Published in final edited form as:

Ther Drug Monit. 2017 June ; 39(3): 235–242. doi:10.1097/FTD.0000000000000402.

A rapid homogeneous immunoassay to quantify gemcitabine in plasma for therapeutic drug monitoring (TDM)

Daniel Kozo, BS¹, Matt W. Ross, PhD¹, Justin Jarrah, BS¹, Michael Barrett, PhD¹, Rebecca L. Harney, BS¹, Jodi B. Courtney, BS¹, Irina Baburina, PhD¹, Julianne L. Holleran, BS², Jan H. Beumer, PhD, PharmD, DABT^{2,3}, Godefridus J. Peters, PhD⁴, Richard J. Honeywell, PhD⁴, and Salvatore J. Salamone, PhD¹

¹Saladax Biomedical, Inc., Bethlehem, PA ²Cancer Therapeutics Program, University of Pittsburgh Cancer Institute, Pittsburgh, PA ³Department of Pharmaceutical Sciences, University of Pittsburgh School of Pharmacy, Pittsburgh, PA 15213 ⁴Department of Medical Oncology, VU University Medical Center, Amsterdam, The Netherlands

Abstract

Background—Gemcitabine (2',2'-difluoro-2'-deoxycytidine) is a nucleoside analog used as a single agent and in combination regimens for the treatment of a variety of solid tumors. Several studies have shown a relationship between gemcitabine peak plasma concentration (C_{max}) and hematological toxicity. An immunoassay for gemcitabine in plasma was developed and validated to facilitate Therapeutic Drug Monitoring (TDM) by providing an economical, robust method for automated chemistry analyzers.

Methods—A monoclonal antibody was coated on nanoparticles to develop a homogenous, agglutination inhibition assay. To prevent *ex vivo* degradation of gemcitabine in blood, tetrahydrouridine (THU) was used as a sample stabilizer. Validation was conducted for precision, recovery, cross-reactivity, and linearity on a Beckman Coulter AU480. Verification was done on an AU5800 in a hospital laboratory. A method comparison was performed with LC-MS/MS utilizing clinical samples. Selectivity was demonstrated by testing cross-reactivity of the major metabolite, 2',2'-difluorodeoxyuridine (dFdU).

Results—Coefficients of variation for repeatability and within-laboratory precision were <8%. Measured values deviated <3% from assigned. Linear range was from 0.40 to 33.02 $\mu\text{g/mL}$ (1.5–125.5 μM). Correlation with validated LC-MS/MS methods was $R^2 = 0.977$. The assay was specific for gemcitabine: there was no cross-reactivity to dFdU, chemotherapeutics, concomitant or common medications tested. THU was packaged in single-use syringes. Gemcitabine stability in whole blood was extended to 8 hours (RT) and in plasma to eight days (2–8 °C).

Conclusions—The assay demonstrated the selectivity, test range, precision, and linearity to perform reliable measurements of gemcitabine in plasma. The addition of stabilizer improved the

sample handling. Using general clinical chemistry analyzers, gemcitabine could be measured for TDM.

Keywords

immunoassay; gemcitabine; oncology; pharmacokinetic-pharmacodynamic; therapeutic drug monitoring

Introduction

Like other cytotoxic agents used in chemotherapy, gemcitabine is dosed by body surface area: a method of dosing that is known not to reduce inter-individual variability in clearance and exposure [1, 2]. High interpatient variability is a crucial challenge in chemotherapy, one with grave consequences. Underdosed patients are not deriving the most benefit from the treatment, and there may be tumor progression; overdosed patients may experience toxicity causing delays and changes in regimens. The work to develop and validate an automated Therapeutic Drug Management (TDM) immunoassay was undertaken to provide a clinically accessible tool for dose monitoring of this drug.

Gemcitabine (2',2'-difluoro-deoxycytidine) is a nucleoside analog [3]. It is used as a single agent and in combination regimens to treat non-small cell lung cancer (NSCLC), as well as pancreatic, breast, bladder, and ovarian cancers [4–7]. The drug is typically administered as a 30-minute infusion.

Gemcitabine is anabolized in cells by deoxycytidine kinase to mono-, diphosphate (dFdCDP), and triphosphate (dFdCTP) nucleotides. The major metabolite 2',2'-difluorodeoxyuridine (dFdU) is produced by the action of the enzyme cytidine deaminase (CDA) (Figure 1) [8]. At prolonged exposure to clinically achievable concentrations, dFdU has cytotoxic effects by itself, but its contribution to gemcitabine's activity is not clear yet [9].

Grade 3–4 hematological toxicities of gemcitabine occur frequently [10–12]. Several studies have shown a relationship between gemcitabine peak plasma concentration (C_{max}) and hematological toxicity [13, 14]. Kroep et al. found a correlation between the decrease in platelets and the gemcitabine C_{max} , with a >50% decrease at C_{max} concentration >20 μ M (5.26 μ g/mL) [13]. A 2001 study of non-small cell lung cancer patients by Fogli et al. found a correlation between gemcitabine C_{max} and reduction in platelet counts [14].

The concentrations to be targeted may depend on the schedule and combinations but are likely to be in the range of 20 μ M (around 5 μ g/mL), and therefore this range may be the concentration of clinical interest to target in TDM [15–18]. Patients with plasma concentrations above ~14 μ g/mL showed significant hematological toxicities [14, 19].

Materials and Methods

Sample Stabilizer

Gemcitabine is rapidly metabolized in blood by CDA. Tetrahydrouridine (THU) is a known inhibitor of CDA (Figure 1). THU has been used by a number of investigators in clinical studies to stabilize gemcitabine in whole blood and plasma [13, 18, 20–22]. To facilitate handling in a clinical setting, a stabilizer kit was developed consisting of a single-use syringe containing 100 μ L of 500 mg/mL THU. The stabilizer was injected into the blood collection tube using a BD Vacutainer Blood Transfer Device (Becton Dickinson, Franklin Lakes, NJ). With the entire contents of the syringe injected into a 4-mL blood collection tube, the final concentration of THU was 125 μ g/mL. All plasma used in the validation of the assay contained the sample stabilizer as would a patient sample.

To verify the effectiveness of the sample stabilizer to stop degradation of gemcitabine by CDA, stability studies were carried out to mimic different handling scenarios for whole blood and its processing into plasma. Human blood was drawn into 4-mL blood tubes, spiked with gemcitabine, and then the sample stabilizer was added within 6 minutes. Portions of the blood were processed at time zero to demonstrate that adding stabilizer within 6 minutes was acceptable, and periodically thereafter to determine the stability of gemcitabine in whole blood at room temperature and in plasma processed from that whole blood and stored at 2–8 °C. The following anticoagulants were tested: K₂EDTA, K₃EDTA, sodium citrate, sodium heparin, lithium heparin, lithium heparin with gel (Plasma Separator Tube, or “PST”), and sodium fluoride.

Assay Method

The principle of the current assay has been described previously and represents a standard approach for a homogeneous nanoparticle immunoassay [23, 24]. The assay is comprised of two liquid reagents. Reagent one contains a large polymer with repeating units of a gemcitabine derivative. Reagent two is comprised of 200 nm polystyrene nanoparticles to which antibodies are covalently attached. When the two reagents are mixed, the antibody particles react with the gemcitabine polymer to form an agglutination complex that can be monitored spectrophotometrically. The larger the complex, the more light that is scattered, resulting in an increase in the net absorbance by the solution. The amount of light scattering is proportional to r^6 where r is the radius of the complex. When free gemcitabine is present in the sample, it competes with the drug conjugate for the limited antibody. The higher the concentration of gemcitabine, the smaller the agglutination complexes that are formed and the less light that is scattered. This results in a decreasing calibration curve: maximum absorbance in the absence of analyte in the sample and decreasing absorbance proportional to the amount of analyte present. The assay was designed to measure the concentration of gemcitabine observed in patient plasma at $C_{\max}$ of a 30-minute infusion. The reagent formulations were optimized to maintain performance over a shelf-life of 24 months.

The major metabolite of gemcitabine is dFdU and is present at concentrations greater than the parent drug at the end of infusion. The gemcitabine monoclonal antibody used in the assay is selective for the parent drug and has low cross-reactivity to the related compounds

dFdU and THU. The immunogen for the monoclonal antibody program was prepared by derivatizing gemcitabine from the 5'-hydroxyl moiety and covalently attaching the compound to the immunogenic protein carrier keyhole limpet hemocyanin. The derivative preparation, immunogen, and antibody generation has been described previously [25].

The gemcitabine reagent kit is manufactured by Saladax Biomedical (Bethlehem, PA, USA). The reagent kit consists of two Beckmann Coulter AU (Brea, CA, USA) compatible bottles: one bottle contains the reaction buffer with a polymeric gemcitabine conjugate (R1), and the other bottle contains the monoclonal antibody-coated nanoparticle (R2). The kit contains enough reagents to perform 100 tests on the analyzer. The AU480 is an open clinical analyzer, and the assay parameters are applicable to other Beckman Coulter instruments in the AU family. The analyzer pipettes 95 μ L of R1 and 2 μ L of sample into a reaction cuvette. After an incubation time of 3.4 minutes, 95 μ L of R2 is added. Mixing occurs after each step. The agglutination reaction is monitored at 600 nm. The calculated reaction end point is inversely proportional to the concentration of gemcitabine present in the sample.

Validation studies were run on an AU480 clinical analyzer. The performance of the assay was verified in a second laboratory on an alternative (AU5800) analyzer.

Calibrators, Controls, Calibration Curve, and Calibration Interval

The gemcitabine concentrations of the calibrators were 0.00, 1.00, 3.00, 8.00, 15.00, and 30.00 μ g/mL (0.00, 3.80, 11.4, 30.4, 56.99, 114.0 μ M). The low, medium, and high assay control concentrations, 2.00, 10.00, and 25.00 μ g/mL, respectively (7.60, 37.99, 94.99 μ M), were selected to ensure accurate quantitation at medical decision points. Calibrators and controls were formulated in a buffer matrix for ease of use and stability. The calibration interval (standard curve stability) was the length of time that a kit generated QC results within specifications without a recalibration. The kits were stored at 2–8 $^{\circ}$ C during the study. The calibration interval was established using three lots of reagents. Calibrations were performed on day 0; thereafter, assay controls of each concentration were run in singlicate at defined intervals (every 3–4 days). The calibration interval was maintained when control results met the recovery specifications of $\pm 15\%$ for the low or $\pm 10\%$ for the medium and high controls.

Precision

Precision was determined according to CLSI Guideline EP05-A3 [26]. To evaluate repeatability and within-laboratory precision, drug-free plasma pools spiked at 1.50, 4.00, 12.50, and 27.50- μ g/mL (5.70, 15.20 47.49, 104.5 μ M) gemcitabine, and the three assay controls were tested in duplicate in two runs per day (separated by at least 2 hours) to generate 20 days of data without outliers. To identify outliers during daily testing, the difference between each pair of replicates within a run was compared to an outlier threshold determined early in the study by testing the precision study samples ($n = 20$) in a single run. The threshold for each precision study sample was equal to 5.5 times the SD of the results from the $n = 20$ run, and differences between replicates that exceeded this threshold caused the run to be repeated on a different day. The repeatability and within-laboratory standard deviation (SD) and coefficient of variation (CV) were calculated with EP Evaluator Release

9, EP5 Precision module (version 9.0.0.366; Data Innovations). Precision performance was verified on an AU5800 in an external hospital laboratory using an abbreviated protocol similar to CLSI guideline EP15-A3[27] For the performance verification, controls were measured in duplicate twice a day, with runs separated by at least 2 hours, for 5 days.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were tested according to CLSI Guideline EP17-A2 [28]. Individual normal human (drug-free) K₂EDTA plasmas spiked with gemcitabine were used to determine the LOD (five plasma samples) and LOQ (four plasma samples). The samples were spiked around the estimated limits. Samples were analyzed n = 3 using three reagent lots on two analyzers. LOD was tested over two days and LOQ over three. Limit of Blank (LOB) was determined in the same experiment by testing the five drug-free human plasmas n = 3 over two days. The LOB was the 95th percentile result for drug-free plasma. The LOD was the gemcitabine concentration at which 95% of the results were greater than the assay LOB. The LOQ was the gemcitabine concentration at which the total analytical error (TE) calculated by the Westgard model ($TE = \text{bias} + 2 \times \text{standard deviation}$) was 35% [29]. For the external LOQ verification, the hospital laboratory ran the four samples containing gemcitabine at the assay LOQ over two days on an AU5800. Fifty-four of the 60 results were required to be between 0.55 and 1.15 µg/mL: $LOQ \pm 35\%TE$.

Linearity

Assay linearity testing was conducted according to CLSI Guideline EP6-A [30]. Drug-free pooled normal human K₂EDTA plasma was selected as the sample matrix because patient samples from individuals being treated with gemcitabine were not available in sufficient quantity. Gemcitabine was spiked into the plasma at 0.40 (1.52 µM) and 33.02 µg/mL (125.46 µM). These were combined in 11 different proportions to generate a 13-level linearity testing set. Three of the 13 levels were below the assay limit of quantitation: two of those samples, 0.04 and 0.06 µg/mL (0.15 and 0.23 µM), were eliminated from the analysis because of the large total error associated with measuring concentrations far below the LoQ; the third sample (0.80 µg/mL) was included in the analysis to demonstrate that the linear range of the assay included the LOQ. The linear range of the assay was determined from the lowest and highest linearity samples between which all linearity samples (tested n = 5) met the mean deviation ($0.8 \leq X < 1 \mu\text{g/mL}$: $\pm 20\%$; $1 \leq X < 1.5 \mu\text{g/mL}$: 16% ; $1.5 \leq X < 5.39$: $\pm 11\%$; $5.39 \leq X \leq 33.02 \mu\text{g/mL}$: $\pm 8\%$) and deviation from linearity ($\leq 15\%$) acceptance criteria across three lots of reagents on two analyzers. In addition, the range of samples meeting those criteria had to contain at least nine levels. For the external assay linearity verification, the hospital laboratory tested the same samples described above on an AU5800.

Recovery of Gemcitabine

To validate the ability of the assay to determine gemcitabine concentrations without patient-to-patient bias, gemcitabine was spiked into five individual drug-free normal plasmas at four concentrations (1.50, 4.00, 12.50, and 27.50 µg/mL) covering the range of the assay. All samples were analyzed n = 5 on two analyzers with three lots of reagents. Replicate recoveries were calculated from spiked gemcitabine results and compared to assigned values. For the external recovery verification, the hospital laboratory tested two plasma pools

(n = 20 on two days) and five patient samples (n = 2) containing known levels of gemcitabine on an AU5800.

Cross-Reactivity

The major gemcitabine metabolite dFdU, the sample stabilizer THU, common prescription and over-the-counter medicines, vitamins, herbal supplements, and structurally related compounds (including cytidine derivative antivirals) were tested for cross-reactivity (see Supplemental Digital Content Table S1 for a complete listing). Testing was conducted according to the CLSI guideline EP07-A2 [31]. Cross-reactivity was assessed in a drug-free plasma pool, and the same pool spiked with 4 µg/mL of gemcitabine. The samples were evaluated with one lot of reagent on two analyzers. Compounds were dissolved in a suitable solvent and spiked into plasma pools at a minimum concentration of 100 µg/mL. The cross-reactivity was calculated from the difference of the mean gemcitabine values with and without cross-reactant divided by the cross-reactant concentration.

Interference

Interference from lipemia, icterus, total protein, hemolysis, and rheumatoid factor was evaluated. Bilirubin, human serum albumin (HSA), and immunoglobulin G (IgG) were spiked into a drug-free plasma pool, and this plasma pool was spiked with gemcitabine to a concentration of 4 µg/mL. To prepare lipemic samples, the lipid layer isolated from fresh human plasma was spiked into the plasma pools for interference testing. The concentrations of bilirubin, HSA, IgG, and lipemia were verified using reagent kits from JAS Diagnostics. Rheumatoid factor (Keystone Biologicals) was spiked into plasmas from a known stock concentration. Hemolysate was prepared from blood obtained from healthy donors, and known hemoglobin concentrations were spiked into plasma samples to simulate hemolyzed samples.

Comparative Methods

Two comparative methods were used for the method comparison. The second one was employed when additional samples were obtained.

In the first method, gemcitabine was quantitated using an Agilent 1100 autosampler and binary pump (Agilent Technologies, Palo Alto, CA, USA) coupled to a Micromass Quatromicro triple-stage bench-top mass spectrometer (Waters, Milford, MA, USA). Analytes were separated on a Shodex Asahipak NH2P-50 2D column (5 µm, 2 × 150 mm). The gradient mobile phase system was comprised of solvent A (acetonitrile with 0.1% formic acid) and solvent B (water with 0.1% formic acid). The initial mobile phase was 98% A and 2% B at a flow rate of 0.2 mL/min. Subsequently, solvent B was increased to 20% over 10 minutes, where it was held for 2 minutes. Solvent B was increased to 60% in 6 seconds for 1 minute 50 seconds with a flow rate of 0.7 mL/min and then decreased to 0% at 14.1 minutes and held until 18.2 minutes. Finally, B increased to 2% from 18.3 to 19.8 minutes when the flow rate was returned to 0.2 mL/min and was followed by injection of the next sample. Mass spectrometer settings were as follows: capillary voltage 4kV; cone voltage 20; and desolvation temperature 350 °C. The MRM *m/z* ratios monitored were *m/z* > 264.1 > 112.1 and 267.1 > 115.1 for dFdC (Figure 2) and ¹⁵N₂, ¹³C-dFdC (internal

standard), respectively. Aliquots of 100 μ L of plasma were mixed with 10 μ L of internal standard (1 μ g/mL in water) and were then extracted with 10 μ L of glacial acetic acid and 1 mL of ethyl acetate. After vortexing and centrifugation, the supernatant was transferred to a 12 $\times$ 75 mm borosilicate glass tube, and the solvents were evaporated under nitrogen at 37 $^{\circ}$ C. Samples were reconstituted with 100 μ L of acetonitrile:water (98:2 v/v), transferred to an autosampler vial, and 5 μ L of the sample was injected into the LC-MS/MS system. The ion chromatograms were integrated and quantified using Micromass Masslynx Version 4.0 (Waters). The linearity of this assay was 10–5,000 ng/mL. This assay had an acceptable accuracy ($\pm$ 15% [31]) based on duplicate QCs (30, 500, 2,000 ng/mL gemcitabine and dFdU) and duplicate calibrators (10, 20, 50, 100, 200, 500, 1,000, 2,000, 5,000 ng/mL of gemcitabine and dFdU) on three separate days resulting in good accuracy (gemcitabine 95.4–107.1%; dFdU 95.9–107.6%) and precision ($\pm$ 15% [32] (gemcitabine $<$ 5.4% CV; dFdU $<$ 8.0% CV).

The second LC-MS/MS method was developed and validated by inVentiv Health Clinical (Princeton, NJ, US) and was proprietary.

Clinical Samples and Method Comparison

Heparinized blood samples collected in BD Vacutainer were de-identified according to an exempt IRB study approved by the University of Pittsburgh Institutional Review Board. These samples were stored at -80° C and analyzed by LC-MS/MS. In addition, archived samples were provided by the VU University Medical Center. These samples were tested by inVentiv Health Clinical. Each sample was tested $n = 1$ using each method. Results for both comparator methods were adjusted to account for differences in standardization. Samples with results below the gemcitabine assay LOQ were immediately excluded, and the remaining data were examined for outliers. Samples with disproportionately high influence were identified by calculating standardized residuals and leverages using Minitab17 (Minitab, State College, PA, 2015). One sample was excluded because its standardized residual of 7.9 was outside of the limits of -3 to $+3$, indicating that it was an outlier [26]. A second sample was excluded because its leverage of 0.435 exceeded the limit of 0.092 (or, $3 \times$ the number of terms in the model $\div$ the number of observations), indicating that it was exerting a disproportionately large influence on the regression [33]. A total of 63 patient samples were used in the final Deming regression analysis [34].

Results

Sample Stability

There was no significant ($\pm$ 8%) loss of gemcitabine in whole blood when Sample Stabilizer was added to the whole blood within 6 minutes; recoveries compared to the theoretical value ranged from 92% to 103% for the following anticoagulants: K₂EDTA, K₃EDTA, sodium heparin, lithium heparin, and lithium heparin in a PST. More degradation ($>$ 10% loss) of gemcitabine was observed in sodium citrate and fluoride tubes, and these were considered unacceptable. After the addition of Sample Stabilizer, the gemcitabine was stable in whole blood for at least 8 hours when stored at room temperature and 24 hours when stored at 2–

8 °C. Gemcitabine was stable for at least seven days in plasma prepared from whole blood with stabilizer stored at room temperature for 8 hours or at 2–8 °C for 24 hours.

Calibration Curve and Calibration Interval

The assay covers the range of expected results using six calibrators, 0.00, 1.00, 3.00, 8.00, 15.00, and 30.00 µg/mL (0, 3.80, 11.4, 30.4, 56.99, 114.0 µM), with a four parameter logistic regression curve fit. The calibration curve is shown in Figure 3. The analyzer was programmed to automatically dilute samples higher than 30 µg/mL by 1:2 (one part specimen + two parts diH₂O). This gives the assay an effective range up to 90 µg/mL (342.0 µM). When the reagents were calibrated on day 0, control values remained within specification for at least six weeks, during which time no recalibration of the instrument was required. Recalibration after controls failed brought control recovery within specifications.

Precision

Repeatability and within-laboratory precision were determined for samples prepared from a drug-free plasma pool spiked with gemcitabine at four concentrations, 1.50, 4.00, 12.50, and 27.50 µg/mL (5.70, 15.20, 47.49, 104.5 µM), and the assay controls at three: 2.00, 10.00, 25.00 µg/mL (7.60, 37.99, 94.99 µM). The CVs for repeatability were between 1.3% and 3.1% (Table 1). The within-laboratory CV ranged from 3.3% to 7.5%. The highest CV occurred at the lowest plasma concentration (Table 1). In the external laboratory, CVs were between 2.2% and 6.0% for repeatability and between 2.2% and 6.7% for within-laboratory precision.

Linearity

The immunoassay was linear from 0.80 to 33.02 µg/mL. The greatest mean deviation from linearity was less than 3% and mean recoveries were between 95% and 118%. For linearity samples in the test range (LoQ–30 µg/mL), the recovery was between 95% and 107%. In the external laboratory, mean recoveries were between 92% and 101%.

Recovery

The recovery of gemcitabine in five individual plasmas ranged from 82% to 118% at 1.50 µg/mL (5.70 µM), 89% to 111% at 4.00 µg/mL (15.2 µM), and 90% to 110% at 12.50 (47.49 µM) and 27.50 µg/mL (104.5 µM). In the external laboratory, the recovery results for five plasmas containing gemcitabine at concentrations ranging from 1.26 to 7.54 µg/mL (4.79 to 28.6 µM), measured twice, were 103%–122%.

Limit of Detection and Limit of Quantification

The assay LOQ was 0.85 µg/mL (3.2 µM), the lowest gemcitabine concentration for which the total analytical error was <35%. The LOD was 0.44 µg/mL (1.7 µM): 95% of the results at this concentration exceeded the assay LOB. The LOQ was confirmed in the external laboratory when 58 of 60 results from the analysis of four plasmas spiked with gemcitabine at 0.85 µg/mL were within ±35% of expected.

Cross-Reactivity

Over 110 compounds were tested for cross-reactivity to determine if they would influence assay results (see Supplemental Digital Content Table S1 for a complete listing). No assay interference from the tested compounds was found. The cross-reactivities of the major metabolite (dFdU) and the stabilizer (THU) were <0.1%. The cross-reactivity of cytosine and cytidine was 0.1%, and the cross-reactivity of deoxycytidine was 0.8%. All compounds tested had <1% influence on the assay.

Interference

The following endogenous interferents were tested in the assay: lipemia, icterus, total protein, hemolysis, and rheumatoid factor. The effect on the assay ranged from –8% to 3% (Table 2). No significant interference was observed from the following interferents: rheumatoid factor at 550 IU/mL, human serum albumin at 6 g/dL, human IgG at 6 g/dL, icteric samples at 34 mg/dL, lipemic samples at 521 mg/dL, and hemolysate samples at 1,100 mg/dL.

Method Comparison

There were 63 patient samples included in the regression analysis. The range of gemcitabine concentrations measured by the immunoassay was 0.87 to 21.79 µg/mL (3.3 to 82.79 µM) with a mean of 7.487 ± 5.460 (SD) µg/mL (28.45 ± 20.74 µM), and the range of concentrations measured by the LC-MS/MS methods was 0.86 to 20.45 µg/mL (3.3 to 77.70 µM) with a mean of 7.661 ± 5.429 (SD) µg/mL (29.11 ± 20.63 µM).

The Deming regression of the data set had a slope of 1.006 (95% confidence interval: 0.962–1.050) with a y-intercept of –0.218 (95% confidence interval: –0.628 to 0.193), a standard error of the estimate of 0.937 and a correlation coefficient of 0.9855 (Figure 4). Bias to the average of the methods ranged from –16% to +6% with an average bias of –0.09 µg/mL (0.3 µM). Sixty-one samples measured by the immunoassay deviated <1 µg/mL (<4 µM) from the average of the methods.

Discussion

Physical methods to quantify analytes of interest are often considered, and with good reason, the gold standard to which immunoassays are compared. To eliminate interference from matrix or related substances, they use pre-treatment of samples and chromatography for separation and various detection methods, such as ultraviolet- or mass-spectrometry. Numerous methods have been used for the detection of antimetabolite chemotherapeutics including gemcitabine [20, 35–39]. Most pharmacokinetic studies not only in oncology and in Phase I studies, but also in TDM are done by chromatographic methods, also because they have the advantage of determining several analytes simultaneously [40]. However, in contrast to a commercial liquid reagent that can be run on a clinical chemistry analyzer, physical methods require method development, additional validation, special equipment, and technical expertise. To make testing of chemotherapeutics easily accessible to any laboratory with a clinical chemistry analyzer with user-defined channels and to support a planned clinical trial, we sought to develop an automated immunoassay for gemcitabine. To ensure

the correct selectivity of the assay, and absence of matrix effects, a method comparison was done to LC-MS/MS. For a lot-to-lot consistency, a monoclonal antibody was selected for the assay. While monoclonal and polyclonal antibodies to uracil, 5-fluorouracil and 5-fluoro-2'-deoxyuridine-5'-monophosphate have been published, this is the first homogeneous immunoassay reported for gemcitabine, which we know of, using a monoclonal antibody [41–43].

Gemcitabine has a very short half-life of about 8 minutes with wide interpatient variability [18, 39]. This makes the use of sample stabilizer necessary. Even with a stabilizer, many clinical studies in the literature report placing samples on ice and/or immediate centrifugation and freezing before measuring [13, 14, 17, 19, 39, 44]. This is not so practical in routine clinical settings. The demonstration of stability in whole blood with a premeasured stabilizer simplifies blood processing. Adding stabilizer to the blood within minutes after the draw extended the window within which plasma must be isolated, eliminating the need for any ice. This is a critical advantage to sample handling for routine TDM testing. We demonstrated that gemcitabine was stable for 8 hours at ambient room temperature and 48 hours at 2–8 °C before being processed into plasma.

The assay was optimized to measure $C_{\max}$ of a 30-minute infusion. As a routine TDM tool, this is the most practical pharmacokinetic parameter: only one blood sample is required at the end of infusion, and there is no requirement for the patients to stay for a second sample to be drawn. While some studies have suggested the clinical utility of monitoring $C_{\max}$, a clinical trial is planned to validate the association of $C_{\max}$ with toxicity and outcomes [13, 14].

The limit of quantitation for the assay was 0.85 µg/mL. This is, as would be expected for an immunoassay, higher than those published for physical methods that incorporate a separation step to remove matrix interference and use sensitive detection techniques such as tandem mass-spectrometry [16, 35, 37, 45]. Measurement of the $C_{\max}$ would not be affected, as values >10 µM (2.63 µg/mL) have been reported for the clinically common doses around 1,000 mg/m² [13, 18, 19, 39].

A turbidimetric immunoassay was developed with a monoclonal antibody selective for gemcitabine that is not affected by the metabolite dFdU and the blood stabilizer THU. The assay was formulated with two reagents so that it can be performed on most clinical chemistry analyzers with utility or open channels.

Performance was demonstrated with gemcitabine-spiked plasma containing stabilizer at concentrations below, within, and above the suggested range of 10–20 µM [46–48]. Having demonstrated acceptable analytical performance according to CLSI protocols and the appropriate selectivity in comparison to LC-MS/MS, the assay is suitable as a tool for TDM. Given the association of gemcitabine $C_{\max}$ with toxicity, and the wide inter-patient variability that has been demonstrated, the management of gemcitabine therapy may be improved with TDM [13, 14].

Conclusion

The assay evaluated in this report is the first automated immunoassay for the management of gemcitabine therapy. A reliable analytical performance validated by CLSI methods was demonstrated. This assay can be used in clinical studies to confirm the utility of therapeutic blood monitoring in patients being treated with gemcitabine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Source of Funding: All work was supported by Saladax Biomedical, Inc. This project used the UPCI Cancer Pharmacokinetics and Pharmacodynamics Facility (CPPF) and was supported in part by award P30-CA47904.

This project used the UPCI Clinical Pharmacology Analytical Facility (CPAF) and was supported in part by award P30CA047904 (Jan H. Beumer).

References

1. Baker SD, Verweij J, Rowinsky EK, et al. A Role of body surface area in dosing of investigational anticancer agents in adults, 1991–2001. *J Natl Cancer Inst.* 2002; 94:1883–1888. [PubMed: 12488482]
2. Felici A, Verweij J, Sparreboom A. Dosing strategies for anticancer drugs: the good, the bad and body-surface area. *Eur J Cancer.* 2002; 38:1677–1684. [PubMed: 12175683]
3. Peters GJ. Novel developments in the use of antimetabolites. *Nucleosides Nucleotides Nucleic Acids.* 2014; 33:358–374. [PubMed: 24940694]
4. Sandler AB, Nemunaitis J, Denham C, et al. Phase III trial of gemcitabine plus cisplatin versus cisplatin alone in patients with locally advanced or metastatic non-small-cell lung cancer. *J Clin Oncol.* 2000; 18:122–130. [PubMed: 10623702]
5. Burris HA 3rd, Moore MJ, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol.* 1997; 15:2403–2413. [PubMed: 9196156]
6. Spielmann M, Llombart-Cussac A, Kalla S, et al. Single-agent gemcitabine is active in previously treated metastatic breast cancer. *Oncology.* 2001; 60:303–307. [PubMed: 11408796]
7. Pfisterer J, Plante M, Vergote I, et al. Gemcitabine plus carboplatin compared with carboplatin in patients with platinum-sensitive recurrent ovarian cancer: an intergroup trial of the AGO-OVAR, the NCIC CTG, and the EORTC GCG. *J Clin Oncol.* 2006; 24:4699–4707. [PubMed: 16966687]
8. Ciccolini J, Serdjebi C, Peters GJ, et al. Pharmacokinetics and pharmacogenetics of Gemcitabine as a mainstay in adult and pediatric oncology: an EORTC-PAMM perspective. *Cancer Chemother Pharmacol.* 2016; 23:23.
9. Honeywell RJ, Ruiz van Haperen VW, Veerman G, et al. Inhibition of thymidylate synthase by 2', 2'-difluoro-2'-deoxycytidine (Gemcitabine) and its metabolite 2',2'-difluoro-2'-deoxyuridine. *Int J Biochem Cell Biol.* 2015; 60:73–81. [PubMed: 25562513]
10. Conroy T, Desseigne F, Ychou M, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med.* 2011; 364:1817–1825. [PubMed: 21561347]
11. Ueno H, Ioka T, Ikeda M, et al. Randomized phase III study of gemcitabine plus S-1, S-1 alone, or gemcitabine alone in patients with locally advanced and metastatic pancreatic cancer in Japan and Taiwan: GEST study. *J Clin Oncol.* 2013; 31:1640–1648. [PubMed: 23547081]
12. Van Cutsem E, van de Velde H, Karasek P, et al. Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. *J Clin Oncol.* 2004; 22:1430–1438. [PubMed: 15084616]

13. Kroep JR, Giaccone G, Voorn DA, et al. Gemcitabine and paclitaxel: pharmacokinetic and pharmacodynamic interactions in patients with non-small-cell lung cancer. *J Clin Oncol.* 1999; 17:2190–2197. [PubMed: 10561275]
14. Fogli S, Danesi R, De Braud F, et al. Drug distribution and pharmacokinetic/pharmacodynamic relationship of paclitaxel and gemcitabine in patients with non-small-cell lung cancer. *Ann Oncol.* 2001; 12:1553–1559. [PubMed: 11822754]
15. Tempero M, Plunkett W, Ruiz Van Haperen V, et al. Randomized phase II comparison of dose-intense gemcitabine: thirty-minute infusion and fixed dose rate infusion in patients with pancreatic adenocarcinoma. *J Clin Oncol.* 2003; 21:3402–3408. [PubMed: 12885837]
16. Veltkamp SA, Beijnen JH, Schellens JH. Prolonged versus standard gemcitabine infusion: translation of molecular pharmacology to new treatment strategy. *Oncologist.* 2008; 13:261–276. [PubMed: 18378536]
17. Grunewald R, Kantarjian H, Du M, et al. Gemcitabine in leukemia: a phase I clinical, plasma, and cellular pharmacology study. *J Clin Oncol.* 1992; 10:406–413. [PubMed: 1740680]
18. Abbruzzese JL, Grunewald R, Weeks EA, et al. A phase I clinical, plasma, and cellular pharmacology study of gemcitabine. *J Clin Oncol.* 1991; 9:491–498. [PubMed: 1999720]
19. Faivre S. Phase I–II and pharmacokinetic study of gemcitabine combined with oxaliplatin in patients with advanced non-small-cell lung cancer and ovarian carcinoma. *Ann Oncol.* 2002; 13:1479–1489. [PubMed: 12196375]
20. Honeywell R, Laan AC, van Groenigen CJ, et al. The determination of gemcitabine and 2'-deoxycytidine in human plasma and tissue by APCI tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2007; 847:142–152.
21. Marangon E, Sala F, Caffo O, et al. Simultaneous determination of gemcitabine and its main metabolite, dFdU, in plasma of patients with advanced non-small-cell lung cancer by high-performance liquid chromatography-tandem mass spectrometry. *J Mass Spectrom.* 2008; 43:216–223. [PubMed: 17941128]
22. Xu Y, Keith B, Grem JL. Measurement of the anticancer agent gemcitabine and its deaminated metabolite at low concentrations in human plasma by liquid chromatography-mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2004; 802:263–270.
23. Wu RS, McNally AJ, Pilcher IA, et al. Synthesis of new d-propoxyphene derivatives and the development of a microparticle-based immunoassay for the detection of propoxyphene and norpropoxyphene. *Bioconjug Chem.* 1997; 8:385–390. [PubMed: 9177845]
24. Li Z, Goc-Szkutnicka K, McNally AJ, et al. New synthesis and characterization of (+)-lysergic acid diethylamide (LSD) derivatives and the development of a microparticle-based immunoassay for the detection of LSD and its metabolites. *Bioconjug Chem.* 1997; 8:896–905. [PubMed: 9404664]
25. Salamone, SJ., Courtney, JB., Sard, H., et al. Saladax Biomedical, Inc. Gemcitabine Immunoassay. US Patent. 8,426,143 B2. Apr 23. 2013
26. CLSI. Evaluation of Precision Performance of Quantitative Measurement Methods; Approved Guideline-Second Edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2014. CLSI document EP05-A3
27. CLSI. User Verification of Precision and Estimation of Bias; Approved Guideline-Third Edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2014. CLSI document EP15-A3
28. CLSI. Evaluation of Detection Capability for Clinical Laboratory Measurement Procedures; Approved Guideline-Second Edition. Wayne, PA: Clinical and Laboratory Standards Institute; 2012. CLSI document EP17-A2
29. Westgard JO, Carey RN, Wold S. Criteria for judging precision and accuracy in method development and evaluation. *Clin Chem.* 1974; 20:825–833. [PubMed: 4835236]
30. NCCLS. Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach; Approved Guideline. Wayne, PA: NCCLS; 2003. NCCLS document EP6-A
31. CLSI. Interference Testing in Clinical Chemistry; Approved Guideline-Second Edition. 2. Wayne, PA: Clinical and Laboratory Standards Institute; 2005. CLSI document EP07-A2
32. Guidance for Industry: Bioanalytical Method Validation. Silver Spring, MD: Food and Drug Administration Center for Drug Evaluation and Research; 2013.

33. [accessed April 29, 2016] Chapter 5: Regression Diagnostics. J Trop Med. http://www.oxfordjournals.org/our_journals/tropej/online/ma_chap5.pdf
34. Method Comparison Alternate (Qualitative) module of EP Evaluator(R). Version 9. South Burlington, VT: Data Innovations, LLC; 2009.
35. Kosovec JE, Egorin MJ, Gjurich S, et al. Quantitation of 5-fluorouracil (5-FU) in human plasma by liquid chromatography/electrospray ionization tandem mass spectrometry. Rapid Commun Mass Spectrom. 2008; 22:224–230. [PubMed: 18085512]
36. Maring JG, Schouten L, Greijdanus B, et al. A simple and sensitive fully validated HPLC-UV method for the determination of 5-fluorouracil and its metabolite 5,6-dihydrofluorouracil in plasma. Drug Monit. 2005; 27:25–30.
37. Meesters RJ, Cornelissen R, van Klaveren RJ, et al. A new ultrafast and high-throughput mass spectrometric approach for the therapeutic drug monitoring of the multi-targeted anti-folate pemetrexed in plasma from lung cancer patients. Anal Bioanal Chem. 2010; 398:2943–2948. [PubMed: 20865407]
38. Lanz C, Fruh M, Thormann W, et al. Rapid determination of gemcitabine in plasma and serum using reversed-phase HPLC. J Sep Sci. 2007; 30:1811–1820. [PubMed: 17638352]
39. Peters GJ, Clavel M, Noordhuis P, et al. Clinical phase I and pharmacology study of gemcitabine (2', 2'-difluorodeoxycytidine) administered in a two-weekly schedule. J Chemother. 2007; 19:212–221. [PubMed: 17434832]
40. Wu AH, French D. Implementation of liquid chromatography/mass spectrometry into the clinical laboratory. Clin Chim Acta. 2013; 420:4–10. [PubMed: 23085380]
41. Honda T, Inagawa H, Fukushima M, et al. Development and characterization of a monoclonal antibody with cross-reactivity towards uracil and thymine, and its potential use in screening patients treated with 5-fluorouracil for possible risks. Clin Chim Acta. 2002; 322:59–66. [PubMed: 12104082]
42. Peters GJ, Laurensse E, Steinbusch HW, et al. Development, characterization and application of an antibody against 5-fluoro-2'-deoxyuridine-5' monophosphate, the active metabolite of 5-fluorouracil. Anticancer Res. 1993; 13:835–839. [PubMed: 8352551]
43. Salamone, SJ., Courtney, JB. Stocker D.5-Fluoro-uracil Immunoassay. US Patent. 7,767,794. Aug 3. 2010
44. Felici A, Di Segni S, Milella M, et al. Pharmacokinetics of gemcitabine at fixed-dose rate infusion in patients with normal and impaired hepatic function. Clin Pharmacokinet. 2009; 48:131–141. [PubMed: 19271785]
45. Bowen C, Wang S, Licea-Perez H. Development of a sensitive and selective LC-MS/MS method for simultaneous determination of gemcitabine and 2,2-difluoro-2-deoxyuridine in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci. 2009; 877:2123–2129.
46. Gandhi V, Plunkett W. Modulatory Activity of 2',2'-Difluorodeoxycytidine on the Phosphorylation and Cytotoxicity of Arabinosyl Nucleosides. Cancer Res. 1990; 50:3675–3680. [PubMed: 2340517]
47. Grunewald R, Abbruzzese JL, Tarassoff P, et al. Saturation of 2', 2'-difluorodeoxycytidine 5'-triphosphate accumulation by mononuclear cells during a phase I trial of gemcitabine. Cancer Chemother Pharmacol. 1991; 27:258–262. [PubMed: 1998982]
48. Soo RA, Lim HL, Wang LZ, et al. Phase I trial of fixed dose-rate gemcitabine in combination with carboplatin in chemo-naïve advanced non-small-cell lung cancer: a Cancer Therapeutics Research Group study. Cancer Chemother Pharmacol. 2003; 52:153–158. [PubMed: 12750842]

Figure 1. Gemcitabine is anabolized by the enzyme cytidine deaminase. The deactivation can be inhibited ex-vivo by the structurally similar tetrahydrouridine.

Figure 2.
LC-MS/MS MRM ($m/z > 264.1 > 112.1$) chromatogram of gemcitabine at the LLQ (top chromatogram) and control plasma (bottom chromatogram).

Figure 3.
Typical instrument calibration curve for the gemcitabine assay: generated on the Beckman Coulter AU480 analyzer absorbance vs. concentration.

Figure 4.
Method comparison results with Deming regression: gemcitabine immunoassay vs. LC-MS/MS for 63 samples.

Table 1

Coefficient of variation for precision studies. Repeatability and within-laboratory precision was calculated from 2 replicates per run, measured for a total of 20 nonconsecutive days.

Sample Type	Assigned Value (µg/mL)	Mean (µg/mL)	Repeatability (%CV)	Within-Laboratory Precision (%CV)
Buffer	2.00	2.00	2.6	6.9
	10.00	9.92	1.7	3.7
	25.00	24.60	1.3	3.4
Plasma	1.50	1.52	3.1	7.5
	4.00	4.22	2.5	5.4
	12.50	12.60	2.1	3.3
	27.50	27.39	1.8	3.7

N=2 replicates per run, determined twice per day for 20 days

Table 2

Interference Results. N=1

Interference Type	Interferent Concentration	Interference
Lipemia (Triglycerides)	521 mg/dL	−4%
Icterus (Bilirubin)	34 mg/dL	3%
Total Protein (HSA)	6 g/dL	−8%
Total Protein (IgG)	6 g/dL	−6%
Hemolysis (Hemoglobin)	1,100 mg/dL	3%
Rheumatoid Factor	550 IU/mL	2%