# Determination of gemcitabine and its metabolite in human plasma using high-pressure liquid chromatography coupled with a diode array detector[1]

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**KEY WORDS** high pressure liquid chromatography; gemcitabine; deoxycytidine; floxuridine

## ABSTRACT

**AIM:** To establish a high pressure liquid chromatography (HPLC) method for determination of the concentration of gemcitabine (dFdC) and its metabolite (dFdU) in human plasma. **METHODS:** Plasma 1.0 mL spiked with floxuridine as an internal standard was extracted with 3.0 mL of methanol-acetonitrile (v/v, 1:9). The supernatant was evaporated at 60 ºC and the residue was reconstituted with 0.5 mL of the solution used as the mobile phase. After centrifugation, 50 μL of the supernatant was injected into the HPLC system. Separation was achieved on a $C_{18}$ (4.6 mm×250 mm, 5 μm) column at 25 ºC with the flow rate of the mobile phase set to 0.8 mL/min. The compounds were detected at 268 nm. The mobile phase consisted of 40.0 mmol/L acetate ammonium buffer solution (pH 5.5) and acetonitrile (v/v, 97.5:2.5). **RESULTS:** The linear range was 0.20-10.0 mg/L ($r$=0.9999) for dFdC and 0.50-50.0 mg/L ($r$=0.9999) for dFdU. The limit of detection (LOD) was 0.10 mg/L for dFdC and 0.25 mg/L for dFdU, while the limit of quantification (LOQ) was 0.20 mg/L (RSD<10 %) for dFdC and 0.50 mg/L (RSD <3 %) for dFdU. The average recovery of dFdC and dFdU by this method were 103.3 % and 98.7 %, respectively. For intra-day and inter-day, the corresponding standard deviations of the measurements of dFdC and dFdU were both less than 5.5 %. **CONCLUSION:** An analytical method was established to measure the concentrations of dFdC and dFdU in human plasma and was effectively applied to the dFdC and dFdU pharmacokinetic studies of 8 Chinese patients with malignant tumors.

## INTRODUCTION

Similar to cytosine arabinoside (Ara-C), gemcitabine (2,2′-difluorodeoxy-cytidine, dFdC) (Fig 1A) is a novel deoxycytidine analogue. However, when compared to Ara-C, dFdC is more potent and has a broader therapeutic index against several solid tumors, of which non-small-cell lung cancer, breast cancer, and pancreas cancer[1-3] are the most responsors. Gemcita-bine also demonstrates good efficacy both administering alone and in combination with other anticancer drugs. Like Ara-C, dFdC is a prodrug which, after intracellular phosphorylation, exerts its cytotoxic effects through its active intracellular metabolites,gemcitabine di- and tri-phosphate. After intravenous administration, dFdC is rapidly metabolized in the liver, kidney, and other tissues to a noncytotoxic metabolite (2,2′-difluorodeoxy-uridine, dFdU)[4,5] (Fig 1B).

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Several methods, including enzyme linked immunosorbance assay (ELISA)[5], $^{19}$F-NMR[6], high-performance liquid chromatography tandem-mass spectrometry (HPLC/NS)[7], derivative-spectrophotometric[8], and HPLC, have been reported for determining the concentrations of dFdC and dFdU in plasma, cerebrospinal fluid, urine, and human carcinoma cells[9-13]. However, in our experience it was technically difficult to precipitate plasma protein and extract dFdC and dFdU using the current reported RP-HPLC methods. Therefore, we have developed a new HPLC method for determining the concentrations of dFdC and dFdU in patient's plasma. In this new method, a mixture of acetonitrile and methanol (9:1 v/v) was found to be an effective solvent to precipitate protein, and floxuridine was used as an alternative internal standard to deoxycytidine since deoxycytidine was proved to have the same retention time to several impurities in plasma.

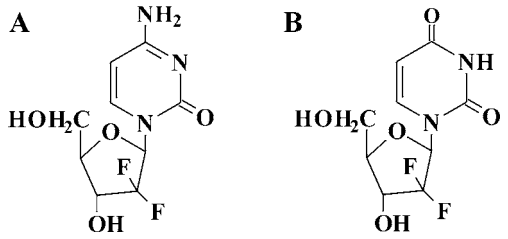


**Fig 1. Chemical structures of gemcitabine (A) and the metabolite, 2,2′-difluorodeoxy-uridine (B).**

## MATERIALS AND METHODS

**Reagents** Gemcitabine mono-hydrochloride was obtained from Eli Lilly Company (Indianapolis, USA). The internal standard (IS), floxuridine, was donated by Haizheng Pharmaceutical Manufacturing Company (Zhejiang, China), while the metabolite, dFdU was obtained from Department of Clinical Pharmacology, Zhejiang Cancer Research Institute (Hangzhou, China). All other chemicals and solvents were of chromatographic or analytical grade and were obtained from several commercial sources. Drug-free heparinized human plasma was obtained from Hangzhou Central Blood Station (Hangzhou, China).

**Chromatographic equipment and conditions** HPLC system (Agilent 1100) was equipped with a G1311A pump, a G1314A programmable diode array detector (DAD) and a G1313A auto-injector. A Hewlett Packard (HP) 1000 computer with in-house developed software was used for on-line data acquisition and subsequent calculations. The analytical column was packed with Lichrospher $C_{18}$ (250 mm×4.6 mm ID, 5 μm). The mobile phase used to run the column consisted of 40 mmol/L acetate ammonium buffer solution (pH 5.5)/ acetonitrile (97.5:2.5, v/v) and the detector operated at 268 nm. The injection volume was 50.0 μL, the flow rate of mobile phase was set to 0.8 mL/min, and the experiment was performed at 25 ºC.

The LC/MS (Esquire, Bruker, Germany) was equipped with an electrospray mass spectrometric detector and operated with a positive ion module.

**Preparation of stock and standard solutions** A reference stock solution of dFdC (200.0 mg/L) was prepared by dissolving 20.0 mg dFdC in 100.0 mL of sterile water, which was subsequently stored at 4 ºC, while the reference stock solution of dFdU (1000.0 mg/L) was prepared by dissolving 2.0 mg dFdU in 2.0 mL of sterile water, which was also stored at 4 ºC. The IS stock solution (202.0 mg/L) was prepared with the same procedure as dFdC and was stored at 4 ºC until use, when it was diluted to 8.0 mg/L.

**Assay procedure** Methanol-acetonitrile 3.0 mL (v/v 1:9) was added to 1.0 mL plasma containing 100.0 μL of IS. The sample was vortexed for 1 min and then centrifuged at 3500 r/min for 10 min, after which the supernatant was transferred to a disposable tube. This solution was evaporated to dryness at 60 ºC in a water bath under a gentle stream of nitrogen. The residue was dissolved in 0.5 mL of the solution used as mobile phase and then centrifuged at 15000 r/min for 10 min. Following this, 50 μL of the supernatant was injected into the HPLC system using a G1313A automatic sample injector.

## RESULTS AND DISCUSSION

**Chromatographic conditions and selectivity** Isopropranol, methanol, acetonitrile, and a mixture of acetonitrile and methanol (9:1 v/v) as solvents were used to precipitate plasma proteins. Our results indicate that isopropanol is a suitable solvent for small volumes of plasma (<0.3 mL). Using methanol or acetonitrile, we found that several impurities in the plasma with the same chromatographic retention times as the dFdC or dFdU were precipitated together with dFdC and dFdU. Therefore, these two solvents were not feasible for isolation of dFdC or dFdU. Our experiments show that a mixture of acetonitrile and methanol (9:1 v/v) is the most effective and suitable solvent to precipitate pro-

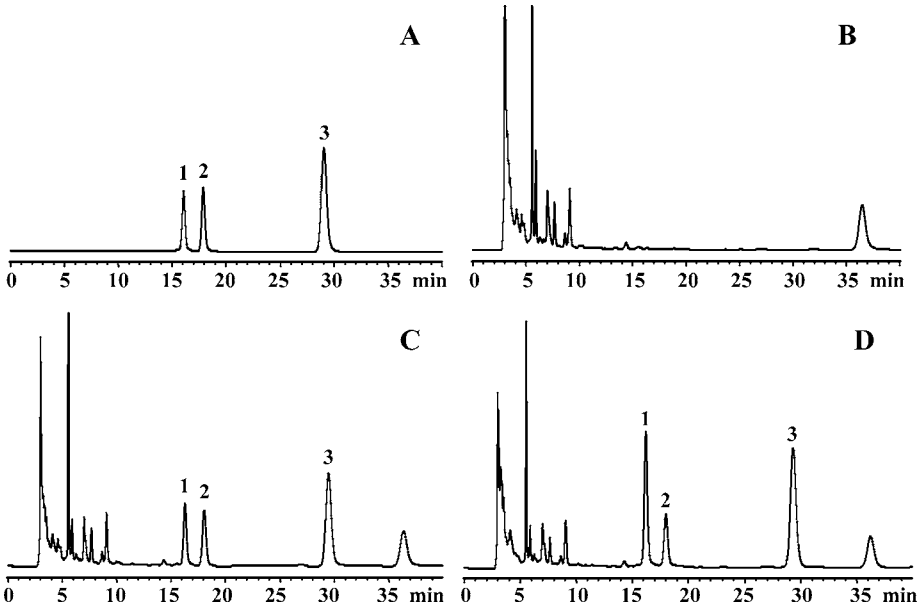


**Fig 2. Typical HPLC chromatograms. (A) Mobile phase spiked with 2.5 mg/L of dFdC, 10.0 mg/L of dFdU, and 2.5 mg/L of internal standard (IS). (B) Blank human plasma; (C) Blank human plasma spiked with 2.5 mg/L of dFdC, 10.0 mg/L of dFdU, and 2.5 mg/L of IS; (D) An example of one of the plasma samples from a patient's plasma at 0 min after infusion of 1000 mg/m$^2$ for 30 min of dFdC (1), floxuridine (2), and dFdU (3).**

tein as no interference was observed in our experiment at the peak positions of dFdC, dFdU, and the IS (Fig 2B). In addition, heating the sample in boiling water can precipitate plasma protein. However, the sensitivity of this method was lower because the sample proved difficult to concentrate. Also, the use of acidic agents to precipitate proteins resulted in hydrolyzation of gemcitabine.

Deoxycytidine is usually used as an internal standard as reported[10-12]. We examined seven compounds that had similar chemical structures to select an alternative IS. The retention time of the seven compounds, fluorouracil, aracytidine, deoxycytidine, gemcitabine, floxuridine, dFdU, and tegafur, were 2, 8.1, 8.8, 16.5, 18.3, 29.2, and 66.8 min, respectively. Fluorouracil, aracytidine, and deoxycytidine had the same retention time to several impurities in plasma, while the retention time of tegafur was too long. Floxuridine proved to be the most suitable compound as an IS (Fig 2C). In addition, floxuridine was stable for at least three months in water but proved to be unstable at pH below 5.0.

The maximum absorbance wavelength of dFdC and floxuridine is 268 nm, while dFdU is 253 nm. In order to increase the sensitivity for dFdC, the detection wavelength was set at 268 nm, as the half-time of gemcitabine in patient's plasma is very short, when compared with that of dFdU.

Using this chromatographic condition, dFdC, dFdU and the IS floxuridine were completely separated with a resolution >1.5. Typical chromatograms of blank plasma sample are shown in Fig 2. Detection of dFdU was confirmed by HPLC-MS (Fig 3). Therefore, this

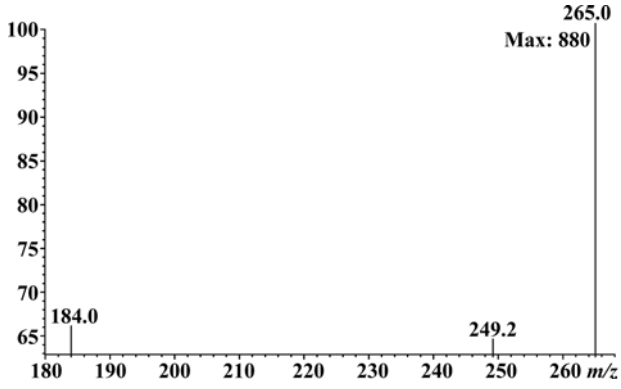


**Fig 3. Mass spectrum of 2,2′-difluorodeoxyuridine ($M_r$: 264). Positive parent ion ([M+1]$^-$ *m/z* 265)**

is a specific method to determine the concentrations of dFdC and dFdU in human plasma.

**Calibration curves** A series of drug-free human plasma spiked with different concentrations of dFdC and dFdU were processed. The final concentrations of

dFdC were 0.2, 0.35, 0.5, 1.0, 2.5, 5.0, and 10.0 mg/L, while those of dFdU were 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 mg/L. The calibration curves of dFdC and dFdU were constructed by plotting peak area ratio ($y$) of dFdC or dFdU over internal standard *vs* dFdC or dFdU concentration ($x$). The regression equations were $y$=1.1094$x$-0.02446 ($r$=0.9999) for dFdC and $y$=0.785$x$+0.03189 ($r$=0.9999) for dFdU.

**Accuracy and precision** The blank plasma samples spiked with different concentrations of dFdC and dFdU were processed. For the method recovery studies, the concentrations of dFdC and dFdU were calculated using the calibration curves. For the extraction recovery studies, the peak areas of dFdC and dFdU were compared with those of the reference solutions.

**Tab 1. Recovery and precision of dFdC and dFdU in human plasma (%, $n$=5).**

| Compound | Theoretical concentration/mg·L$^{-1}$ | Extraction recovery/% | Method recovery/% | Precision (RSD)/% intra-day | inter-day |
|---|---|---|---|---|---|
| dFdC | 0.2 | 98.9±9.3 | 106.6±2.9 | 2.7 | 4.1 |
| | 1.0 | 97.8±2.4 | 103.2±2.4 | 2.3 | 5.2 |
| | 10.0 | 91.8±3.5 | 100.1±4.0 | 4.0 | 3.2 |
| dFdU | 0.5 | 93.4±2.6 | 94.8±2.8 | 3.0 | 3.5 |
| | 5.0 | 98.6±3.5 | 100.8±3.6 | 3.6 | 3.6 |
| | 50.0 | 96.1±2.1 | 100.4±2.2 | 2.2 | 4.0 |

The results and accuracy of the analytical method were shown in Tab 1.

Drug-free plasma spiked with different concentrations of dFdC (0.2, 1.0, and 10.0 mg/L) and dFdU (0.5, 5.0, and 50.0 mg/L) were used for intra-day and inter-day precision studies. Five samples were taken within a day for intra day study and each day over five consecutive days for inter-day study. These samples were analyzed. The corresponding standard deviations for intra-assay and inter-assay were all less than 5.5 % for dFdC and 4.0 % for dFdU (Tab 1).

**Stability of samples**

Stability of stock solutions The stability of the stock solutions of dFdC, dFdU as well as the internal standard were evaluated at d 1, d 7, d 14, d 30, d 60, and d 90. Before analysis, the stock solutions of dFdC, dFdU, and the internal standard were diluted to 5.0, 10.0, and 0.8 mg/L with the solution used as mobile phase. The corresponding standard deviations of the concentrations measured from five repeated assays were all less than 0.5 %. These results show that the stock solution of dFdC, dFdU, and internal standard are stable for at least 90 d at 4 ºC.

Stability of plasma sample Plasma samples spiked with different concentrations of dFdC (0.2 and 10.0 mg/L) and dFdU (0.5 and 50.0 mg/L) were processed. The concentrations of dFdC, dFdU, and the IS in the plasma samples were measured at 0, 4, 8, 12, and 16 h by recording and calculating the peak areas of HPLC chromatography. The results indicate that the plasma samples are stable for at least 16 h.

**Sensitivity** Blank human plasma samples spiked with low concentrations of dFdC and dFdU were prepared. HPLC of these samples was taken and the peak areas corresponding to dFdC and dFdU were measured. The results showed that the limit of detection (LOD) was 0.1 mg/L for dFdC and 0.25 mg/L for dFdU ($S/N$>3), and the limit of quantification (LOQ) was 0.20 mg/L for dFdC (RSD<10 %, $n$=5) and 0.50 mg/L for dFdU (RSD<3 %, $n$=5).

**Application of the method to gemcitabine pharmacokinetic study** With approval of the Ethics Committee of Zhejiang Cancer Hospital and College of Pharmaceutical Sciences, Zhejiang University, eight patients (49 to 61 a) with malignant tumors participated the pharmacokinetic studies. Before the treatment, informed consent was obtained from all the patients for the treatment regime and for obtaining blood samples from them for pharmacokinetic studies. Each of the patients was infused with 1000 mg/m$^2$ of gemcitabine for 30 min. Approximately, 2.5 mL of whole blood samples were collected from forearm veins of the patient into heparinized polypropylene centrifuge tubes at 0, 0.25, 0.5, 1, 1.5, 2, 4, 8, 16, 24, 48, 96, and 168 h after infusion. The blood samples were placed on ice and immediately transported to the laboratory, where they were centrifuged for 10 min at 3000 r/min at 4 ºC. Following this, the plasma was stored at -70 ºC until assay. The plasma was prepared using the procedures described in section 2.4. Due to a wide range of concentrations of dFdC and dFdU seen in patient plasma samples, it was often necessary to dilute the samples to achieve measurable concentrations using the standard curve. The concentration-time curves of of dFdC and dFdU from 8 patients' plasma were plotted from the data of the studies and shown in Fig 4.

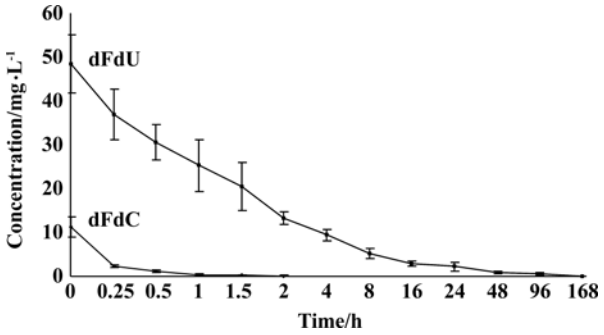


**Fig 4. Concentration-time curves of dFdC and dFdU after infusion of 8 patients with 1000 mg/m² gemcitabine for 30 min.**

Data from pharmacokinetic studies were calculated using PKS software (Pharmacokinetic Calculation System-version 1.0.2, Hongneng Software Company, Shanghai, China). The results for both dFdC and dFdU fit biphasic kinetic models and their pharmacokinetic parameters were shown in Tab 2. Compared with the published pharmacokinetic data[5,14], no apparent difference was found with respect to peak plasma concentration, elimination half-life, clearance, or AUC (Tab 2).

## CONCLUSION

A new reversed phase HPLC method was developed for the detection of dFdC and dFdU in human plasma. This analytical method was proved to be simple and efficient. In addition, it also showed good sensitivity, accuracy, and precision. This method was effectively applied to a dFdC pharmacokinetic study of 8 Chinese patients with malignant tumors.

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**Tab 2. Pharmacokinetic parameters of dFdC and dFdU (*n*=8).**

| Compound | $C_{max}$/mg·L$^{-1}$ | $T_{1/2\alpha}$/h | *Cl*/mL·min$^{-1}$ | $V_d$/L | AUC/mg·L$^{-1}$·h |
|---|---|---|---|---|---|
| dFdC | 11.3±2.4 | 0.42±0.13 | 4780±850 | 270±53 | 2.2±0.4 |
| dFdU | 48±7 | 8±4 | 103±25 | 66±24 | 170±39 |

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