chosen as it provided high recovery with minimal variation, ensuring efficient cleanup and consistent recovery of 5-FU.

3.4. Effect of 5-FU Concentration and Plasma Volume on Recovery

This experiment investigated the impact of the concentration of 5-FU and plasma volume in plasma samples on the recovery efficiency during the analysis process. Plasma samples were spiked with 5-FU at three different concentrations: 50 $\mu\text{g/L}$, 200 $\mu\text{g/L}$, and 1000 $\mu\text{g/L}$ (low, medium, and high concentration) with a similar sample preparation procedure. Different concentrations of 5-fluorouracil (5-FU) showed an influence on the rate of recovery (Fig. 5A). At low concentrations (50 $\mu\text{g/L}$), the recovery is only 37.6% with a high standard deviation (SD) (9.0). This is because the signal from 5-FU is weak and masked by background noise, leading to lower recovery and higher variability [19]. As the concentration increases to 200 $\mu\text{g/L}$, recovery improves to 54.7% with a lower SD (4.5). At 1000 $\mu\text{g/L}$ (highest concentration), the recovery reaches 97.9% with a very low SD (0.2). This method performs well for quantifying 5-FU in plasma at higher concentrations.

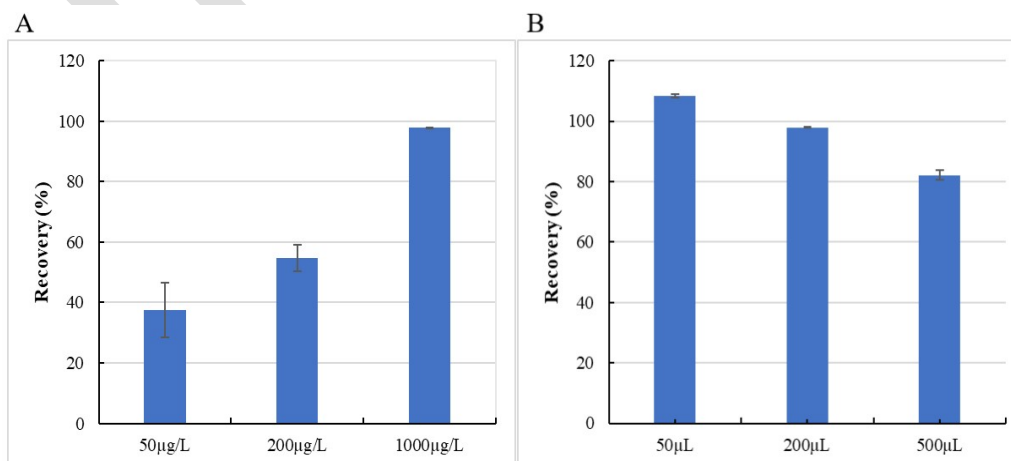


Fig. 5. A) The recovery of 5-FU in plasma at different concentrations; B) The effect of plasma volume on recovery

Three different plasma volumes (50 μ L, 200 μ L, and 500 μ L) spiked at 1000 μ g/L 5-FU via analysis were extracted and purified by the above optimization processes (Fig. 5B). The results showed that 50 μ L plasma shows the highest recovery (108.4%) with a very low *SD* (0.5). However, 500 μ L plasma shows the recovery decreased significantly (82.1%) with a slightly higher (*SD* \pm 1.6). This suggests that increased matrix effects from larger amounts of plasma components interfere with 5-FU detection (masked by background noise) [19].

3.5. In Vivo Study of 5-Fluorouracil in Mice

In vivo, 5-fluorouracil (5-FU) studies provide important information on therapeutic efficacy and dosage requirements. This study confirmed the effectiveness of the method in the analysis. The experiments were performed on 25 individual mice. At each interval time, five mice were used to collect plasma. The 5-FU concentration was presented as the average value of 5 individual with standard variation. Fig. 6 shows the variation of 5-FU concentration in mice plasma collected after 256 minutes (or approximately 4 hours and 16 minutes), which is considered to be moderate. This means the drug stays in the body for a reasonable amount of time, allowing for less frequent dosing compared to drugs with a shorter half-life. 5-FU is rapidly absorbed into the bloodstream after oral administration in mice. The drug reaches its peak concentration relatively quickly (90 minutes), consistent with 5-FU, typically characterized by rapid absorption and distribution [10].

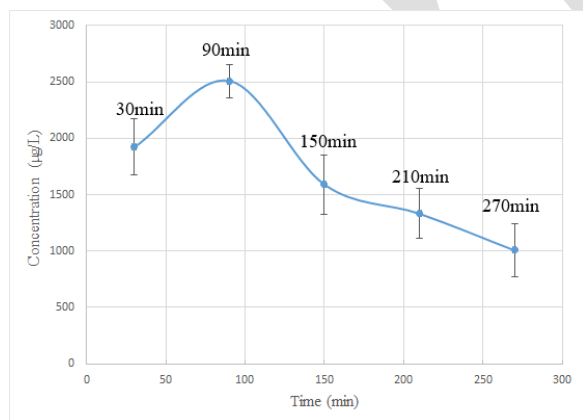


Fig. 6. Variation of 5-FU concentration in mouse plasma at interval times t_0 , t_1 , t_2 , t_3 , t_4 (30 minutes, 90 minutes, 150 minutes, 210 minutes, 270 minutes, respectively)

The measured 5-FU concentration increased from 1922.2 μ g/L at t_0 to 2505.9 μ g/L at t_1 . This is an absorption phase where 5-FU enters the bloodstream from the administration site. The concentration then decreases progressively from t_1 (2505.9 μ g/L) to t_4 (1011.4 μ g/L), indicating an elimination phase during which metabolic and excretory processes reduce the drug's concentration in the plasma [20].

4. Conclusion

This study successfully validated a selectivity and sensitivity analytical method for quantification of 5-fluorouracil (5-FU) in plasma sample. The method demonstrated good linearity, precision, accuracy, limits of detection, and quantification (LOD and LOQ) within the specified concentration range. The ethyl acetate (2 \times 3mL) was used as the optimal extraction solvent; PSA/C18 was used as the clean-up agent and a slight effect of PSA/C18 mass was found in the extraction recovery; and the plasma volumes influenced recovery. Furthermore, the initial *in vivo* observations based on the measured plasma concentrations suggest rapid absorption and elimination of 5-FU in mice. Therefore, personalized dosing regimens are important to maintain therapeutic levels, especially in patients with variable metabolic rates or compromised organ function. Future research should explore the mechanisms of interactions further and develop strategies to optimize 5-FU therapy, including the use of continuous infusion and tailored dosing schedules to improve patient outcomes. Additionally, investigating the application of LC-MS/MS techniques could potentially provide more revealing additional details about fate of 5-FU metabolites, its transformation products, and potential interactions with other drugs or biological matrices.

References

- [1] S. V. S. Deo, J. Sharma, and S. Kumar, GLOBOCAN 2020 Report on global cancer burden: Challenges and opportunities for surgical oncologists, *Annals of Surgical Oncology*, vol. 29, no. 11, pp. 6497-6500, 2022. <https://doi.org/10.1245/s10434-022-12151-6>
- [2] R. Ladner, The role of dUTPase and uracil-DNA repair in cancer chemotherapy, *Current Protein and Peptide Science*, vol. 2, iss. 4, pp. 361-370, 2001. <https://doi.org/10.2174/1389203013380991>
- [3] H. liang Wu, Y. Gong, P. Ji, Y. fan Xie, Y. Z. Jiang, and G. yu Liu, Targeting nucleotide metabolism: a promising approach to enhance cancer immunotherapy, *Journal of Hematology and Oncology*, vol. 15, no. 1, Apr. 2022. <https://doi.org/10.1186/s13045-022-01263-x>
- [4] E. C. Gamelin, E.M. Danquechin-Dorval, Y. F. Dumesnil, P. J. Maillart, M. J. Goudier, P. C. Burtin, R. G. Delva, A. H. Lortholary, P. H. Gesta, and F. G. Larra., Relationship between 5-fluorouracil (5-FU) dose intensity and therapeutic response in patients with advanced colorectal cancer receiving infusional therapy containing 5-FU, *American Cancer Society*, vol. 77, iss. 3, pp. 441-451, Feb. 1996. [https://doi.org/10.1002/\(SICI\)1097-0142\(19960201\)77:3<441::AID-CNCR4>3.0.CO;2-N](https://doi.org/10.1002/(SICI)1097-0142(19960201)77:3<441::AID-CNCR4>3.0.CO;2-N)
- [5] R. Sabourian, S. Z. Mirjalili, N. Namini, F. Chavoshy, M. Hajimahmoodi, and M. Safavi, HPLC methods for quantifying anticancer drugs in human samples: a systematic review, *Analytical Biochemistry*, vol. 610, Dec. 2020. <https://doi.org/10.1016/j.ab.2020.113891>

- [6] A. Marcillo, B. M. Weiß, A. Widdig, and C. Birkemeyer, Challenges of fast sampling of volatiles for thermal desorption gas chromatography - mass spectrometry, *Journal of Chromatography A*, vol. 1617, Apr. 2020. <https://doi.org/10.1016/j.chroma.2019.460822>
- [7] A. Escarpa and M. C. González, High-performance liquid chromatography with diode-array detection for the determination of phenolic compounds in peel and pulp from different apple varieties, *Journal of Chromatography A*, vol. 823, no. 1-2, pp. 331-337, Oct. 1998. [https://doi.org/10.1016/S0021-9673\(98\)00294-5](https://doi.org/10.1016/S0021-9673(98)00294-5)
- [8] E. Gamelin, M. Boisdron-Celle, A. Turcant, F. Larra, P. Allain, and J. Robert, Rapid and sensitive high-performance liquid chromatographic analysis of halogenopyrimidines in plasma, *Journal of Chromatography B: Biomedical Science and Applications*, vol. 695, no. 2, pp. 409-416, Aug. 1997. [https://doi.org/10.1016/S0378-4347\(97\)00211-9](https://doi.org/10.1016/S0378-4347(97)00211-9)
- [9] J. M. Joulia, F. Pinguet, P. Y. Grosse, C. Astre, and F. Bressolle, Determination of 5-fluorouracil and its main metabolites in plasma by high-performance liquid chromatography: application to a pharmacokinetic study, *Journal of Chromatography B: Biomedical Sciences and Applications*, vol. 692, iss. 2, pp. 427-435, May 1997. [https://doi.org/10.1016/S0378-4347\(96\)00518-X](https://doi.org/10.1016/S0378-4347(96)00518-X)
- [10] Y. S. Sim, Z. Y. Chong, J. Azizi, and C. F. Goh, Development and validation of a gradient HPLC-UV method for mitragynine following in vitro skin permeation studies, *Journal of Chromatography B*, vol. 1204, Jul. 2022. <https://doi.org/10.1016/j.jchromb.2022.123316>
- [11] S. Handali, Z. Ramezani, E. Moghimipour, M. Rezaei, and F. A. Dorkoosh, A novel method for the simultaneous determination of 5-fluorouracil and oxaliplatin in new biodegradable PHBV/PLGA nanoparticles, *Journal of the Iranian Chemical Society*, vol. 16, no. 3, pp. 609-615, Oct. 2018. <https://doi.org/10.1007/s13738-018-1538-1>
- [12] F. T. Peters, O. H. Drummer, and F. Musshoff, Validation of new methods, *Forensic Science International*, vol. 165, iss. 2-3, pp. 216-224, Jan. 2007. <https://doi.org/10.1016/j.forsciint.2006.05.021>
- [13] R. R. Larson, M. B. Khazaeli, and H. K. Dillon, Development of an HPLC method for simultaneous analysis of five antineoplastic agents, *Applied Occupational and Environmental Hygiene*, vol. 18, iss. 2, pp. 109-119, 2003. <https://doi.org/10.1080/10473220301432>
- [14] A. M. P. de P. Alcântara, R. V. Vitor, E. P. Vieira, and I. Martins, Simultaneous detection of three antineoplastic drugs on gloves by liquid chromatography with diode array detector, *Brazilian Journal of Pharmaceutical Sciences*, vol. 46, no. 4, pp. 731-740, Dec. 2010. <https://doi.org/10.1590/s1984-82502010000400015>
- [15] A. Di Paolo, R. Danesi, L. Ciofi, F. Vannozzi, G. Bocci, M. Lastella, F. Amatori, B. M. Martelloni, T. Ibrahim, D. Amadori, A. Falcone, and M. Del Tacca, Improved analysis of 5-fluorouracil and 5,6-dihydro-5-fluorouracil by HPLC with diode array detection for determination of cellular dihydropyrimidine dehydrogenase activity and pharmacokinetic profiling, *Therapeutic Drug Monitoring*, vol. 27, no. 3, pp. 362-368, Jun. 2005. <https://doi.org/10.1097/01.ftd.0000162016.11148.1b>
- [16] A. Krueve, R. Rebane, K. Kipper, M.L. Oldekop, H. Evard, K. Herodes, P. Ravio, and I. Leito, Tutorial review on validation of liquid chromatography-mass spectrometry methods: part II, *Analytica Chimica Acta*, vol. 870, no. 1, pp. 8-28, Apr. 2015. <https://doi.org/10.1016/j.aca.2015.02.016>
- [17] N. F. Semail, A. S. Abdul Keyon, B. Saad, S. S. Noordin, N. N. S. Nik Mohamed Kamal, N. N. Mohamad Zain, J. Azizi, S. Kamaruzaman, and N. Yahaya., Analytical method development and validation of anticancer agent, 5-fluorouracil, and its metabolites in biological matrices: An updated review, *Journal of Liquid Chromatography and Related Technologies*, vol. 43, iss. 15-16, pp. 562-579, Jul. 2020. <https://doi.org/10.1080/10826076.2020.1781654>
- [18] A. Vermes, H. J. Guchelaar, and J. Dankert, Flucytosine: A review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions, *Journal of Antimicrobial Chemotherapy*, vol. 46, no. 2, pp. 171-179, Aug. 2000. <https://doi.org/10.1093/jac/46.2.171>
- [19] K. Gong, P. Sun, Y. Cai, X. Wang, Y. Pang, C. Liu, J. Guo, and L. Fang, Water-compatible cross-linked pyrrolidone acrylate pressure-sensitive adhesives with persistent adhesion for transdermal delivery: Synergistic effect of hydrogen bonding and electrostatic force, *Acta Biomaterialia*, vol. 179, pp. 130-148, Apr. 2024. <https://doi.org/10.1016/j.actbio.2024.02.045>
- [20] A. C. De Mattos, C. Altmeyer, T. T. Tominaga, N. M. Khalil, and R. M. Mainardes, Polymeric nanoparticles for oral delivery of 5-fluorouracil: formulation optimization, cytotoxicity assay and pre-clinical pharmacokinetics study, *European Journal of Pharmaceutical Sciences*, vol. 84, pp. 83-91, Mar. 2016. <https://doi.org/10.1016/j.ejps.2016.01.012>