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Development of a sensitive and selective LC–MS/MS method for simultaneous determination of gemcitabine and 2,2-difluoro-2-deoxyuridine in human plasma

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ABSTRACT

A sensitive, selective, and quantitative method for the simultaneous determination of gemcitabine and 2,2-difluoro-2-deoxyuridine (dFdU) has been developed and validated in human plasma in the presence of tetrahydrouridine, a cytidine deaminase inhibitor. The method employs derivatization of gemcitabine and dFdU with dansyl chloride to improve the chromatographic retention and separation. The derivatization was performed in plasma without prior sample clean-up, followed by extraction of the dansyl-derivatives using methyl tertiary-butyl ether (MTBE). Ultra performance liquid chromatography (UPLC) technology on a BEH C18 stationary phase column with 1.7 μm particle size was used for chromatographic separation coupled to tandem mass spectrometry. The method was validated over the concentration ranges of 20–5000 and 100–25,000 ng/mL for gemcitabine and dFdU, respectively. The results from assay validation show that the method is rugged, precise, accurate, and well-suited to support pharmacokinetic studies. In addition, the relatively small sample volume (50 μL) and a run time of 1.5 min facilitate automation and allow for high-throughput analysis.

1. Introduction

Gemcitabine (2,2-difluoro-2-deoxycytidine, Gemzar®) is a novel deoxycytidine analog and pyrimidine antimetabolite [1,2]. Gemcitabine is either excreted in urine or deaminated to 2,2-difluoro-2-deoxyuridine (dFdU) in the liver, kidney or plasma by cytidine deaminase (Fig. 1) [3,4]. Gemcitabine enters cancer cells by nucleoside transporters and undergoes intracellular phosphorylation by deoxycytidine kinase to form 2,2-difluoro-2-deoxycytidine 5-monophosphate (difluoro-dCMP), 2,2-difluoro-2-deoxycytidine 5-diphosphate (difluoro-dCDP), and 2,2-difluoro-2-deoxycytidine 5-triphosphate (difluoro-dCTP) [2–4]. Difluoro-dCDP and difluoro-dCTP are responsible for the cytotoxic effects of gemcitabine [5,6]. Difluoro-dCDP is known to cause depletion of endogenous deoxyribonucleoside triphosphate pools by inhibiting ribonucleoside reductase [7]. Difluoro-dCTP is known to be incorporated into DNA strands inducing cross-links and effectively inhibiting cross-linked DNA repair [8]. Gemcitabine has been used for treatment of a variety of cancer types including lung, pancreatic, ovarian, bladder, breast, urothelial, and hematological malignancies [3,9–12]. To improve therapeutic efficacy, gemcitabine has also been used in combination with other cytotoxic drugs, such as paclitaxel and cisplatin [9]. As a single therapy, gemcitabine is commonly administered intravenously at a weekly dose of 1000 mg/m^2 by 30 min infusion [6] with peak plasma concentration within 15–30 min [13]. Preclinical data suggest that a weekly high dose of gemcitabine is better tolerated and less toxic than a daily low dose [14]. While gemcitabine is generally well tolerated in humans, some patients can develop adverse reactions including myelosuppression, constipation, fever, malaise, headache, anorexia, skin rashes, and reversible thrombocytopenia [14]. To monitor the pharmacokinetics of gemcitabine following chemotherapy, a rapid, sensitive, and accurate bioanalytical method for the analysis of gemcitabine and dFdU is required.

The development of a method for analysis of gemcitabine and dFdU is a challenge due to the instability of gemcitabine in biological matrices leading to the formation of dFdU. Tetrahydrouridine (THU), a potent inhibitor of cytidine deaminase, is commonly used to prevent deamination [3,4,6,15–19]. Beumer et al. [19] showed that oral administration of gemcitabine in combination with THU prolongs the half-life of gemcitabine and decreases first-pass metabolism in the gut and liver, thus increasing the bioavailability of gemcitabine. The concentration of THU recommended in previously validated assays varies from 0.250 up to 100 μg/mL [3,4,6,15–19]. In the present work, an experiment was designed to

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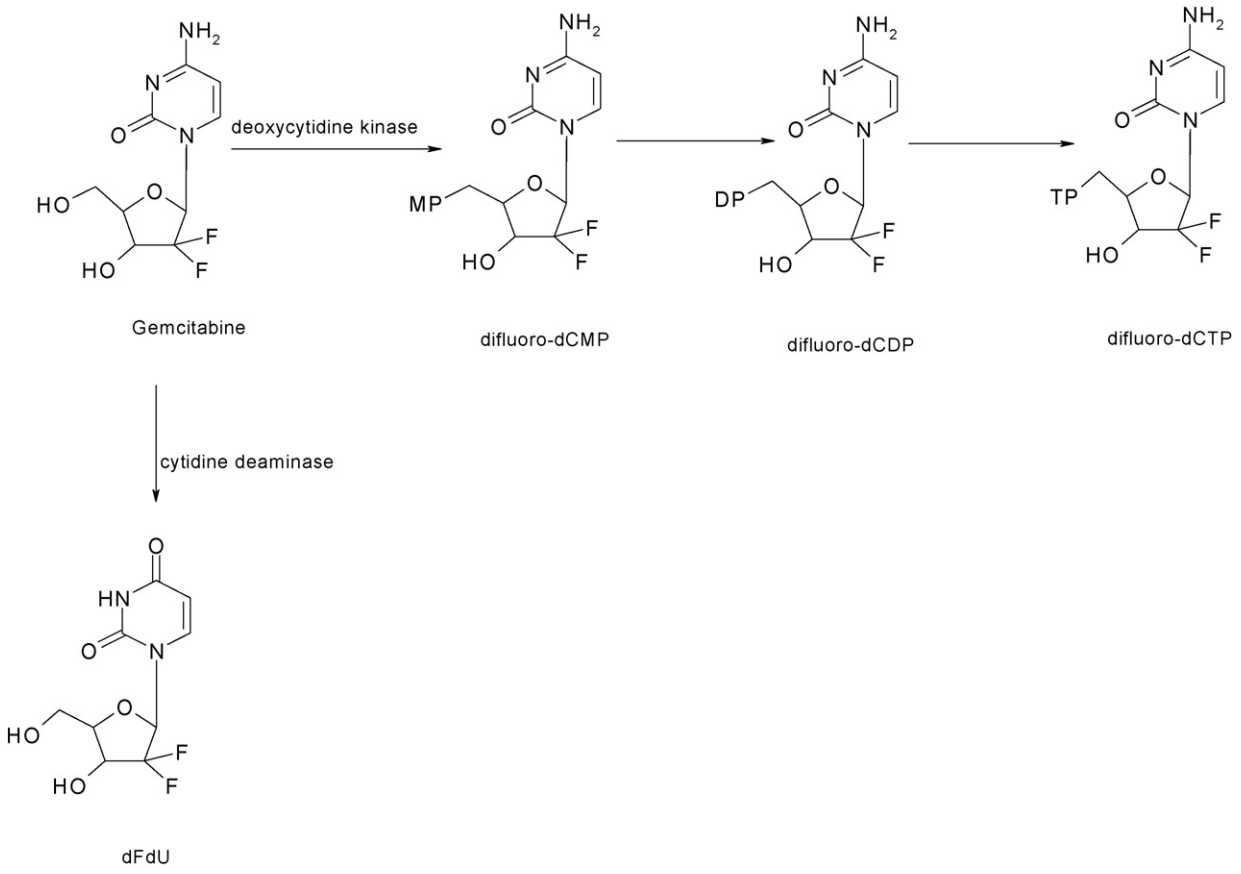

Fig. 1. Metabolic scheme for gemcitabine (adopted from Kirstein et al. [4]).

determine the optimal concentration of THU (≥10 μg/mL) to prevent the deamination of gemcitabine.

Published methods for the analysis of gemcitabine and dFdU include sample extraction using direct injection [5], liquid–liquid extraction (LLE) [15,16,19], protein precipitation (PPT) [3,4,7,16,17], and solid phase extraction (SPE) [6] with either ultraviolet (UV) or mass spectrometric (MS) detection. Methods that employ UV detection often require a sample volume of at least 200 μL of plasma and an analysis run time exceeding 20 min, thus making it difficult for automation and high-throughput analysis [4,5,7,15,18]. MS detection increases selectivity and signal to noise ratio, therefore allowing the reduction of sample volume and analysis time using multiple reaction monitoring (MRM). However, these existing methods for the analysis of gemcitabine and dFdU using MS detection have been reported with a plasma volume of at least 125 μL and run time of 5 min [3,6,16,19].

Another challenge associated with the development of a method for the analysis of gemcitabine and dFdU using MS detection is the one mass unit difference between the analytes. To prevent overestimation of the concentration of dFdU due to the isotopic contribution from gemcitabine, chromatographic separation between the analytes is required. However, the relatively high polarity of gemcitabine and dFdU make it difficult to achieve good resolution between the analytes on reverse phase columns. In the present work, a simple and rapid (5 min) derivatization procedure with dansyl chloride was introduced to increase the retention time and achieve baseline separation between the analytes on a reverse phase column. In addition, the MS sensitivity of the investigated analytes was greatly improved using the described derivatization procedure. The relatively small plasma volume (50 μL) allows sample extraction in 96-well format, thereby increasing throughput analysis, over previously validated methods. Moreover, the relatively short run time of 1.5 min, afforded by UPLC separation, allows for the analysis of about 300 samples per day.

2. Experimental

2.1. Chemicals and reagents

Gemcitabine, [$^{13}C_1$,$^{15}N_2$]-gemcitabine, dFdU, and [$^{13}C_1$,$^{15}N_2$]-dFdU were purchased from Toronto Research Chemicals (North York, ON, Canada). Dansyl chloride was purchased from MP Biomedicals (Solon, OH, USA). HPLC grade acetone, acetonitrile, and methanol were purchased from Burdick and Jackson (Muskegon, MI, USA). MTBE, THU, and formic acid were purchased from EMD Scientific (Gibbstown, NJ, USA). Sodium bicarbonate was purchased from Sigma–Aldrich (St. Louis, MO, USA). Human blood was obtained from Bioreclamation Inc. (East Meadow, NY, USA).

2.2. Equipment

An Eppendorf 5810R centrifuge with a rotor capacity for four 96-well plates (Brinkmann Instrument, Westbury, NY, USA) and a Mettler UMX2 balance (Columbus, OH, USA) were used. A Hamilton Mircrolab STAR liquid handler (Reno, NV, USA) was used for plasma transfer, and a TomTec Quadra 3 SPE (Hamden, CT, USA) was used for liquid transfer. Arctic White LLC 96-well round 2 mL polypropylene plates and ArctiSeal silicone mats with PTFE film (Bethlehem, PA, USA) were used to extract the dansyl-derivatives of the analytes and their internal standards from plasma. One milliliter glass vials along with 96-well plate covers (Blue CapMat with Pre-Cut T/S Septa) from MicroLiter Analytical Supplies (Suwanee, GA, USA) were used

for sample introduction to the LC–MS/MS. An ACQUITY™ UPLC integrated system from Waters (Milford, MA, USA) consisting of a sample manager combined with a sample organizer, capable of holding ten 96-deep well plates, and a binary solvent manager were used. A triple quadrupole mass spectrometer API 4000 (Applied Biosystems/MDS-Sciex, Concord, Ontario, Canada) was used.

2.3. Stability of gemcitabine in the presence and absence of THU

An appropriate amount of gemcitabine was added to a known volume of fresh human blood, containing EDTA, to give a final concentration of 4000 ng/mL. Spiked blood was kept at ambient temperature and aliquots were removed at 0, 0.25, 0.5, 1, 2, 3, 5, and 24 h. This was followed by the immediate addition of THU (50 μg/mL) at each time point to stop the deamination of gemcitabine. After addition of THU, the plasma was isolated by centrifugation at 3200 × *g* for 5 min and then frozen at −20 °C. In a parallel experiment, fresh human blood was treated with THU (50 μg/mL) and gemcitabine was spiked into the pre-treated blood to give a final concentration of 4000 ng/mL. The blood was kept at ambient temperature and aliquots were collected at the same time points as listed above and similarly processed into plasma and stored. The concentration of gemcitabine and dFdU in plasma samples from both experiments were determined according the procedure described in Section 2.6.

2.4. Optimization of the THU concentration

The amount of THU added to whole blood was optimized by addition of THU over the range of 0.1–100 μg/mL, while holding the concentration of gemcitabine constant at 4000 ng/mL. These samples remained at ambient temperature for 24 h followed by centrifugation at 3200 × *g* for approximately 5 min to isolate the plasma. The treated plasma was stored at −20 °C before analysis. The concentration of gemcitabine and dFdU was determined using the procedure described in Section 2.6.

2.5. Preparation of calibration standards and quality control (QC) samples

Stock solutions of gemcitabine, [$^{13}C_1$,$^{15}N_2$]-gemcitabine, and [$^{13}C_1$,$^{15}N_2$]-dFdU were individually prepared in water at a concentration of 1.0 mg/mL. Stock solutions of dFdU were prepared in water at a concentration of 5.0 mg/mL. All stock solutions were stored at 4 °C. Two separate working solutions (WS and WQ) were prepared fresh on the day of analysis containing both gemcitabine and dFdU in 25/75 (v/v) acetonitrile/water. The concentration of gemcitabine and dFdU in these working solutions was 100 and 500 μg/mL, respectively. The WS is used for the preparation of the calibration standard while WQ is used for the preparation of the quality control samples.

An aqueous solution of 10 mg/mL THU was used to prepare a 50 μg/mL solution of THU in EDTA human whole blood. The test tube containing the treated blood was gently inverted several times and the plasma was isolated from the red blood cells by centrifugation at 3200 × *g* for approximately 5 min. The treated plasma was used for the preparation of the standards and QC samples.

The WS was used to make calibration standards in treated plasma at 5000/25,000, 2500/12,500, 1000/5000, 500/2500, 250/1250, 100/500, 50/250 and 20/100 ng/mL of gemcitabine/dFdU using a serial dilution procedure. The WQ was used to make QC samples in treated plasma at 20,000/1,00,000 (dilution QC, analyzed after 10-fold dilution), 5000/25,000, 4000/20,000, 500/2500, 80/400, and 20/100 ng/mL of gemcitabine/dFdU. QC samples were divided into 0.5 mL aliquots and frozen at −20 °C or extracted immediately. In the first validation run, freshly prepared QC samples were analyzed against freshly prepared calibration standards. For each subsequent validation run, frozen replicate aliquots of the QC samples were thawed at ambient temperature and analyzed against a freshly prepared standard curve.

2.6. Sample preparation

MTBE (0.5 mL) was added to each well of the 2 mL ArcticWhite 96-well polypropylene plate. The plate was sealed with the ArctiSeal mat and vortex-mixed in an inverted position for approximately 3 min. Subsequently, the MTBE was discarded and the plate was left to dry in a chemical hood. This wash step was used to remove any plastic residue from the plates and plate seals. Plasma samples (50 μL) were transferred to the washed 96-well plate using a Hamilton STAR liquid handler. A 50 μL aliquot of internal standard solution (2000 ng/mL of [$^{13}C_1$,$^{15}N_2$]-gemcitabine, and [$^{13}C_1$,$^{15}N_2$]-dFdU in 25/75 (v/v) acetonitrile/water) was added to all wells with the exception of the blanks, which instead received 50 μL of 25/75 (v/v) acetonitrile/water. The plate was capped and vortex-mixed for approximately 1 min. After vortex-mixing, 100 μL of 10 mg/mL dansyl chloride in acetone and 100 μL sodium bicarbonate (100 mM; pH 11) were added to all wells. The plate was sealed and vortex-mixed for approximately 3 min followed by incubation at 60 °C for 5 min. After incubation, 1 mL of MTBE was added to all wells to extract the dansyl-derivatives of gemcitabine, dFdU, and their internal standards. After vortex-mixing and centrifugation, the MTBE layer was transferred to a 2 mL polypropylene 96-well plate containing 1 mL glass-inserts using a TomTec liquid handler and evaporated under a stream of nitrogen at 45 °C. The extract was then reconstituted in 150 μL of 25/75 (v/v) acetonitrile/water.

2.7. Chromatographic conditions for gemcitabine and dFdU

The analytical column used was a BEH C18, 2.1 mm × 50 mm with 1.7 μm particle size from Waters Co. The column temperature was held at 50 °C and the sample compartment was at ambient temperature. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B was acetonitrile. A linear gradient ran from 30% B to 60% B for 1.0 min and held at 60% B until 1.25 min to remove late eluting substances from the column, after which the system was returned to the initial condition. The total run time, including sample loading, was approximately 1.5 min and the flow rate was maintained at 0.7 mL/min throughout the run. A typical injection volume of 2 μL in a 10 μL loop (partial loop injection mode) was used.

2.8. Mass spectrometric conditions

An API 4000 with a TurboIonspray interface (TIS) was operated in the positive ionization mode. The instrument was optimized for the dansyl-derivatives of gemcitabine, [$^{13}C_1$,$^{15}N_2$]-gemcitabine, dFdU, and [$^{13}C_1$,$^{15}N_2$]-dFdU by infusing a 10 ng/mL solution of purified derivatives in acetonitrile/water (50/50, v/v) at 0.7 mL/min through an Agilent pump 1100 series (Palo Alto, CA, USA) directly connected to the mass spectrometer. The MRM transitions of *m*/*z* 497–112, *m*/*z* 500–115, *m*/*z* 498–113, and *m*/*z* 501–116 were chosen for the dansyl-derivatives of gemcitabine, [$^{13}C_1$,$^{15}N_2$]-gemcitabine, dFdU and [$^{13}C_1$,$^{15}N_2$]-dFdU, respectively. Dwell times of 150 ms were used for the dansylated derivatives of gemcitabine and dFdU, while 100 ms were used for the internal standards dansylated derivatives. The optimized mass spectrometric conditions for the dansyl-derivatives of gemcitabine and dFdU included the following MS conditions: TIS source temperature, 700 °C; TIS voltage, 5000 V; curtain gas, 30 psi (nitrogen); nebulizer gas (GS1), 60 psi (zero air); turbo gas (GS2), 70 psi (zero air); collision energy, 37 eV.

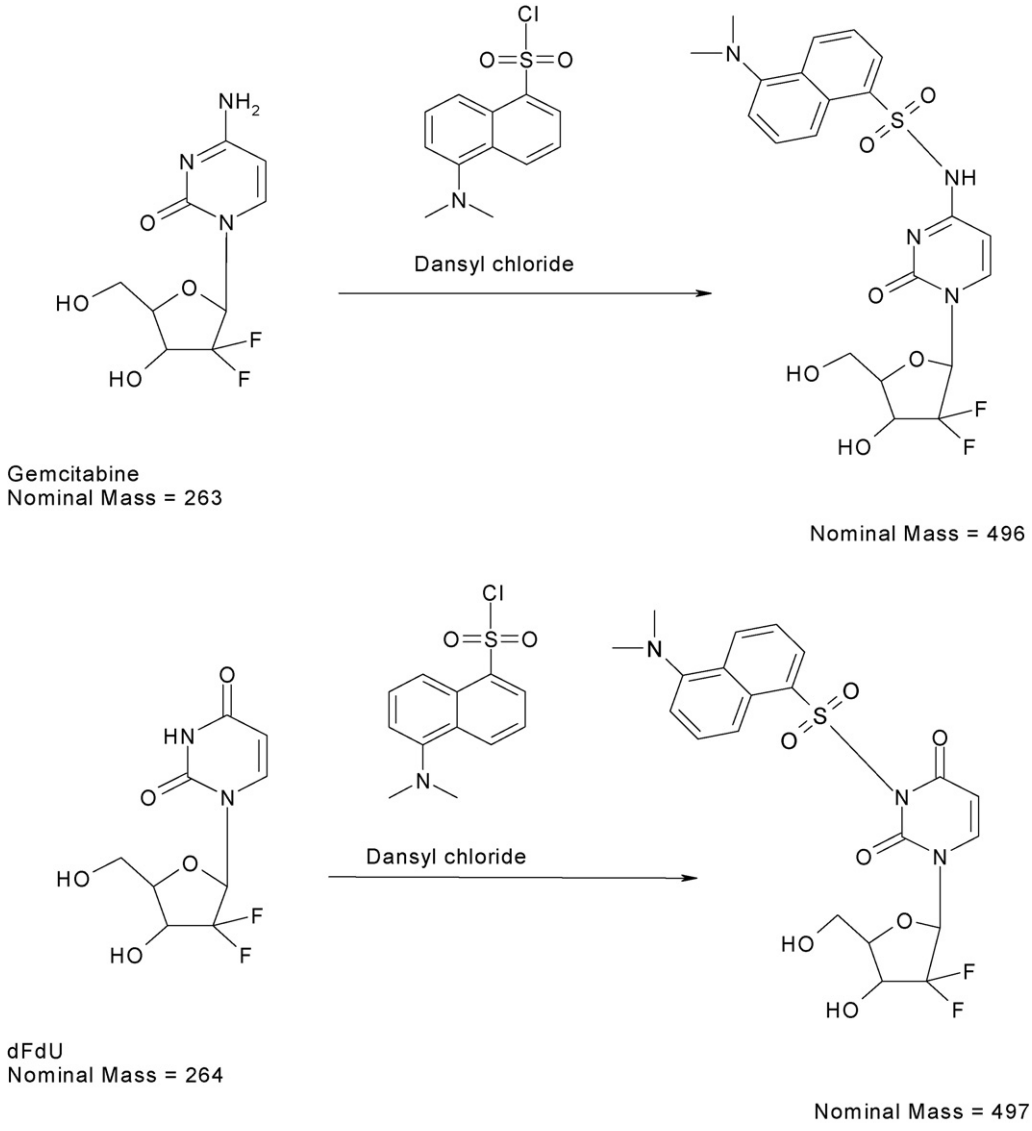

Fig. 2. Scheme for the reaction of gemcitabine and dFdU with dansyl chloride.

2.9. Data analysis

MS data were acquired and processed (integrated) using the proprietary software application Analyst™ (Version 1.4.2, Applied Biosystems/MDS-Sciex, Canada). Calibration plots of analyte/internal standard peak area ratio versus gemcitabine and dFdU concentrations were constructed and a weighted $1/x^2$ linear regression was used. Concentrations of gemcitabine and dFdU in validation samples were determined from the appropriate calibration line and used to calculate the bias and precision of the method with an in-house LIMS (Study Management System, SMS2000, version 2.0, GlaxoSmithKline).

3. Results and discussion

3.1. Challenges during method development

The objective was to develop a rugged, sensitive, and relatively high-throughput LC–MS/MS method allowing simultaneous determination of gemcitabine and dFdU with a run time of less than 2 min. Three major challenges were faced during method development: (1) only one mass unit difference between gemcitabine and dFdU, which required LC base line separation, (2) poor chromatographic retention of both compounds on reversed phase columns, and (3) stability of gemcitabine in biological matrices. The approaches used to resolve these challenges are discussed in detail below.

3.2. Underivatized gemcitabine and dFdU

All attempts within this laboratory to develop an assay for simultaneous determination of gemcitabine and dFdU with a run time of less than 2 min without chemical derivatization were unsuccessful. The failures were attributed to the polar characteristics of the analytes and difficulties finding an analytical column to retain and separate them. To avoid an overestimation of the concentration of dFdU due to MS isotopic contribution from gemcitabine, baseline LC separation between the analytes was necessary. A number of analytical columns with different chemistries and mobile phase combinations were tested without success. Specifically, poor retention and chromatographic separation between the analytes were observed using C18 and C5 stationary phase columns in the presence or absence of mobile phase additives, while Waters HILIC and mixed-mode Primesep® HPLC columns from SIELC Technolo-

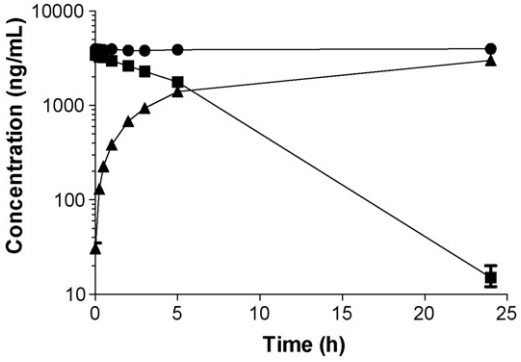

Fig. 3. Deamination of gemcitabine at 4000 ng/mL in human blood in the presence (●) and absence of THU (■), and the formation of dFdU (▲) in the absence of THU.

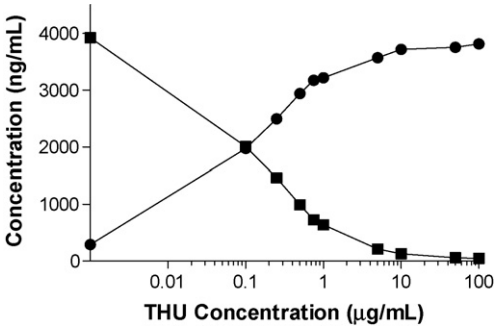

Fig. 4. The optimization of THU concentration to prevent deamination of gemcitabine (●) to dFdU (■) in human blood.

gies were also evaluated for separation of underivatized analytes without success.

3.3. Derivatization of gemcitabine and dFdU

To increase the hydrophobicity and chromatographic retention of the analytes on reverse phase columns, a chemical derivatization with dansyl chloride was investigated (Fig. 2). Dansyl chloride has been widely used for derivatization of primary and secondary amines, as well as phenols in bioanalysis [20–22]. In the present method, derivatization was performed in plasma without prior sample clean-up. The dansyl-derivatives were purified by LLE using MTBE before LC–MS/MS analysis. Selection of an appropriate BEH C18 stationary phase column with 1.7 μm particle size allowed a resolution of approximately 4.1 between dansyl-derivatives with a run time of less than 2 min. The average values for retention factor (k) of 3.1 and 3.5 for dansyl-gemcitabine and dansyl-dFdU, respectively, were calculated using an equation for a linear gradient profile as described by Neue [23]. The peak width values at the base of the peaks were 2.6 and 3.2 s for dansylated gemcitabine and dFdU, respectively.

3.4. Blood treatment with THU

The results from the experiment described in Section 2.3 show that in the absence of THU, approximately 50% of the gemcitabine was converted to dFdU in whole blood after 5 h ($T_{1/2}$), and complete conversion was observed after 24 h (Fig. 3). Therefore, the presence of a potent inhibitor of cytidine deaminase is required for the analysis of gemcitabine and dFdU.

Although most of the existing published analytical methods for the analysis of gemcitabine and dFdU employ THU to inhibit cytidine deaminase, there is not a general consensus on the concentration of THU required to prevent the deamination of gemcitabine leading to the formation of dFdU. The experiment described in Section 2.4 was designed to determine the optimum concentration of THU required to prevent deamination. Whole blood was used instead of plasma to mimic the conditions for sample collection at clinical site and to minimize the degradation of gemcitabine to dFdU during blood and plasma handling. For the same reason, the elimination of gemcitabine and formation of dFdU in human blood was investigated at ambient temperature. The experimental results showed that at a THU concentration of 10 μg/mL in human plasma approximately 3% of gemcitabine was converted to dFdU after 24 h. Even less conversion was noted at THU concentrations exceeding 10 μg/mL (Fig. 4). Therefore, in the present assay validation a concentration of 50 μg/mL THU was used. At this THU concentration, gemcitabine was stable for at least 24 h at ambient temperature in human blood (Fig. 3).

3.5. Selectivity and linearity

The characteristic precursor $[M+H]^+$ to product ion transitions m/z 497–112, m/z 500–115, m/z 498–113, and m/z 501–116 are consistent with the structures of the dansyl-derivatives of gemcitabine, $[^{13}C_1,^{15}N_2]$-gemcitabine, dFdU, and $[^{13}C_1,^{15}N_2]$-dFdU, respectively. The selectivity of the method was established by the analysis of samples of control human blood from six individual volunteers. UPLC–MS/MS chromatograms of the blanks and QC samples were visually examined and compared for chromatographic integrity and potential interferences. Representative UPLC–MS/MS chromatograms for the dansyl-derivatives of gemcitabine and dFdU of a blank sample, with and without the internal standards, and QC samples at the limit of quantification (LOQ) and high limit of quantitation (HLQ) are shown in Figs. 5 and 6, respectively. No unacceptable interferences at the retention times of the dansyl-derivatives of gemcitabine, dFdU, or the internal standards were observed. Post-column infusion experiments were also performed to investigate potential ion suppression effects from endogenous plasma interferences on the MRM transitions of the dansyl-derivatives. For this purpose, a control human plasma sample, in the presence of THU, was processed as described in Section 2.6. Two microliters of the extracted control plasma was injected into the LC system with a continuous post-column infusion at 20 μL/min of a solution containing 10 ng/mL dansylated gemcitabine and dFdU. No evidence of ion suppression was observed at the retention times for the investigated dansyl-derivatives.

The linearity of the method was evaluated by analyzing eight calibration standards in duplicate over the nominal concentration ranges of 20–5000 and 100–25,000 ng/mL for gemcitabine and dFdU, respectively. The correlation coefficients obtained using $1/x^2$ weighted linear regressions were better than 0.9991 for gemcitabine and 0.9986 for dFdU.

3.6. Bias and precision

The maximum bias observed for gemcitabine was −7.9% and 6.2% for dFdU (see Table 1). The maximum within-run precision values observed were 11.3% for gemcitabine and 5.6% for dFdU. The maximum between-run precision values observed were 4.2% for gemcitabine and 2.8% for dFdU. As defined by the lower and upper QC concentrations possessing acceptable accuracy and precision, the validated range of this method based on 50 μL of EDTA human plasma is 20–5000 and 100–25,000 ng/mL for gemcitabine and dFdU, respectively.

3.7. Stability of gemcitabine and dFdU in biological matrix

The ambient temperature stability of gemcitabine and dFdU in spiked human plasma samples in the presence of THU was assessed

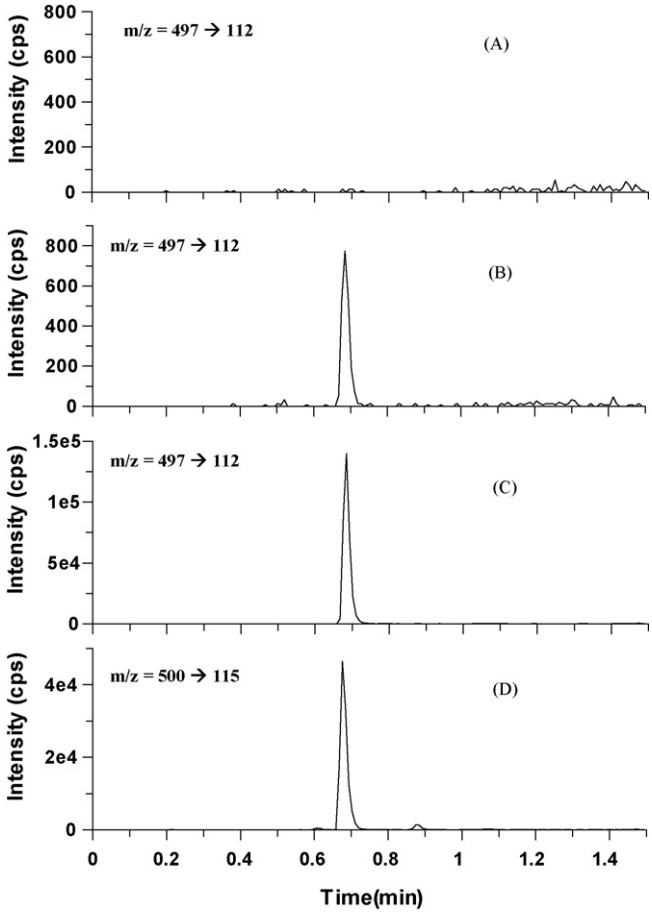

Fig. 5. Chromatograms for dansyl-derivatives of gemcitabine from blank human plasma (A), plasma spiked with 20 ng/mL (B), 5000 ng/mL (C) and the internal standard (D).

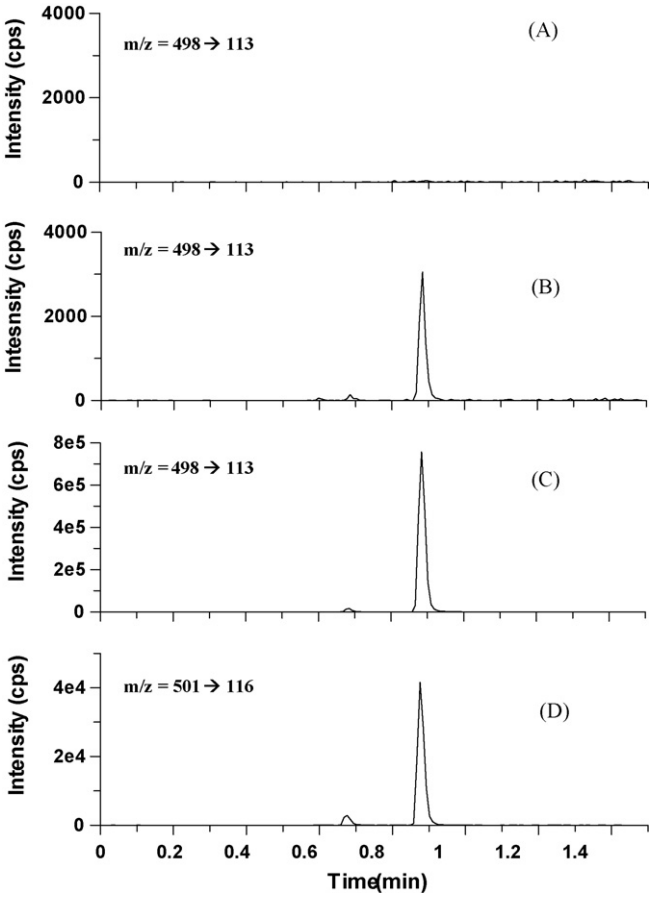

Fig. 6. Chromatograms for dansyl-derivatives of dFdU from blank human plasma (A), plasma spiked with 100 ng/mL (B), 25,000 ng/mL (C) and the internal standard (D).

at 80/4000 ng/mL for gemcitabine, and at 400/20,000 ng/mL for dFdU (in replicates of six) by comparing the mean concentrations of samples extracted after storage for 24 h against those of the samples extracted immediately upon thawing. The percentage differences between the stored and the reference QC samples were −1.2% and −6.1% for 80 and 4000 ng/mL gemcitabine and 0.3% and −9.7% for 80 and 4000 ng/mL dFdU, which indicates gemcitabine and dFdU are stable in human plasma in the presence of THU after storage at ambient temperature for at least 24 h.

The long-term freezer stability of gemcitabine and dFdU in human plasma was thoroughly investigated by Freeman et al. [15] and was not investigated in this study. According to Freeman et al. [15], both gemcitabine and dFdU are stable at −20 °C for at least 8 months and at −70 °C for at least 21 months in human plasma in the presence of THU.

Table 1
Bias, precision, and mean validation sample concentrations for gemcitabine and dFdU in human plasma in the presence of THU.

Concentration (ng/mL)	Gemcitabine					dFdU				
	20	80	500	4000	5000	100	400	2500	20,000	25,000
RUN 1, $n = 6$										
Mean	20.6	76.2	501.2	4046.3	5001.8	101	387	2468	20,292	24,815
CV (%)	11.3	6.0	3.5	2.3	2.0	5.6	3.4	2.2	4.0	2.3
Bias %	3.1	−4.8	0.2	1.2	0.0	1.2	−3.2	−1.3	1.5	−0.7
RUN 2, $n = 6$										
Mean	20.1	79.1	499.8	4049.4	4908.8	98	401	2606	21,241	25,227
CV (%)	9.4	3.5	2.2	3.7	4.3	4.2	1.9	2.8	3.2	2.7
Bias %	0.7	−1.1	0.0	1.2	−1.8	−1.8	0.2	4.2	6.2	0.9
RUN 3, $n = 6$										
Mean	18.4	77.1	495.7	4055.2	5027.5	98	391	2548	20,949	26,278
CV (%)	8.6	1.5	3.9	2.4	2.3	3.1	4.7	2.5	1.7	2.4
Bias %	−7.9	−3.7	−0.9	1.4	0.5	−2.0	−2.2	1.9	4.7	5.1
Overall totals, $n = 18$										
Mean	19.7	77.4	498.9	4050.3	4979.4	99	393	2541	20,827	25,440
Average bias (%)	−1.4	−3.2	−0.2	1.3	−0.4	−0.9	−1.7	1.6	4.1	1.8
Between-run CV (%)	4.2	1.0	1.2	1.2	0.2	0.8	1.0	2.5	2.0	2.8

3.8. Stability of gemcitabine and dFdU during freeze–thaw cycles

The stability of gemcitabine and dFdU in human plasma in the presence of THU after three freeze–thaw cycles from −20 °C to ambient temperature was assessed at 80/4000 ng/mL for gemcitabine and at 400/20,000 ng/mL for dFdU (in replicates of six) by comparing the mean concentrations against those of the freshly prepared spiked samples. The difference between the freeze–thaw samples and the freshly spiked plasma expressed as percent difference and CV (%) between replicates were less than 15%, which indicates that both gemcitabine and dFdU are stable in human plasma in the presence of THU after at least three freeze–thaw cycles from −20 °C to ambient temperature.

3.9. Matrix dilution

The ability to dilute samples containing gemcitabine and dFdU at concentrations above the HLQ was demonstrated by performing a 10-fold dilution of human plasma QC sample in the presence of THU spiked at 20,000/1,00,000 (gemcitabine/dFdU) ng/mL. Concentrations of gemcitabine and dFdU in these samples were determined and corrected for the dilution factor. The bias and precision values using human plasma as the diluent were less than 15%, indicating that a 10-fold dilution of human plasma samples in the presence of THU containing gemcitabine and dFdU up to 4 times the HLQ is acceptable.

3.10. Stability in processed samples

The stability of dansylated gemcitabine and dFdU in processed samples of human plasma was assessed by re-injecting extracted QC samples from a previous run after storage at ambient temperature for 6 days along with a freshly prepared standard curve. The accuracy and precision of these samples were found to be acceptable (less than 15%) and no changes in the sensitivity for gemcitabine or dFdU dansyl-derivatives were observed on re-injection, indicating that processed samples are stable when stored at ambient temperature for at least 6 days.

4. Conclusion

A semi-automated sample preparation method in 96-well plate format for simultaneous determination of gemcitabine and dFdU in human plasma was developed and validated over the concentration range of 20–5000 and 100–25,000 ng/mL for gemcitabine and dFdU, respectively. The method employs a simple and rapid (5 min) derivatization of gemcitabine and dFdU with dansyl chloride to improve the chromatographic retention, peak resolution, and LC–MS/MS sensitivity. The LOQ of 20 ng/mL for gemcitabine and 100 ng/mL for dFdU can be easily lowered if desired by decreasing the reconstitution volume and increasing the LC injection volume. The results reported herein suggest this method is rugged, precise, accurate, and well-suited to support pharmacokinetic studies. The relatively small plasma volume (50 μL) allows sample extraction in 96-well format, thereby increasing throughput analysis, over previously validated methods. Moreover, the relatively short run time of 1.5 min, afforded by UPLC separation, allows for the analysis of about 300 samples per day.

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