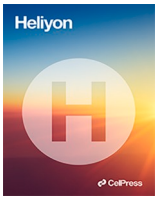
Heliyon 10 (2024) e29990

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Research article

Development and validation of UPLC-MS/MS method for 5-Fluorouracil quantification in dried blood spot

Yahdiana Harahap [a,b,*], Vanessa Amarta [a], Febrina Amelia Saputri [a]

[a] Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, West Java, Indonesia
[b] Faculty of Military Pharmacy, Republic of Indonesia Defense University, Bogor, 16810, Indonesia

ARTICLE INFO

Keywords:
5-Fluorouracil
Propylthiouracil
UHPLC-MS/MS
Dried Blood Spot (DBS)

ABSTRACT

5-Fluorouracil is an antimetabolite drug indicated for cancer treatment. Therapeutic drug monitoring of 5-Fluorouracil is necessary because 5-Fluorouracil has narrow therapeutic window and its concentration in blood is affected by individual conditions, like gene polymorphisms. Dried Blood Spot (DBS) is one of the biosampling methods used for therapeutic drug monitoring. Asides from reducing patients' discomfort, the use of DBS can increase 5-Fluorouracil stability by stopping the enzymes activity in blood. Therefore, this research developed a method to monitor 5-Fluorouracil levels in DBS using ultra-high performance liquid chromatography-tandem mass spectrometry. Sample preparation was carried out by extracting DBS using 2-Propanol: ethyl acetate (16:84). Reconstituted samples were analyzed using ultra high performance liquid chromatography equipped with Acquity® UPLC BEH C18 column (2.1 × 100 mm; 1.7 μm). The ionization process was carried out in negative electrospray ionization mode. Multiple Reaction Monitoring (MRM) values were set at m/z 128.97 > 41.82 for 5-Fluorouracil and 168.97 > 57.88 for propylthiouracil as the internal standard. Optimum analytical conditions were obtained with acetonitrile-ammonium acetate 1 mM (95:5) as mobile phase, flow rate of 0.15 mL/min, and column temperature of 40 °C. The lowest level of quantification obtained from this method was 0.1 μg/mL with a calibration curve range of 0.1 μg/mL-60 μg/mL. This method was proven to be valid according to the requirements set by the US Food and Drug Administration and the European Medicines Agency.

1. Introduction

5-Fluorouracil is a pyrimidine analog compound indicated for cancer therapy [1]. 5-Fluorouracil is commonly used to treat stomach cancer, pancreatic cancer, breast cancer, and colorectal cancer [2]. Cytotoxic activity of 5-Fluorouracil comes from its incorporation with RNA and DNA, along with Thymidylate Synthetase (TS) inhibition [1]. The structure of 5-Fluorouracil is shown in Fig. 1. 5-Fluorouracil regimen is generally given through the parenteral route because of its unpredictable oral absorption and low bioavailability [1,2].

The use of 5-Fluorouracil requires drug levels monitoring given the narrow therapeutic window of 5-Fluorouracil. Area Under Curve (AUC) of 5-Fluorouracil should be in the range of 20–30 mg h/L [3]. In addition, several individual conditions such as Dihydropyrimidine Dehydrogenase (DPD), Thymidylate Synthetase (TS), and Metilentetrahydrofolate Reductase (MTHFR) polymorphisms

* Corresponding author. Faculty of Pharmacy, Universitas Indonesia, Depok, 16424, West Java, Indonesia.
E-mail address: yahdiana03@yahoo.com (Y. Harahap).

https://doi.org/10.1016/j.heliyon.2024.e29990
Received 23 October 2023; Received in revised form 17 April 2024; Accepted 18 April 2024
Available online 19 April 2024

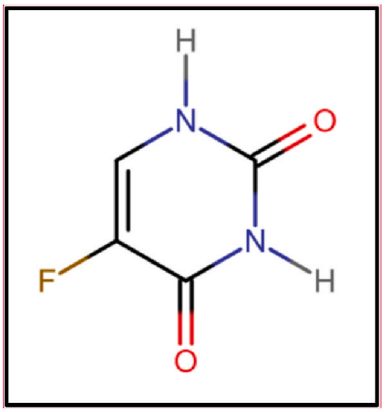


Fig. 1. 5-Fluorouracil chemical structure.

can affect blood levels of 5-Fluorouracil [4]. Increased concentration of 5-Fluorouracil can trigger the emergence of side effects. Side effects that can be caused by 5-Fluorouracil include nausea, mucosal ulceration, myelosuppression, leukopenia, and immunocompromised [1,2].

In previous studies, monitoring of 5-Fluorouracil levels in blood was mostly done in plasma matrices. However, this method required the blood sample to be placed on ice, centrifuged under cold conditions, and the separated plasma had to be frozen immediately [5–7]. This is due to the nature of 5-Fluorouracil that is unstable and rapidly metabolized by enzymes in the blood [5].

In the development of bioanalytical methods, there is a biosampling technique known as DBS. DBS can be used for quantifying analytes that are distributed in peripheral veins, such as 5-Fluorouracil [8]. This biosampling method can simplify sample preparation because samples in DBS do not need to be centrifuged and can be prepared at room temperature [5,9,10]. DBS also has several advantages over the plasma method. Sampling with DBS requires smaller blood sample volume (10–100 μL) compared to the plasma method (5–15 mL), resulting in less biohazard and complying with the green chemistry principles. When implemented, sampling with DBS can increase patient comfort because it only requires a finger prick compared to plasma sampling, which requires venipuncture [11]. DBS is also easier to transport since the blood is already dried, thereby minimizing the risk of spillage [11].

Based on this background, this study aimed to develop and validate an analysis method for 5-Fluorouracil in DBS using UPLC-MS/MS. This study is expected to provide an easier and time-efficient bioanalytical method. The result of this research is expected to be implemented for therapeutic drug monitoring of 5-Fluorouracil in cancer patients.

2. Materials and methods

2.1. Chemical and reagents

5-Fluorouracil standard and propylthiouracil as internal standard were purchased from Indonesian Food and Drug Authority (Jakarta, Indonesia). Reagents such as acetonitrile, methanol, ammonium acetate, and ammonium solution were purchased from Merck Co. Ltd. (Darmstadt, Germany). Ultrapure water was provided by the Sartorius Water Filter System. Six whole blood bags from different donor sources were purchased from the Indonesian Red Cross (Jakarta, Indonesia). Dried Blood Spot card Whatman 903 were purchased from Sigma-Aldrich (USA).

2.2. UPLC-MS/MS

This research was performed using Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry consisting of the Quartenary Solvent Manager Acquity® UPLC H-Class (Waters Xevo TQD Triple Quadropole, USA); Sample Manager FTN Acquity® UPLC (Waters, USA); Acquity® UPLC BEH C_{18} column (2,1 × 100 mm; 1,7 μm) (Waters, USA); mass analyzer triple quadrupole Xevo TQD with Zspray™ ionization source (Waters, USA); and data processing software MassLynx (Waters, USA).

2.3. Preparation of stock and working standard solution

The stock solution of 5-Fluorouracil with a concentration of 4000 μg/mL was prepared by dissolving 20 mg of 5-Fluorouracil standard in 5 mL of ultrapure water. A stock solution of propylthiouracil with a concentration of 1000 μg/mL was prepared by dissolving 10 mg propylthiouracil in 10 mL of methanol. The 5-Fluorouracil stock solution was diluted in water to obtain a working standard. The working standard solution was then further diluted with blood to obtain the standard calibration curve solutions with concentrations of 0.1 μg/mL, 0.5 μg/mL, 5 μg/mL, 20 μg/mL, 40 μg/mL, and 60 μg/mL. Quality control sample solutions were prepared by diluting different stock solutions of 5-Fluorouracil. The stock solution was diluted with ultrapure water to a concentration of 100 μg/mL. Then, further dilutions were performed with blood to obtain three concentrations: QCL (0.3 μg/mL), QCM (25 μg/mL), and

QCH (50 μg/mL).

2.4. Optimization of analytical condition

Optimization of the analysis conditions was carried out using standard solutions of 5-Fluorouracil and internal standard propylthiouracil, each at a concentration of 1 μg/mL. Optimization was conducted for mass spectrometry detection, mobile phase composition and combination, flow rate, and column temperature. Optimization of mass spectrometry detection was performed by infusing the mixture of 5-Fluorouracil standard solution and propylthiouracil internal standard solution into the mass spectrometry. During the process, the voltage on the capillary tube, temperature, desolvation gas flow rate, voltage at the entrance, and voltage in the collision chamber for negative ESI (−) which gave the highest 5-Fluorouracil and propylthiouracil response were recorded and used during analysis.

The mobile phase optimization was carried out by testing a variety of combinations, namely, acetonitrile – water 95:5 (%v/v); acetonitrile – water 5:95 (%v/v); acetonitrile – 0.1 % formic acid in water 95:5 (%v/v); acetonitrile – 1 mM ammonium formate in water (pH 8) 95:5 (%v/v); acetonitrile – 1 mM ammonium acetate in water (pH 8) 95:5 (%v/v); and acetonitrile – 1 mM ammonium acetate in water (pH 8) – methanol 90:5:5 (%v/v). Flow rate optimization was carried out for flow rate variations of 0.10 mL/min; 0.15 mL/min; and 0.20 mL/min. Column temperature optimization was performed for column temperature variations of 30 °C, 40 °C, and 50 °C. The most optimal conditions were selected based on the highest response and the best chromatogram quality.

2.5. System suitability test

System suitability test was conducted daily before the analysis procedure to ensure the instrument operated optimally. 10 μL of a mixture containing 5-Fluorouracil and propylthiouracil at a concentration of 1 μg/mL was injected into the UPLC-MS/MS system for six times. The elution process was performed according to the previously optimized analysis conditions. The system suitability test was considered acceptable if the percent coefficient of variation (%CV) of the generated responses was below 6 % [12].

2.6. Optimization of sample preparation

Optimization of sample preparation in DBS was performed using blood containing 2 μg/mL of 5-Fluorouracil. Aspects that were optimized included extraction methods, addition of acid or base, volume of extraction solvent, nitrogen evaporator temperature, volume of blood spotting in DBS, vortex mixing time, sonication time, and centrifugation time.

Optimization of extraction methods was carried out by comparing protein precipitation method, extraction with ethyl acetate: 2-Propanol, and salting-out liquid-liquid extraction. Optimization of acid or base addition was performed with the extracting solution containing 0.1 % formic acid, extracting solution containing 0.01 % NH4OH, and extracting solution without pH adjustment. Optimization of extraction solution volume was conducted with variations of 250 μL, 500 μL, and 750 μL. Evaporator temperature optimization was achieved by evaporating the supernatant at 30 °C for 30 min, 40 °C for 20 min, and 50 °C for 15 min. Optimization of blood spotting volume was done at 30 μL, 40 μL, and 50 μL. Vortex mixing time optimization was carried out for 5 s, 10 s, and 15 s. Sonication time optimization was performed for durations of 5 min, 10 min, and 15 min. Centrifugation time optimization was conducted for durations of 5 min, 10 min, and 15 min at a speed of 4000 rpm. Optimal conditions were chosen based on the highest response and the effectiveness of sample preparation time.

2.7. Method validation

The validation of the analysis method was conducted based on the requirements set by the U.S. Food and Drug Administration [13] and the European Medicines Agency [14].

2.7.1. Lower Limit of Quantification

5-Fluorouracil standard solution was diluted in blood to obtain a concentration of 0.1 μg/mL. The analysis was performed with five replicates and one blank sample. The analyte responses from the LLOQ sample must be at least 5 times the response of the blank sample [13,14]. Results from 5 replicates must have an accuracy within ±20 % of the nominal concentration and precision (%CV) should not exceed 20 % [11,12].

2.7.2. Calibration curve

Samples with six concentration levels (including LLOQ), zero sample, and blank sample were spotted onto DBS paper and prepared under optimal conditions. The testing was repeated until three acceptable calibration curves were obtained. The back-calculated concentrations for the standard calibration should be within ±15 % of the nominal concentration, except for LLOQ which should be within ±20 % of nominal concentration [13,14].

2.7.3. Selectivity

Selectivity was demonstrated using 6 blank matrices from different sources, which were individually analyzed and evaluated for interference. Analysis was performed on 6 blood samples containing 5-Fluorouracil with a concentration of 0.1 μg/mL (LLOQ). Selectivity validation met the acceptance criteria if the response of interfering components is less than 20 % of the Lower Limit of

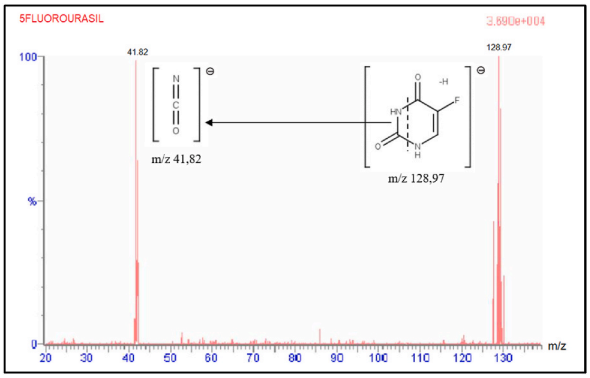


Fig. 2. Fragmentation spectrum of 5-Fluorouracil.

Quantification (LLOQ) for 5-Fluorouracil and less than 5 % for the internal standard propylthiouracil [13,14].

2.7.4. Accuracy and precision

Intraday accuracy and precision were determined by analyzing 5 replicates at the LLOQ, QCL, QCM, and QCH concentrations. Interday accuracy and precision were performed by repeating the analysis process at least three times on different days [13,14]. The back-calculated concentrations obtained should be within ±15 % of the nominal concentration, except for the LLOQ, which was required to be within ±20 % of the nominal concentration. The %CV values obtained should not exceed 15 %, except for the LLOQ which should not exceed 20 %.

2.7.5. Recovery

Recovery testing was conducted by comparing the analysis response of sample extracts with blank extracts spiked with the analyte at QCL, QCM, and QCH concentrations. Recovery was not required to be 100 %, but the analysis method should exhibit consistent and reproducible recovery (%CV $\leq$ 15 %) [13,14].

2.7.6. Carry over

Carry over was tested by measuring the analyte response in a blank sample after analyzing a sample with ULOQ concentration. The blank response should not exceed 20 % of the LLOQ for the analyte and 5 % for the internal standard [13,14].

2.7.7. Dilution integrity

Dilution integrity validation was conducted by preparing blood samples with a concentration twice higher than QCH (100 μg/mL). During preparation, the samples were reconstituted with twice and four times the volume of the intended mobile phase, resulting in reconstituted samples with QCH and ½ QCH concentrations. The analysis was repeated until 5 replicates were obtained for each dilution factor. The method was considered to pass the dilution integrity validation if the obtained accuracy and precision remained within the acceptance criteria, which was within a range of 15 % [13,14].

2.7.8. Matrix effect

Matrix effects were tested using six blank matrices from different donors. Each matrix source was spiked with 5-Fluorouracil to obtain QCL and QCH concentrations. The samples were then prepared and analyzed according to the optimized method. The testing was performed in triplicate for each concentration from each different matrix source. The method met the requirements if the back-calculated concentrations fell within the range of ±15 % of the nominal concentration and %CV was not more than 15 % [13,14].

2.7.9. Stability

Stability tests evaluation include stock solution stability, short-term analyte stability, long-term analyte stability, and autosampler stability. Stock solution stability was evaluated at 25 °C for short-term stability and 4 °C for long-term stability. The stock solution is considered stable if the concentration difference and %CV obtained are not more than 10 % [15]. Short-term analyte stability in DBS was tested at 25 °C for short-term stability and −20 °C for long-term stability. Autosampler stability was tested according to autosampler conditions. Analyte stability in DBS and autosampler stability were considered acceptable if the average concentration difference at each level fell within the ±15 % of the true concentration [13,14].

2.7.10. Reinjection reproducibility

Reinjection reproducibility test was conducted by performing reinjection of the calibration curve and five replicates of QC samples that have been stored for 24 h. The method passed the reinjection reproducibility test if the back-calculated concentrations fell within the range of ±15 % of the nominal concentration and %CV was not more than 15 % [13,14].

Table 1
System suitability test.

Data number	Area (μV/s)		Retention time (min)	
	5-Fluorouracil	PTU	5-Fluorouracil	PTU
1	5474.16	18,770.25	1.56	1.61
2	5548.99	19,560.84	1.56	1.61
3	5695.74	19,035.05	1.56	1.61
4	5712.63	19,946.13	1.56	1.61
5	5804.57	19,024.90	1.56	1.61
6	5740.44	20,132.47	1.56	1.61
Mean	5.662,76	19411,61	1,56	1,61
SD	125,09	553,23	0	0
%CV	2,21	2,85	0	0

Table 2
Data of interday calibration curve of 5-Fluorouracil.

Interday Replicate	Slope	Intercept	R
1	0.5497	0.3655	0.9996
2	0.5397	0.3631	0.9986
3	0.5156	0.4233	0.9999

3. Results and discussion

3.1. Optimization of analytical condition

The detection method was performed using Multiple Reaction Monitoring (MRM). Mass spectrometry detection optimization was carried out at a capillary voltage of 5.50 kV, desolvation gas temperature of 350 °C, and desolvation gas flow rate of 650 L/h. The highest response was obtained from the fragmentation m/z 128.97 > 41.82 for 5-Fluorouracil and m/z 168.97 > 57.88 for propylthiouracil. The fragmentation spectrum of 5-Fluorouracil obtained is presented in Fig. 2. The elution was performed for 3 min using the mobile phase of acetonitrile-1 mM ammonium acetate (95:5), at a flow rate of 0.15 mL/min, and a column temperature of 40 °C.

3.2. System suitability test

The %CV of the area obtained from system suitability test for 5-Fluorouracil and propylthiouracil was 2.21 % and 2.85 % respectively. %CV for the retention time of both 5-Fluorouracil and propylthiouracil was 0. Both %CV values for the area and retention time were below 6 %, indicating that the chromatographic system met the criteria and was ready for analysis. The responses generated from the system suitability test are shown in Table 1.

3.3. Optimization of sample preparation

50 μL of previously prepared blood sample was spotted onto DBS paper and was dried for at least 4 h at 25 °C. After drying, the paper was cut and placed into a sample cup. Then, 20 μL of internal standard propylthiouracil (0.5 μg/mL) were added to the sample cup. The sample in DBS was subsequently extracted with 500 μL of ethyl acetate: 2-Propanol (84:16). Ethyl acetate has a large dipole moment and the addition of 2-Propanol resulted in better 5-Fluorouracil extraction [14]. Combination of ethyl acetate and 2-propanol prevented the extraction of polar impurities in blood, thereby created a cleaner chromatogram. In addition, ethyl acetate: 2-Propanol provided faster evaporation times and improved internal standard extraction, consistent with prior research findings by Stephen and Road [15].

The sample cup was vortex-mixed for 15 s, sonicated for 10 min, and centrifuged for 10 min. A total of 300 μL of the obtained extract was evaporated using an evaporator at 40 °C for 20 min. The evaporated residue was reconstituted with 100 μL of the mobile phase and vortex-mixed for 5 s. Finally, 10 μL of the reconstituted sample was injected into the instrument.

3.4. Method validation

3.4.1. Lower Limit of Quantification

At a concentration of 0.1 μg/mL, %diff ranged between −11.47 % and 4.39 % was obtained with %CV of 7.69 %. The response obtained at the LLOQ concentration was also five times greater than the response from the blank sample. These results indicated that the LLOQ at a concentration of 0.1 μg/mL met the validation requirements.

Table 3
Stability test result.

Stability test		Stable to-	Accuracy (%diff)	Precision (%CV)
Short term stock stability	5-Fluorouracil	24 h	−8.19 % to −7.12 %	0.66 %
	propylthiouracil		−2.21 % to −1.99 %	0.12 %
Long term stock stability	5-Fluorouracil	30 days	−5,63 % to −3,46 %	1.15 %
	propylthiouracil		−7.60 % to −7.16 %	0.06 %
Short term sample in DBS stability		24 h	−12.10 % to −4.04 %	3.28 % (QCL)
				2.54 % (QCH)
Long term sample in DBS stability		30 days	−7.09 %–3.39 %	2.26 % (QCL)
				6.28 % (QCH)
Autosampler stability		24 h	−11.41 % to −0.16 %	2.19 % (QCL)
				5.72 % (QCH)

3.4.2. Calibration curve

A calibration curve was generated with six concentration points: 0.1 μg/mL; 0.5 μg/mL; 5 μg/mL; 20 μg/mL; 40 μg/mL; and 60 μg/mL. The test results indicated that all six concentration points on the calibration curve met the back-calculated concentration (%diff) criteria. The calibration curve exhibited good linearity with $R^2 \geq 0.990$ ($R \geq 0.9950$) [5]. Interday calibration curve data is presented in Table 2.

3.4.3. Selectivity

The blank response yielded zero values for both 5-Fluorouracil and propylthiouracil. This results indicated the absence of matrix interference in the analysis outcomes. Hence, the method met the criteria for selectivity [13,14].

3.4.4. Accuracy and precision

The intraday accuracy ranged from 87.26 % to 110.54 % with %CV ≤ 6.94 % (QCL, QCM, and QCH concentrations) and ranged from 91.07 % to 107.09 % with %CV of 6,74 % (LLOQ concentration). The interday accuracy ranged from 87.26 % to 112.80 % with %CV ≤ 4.79 (QCL, QCM, and QCH concentrations) and ranged from 88.94 % to 113.78 % with %CV of 4.19 % (LLOQ concentration). These %diff and %CV values obtained during the accuracy and precision validation met the specified requirements [13,14].

3.4.5. Recovery

During the validation, the average recovery of 5-Fluorouracil at QCL, QCM, and QCH concentrations were 65.65 % ± 5.58 %, 62.04 % ± 2.11 %, and 61.53 % ± 0.72 % respectively, with %CV of 8.60 %, 3.40 %, and 1.17 % respectively. As for the propylthiouracil internal standard, a recovery value of 39.49 % ± 3.82 % was obtained with a %CV of 9.68 %. These values indicated that the method met the recovery requirements because of the proven consistency and reproducibility (%CV didn't exceed 15 %) [13,14].

3.4.6. Carry over

Carry over testing aims to determine the presence of analytes carried over to subsequent analyses. In this method, the carry over generated by 5-Fluorouracil and propylthiouracil respectively fell within the range of 1.77 %–2.92 % and 1.35 %–2.32 %. These values met the carry over requirements and it is concluded that high concentration injection would not interfere with the subsequent injection [13,14].

3.4.7. Dilution integrity

In the twofold dilution, a %CV of 4.83 % and %diff within the range of −11.14 % to −1.46 % were obtained. In the fourfold dilution, a %CV of 4.22 % and %diff within the range of −11.05 % to −1.84 % were obtained. The %diff and %CV values met the requirements and indicated that twofold and fourfold dilutions can be performed for samples with concentrations exceeding the ULOQ [13,14].

3.4.8. Matrix effect

At the QCL concentration, the %CV from each matrix fell within the range of 2.57 %–11.20 % and %diff was within the range of −12.45 %–10.41 %. At the QCH concentration, the %CV from each matrix was within the range of 1.85 %–6.07 % and %diff was within the range of −9.49 %–3.33 %. These values met the validation requirements and indicated that the matrix effect (ion suppression or enhancement) from sample would not interfere analysis results with this method [13,14].

3.4.9. Stability

The validation results indicated that the stock solutions of 5-Fluorouracil and propylthiouracil were stable when stored for 24 h at room temperature (25 °C). For long-term storage, the stock solutions of 5-Fluorouracil and propylthiouracil remained stable for 30 days at 4 °C. The difference in response obtained met the requirements as %diff was not greater than 10 % [15]. Analyte stability testing in DBS samples showed that samples in DBS remained stable when stored for 24 h at 25 °C. For long-term storage, DBS samples remain stable for 30 days when stored in a −20 °C freezer. The test results met the stability requirements as %diff and %CV obtained were not greater than 15 % [13,14]. Autosampler stability testing indicated that the samples remained stable during the autosampling

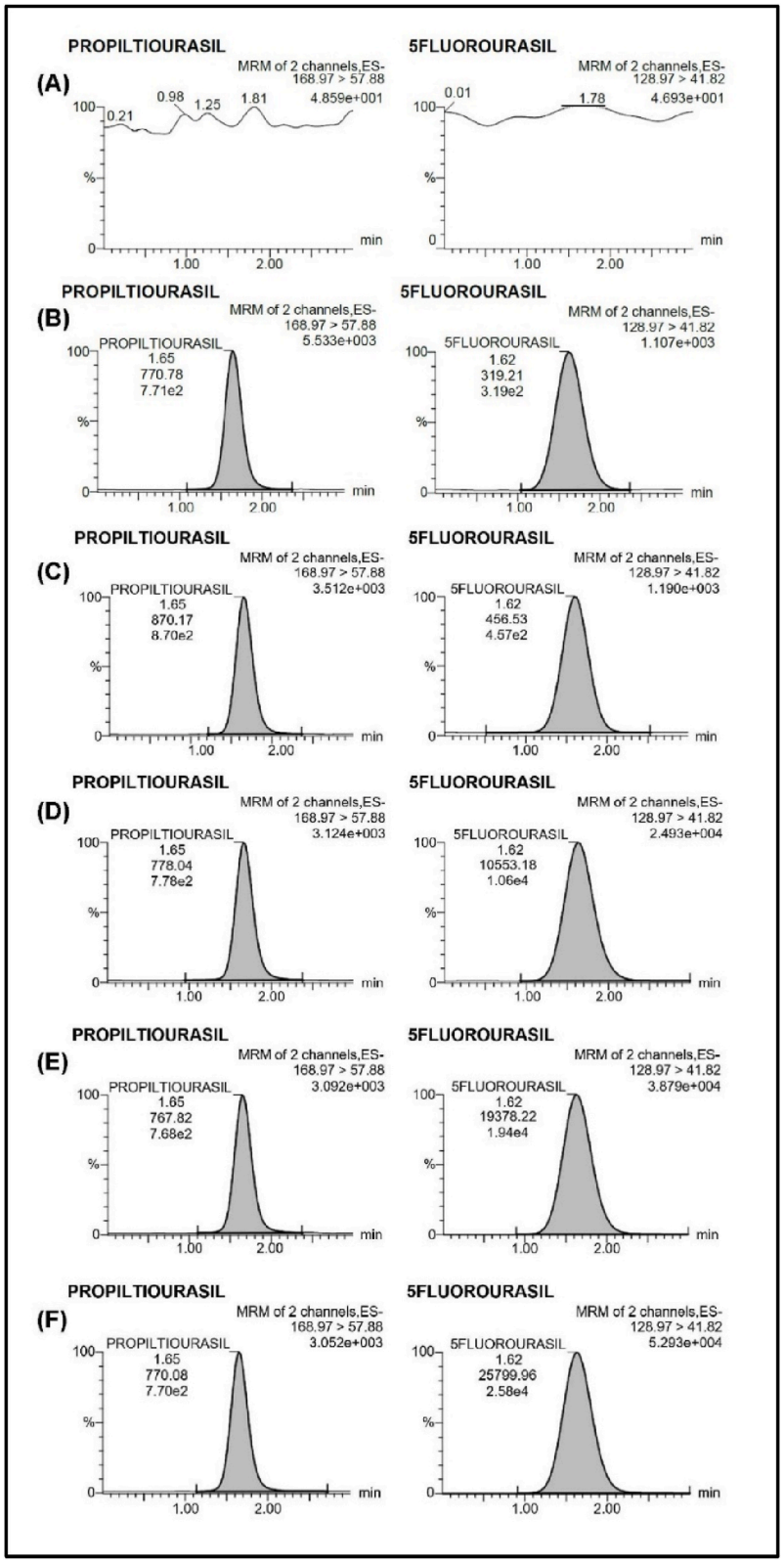


Fig. 3. Representative chromatograms at concentrations (A) blank, (B) LLOQ, (C) QCL, (D) QCM, and (E) QCH, and (F) ULOQ.

Table 4
Comparison of previous research and conducted study.

	Previous Research			Conducted Resesach
	[16]	[7]	[17]	
Instrument	KCKT-UV	KCKT-SM/SM	KCKT-SM/SM	KCKUT-SM/SM
Matrix	Plasma	Plasma	Plasma	DBS
Preparation time (without evaporation)	±15 min	±15 min	±30 min	±20 min
Analysis time	25 min	10 min	10 min	3 min
Extraction solution	500 μL of saturated ammonium sulfate	10 μL glacial acetic acid and 1 mL ethyl acetate	Protein precipitation: 200 μL saturated ammonium sulfate Extraction: 2,5 mL and 2 mL ethyl acetate: 2-Propanol (10:1)	500 μL of ethyl acetate: 2-Propanol (84:16)
Calibration Curve Range	0,5 μg/mL-100 μg/mL	0.01 μg/mL-10 μg/mL	0.1–75 μM (0.013 μg/mL-9.75 μg/mL)	0.1 μg/mL-60 μg/mL
Recovery	62–65 %	46.0–72.6 %	103.0–104.8	61.53–65.65 %
Accuracy	96.5–106.3 %	96.0–102.2 %	Biased 1.22–13.9 %	87.26–112.34 %
Precision	6,6–11,2 %	2,1–7,5 %	1.95–6.44 %	2.70–11.93 %

process, as %diff and %CV obtained were not greater than 15 % [13,14]. Table 3 shows the accuracy and precision of each stability test.

3.4.10. Reinjection reproducibility

The reinjection calibration curve demonstrated good accuracy with a %diff of 11.18 % for the LLOQ and %diff within the range of −3.15 %–7.43 % for other concentrations. The anaylisis of QCL, QCM, and QCH samples yielded %diff within the range of −5.40 %–8.28 %, −8.76 %–0.79 %, and −9.38 % to −5.67 % with %CV values of 5.41 %, 4.20 %, and 1.82 % respectively. These values met the reinjection reproducibility requirements, thus recalibrating and reinjecting both the calibration curve and samples could be performed if an instrument error occurred during anaylisis [13,14]. Fig. 3 displays chromatograms obtained during the analysis for blank, LLOQ, QCL, QCM, QCH, and ULOQ samples.

4. Conclusion

The 5-Fluorouracil analysis method developed in this study has been shown to meet the validation requirements set by the U.S. Food and Drug Administration (2022) and the European Medicines Agency (2022). Compared to the previous methods, the method in this study requires lower volume of mobile phases, significantly less extraction solution, and shorter preparation time, enabling large-scale analysis while still supporting green chemistry. The comparison of the method conducted with the previous methods is shown in Table 4. The method has proven to be accurate, precise, and selective, with a calibration curve range of 0.1 μg/mL to 60 μg/mL. Further clinical research can be conducted to demonstrate that plasma matrix can be substituted with DBS.

Data availability statement

Data included in article/supplementary material/referenced in article.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Yahdiana Harahap: Methodology, Conceptualization. **Vanessa Amarta:** Writing – original draft, Formal analysis. **Febrina Amelia Saputri:** Writing – review & editing, Visualization.

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.

References

[1] L.L. Brunton, R. Hilal-Dandan, B.C. Knollmann, Goodman & Gilman's: the Pharmacological Basis of Therapeutics, thirteenth ed., McGraw-Hill Education, New York, 2018.
[2] J. Casale, P. Patel, Fluorouracil, StatPearls Publishing, 2022. https://www.ncbi.nlm.nih.gov/books/NBK549808/. (Accessed 28 August 2022).

[3] S. Mindt, S. Aida, K. Merx, A. Müller, T. Gutting, M. Hedtke, et al., Therapeutic drug monitoring (TDM) of 5-fluorouracil (5-FU): New preanalytic aspects, Clin. Chem. Lab. Med. 57 (2019) 1012–1016, https://doi.org/10.1515/cclm-2018-1177.
[4] A. Amirfallah, G.C. Kocal, O.U. Unal, H. Ellidokuz, I. Oztop, Y. Basbinar, DPYD, TYMS and MTHFR genes polymorphism frequencies in a series of Turkish colorectal cancer patients, J. Personalized Med. 8 (2018), https://doi.org/10.3390/JPM8040045.
[5] M. Radovanovic, J.J. Schneider, M. Shafiei, J.H. Martin, P. Galettis, Measurement of 5- fluorouracil, capecitabine and its metabolite concentrations in blood using volumetric absorptive microsampling technology and LC-MS/MS, J. Chromatogr. B 1188 (2022) 123075, https://doi.org/10.1016/J.JCHROMB.2021.123075.
[6] F. Casale, R. Canaparo, L. Serpe, E. Muntoni, C. Della Pepa, M. Costa, et al., Plasma concentrations of 5-fluorouracil and its metabolites in colon cancer patients, Pharmacol. Res. 50 (2004) 173–179, https://doi.org/10.1016/J.PHRS.2004.01.006.
[7] J.E. Kosovec, M.J. Egorin, S. Gjurich, J.H. Beumer, Quantitation of 5-fluorouracil (5-FU) in human plasma by liquid chromatography/electrospray ionization tandem mass spectrometry, Rapid Commun. Mass Spectrom. 22 (2008) 224–230, https://doi.org/10.1002/RCM.3362.
[8] A. Sharma, S. Jaiswal, M. Shukla, J. Lal, Dried blood spots: concepts, present status, and future perspectives in bioanalysis, Drug Test. Anal. 6 (2014) 399–414, https://doi.org/10.1002/DTA.1646.
[9] D.E. Raynie, Supporting bioanalysis with dried blood spots, LCGC North Am. 34 (2016) 834–836.
[10] C.J. Briscoe, M.R. Stiles, D.S. Hage, System suitability in bioanalytical LC/MS/MS, J. Pharm. Biomed. Anal. 44 (2007) 484–491, https://doi.org/10.1016/J.JPBA.2007.03.003.
[11] U.S Food and Drug Administration, M10 Bioanalytical Method Validation and Study Sample Analysis Guidance for Industry, 2022.
[12] European Medicines Agency, ICH guideline M10 on bioanalytical method validation, Science Medicines Health 44 (2022) 57.
[13] N. Van De Merbel, N. Savoie, M. Yadav, Y. Ohtsu, J. White, M.F. Riccio, et al., Stability: recommendation for best practices and harmonization from the global bioanalysis consortium harmonization team, AAPS J. 16 (2014) 392–399, https://doi.org/10.1208/S12248-014-9573-Z.
[14] R.C. Paul, D. Singh, K.C. Malhotra, Ethyl acetate as a polar solvent, II formation of acids and bases due to solvation of proton in ethylacetate, Z. Anorg. Allg. Chem. 321 (1963) 70–80, https://doi.org/10.1002/ZAAC.19633210107.
[15] S. Stephen, F. Road, Determination of 5-fluorouracil in Human Plasma by High-Pressure Ion-Exchange Chromatography, 1980, pp. 272–276.
[16] J. Gao, R. Zhen, H. Liao, W. Zhuang, W. Guo, Pharmacokinetics of continuous transarterial infusion of 5-fluorouracil in patients with advanced hepatocellular carcinoma, Oncol. Lett. 15 (2018) 7175–7181, https://doi.org/10.3892/OL.2018.8242/HTML.
[17] B. Büchel, P. Rhyn, S. Schürch, C. Bühr, U. Amstutz, C.R. Largiadèr, 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 (2013) 7–16, https://doi.org/10.1002/bmc.2741.