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Measurement of the Anti-Cancer Agent Gemcitabine in Human Plasma by High-Performance Liquid Chromatography

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Introduction

Gemcitabine (2′-2′-difluorodeoxycytidine, dFdC) is a deoxycytidine analog in which the deoxyribose moiety contains two fluorine atoms in place of hydrogen at the 2′-position (Fig. 1). Its anti-neoplastic activity is mediated though inhibition of DNA
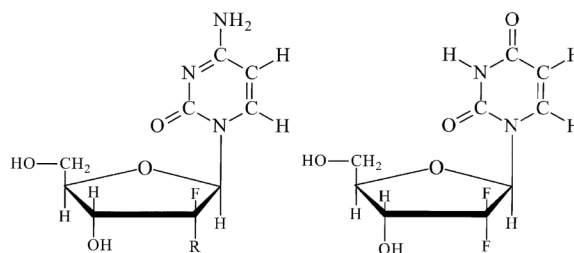

Fig. 1. The chemical structures dFdC (R=F), FdC (R=H), and dFdU.

synthesis [1–3]. The diphosphate derivative inhibits ribonucleotide reductase, thereby depleting the endogenous deoxyribonucleotide triphosphate pools. The triphosphate derivative is incorporated into DNA and subsequently interferes with DNA chain elongation. Gemcitabine is deaminated to the inactive metabolite 2′,2′-difluorodeoxyuridine (dFdU) by cytidine deaminase. Gemcitabine is used clinically to treat a variety of solid tumors, particularly pancreatic, bladder and non-small cell lung cancers.

Preclinical studies conducted in our laboratory suggested that the combination of dFdC for 4 h followed by a 24-h exposure to 5-fluoro-2′-deoxyuridine (FdU) resulted in more than additive cytotoxicity and enhanced DNA damage in a human colon cancer cell line [4]. Therefore, a Phase I trial of weekly dFdC followed by a 24-h infusion of FdU was designed. To permit determination of the plasma levels of dFdC and dFdU in patient samples, a reversed-phase HPLC assay was developed.

Experimental conditions

Chemicals

The commercial formulation of gemcitabine hydrochloride (each vial contains 200 mg of gemcitabine as the free base, 262.9 g/mol) was purchased from Eli Lilly and Company (Indianapolis, IN, USA). dFdU was generously provided by Lilly Research Laboratories though a material transfer agreement with the National Cancer Institute (NCI). FdC (HPLC internal standard), was purchased from Sigma (St Louis, MO, USA). Tetrahydrouridine (THU), an inhibitor of cytidine deaminase, was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, NCI, National Institutes of Health (NIH), Bethesda, MD, USA [5]. Glacial acetic acid (analytic reagent (AR) grade) and sodium acetate (AR grade) were from Mallinckrodt (Paris, KY, USA). HPLC grade water, methanol and acetonitrile were from Fisher (Fair Lawn, NJ, USA). All other chemicals were AR grade. Pooled donor plasma was obtained from the Department of Transfusion Medicine, Warren G.

Magnusen Clinical Center, NIH (Bethesda, MD, USA).

Standard solutions

Stock solutions of dFdC, dFdU and FdC at concentrations of 10 mM (2.63 mg/ml), 7.57 mM (2 mg/ml) and 10 mM (2.44 mg/ml), respectively, were prepared in water; aliquots were stored at −30 °C. Calibration standards were prepared by appropriate dilutions of the dFdC stock solutions with water to generate 10× calibration standards at concentrations ranging between 5 and 1500 μM; aliquots were stored at −30 °C; dilutions of the dFdU stock solutions with water generated 10× calibration standards ranging from 5 to 2270 μM. Plasma calibration standards were made by adding 50 μl of a 10× calibration standard solution to 0.5 ml of plasma to give final concentrations upon reconstitution of the extracted plasma sample that ranged between 0.5 and 150 μM for dFdC or 0.5 and 227 μM for dFdU. Fifty microliters of 250 μM FdC or 20 μl of 1 mM FdC was added to 0.5 ml plasma to give a final concentration of 25 or 40 μM (see Section 3.1).

Sample preparation

Standard curves were prepared in pooled plasma which was placed on ice immediately upon thawing. Aliquots of 0.5-ml test plasma were placed in 12×75 mm glass tubes on ice; calibration standards and 20 μl of 1 mM internal standard were added. Glacial acetic acid (50 μl) was added to decrease hydrogen bonding between the nucleosides and proteins. After the addition of 1 ml of acetonitrile, the samples were vortex-mixed. Following centrifugation at 800 g for 15 min at 4 °C, the supernatant was removed. To improve the recovery (see Section 3.2), 1 ml of acetonitrile was added to the precipitate, the sample was vortex-mixed followed by repeat centrifugation. The supernatants were combined, and the sample was evaporated to dryness using filtered compressed air in a 42 °C water bath in a Zymark TurboVap® (Hopkinton, MA, USA) and stored at −30 °C. On the day of analysis, the residue was re-suspended in 0.5 ml HPLC water, incubated for 5 min in a 37 °C water bath, and then clarified by centrifugation at

12 000 g for 10 min at 20 °C. The resultant supernatant was filtered though a 1-ml syringe fitted with a GHP 13-mm Acrodisc, 2-μm pore size, with mini-spike outlet (Pall Gelman Laboratory, Ann Arbor, MI, USA). An aliquot of each sample was placed in 250-μl low volume glass inserts placed in 4-ml glass vials (Waters, Milford, MA, USA) and loaded into a 48-position carousel tray. The injection volume was 190 μl. The residuals of the reconstituted samples were stored at −30 °C in the event that repeat injection was needed. Because the calibration standards were prepared in pre-chilled plasma on ice, the inclusion of THU (final concentration 1 μM) during their preparation did not affect either the recovery or peak heights of the analytes or internal standard.

Blood samples from patients receiving 1000 mg/m^2 of dFdC were obtained using 10-ml heparinized tubes containing 10 nmol THU. The blood collection tubes were immediately placed on ice until centrifugation at 1000 g for 10 min at 4 °C. Aliquots of plasma were stored at −70 °C until analysis. Twenty microliters of 1 mM FdC were added to 0.5-ml aliquots of plasma, and the samples were then prepared as described above.

High-performance liquid chromatography with photodiode array detection

The Waters chromatographic system (Milford, MA, USA) was used consisting of a 600E Multisolvent Delivery System, a 717 plus Autosampler set at 4 °C, a 996 Photodiode Array Detector and an in-line vacuum degasser. A Columbus™ C$_{18}$ column (5-μm particle size, 110 Å pore size, 150×4.6 mm) was employed for the separations (Phenomenex, Torrance, CA, USA). The pre-column was a Waters C$_{18}$ Nova-Pak Sentry guard column (4-μm particle size). A Micron Millennia computer (Nampa, ID, USA) was used to acquire data and perform the initial data analysis using Millenium 32 software version 3.2 (Waters).

HPLC solvent A was 50 mM sodium acetate (pH 5.0) with 2% (v/v) methanol; solvent B was the same buffer with 10% (v/v) methanol. Total flow-rate was 1.5 ml/min and the column was at ambient temperature. The following program was run: 100% A for 17 min, a linear gradient of 100% A to 100% B over 14 min, 100% B for 2 min, and immediate return to 100% A and re-equilibration for 6 min. FdC, dFdC and dFdU were monitored at wavelengths of 282, 269 and 258 nm, respectively.

Calculations

The ratios of the peak heights of dFdC or dFdU to FdC were calculated, which were then plotted versus the nominal total amount of dFdC or dFdU in the sample. The data were fitted using least squares linear regression with a weighting factor of $1/y$, not including the origin, using SigmaPlot 2001 (SPSS, Chicago, IL, USA). Analyte concentrations were determined using the calibration lines thus generated.

Recovery

Plasma samples were prepared in triplicate containing six concentrations ranging from 0.5 to 100 μM of dFdC and dFdU; each sample contained a fixed concentration of FdC (25 μM). Non-treated standards were samples in which the same concentration of the internal standard, dFdC or dFdU were prepared in water and not subjected to the precipitation procedure. The percent absolute recovery was defined as the (peak height of the precipitated plasma standard÷peak height of the non-treated standard)×100%.

Validation

Concentrations of dFdC and dFdU were determined by three calibration curves run over a 3-week period to compare the observed with the theoretical concentration. To determine within-run variation, triplicate samples at six different concentrations of dFdC and dFdU were prepared in human plasma, and the samples were injected on the same day. Between-run variation was determined by injecting triplicate samples prepared at three different concentrations on three separate occasions.

Stability of samples

The stability of the stock solutions of dFdC, dFdU and FdC was tested by preparing three concentrations of the compounds in water, and analyzing an

aliquot immediately and after storage at room temperature and at $-30\,°C$.

Analysis of patient plasma samples

Patients were participating in an Institutional Review Board-approved Phase I clinical trial involving dFdC given as either a 1- or 2-h infusion followed by a 24-h infusion of FdU weekly for 3 of 4 weeks. All patients gave written, informed consent. A baseline plasma sample was obtained pre-therapy. To provide an estimate of steady-state gemcitabine plasma concentrations, samples were obtained at 30, 40 and 50 min during the 1-h infusion, and at 90 and 105 min during the 2-h infusion.

Results

High-performance liquid chromatography

FdC, dFdC and dFdU were resolved from endogenous compounds with resulting retention times of $13.6±0.5$, $18.1±1.1$ and $29.0±0.6$ min, respectively. dFdC and dFdU were monitored at their optimal UV wavelengths, 269 and 258 nm, respectively, under the experimental conditions as determined by spectral analysis using the photodiode array detector. Because an endogenous peak (that was not present when FdC stock solution was directly injected) eluted shortly after FdC, we compensated by increasing the amount of the internal standard from 25 to 40 μM, and monitoring the peak height at 282 nm. These measures provided a greater peak height for FdC and allowed baseline resolution between FdC and the adjacent endogenous peak (Fig. 2). THU is not detectable under the conditions used.

Recovery

Absolute recovery of FdC, dFdC and dFdU from plasma was compared to non-extracted samples prepared in water under identical concentrations. In preliminary studies, it was determined that the recovery was about 10% greater when the samples were filtered though a GHP 0.2-μm syringe filter compared to the use of a PVDF filter. Application of

a second acetonitrile precipitation improved the absolute recoveries by about 17% over that achieved with a single precipitation with 1 ml acetonitrile; recovery with two sequential 1-ml precipitations was also greater than a single precipitation with 2 ml acetonitrile (data not shown). With the sample preparation described herein, the overall recovery of all three analytes from 0.5 ml plasma was $≥70\%$ for dFdC, $≥68\%$ for FdC, and $≥82\%$ for dFdU (Table 1).

Accuracy and precision

Data from three calibration curves prepared with six standard concentrations of dFdC and dFdU with a fixed FdC concentration that were examined over a 3-week period are shown in Table 2. The percent coefficients of variation (% CV) for dFdC were below 3% at concentrations $≥5$ μM, and were below 10.2% for 1 and 0.5 μM. There was greater variability with dFdU: the %CV was 22.2% with 0.5 μM, but was below 7% for higher concentrations with the exception of 5 μM (%CV 11.5%). The average r^2 values for the three calibration curves were above 0.998 for both dFdC and dFdU.

Within-run variation was assessed using six concentrations of dFdC and dFdU with fixed concentrations of FdC (25 μM) and THU (1 μM) prepared in triplicate. The results of within-run variation were determined by comparing the peak heights for dFdC, dFdU and FdC for these nine separate samples injected into the HPLC system on the same day. The coefficients of variation ranged from 1.1 to 6.0% for dFdC, and were $<4.3\%$ for dFdU and FdC (Table 3). Assessment of between-run variability was carried out subsequently using concentrations of dFdU and dFdC that would encompass the expected plasma concentrations taken from patients during the 1- to 2-h dFdC infusion. The coefficients of variation were $≤5.1\%$ for three compounds (Table 4).

Linearity

The assay was linear between 0.5 and 150 μM (0.13–39.4 μg/ml) for dFdC and 1 to 227 μM (0.26–60.0 μg/ml) for dFdU.
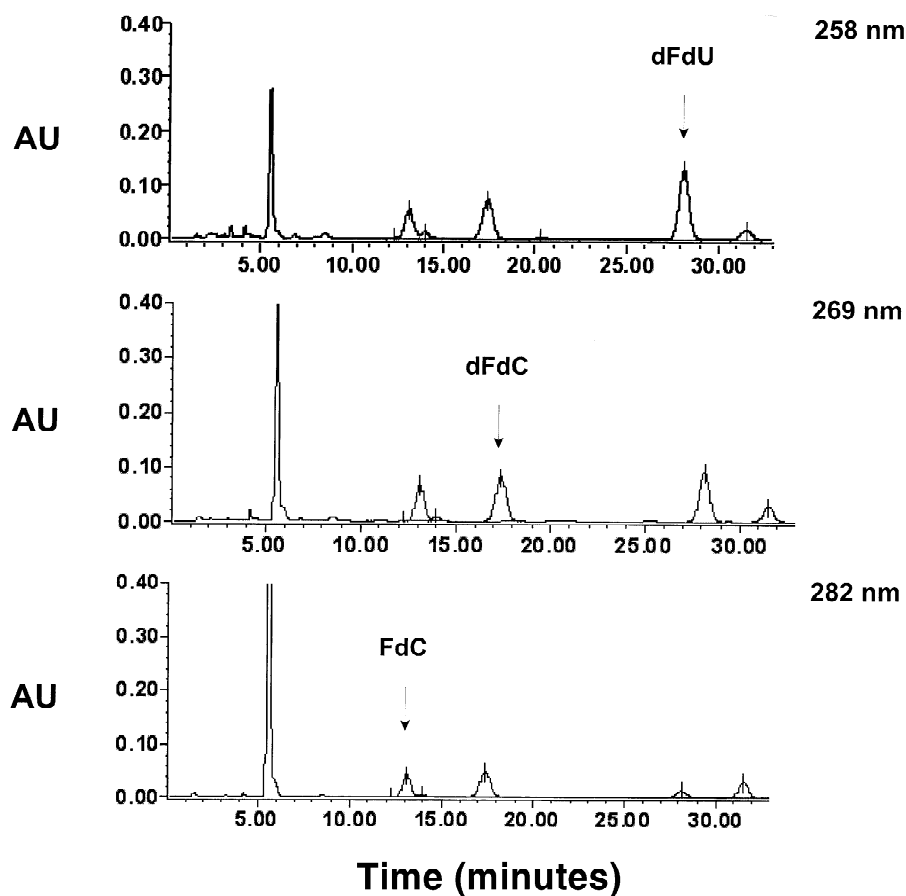
Fig. 2. Chromatography and UV detection of dFdC and dFdU in the plasma of a patient 40 min after the start of a 1-h infusion of 1000 mg/m^2 of dFdC. Identified peaks include the internal standard, dFdC and dFdU. The calculated plasma concentrations of dFdC and dFdU were 23.7 and 59.2 μM, respectively.

Table 1
Absolute recovery of internal and calibration standards

Analyte		0.5 μM	1 μM	5 μM	10 μM	50 μM	150 μM
dFdC	Mean	75.8	78.8	80.2	86.3	70.0	78.5
	SD	1.2	0.9	1.2	3.5	2.6	4.7
	%CV	1.6	1.1	1.5	4.1	3.8	6.0
dFdU	Mean	95.2	86.4	87.4	81.8	87.5	101.4
	SD	2.6	1.0	1.7	3.5	0.8	2.2
	%CV	2.8	1.2	2.0	4.3	0.9	2.2
FdC	Mean	68.3	76.2	67.9	83.4	68.0	75.8
	SD	1.8	1.5	1.5	3.5	2.1	0.9
	%CV	2.7	2.0	2.2	4.2	3.1	1.2

Triplicate samples of internal standard (fixed concentration of 25 μM) and calibration standards were prepared in both plasma and water. The plasma was subjected to the sample preparation described in Section 2, whereas aliquots of the standards prepared in water were directly injected into the HPLC without further processing.

Table 2
Gemcitabine (dFdC) and 2′,2′-difluorodeoxyuridine (dFdU) calibration standards in human plasma

Concentration (μM)	Measured dFdC (μM) (mean±SD)	dFdC %CV	Measured dFdU (μM) (mean±SD)	dFdU %CV
150	152.1±3.0	2.0	152.4±3.4	2.2
50	48.7±1.2	2.5	48.2±1.5	3.1
10	9.4±0.3	2.7	9.8±0.7	6.7
5	4.8±0.10	2.0	4.7±0.5	11.5
1	1.0±0.1	10.2	1.1±0.0	1.6
0.5	0.53±0.05	8.8	0.52±0.11	22.2

Calibration curve parameters	dFdC	dFdU
r^2	0.9989±0.0009	0.9985±0.0014
X-coefficient	0.075±0.005	0.067±0.016
Y-intercept	−0.0038±0.0095	0.0262±0.0360

The data are from three calibration curves in human plasma done over a 3-week period. The internal standard concentration was 40 μM.

Table 3
Within-run precision for gemcitabine (dFdC) and 2′,2′-difluorodeoxyuridine (dFdU) in human plasma

Concentration (μM)	dFdC			dFdU			25 μM FdC		
	Peak height	SD	%CV	Peak height	SD	%CV	Peak height	SD	%CV
0 5	1917	73	3 8	2232	62	2 8	76 717	627	0 8
1	4614	274	5 9	5413	119	2 2	79 946	1571	2 0
5	22 625	334	1 5	21 226	422	2 0	76 761	1954	2 6
10	53 242	2186	4 1	40 011	1732	4 3	77 222	3214	4 2
50	223 332	3488	1 6	229 301	2037	0 9	73 603	5146	7 0
150	896 568	9901	1 1	600 932	7148	1 2	68 415	831	1 2

The data are from samples prepared in triplicate and injected during the same HPLC run.

Stability

It has been reported that both gemcitabine and dFdU are stable for at least 21 months at −70 °C in THU-treated human plasma, and that both analytes were unaffected by three freeze–thaw cycles [8]. Therefore, we did not repeat this analysis. In the current study, stock solutions of 1 mM FdC, 1 mM, 100 and 10 μM dFdC, and 7.57 mM, 378.5 and 37.85 μM dFdU were stable at −30 °C for at least 1 month, and at both 4 °C and room temperature for at least 1 week (data not shown).

Testing of patient plasma samples

Plasma dFdC and dFdU concentrations were determined as part of a Phase I trial of weekly dFdC followed by infusional FdU. The plasma concentrations for six patients each receiving 1000 mg/m^2 dFdC as either a 1- or 2-h infusion are shown in Fig.

Table 4
Between-run precision for gemcitabine (dFdC) and 2′,2′-difluorodeoxyuridine (dFdU) in human plasma

Concentration (μM)		dFdC			dFdU			40 μM FdC		
dFdC	dFdU	Peak height	SD	%CV	Peak height	SD	%CV	Peak height	SD	%CV
5	15	25 449	389	1 5	74 850	1755	2 4	107 342	3007	2 4
25	95	125 054	962	0 8	428 440	21 737	5 1	108 677	3053	2 8
100	227	524 059	10 345	2 0	1 009 497	39 371	3 9	112 268	1977	1 8

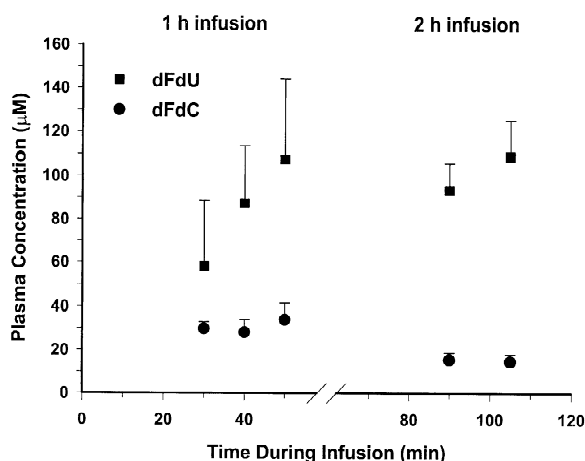The data are from samples prepared in human plasma in triplicate and injected on three different days.

Fig. 3. Plasma concentrations of dFdC and dFdU in patients receiving 1000 mg/m^2 gemcitabine. The data, shown as the mean±SD, are from six patients who each received either a 1-h or 2-h infusion.

3. There were no interfering endogenous peaks in the pre-therapy samples under the conditions used. In all patients, dFdC and dFdU were detected at the planned sampling times. The dFdC levels were similar at 30, 40 and 50 min in patients receiving a 1-h infusion, and at 90 and 105 min in those receiving a 2-h infusion, suggesting the drug was at or near steady-state. The average plasma levels for all time points for the 1-h infusion, 30.7 μM, was about twofold higher than that observed with the 2-h infusion, 15.2 μM. In contrast, the dFdU levels appeared to be increasing throughout the infusion, and were on average 3.2- and 7.6-fold higher than the corresponding dFdC levels at the 50- and 105-min sampling times after the start of a 1- and 2-h infusion, respectively. This observation is consistent with a longer half-life for dFdU, which is cleared by renal excretion, as opposed to the predominantly metabolic clearance of dFdC.

Discussion

In the process of developing the current method, we evaluated several methods for sample preparation, including solid-phase extraction using different types of cartridges (phenylboronate, C$_{18}$), and liquid–liquid extraction using either methanol, ethyl acetate, or acetonitrile. We found that liquid–liquid extraction with two sequential acetonitrile applications yielded the best recovery.

Several studies have been published that describe analysis of dFdC and dFdU in plasma samples obtained from cancer patients receiving gemcitabine [6–10]. However, only a few employed an internal standard and provided sufficient information to ensure method reproducibility [6,9]. The first validated assay reported from researchers at Lilly Research Laboratories used normal-phase HPLC with an Alltech™ amino column [6]. The procedure required sequential liquid–liquid extraction using both iso-propanol and ethyl acetate followed by concentration to dryness; the reconstituted residue was then filtered though an ultra-free MC filter to remove insoluble material. The mobile phase represented an aqueous mixture containing five different solvents.

An additional published method used acetonitrile precipitation for sample preparation, and an isocratic mobile phase of 15 mM ammonium acetate, pH 5.0, and acetonitrile (97.5:2.5, v/v) for 47 min followed by a linear increase to 15 mM ammonium acetate, pH 5.0, and acetonitrile (95:5, v/v) over 5 min [9]. The latter solvent was run isocratically for 10 min followed by a linear return over 5 min to initial conditions. The column was allowed to equilibrate for 10 min prior to the next injection, for an overall cycle time of 77 min to resolve dFdC, dFdU and the internal standard, 2′,2′-difluorodeoxythymidine.

The current method employs reversed-phase HPLC that offers a shorter cycling time (39 min), partly due to the selection of the internal standard FdC, which elutes sooner than dFdC and has similar recovery. This method has proven useful in the analysis of samples from patients receiving dFdC given as a 1- or 2-h infusion weekly for 3 of 4 weeks. By using a smaller volume of plasma (0.1–0.2 ml), the methodology described herein can also be employed in the analysis of samples obtained during a 30-min infusion of dFdC.

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