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Rapid Determination of Gemcitabine and Its Metabolite in Human Plasma by LC-MSMS through Micro Protein Precipitation with Minimum Matrix Effect

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ABSTRACT

A novel micro protein precipitation was proposed and employed effectively for bio-sample preparation of only 5 µl of human plasma for rapid liquid chromatography- tandem mass spectrometric (LC-MSMS) determination of gemcitabine (dFdC) and its deaminated metabolite, 2', 2'-difluorodeoxyuridine (dFdU).). Gemcitabine-13C, 15N2 Hydrochloride was the internal standard. Baseline chromatographic separation was achieved with an Alltima C_{18} column (2.1×100 mm, 5µm) using isocratic mobile phase [10 mM ammonium acetate (pH = 6.8): methanol, 90:10]. The run time was only 5 min. The mass spectrometer was operated under a positive electrospray ionization condition and a multiple reaction monitoring mode. The linear calibration ranges were 2-2,000 ng/ml for gemcitabine and 20-20,000 ng/ml for dFdU. The recoveries for three analytes were in the same magnitude range from 87.7 to 89.7% with small variation**.** The intra and inter-day precisions for dFdC and dFdU were ≤ 5 and ≤ 7 and their accuracy ranged from 98 to 105.3% for dFdC and 93.8 to 104.9% for dFdU, respectively. Ion suppression effect was near negligible. This well validated assay has been applied for quantification of gemcitabine and dFdU in our gemcitabine phase II clinical trial study on Asian non-small cell lung cancer patients treated with different infusional rates of gemcitabine.

Keywords: LC-MSMS, gemcitabine, 2', 2'-difluorodeoxyuridine, human plasma

1. Introduction

Gemcitabine (2', 2'-difluoro deoxycytidine), is probably one of the most valuable cytotoxic drugs for several solid tumors, e.g. pancreatic, lung and breast cancer.[1] Gemcitabine, a pyrimidine analog, is used in combination with cisplatin for the treatment of advanced non small cell lung cancer in the first-line setting. [2] Gemcitabine's unique mechanism of action and its lack of overlapping toxicity with other cytotoxic agents render it an ideal candidate for combination therapy. [3] Many new gemcitabine combinations are being tested in clinical trials to find the relationship between response rate, toxicity and pharmacokinetics in patients. [4] A rapid and sensitive quantitative method is needed to be developed in order to provide an analytical platform for clinical plasma samples. Plasma quantification of gemcitabine is very challenging for bioanalysis using HPLC-UV. This is because gemcitabine has an extremely short half-life due to rapid deamination to dFdU by cytidine deaminase.[5,6] In addition, simultaneous analysis of gemcitabine and dFdU is important for the characterization of pharmacokinetic profile of gemcitabine to accurately define the elimination pathway of the parent drug and its metabolite. [7] Several methods have been published for quantification of gemcitabine and dFdU in plasma, urine and tissues using high-performance liquid chromatography coupled with UV detection. [8-18]

However, it is very difficulty to monitor the very low levels of the parent drug at later sampling points because of the limitation of UV detection sensitivity. [19] In our previous report using UV detection, [16] the LLOQ was 80 ng/mL. Unfortunately, most of the last sampling points from our Phase II trial study were below 80 ng/ml and this initiated the development of the current more sensitive and simpler bioanalytical method. Although two mass spectrometric assays have been published for gemcitabine and dFdU in human plasma and urine, [20-21] the tedious solid-phase extraction (SPE) for sample preparation as well as a relative long run time (≥ 10 min) preclude rapid quantifications of gemcitabine and its metabolite in patient plasma samples. Due to the big difference in polarity, a gradient elution mode was commonly utilized to simultaneously quantify both gemcitabine and dFdU in order to avoid interference between these two compounds. This potential cross talk interference between these two analytes is expected because of the close molecular mass (only 1 amu difference) and similar fragmentation. Simplicity of the sample preparation is also an important factor for rapid and high throughput analyses.

In this study, we developed and validated a highly sensitive and rapid LC tandem Mass Spectrometry (LC-MSMS) method for gemcitabine and dFdU with 5 μL of human plasma using protein precipitation and isocratic elution. The novel strategy was to use a very small volume of plasma to minimize ion-suppression in the mass analyzer. We believe that this is the first report of the micro protein precipitation (mPPT) method as a robust, fast and convenient tool for bio-sample preparation for LC-MSMS quantitative analysis. This should pave the way for high throughput analyses of samples.

2. Materials and Methods

2.1. Chemicals and reagents

Gemcitabine hydrochloride (dFdC, LY 264368) and 2', 2'-difluorodeoxyuridine (dFdU, LY 198791) were kindly provided by Eli Lilly & Co. (Indianapolis, IN, USA). The internal standard, dFdC-13C, 15N2 hydrochloride (Toronto, Canada), was purchased from Toronto Research Chemicals, Canada. Tetrahydrouridine (THU) was purchased from Biosciences, inc. La Jolla, CA 92039-2087, an affiliate of Merck KGaA, Darmstadt, Germany. HPLC grade methanol and acetonitrile were purchased from Merck Darmstadt, Germany. Milli Q water was used for mobile phase preparation.

2.2. Sample collection and preparation

Blood samples were collected from patients before initiation of a dFdC infusion (baseline), 10 minutes, 30 minutes during infusion, 10 minutes before the end of the infusion, and 30 minutes, 1 hour, 2 hours after the end of the infusion. At each point, 8 ml of blood was drawn into 15-ml heparined plastic tubes that had been preloaded with 0.1 ml of a 10mg/ml solution of tetrahydrouridine, the cytidine deaminase inhibitor. Blood samples were centrifuged for 5 minutes at approximately 1,200 ×g at room temperature. The plasma portion of the samples were removed and kept at –20 °C until analysis. The buffy coat remaining was for mononuclear blood cell isolation for quantification of dFdCTP, the intracellular main active metabolite.

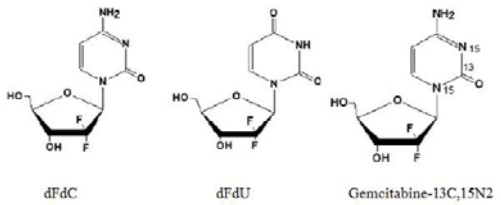

Fig.1. The chemical structures of dFdC, dFdU and Gemcitabine-13C, 15N2

Micro protein precipitation (mPPT) with acetonitrile was used for sample preparation rather than the published SPE method.[20,21] To a 1.5 ml Eppendorf tube was added 5 µl of patients' plasma, 10 µl of water and 45 µl of acetonitrile containing 50 ng/mL of internal standard. The tube was tightly capped and immediately vortex-mixed for 1 minute, and then centrifuged at 10,000×g for six minutes at 4 °C. Fifty µl of supernatant was transferred into another Eppendorf tube and dried under nitrogen and reconstituted with 50 µl of mobile phase containing 10% methanol in 10 mM ammonia acetate buffer solution pH 6.8 (10:90, V/V). After mixing, the reconstituted supernatant was loaded onto the auto-injector of the HPLC column for LC-MSMS analysis.

2.3. HPLC-MSMS instrumentation

The high-performance liquid chromatographic system consisted of an Agilent 1100 Binary pump equipped with an Agilent 1100 auto-sampler injector with 100 µl loop and 1100 column oven (Germany) set at 20°C. Chromatographic separations were achieved using a Alltima C_{18} column (2.1×100 mm, 5µ) (Alltech Associates, Inc. USA) following an Eclipse XDB-C8 guard column (2.1 mm x 50 mm, 5 µm) (Agilent Technologies, USA) with isocratic elution of the analytes with a constant composition of 10% methanol in 10 mM ammonium acetate buffer pH 6.8 (10:90, v/v). The flow rate was set at 0.5 ml /min. Ten µl of reconstituted

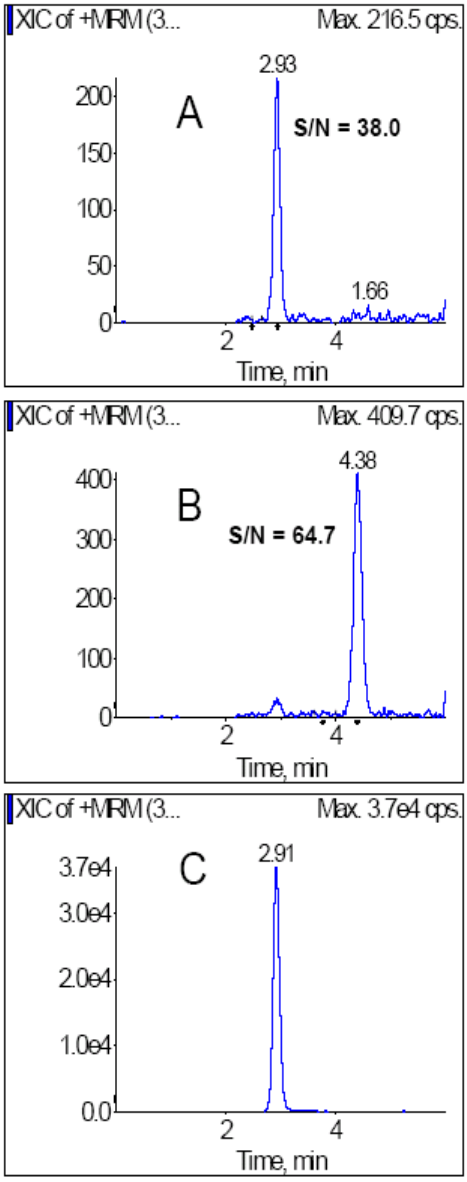

Fig 2. The representative chromatograms of dFdC, dFdU and Gemcitabine-13C, 15N2 at the LLOQ A: dFdC (2ng/mL, 2.93 min); B: dFdU (20 ng/mL, 4.38 min); C: the internal standard (450 ng/mL; 2.91 min) in human plasma

supernatant was injected to the HPLC column and the elutant directed to the mass spectrometer turboionspray source without splitting. In order to avoid contaminating the ion source detector, the solvent front eluting in the first 2.2 min was switched to waste container.

LC-MSMS analyses were performed using an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). The instrument was operated in positive ion mode calibrated by polypropylene glycol. The plasma samples were analyzed by tandem MS using the IonSpray needle at +5500 V and the cluster breaking orifice voltage at 30 V. The ions of dFdC at m/z 264, dFdU at m/z 265 and internal standard at m/z 267 were passed through the first quadropole (Q1) and into the collision cell (Q2). The product ions for dFdC (m/z 112), dFdU (m/z 113) and internal standard (m/z 115) were monitored through the third quadrupole (Q3). The dwell time per channel was 200 ms for data collection.

2.4. Construction of standard curve

Standard stock solutions of dFdC, dFdU and internal standard prepared in methanol at 1 mg/ml and were kept at –20 °C. These stock solutions were diluted with water to obtain the concentrations required for preparation of standard working solutions. For dFdC, working solutions of 0.002, 0.004, 0.02, 0.1, 0.25, 0.5, 1, 2, μg/ml and for dFdU, working solutions of 0.02, 0.04, 0.2, 0.5, 1, 5, 10, 20 μg/ml were prepared. A working solution of internal standard was prepared at 50 ng/ml using acetonitrile.

For quality control (QC), solutions of dFdC at concentrations of 0.006, 0.2, 0.75 and 1.5 μg/ml and QC solutions of dFdU at concentrations of 0.06, 2, 7.5 and 15 μg/ml in 10% methanol solution were prepared.

Least-squares regression and standard curves weighted according to $1/x^2$ for gemcitabine and 1/x for dFdU (x = concentration) were drawn using linear regression of the peak area ratios of gemcitabine or dFdU against internal standard obtained from LC-MS/MS analysis of standard solution against actual standard concentrations.

The limit of detection (LOD) was defined as the lowest concentration that the analytical assay can reliably differentiate from background levels (S/N > 3). The lower limit of quantification (LLOQ) was defined as the lowest calibrator with a inter-day coefficient of variation < 20%. [22]

The specificity of the method was evaluated by checking chromatograms of six blank plasma samples from clinical trial subjects and comparing standard curves produced from healthy human plasma and patients' blank plasma which contained co-administered medicines such as other pre-chemotherapy adjunct medications as well as carboplatin.

2.5. Validation description

Quantification was based on the ratios of the peak areas of dFdC and dFdU against that of internal standard. Validation was performed through establishing intra and inter-day precision and accuracy of the method on quality control samples (QCs). The calibration curves were constructed using 8 different calibrator concentrations of dFdC and dFdU. Four quality control samples were prepared at the nominal concentrations of 6, 200, 750 and 1500 ng/ml for dFdC and 60, 2000, 7500 and 15000 ng/ml for dFdU. Intra-day variability was determined by analyzing 5 x the QCs using the same calibration curve. Inter-day variability was determined by analyzing the QCs on five different days using calibration curves obtained daily. The precision of the method at each QC concentration was expressed as a coefficient of variation (%C.V.) by calculating the standard

deviation as a percentage of the mean calculated concentration, while the accuracy of the assay was determined by expressing the percentage of the mean from the true value.

2.6. Matrix and recovery

Matrix effect is a common and harmful phenomenon in LC-MS or LC-MSMS procedure. According to FDA bio-analytical method validation guidance for industry, matrix effect should be investigated to achieve good precision and accuracy. [22] The matrix effect [ME(%)] was evaluated according to the following formula: [23]

ME(%) = [Peak Area in control matrix/ Peak Area in neat standard]×100 …… ***Equation 1***

If ME (%) is less than 100, it represents ion suppression. If ME (%) is greater than 100,, it represents ion enhancement. If [Me(%)-100] is employed, negative value and positive value represent ion suppression and ion enhancement respectively. In our current validation procedure, six patient control plasma samples were used for evaluation of matrix effects and recovery for the three compounds. The complete set of concentration levels evaluated were QC1, QC2 and QC3 for both dFdC and dFdU (dFdC at 6, 200, 750 ng/ml and dFdU at 60, 2000, 7500 ng/ml) and 450 ng/mL for the internal standard. Three separate complete sets were prepared. The first set (set A) was to determine the MS/MS response for neat standards dFdC and dFdU and internal standard. The second set (set B) was spiked into plasma from six different donors after extraction. By comparing absolute area of set B against those of set A, the matrix effect (ion suppression or enhancement) associated with a given lot of plasma can be measured. The third set (set C) was prepared in the same six plasma sources as in set B, but the analytes were spiked into plasma before extraction and brought through the whole extraction process. Absolute recovery was determined by comparing the peak area of set C with those of set B (in post extracted plasma).

3. Results and Discussion

3.1. Chromatographic separation

Liquid chromatography-tandem mass spectrometry has been commended for its excellence in specificity in biopharmaceutical analysis. [24] In most cases, chromatographic separation is not an important consideration but is critical for gemcitabine and dFdU quantification because of very small mass difference between both their parent and daughter ions with mass transition as 264/112 for gemcitabine and 265/113 for dFdU. A small mass difference will result in a "cross talk" between gemcitabine and dFdU. Hence, chromatographic conditions had to be optimized to achieve baseline separation and nice peak shape according to following aspects.

Isocratic elution mode is ideal for a rapid HPLC analysis because no equilibrium time for mobile phase is needed. However, gemcitabine with an ammonia group in cytosine ring is much more hydrophilic than dFdU (Fig.1). To elute these 2 compounds of different polarities in a short isocratic run of 5 min is challenging. Several HPLC columns were used for optimization and Alltima C_{18} column was identified as the best choice among them. With the Alltima C18, both gemcitabine and dFdU were appropriately retained during HPLC separation. This maybe attributable to the larger double end-capped surface area for this HPLC column compared to

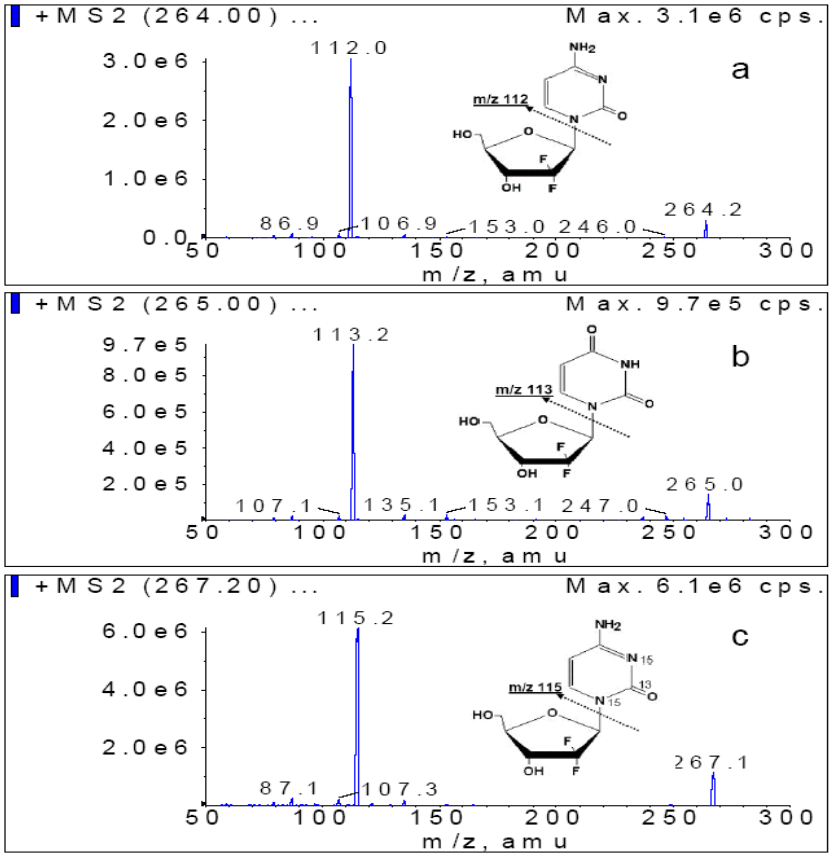

Fig.3. MS/MS product ion scan of a: gemcitabine (precursor ion m/z 264); b: dFdU (precursor ion m/z 265); c: Gemcitabine-13C, 15N2 (precursor ion m/z 267).

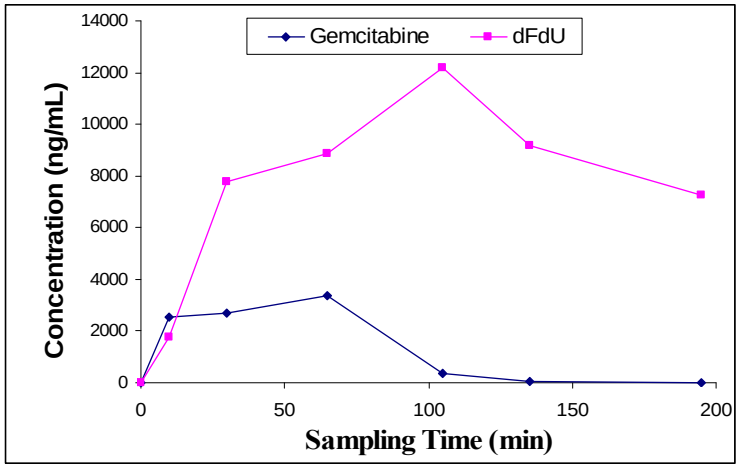

Fig. 4. Pharmacokinetic profile from one of study subjects in 75-min infusion arm

Table 1. Matrix effect tested in patient control plasma with 5 µL or 50 µL at three concentration levels (n = 6).

Analyte	Nominal Conc. (ng/mL)	ME (%)			
		5 µL plasma		50 µL plsama	
		Mean ± SD	CV(%)	Mean ± SD	CV(%)
dFdC	6	94.0 ± 1.9	2.0	78.8 ± 2.0	2.6
	200	94.1 ± 3.7	3.9	78.2 ± 1.5	1.9
	750	94.9 ± 3.5	3.7	77.5 ± 1.1	1.4
dFdU	60	94.7 ± 4.1	4.3	80.2 ± 2.2	2.7
	2000	95.3 ± 2.1	2.2	82.4 ± 1.1	1.3
	7500	96.1 ± 1.6	1.7	79.0 ± 2.2	2.8
IS	450	94.2 ± 3.3	3.5	77.1 ± 1.4	1.8

Table 2. Intra-day and inter-day precision and accuracy for dFdC and dFdU

dFdC			Nominal Concentration (ng/mL)			
			6	200	750	1500
Intraday (n = 5)	Observed (Mean ± SD)	(ng/mL)	6.22 ± 0.22	198.2 ± 2.4	774.0 ± 4.9	1470 ± 21
	Accuracy	(%)	103.7	99.1	103.2	98
	CV	(%)	3.5	1.2	0.6	1.4
Interday (n = 5)	Observed (Mean ± SD)	(ng/mL)	6.32 ± 0.28	200.6 ± 2.7	782.6 ± 8.6	1496 ± 21
	Accuracy	(%)	105.3	100.3	104.3	99.7
	CV	(%)	4.4	1.3	1.1	1.4
dFdU			Nominal Concentration (ng/mL)			
			60	2000	7500	15000
Intraday (n = 5)	Observed (Mean ± SD)	(ng/mL)	56.4 ± 3.3	2098 ± 72	7620 ± 285	14760 ± 261
	Accuracy	(%)	93.8	104.9	101.6	98.4
	CV	(%)	5.9	3.4	3.7	1.8
Interday (n = 5)	Observed (Mean ± SD)	(ng/mL)	62.0 ± 4.0	2048 ± 109	7686 ± 372	15120 ± 672
	Accuracy	(%)	103.3	102.4	102.5	100.8
	CV	(%)	6.5	5.3	4.8	4.4

others. [25] As for isocratic mobile phase, various pH values and organic modifiers had to be considered to achieve a baseline separation. Since gemcitabine is weak base with pKa values of 3.6 [26] it exists mainly in non-ionic forms in an environment with pH values above 5.6. Hence, ammonia acetate buffer (10 mM, pH = 6.8) was selected as the main component of mobile phase. Methanol rather than acetonitrile was used as the organic modifier because acetonitrile elutes gemcitabine too rapidly. With the optimized chromatographic conditions described, gemcitabine and dFdU eluted at about 2.91, 4.38 min, respectively. The isotope internal standard of gemcitabine eluted at 2.93 (Fig 2). No interference was detected between gemcitabine and the internal standard even though both of them co-eluted from the column. This is due to their mass difference (3 amu) being large enough to avoid interference. The chromatograms showed excellent specificity as endogenous compounds and the usual co-administered medications in plasma did not interfere with these analytes. The run time was only 5 min.

3.2. Method validation

Sample preparation is a very critical factor in the development of LC-MSMS methods. This is because the peak intensity from mass spectrometer can be heavily suppressed by endogenous substances and co-administered medications within the bio-samples. Among the three common sample preparation procedures (solid-phase extraction (SPE), liquid-liquid extraction (LLE) and protein precipitation (PPT)), SPE is usually the choice for bio-sample clean-up since it is the most efficient process of removing unwanted substances. However, it involves another developmental and validation step. LLE is suitable for hydrophobic compounds, but is considered less efficient than SPE in terms of ion suppression and removal of potential interfering compounds. In addition, LLE is not appropriate for highly hydrophilic gemcitabine and dFdU. Lastly, PPT is a fast and convenient procedure for bio-sample preparation. However, PPT is generally the least effective in sample clean-up due to the presence of many residual matrix components which will cause bad ion suppression for LC-MSMS analysis and contaminate the ion source. [27] Analytical scientists are usually very concern about this phenomenon when they use LC-MS. In order to overcome these problems, we used a novel strategy to decrease the volume of plasma to 5 µL during sample preparation. This would be expected to reduce matrix effect efficiently. In order to demonstrate this strategy, two different plasma volumes (50 µL and 5 µL) were used to compare the ion suppression for gemcitabine, dFdU and internal standard. The matrix effect, ME (%), was calculated according to the formula mentioned (Equation 1). With 50 µL of plasma, the ion suppression for gemcitabine, dFdU and internal standard were -21.8%, -19.5% and -22.9% respectively (Table 1). When the sample volume was reduced to 5 µL, ion suppression was greatly reduced correspondingly to -5.7 %, -4.6% and -5.8%. Hence, the obstacle of PPT sample preparation can be overcome efficiently by using mPPT. Based on our knowledge, this is the first report of using this micro-plasma volume in PPT for LC-MSMS analysis. Our experimental data proved that 5 µL of plasma can efficiently eliminate ion

suppression caused by routine PPT. Our strategy of this novel micro protein precipitation (mPPT) significantly minimized ion suppression and may initiate a special method for bio-sample preparation for LC-MSMS. Another advantage is that mPPT is rapid and simple and is suitable for dealing with a large sample size in a short period. Most importantly, it is very useful for pediatric patients as well as preclinical pharmacokinetic studies where plasma samples and volume are limited from infants and small animals like mice and rats. Although drying with nitrogen gas is needed for the hydrophilic analytes following the mPPT, the process is fast (about 10 minutes). Moreover, this method of sample preparation resulted in good recovery for all three compounds. The recoveries of gemcitabine, dFdU and internal standard were 87.9 ± 3.8 (4.3%), 89.7 ± 4.1 (4.6%) and 87.7 ± 3.1 (3.5%).

Good linearity was achieved for concentration ranges of 2-2000 ng/ml for dFdC and 20-20000 ng/ml for dFdU based on the current LC-MSMS conditions. The correlation coefficients (r) for dFdC and dFdU were higher than 0.9991. The lower limit of detection (LOD) was 0.2 ng/ml and 1 ng/ml for dFdC and dFdU, respectively. The assay sensitivity was more than adequate for all clinical samples with the last sampling time of 120 min after the end of gemcitabine infusion.

The accuracy and precision of this method were evaluated from the four QC samples. The precision and accuracy of dFdC and dFdU for QCs were listed in Table 2. The intra-day and inter-day precisions for dFdC and dFdU were ≤ 5 and ≤ 7 and their accuracy ranged from 98 to 105.3 for dFdC and 93.8 to 104.9 for dFdU, respectively.

The sample stability was judged by decrease of sample concentrations after one year storage of plasma samples at -20 ºC. Our results showed that both dFdC and dFdU showed good stability with decreases in concentrations being less than 10% after one year storage period.

Needless to say, this LC-MSMS method is much more sensitive than our previous ion-pair HPLC method[19] where most concentrations of the last sampling point (2 h) were below its LLOQ.

The method has been successfully used in phase II clinical trial of dFdC at a dosage of infusional dFdC given at a constant rate of 10 mg/m^2/min over 75 min or at 1000 mg/m^2 in 30-min, when combined with a fixed dose of carboplatin. Figure 4 showed a pharmacokinetic profile from one of study subjects in 75-min infusion arm.

4. Conclusions

A rapid LC-MSMS method was well validated with 5 μL of human plasma. A novel bio-sample preparation strategy was proposed by using mPPT method with minimum ion suppression effect for LC-mass spectrometers. The micro-plasma volume employed did not compromise the accuracy or the precision of the method. This robust method was applied to Phase II clinical sample quantification of gemcitabine and its deaminated metabolite. Its simplicity and sensitivity contributed to the clinical applicability.

Acknowledgments

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REFERENCES

[1] Noble S., Goa K. L., *Drugs*1997, 54, 447-472.

[2] Ramalingam S., Belani C. P., *Oncology,* 2004, 18, 21-26.

[3] Manegold C., *Expert Rev Anticancer Ther*. 2004, 4, 345-360.

[4] Soo R. A., Lim H. L., Wang L. Z., et al., *Cancer Chemoth er Pha rmacol.* 2003, 52, 153-158.

[5] Heinemann V., Xu Y. Z., Chubb S., et al., *Cancer Res.* 1992, 52, 533-539.

[6] Xu Y. Z., Plunkett W., *Biochem Pharmacol.* 1992, 44, 1819-1827.

[7] Venook A. P., Egorin M. J., Rosner G. L., et al., *J Clin Oncol.* 2000, 18, 2780-2787.

[8] Abbruzzese J. L., Grunewald R., Weeks E.A., et al. *J Clin Onco l.* 1991,9, 491-498.

[9] Grunewald R., Kantarjian H., Du M., et al. *J Clin Oncol.* 1992,10, 406-413.

[10] Veerman G., vanHaperen V. W. T. R., Vermorken J. B., et al. *Cancer Chemoth Pharmacol.* 1996, 38, 335-342.

[11] Edzes H. T., Peters G. J., Noordhuis P., et al. *Anal Biochem.* 1993, 214, 25-30.

[12] Guo P. G., Ma J. G., Li S. L., et al. *Cancer Chem oth Pharm acol.* 2001, 48, 169-76.

[13] Venook A. P., Egorin M. J., Rosner G. L., et al. *J Clin Oncol.* 2000, 18, 2780-2787.

[14] Kroep J. R., Giaccone G., Voorn D. A., et al. *J Clin Oncol.* 1999, 17, 2190-2197.

[15] Freeman K. B., Anliker S., Hamilton M., et al. *J Chromatogr B.* 1995, 665, 171-181.

[16] Wang L. Z., Goh B. C., Lee H. S., et al. *Ther Drug Monit.* 2003,25, 552-557.

[17] Lin N. M., Zeng S., Ma S. L., et al. *Acta Pharmacol Sin.* 2004, 25, 1584-1589.

[18] Losa R., Sierra M. I., Guardado C., Fernandez A., et al. *Anal Ch im Acta* . 2005, 528, 255-260.

[19] Yilmaz B., Kadioglu Y., Aksoy Y., *Anal Biochem.* 2004,332, 234-237.

[20] Xu Y., Keith B., Grem J. L., *J. Chromatography B*. 2004, 802, 263-270.

[21] Sottani C., Zucchetti M., Zaffaroni M., et al. *Rapid Commun M ass Spectro m.* 2004, 18, 1017-1023.

[22] Guidance for Industry-Bioanalytical Method Validation. Internet at Internet at http://www.fda.gov/cder/guidance/4252fnl.htm

[23] Matuszewski B. K., Constanzer M. L., Chavez-Eng C. M., *Anal. Ch em.* 2003, 75, 3029-30.

[24] Kostiainen R., Kotiaho T., Kuuranne T., et al. *J Mass Spectrom.* 2003, 38, 357-372.

[25] Karasová G., Kowalska S., Lehotay J., et al. *J Sep Sci.* 2006, 29, 1074-81.

[26] Colin Kellery. Therapeutic Drugs (Second edition) 1998.

[27] Chambers E., Wagrowski-Diehl D. M., Lu Z., et al. *J. Chromatography B.* 2007, 852, 22-34.