Short communication

High-performance liquid chromatographic method for the determination of gemcitabine and 2′,2′-difluorodeoxyuridine in plasma and tissue culture media

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Abstract

Gemcitabine, a pyrimidine antimetabolite undergoes metabolism by plasma and liver cytidine deaminase to form the inactive compound, 2′,2′-difluorodeoxyuridine (dFdU). The parent molecule is activated by intracellular phosphorylation. To evaluate the population pharmacokinetics in patients receiving gemcitabine, and to test the relation between gemcitabine infusion rate and antitumor activity in an in vitro bioreactor cell culture system, we developed and validated a sensitive and specific HPLC-UV method for gemcitabine and dFdU. Deproteinized plasma is vortexed, centrifuged, and 25 μL of the acidified extract sample is injected onto a Waters Spherisorb 4.6 mm × 250 mm, 5 μm C18 column at 40 °C. The mobile phase (flow rate, 1.0 mL/min) consists of 10:90 (v/v) acetonitrile-aqueous buffer (50 mM sodium phosphate and 3.0 mM octyl sulfonic acid, pH 2.9). Gemcitabine, dFdU, and the internal standard, 2′-deoxycytidine (2′dC) were detected with UV wavelength set at 267 nm. The standard curves for gemcitabine in both matrices ranged from 2 to 200 μM, and for dFdU in plasma, from 2 to 100 μM. Within-run and between-run component precision (CV%) was ≤6.1 and 5.7%, respectively for both human plasma and tissue culture media, and for dFdU, 2.3 and 2.7%. Total accuracy ranged from 98.7 to 106.2% for human plasma and from 96.9 to 99.2% for tissue culture media, respectively, and for dFdU, from 96.5 to 99.6%. Tetrahydrouridine (THU), an inhibitor of cytidine deaminase is used to prevent breakdown in human plasma. With one method we can measure gemcitabine in both plasma and tissue culture media. Utility is demonstrated by evaluation of the disposition of gemcitabine in an in vitro bioreactor cell culture system.
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1. Introduction

Gemcitabine (2′,2′-difluorodeoxycytidine, dFdC, Gemzar) a pyrimidine antimetabolite, is approved for treatment of pancreatic and non-small cell lung cancer, and second-line therapy in combination with paclitaxel for treatment of breast cancer. After intravenous injection, gemcitabine undergoes metabolism (Fig. 1) by plasma and liver cytidine deaminase to form 2′,2′-difluorodeoxyuridine (dFdU), a compound with little antitumor activity. Overall, approximately 77% of administered gemcitabine is excreted either unchanged, or as the dFdU metabolite into the urine within 24 h. Gemcitabine also undergoes intracellular phosphorylation by deoxycytidine kinase at the tumor site to form difluoro-dCMP, and is phosphorylated further by other intracellular kinases to form difluoro-dCDP and difluoro-dCTP [1–3]. The diphosphate metabolite (dFdCDP) inhibits ribonucleotide reductase, an enzyme that catalyzes formation of deoxynucleosides required for DNA synthesis [4]. The triphosphate (dFdCTP) is incorporated into DNA, resulting in chain termination [5,6].

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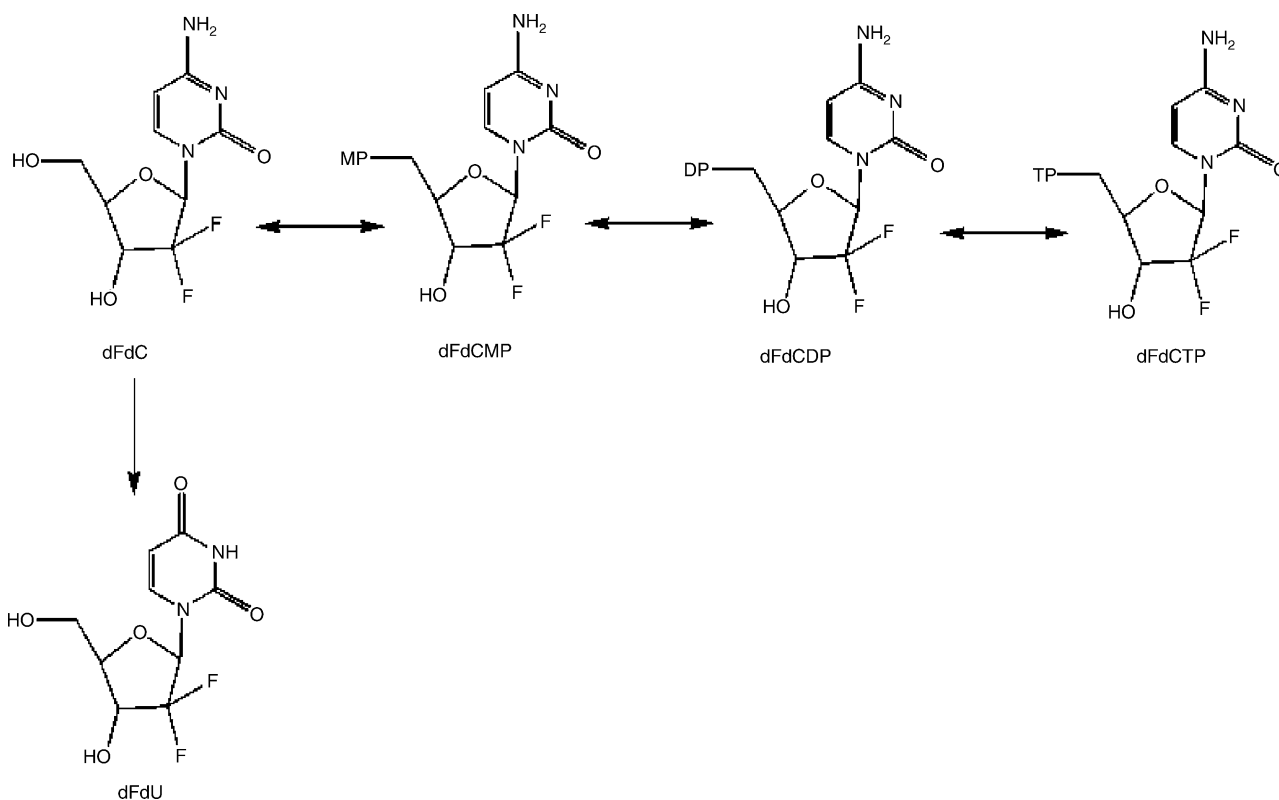*E-mail address:* kirst002@umn.edu (M.N. Kirstein).

Fig. 1. Metabolic schema for gemcitabine showing conversion to its inactive metabolite, dFdU by cytidine deaminase, and also phosphorylation to the mono-, di-, and triphosphate metabolites.

Plasma gemcitabine clearance varies 4–30-fold between patients receiving the same dose [7,8]. It is unclear how this variability affects active metabolite production and antitumor activity, since dFdU production also varies 2–11-fold between patients [8]. Patient-specific characteristics (i.e., covariates, genetic polymorphisms, etc.) can influence drug and metabolite disposition [9], and could affect pharmacologic activity (i.e., antitumor activity, toxicity). Thus, identification of covariates associated with variable gemcitabine distribution in patients may provide a rationale for individualized drug dosing and treatment regimens. Hence, further pharmacokinetic studies that evaluate the importance of gemcitabine disposition on pharmacologic activity in human subjects are warranted.

Infusion rates also influence gemcitabine antitumor activity. Tempero and colleagues compared gemcitabine efficacy when administered as a 30-min infusion versus a fixed dosage rate infusion (10 mg/m^2/min) to patients with pancreatic adenocarcinoma [10]. It is theorized that the prolonged infusion results as an increase of active metabolite production at the tumor site, leading to an improved survival rate. While these results appear promising, additional investigation to clarify underlying mechanism(s) responsible for undefined dFdCTP concentration-effect relationships of gemcitabine are needed, especially in tumor cells. Studies that establish optimal dose-infusion rates that lead to maximum intracellular active metabolite concentrations are needed to enhance response rates during therapy with gemcitabine. To allow for testing of multiple gemcitabine treatment regimens and measurement of active metabolite production

under controlled conditions, an in vitro bioreactor cell culture system will be used [11–13]. Mammalian cells are grown in a sterile-enclosed space that receives media driven by a pump. This system will allow for controlled gemcitabine infusion, which simulates human concentration–time profiles. The resultant accumulation of gemcitabine intracellular phosphorylated metabolites leading to cell death can then be studied. From these studies, we anticipate that we can develop dosing regimens that will lead to greater intracellular active metabolite exposure and enhanced antitumor activity. Once monotherapies are optimized, work can begin to test the utility of adjunct agents in improving efficacy. For phase I trials, Abbruzzese et al. [7] developed an ELISA method for determination of gemcitabine in plasma. Several high performance liquid chromatography assays with ultraviolet detection to measure gemcitabine and dFdU in human plasma have been described [14–18]. Reversed-phase columns were used by most investigators, although Freeman et al. initially employed a normal phase aminopropyl silica column, an approach also used by other investigators [16]. Sottani et al. [20] and Xu et al. [19] developed an LC–MS methods for gemcitabine and its major metabolite, 2′,2′-difluorodeoxyuridine. Many of these reported assays require solid-phase [19,20], or liquid–liquid extractions [16]. Direct plasma protein precipitation procedures with acids [15] or water-miscible organic solvents [14] have also been used for sample preparation. Mobile phases used for reversed-phase columns employ either gradient elution [8,17,19] or ion-pairing procedures [15] for efficient separation of gemcitabine, dFdU, and an internal standard. To

verify our targeted gemcitabine concentration–time exposure in the bioreactor system, we developed a rapid, reliable, and accurate HPLC assay to measure gemcitabine in tissue culture media. Furthermore, this assay will also be used to evaluate plasma gemcitabine concentrations for our population pharmacokinetics study in human subjects.

2. Experimental

2.1. Chemicals and reagents

Gemcitabine (Gemzar) and difluorodeoxyuridine used for preparation of standards or quality control samples were supplied by Eli Lilly (Indianapolis, IN, USA). Internal standard, $2'$-deoxycytidine ($2'$dC), was obtained from Sigma (St. Louis, MO, USA). Perchloric acid 70% was from Sigma. HPLC grade acetonitrile was obtained from Fisher (Fairlawn, NJ, USA). Sodium octyl sulfonate, an ion-pairing reagent, ≥99% was obtained from Sigma. RPMI 1640 medium, penicillin, streptomycin, and glutamine were from Invitrogen (Carlsbad, CA, USA). Fetal calf serum was from Biosource (Camarillo, CA, USA). Heparinized human plasma was from Biological Specialties (Colmar, PA, USA). Tetrahydrouridine was from Calbiochem (San Diego, CA, USA).

2.2. Instrumentation and chromatographic conditions

The HPLC system consisted of an Agilent quaternary pump with on-line degasser, an 1100 variable wavelength detector with deuterium lamp (Agilent Technologies, Palo Alto, CA, USA), a thermostatted autosampler and column compartment, an 1100 control module. Data acquisition and processing were performed with Chrom Perfect Spirit software, v. 5.2 (Justice Laboratories, Denville, NJ, USA).

A 25 μL volume of the standard and sample extracts was injected onto a Waters Spherisorb 4.6 mm × 250 mm, 5 μm ODS2 column (Waters Corporation, Milford, MA, USA) with A-102X in-line filter, 0.5 μM Frit (Chromtech, Apple Valley, MN, USA). Analytes were isocratically eluted with a mobile phase of acetonitrile (10:90, v/v) 50 mM sodium phosphate, 3.0 mM octyl sulfonic acid. The aqueous buffer was adjusted to pH 2.9 with 85% *o*-phosphoric acid. The mobile phase was filtered with 0.45 μ nylon filter (Chromtech, Apple Valley, MN, USA). The flow rate was 1.0 mL/min and the column temperature was 40 °C.

2.3. Sample preparation

2.3.1. Preparation of calibration standards

The gemcitabine stock solution was made by dissolving 1.0 mg of gemcitabine in 1.0 mL methanol. The dFdU stock solution was made by dissolving 1.3 mg in 1 mL methanol. The internal standard, $2'$dC was also made by dissolving 1.0 mg of $2'$dC in 1.0 mL methanol. Aliquots of the stock solutions were stored at −20 °C. Gemcitabine calibration standards were prepared at the time of assay from the 1.0 mg/mL stock solution of gemcitabine by making dilutions with heparinized human plasma or tissue culture media to obtain calibrators for the stan-

dard curve at the following concentrations: 2, 10, 25, 75, 150, and 200 μM. Tissue culture media was prepared from RPMI 1640 supplemented with 5% fetal calf serum, 50 units/mL penicillin, 50 μg/mL streptomycin and 2 mM glutamine. The dFdU calibration standards were prepared similarly with human plasma to obtain calibrators for the standard curve at the following concentrations: 2, 10, 25, 75, and 100 μM.

2.3.2. Preparation of quality control samples

Gemcitabine and dFdU quality control samples were prepared prior to the validation with tetrahydrouridine, an inhibitor of cytidine deaminase (40 μL of 10 mg/mL THU in water were added to each 5.0 mL of plasma prior to addition of gemcitabine) human plasma. The samples were then stored at −80 °C. The internal standard, $2'$dC was added to obtain a final concentration of 100 μM at the time of analysis. Quality control samples with tissue culture media were prepared at the time of analysis. Gemcitabine quality control concentrations in both matrices were 5, 50, and 175 μM. Quality control samples for tissue culture media were prepared similarly, but without THU. The dFdU concentrations in plasma were 5 and 50 μM.

2.4. Assay validation

Gemcitabine calibration standards were analyzed in triplicate for plasma samples and in duplicate for tissue culture samples. The linear regression of the peak height of gemcitabine to peak height of the internal standard, $2'$dC, was weighted by $1/x$. The squared correlation coefficient was used to evaluate the linearity of the calibration curve. The procedures developed for the quantitation of gemcitabine were validated over 5 days in plasma, and 3 days in tissue culture media by analysis of quality control samples to determine the within-day and between-day components of variability and the total precision and accuracy. Quality control samples were analyzed in triplicate for plasma samples, and in quintuplicate for tissue culture samples.

The limit of detection (LOD) was defined as the peak height ratio of signal/noise ≥3. The LOD was determined by triplicate analysis of samples based upon an estimate of the signal to noise ratio from prior analyses. Peak height was used for analysis in plasma and peak area was used for samples in tissue culture media. Gemcitabine peak broadening was observed after injection of the calibrator samples in tissue culture media. Thus, peak area was used to evaluate for this matrix. We speculate that plasma and media have different buffering capacity when acidified, and this may affect gemcitabine's ion pairing interaction on the column. Since we will plan to use this assay to assess gemcitabine pharmacokinetics in patients and in the hollow fiber system, we are aware of the rapid clearance of the parent molecule, and thus we did not assign a limit of quantitation (LOQ) below the lowest concentration calibrator. We assessed our lowest concentration (2 μM) in quintuplicate for 2 days during the validation of our tissue culture assay. The LOQ was defined as precision and accuracy of ≤10%. Accuracy parameters were determined with analysis of variance (ANOVA).

The dFdU calibration standards were analyzed in single line. The linear regression of the peak area of dFdU to the peak

area of 2′dC was weighted by $1/x$, and the squared correlation coefficient was used to evaluate linearity. The procedures for dFdU quantitation were validated over 3 days in human plasma by analysis of quality control samples to determine within-day and between-day components of variability and the total precision and accuracy. Quality control samples were analyzed in quintuplicate. We did not assign an LOQ below the lowest concentration calibrator.

2.5. Tissue culture sample collection

2.5.1. Bioreactor tissue culture samples

A polysulfone Plus cartridge (FiberCell Systems, Frederick, MD, USA) with a M_r (at 50%) 0.1 μm cutoff and 70 cm^2 surface area was used. The in vitro bioreactor culturing system consists of a cartridge containing hollow fiber capillaries made from semi-permeable polysulfone within a clear plastic chamber. The tumor cells are grown on the outside of these fibers. Gas permeable tubing connects this chamber to a source of growth media [11,12]. The semipermeable fibers permit constant perfusion of medium nutrients and oxygen for the cells and dialysis of metabolic waste products away from the cells. Drug delivery is precisely controlled with a programmable syringe pump (Cole Parmer, Vernon Hills, IL, USA) into the central reservoir, which also receives media from a diluent reservoir. The media is mixed in the central reservoir, and is kept at a constant volume. Media then travels the length of the fibers, delivering the drug. Waste is collected in the elimination reservoir. Flow rates from the diluent reservoir and central reservoir are controlled with a Masterflex L/S digital drive peristaltic pump (Cole Parmer). To determine whether this method could reliably detect gemcitabine media concentrations in samples collected from the hollow fiber system pharmacokinetic studies during and after gemcitabine infusion, we infused gemcitabine as 5.3 mg over 30 min through a cartridge without cells. Samples were collected serially after the end of the injection and media samples (250 μL) were immediately frozen at −80 °C until analysis. Drug-free blank media for calibration curves and quality control samples were prepared similarly.

3. Results and discussion

3.1. Sample preparation

Precipitation of plasma proteins was the most practical sample preparation procedure, since these nucleosides are polar and are not readily extractable by solid phase or liquid–liquid extraction. To optimize the plasma deproteinization step in our sample pretreatment method, various concentrations of perchloric acid (0.1, 0.2, 0.4, and 0.82 M) were examined. We limited the volume of perchloric acid to 10% of the plasma volume to minimize effects of dilution on gemcitabine peak height. We evaluated for the presence or absence of a clear supernatant by visual inspection after various concentrations of perchloric acid were added to plasma (v/v = 1:10). After addition of perchlorate, the samples were mixed with a vortex mixer, incubated on ice for 10 min, and then centrifuged at $16,000 \times g$ and 4 °C for 5 min. The gemc-

itabine peak height in these samples was determined to assess the effect of increased perchloric acid molarity on gemcitabine stability and recovery. The lowest two perchloric acid concentrations that we tested resulted in a cloudy supernatant 24 h after centrifugation. The highest two perchloric acid concentrations tested resulted in clear supernatants with greater peak heights. Based upon the results from these studies we chose a concentration of 0.82 M perchloric acid for our sample pretreatment. This extraction method enables us to process many samples more rapidly and more cost effectively than would be possible with the liquid–liquid and solid phase extractions described in previous reports [14,16,20].

3.2. Separation

Since gemcitabine and 2′dC are polar nucleoside analogues and primary amines, we used reversed-phase chromatography with a mobile phase at pH 2.9 and 3.0 mM sodium octyl sulfonate as an ion-pairing reagent for best results. Wang et al. [15] have previously described a similar assay, with heptanesulfonate as an ion-pairing agent. We initially tested heptanesulfonate as an ion-pairing agent, but we observed better separation of the internal standard, 2′dC from the non ion-pairing dFdU metabolite with sodium octyl sulfonate. We were not able to resolve the dFdU peak from an endogenous peak in human plasma, on a Waters Spherisorb 4.6 mm × 250 mm, 5 μm C8 column. We observed improved separation with the Waters Spherisorb 4.6 mm × 250 mm, 5 μm C18 column, hence further development and validation were completed with this column. No interfering peaks were detected at the retention times for gemcitabine, 2′dC, or dFdU. Acetonitrile 10% (test range 8–25%) in the ion-pairing buffer with a column temperature of 40 °C produced the best resolution from endogenous substances and a favorable retention time. Depicted in Fig. 2 is a chromatogram of deproteinized plasma with and without gemcitabine, 2′dC, and the inactive metabolite, dFdU. Shown in Fig. 3 is a chromatogram of deproteinized tissue culture media with and without 2′dC and gemcitabine. Gemcitabine is the last compound to elute from the
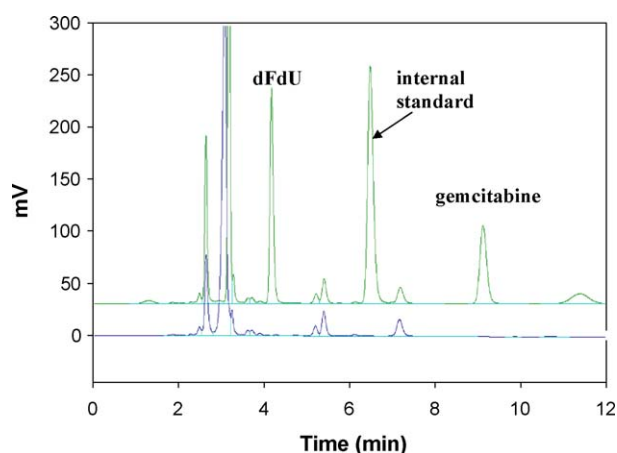

Fig. 2. Representative chromatograms of deproteinized human plasma with and without internal standard (2′dC), dFdU (100 μM), and gemcitabine (50 μM).

Table 1
Accuracy and precision data for the gemcitabine calibrators

Conc[a] (μM)	Accuracy (%)	S_W	CV_W (%)	S_b	CV_b (%)	S_t	CV_t (%)
Plasma							
2	104.7	0.07	6.90	0.04	2.02	0.08	3.90
10	91.4	0.26	2.80	0.23	2.54	0.11	1.20
25	101.5	0.63	2.50	0.40	1.57	0.50	2.00
75	101.8	1.65	2.20	0.76	1.00	1.46	1.90
150	102.2	2.43	1.60	1.48	0.96	1.93	1.30
200	97.4	4.77	2.40	3.36	1.72	3.38	1.70
Tissue culture media							
2	101.5	0.20	10.0	0.01	0.49	0.20	10.0
10	96.6	0.28	2.90	0.21	2.14	0.19	10.0
25	93.5	0.57	2.40	0.35	1.50	0.45	1.90
75	96.8	1.62	2.20	1.00	1.38	1.91	2.60
150	96.5	3.91	2.70	3.41	2.35	5.19	3.60
200	98.6	8.69	4.40	0.22	0.11	8.69	4.40

S_W, within-run component S.D.; CV_W, within-run component CV; S_b, between-run component S.D.; CV_b, between-run component CV; S_t, total S.D.; CV_t, total CV.
[a] Conc, concentration.

column; however, we observe a later endogenous peak in plasma that requires us to use a 15-min run time for each sample.

3.3. UV wavelength determination

To determine the optimal detection wavelength for gemcitabine, UV spectra of the reference gemcitabine was scanned on a HP diode array detector. The peak height of gemcitabine was at its maximum at 275 nm, compared to other wavelengths at the same concentration of gemcitabine. The peak height of $2'$dC was at its maximum at 282 nm, compared to other wavelengths at the same concentration of $2'$dC. The peak height of dFdU was at its maximum at 262 nm, compared to other wavelengths at the same concentration of dFdU. Keith et al. [17] reported similar UV maxima for gemicitabine (268 nm), dFdU (258 nm), and FdC (282 nm) with a diode array detector. Based on these results, we selected 267 nm as our optimal wavelength for method development.
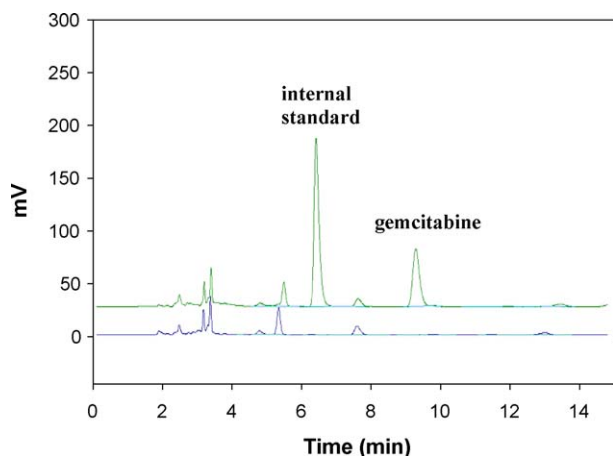

Fig. 3. Representative chromatograms of deproteinized tissue culture media with and without internal standard ($2'$dC) and gemcitabine (50 μM).

3.4. Validation of the assay

Shown in Table 1 are the accuracy and precision data for the gemcitabine calibrators. The calibrators for human plasma were analyzed in triplicate over 5 days, whereas for media, in duplicate over 3 days ($n = 6$). For the plasma matrix, the mean ($\pm$S.D.) weighted linear regression slopes, intercepts, and coefficients of determination (r^2 values) were as follows: slope = 0.0084 ± 0.0005, y-intercept = -0.0004 ± 0.00116, $r^2 = 0.9964 \pm 0.00186$. For the tissue culture matrix, the mean ($\pm$S.D.) weighted linear regression slopes, intercepts, and coefficients of determination (r^2 values) were as follows: slope = 0.0087 ± 0.0019, y-intercept = -0.0012 ± 0.00338, $r^2 = 0.9995 \pm 0.0004$. To assess within-day and between-day variability in assay performance, precision, and accuracy, an analysis of variance procedure was used for gemcitabine in human plasma (Table 2). Three injections of low, medium, and high quality control samples as described previously were made on days one through five to determine the within-run and between-run components of variability [21]. The samples were assessed with calibration curves prepared in human plasma and run in triplicate. The LOD of gemcitabine was 20 nM. The limit of quantitation was assessed as the lowest concentration of the calibration curve with a precision and accuracy of $\leq$10% (2 μM). The calibration curves were linear up to 200 μM, and were weighted by $1/x$. We selected this range for the calibrators, based upon published gemcitabine pharmacokinetic parameters. When gemcitabine is administered as 1000 mg/m^2, peak concentrations range up to 125 μM, and since the parent compound has a half-life of approximately 10 min, gemcitabine is usually not measurable beyond 2 h after administration. The dFdU metabolite concentrations can range up to 190 μM, and is more slowly eliminated than the parent molecule (i.e., half-life > 24 h) [7,8,22]. Hence, for samples with dFdU concentrations that exceed the highest concentration calibrator, we plan to dilute those samples accordingly.

Table 2
Accuracy and precision data for the gemcitabine quality controls

Conc[a] (μM)	Accuracy (%)	S_W	CV_W (%)	S_b	CV_b (%)	S_t	CV_t (%)
Plasma							
5	98.7	0.30	6.10	0.28	5.70	0.10	2.10
50	103.6	1.24	2.40	2.05	3.96	2.40	4.60
175	106.2	4.27	2.30	9.67	5.20	10.57	5.70
Tissue culture media							
5	97.4	0.21	4.30	0.11	2.24	0.18	3.70
50	96.9	0.83	1.70	0.67	1.38	1.07	2.20
175	99.2	1.37	0.80	4.32	2.49	4.54	2.60

S_W, within-run component S.D.; CV_W, within-run component CV; S_b, between-run component S.D.; CV_b, between-run component CV; S_t, total S.D.; CV_t, total CV.
 [a] Conc, concentration.

To assess within-day and between-day variability in assay performance, precision, and accuracy, we evaluated validation parameters for gemcitabine in tissue culture media (Table 2). Five injections (i.e., quintuplicate) of low and high quality control samples as described previously were made on Days 1 through 3 to assess within-assay variability and total assay variability. The samples were assessed with calibration curves prepared in tissue culture media and run in duplicate. The LOQ of gemcitabine was 2 μM as determined by five injections of the lowest calibrator on Days 2 and 3. The calibration curves were linear up to 200 μM.

To assess within-day and between-day variability in assay performance, precision, and accuracy, we evaluated validation parameters for dFdU in human plasma. Five injections (i.e., quintuplicate) of low and medium quality control samples as described previously were made on Days 1 through 3 to assess within-assay variability and total assay variability. The samples were assessed with calibration curves prepared in plasma and run in singlet. For the plasma matrix, the mean ($\pm$S.D.) weighted linear regression slopes, intercepts, and coefficients of determination (r^2 values) were as follows: slope = 0.0066 ± 0.006, y-intercept = -0.0026 ± 0.00017, $r^2 = 0.9999 \pm 10^{-5}$. To assess within-day and between-day variability in assay performance, precision, and accuracy, an analysis of variance procedure was used for dFdU in human plasma. For the 5 μM quality control, the accuracy was 96.5%, the within run component was $\leq$2.3%, and the between run component was $\leq$2.7%. For the 50 μM quality control, the accuracy was 99.6%, the within run component was $\leq$2.3%, and the between run component was $\leq$1.8%. The calibration curves were linear up to 100 μM.

3.5. Sample stability

To test for sample stability at $-80\,^{\circ}$C after processing as described, we measured gemcitabine concentrations (5 and 175 μM) after 1-month storage. Quality control samples were prepared at the time of validation, and frozen at $-80\,^{\circ}$C. Upon thawing, the internal standard, 2'dC, was added, and the samples processed and analyzed as described previously. The mean $\pm$ standard deviation for the 5 μM samples was 4.97 ± 0.11, and the accuracy was 99%. The corresponding values for the 175 μM samples were 173.13 ± 4.62, and the accuracy was 99%. Others have also demonstrated sample stability

when stored at $-80\,^{\circ}$C for up to almost 2 years [17,18]. Gemcitabine has been shown to rapidly degrade in human plasma. Tetrahydrouridine, an inhibitor of cytidine deaminase was used to prevent breakdown [7,23]. We tested gemcitabine (5 μM) stability in 250 μL of human plasma (triplicate samples) after the addition of 20 μg THU. After incubation for 2 h at 37 $^{\circ}$C, gemcitabine peak heights were 97% of the peak height values observed for control (non-incubated) samples. Thus, 20 μg THU are added to plasma prior to gemcitabine for each calibrator and quality control. Since gemcitabine may also degrade in fetal calf serum-containing media, we also tested gemcitabine (5 μM) stability in 250 μL of tissue culture media (quintuplicate) without THU. After incubation for 2 h at 37 $^{\circ}$C, gemcitabine peak heights were 98% of the peak height values observed for control (non-incubated) samples. Thus, THU is not added to media for the experiments with the in vitro hollow fiber system.

To test for freeze-thaw stability, five quality control samples were also re-frozen and thawed to room temperature three times and then analyzed. The recovery for the 5 μM gemcitabine samples was (mean $\pm$ standard deviation) 4.78 ± 0.58 (accuracy = 96%). The recovery for the 175 μM gemcitabine samples was 185.57 ± 14.31 (accuracy = 106%).

To test for plasma post-processing stability, quality control samples were processed as described previously, and allowed to incubate at room temperature for 72 h. The samples were then analyzed against newly prepared calibration curves. The recovery for the 5 μM gemcitabine samples was 5.30 ± 0.20 (accuracy = 106%). At the 175 μM level, the gemcitabine recovery was 178.67 ± 3.83 (accuracy = 102%). Thus, it is recommended to analyze the samples within 72 h, once they have been processed as described. To test for tissue culture media post-processing stability, quality control samples were processed as described previously, and allowed to incubate at 4 $^{\circ}$C temperature for 1 month. The samples were then analyzed against newly prepared calibration curves. For measurement in tissue culture media, the recovery for the 5 μM gemcitabine samples was 4.69 ± 0.07 (accuracy = 93.9%). At the 175 μM level, the gemcitabine recovery was 182.88 ± 4.82 (accuracy = 104.5%).

3.6. Bioreactor concentration–time profile

Depicted in Fig. 4 is a tissue culture media concentration–time profile for gemcitabine measured from a hollow fiber car-
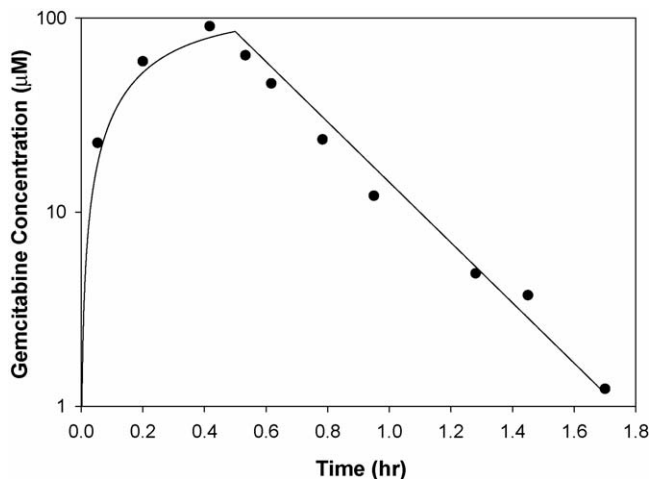
Fig. 4. Concentration–time plot of tissue culture samples collected from the lumen of a hollow fiber culture after treatment with 5.3 mg gemcitabine infused over 30 min.

tridge (without cells) after treatment with a 30 min infusion of 5.3 mg gemcitabine. All samples were analyzed within 24 h. The pharmacokinetic parameter values for gemcitabine were as follows: elimination rate constant (K_e), 3.6 h^{-1}; volume of the central compartment (V_c), 94.2 mL; clearance (Cl), 5.6 mL/min; maximum concentration (C_{max}), 85.2 μM; area under the curve (AUC), 51.2 μM h; and elimination half-life, 11.6 min. The calculated V_c parameter closely approximates the volume of media in the central reservoir plus the tubing (80 mL). Our pump flow rate was set to 5 mL/min, and we recovered 560 mL in the elimination reservoir over 2 h (theoretical yield is 600 mL). Thus, our calculated clearance was 120% of the measured clearance. The values for C_{max}, AUC, and half-life are close to clinically-observed pharmacokinetic exposures [7,8,22].

4. Conclusions

The goal of this study was to develop a simple and reliable method for the separation and quantitation of gemcitabine in human plasma and tissue culture media. The proposed method is suitable for the specific determination of gemcitabine in both matrices, and for dFdU in plasma. The plasma and media pretreatment, which is based upon a simple deproteinization with perchloric acid, requires a small amount of either matrix (250 μL) and still has the advantage of satisfactory sensitivity and precision. Prior to processing of plasma, it is important to add 20 μg THU, an inhibitor of cytidine deaminase. As we showed with the gemcitabine pharmacokinetic profile in the in vitro bioreactor system, this method will be very useful in determining and verifying controlled gemcitabine concentration–time profiles. The limit of quantitation for our assay in plasma samples was ≤2 μM (accuracy and precision <7% for the 2 μM standard) and is similar to that reported for other HPLC-UV assays of ~0.5 μM [17,24], but not as low as

the method by Xu et al. [19] with LC–MS. Thus, this assay will be an extremely useful tool as we begin to test the importance of gemcitabine infusion rates on antitumor activity, and in combination with other cytotoxic and targeted agents.

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