Method Validation of an LC-MS/MS Method for the Determination of Gemcitabine and 2´-deoxy-2´, 2´-Difluorouridine (dFdU) in Tetrahydrouridine (THU)-Treated Human Plasma

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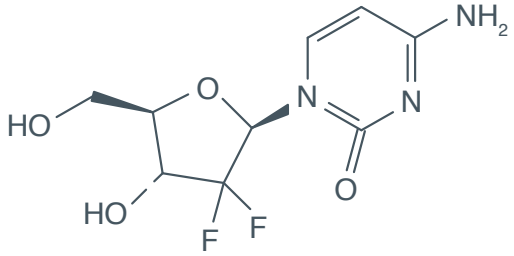
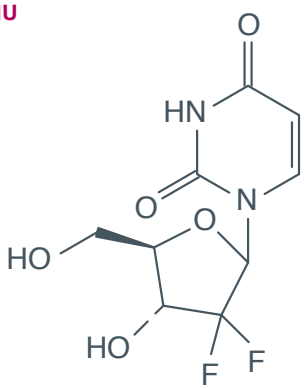
Introduction

Gemcitabine is a chemotherapeutic agent that works by slowing or stopping the growth of cancer cells. 2´-deoxy-2´,2´-difluorouridine (dFdU) is the inactive uracil metabolite. Gemcitabine rapidly converts to dFdU in blood so tetrahydrouridine, a cytidine deaminase inhibitor, must be added to the sample collection tubes prior to collection.

Gemcitabine



dFdU



Methods

Sample Preparation

1. Aliquot 0.0500 mL of plasma sample and 0.0500 mL of ISTD
2. Add acetonitrile to all samples, mix, and centrifuge
3. Supernatant was transferred and diluted with ultrapure water in a 96-well plate for gemcitabine analysis
4. A second portion of the supernatant was transferred to a separate 96-well plate, evaporated to dryness and reconstituted with ultrapure water for dFdU analysis

HPLC

Column: Thermo Scientific, AQUASIL C_{18}

Mobile phase: 15:85 MeOH:5 mM CH_3COONH_4

Run time: 1.5 minutes

Retention time: 0.7 minutes (gemcitabine)
0.9 minutes (dFdU)

LC-MS/MS

Mass spectrometer: API 4000

Source: APCI + (gemcitabine)
APCI - (dFdU)

Resolution: Unit for both gemcitabine and dFdU

Ions monitored: Gemcitabine (264.1 → 112.1 m/z)
dFdU (263.1 → 220.0 m/z)
$^{13}C,^{15}N_2$–gemcitabine (IS) (267.1 → 115.2 m/z)
$^{13}C,^{15}N_2$-dFdU (IS) (266.1 → 221.0 m/z)

Results

Table 1.

Inter-Batch Statistics for Precision and Accuracy

Gemcitabine Quality Control Samples		Precision (% CV)	Accuracy (% Bias)
Inter-Batch	LLOQ	9.2	-3.7
	Low	3.5	0.3
	Medium	1.8	2.5
	High	1.7	1.3
dFdU Quality Control Samples		**Precision (% CV)**	**Accuracy (% Bias)**
Inter-Batch	LLOQ	6.4	-3.6
	Low	4.5	0.7
	Medium	2.2	1.6
	High	2.6	1.1

Table 2.

Matrix Effect for Gemcitabine in Human Plasma Collected from THU-Treated Whole Blood

		LLOQ		High	
	Lot#	100 ng/mL	% Bias	15,000 ng/mL	% Bias
	1	98.8	-1.2	15,100	+0.7
	2	96.3	-3.7	14,500	-3.3
	3	97.1	-2.9	14,400	-4.0
	4	95.3	-4.7	14,700	-2.0
	5	92.7	-7.3	14,500	-3.3
	6	106	+6.0	14,800	-1.3
	7	94.8	-5.2	15,100	+0.7
	8	96.8	-3.2	14,900	-0.7
	9	98.2	-1.8	14,600	-2.7
	10	93.4	-6.6	15,400	+2.7
Mean		96.9		14,800	
% CV		3.9		2.2	
% Bias		-3.1		-1.3	
n		10		10	

Table 3.

Matrix Effect for dFdU in Human Plasma Collected from THU-Treated Whole Blood

	Lot#	LLOQ 247 ng/mL	% Bias	High 37,000 ng/mL	% Bias
	1	241	-2.4	38,500	+4.1
	2	256	+3.6	38,000	+2.7
	3	248	+0.4	37,700	+1.9
	4	237	-4.0	38,200	+3.2
	5	259	+4.9	37,900	+2.4
	6	270	+9.3	39,000	+5.4
	7	254	+2.8	38,000	+2.7
	8	242	-2.0	37,300	+0.8
	9	253	+2.4	38,600	+4.3
	10	275	+11.3	38,800	+4.9
Mean		254		38,200	
% CV		4.8		1.4	
% Bias		2.8		3.2	
n		10		10	

Figure 1. *Representative Chromatograms of Gemcitabine and ISTD (A) and dFdU and ISTD (B)*

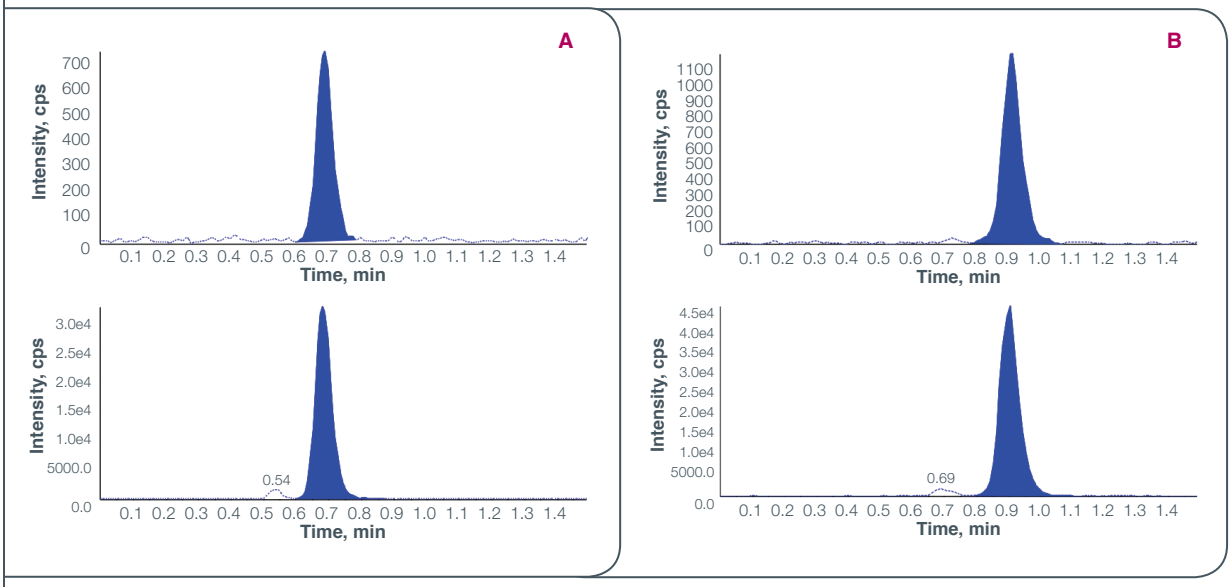
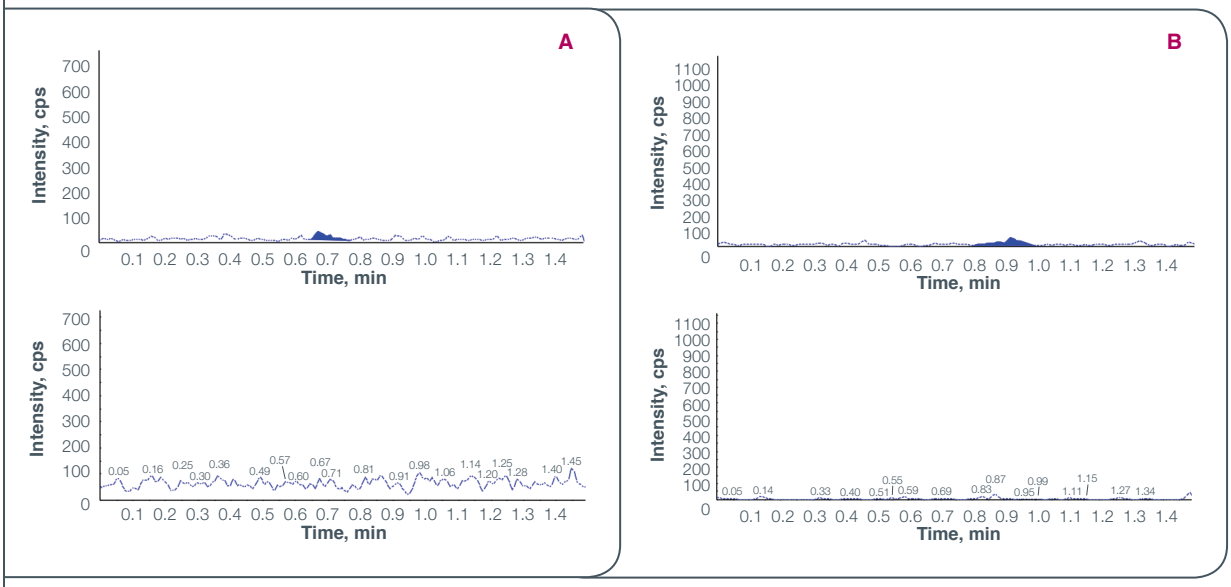


Figure 2. Representative Chromatograms of Gemcitabine and ISTD (A) and dFdU and ISTD (B)



Validation Summary

Assay Volume Required	0.0500 mL
Standard Curve Range	100 to 20,000 ng/mL for gemcitabine 247 to 49,300 ng/mL for dFdU
Dilution Integrity	Up to 100,000 ng/mL for gemcitabine Up to 198,000 ng/mL for dFdU
Regression Type	Linear (1/concentration^2)
Batch Size	192 injections
Mean Extraction Recovery	61% for gemcitabine 86% for dFdU
Short-term Stability	52 hours at ambient temperature
Freeze and Thaw Stability	6 cycles in polypropylene tubes at -80°C
Processed Sample Integrity	148 hours for gemcitabine in polypropylene at 5°C 165 hours for dFdU in polypropylene at 5°C
Post-preparative Stability	151 hours for gemcitabine in polypropylene at 5°C 195 hours for dFdU in a polypropylene at 5°C
Sample Collection and Handling Stability	Up to 30 minutes in THU-treated human whole blood (heparin) at ambient temperature

Conclusion

The bioanalytical assay for the quantitation of gemcitabine and dFdU in human plasma met acceptance criteria for precision, accuracy, sensitivity, selectivity and stability. Assay selectivity was demonstrated by the quantitation of ten separate lots of human plasma fortified with known concentrations of gemcitabine and dFdU. The method utilized a small sample volume and extraction automation to achieve high throughput.