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ORIGINAL RESEARCH

Determination of 5-Fluorouracil in Dried Blood Spots for Therapeutic Drug Monitoring and Adverse Event Assessment in Breast Cancer Patients

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Background: 5-Fluorouracil (5-FU) is a chemotherapy drug used to treat breast cancer. Monitoring 5-FU levels in blood is essential due to its narrow therapeutic range, high individual variability, nonlinear pharmacokinetics, dosage calculations based on body surface area, and susceptibility to toxicity influenced by individual factors such as enzyme polymorphisms.

Methods: An observational study was conducted in 2 types of patients: patients receiving intravenous 5-FU chemotherapy and those receiving oral chemotherapy with capecitabine as the 5-FU prodrug. 5-Fluorouracil blood levels in those patients were monitored using dried blood spots (DBS) as a biosampling method to correlate them with adverse events experienced by patients. Samples from DBS were analyzed using LC-MSMS which has been fully validated with propylthiouracil as an internal standard. All patients were also interviewed to determine adverse events experienced during 5-FU treatment. These adverse events were evaluated based on Common Terminology Criteria for Adverse Events version 5.0.

Results: Among the five patients who received intravenous 5-FU, only one patient had concentrations within the target range of 2–3 µg/mL, two patients were below the target range, and two patients were slightly above the target range. For the patients receiving oral capecitabine, 5-FU concentrations were detectable in only 3 out of 8 patients, with values near the LLOQ. The most common adverse events observed in intravenous chemotherapy patients were constipation, fatigue, and alopecia. Hand-foot syndrome was the most common syndrome in patients receiving oral chemotherapy.

Conclusion: The validated DBS method can be applied for therapeutic drug monitoring of 5-FU, offering advantages such as reduced invasiveness compared to traditional venipuncture. However, no significant relationship was found between the administered drug dosage, 5-FU blood levels, and adverse events. This study's limitations include its small sample size, which requires further research with larger cohorts to validate these observations and their clinical relevance.

Keywords: breast cancer, fluorouracil, liquid chromatography-mass spectrometry, therapeutic drug monitoring, dried blood spot

Introduction

According to the Global Burden of Cancer 2020 database from the International Agency for Research on Cancer, female breast cancer is the most prevalent cancer with 16.6% new cases and 9.6% mortality.[1] This type of cancer can be treated with systemic therapy such as chemotherapy. Chemotherapy involves the administration of anticancer drugs that are given directly into the blood vessels or orally so that they can spread throughout the body. One of the anticancer agents used to treat breast cancer is the antimetabolite class, which is 5-fluorouracil (5-FU). 5-FU is a pyrimidine analog commonly used to treat breast cancer, colorectal cancer, stomach cancer, and pancreatic cancer. The cytotoxic activity of 5-FU results from the combination of fluoronucleotides with RNA or DNA and the inhibition of thymidylate synthetase. 5-FU is generally administered parenterally or orally, as in the case of 5-FU prodrugs such as Capecitabine.[2]

5-FU levels in the blood should be monitored given its narrow therapeutic window, high individual variability, nonlinear pharmacokinetics, dosing based on body surface area, and ability to be affected by individual conditions like enzyme polymorphisms.[3] A lack of 5-FU can increase the probability of cancer relapse, but an excess concentration of

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5-FU can trigger the emergence of toxic effects. Early symptoms of 5-FU toxicity include anorexia and nausea, followed by stomatitis and diarrhea. Leukopenia caused by 5-FU toxicity usually occurs from day 9 to day 14 after the first day of drug injection and can cause an immunocompromised condition that can progress to secondary pneumonia or sepsis.[4] 5-FU toxicity can be severe in patients with dihydropyrimidine dehydrogenase (DPD) enzyme deficiency, which decreases the elimination of the drug and results in life-threatening toxicity in 10%–30% of patients and death in approximately 1% of patients.[5,6]

Capecitabine, a prodrug of 5-FU, is first metabolized by hepatic carboxylesterase into 5'-deoxy-5-fluorocytidine (5'-DFCR), which is then converted to 5'-deoxy-5-fluorouridine (5'-DFUR) by cytidine deaminase, primarily in liver and tumor tissues. Then, 5'-DFUR is activated into 5-FU by thymidine phosphorylase, an enzyme involved in catalytic activation in both tumor and normal tissues.[7] Capecitabine commonly causes hand-foot syndrome as a major side effect. Other side effects include diarrhea, hyperbilirubinemia, and leukopenia, but they are not severe.[8] Owing to these side effects, many doctors subsequently lower the initial dosage for most patients until a dose that can be tolerated individually is reached.[9]

5-FU shows significant interindividual variability in blood concentrations, which can be caused by the enzyme that metabolizes capecitabine or pharmacogenomic factors such as polymorphisms in the *DPYD* gene encoding DPD. Variability in blood concentrations of the drug can lead to unpredictable therapeutic outcomes, which may result in reduced efficacy or increased risk of toxicity.[10] Therefore, therapeutic drug monitoring (TDM) is essential to measure the actual drug concentration in the blood, allowing for individualized dose adjustments to ensure concentrations remain within the therapeutic range and to optimize both safety and efficacy.

TDM of 5-FU has been extensively researched in various types of cancer. Meta-analysis studies in several types of cancer have shown that pharmacokinetic-based 5-FU dosing has better response rates then does body surface area-based dosing.[11] TDM enable individualization of drug dosing by observing drug pharmacokinetics to maintain drug concentrations in the blood within the targeted therapeutic range.[12] These studies and other dose–response relationship studies regarding side effects and blood 5-FU levels in the context of breast cancer are still relatively rare in terms of the number of available articles.

Currently, the monitoring of intravenous 5-FU therapy in patients with colorectal and head and neck cancer has been recommended by the International Association of Therapeutic Drug Monitoring and Clinical Toxicology based on literature evidence and several studies.[13] Existing research on 5-FU monitoring typically involves the collection of blood samples from a vein by a phlebotomist in a health facility, which may cause discomfort for patients, especially for cancer patients, including large sample volumes and pain.[14] The innovation of the biosampling technique with microsampling such as dried blood spot (DBS) has better 5-FU stability because the blood is dried on DBS cards, enabling analysis at room temperature, less biohazard because of the smaller sample volume, a less invasive sampling method (using finger prick), does not require skilled experts for operation and the ability to support green chemistry by using less solvent for extraction.[15]

Methods for analyzing 5-FU levels have been extensively developed in plasma samples using high-performance liquid chromatography-tandem mass spectrometry.[16–18] Analytical methods for microsampling have been established in VAMS[19] and DBS.[20] Therefore, this study will carry out TDM for 5-FU using the DBS method and analyze the relationship between 5-FU concentrations and adverse events in breast cancer patients. The results of this study are expected to be used as a reference for using DBS as a less invasive alternative for blood collection in TDM of 5-FU, helping to check drug concentrations in breast cancer patients undergoing 5-FU chemotherapy.

Materials and Methods

Patients

The present study was approved by the Ethics Committee of Dharmais Cancer Hospital (Jakarta, Indonesia), and all patients provided written informed consent. In this study, 13 patients were included (including: 5 patients treated with IV 5-FU and 8 patients treated with oral capecitabine) from March 2024 to May 2024. Eligible participants were aged ≥18 years with a histologically or cytologically confirmed diagnosis of breast cancer who were seen at the participating sites and were receiving treatment with 5-FU chemotherapy or capecitabine (adjuvant or palliative) either as monotherapy or in combination with other anticancer drugs. The exclusion criteria were the patients who underwent treatment with propylthiouracil.

Sampling and Sample Preparation

Patients were interviewed to obtain demographic data and adverse events experienced during chemotherapy based on CTCAE Version 5.0. All adverse events experienced by patients were categorized by severity level based on the questions asked in the interview. Blood samples from participants receiving intravenous 5-FU chemotherapy were taken after 5-FU administration and samples from participants receiving oral capecitabine chemotherapy were taken no more than 4 hours after dosing. Blood samples were taken via finger prick using a lancet by experienced staff. Blood was dripped onto DBS cards and dried at 25°C. The dried DBS cards were sealed in a plastic holder and labeled before being stored at −20°C until analysis.

The analyte was extracted using ethyl acetate and 2-propanol from DBS cards. The samples were vortexed, sonicated, centrifuged, evaporated, and resuspended in mobile phase before injection into the LC MS-MS. The injection volume was 10 µL with a total run time of 3 min.[20]

Bioanalysis

The samples were analyzed for 5-FU concentration using the LC MS-MS and the preparation method of Harahap et al which was validated according to the US Food and Drug Administration guidelines for bioanalytical method validation.[21] Before sample analysis, partial validation was conducted to verify the validity of the existing method. A Waters TQD LC-MS/MS instrument equipped with an electrospray ionization source interface that operates in negative ion mode. Multiple reaction monitoring (MRM) values were set at m/z 128.97 > 41.82 for 5-FU and 168.97 > 57.88 for propylthiouracil as the internal standard. Analysis was conducted with reversed-phase chromatographic separation and acetonitrile-ammonium acetate as the mobile phase.

Partial validation was performed for linearity, accuracy, and precision. Linearity was assessed at concentrations of 0.1, 0.5, 5.0, 20.0, 40.0, and 60.0 µg/mL. The back-calculated concentrations for the standard calibration should be within ±15% difference from the nominal concentration, except for the LLOQ which should be within ±20% difference from the nominal concentration. Accuracy and precision were evaluated by measuring control quality samples, specifically 0.3 µg/mL, 25 µg/mL, and 50 µg/mL. The intraday accuracy and precision were determined by analyzing 5 replicates at the LLOQ, QCL, QCM, and QCH concentrations. The back-calculated concentrations obtained should be within ±15% of the nominal concentration, except that the LLOQ should be within ±20% of the nominal concentration. The %CV values obtained should not exceed 15%, except for the LLOQ which should not exceed 20%.

Data Collection and Analysis

The LC-MSMS data were acquired using Masslynx 4.1 software. Using the Statistical Package for the Social Science (SPSS) software, the dosage, concentration data for each drug (intravenous 5-FU and oral capecitabine), and adverse events were analyzed and investigated using non-parametric tests because of the small number of participants. If the significance value is greater than 0.05 then there is not enough evidence to reject H0, indicating that there is no significant difference between each variable.

Results

Patients and Dose Administration

The population of patients examined consisted of females with breast cancer in any stadium. Among the age groups, 30% of the patients were in their 40s, 62% were in their 50s, and 8% were in their 60s. Patient characteristics can be seen in Table 1.

The patient received 5-FU according to their body surface area (BSA). Patients who participated in this study received a 5-FU dose of 500 mg/m2 in chemotherapy regimens of FAC (fluorouracil, doxorubicin, cyclophosphamide), every 3 weeks for 6 cycles. There was no significant difference between the dose received by patients and the dose calculated based on BSA by the researchers based on paired t-test correlations. A significant correlation was found between the dose received by patients and their BSA with a Spearman correlation (r) of 0.900 (p = 0.037).

For capecitabine, patients receive regimens that vary in monotherapy or with additional anticancer therapy such as lapatinib, anastrozole, and letrozole. The dose of capecitabine used was 1250 mg/m², which was taken orally twice daily for 2 weeks,

Table 1 Patient Characteristics

Characteristic	IV 5-Fluorouracil	Oral Capecitabine
Numbers	5	8
Age (y)	56.0 (45–69)	53.5 (43–58)
BMI (kg/m^2)	28.13 (20.36–36.20)	22.60 (16.94–35.16)
BSA (m^2)	1.66 (1.34–2.07)	1.47 (1.29–2.00)
Dose (mg)	815 (665–995)	1500 (1500–2000), 2x1
Stadium (n)	Stadium 2 = 2 Stadium 3 = 3	Metastasis

Note: Data are presented as median (range).

followed by a 1-week rest period in 3-week cycles. The physician evaluated the tumor by CA15-3 values, radiological tests, and patient conditions every 3 cycles to determine whether to continue or discontinue the treatment. On this basis, physicians subsequently reduce or adjust the initial dose for patients.[9] In this study, the number of cycles varied from 2 to 11.

Sample collection times varied (2–4 hours post-capecitabine, 2–7 minutes post-IV), which may have influenced concentration variability. This variability was considered in the analysis, though its impact remains a potential limitation.

All participants were asked about the sampling method they preferred between microsampling for this study and their usual venipuncture for routine checkup. It was reported that they preferred the microsampling method over venous blood sampling (microsampling strongly preferred: 12/13, microsampling slightly preferred: 1/13). The patient's level of pain showed that the most reported was no to less pain compared to venipuncture sampling (median 0, range 0–1).

Partial Validation

5-FU is unstable in whole blood at room temperature because of ex vivo catabolism by DPD in RBCs; therefore, collected blood samples should be placed on ice, and plasma should be separated as soon as possible.[22] Therefore, the blood samples were immediately spotted onto DBS cards and once dried, stored at −20°C for long-term storage.

The linearity was calculated by a calibration curve 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 results met the acceptance criteria of linearity with a correlation coefficient ≥0.99 and all six concentration points on the calibration curve met the back-calculated concentration (%diff) criteria. In addition, the intraday accuracy calculated by the percentage of difference from calculated concentration and precision by the coefficient of variance was within acceptable criteria. The results of the partial validation are presented in Table 2.

Sample Analysis

The highest concentration of 5-FU was 3.18 μg/mL (0.16–3.18 μg/mL) for IV 5-FU and 0.23 μg/mL (0–0.23 μg/mL) for oral capecitabine. The concentrations in 5 patients who received oral capecitabine were not detected.

Table 2 The Result of Partial Validation

Parameter	Criteria	Result
Linearity	R > 0.9900 %diff LLOQ < ±20% %diff QC < ±15%	R = 1.0000 LLOQ = 3.91% QC = −0.90–5.27%
Accuracy	%diff LLOQ < ±20% %diff QC < ±15%	LLOQ = −14.22–14.67% QC = −9.06–12.21%
Precision	CV LLOQ < ±20% CV QC < ±15%	LLOQ = 11.59% QC = 3.60–8.21%

Adverse Events

Fatigue, nausea, and constipation were the most common side effects in patients who received 5-FU IV chemotherapy. The level of toxicity in all patients remained low and did not require treatment or follow-up. Patients who take capecitabine often experience hand-foot syndrome, fatigue, and pain. Hand-foot syndrome occurred in all patients, with toxicity levels ranging from 1 to 3. Capecitabine had a more tolerable level of toxicity and did not cause myelosuppression or alopecia in the study patients. The level of toxicity data that was adjusted to the CTCAE version 5.0 can be seen in Figure 1.

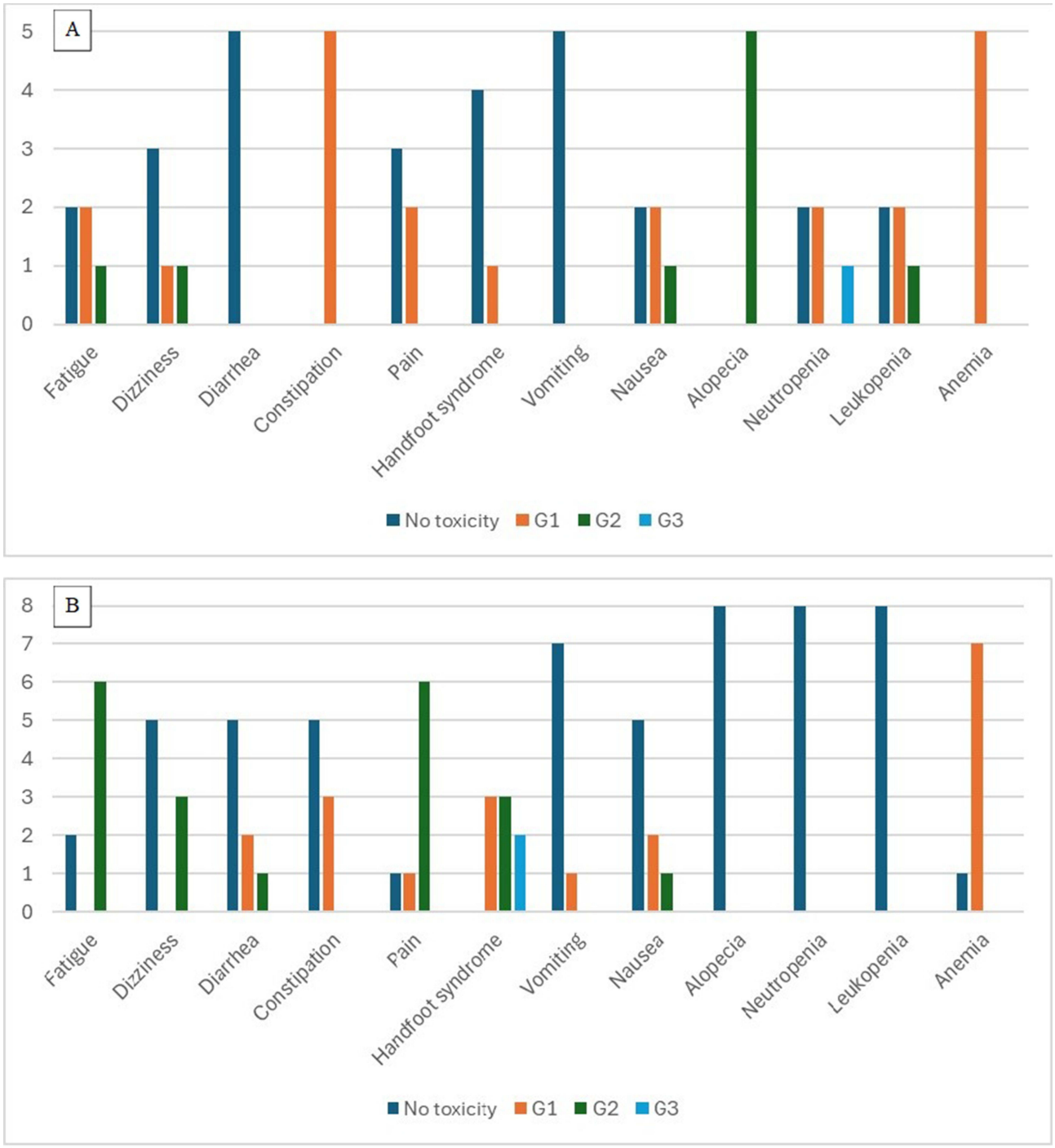

Figure 1 The number of patients at each toxicity grade based on CTCAE who received IV 5-FU (**A**) and oral capecitabine (**B**).

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The results of the correlation analysis between dose, 5-FU blood concentration, and adverse event toxicity level in patients receiving IV and oral capecitabine are not statistically significant ($\alpha > 0.05$). However, in patients receiving IV 5-FU, there was a correlation between the dose administered with the confounding variable (BSA) (Spearman 0.900, p=0.037) and the 5-FU blood concentration with patient age (Spearman −1.000, p<0.01).

Discussion

Compared with venipuncture, the DBS method requires fewer blood samples. Additionally, the use of filter paper in DBS cards can minimize the risk of bacterial contamination in the samples to laboratory personnel and can support green chemistry by using less solvent for extraction.[15] The application of green chemistry can help achieve this goal by eliminating or reducing hazardous chemical pollution, one of which is by using the smallest possible sample and reducing the use of toxic solvents.[23]

No studies have established target 5-FU concentrations in capillary blood. Research has been conducted on metastatic colon cancer patients receiving an 8-hour infusion of 5-FU adjusted doses to achieve a target plasma concentration range of 2–3 μg/mL.[24] The target AUC range for the 5-FU infusion regimen with leucovorin is 20–25 μg·h/mL, which is administered either as a bolus or infusion, with schedules varying from a few hours to several days.[25] In pharmacokinetic studies, the maximum concentration of 5-FU after an IV bolus of 600 mg/m² over 30 minutes was 15.7–29.2 μg/mL in whole blood, 14.2–27.5 μg/mL in plasma, and 1.1–1.4 μg/mL in red blood cells, and the 5-FU concentrations in plasma were similar to those in whole blood (106–115%).[26] Based on this, it can be assumed that the patient's 5-FU concentrations are near the target range of 2–3 μg/mL, with one within the target range, two slightly above it, and two below it. Two patients with concentrations above the target range are more likely to have a greater risk of experiencing drug side effects.

In a study using capillary blood with VAMS, 5-FU concentrations ranged from 4.24 to 47.9 μg/mL among 20 cancer patients. The researchers noted significant variability in patient sample concentrations, which may be attributed to the very short half-life of 5-FU, meaning that even slight differences in sample collection times could contribute to this variability.[19]

Out of 8 patients who received capecitabine, 5 were not detected by the instrument. Based on previous pharmacokinetic studies, the Cmax of 5-FU occurs 2 hours (0.5–4 hours) after capecitabine administration, with concentrations ranging from 0.22 to 0.31 μg/mL in plasma.[27] Another TDM study revealed that 5-FU levels in blood collected 2 hours after capecitabine administration could be undetectable (0–0.4966 μg/mL). A comparison of 5-FU concentrations in patients receiving capecitabine between plasma samples and VAMS microsampling revealed that the concentrations in blood samples were lower than those in plasma but correlated (Pearson: 0.89, r^2: 0.69, p = 0.05).[28]

In this study, there were variations in sample collection times, ranging from 2 to 4 hours after morning capecitabine consumption or 2–7 minutes after the IV dose had been administered. This variability was not considered in the statistical analysis and represents a limitation of this study. The sample collection time is crucial for TDM studies because of significant variability among patients in the time required for 5-FU to reach peak concentration. Intraindividual variability may also be influenced by other factors such as cancer severity, genetic variations, drug interactions, ethnicity, age, kidney and liver function, comorbidities, nutritional status, and lifestyle.[29] Therefore, a pharmacokinetic analysis should be conducted for each patient before the TDM study to determine the optimal sample collection time.

The variability of the pharmacokinetic parameters of capecitabine and its metabolites is high (27–89%).[27,30] TDM study of this analyte also showed a high interpatient variability.[19,31] The interpatient variability is likely due to variability in the activity of the enzymes involved in capecitabine metabolism. The variability of pharmacokinetic results can also pose a challenge in bioequivalence studies, even when conducted within the same patient.

The lower blood concentrations of 5-FU at the capecitabine dose are also influenced by the activation of capecitabine to 5-FU, which occurs primarily in tumor tissue, resulting in lower 5-FU blood concentrations. The average concentration of 5-FU in colorectal tumor tissue is known to be 20 times or greater than that in plasma after oral capecitabine administration. For healthy colorectal tissue, the concentration is approximately 9 times greater, and for liver metastases and healthy liver tissue, it can be up to 10 times greater.[27,32]

A study with a Caucasian population showed that four DPYD risk variants are identified as contributing to individual differences in the development of 5-FU toxicity.[10] A study conducted on the Japanese population found that DPYD polymorphisms in this nine DPD variants (C29R, Y304H, R353C, F438V, R592W, G748D, T768K, G926V, and T990I)

reduced enzymatic activity to less than 50% of the normal DPD levels. As a result, patients may experience severe toxicity due to higher concentrations of 5-FU during chemotherapy.[33]

Not all adverse events are solely due to 5-FU. Other medications administered concurrently, such as doxorubicin and cyclophosphamide in chemotherapy regimens, can also contribute to these effects. Additionally, patients might have other conditions requiring treatment, such as hypertension or diabetes, or the ADRs might be related to the cancer itself or other comorbidities, such as pain experienced by the patient.

In the FAC chemotherapy regimen, all the drugs given commonly cause side effects such as nausea, vomiting, and alopecia. Three patients receiving IV 5-FU chemotherapy experienced neutropenia and leukopenia at levels 1 and 2, with some cases of grade 3 neutropenia. In a study of 5-FU toxicity in colorectal cancer patients, the most common side effects were neutropenia (69%), mucositis (58%), and leukopenia (46%), with 70% of patients experiencing neutropenia and/or leukopenia of grade 1 or higher.[34]

Hand-foot syndrome is a unique adverse event associated with 5-FU. This syndrome occurred in all patients receiving oral chemotherapy and one patient receiving IV chemotherapy. Research on cancer patients receiving both infusion and oral capecitabine revealed that 76.90% of them experienced hand-foot syndrome. The onset of this toxicity varies among patients, with most cases of grade 1 toxicity occurring by the third cycle.[35]

Currently, no studies have specifically compared 5-FU levels and adverse events in breast cancer patients. This study attempted to address this gap but did not find a significant relationship between blood concentration and adverse events. The small sample size was a limitation for statistical analysis and resulted in nonsignificant findings.

Conclusions

The method for analyzing 5-FU from DBS can be used to assess 5-FU levels in patients receiving both IV 5-FU and oral capecitabine. This study can be valuable for facilitating more comfortable TDM through less invasive blood sampling, so that personalized treatment adjustments can be provided to maintain 5-FU levels within the therapeutic target range and potentially reduce adverse events. However, this research has limitations, including a small sample size that affects the statistical power to detect the relationships between the 5-FU blood concentration and adverse events. Additionally, this study has considerable variation, like sampling time, potential polymorphism, and concomitant drugs, which requires caution in interpreting the data and prevents drawing relevant or conclusive findings. Therefore, further studies with a larger and more standardized cohort are needed to gain a better understanding of the relationship between the 5-FU blood concentration from DBS and adverse events.

Data Sharing Statement

The authors confirm that the data supporting the findings of this study are available within the article. Raw data that support the findings of the study are available from the corresponding author, upon reasonable request.

Ethics Approval and Consent to Participate

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Dharmais Cancer Hospital, Jakarta. Informed consent was obtained from all patients involved in the study.

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Disclosure

The authors declare that they have no conflicts of interest in this work.

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