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Research Article

Comparison between a single- and a multi-point calibration method using LC-MS/MS for measurement of 5-fluorouracil in human plasma

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ABSTRACT

When quantifying therapeutic drugs using LC-MS/MS instrumentation in clinical laboratories, batch-mode analysis with a calibration curve consisting of 6–10 concentrations for each analyte is the most widely used approach. However, this is an inefficient use of this technology since it increases cost, delays result availability and precludes random instrument access. Various alternative methods to reduce the calibrator use and improve efficiency without compromising analytical quality have been investigated, and a single-point calibration has been reported to be the simplest, least expensive and the quickest approach.

This study compares a single and a multi-point calibration method using LC-MS/MS with 5-fluorouracil (5-FU) as a model drug. The method was validated for quantitative analysis of 5-FU over a concentration range of 0.05–50 mg/L. Patients undergoing cancer treatment with intravenous 5-FU had plasma 5-FU concentrations measured, and their dose adjusted in real time based on the calculated area under the time-concentration curve (AUC). Subsequently, a single point calibration method using a concentration at 0.5 mg/L was compared to the multi-point calibration method in terms of accuracy and precision. A Bland-Altman bias plot and a Passing-Bablok regression analysis showed a good agreement between the two methods (mean difference = −1.87 %, slope = 1.002, respectively) when comparing patient plasma 5-FU concentrations. The calibration method did not impact the AUC results nor the decision on 5-FU dose adjustments. Our study demonstrated that a single point calibration method produced analytically and clinically comparable results to those produced by a multi-point method when quantifying 5-FU and is feasible to be used clinically.

1. Introduction

In the past two decades, there has been an increasing adoption of liquid chromatography tandem mass spectrometry (LC-MS/MS) instrumentation in clinical laboratories owing to, particularly, the specificity and sensitivity of this technology compared to conventional assays. To fully leverage the potential of LC-MS/MS, facilitate greater inclusion of this type of instrumentation in clinical laboratories, and establish them as automated chemical analyzers, some limitations need to be addressed. Aside from instrument complexity requiring high operator skill, high instrument costs, and the need for in-house method development due to limited commercial kit availability, a limitation that can be addressed is the typical batch-mode analysis approach, which creates a bottleneck to greater efficiency and the flexibility of random instrument access.

In the routine clinical bioanalysis setting, the most widely used approach with LC-MS/MS is inclusion of a calibration curve, generally consisting of 6–10 concentrations, with every batch of samples. This is based on guidelines set by the governing authority [1], which recommends a minimum of six non-zero concentrations for each analyte be

Abbreviations: 5-FU, 5-fluorouracil; AUC, area under the time-concentration curve; CE, collision energy; CI, confidence interval; ESI, electrospray ionisation; K_3-EDTA, potassium ethylenediamine tetra-acetic acid; LC-MS/MS, liquid chromatography tandem mass spectrometry; LLOQ, lower limit of quantitation; ME, matrix effect; MRM, multiple reaction monitoring; QC, quality control; r^2, coefficient of determination; Rec, recovery; RT, room temperature; R*t*, retention time; SD, standard deviation; SIL-IS, stable isotope labelled internal standard; TDM, therapeutic drug monitoring.

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included in each analytical run. However, performing a calibration curve with each assay batch generates a significant amount of data, prolongs the analysis time, delays the availability of the first patient result, and markedly increases costs. This practice is an inefficient use of the technology.

Alternative calibration approaches have been investigated for LC-MS/MS [2–8] to reduce calibrator use and improve efficiency without compromising analytical quality. For example, utilizing a pre-determined response factor based on the analyte and its stable isotope-labeled internal standard (SIL-IS) ratio to quantify the unknowns was reported by Nilsson and Eklund in 2007 [2]. A similar concept was used later by others [3,4]. However, to utilize this approach, establishing the optimal internal standard (IS) concentration that ensures the best accuracy and precision within the clinical and desired analytical range is essential. Additionally, it is crucial that the response is independent of the analyte concentration, and there should be no cross-signal contribution present.

More recently, scientists have taken advantage of the presence of multiple naturally occurring isotopes to generate an internal calibration curve [5–8]. The calibration curve is either generated by adding a mixture of various, commercially available, isotopically-labelled internal standards (e.g., ^{13}C, ^{2}H, ^{15}N) [7,8] for an analyte of interest at various concentrations or, alternatively, by measuring the response of multiple isotopes of the selected precursor and product ions relative to their respective isotopic abundance [5,6].

While these methods have the advantage of overcoming the potential matrix effects, as each sample will have a calibration curve generated using its own matrix [5–8], it requires the use of at least three or more available SIL-IS [7,8] for each analyte of interest, which is not always available and can be costly. The latter method of utilising multiple naturally occurring isotopes is predominantly aimed at quantifying large molecules, such as proteins and peptides [6] and is less applicable to quantifying small molecules, such as therapeutic drugs [9]. Although various approaches have been published, utilising a single point calibration appears the simplest, the least expensive and the quickest. A single-point calibration method has been demonstrated to yield results as reliable as those obtained from a multi-point calibration method [10]. While the ability to use single-point calibration as an alternative approach was demonstrated in the late nineties by Taylor and co-workers [11], two decades later, it is still not routinely used in clinical laboratories, and even its use in research is limited.

For a single-point calibration method to be utilised, the following criteria need to be met: no presence of a cross-signal contribution, calibration curve (response-concentration relationship) must be linear, and a single-point calibration should produce as reliable a result as a multi-point calibration method. During method validation, it is crucial to ensure that these criteria are met. The accuracy and precision of the single-point method should be compared to a fully validated multi-point calibration method to verify its suitability for use.

We aimed to investigate the use of a single-point calibration method for quantifying therapeutic drugs in patient samples and evaluate the feasibility of random instrument access. As a model for this research, we selected the therapeutic 5-fluorouracil (5-FU). 5-FU is an anti-neoplastic agent used to treat various types of cancers. Large variations in 5-FU exposure have been observed [12] when patients are dosed based on their body surface area, with only 20–30 % achieving their therapeutic targets [12,13]. Over the decades, studies have shown that individual 5-FU dose adjustment based on using a steady-state plasma 5-FU concentration measured while a patient receives their infusion is associated with improved clinical outcome. The target 5-FU exposure, measured as the area under the time-concentration curve (AUC), is 20–30 mg h/L [14] in patients with colorectal cancer. Considering that chemotherapy regimens of 5-FU are given in cycles two weeks apart, dosing review and adjustment before the next cycle is possible. To assess the applicability of a single-point calibration method, both calibration methods were applied to clinical samples obtained from cancer patients treated with 5-FU, for which dose adjustments were made in real-time.

To determine the feasibility of the proposal, we: a) developed and validated an LC-MS/MS method for measurement of 5-FU in plasma matrix using multi-point calibration according to the guidelines, b) compared a single-point calibration method to a fully validated multi-point method, in terms of accuracy and precision, c) quantified 5-FU concentrations in patient samples using a multi-and a single-point calibration methods, and d) assessed if a decision on dose adjustments of 5-FU in patients differed based on the calibration approach.

2. Materials and methods

2.1. Chemicals and reagents

5-FU (≥99 % chemical purity) and formic acid (>95 % chemical purity) were purchased from Sigma Aldrich (Sigma, Australia) while 5-FU $^{13}C^{15}N_2$ (IS, 99.6 % isotopic purity) was purchased from Toronto Research Chemicals (PM Separation, Australia). LC-MS grade acetonitrile and methanol was from VWR (VWR, Australia) while water was prepared in-house using a Milli-Q (Q-POD ®) Advantage A10 purification system (Merck, Australia). Expired, drug-free human plasma was obtained from Australian Red Cross Blood Services, Sydney.

2.2. LC-MS/MS equipment and conditions

The LC-MS/MS system was a Shimadzu Prominence Ultra-Fast Liquid Chromatography coupled to a tandem mass spectrometry, Shimadzu 8060 (Shimadzu Oceania, Rydalmere, NSW, Australia). The LC consisted of a solvent delivery system, an autosampler maintained at 15 °C, a vacuum degasser, a column oven set to 30 °C and a system controller. Compounds were chromatographically separated on a Phenomenex Luna Omega Polar C_{18} (50 × 3.0 mm, 3 μm) column using an isocratic elution of acetonitrile, water, and formic acid (1/98.9/0.1 *v/v/v*). Flow rate was 0.5 mL/min and the run time was 3 min.

A Shimadzu 8060 tandem mass spectrometer equipped with an electrospray ionization source (ESI) interface, operated in negative ion mode, was used for multiple reaction monitoring (MRM) analysis. At least two ion transitions were selected for the analyte and the IS. Details of the optimised parameters are presented in Table 1. For data acquisition and processing, Shimadzu LabSolution software, version 5.96, was used. Optimised interface parameters were as follows: 1) nitrogen was used as the nebulising, heating and drying gas set to 2.0, 10 and 10 L/min, respectively 2) capillary voltage applied to the ESI probe was set to 3 kV 3) for the collision induced dissociation, high purity argon was used at a pressure of 270 kPa and 4) interface, heating block and desolvation line temperatures were set to 300 °C, 400 °C and 250 °C, respectively.

2.3. Preparation of calibrators and quality control (QC) samples

Stock solutions of 5-FU and IS at concentrations of 1 mg/mL were prepared in water and stored at −30 °C. Intermediate calibrators and QCs in methanol were prepared at concentrations ten times higher than working calibrators and QCs and stored at −30 °C. The intermediate calibrator concentrations were 0.5, 1.0, 5.0, 50, 250, and 500 mg/L while the concentrations of the intermediate QCs were 2.0, 20 and 400 mg/L. For preparation of working calibrators, intermediate calibrators, prepared in methanol, were diluted one in ten with drug-free plasma on the day of analysis. The resulting calibrator concentrations were 0.05, 0.1, 0.5, 5.0, 25 and 50 mg/L. For calibrator 0, methanol was added to blank plasma equivalent to the amount of methanol used to prepare working calibrators. This was used to prepare a double blank sample containing neither the analyte nor the internal standard, and a zero calibrator (blank plasma with the IS). Plasma QCs were prepared at concentrations of 0.2, 2.0 and 40 mg/L and stored at −30 °C. Working-IS at concentration of 1 mg/L was prepared in acetonitrile and stored at −30 °C.

Table 1
Optimization parameters, MRM transitions and retention times of 5-fluorouracil and the stable isotope labelled internal standard.

Analyte	Molecular weight (g/mol)	R*t* (min)	MRM (*m/z*)	Collision energy (eV)	Dwell time (ms)	Ion mode
5-FU	130.08	1.31	129.30 → 42.05	22	20	Negative
			129.30 → 86.00	19	20	
			129.30 → 59.05	26	20	
5-FU $^{13}C^{15}N_2$ (SIL-IS)	133.06	1.30	132.10 → 44.00	17	20	Negative
			132.10 → 43.00	21	20	
			132.10 → 87.90	19	20	
			132.10 → 60.00	24	20	

R*t*, retention time; MRM, multiple reaction monitoring; 5-FU, 5-fluorouracil; SIL-IS, stable isotope labelled internal standard.

2.4. Sample extraction procedure

To 50 μL of each calibrator and QC, 50 μL of the working-IS and 400 μL of acetonitrile were added to 1.5 mL polypropylene tubes. These were vortex mixed (10 s), centrifuged (15,000 × *g*, 5 min) and 300 μL of the supernatant was transferred into plastic vials and dried under vacuum (30 min, 60 °C). Samples were resuspended in formic acid/water (0.1/99.9 *v/v*, 20 μL), vortex mixed (10 sec) and 1 μL was injected into the LC-MS/MS system.

2.5. Validation protocol

The multi-point calibration method was developed and validated in accordance with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) [1] guidelines in terms of linearity, accuracy and precision, specificity and selectivity, carry-over and stability.

2.5.1. Linearity

Six non-zero calibrators, as well as a double blank and a zero calibrator were assayed on four different days in duplicate. Linear regression analysis, weighted 1/x using the peak area ratio (analyte/IS) versus the concentration, was used to determine the analyte concentration. Acceptance criteria of the interpolated values of each calibrator were set to ≤15 % from the weighed-in values, except the lower limit of quantitation (LLOQ) for which ≤20 % was allowed. Correlation coefficient (r^2) of the calibration curves was set to ≥0.990.

2.5.2. Accuracy and precision

QCs at three concentrations were used to evaluate accuracy (mean obtained concentration/weighted-in concentration * 100) and precision (defined by the coefficient of variation, CV % = 100*Standard deviation (SD)/Mean) on four different days in triplicate. For intra-day accuracy and precision, seven sets of QCs at three concentrations were tested in one batch. To determine the accuracy and precision of the LLOQ, 5 replicates were tested in one batch. Acceptance criteria for each QC was set to ≤15 % from the weighed-in values and ≤20 % for the LLOQ.

2.5.3. Carryover

Carryover was assessed by injecting the highest calibrator followed by a double blank extract. Response should not exceed 20 % of the analyte LLOQ response.

2.5.4. Specificity and selectivity

2.5.4.1. Blank plasma matrix. Blank plasma matrices from five different sources without the addition of either the analyte or the IS were extracted as outlined. The presence of peaks at the analyte or IS retention times (R*t*) and their MRMs were visually assessed. The peak response should be ≤20 % of LLOQ response and ≤5 % of the IS.

2.5.4.2. Cross-signal contribution. Cross-signal contribution between the analyte and SIL-IS from naturally occurring isotopes and isotopically impure SIL-IS was assessed. The analyte, prepared in pure solution at the upper limit of quantification, and IS at concentration expected in the extracted samples, were individually injected into the LC-MS/MS while their responses were monitored. Any peaks observed at the R*t* and MRM, except for the analyte being injected, were considered a cross-signal contribution. Acceptance criteria were set to the response being ≤20 % of the analyte LLOQ and ≤5 % of the IS response.

2.5.5. Matrix effect and recovery

Matrix effect (ME, analyte response in a matrix compared to a pure solution) and recovery (Rec, extraction efficiency independent of matrix effect) were evaluated as described by Matuszewski et al., 2003 [15]. Three sets of calibrators were prepared. Set 1 was prepared in a pure solution and injected directly into the LC-MS/MS analyser; set 2 calibrators were extracted as outlined in the method (pre-addition of the analyte and IS) and set 3 contained blank extracts of the same matrix (double blank samples) that were spiked with a solution used in set 1 (post-addition). Percentage of the absolute and normalised (for IS) ME and Rec were calculated in the following way: ME = set 3/set 1 * 100; Rec = set 2/set 3 * 100. Results between 85–115 % indicate absence of suppression or enhancement of ionisation by the matrix.

2.5.6. Stability

5-FU stability was evaluated in plasma and whole blood matrices at room temperature (RT), 4 °C and on ice for up to 4 h. Fresh blank plasma and whole blood were spiked with a pure solution of 5-FU at three concentrations. Samples were stored at various temperature conditions and aliquots were taken at various time points for up to 4 h. Stability was assumed if the concentrations at the defined temperature conditions were within ± 15 % of the spiked concentrations. Stability of the extracted samples in the autosampler (15 °C) was evaluated for up to 40 h. Calibrators and QCs were prepared and analysed fresh and then reinjected after sitting in the autosampler. The obtained concentrations of the reinjected samples were compared to the results when samples were freshly prepared and analysed. Long-term stability of 5-FU in plasma and in pure solution at −30 °C was assessed for up to 18 months. The freeze–thaw stability (−30 °C to RT) was assessed for three cycles.

2.6. Patient samples collection

Patients with cancer undergoing treatment with an intravenous infusion of 5-FU had their steady-state plasma 5-FU concentration quantified using the described multi-point calibration LC-MS/MS method and dose adjustments were made in real-time before the next treatment cycle. Informed consent from patients was obtained prior to their participation. Thirty-six patients receiving a 46-hour 5-FU infusion were included in the study. Each patient underwent one or more TDM cycles (i.e., had at least one 5-FU concentration measured) resulting in a total of 53 measured 5-FU plasma concentrations. Venous blood samples were collected using K_3-EDTA tube, which were kept on ice before cold centrifugation and separation of plasma from red cells. Plasma samples were stored, transported, and kept frozen until the time of analysis.

2.7. Ethical considerations

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Hunter New England and University of Newcastle of Australia (project codes: HREC/58470/PMCC-2019; UON: H/2022-0145). Informed consent was obtained.

2.8. Method comparison

For the method comparison, 5-FU concentrations from patient samples were determined with the multi- and a single-point calibration method. The analytical method for which the validation data is reported herein had an analytical range between 0.05–50 mg/L. For the comparison study, the analytical range of the method was narrowed to 0.05–5 mg/L, aligning it more closely with the concentrations typically encountered in clinical samples. The concentrations of 0.05, 0.1, 0.25, 0.5, 1 and 5 mg/L were used to generate a multi-point calibration curve. For the single-point method, a calibrator at concentration of 0.5 mg/L was selected since a 5-FU plasma concentration of 0.5 mg/L will result in a target 5-FU AUC of 20–30 mg h/L for a 46-hour infusion. Linear regression analysis using the peak area ratio (analyte/IS) versus the concentration was used to construct a calibration curve. A weighting factor of 1/x and an intercept not forced through zero were implemented in both calibration methods. Methods were compared in terms of accuracy and precision using the QCs at three concentrations (0.2, 0.6 and 2 mg/L). Thirty-seven calibration curves, generated from 37 analytical batches, and 50 QCs at each concentration were acquired over the period of January 2021 to April 2022. Patient samples were analysed in batches using a multi-point calibration method consisting of six concentrations and bracketed with QCs. A single-point calibration curve was regenerated from the same acquired data using only the calibrator at 0.5 mg/L concentration. 5-FU results obtained with each method were used to calculate the patient AUCs (AUC = 5-FU concentration (mg/L) multiplied by the time length (h) of the infusion) and assessed if it impacted the decision on 5-FU dose adjustment.

3. Results

3.1. Chromatography and method validation

5-FU and the IS eluted at 1.15 min under the chromatographic conditions described above (Fig. 1). The assay demonstrated linearity across the tested concentration range of 0.05–50 mg/L, as confirmed by a runs test for linearity determination. The mean coefficient of determination (r^2) of the calibration curves (n = 8) was 0.998 (SD = 0.000159, CV = 11.7 %). Inter- and intra-day imprecision were ≤9.1 % and ≤4.8 %, respectively while accuracy ranged from 102-111 % (Table 2). The LLOQ with an imprecision of < 6.0 % and accuracy of 110 % was determined to be 0.05 mg/L (Table 2). No carry over was observed for the analyte or the IS. Blank plasma matrix (n = 5) showed no interfering peaks at the R*t* of the analyte and the IS. The absolute observed ME ranged from 48-78 % across the concentration range. This was normalised by using an IS (ionisation suppression ranged from 3-7 %). Freeze-thaw stability (−30° C to RT) for three cycles was acceptable at three concentrations with mean degradation of up to 12 %, (CV<3.1 %, n = 3). Extracted plasma sample were stable in the autosampler for up to 42 h (maximum degradation across the concentration range was <4 %, CV <4.3 %). Long-term stability of 5-FU in plasma at −30 °C was demonstrated for up to 18 months (recoveries between 96.4 to 102.2 %, CV 0.6–3.2 %, n = 3 at 3 concentrations). 5-FU in plasma remained stable when kept on red cells on ice for up to 4 h. However, degradation was observed, with a 17 % decrease at low concentration, after 30 min when blood was stored at room temperature (RT) and after 1 h when

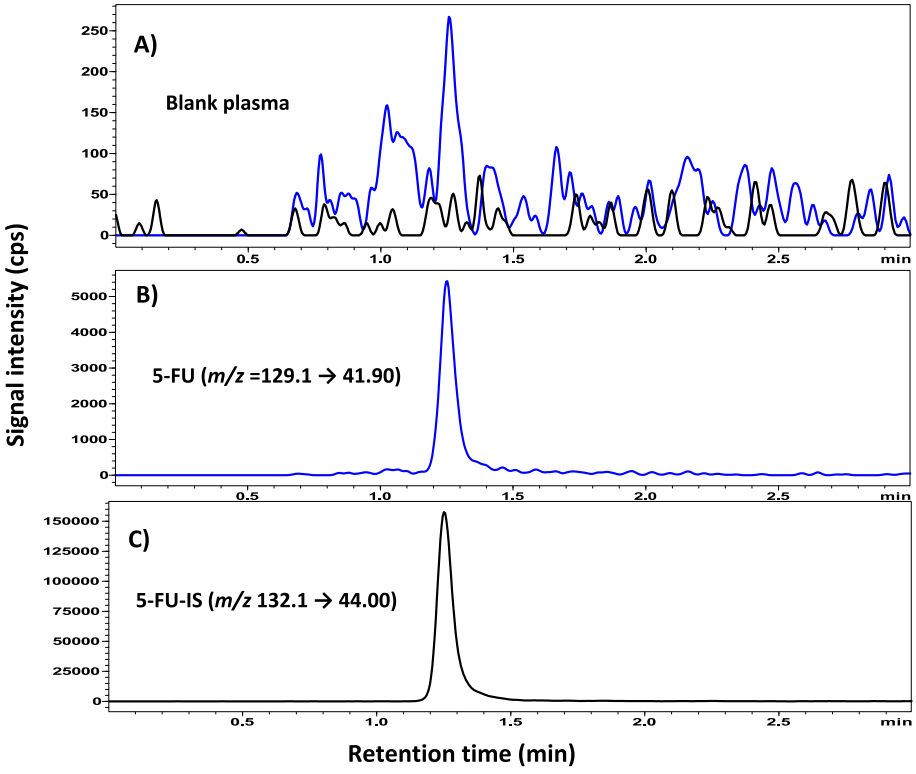

Fig. 1. Chromatograms of a blank plasma (A), 5-fluorouracil (5-FU) (B), and the stable isotope labelled internal standard (5-FU-IS) (C). The concentrations of 5-FU and 5-FU-IS were 0.05 mg/L (lower limit of quantification) and 1 mg/L, respectively. Cps, counts per minute; blue colour on the blank chromatogram (A) represents the background of the 5-FU (m/z = 129.1 → 41.90) while black represents the 5-FU-IS (m/z 132.1 → 44.00). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Intra and inter-day precision and accuracy validation data for a multi-point calibration method.

Analyte concentration (mg/L)	Intra-batch precision and accuracy data (n = 7)			Inter-batch data (n = 20)		
	Mean concentration (mg/L) ± SD	CV (%)	Accuracy (%) ± SD	Mean concentration (mg/L) ± SD	CV (%)	Accuracy (%) ± SD
0.05*	0.055 ± 0.006	6.0	110 ± 12			
0.2	0.21 ± 0.01	4.8	105 ± 5.1	0.21 ± 0.017	8.0	104 ± 8.3
2.0	2.22 ± 0.074	3.3	111 ± 3.3	2.03 ± 0.185	9.1	102 ± 9.2
40.0	41.8 ± 1.48	3.5	105 ± 3.5	41.8 ± 2.45	5.9	105 ± 6.1

SD; standard deviation; CV, coefficient of variation *n = 5.

stored at 4 °C (Fig. 2C). Similarly, 5-FU in whole blood is stable for up to 2 h if samples are kept on ice and at 4 °C but are only stable for 30 min if kept at RT (Fig. 2B). Further, 5-FU stability in plasma which was separated from the red cells was acceptable for up to 4 h when stored at RT (Fig. 2A).

3.2. Method comparison

The relationship between a single- and a multi-point calibration method used to measure 5-FU concentrations in plasma samples from cancer patients is represented by a Bland-Altman bias plot of differences and a Passing-Bablok analysis in Fig. 3A and B, respectively. The mean difference between the two methods, represented by a Bland-Altman bias plot, was −1.87 % (95 % limit of acceptance (LoA) −10.5 % to 6.8 %), Fig. 3A. The relationship between the two methods defined by the Passing-Bablok regression analysis had a slope of 1.002 and 95-% CI of 0.9718 to 1.043 (Fig. 3B).

Thirty-six patients underwent either one or more 5-FU TDM cycles resulting in 53 measured 5-FU concentrations. The 5-FU concentrations ranged from 0.117 to 0.782 and 0.124 to 0.807 mg/L when quantified utilising a single- and a multi-point calibration method, respectively. The maximum imprecision of the methods based on three QC concentrations defined by % CV were 9.9 % and 7.6 % for a single- and a multi-point method, respectively (Table 3). The calculated 5-FU AUCs based on the two calibration methods are shown in Supplementary Table 1. The maximum percent difference (%) in the AUCs was <11 % (one was 20 %).

4. Discussion

The developed LC-MS/MS method for measurement of 5-FU in plasma matrix was demonstrated to be accurate and precise based on standard definitions for this technology in clinical practice. As stated previously, the method was used for quantification of 5-FU concentrations in samples from patients undergoing cancer treatment, with dose adjustments made in real-time. 5-FU results obtained with each method were compared using a Bland-Altman bias plot of differences and a Passing-Bablok regression analysis. The data shown in Fig. 3A

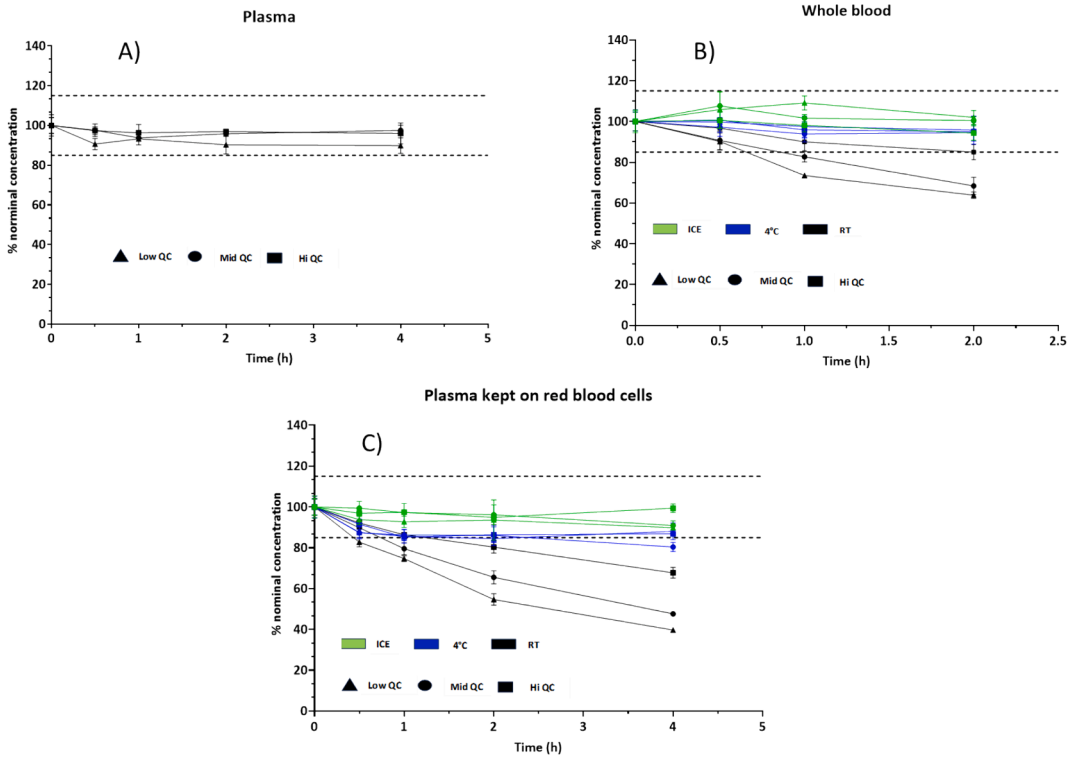

Fig. 2. 5-FU stability in plasma and whole blood. Stability of 5-FU in plasma (A), whole blood (B) and plasma kept on red cells (C) stored at room temperature (RT), 4 °C and on ice for up to 4 h. 5-FU was stable in plasma at RT for up to 4 h (2A), but degradation of 5-FU at low concentration was observed after 30 min if samples were stored at RT and plasma was kept on red cells (2C). Similarly, degradation of 5-FU in whole blood was observed after an hour when kept at RT (2B). Stability of 5-FU, either as whole blood or plasma kept on red cells, was acceptable at all concentrations when samples were stored on ice (2B and 2C). Values represent mean ± SD, n = 3. Low QC (0.2 mg/L), Mid QC (0.6 mg/L), High QC (2.0 mg/L). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

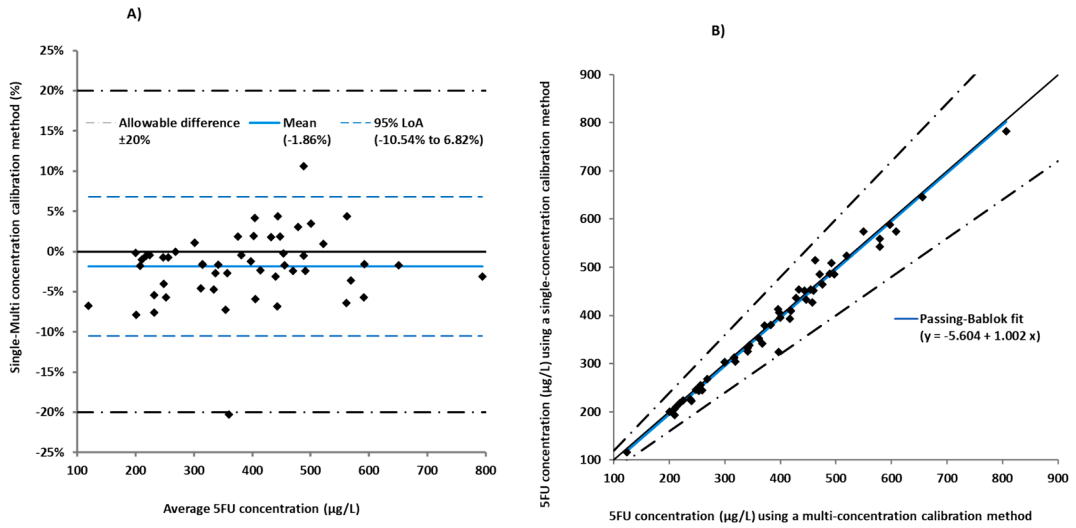

Fig. 3. A) Bland-Altman bias plot of differences (A) and a Passing-Bablok analysis (B) of 5-FU concentrations obtained using a multi- and a single point calibration method. 5-FU concentrations from cancer patients (n = 53) were quantified over 15 months period using a multi-point calibration method (range 50–5000 µg/L) and reanalysed using a single concentration at 500 µg/L for comparison. Bland-Altman bias plot of differences showed a good agreement between the methods with all the results being within ± 10 % difference (except one, 20 % difference) (A). Slope of a Passing-Bablok regression line was 1.002 with 95 % confidence interval (CI) range 0.9718 to 1.043. Blue solid line denotes a mean, the black solid line is the identity line while the blue and the black dotted lines denote 95 % limit of acceptance (LoA) and allowable range (±20 % difference), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Precision and accuracy data for a multi- and a single point calibration method (n = 50). Single point calibration was based on a concentration of 0.5 mg/L.

	Multi-point calibration method			Single point calibration method		
Analyte concentration (mg/L)	Mean concentration (mg/L) ± SD	CV (%)	Accuracy (%) ± SD	Mean concentration (mg/L) ± SD	CV (%)	Accuracy (%) ± SD
0.2	0.21 ± 0.016	7.6	105 ± 8.0	0.21 ± 0.017	8.3	102 ± 8.5
0.6	0.67 ± 0.035	5.2	112 ± 5.8	0.67 ± 0.062	9.3	111 ± 10
2.0	2.21 ± 0.137	6.2	111 ± 6.8	2.23 ± 0.221	9.9	111 ± 11

SD; standard deviation; CV, coefficient of variation.

demonstrates that a single-point calibration method performed equally to a multi-point method. The mean difference between the two methods was small (−1.87 %) and the 95 % limit of acceptance was < 10 % (−10.5 % to 6.8 %) with almost all the results falling within the 10 % range (1 result had a 20 % difference). Similarly, a good relationship was demonstrated between the two methods using a Passing-Bablok analysis (slope = 1.002 and 95 % CI 0.9718 to 1.043 (Fig. 3B)). Previously, a single-point calibration was shown to be superior in terms of accuracy compared to a 8-point calibration method for quantifying sirolimus [10]. The observation in this study agreed with what was previously shown with a multi-point calibration method being marginally better in terms of precision (CV < 8 % for all 3 QCs compared to CV<10 %, Table 3). QC failure was observed in one instance when results were quantified using a single-point calibration method. Given that the two methods agreed, the AUC was calculated based on the 5-FU concentration obtained with each method to determine if the outcome would result in a dose change. The maximum percent difference in the AUC based on the two methods was <11 % with one being 20 % (Supplementary Table 1). Based on the recommended target AUC for patients on 5-FU infusion (20–30 mg h/L), 3/35 patients had an AUC below the recommended target (AUC = 19 mg h/L) when the results were calculated using a multi-point calibration method. The corresponding AUCs for the three patients based on a single-point calibration method were 20 mg h/L, which would have resulted in no dose adjustments. The discrepancy in the calculated AUCs based on the calibration method was not due to the percentage difference in the 5-FU concentrations or AUC values (maximum difference <5 %), but rather the reporting of the AUC values (AUC = 19.5, 19.3 and 18.9 *vs.* 19.8, 20.1 and 19.7 mg h/L, for a multi- and a single-point calibration method, respectively). The 5-FU result with a 20 % difference had the calculated AUCs below the recommended target when quantified with either of the methods, hence, the dose recommendation would have remained the same. However, QCs quantified using a single-point method were outside the acceptance criteria (up to 19 % lower), so in this instance the batch would have been rejected.

The current LC-MS/MS method described herein, had a shorter run time (3 min) compared to the previously published methods [16,17] and used either the same sample volume or less (50 µL). When combined with a single-point calibration method, the time taken for sample preparation, analysis, and availability of the first patient result is just over an hour, important for therapeutic drugs where quick decision making is required (e.g., antimicrobials and anti-fungal therapies). With the average run time for an LC-MS/MS analytical method typically between 7 and 10 min, reducing the number of calibrators can decrease the turnaround time and increase instrument availability, even though this may not apply specifically to this method. Moreover, a significant reduction in the number of calibrators not only decreases the analyst time needed for preparation and analysis, but also lowers the associated storage costs. This efficiency is particularly evident when preparing calibrators for a simultaneous analysis involving multiple analytes. Furthermore, cost savings can be achieved through a reduction in the quantity of SIL-IS utilized. Short- and long-term stability of 5-FU in plasma and pure solution was in agreement with previously published work [18]. It is worth noting that the analyte remains stable at RT once

the plasma has been separated from the red cells. This separation eliminates the dihydropyrimidine dehydrogenase enzyme, primarily present in the leukocytes, which is responsible for 5-FU degradation. In contrast, degradation occurs quickly (within 30 min), particularly at low concentration, if samples are not centrifuged immediately or kept on ice (Fig. 2B and C).

Our study results indicate that a single-point calibration method for quantifying 5-FU in cancer patients is as effective as a multi-point method. This calibration approach should be encouraged for adoption in clinical laboratories as it reduces result turnaround time, minimizes costs by decreasing the preparation, testing, and storage of calibrators, all without compromising quality. Utilizing a single-point calibration model for measuring 5-FU did not influence clinical decision-making. However, it is essential to test and validate the model for each target analyte. Caution is necessary each time a new calibrator is prepared to guarantee accuracy, which can be achieved by simultaneously analyzing the current calibrator and the newly prepared one over multiple analytical runs. Moreover, a new calibrator can be compared to a set of multi-concentration calibrators that were previously prepared, tested, and stored for verification and validation. This comparison can be complemented by measuring QCs with known target values. Implementing QCs that cover the validated analytical range in every sample batch is crucial to minimize the risk of errors resulting from system drift and deviations from linearity.

5. Conclusion

While the use of a single-concentration calibration method for quantifying analytes is not a new concept, our study has highlighted its potential for broader utilization in clinical laboratories to quantify therapeutic drugs. This approach can lead to increased efficiency in the laboratory, translating to cost savings, reduced analysis time, and quicker availability of results. These benefits are particularly significant for measuring antimicrobials and anti-infective drugs, where rapid decision-making is crucial. Our study demonstrated that a single-point calibration method provided results that were analytically and clinically comparable to those obtained from a multi-point method when quantifying 5-FU, supporting its feasibility for clinical application.

Ethics statement

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Hunter New England and University of Newcastle of Australia (project codes: HREC/58470/PMCC-2019; UON: H/2022–0145). Informed consent was obtained.

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CRediT authorship contribution statement

Mirjana Radovanovic: Writing – original draft, Validation, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Jennifer J. Schneider:** Writing – review & editing, Resources, Funding acquisition. **Jennifer H. Martin:** Writing – review & editing, Resources, Funding acquisition. **Ross L.G. Norris:** Writing – review & editing. **Peter Galettis:** Writing – review & editing, Supervision, Project administration.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2024.07.003.

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