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Simultaneous determination of gemcitabine and its metabolite in human plasma by high-performance liquid chromatography

Bilal Yılmaz[a], Yücel (Yaşar) Kadıoğlu[a,*], Yılmaz Aksoy[b]

[a]*Atatürk University, Faculty of Pharmacy, Department of Analytical Chemistry, 25240, Erzurum, Turkey*
[b]*Atatürk University, Faculty of Medicine, Department of Urology 25240, Erzurum, Turkey*

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Abstract

Gemcitabine (dFdC) is a pyrimidine antimetabolite with broad spectrum activity against tumors. In this paper, a normal-phase high-performance liquid chromatographic method was developed for the determination of the parent drug (dFdC) and its metabolite (dFdU) in human plasma. The described sample preparation procedure for determination of dFdC and dFdU is rapid, sensitive, reproducible and simple. The linear regression equations obtained by least square regression method, were area under the curve=0.0371 concentration (ng ml^{-1})+192.53 and $1.05 \cdot 10^{-4}$ concentration (ng ml^{-1})−1.2693 for dFdC and dFdU, respectively. The assay for dFdC and dFdU described in the present report has been applied to plasma samples from a bladder cancer patient.

Keywords: Gemcitabine; 2′-Difluoro-2′,2′-deoxyuridine

1. Introduction

2′,2-Difluorodeoxycytidine (gemcitabine, dFdC, Gemzar) is a deoxycytidine analoque [1] with clinical activity against several solid tumors, including ovarian cancer, non-small cell lung cancer, head and neck squamous cell carcinoma, pancreatic cancer and bladder cancer [2]. dFdC is a prodrug that undergoes metabolism by cytidine deaminase to from an inactive metabolite (Fig. 1a and b) [3]. The metabolite (dFdU, 2′-difluoro-2′,2′-deoxyuridine) was also the primary metabolite observed in human plasma and urine [4].

Several methods have been reported for the determination of dFdC and dFdU [3–10]. An ELISA assay [4] has been used for the determination of dFdC and dFdU in human plasma and urine, while ^{19}F-NMR [5] and HPLC techniques [3,6–10] have been used for the quantitation of dFdC and dFdU in biological fluids. But no HPLC method with a diode array detection (DAD) system in bladder cancer has been reported in the literature. We wanted to develop a new HPLC method for the determination of dFdC and its metabolite in plasma.

The aim of this work was to improve a method using a smaller sample volume and a more sensitive and specific DAD system, and to validate the whole

*Corresponding author. Tel.: +90-442-231-1539; fax: +90-442-236-0962.
E-mail address: ykadiogli@yahoo.com (Y.(. Kadıoğlu).

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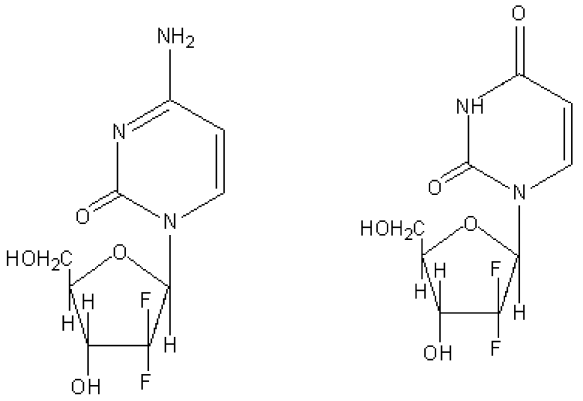

(a) **(b)**

Fig. 1. Chemical structures of (a) gemcitabine (dFdC) and (b) its metabolite (dFdU).

analytical method, according to international guidelines in order to obtain an efficient tool for further pharmacokinetic studies.

2. Experimental

2.1. Instruments

HPLC was carried out using a Thermoseparations Spectra Series P 4000 gradient pump coupled with a Spectra System UV 6000 LP photodiode array detection system and a Thermoseparations AS 3000 autosampler. The detector was set to scan from 200 to 500 nm and had a discrete channel set at 272 nm, which was the wavelength used for quantification.

2.2. Reagent and standards

Gemcitabine and dFdU were kindly provided by Lilly Research (Eli Lilly, Indianapolis, IN, USA). HPLC grade methanol, cyclohexane and 1,2-dichloroethane were purchased from Merck. All other chemicals were obtained from commercial sources and were of analytical grade.

2.3. Chromatographic conditions

The analytical column was Nucleosil 5 NH_2 (5 μm, 250× 4.0 mm). The column temperature was 30 °C. CHROMQUEST software was used for on-line data acquisition. The mobile phase consisted of methanol–cyclohexane–1,2-dichloroethane (30:50:20, v/v/v). The mobile phase was filtered through nylon membrane of 0.45-μm pore size (47 mm filter membrane, USA). The flow-rate was set at 1.0 ml min^{-1}. The peaks of interest eluted within 10 min. An injection volume of 10 μl was used.

2.4. Preparation of plasma standards and controls

A standard stock solution containing dFdC and dFdU was prepared in methanol at a concentration of approximately 50 μg ml^{-1} of each compound. The stock solutions were stable for at least 3 weeks when stored refrigerated.

Standard calibration solutions were prepared by spiking drug-free human plasma with stock standard solutions, which were then further diluted to achieve final concentrations of between 0.2 and 50 μg ml^{-1} of dFdC and 0.2 and 40 μg ml^{-1} of dFdU. dFdC plasma control samples were prepared from a separate stock solution at concentrations of approximately 3, 10, 20 and 50 μg ml^{-1} while those of dFdU were 0.5, 5.0, 20 and 40 μg ml^{-1}.

2.5. Plasma sample preparation procedure

Individual 0.2-ml aliquots of plasma standards, controls and subject samples were pipetted into 12-ml disposable glass tubes. Standard solutions (0.2 ml) were added, into the plasmas and the solutions were briefly vortexed. Then 1 ml of isopropanol was added, followed by vortex-mixing. The samples were allowed to sit for 5 min. A 2.5-ml aliquot of ethyl acetate was added and vortex-mixed and then the samples were centrifuged for 10 min at approximately 2500 *g* at 4 °C. The organic phase was transferred to a 5-ml tube and evaporated to dryness at 40 °C under a stream of nitrogen. The dried residues were reconstituted in 1 ml of methanol. All samples were filtered through a Phenomenex membrane of 0.45-

μm pore size before injection. Injection volumes of samples and standards (10 μl) were performed with an automatic sample injector.

3. Results and discussion

3.1. HPLC measurements and validation

The criteria established for the development of our analytical procedure include: (1) using the smallest amount of mobile phase possible; (2) restricting k' values to between 1 and 10; (3) ionization suppression between drug molecule and residual amino groups of the surface of silica.

It is known that HPLC–DAD is a highly effective screening method. Criterion for identification of the analyte is that the maximum absorption wavelength in the UV spectrum of the analyte should be the same as that of the standard material within ±2 nm. The use of the photo-DAD also confers the advantage of identifying the analyte both by retention time and UV spectrum.

The following parameters were determined for the validation of analytical method developed for dFdC and dFdU in human plasma; linearity, precision, accuracy, LOQ, recovery, ruggedness and stability [11]. Peak purities for dFdC and dFdU were further confirmed by means of a photo-DAD system.

A standard stock solution containing dFdC and dFdU was prepared in methanol at a concentration of approximately 50 μg ml^{-1} of each compound. The stock solutions were stable for at least 3 weeks when stored refrigerated. dFdC concentrations in the working standard solutions chosen for the calibration curve were 0.2, 2.0, 5.0, 10, 15, 25, 35 and 50 μg ml^{-1} while that of dFdU were 0.2, 0.3, 0.5, 1.0, 2.5, 5, 10, 20, 30 and 40 μg ml^{-1}. These working solutions were made by further dilution of the stock solutions in methanol. dFdC plasma control samples were prepared from a separate stock solution at concentrations of approximately 3, 10, 20 and 50 μg ml^{-1} while those of dFdU were 0.5, 5.0, 20 and 40 μg ml^{-1}.

Under the described chromatographic conditions dFdC and dFdU are well separated. Typical chromatograms of blank plasma sample, spiked plasma sample containing 10 μg ml^{-1} dFdU and dFdC are shown in Fig. 2a–c. The mean retention times were 7.5 and 4.3 min for dFdC and dFdU, respectively. A chromatogram of 10 μg ml^{-1} of dFdC and its metabolite (dFdU) spiked in plasma is shown in Fig. 3. A chromatogram of 15 μg ml^{-1} of the injectable dosage form of commercial Gemzar spiked in plasma is shown in Fig. 4.

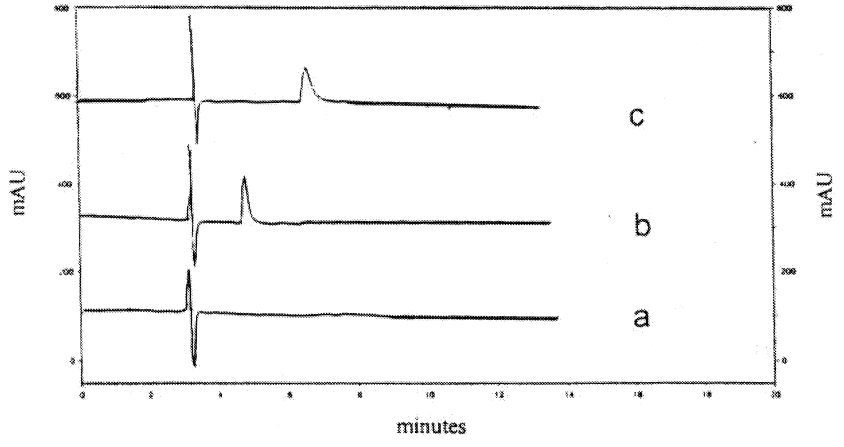

Fig. 2. Representative chromatograms of (a) blank human plasma without compounds, (b) human plasma standard spiked with 10 μg ml^{-1} of dFdU, (c) human plasma standard spiked with 10 μg ml^{-1} of dFdC.

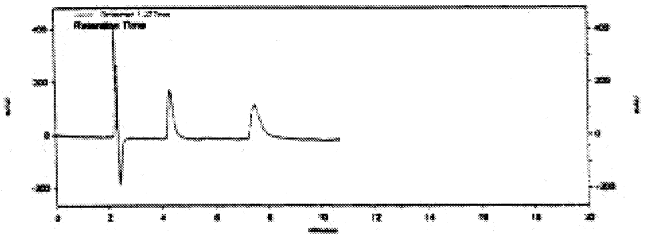

Fig. 3. Chromatogram of spiked human plasma sample containing 10 μg ml^{-1} dFdC and its metabolite 10 μg ml^{-1} dFdU.

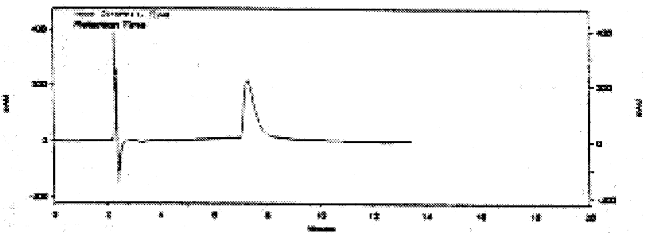

Fig. 4. Chromatogram of 15 μg ml^{-1} of the injectable dosage form of commercial Gemzar spiked in human plasma.

3.2. Linearity

The linearity of the response for the plasma assay was established over the concentration range 0.2–50 μg ml^{-1} for each dFdC and 0.2–40 μg ml^{-1} for each dFdU. Typical correlation coefficients were >0.99 (Table 1).

3.3. Precision and accuracy

The precision of a quantitative method is the degree of agreement among individual test results when the procedure is applied repeatedly to multiple samplings. It is measured by repeatedly injecting a ready-made sample pool and expressed as relative standard deviation of the results.

The precision of the test was evaluated by determining the inter- and intra-day RSD of the measured peak areas for different concentrations.

The precision and accuracy for each procedure was determined by spiking dFdC and dFdU into drug-free human plasma at four concentrations. Six replicates from each pool were assayed on each of 3 days so that both intra- and inter-day precision and accuracy could be determined. The results for dFdC and dFdU in human plasma are shown in Tables 2 and 3, respectively. For all the concentrations studied, the RSD values and the relative errors for the plasma method were <10% and for all concentrations of compounds the accuracy was >90%.

3.4. Limits of quantitation

The LOQ, defined in the presented experiment as the lowest plasma concentration in the calibration curve that can be measured routinely with acceptable precision (RSD<20%)

The lower LOQ for dFdC and dFdU assays were

Table 1
Features of the calibration curves of dFdC and its metabolite (dFdU)

Method	Sample	Plot	Wavelength (nm)	Linear range (μg ml^{-1})	Regression equation	*r*
HPLC	dFdC	8	272.0	0.2–50	$y = 0.0371x + 192.532$	0.992
	dFdU	10	272.0	0.2–40	$y = 1.05 \cdot 10^{-4}x - 1.2693$	0.996

Table 2
Summary of assay precision and accuracy data for dFdC in human plasma

Added ($\mu g\ ml^{-1}$)	Intra-day			Inter-day		
	Found ($\mu g\ ml^{-1}$)	Precision RSD (%)	Accuracy relative error (%)	Found ($\mu g\ ml^{-1}$)	Precision RSD (%)	Accuracy relative error (%)
3.0	2.7	10.38	−9.67	2.8	12.40	−5.60
10	9.7	4.72	−2.70	9.8	6.17	−2.00
20	17.8	5.16	−10.90	18.7	4.85	−6.27
50	47.6	3.52	−4.80	47.8	3.72	−4.80

Table 3
Summary of assay precision and accuracy data for dFdU in human plasma

Added ($\mu g\ ml^{-1}$)	Intra-day			Inter-day		
	Found ($\mu g\ ml^{-1}$)	Precision RSD (%)	Accuracy Relative error (%)	Found ($\mu g\ ml^{-1}$)	Precision RSD (%)	Accuracy Relative error (%)
0.5	0.46	6.37	−8.0	0.47	5.37	−6.0
5.0	4.78	4.92	−4.4	4.58	13.0	−8.4
20	19.66	4.50	−3.7	19.12	6.10	−4.4
40	38.80	5.07	−3.0	38.48	4.91	−3.8

Accuracy = [(found − added)/added] × 100; RSD, relative standard deviation.

0.15 and 0.18 $\mu g\ ml^{-1}$, respectively. The limits of detection for dFdC and dFdU were 0.10 and 0.12 $\mu g\ ml^{-1}$, respectively, at a signal-to-noise ratio of 3.

3.5. Recovery

The absolute recovery was calculated by comparing peak areas obtained from freshly prepared sample extracts with those found by direct injection of aqueous standard solutions of the same concentration [12]. The recoveries of dFdC and dFdU from plasma averaged 91.52 and 94.08%, respectively (Tables 4 and 5).

Table 4
Summary of assay recovery data for dFdC in human plasma ($n = 6$)

Concentration ($\mu g\ ml^{-1}$)	Recovery ratio[a] (%)	RSD (%)
3	90.6±9.40	10.38
10	97.3±4.59	4.72
20	89.1±4.59	5.16
50	95.2±3.35	3.52

RSD = [(standard deviation/mean)] × 100.
[a] Mean values.

3.6. Specificity

Specificity is the ability of a method to determine accurately and specifically the analyte of interest in the presence of other components in a sample matrix under the stated conditions of the test [13].

Chromatograms representing the separation of the analytes from the matrix are shown in Fig. 3. No plasma components were detected at the retention times for dFdC or dFdU in blank plasma samples. dFdC and dFdU were well retained from the void with k' values of 2.3 and 0.9, respectively.

System specificity was gauged by selectivity (α) and resolution (R_s) in sample chromatograms. dFdC

Table 5
Summary of assay recovery data for dFdU in human plasma ($n = 6$)

Concentration ($\mu g\ ml^{-1}$)	Recovery rate (%)[a]	RSD (%)
0.5	92.9±5.92	6.37
5.0	91.6±4.51	4.92
20	95.6±4.30	4.50
40	96.2±4.88	5.07

RSD = [(standard deviation/mean)] × 100.
[a] Mean values.

Table 6
System suitability parameters for dFdC and dFdU in human plasma

Parameter	Metabolite (dFdU)	Parent drug (dFdC)
Retention time, $t_{R'}$ (min)	4.3	7.5
Capacity factor, k'	0.9	2.3
Resolution, R_S	–	8.0
Selectivity, α	–	2.6
Number of theoretical plates, N	3328	3643

and dFdU were sufficiently well resolved ($R_s > 8.0$) from each other Table 6.

3.7. Ruggedness and stability

The formal ruggedness test was conducted when the method were validated on another HPLC system by another analyst. Using the optimized parameters, the method was found to be equally robust. dFdC and dFdU were found to be exceedingly stable compounds under all the conditions examined.

3.8. Application of the method

The developed method was applied to plasma samples from a bladder cancer patient. A patient with advanced cancer was treated with 1000 mg m^{-2} Gemzar infused over a 30-min period. The patient received a Gemzar infusion once per week for 3 weeks followed by a rest week. Blood samples were collected at different times after drug administration and blood samples were analyzed immediately. A typical chromatogram of a plasma sample collected from the bladder cancer patient 15, 40, 7200 min after intravenous administration of 1000 mg m^{-2} Gemzar is shown in Fig. 5a –d. Fig. 6a and b represent the concentration–time profiles of dFdC and dFdU metabolite in the patient with bladder cancer.

4. Conclusion

In the developed method, 0.2 ml of plasma was used in the sample preparation. Only 10 μl of the reconstituted solution (1 ml) was injected into the system. This method was utilized in the analysis of plasma samples collected from a bladder cancer patient who was administered Gemzar (1000 mg m^{-2}). Differences in the pharmacokinetic properties of the parent drug (dFdC) and its metabolite (dFdU) were observed.

The whole HPLC procedure described here for the simultaneous determination of dFdC and dFdU is widely available in biochemical laboratories.

In particular, the method has satisfactory specificity, linearity, recovery, accuracy and precision range over the concentration range examined.

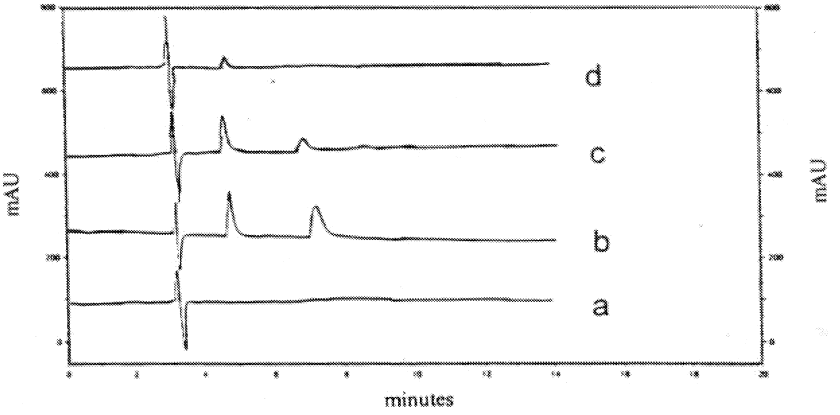

Fig. 5. Chromatograms from patient administered a 30-min infusion of 1000 mg m^{-2} of gemzar (a) patient sample prior to infusion, (b) patient sample at 15 min postinfusion, (c) patient sample at 40 min postinfusion, (d) patient sample at 7200 min postinfusion.

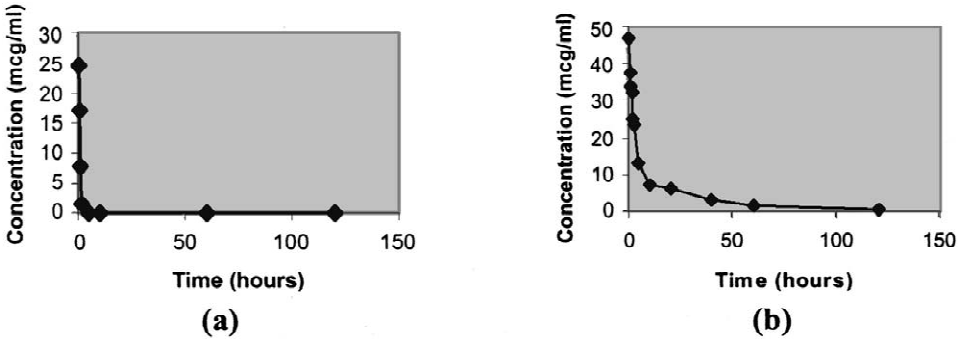

Fig. 6. Profile of the concentrations in patient plasma of (a) dFdC, (b) dFdU determined by the HPLC method.

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