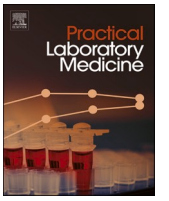
Practical Laboratory Medicine 40 (2024) e00415

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm



Registered Report Stage II

Optimizing cancer patient care with a robust assay for 5-fluorouracil quantification and in-vitro stability in human blood for therapeutic drug monitoring

Murari Gurjar [a,c,e,1], K. Ambedkar Priyan [b,1], Priyanka Asia [c,e], Uday Kumar [c], Kajal Shukla [a], Bal Krishna Mishra [d,e], Akhil Kapoor [d,e,**], Pratibha Gavel [c,e,*]

[a] Department of Clinical Pharmacology, Homi Bhabha Cancer Hospital & Mahamana Pandit Madan Mohan Malaviya Cancer Centre, Tata Memorial Centre, Varanasi-221005, India
[b] Department of Biochemistry, Christian Medical College, Vellore-6326002, Tamil Nadu, India
[c] Department of Biochemistry, Homi Bhabha Cancer Hospital & Mahamana Pandit Madan Mohan Malaviya Cancer Centre, Tata Memorial Centre, Varanasi-221005, India
[d] Department of Medical Oncology, Homi Bhabha Cancer Hospital & Mahamana Pandit Madan Mohan Malaviya Cancer Centre, Tata Memorial Centre, Varanasi-221005, India
[e] Homi Bhabha National Institute (HBNI), Mumbai, India

ARTICLE INFO

Keywords:
DPD inactivator
Colorectal cancer
Method development
Personalized therapy
UV-Detection

ABSTRACT

Background: The plasma concentration of 5-Fluorouracil (5-FU) is affected by numerous factors, thereby limiting its efficacy. The current therapeutic regimen's doses based on body surface area (BSA) are linked to increased toxicity and sometimes inadequate drug exposure.
Aim and objectives: The study aims to develop an in-vitro assay to monitor 5-Fluorouracil's therapeutic efficacy in cancer patients' blood samples, focusing on pharmacokinetics to improve therapy precision.
Materials and methods: Drug levels were determined from standards, quality controls, and experimental samples using protein precipitation, liquid-liquid extraction, and separation using a C18 analytical column with an isocratic program.
Result: In EXP-1A, the mean concentration of 5-Fluorouracil was 1.15 μg/ml; in EXP-1B, it was 1.16 μg/ml, while in EXP-1C, the mean concentration was 0.9 μg/ml. The percentage difference in mean 5-Fluorouracil concentration between the experiment sample containing a DPD inactivator and EXP-1C (without a DPD inactivator) was 21.5 % higher for EXP-1A and 0.68 % higher for EXP-1B. In the second phase of the experiment, the overall stability of 5-Fluorouracil in samples containing a DPD inactivator was 24.5 % superior compared to samples without a DPD inactivator.
Conclusion: A modified extraction technique has been developed to accurately measure 5-Fluorouracil concentration in blood, preserving its stability and concentration by adding a DPD inactivator.

* Corresponding author. Department of Biochemistry, Homi Bhabha Cancer Hospital & Mahamana Pandit Madan Mohan Malaviya Cancer Centre, Tata Memorial Centre, Varanasi, India.
** Corresponding author. Department of Medical Oncology, Homi Bhabha Cancer Hospital & Mahamana Pandit Madan Mohan Malaviya Cancer Centre, Tata Memorial Centre, Varanasi, India.
E-mail addresses: kapoorakhil1987@gmail.com (A. Kapoor), pratibhagavel@gmail.com (P. Gavel).
[1] Joint first authorship. The authors contributed equally to the manuscript.

https://doi.org/10.1016/j.plabm.2024.e00415
Received 12 April 2024; Received in revised form 24 May 2024; Accepted 3 June 2024
Available online 8 June 2024

1. Introduction

Five decades since its introduction, 5-Fluorouracil (5-FU) has been extensively employed as an anticancer medication. It is a cytotoxic drug most widely used for the Treatment of gastrointestinal (G.I.) cancers, head and neck cancer, breast cancer, and skin cancer [1]. Chemically, 5-FU is a structural analogue of uracil, which deviates by incorporating a single fluorine atom at the C-5 position instead of hydrogen. 5-FU administered intravenously inhibits the thymidylate synthase (T.S.) enzyme, which has an anti-tumor impact. During the S-phase of the cell cycle, this enzyme is essential for catalyzing the production of thymidylate. It does this by using reduced 5,10-methylenetetrahydrofolate, which is a critical cofactor for DNA replication and repair [2–4]. Recent evidence shows that pharmacokinetic-based dose adjustment of plasma 5-FU levels has improved drug response and significantly reduced systemic severe toxicity in cancer patients [5–8].

Body surface area (BSA) is the basis for 5-FU dosing as it is currently common practice. Notably, BSA-based 5-FU regimens exhibit considerable variability among individuals and between cohorts significantly influenced by various factors, encompassing age, gender, diseases, drug interactions, metabolism, pharmacogenetics, and many more. Consequently, the dependability of therapeutic drug monitoring (TDM) is dynamically tied to the precision of the chosen analytical method [8–11]. Specimen type and the accurate timing of sample collection are additional critical factors influencing drug concentration, particularly when estimating the monitoring of peak concentration or trough levels of drugs [12].

Therapeutic drug monitoring (TDM) is infrequently implemented in oncology settings for various reasons. However, it has demonstrated a particular advantage for drugs with a narrow therapeutic index, such as 5-fluorouracil. When evaluating drug exposure vs response relationship to monitor clinically useful drug agents, there are undoubtedly many endogenous and exogenous conditions factors responsible for the perturbation in optimizing dose for specific treatments. While the evolving approach of targeted drug therapy in cancer management has addressed these challenges, monitoring the intricate pharmacokinetic properties of these drugs remains challenging. Even though the emerging trend in targeted drug therapy in cancer management has countered these difficulties, monitoring the complex pharmacokinetic properties of these drugs is again a challenging task [13,14]. Considering cytotoxic chemotherapy compared to targeted therapy has shown a large difference in the pharmacokinetics of a drug regarding percentage variation in clearance, which was observed to be above 50 %. Most of this variability is attributed to genetic polymorphism seen in the population [15,16].

Evaluating the complex pharmacokinetics of a drug like 5-FU requires specific expertise in interpreting the drug exposure-response relationship. However, an understanding of steady-state concentration is sufficient to adjust the dose of the medicine by simply measuring its plasma trough concentration. In the background of this phenomenon, we have developed high throughput with a highly sensitive UHPLC method to determine the stability and concentration of 5-FU in plasma.

2. Materials and methods

This study was strictly adhered to the Declaration of Helsinki and in compliance with the Guideline for Good Clinical Practice standards. The study protocol was approved by the responsible Ethics Committee (Project No. :11000660).

2.1. Chemicals and Reagents

Analytical standards of 5-Fluorouracil (synthesis purity 99 %) were purchased from Merck (India); Internal standards 5-Bromouracil (synthesis purity 99 %) were purchased from Sigma Aldrich (India); Gimeracil (inactivator) (synthesis purity 99 %) was purchased from Tokyo Chemical Industry Co. Ltd (Japan); HPLC grade solvents acetonitrile, methanol, ethyl acetate were purchased from Merck (India); di-methyl sulfoxide (DMSO) was procured from Sisco Research Laboratories (Mumbai, India); Perchloric Acid -Analytical grade were purchased from Merck (India). Ultra-pure water $\geq$18.2 MΩ was obtained from PURELAB® flex 2 plus water purification system (ELGA Lab Water, USA).

2.2. Instrumentation and chromatography conditions

The Shimadzu-I Series LC-2030C, 3D plus UHPLC system was used to quantify the analytes. The apparatus consists of a PDA detector, an autosampler, an oven, and an LC-2030C-plus solvent supply system. With LabSolution software, data analysis was done. At 40 °C, a Shim-pack C18 column (4.6 × 250 mm, 5 μm) was used to achieve separation. Methanol (Sol. A) and 0.05 % perchloric acid (Sol. B), supplied at a rate of 0.6 mL/min, made up the mobile phase. Throughout the duration, a separation with a mobile phase composition of 3 % (Sol. A) and 97 % (Sol. B) was achieved using isocratic elution. A temperature of +15 °C was maintained for the autosampler, and an injection volume of 20 μL. At 260 nm, the detecting wavelength was chosen. The protocols were adopted and modified from previous literature [17–21].

2.3. Standard preparation stock solutions

5-FU and 5-Bromouracil (1 mg/mL): 10 mg of pure powder of 5-FU was dissolved in 5 mL of Milli Q water and final volume was raised to 10 mL q.s, aliquoted and stored at −20 °C.

At the time of analysis, stock solutions were diluted with water to create spike-in working stock solutions containing 2, 5, 10, 20,

50, 100, and 200 μg/mL of 5-FU. To generate calibration standards, 950 μL of drug-free plasma were mixed with 50 μL of each spike-in from the working stock solution. This yielded the following concentrations: 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, and 10.00 μg/mL. Comparably, four distinct concentrations (lower limit of quantification) of quality control samples were produced independently in blank plasma for 5-FU: LLOQ, 0.10 μg/mL; low-quality controls, 1 μg/mL; medium-quality controls, 2.5 μg/mL; and high-quality controls, 5 μg/mL. Samples of spiked plasma were all kept at −20 °C. The internal standard (I.S.) stock solution was made with a concentration of 1 mg/mL in water. I.S. working stock was further diluted with water and kept at −20 °C. Supplementary Materials and Procedures contain more information about preparing stocks.

2.4. Study methodology and experimental design

To mimic the patient sample, we collected residual whole blood sample in an EDTA vacutainer of 60 mL which was pooled and mixed in a container for the experiment. The pooled blood sample was spiked with the 5-FU standard stock solution and mixed vigorously. The sample was further divided into two parts for the experiment, as mentioned below.

EXPERIMENT-1 (Exp1): The container was divided into 13 sets of 5 mL tubes; these 13 sets are divided into 3 groups (group-1, group-2, and group-3). First group of 5 tubes were marked as set-1 to set- 5 and each tube had 2 mL blood already spiked with 5-FU drug (1000 ng/mL) followed by addition of DPD inactivator. Set-1 was supplemented with DPD inactivator - 600 ng/mL; set-2 650 ng/mL; set-3 700 ng/mL; set-4 750 ng/mL, and set-5 with 800 ng/mL DPD inactivator.

In the 2nd group of 5 tubes labelled as set-6 to set-10, each tube comprised 1 mL blood spiked with 5-FU (1000 ng/mL) and subsequently mixed with DPD inactivator as follows: set-6 with DPD inactivator 600 ng/mL, set-7 with 650 ng/mL, set-8 with 700 ng/mL, set-9 with 750 ng/mL, and set −10 with DPD inactivator at 800 ng/mL concentration.

The final group (3rd group) set of 3 tubes were marked as set-11 to set-13 and to each we added 1 mL blood spiked with 5-FU drug (1000 ng/mL) and without DPD inactivator. At the end, we took all group samples, separated the plasma after 30 min and proceeded with sample analysis using UHPLC.

EXPERIMENT-2 (Exp2): We divided one group into 10 sets of 5 ml; where 1st set of 5-tubes with blood samples were spiked 5- FU drug 2000 ng/mL in 1 ml blood sample. Further we added DPD inactivator to make the final concentration of 650 ng/mL in the tube. We separated the plasma at different time points ranging from 0 *h*, 1 *h*, 4 *h*, 12 *h*, 24 *h*, 48 *h*, after storing at −20 °C till analysis by UHPLC.

The 2nd set of 5-tube contained blood samples spiked 5-FU drug (2000 ng/mL) in 1 ml blood sample in each tube. Without adding the DPD inactivator, we separated the plasma at different points as mentioned before, followed by storage at −20 °C till analysis by UHPLC. Additional details pertaining to study methodology and experimental design is provided as Supplementary Materials and Methods.

2.5. Sample preparation

After thawing at room temperature, the frozen samples were vortexed. Volume of 20 μL of the internal standard (5 μg/mL 5-Bromourecil solution in Milli Q water) and an aliquot of 200 μL of the standard, quality control, or study samples were spiked into 2 mL tubes. After adding 200 μL of 100 % acetonitrile the mixture was vortexed for 40 s. Next, we filled one set of pre-labelled 2 mL tubes with 1000 μL of ethyl acetate. These tubes with labels were centrifuged for 15 min at 17000 rpm after being vortexed for 2 min. Supernatant (1000 μL) was transferred into the corresponding second set of pre-labelled 2 mL tubes, and it was then dried for 45 min at room temperature in a speed vacuum. Following total drying, 200 μL of reconstitution solution was used to reconstitute the samples, which were then vortexed for 5 min and centrifuged for 5 min at 17000 rpm. For UHPLC analysis, we added 90 μL of supernatant into autosampler vials following the previous studies [22–25]. Additional details are in Supplementary Materials and Methods.

2.6. Validation

In accordance with the US-FDA Guidance for Industry for Bioanalytical Method Validation, the methodology was validated [26–28].

2.6.1. Selectivity

Using six distinct lots of blank plasma (containing EDTA), the method's selectivity against endogenous chemical interferences was evaluated. The detector response in blank plasma not exceeding 20 % of LLOQ at the 5-FU retention duration verified the lack of interfering components.

2.6.2. Sensitivity

At LLOQ levels, the method's sensitivity to identify 5-FU was calculated. The calibration curve was performed in conjunction with six LLOQ replicates. If the precision was within ±20 % and the accuracy was between 80 and 120 %, sensitivity was determined.

2.6.3. Carry-over

By injecting blank samples six times following the highest calibration standard, carry-over was evaluated. After applying the high standard, the blank sample's signal did not exceed 20 %.

2.6.4. Calibration and linearity

In order to cover a wide range of plasma values, three sets of calibration standards between 0.1 μg/mL and 10 μg/mL were analyzed for a linearity analysis. Using the peak area ratio of the analyte/I.S. (y) versus the amount (x), the calibration curve was created. Using linear regression analysis, the slope, intercept, and regression coefficient (r^2) were calculated. The theoretical value cut-off deviation was ±15 % for back-calculated standard amounts, with the exception of ±20 % for the lowest standard.

2.6.5. Accuracy

The accuracy of the obtained concentration in comparison to the known concentrations was assessed for the method. In one analytical run, the seven levels of calibration standards ranging from 0.1 to 10 μg/mL were assayed for the investigation. If the back-calculated standard amounts did not deviate from ±15 % of the theoretical value (with the exception of ±20 % for the lowest standard), then the accuracy was considered to be within the range of 80–120 %.

2.6.6. Precision (intraday precision and inter-day precision)

The four quality control levels (LLOQ, LQC, MQC, and HQC) (n = 9) were assayed in a single analytical run for the within-run study, while the four quality control levels were assayed in duplicate on different days for the between-run comparison. The following were the acceptance criteria: With the exception of the lowest standard, precision bias within- and between-run should be less than ±15 % of the theoretical value.

2.6.7. Lower limit of quantification (LLOQ)

The LLOQ sample's analyte signal ought to be at least five times stronger than a blank sample's signal. By comparing measured signal samples with known low concentrations of analysis of blank samples, a lower limit of quantification (LOQ) was established, indicating the lowest concentration at which the analysis can be consistently detected.

2.6.8. Extraction recoveries

The concentration levels of 1.0, 2.5, and 5.0 μg/mL for 5-FU were used to measure the extraction recoveries, which were computed by comparing the peak area of the analytes spiked prior to extraction to the peak area of the unextracted standard solutions at the same concentration.

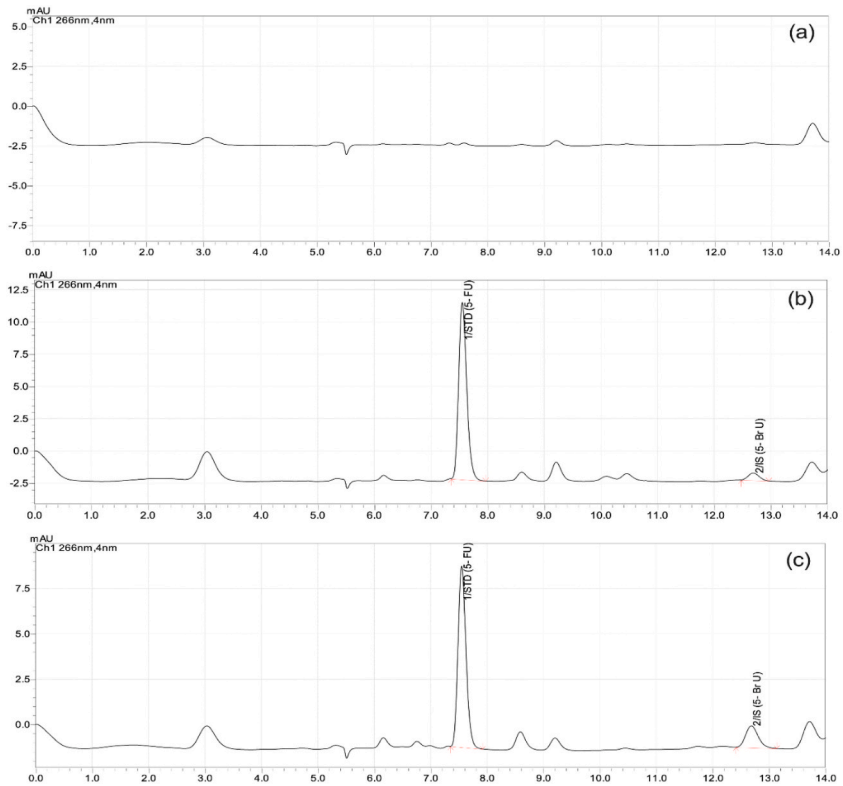


Fig. 1. A chromatogram of (a) human blank plasma sample (b) Plasma sample spiked with 2.5 μg/mL of 5- FU (c) chromatogram of experimental sample.

2.6.9. Robustness

An unexpected source of variance in the instrument response can arise from the experimental parameters changing irregularly within a restricted range during instrument operation. To verify the magnitude of this phenomenon, retention time changes were analyzed at the minimum and maximum of the variable range of the chromatographic parameters: The amounts of perchloric acid buffer (0.04, 0.05, and 0.06); column type; temperature (35–40 °C); and wavelength absorbance (±5 nm).

2.7. Stability of quality controls

Three quality control levels (low, medium, and high) underwent stability experiments in triplicate. The quality controls were kept at −20 °C for a month before their stability was evaluated. The stability of the freeze-thaw process on quality controls was assessed in three cycles. After a 24 *h* storage period, the quality control extracts' stability in the autosampler maintained at +4 °C was evaluated. Within 15 % of the nominal concentrations should be the range for all stability sample findings.

3. Results

3.1. Validation result

3.1.1. Selectivity

Analyte retention durations of 5-FU (2.5 μg/mL) spiked in plasma, IS spiked, and blank plasma chromatograms were compared, and the results showed no substantial interferences (Fig. 1). The method's selectivity was optimal for each blank sample as the signal was less than 20 % of the LLOQ for 5-FU and 15 % for IS.

3.1.2. Sensitivity

At LLOQ values, the sensitivity of 5-FU was computed. For 5-FU, the precision and accuracy of the back calculated concentrations were determined. The accuracy ranged between 96.26 % and 101.79 %, and the precision ranged from −3.75 to +2.68 %. These findings indicated that this approach can produce an LLOQ of 0.1 μg/mL. The outcome showed that our method's sensitivity is sufficient for performing plasma drug monitoring in routine clinical practice (Table -S1).

3.1.3. Carry-over

The acceptance criteria were successfully confirmed when six injections of blank samples were made immediately following the highest calibration standard. These injections produced a signal that was less than 20 % of the LLOQ for the 5-FU injections. Finally, after injecting 10 μg/mL of 5-FU, there was no notable carry-over.

3.1.4. Calibration and linearity

It was discovered that the procedure was linear in the range of 0.1–10 μg/mL with 1/ × was the optimal weighting factor. For analytes, the linear correlation values were more than 0.99. The largest bias detected was 5 % for the lower calibration standard, and all back-calculated values were within ±15 % of the theoretical concentration (Figs. S–2 and Tables S–2).

3.1.5. Accuracy

The accuracy of the obtained concentrations in comparison to the known concentration was assessed for the procedure. Table-S1 list the outcome of testing calibration standards ranging from 0.1 to 10 μg/mL in a single analytical run. The accuracy range is 96.26 %–103.75 %, while the range of CV is −1.78 % to +3.75 %. The outcome shows that a wide range of calibration curves have good accuracy.

3.1.6. Precision (within-run and between-run)

Four quality control samples (LLOQ, LQC, MQC, and HQC) were analyzed in triplicate at each concentration level on three separate days in order to evaluate the assay's accuracy and precision. This allowed the evaluation of both intra-day and inter-day precisions. In every instance, the requirements for precision and accuracy were satisfied. Tables S3 and S4 show that the within-run bias varied from −1.45 % to +3.60 % and the accuracy bias from 96.40 % to 101.45 %; the between-run bias varied from −0.25 % to +10.05 % and the accuracy bias from 89.95 % to 100.25 %. Thus, the existing strategy verified the accuracy of 5-FU monitoring by the use of the UHPLC technique.

3.1.7. Lower limit of quantification (LLOQ)

The accuracy and precision results meet the acceptance standards with an LLOQ of 103.75 % and a CV% of −3.75 %. The sensitivity of LLOQ 0.1 μg/mL was revealed by the findings shown in Table -S1. As a result, the suggested UHPLC approach can be used to quantitatively determine the 5-FU concentration in clinical and regular laboratory samples.

3.1.8. Extraction recoveries

The response ratio of the analyte in extracted vs unextracted samples (neat solutions) was used to compute the percent recovery [29]. For 5-FU, the overall extraction efficiency ranged from 79.77 to 87.36 % (Table –S5).

3.1.9. Robustness

The outcome showed that the variation fell within an acceptable range. Since there was no discernible variance in retention time for either of the 5-FU, the approach was deemed robust.

3.1.10. Stability

The quality control stability results under various storage settings showed that control samples, kept in a polypropylene tube at −20 °C, were stable for at least two months (with a percentage CV ranging from −2.50 % to +3.88 % for each of the three levels) (Table -S1). There was no discernible deterioration of 5-FU after three consecutive freeze-thaw cycles (% CV varied from −2.37 % to −8.46 %). In contrast, the control sample extracts remained stable in the autosampler maintained at +4 °C for a minimum of 24 *h*, as indicated by the % CV, which varied from −1.06 % to −2.93 % (Table -S1).

3.2. Experiment results

The experiment was conducted in two phases, in experiment-1 the stability of 5-FU was assessed in the presence of a DPD inactivator of various concentrations added in 2 mL and 1 mL of blood to study the effective concentration of 5-FU. Experimental results are given below in Table-1 and Figure-2.

In experiment-1, 5-FU concentration was affected by the presence of a DPD inactivator whereas in the first two groups (Experiment-1A and Experiment-1B) the concentration of 5-FU was found to be higher in the presence of a DPD inactivator at variable concentration. The observed variability in the stability of 5-FU within a group of Experiment-1A and Experiment-1B (shown in figure-2) can be explained by two factors; 1) DPD inactivator is completely saturated with the larger quantity of enzyme present in blood, 2) efficiency of the DPD inactivator to inhibit the enzyme degradation is not effective. Notably, in Experiment-1C the concentration of the 5-FU significantly reduced without the DPD inactivator thus signifies the importance of treating the blood sample with the DPD inactivator for accurate measurement of 5-FU before storage as a part of the pre-analytical step. The mean concentration of 5-FU in EXP-1A was 1.15 μg/ml, EXP-1 B was 1.16 μg/ml and EXP-1 C was 0.9 μg/ml respectively.

In experiment −2, the stability of 5-FU over 48 h was measured. Experiment results are given below in Table-2 and Figure-3.

The stability of 5-FU was found to be consistent over time till 48 h post separation and storage at −20 °C. The consistency was variables in both the sets (EXP-2A and EXP-2B). Within the group 0 h–4 h stability of 5-FU was similar in both cases. The mean and standard deviation of Experiment-1 and Experiment-2 are 1.65 μg/ml and 1.25 μg/ml, also SD is ±0.38 and ± 0.32. The percentage difference obtained between the groups from 0 h to 48 h was 23.5 %, 13.7 %, 27.4 %, 29.3 %, 32.5 %, and 20.7 % respectively. In summary, the overall stability of 5-FU in samples containing DPD inactivator in comparison to samples without DPD inactivator was observed to be 24.5 % superior.

4. Discussion

We developed a method as specified in Experiment: 1 (dose-dependent activity) & Experiment 2: to determine the time-dependent activity of 5-FU (for details refer to Supplementary Materials and Methods)

In order to monitor the therapeutic efficacy of 5-FU and reduce its toxicity in cancer patients, the primary goal of this study is to ascertain the effective concentration and stability of 5-FU in plasma. HPLC-UV method-based assay was developed to quantify the concentration of 5-FU in whole blood. There are several methods described for the measurements of 5-FU in biological samples including earlier methods like cell-based culture assay & spectrophotometric method, letter replaced with high sensitivity methods like GCMS, HPLC, and most recently nanoparticle immunoassay-based technique was also introduced with variable sensitivity [30]. However, HPLC with U.V. or electrochemical detectors or integrated with mass spectrometry-based techniques remains the most sensitive and reliable for the determination of 5-FU and its metabolites [28,31].

This experiment was conveniently designed to determine the activity of 5-FU at different concentrations of DPD inactivator and evaluate the compound's stability over time in the presence of DPD inactivator, which is the primary enzyme that catabolizes 5-FU in the liver. It is anticipated that DPD located in hepatic and extrahepatic locations degrades around 80–85 % of given 5-FU by conjugation, hence reducing the drug's systemic toxicity [32,33].

It has also been reported that the therapeutic effect of 5-FU is significantly affected in patients with DPD polymorphism or deficiency of the enzyme and consequently its reduced function is responsible for severe systemic drug toxicity in such cases it is important to monitor the activity of DPD enzyme or endogenous metabolites like uracil and dihydrouracil in plasma of patients before initiating the treatment [34–37].

Table −1
Result of Experiment −1 indicating the concentration of Gimeracil used and 5-FU concentration measured in the sample.

DPD inactivator concentration in ng/mL	Experiment-1 A (2 mL whole blood)	Experiment-1 B (1 mL whole blood)	Experiment-1 C (1 mL whole blood)
600	1.31	1.12	0.89
650	1.11	1.15	0.91
700	1.14	1.17	0.90
750	1.12	1.17	
800	1.08	1.17	

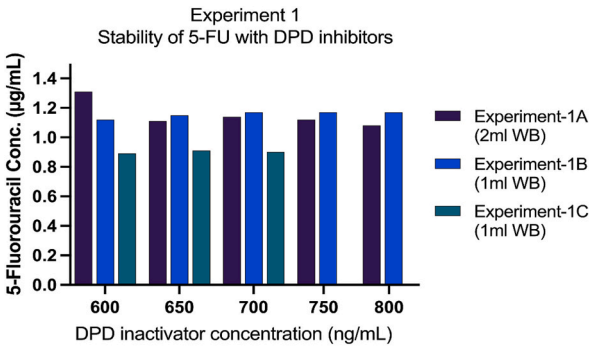


Figure- 2. Concentration of Gimeracil used and 5-FU concentration measured in the sample (Experiment −1).

Table 2
Time dependent stability of 5-FU and percent variation among two tests.

Time point (h)	Experiment-2 A (1 mL whole blood)	Experiment-2 B (1 mL whole blood)	% Difference (Exp 2B vs 2A)
0	2.18	1.67	−23.53
1	1.73	1.49	−13.68
4	1.87	1.36	−27.38
12	1.71	1.21	−29.34
24	1.23	0.83	−32.47
48	1.19	0.95	−20.66
N	**6**	**6**	
Mean	**1.65**	**1.25**	
SD	**0.38**	**0.32**	

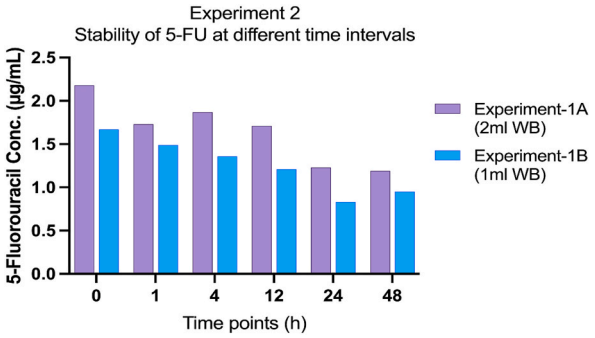


Figure- 3. Stability of 5-FU at different time intervals (Experiment −2).

In this study, we demonstrated *in-vitro* stability of the 5-FU with and without the presence of DPD inhibitor in experiment-1 where the 5-FU concentration was kept constant and the DPD inhibitor was added in increasing concentration in each 2 mL of blood the same was repeated with 1 mL of blood containing the same concentration of both 5-FU and DPD inhibitor showed no significant difference, but when the DPD inhibitor was excluded (experiment 1 group 3 set) we observed a significant difference in the concentration of the 5-FU in plasma with difference of 31.8 %,17.7 % and 20.6 % in each group respectively. From this experiment, it is apparent that therapeutic monitoring of 5-FU could be very useful in optimizing the drug exposure including effective monitoring of the drug efficacy and its preventing toxicity.

As therapeutic drug monitoring is not routinely practiced in chemotherapy in addition to the potential limitation of BSA-based dosing of cytotoxic agents including 5-FU in maintaining the optimal drug exposure and limiting its toxicity can be subdued with the help of adopting an efficient method for TDM of chemotherapeutic agents.

Similarly, in experiment-2 we demonstrated the stability of 5-FU at various time intervals in which the activity of the drug is maintained by adding a DPD inhibitor when the degradation of the drug is significantly high in plasma without the presence of a DPD inhibitor. There was a significant difference between the mean of the two groups where the stability of 5-FU was appreciable in EXP - 2A compared to the concentration estimated in EXP – 2B without the DPD inactivator in which the concentration 5-FU at 0 h, 4 h, 24 h, and 48 h was 23.5 %, 27.4 %, 32.5 % and 20.7 % respectively (table: 2). It is to be noted that the stability of 5-FU is lost after 48 h.

5. Conclusion

We have demonstrated the stability of 5-FU activity and its concentration in blood samples by HPLC-UV-based method. The accurate measurements of 5-FU in the blood are highly influenced in the pre-analytical stage of the samples with presence of DPD enzyme that can be effectively suppressed with the help of specific DPD inactivator like gimeracil. Compared to LCMS, LCMS/MS techniques this HPLC-UV based assay is considerably flexible, cost-effective and can be easily adapted with high sensitivity and reproducibility. Our standardized method for the determination of 5-FU in blood will undeniably improve the quality of management for monitoring the drug efficacy and will help in reducing toxicity in cancer patients undergoing systemic cytotoxic therapy.

CRediT authorship contribution statement

Murari Gurjar: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **K. Ambedkar Priyan:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Formal analysis, Data curation. **Priyanka Asia:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Formal analysis. **Uday Kumar:** Validation, Formal analysis, Data curation. **Kajal Shukla:** Methodology, Formal analysis. **Bal Krishna Mishra:** Writing – review & editing, Writing – original draft, Visualization, Validation. **Akhil Kapoor:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Formal analysis, Conceptualization. **Pratibha Gavel:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Investigation, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

Authors are thankful to Dr Rohit Kumar, SO'E' for his assistance in independent review and proofreading of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2024.e00415.

References

[1] A. Mafi, M. Rezaee, N. Hedayati, S.D. Hogan, R.J. Reiter, M.H. Aarabi, et al., Melatonin and 5-fluorouracil combination chemotherapy: opportunities and efficacy in cancer therapy, Cell Commun. Signal. 21 (1) (2023) 33.
[2] F. Lewin, S. Skog, B. Tribukait, U. Ringborg, Effect of 5-fluorouracil on the cell growth and cell cycle kinetics of a mouse ascites tumor growing in vivo, Acta Oncol 26 (2) (1987) 125–131.
[3] D.B. Longley, D.P. Harkin, P.G. Johnston, 5-fluorouracil: mechanisms of action and clinical strategies, Nat. Rev. Cancer 3 (5) (2003) 330–338.
[4] C.M. Stirk, W.D. Thompson, Artificial exudate: stimulation of cell proliferation by plasma not serum is associated with fibrinolysis, Blood Coagul. Fibrinolysis 1 (4–5) (1990) 537–541.
[5] G. Bocci, C. Barbara, F. Vannozzi, A. Di Paolo, A. Melosi, G. Barsanti, et al., A pharmacokinetic-based test to prevent severe 5-fluorouracil toxicity, Clin. Pharmacol. Ther. 80 (4) (2006) 384–395.
[6] J.J. Lee, J.H. Beumer, E. Chu, Therapeutic drug monitoring of 5-fluorouracil, Cancer Chemother. Pharmacol. 78 (3) (2016) 447–464.
[7] R. Yang, Y. Zhang, H. Zhou, P. Zhang, P. Yang, Q. Tong, et al., Individual 5-fluorouracil dose adjustment via pharmacokinetic monitoring versus Conventional body-area-surface method: a meta-analysis, Ther. Drug Monit. 38 (1) (2016) 79–86.
[8] F. Goirand, F. Lemaitre, M. Launay, C. Tron, E. Chatelut, J.C. Boyer, et al., How can we best monitor 5-FU administration to maximize benefit to risk ratio? Expert Opin Drug Metab Toxicol 14 (12) (2018) 1303–1313.
[9] G. Redlarski, A. Palkowski, M. Krawczuk, Body surface area formulae: an alarming ambiguity, Sci. Rep. 6 (2016) 27966.
[10] Z. He, J. Yang, C. Sui, P. Zhang, T. Wang, T. Mou, et al., FAM98A promotes resistance to 5-fluorouracil in colorectal cancer by suppressing ferroptosis, Arch. Biochem. Biophys. 722 (2022) 109216.
[11] J.H. Beumer, E. Chu, C. Allegra, Y. Tanigawara, G. Milano, R. Diasio, et al., Therapeutic drug monitoring in oncology: international association of therapeutic drug monitoring and clinical toxicology recommendations for 5-fluorouracil therapy, Clin. Pharmacol. Ther. 105 (3) (2019) 598–613.
[12] W. Wattanatorn, H.L. McLeod, F. Macklon, M. Reid, K.E. Kendle, J. Cassidy, Comparison of 5-fluorouracil pharmacokinetics in whole blood, plasma, and red blood cells in patients with colorectal cancer, Pharmacotherapy 17 (5) (1997) 881–886.
[13] C. Sawyers, Targeted cancer therapy, Nature 432 (7015) (2004) 294–297.
[14] L.A. Decosterd, N. Widmer, K. Zaman, E. Cardoso, T. Buclin, C. Csajka, Therapeutic drug monitoring of targeted anticancer therapy, Biomark Med 9 (9) (2015) 887–893.

[15] A. Paci, G. Veal, C. Bardin, D. Leveque, N. Widmer, J. Beijnen, et al., Review of therapeutic drug monitoring of anticancer drugs part 1–cytotoxics, Eur. J. Cancer 50 (12) (2014) 2010–2019.
[16] A. Lee, H. Ezzeldin, J. Fourie, R. Diasio, Dihydropyrimidine dehydrogenase deficiency: impact of pharmacogenetics on 5-fluorouracil therapy, Clin. Adv. Hematol. Oncol. 2 (8) (2004) 527–532.
[17] M.A. Nassim, F.H. Shirazi, C.M. Cripps, S. Veerasinghan, M.J. Molepo, M. Obrocea, et al., An HPLC method for the measurement of 5-fluorouracil in human plasma with a low detection limit and a high extraction yield, Int. J. Mol. Med. 10 (4) (2002) 513–516.
[18] J. Chen, M. Zhou, Determination of eniluracil and 5-fluorouracil in human plasma by LC-MS/MS, Bioanalysis 2 (12) (2010) 2011–2017.
[19] M. Barberi-Heyob, J.L. Merlin, B. Weber, Analysis of 5-fluorouracil in plasma and urine by high-performance liquid chromatography, J Chromatogr 581 (2) (1992) 281–286.
[20] E. Matsushima, K. Yoshida, R. Kitamura, K. Yoshida, Determination of S-1 (combined drug of tegafur, 5-chloro-2,4-dihydroxypyridine and potassium oxonate) and 5-fluorouracil in human plasma and urine using high-performance liquid chromatography and gas chromatography-negative ion chemical ionization mass spectrometry, J. Chromatogr. B Biomed. Sci. Appl. 691 (1) (1997) 95–104.
[21] P. Gopinath, S. Veluswami, R. Thangarajan, G. Gopisetty, RP-HPLC-UV method for estimation of fluorouracil-epirubicin-cyclophosphamide and their metabolite mixtures in human plasma (matrix), J. Chromatogr. Sci. 56 (6) (2018) 488–497.
[22] J. Ciccolini, C. Mercier, M.F. Blachon, R. Favre, A. Durand, B. Lacarelle, A simple and rapid high-performance liquid chromatographic (HPLC) method for 5-fluorouracil (5-FU) assay in plasma and possible detection of patients with impaired dihydropyrimidine dehydrogenase (DPD) activity, J Clin Pharm Ther 29 (4) (2004) 307–315.
[23] B. Buchel, P. Rhyn, S. Schurch, C. Buhr, U. Amstutz, C.R. Largiader, LC-MS/MS method for simultaneous analysis of uracil, 5,6-dihydrouracil, 5-fluorouracil and 5-fluoro-5,6-dihydrouracil in human plasma for therapeutic drug monitoring and toxicity prediction in cancer patients, Biomed. Chromatogr. 27 (1) (2013) 7–16.
[24] C. Pi, Y. Wei, H. Yang, Y. Zhou, J. Fu, S. Yang, et al., Development of a HPLC method to determine 5-fluorouracil in plasma: application in pharmacokinetics and steady-state concentration monitoring, Int J Clin Pharmacol Ther 52 (12) (2014) 1093–1101.
[25] R.A. Coe, R.A. Earl, T.C. Johnson, J.W. Lee, Determination of 5-fluorouracil in human plasma by a simple and sensitive reversed-phase HPLC method, J. Pharm. Biomed. Anal. 14 (12) (1996) 1733–1741.
[26] U. Fda, Guidance for Industry, bioanalytical method validation, Available from: https://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf, 2018.
[27] L.D. Vainchtein, H. Rosing, J.H. Schellens, J.H. Beijnen, A new, validated HPLC-MS/MS method for the simultaneous determination of the anti-cancer agent capecitabine and its metabolites: 5'-deoxy-5-fluorocytidine, 5'-deoxy-5-fluorouridine, 5-fluorouracil and 5-fluorodihydrouracil, in human plasma, Biomed. Chromatogr. 24 (4) (2010) 374–386.
[28] J.G. Maring, L. Schouten, B. Greijdanus, E.G. de Vries, D.R. Uges, A simple and sensitive fully validated HPLC-UV method for the determination of 5-fluorouracil and its metabolite 5,6-dihydrofluorouracil in plasma, Ther. Drug Monit. 27 (1) (2005) 25–30.
[29] L. Zufia, A. Aldaz, C. Castellanos, J. Giraldez, Determination of 5-fluorouracil and its prodrug tegafur in plasma and tissue by high-performance liquid chromatography in a single injection: validation for application in clinical pharmacokinetic studies, Ther. Drug Monit. 25 (2) (2003) 221–228.
[30] M. Breda, S. Baratte, A review of analytical methods for the determination of 5-fluorouracil in biological matrices, Anal. Bioanal. Chem. 397 (3) (2010) 1191–1201.
[31] T. Dodevska, D. Hadzhiev, I. Shterev, Recent advances in electrochemical determination of anticancer drug 5-fluorouracil, ADMET DMPK 11 (2) (2023) 135–150.
[32] R.B. Diasio, B.E. Harris, Clinical pharmacology of 5-fluorouracil, Clin. Pharmacokinet. 16 (4) (1989) 215–237.
[33] M.W. Saif, K.N. Syrigos, N.A. Katirtzoglou, S-1: a promising new oral fluoropyrimidine derivative, Expert Opin Investig Drugs 18 (3) (2009) 335–348.
[34] J.G. Maring, A.B. van Kuilenburg, J. Haasjes, H. Piersma, H.J. Groen, D.R. Uges, et al., Reduced 5-FU clearance in a patient with low DPD activity due to heterozygosity for a mutant allele of the DPYD gene, Br. J. Cancer 86 (7) (2002) 1028–1033.
[35] B.A. Jacobs, H. Rosing, N. de Vries, D. Meulendijks, L.M. Henricks, J.H. Schellens, et al., Development and validation of a rapid and sensitive UPLC-MS/MS method for determination of uracil and dihydrouracil in human plasma, J. Pharm. Biomed. Anal. 126 (2016) 75–82.
[36] T.J. Wigle, E.V. Tsvetkova, S.A. Welch, R.B. Kim, DPYD and fluorouracil-based chemotherapy: mini review and case report, Pharmaceutics 11 (5) (2019).
[37] B. Wormann, C. Bokemeyer, T. Burmeister, C.H. Kohne, M. Schwab, D. Arnold, et al., Dihydropyrimidine dehydrogenase testing prior to treatment with 5-fluorouracil, capecitabine, and tegafur: a consensus paper, Oncol. Res. Treat. 43 (11) (2020) 628–636.