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Determination of gemcitabine and its metabolite in extracellular fluid of rat brain tumor by ultra performance liquid chromatography–tandem mass spectrometry using microdialysis sampling after intralesional chemotherapy

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ABSTRACT

The cytotoxic agent Gemcitabine (2′,2′-difluoro-2′-deoxycytidine) has been proved to be effective in the treatment of malignant gliomas. A rapid, sensitive and specific ultra performance liquid chromatography with tandem mass spectrometry (UPLC–MS/MS) assay using microdialysis sampling was developed and validated to quantify gemcitabine and its major metabolite 2′,2′-difluoro-2′-deoxyuridine (dFdU) in Sprague–Dawley rat bearing 9 L glioma. Microdialysis probes were surgically implanted into the area of rat brain tumor in the striatal hemisphere, and artificial cerebrospinal fluid was used as a perfusion medium. The samples were analyzed directly by UPLC–MS/MS after the addition of 5-bromouracil as an internal standard (IS). Separation was achieved on Agilent SB-C_{18} (50 mm × 2.1 mm I.D., 1.8 μm) column at 40 °C using an isocratic elution method with acetonitrile and 0.1% formic acid (4:96, v/v) at a flow rate of 0.2 mL/min. Detection was performed using electrospray ionization in positive ion selected reaction monitoring mode by monitoring the following ion transitions m/z 264.0 → 112.0 (gemcitabine), m/z 265.1 → 113.0 (dFdU) and m/z 190.9 → 173.8 (IS). The calibration curves of gemcitabine and dFdU were linear in the concentration range of 0.66–677.08 ng/mL and 0.31–312.00 ng/mL, respectively. The lower limit of quantification of gemcitabine and dFdU were 0.66 ng/mL and 0.31 ng/mL, respectively. The lower limit of detection of gemcitabine and dFdU were calculated to be 0.2 ng/mL and 0.1 ng/mL, respectively. All the validation data, such as intra- and inter-day precision, accuracy, selectivity and stability, were within the required limits. The validated method was simple, precise and accurate, which was successfully employed to determinate the concentrations of gemcitabine and dFdU in the extracellular fluid of rat brain tumor.

1. Introduction

With the changes of lifestyle, the incidence of malignant glioma is increasing. Although radiotherapy following surgical resection is the most common treatment, survival in these patients is still unsatisfactory. In order to improve survival in patients with malignant glial tumor, intracranial injection of anticancer drug which can deliver drug to brain tumor tissue has been focused on by more and more research departments.

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine) has been clinically proved to be an effective anticancer drug for the treatment of various types of solid tumor, including small cell lung cancer, head and neck squamous cell cancer, and bladder, breast, ovary, cervix and pancreas tumors [1–4]. Recently, studies have shown that the cytotoxic agent gemcitabine is also effective when treating central nervous system malignancies including malignant glioma cell lines [5–7]. Despite of its small molecular weight (263.2 Da) and low protein binding, gemcitabine has low ability to across the blood-brain barrier. Therefore intravenously injecting gemcitabine is not a common way to treat glioblastoma multiforme [8–10]. Consequently, intracranial injection of gemcitabine may improve its therapeutic

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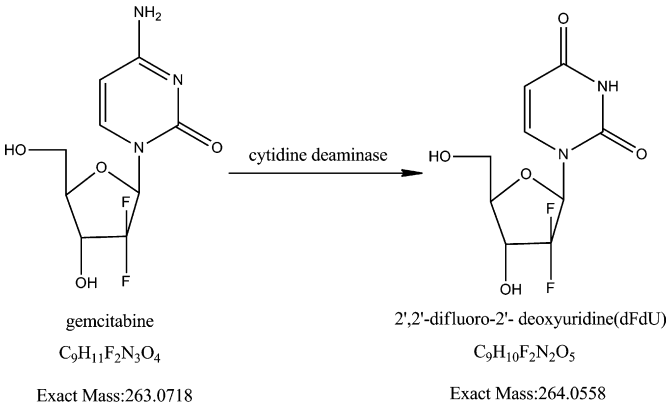

gemcitabine

$C_9H_{11}F_2N_3O_4$

Exact Mass:263.0718

2',2'-difluoro-2'- deoxyuridine(dFdU)

$C_9H_{10}F_2N_2O_5$

Exact Mass:264.0558

Fig. 1. Chemical structures of gemcitabine and dFdU.

effect for the treatment of brain malignant glioma and attenuate systemic adverse reaction caused by intravenous injection.

Gemcitabine is quickly metabolized to 2′,2′-difluoro-2′-deoxyuridine (dFdU) (Fig. 1) by cytidine deaminase in the liver, kidney, plasma and is excreted in urine [11]. Since gemcitabine has a narrow therapeutic index and dFdU may contribute to toxicity [12], quantification of gemcitabine and dFdU in brain tumor tissue is meaningful. Apparaju et al. has investigated the pharmacokinetics of gemcitabine in tumor and non-tumor extracellular fluid of rat brain after intracerebral microdialysis [13]. However, gemcitabine was administrated by intravenous injection in their work. As far as we are concerned, the determination of gemcitabine and dFdU in rat brain tumor after intracranial injection is still not focused on, which may be more significant for the treatment of malignant glioma.

Microdialysis has been proved to be an excellent method for *in vivo* sampling and determining the concentrations of unbound drugs in plasma [14], tissue samples [15], and other biological fluids [16]. Among them is brain microdialysis, a unique catheter-based sampling device for studying changes in brain biochemistry [17], which has many advantages including minimizing potential tissue damage and interrogating near real time metabolism [18], and has been employed to collect samples from extracellular fluid of rat brain tumor [13]. Normally, the collection of brain tissues samples for analysis is limited by its requirement of at least one animal per time point, and the tissue must be also homogenized and samples need clean-up before analysis. However, the size exclusion properties of microdialysis probe eliminate the need for complex protein removal which is normally required for brain tissue samples before analysis. The analytical molecules can be separated from enzymes by the dialysis membrane; therefore the analytes will not be subjected to further metabolism after collection. In addition, instead of obtaining data at discrete time points in traditional methods, microdialysis is a continuous process.

The use of microdialysis is accompanied with challenges, for example the large number of samples and the small sizes of microdialysate samples with low probe recoveries. In order to overcome these problems during the determination of the concentrations of gemcitabine and dFdU from the tumor area of glioma-implanted rats, a rapid, accurate and sensitive analysis method is needed. Several assays have been developed for the determination of gemcitabine and dFdU in plasma, urine and tissue using normal or reverse phase high-performance liquid chromatography (HPLC) separation with UV detection [19] or diode array detection [20]. However, these methods may not be suitable for the study of microdialysis samples. Liquid chromatography coupled with mass spectrometry (LC–MS) is regarded as a rapid, accurate and sensitive analysis method in biological fluid and has been applied for determining gemcitabine and dFdU in plasma and tissue. However, these methods have not quantified their concentrations in brain tumor-region or require 5 min or more for analysis [21–25]. Therefore, the development of ultra performance liquid chromatography with tandem mass spectrometry (UPLC–MS/MS) for the rapid, accurate and sensitive determination of gemcitabine and dFdU in microdialysis samples from brain tumor-region is strongly needed.

In this experiment, 9 L glioma was implanted into rat brain and intralesional chemotherapy by directly injecting gemcitabine into rat brain tumor area was firstly used. Microdialysis was selected to collect extracellular fluid in the focus of infection, and the UPLC–MS/MS was developed and validated to determine the concentrations of gemcitabine and dFdU in rat brain tumor. The results from assay validation showed that the developed UPLC–MS/MS method was precise and accurate, and could be used to optimize dosing regimen and monitor drug toxicity and efficacy during the course of treatment.

2. Experiments

2.1. Chemicals and reagents

Gemcitabine powder for injection was provided by Haosoh Inc. (Lian Yungang, China), and the drug was reconstituted for intralesional chemotherapy in artificial cerebrospinal fluid (aCSF, Gibco, Grand Island, Nebraska, USA) to a final concentration of 0.64 mg/kg. The standard references of gemcitabine (No. MFCD01735988) and 5-bromouracil (Internal Standard, IS, No. MKBH2631V) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dFdU (No. D445740) was provided by Toronto Research Chemicals (North York, ON, Canada). $CaCl_2$, Na_2HPO_4, $MgCl_2$, KCl and NaCl were purchased from Wulian Chemical Factory (Shanghai, China). The aCSF was used as a perfusate for microdialysis probes, which consisted of 0.13 M NaCl, 0.98 mM $MgCl_2$, 2.65 mM KCl, 1.2 mM $CaCl_2$, 0.25 mM ascorbic acid, 10 mM glucose. The perfusate was passed through a 0.22 μm nylon filter before use. The UPLC–MS/MS grade formic acid and acetonitrile were obtained from Fisher Scientific (Pittsburgh, PA, USA). Ultra-water was purified in a Milli-Q system (Millipore, Bedford, MA, USA). Mobile phase was degassed by Ultrasonic Generator (Wuxi Ultrasonic Generator Electronic Equipment Company, Wuxi, China) and filtered by a 0.45 μm filter (Autoscience Instrument Co. Ltd., China). Other reagents used in this work were analytical grade.

2.2. Microdialysis apparatus

The brain microdialysis systems is composed of a microdialysis syringe pump, microdialysis probes and a stereotaxic frame which were purchased from Baimai Company (Sichuan, China). They were used for *in vivo* studies and had a molecular weight cutoff of 5000 Da.

2.3. Animals

Six male Sprague–Dawley rats (250–300 g) were supplied by the Animal Experimental Center of Xuzhou Medical College (Xuzhou, China). The animals were housed under barrier conditions and kept in a room at 22–25 °C with 55% relative humidity under a 12/12 h light/dark cycle. Rats were allowed free access to water and food. Animals were acclimatized to their environment for 1 week before the experiments. All the experimental protocols were approved by the Animal Care and Use Committee of the College and were strictly consistent with institutional guidelines.

2.4. UPLC–MS/MS apparatus and conditions

Samples were analyzed on an UPLC–MS/MS (Agilent Technologies, Wilmington, DE, USA) equipped with an Agilent 1260 UPLC and Agilent 6460 Triple Quadrupole mass spectrometer. The mass spectra were acquired on a "triple" quadrupole instrument equipped with electrospray ionization (ESI) source, which was operated in a positive mode. The software program "Mass Hunter" was used to control the UPLC and mass spectrometer and to capture mass spectrometer data, perform linear regression analysis and calculate sample concentrations. The column of the UPLC system was an Agilent SB-C_{18} (50 mm × 2.1 mm I.D., 1.8 μm). The auto sampler was maintained at 4 °C. The column temperature was kept at 40 °C. The mobile phase consisted of a mixture of acetonitrile and 0.1% formic acid (4:96, v/v) at a flow rate of 0.2 mL/min. The retention time of gemcitabine, IS and dFdU were approximately 1.3 min, 1.9 min and 2.9 min, respectively. The injection volume was 10 μL and the analysis time was 3.5 min per sample. Acquisition was performed in a selected reaction monitoring mode (SRM) using m/z 264.0 → 112.0 (gemcitabine), m/z 265.1 → 113.0 (dFdU) and m/z 190.9 → 173.8 (5-bromouracil). The drying gas flow was 8 L/min and the nebulizer pressure was 15 Psi. The capillary voltage was 4.0 kV and the ion spray temperature was 350 °C. The optimal fragmentation voltages for gemcitabine, dFdU and 5-bromouracil were 110 V, 100 V and 70 V, respectively. The collision gas was nitrogen and the collision voltages for gemcitabine, dFdU and 5-bromouracil were set at 13 V, 9 V and 20 V, respectively.

2.5. Stock solutions, calibration standards and quality control samples

The stock solutions of gemcitabine, 5-bromouracil and dFdU were respectively prepared by dissolving the drugs in ultra-water at the concentrations of 1.625 mg/mL, 1.130 mg/mL and 0.585 mg/mL, and were stored in glass tubes at −20 °C before use.

The above stock solutions containing gemcitabine and dFdU were serially diluted with ultra-water and stored in glass vials at −20 °C which were then used for the preparing calibration and quality control (QC) samples. The stock solution of 5-bromouracil (IS) was further diluted with ultra-water to give the working IS solution containing with 37.6 μg/mL of 5-bromouracil.

For each analytical run, calibration standards in drug-free rat brain blank microdialysate were freshly prepared in triplicate at the concentrations of 0.66, 2.65, 10.58, 21.16, 42.32, 84.64, 169.27 and 677.08 ng/mL for gemcitabine, and 0.31, 1.22, 2.44, 4.88, 9.75, 19.50, 156.00 and 312.00 ng/mL for dFdU. QC samples were prepared in triplicate at the concentrations of 1, 50 and 500 ng/mL for gemcitabine and 0.5, 25 and 250 ng/mL for dFdU by adding the blank microdialysate to the required amount of working stock cocktail solution in a volumetric flask. The QC samples were vortexed-mixed, then subdivided into aliquots and stored at −20 °C. Both calibration and QC standards contained gemcitabine and dFdU.

2.6. Microdialysis experiment

2.6.1. Brain probe implantation

The hair on the top of rat skull was shaved and the skull was disinfected with ethanol (Xilong Chemical industry Co. Ltd., Shantou, Guangdong, China) and betadine (Shandong Lircon Co. Ltd., Dezhou, Shandong, China). The rats were then placed in a stereotaxic apparatus with an incisor bar set at 3.3 mm from the interaural line. The bregma line was identified for use as a reference point. The coordinate for striatum relative to bregma was 1 mm anteroposterior, 3.0 mm lateral and 5.0 mm ventral for tumor implantation with 2×10^5 tumor cell and intralesional administration at the fifth day after tumor inoculation [26]. Then, a hole (1.5 mm posterior; 3.0 mm lateral; 5.0 mm ventral) was drilled through the cranium, dorsal to the striatum. An intracerebral guide cannula was lowered into the area attached to the stereotaxic apparatus. A microdialysis probe was placed within the guide cannula for collection. The cannula was secured to the skull with screws and dental cement and was capped with a dummy stylet. Brain microdialysis probes with 3 mm active membranes were implanted into specific regions of brain. The animals were allowed to recover for a period of 48 h before microdialysis experiment. The rats were decapitated after the experiment. The localization of the probe was verified and only the rats with correctly placed probes were included in the experiment.

2.6.2. In vivo relative recovery analysis of gemcitabine and dFdU

In vivo relative recoveries of gemcitabine and dFdU were estimated by determining the loss of the drug *in vivo* using reverse dialysis technique. Microdialysis probes were inserted into the tumor-regions of rat brain. After one hour of stabilization, an aCSF solution containing gemcitabine (0.01 mg/mL) and dFdU (0.01 mg/mL) was perfused through the probe at a rate of 1 μL/min. The dialysis samples were collected at each 10 min for 6 h. The dialysates entering (C_{inlet}) and leaving (C_{outlet}) the probe were analyzed by UPLC–MS/MS. The *in vivo* recovery was then calculated as the % loss of gemcitabine and dFdU from the probe inlet into the brain, assuming that the identical flux of molecules were across the dialysis membrane in both directions. The *in vivo* recovery of drug was estimated by the following equation: $\text{recovery}_{in\ vivo} = [(C_{inlet} - C_{outlet})/C_{inlet}] \times 100\%$, where, C_{inlet} and C_{outlet} were the concentrations of gemcitabine and dFdU in the perfusate (inlet) and dialysate (outlet) fluids, respectively.

2.6.3. Microdialysis samples

After insertion, the microdialysis probe was perfused with aCSF using a microliter syringe pump at a flow rate of 1 μL/min. Then, the rat received a single intracranial injection of gemcitabine (0.64 mg/kg) after 2 h of stabilization. The dialysis samples were collected in a 200 μL Eppendrof® tubes every 10 min for 6 h. The collected samples were wrapped with aluminum foil and stored in a refrigerator at −20 °C before analysis.

2.7. Sample preparation

The samples were thawed at room temperature before processing. The solutions of 185 μL aCSF and 5 μL IS were added to 10 μL of microdialysis sample in a micro centrifuge tube. The samples were vortexed for 20 s. The mixture was transferred to an auto sampler vial and an aliquot of 10 μL was injected into the UPLC–MS/MS for analysis.

2.8. Method validation

The method was validated for matrix effect, selectivity, linearity, precision, accuracy, and stability according to the FDA guidance for validation of bioanalytical methods [27]. Validation runs were conducted during three consecutive days. The peak area of gemcitabine and dFdU of QC samples were interpolated from the calibration curve on the same run to give the concentration of gemcitabine and dFdU. The results from QC samples in three runs were used to evaluate the precision and accuracy of the method developed.

3. Results and discussion

3.1. UPLC–MS/MS optimization

In UPLC–MS/MS, analytes could be identified by both their retention time and molecular weights. Also, triple quadrupole MS

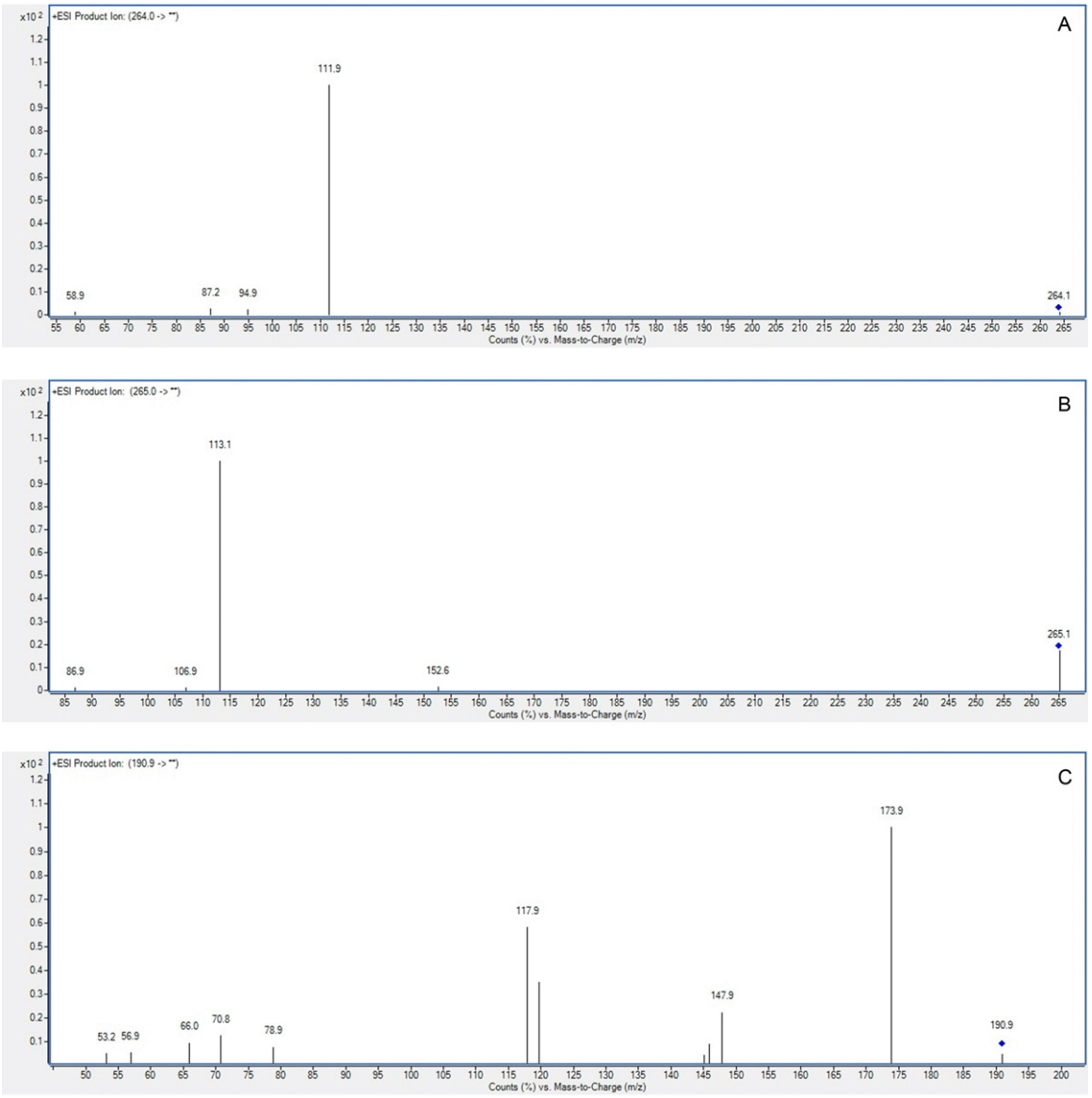

Fig. 2. Product ion scan spectra of $[M+H]^+$ of gemcitabine (A), dFdU (B), and 5-bromouracil (C).

allowed specific product ions to be monitored. The SRM function provided an additional filtering for individual analyte. As a result, the signal-to-noise ratio of a total ion chromatographic peak using SRM mode was significantly higher than that obtained using selected ion monitoring mode. UPLC–MS/MS provided a direct, structurally specific measurement of individual components with high sensitivity.

Parameters of the mass spectrometer detector were tuned according to the MS signal response of the target compound and the results indicated that the positive mode was much more sensitive than the negative mode. The positive ion scan of standard solutions of gemcitabine, IS and dFdU indicated that gemcitabine, IS and dFdU had protonated molecular ions $[M+H]^+$ of *m*/*z* 264.0, *m*/*z* 190.9 and *m*/*z* 265.1 in full scan mass spectra, respectively. In the product ion mass spectra, the fragment ion at *m*/*z* 112.0 for gemcitabine, *m*/*z* 173.8 for IS and *m*/*z* 113.0 for dFdU were shown in Fig. 2.

The chromatographic conditions, especially the composition of mobile phase, were optimized through several tests to achieve good resolution and symmetric peak shapes of the analytes, as well as a short run time. Modifiers, such as ammonium acetate aqueous solution (13 mmol/L and 65 mmol/L) and formic acid aqueous solution (0.1% and 0.5%), were added. The results indicated that when mobile phase containing formic acid (0.1%) was used, the ionization efficiency of gemcitabine, IS and dFdU could be enhanced, whereas ammonium acetate in the mobile phase did not affect the ionization efficiency of analytes. Finally, acetonitrile and 0.1% formic acid (4:96, v/v) were adopted as the mobile phase. After the comparison of several columns (Thermo Syncronis C_{18} column, 50 mm × 2.1 mm I.D., 1.7 μm; Acquity BEH C_{18} column, 50 mm × 2.1 mm I.D., 1.7 μm; Agilent SB-C_{18} column, 50 mm × 2.1 mm I.D., 1.8 μm), Agilent SB-C_{18} column was finally used with a flow rate of 0.2 mL/min to produce good peak shapes and permit a run time of 3.5 min.

3.2. Method validation

3.2.1. Matrix effects

Matrix effects are generally caused by the molecules originating from the sample matrix that co-elute with the compounds of interest. These molecules can interfere with an ionization process in a mass spectrometer, resulting in ionization suppression

or enhancement. These unpredictable effects can produce the response change of a compound when the compound is analyzed in a biological matrix compared with a standard solution. Consideration should be given to evaluate and eliminate matrix effects when developing an LC–MS/MS assay [28]. There are two common methods to assess matrix effects: the post-column infusion method [29] and the post-extraction spike method [30]. Although large compounds (6–20 kDa) like proteins and enzymes in the microdialysate samples were physically removed by the membrane dialysis probe, co-elution of both aCSF inorganic salts and the numerous endogenous compounds in the dialysates could make quantitative analysis difficult due to matrix-induced interference.

The matrix effects due to endogenous compounds and inorganic salts in aCSF during the measurement of investigated compounds were evaluated by comparing the peak area of analytes dissolved in blank microdialysates with those in mobile phase. Three different QC concentration levels of gemcitabine (1, 50 and 500 ng/mL) and dFdu (0.5, 25, 250 ng/mL) were evaluated by analyzing five samples at each level. The matrix effect of IS (5 μL × 37.6 μg/mL) was evaluated using the same method. Matrix effect values were calculated by analyzing the samples at QC concentrations. The average values of matrix effects were 92.1% (relative standard deviation (RSD) = 2.4%, $n = 5$), 99.1% (RSD = 2.2%, $n = 5$) and 100.5% (RSD = 1.6%, $n = 5$) for gemcitabine (1, 50, 500 ng/mL, respectively) and 93.5% (RSD = 2.9%, $n = 5$), 102.2% (RSD = 1.1%, $n = 5$) and 104.3% (RSD = 6.3%, $n = 5$) for dFdU (0.50, 25.00, 250.00 ng/mL, respectively). The average values of matrix effect of IS was 94.6% (RSD = 5.8%, $n = 15$). These data indicated that the matrix effects from endogenous compounds were negligible for the present method.

3.2.2. Selectivity

The selectivity of this method was tested by comparing the chromatograms of blank microdialysates (Fig. 3A), mid quality control sample (Fig. 3C), and rat brain tumor microdialysate samples at 6 h after intralesional chemotherapy of gemcitabine (Fig. 3D). All the blank microdialysates were found to be free of interferences within the retention window of gemcitabine, IS and dFdU using UPLC–MS/MS conditions. Representative chromatograms were shown in Fig. 3 and no other endogenous peaks were observed. Under the above conditions, the retention time of

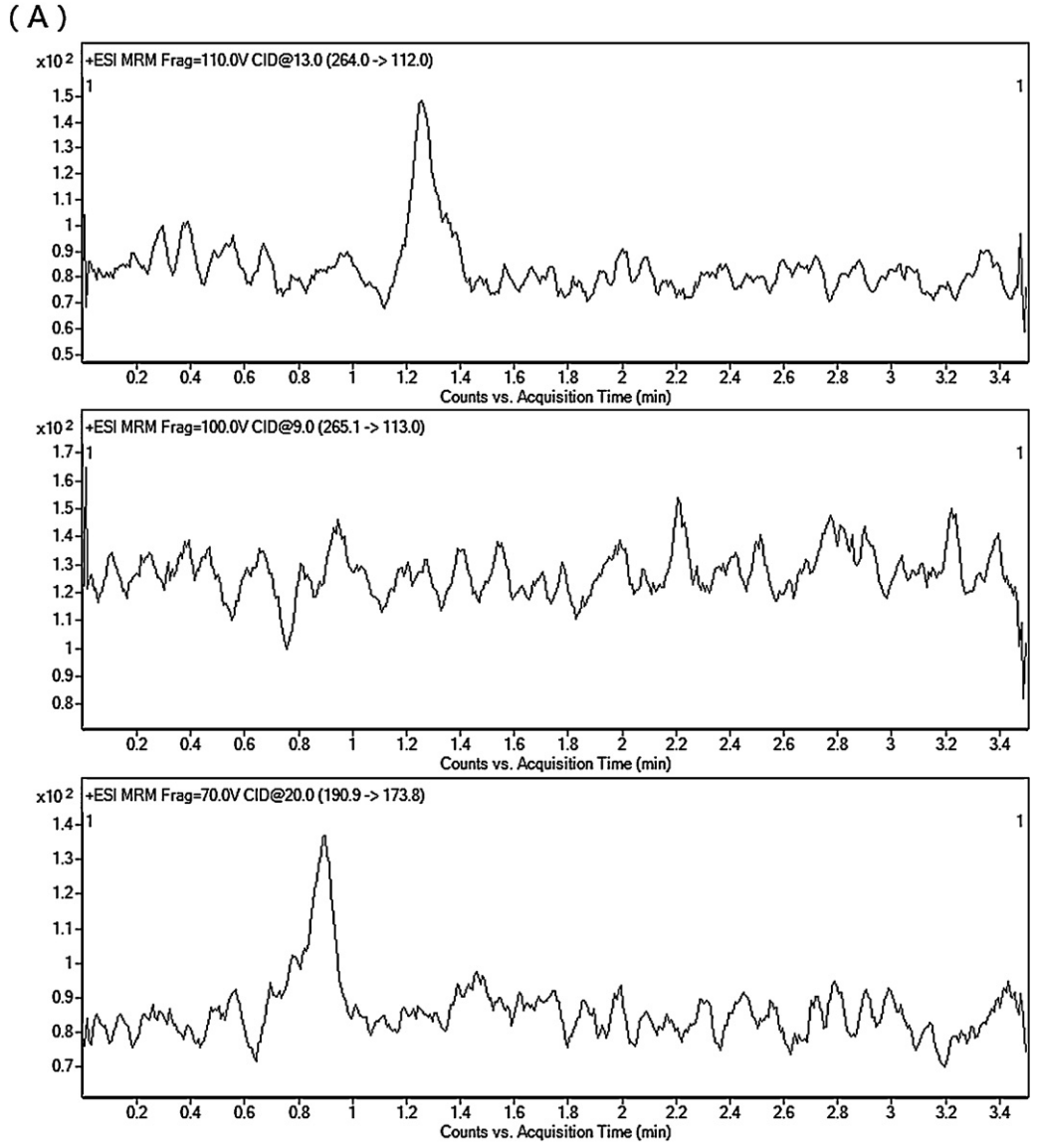

Fig. 3. The SRM (+) chromatogram of (A) blank microdialysates sample, (B) LLOQ of gemcitabine (0.66 ng/mL) and dFdU (0.31 ng/mL), (C) mid quality control sample, and (D) rat brain tumor microdialysate samples at 6 h after intralesional chemotherapy of gemcitabine (0.64 mg/kg). The bottom pane represents gemcitabine (m/z 264.0 → 112.0); the middle pane represents dFdU (m/z 265.1 > 113.0); and the top pane represents the internal standard 5-bromouracil (m/z 109.9 > 173.8).

gemcitabine, IS and dFdU were 1.3 min, 1.9 min and 2.9 min, respectively.

3.2.3. Calibration curve and limits of detection and quantitation

The method exhibited good linear response over the selected concentration range using linear regression analysis. The standard curves were typically described by the following least-square equation $y = 0.7455x - 7.2063$ ($r = 0.9991$) for gemcitabine and $y = 0.0862x + 0.0418$ ($r = 0.9997$), where y corresponded to the mean peak-area ratio of gemcitabine or dFdU to the IS and C referred to the mean concentration of gemcitabine or dFdU added to the blank microdialysate within a concentration range of 0.66–677.08 ng/mL or 0.31–312.00 ng/mL, respectively. The lower limit of quantification (LLOQ) in microdialysis samples was defined as the lowest concentration of the calibration curve that could be quantitatively determined with acceptable precision and accuracy, whereas the lower limit of detection (LLOD) in microdialysis samples was defined as the lower concentration at where the signal-to-noise (S/N) ratio was 3:1. The LLODs of gemcitabine and dFdU were determined as 0.2 ng/mL and 0.1 ng/mL, respectively. The LLOQs of gemcitabine and dFdU were 0.66 ng/mL and 0.31 ng/mL, respectively. The SRM (+) chromatograms of LLOQs (gemcitabine at 0.66 ng/mL and dFdU at 0.31 ng/mL) were shown in Fig. 3B.

3.2.4. Precision and accuracy

The precision and accuracy of the assay were determined using the QC samples by replicate analyses of three concentration levels of gemcitabine (1, 50 and 500 ng/mL) and dFdU (0.5 ng/mL, 25 ng/mL and 250 ng/mL). Intra-day precision and accuracy were determined by repeated analysis of five spiked samples of gemcitabine and dFdU at each QC level on one day ($n = 5$). Inter-day precision and accuracy were determined by repeated analysis on three consecutive days ($n = 5$ series per day). The concentration of each sample was determined using standard curves prepared and analyzed on the same day. An RSD value of a measured concentration was used to evaluate precision and accuracy values were calculated as accuracy (%) = (mean of measured concentration/nominal concentration) × 100. The data of intra-day and inter-day precision and accuracy of the method for gemcitabine and dFdU were presented in Table 1. For gemcitabine, the RSD% values of intra-day precision and accuracy were in the range 2.9–5.5 and 94.29–105.4% respectively, whereas the

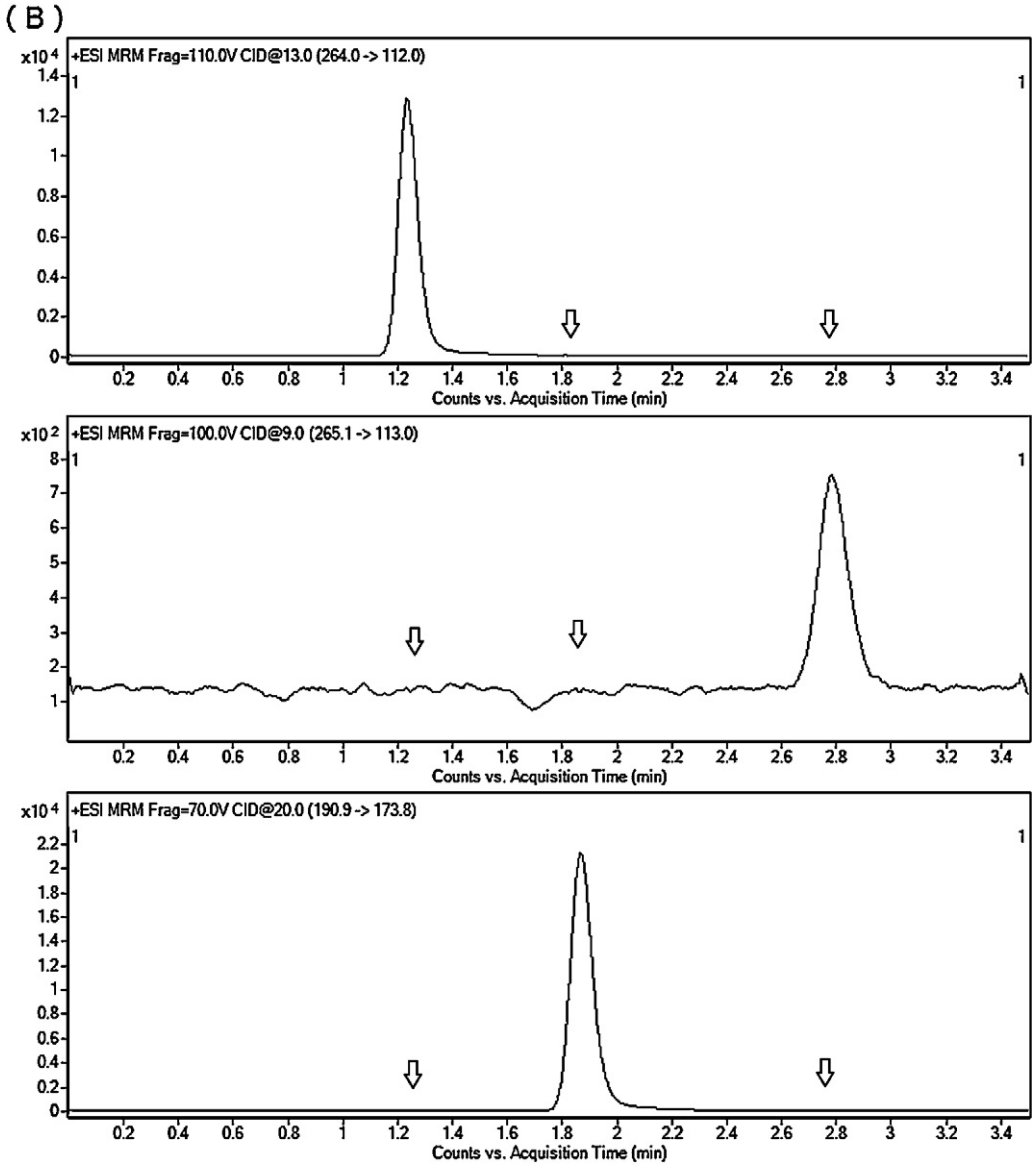

Fig. 3. (*Continued*)

Table 1
The intra- and inter-day precision and accuracy of the method for determination of gemcitabine and dFdU (intra-day, $n = 5$; inter-day, $n = 5$ series per day).

Added concentration (ng/mL)	Intra-day			Inter-day		
	Detected concentration (mean ± SD, ng/mL)	Accuracy (%)	RSD (%)	Detected concentration (mean ± SD, ng/mL)	Accuracy (%)	RSD (%)
Gemcitabine						
1	1.05 ± 0.03	104.5 ± 3.04	2.9	0.89 ± 0.06	89.16 ± 6.04	6.8
50	47.14 ± 1.69	94.29 ± 3.39	3.6	48.64 ± 3.39	97.27 ± 6.79	7.0
500	527.20 ± 28.92	105.4 ± 5.78	5.5	495.10 ± 12.28	99.02 ± 2.46	2.5
dFdU						
0.5	0.49 ± 0.03	98.40 ± 5.23	5.3	0.51 ± 0.04	101.3 ± 7.90	7.8
25	26.38 ± 0.89	105.5 ± 3.56	3.4	25.96 ± 1.45	103.8 ± 5.80	5.6
250	251.39 ± 14.15	100.6 ± 5.60	5.7	256.07 ± 24.26	102.4 ± 9.70	9.5

corresponding inter-day values were 2.5–7.0 and 89.16–99.02%. For dFdU, the RSD% values for intra-day precision and accuracy were in the range 3.4–5.7 and 98.40–105.5% respectively, whereas the corresponding inter-day values were 5.6–9.5 and 101.3–103.8%. These results revealed good precision and accuracy.

3.2.5. Stability

The stabilities of gemcitabine and dFdU in blank microdialysate were evaluated by exposing the QC samples under different temperatures and storage conditions. The QC samples of gemcitabine and dFdU were exposed to short term room temperature conditions for 4 h, long term storage conditions for 20 days (−20 °C), and

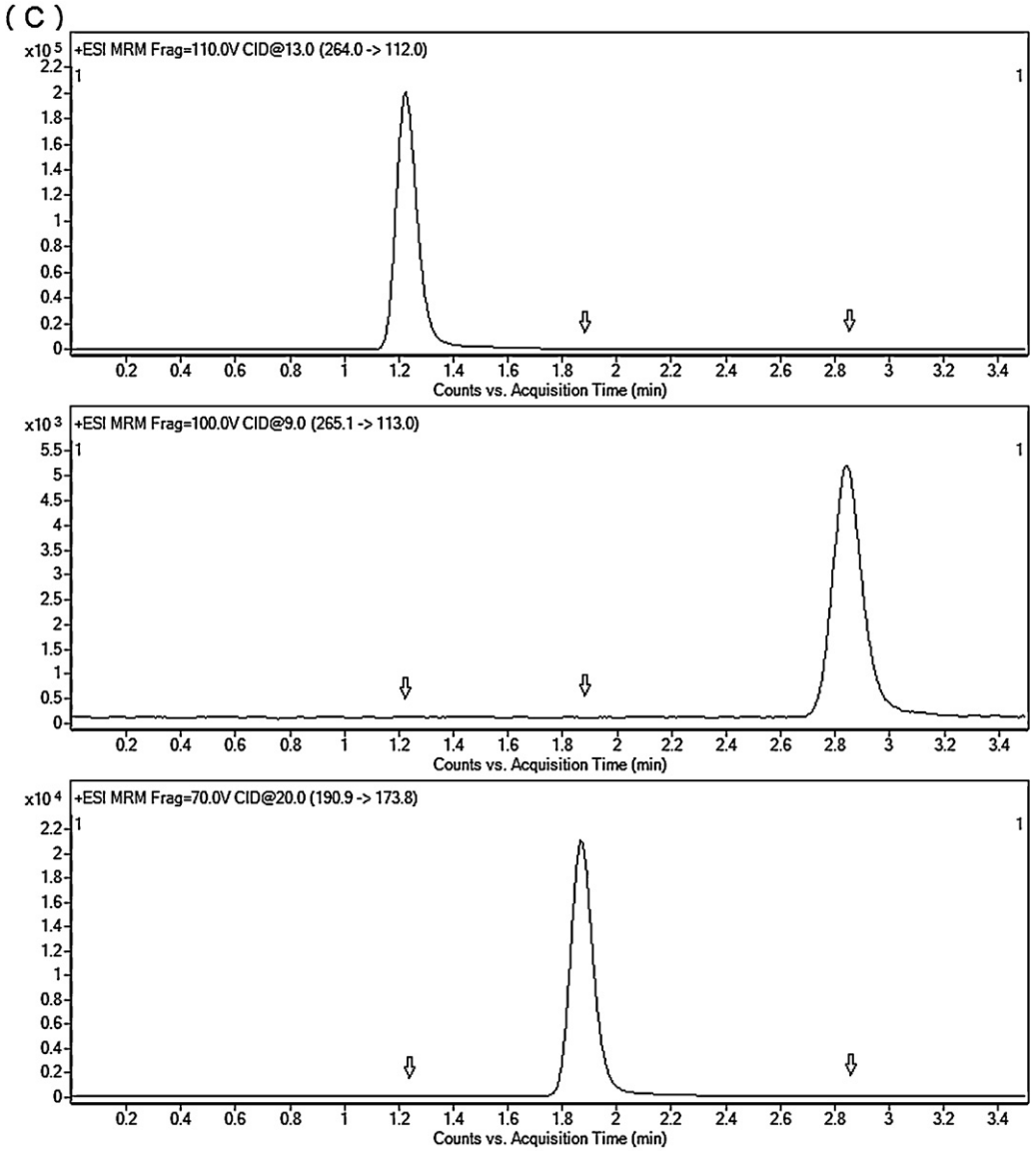

Fig. 3. (*Continued*)

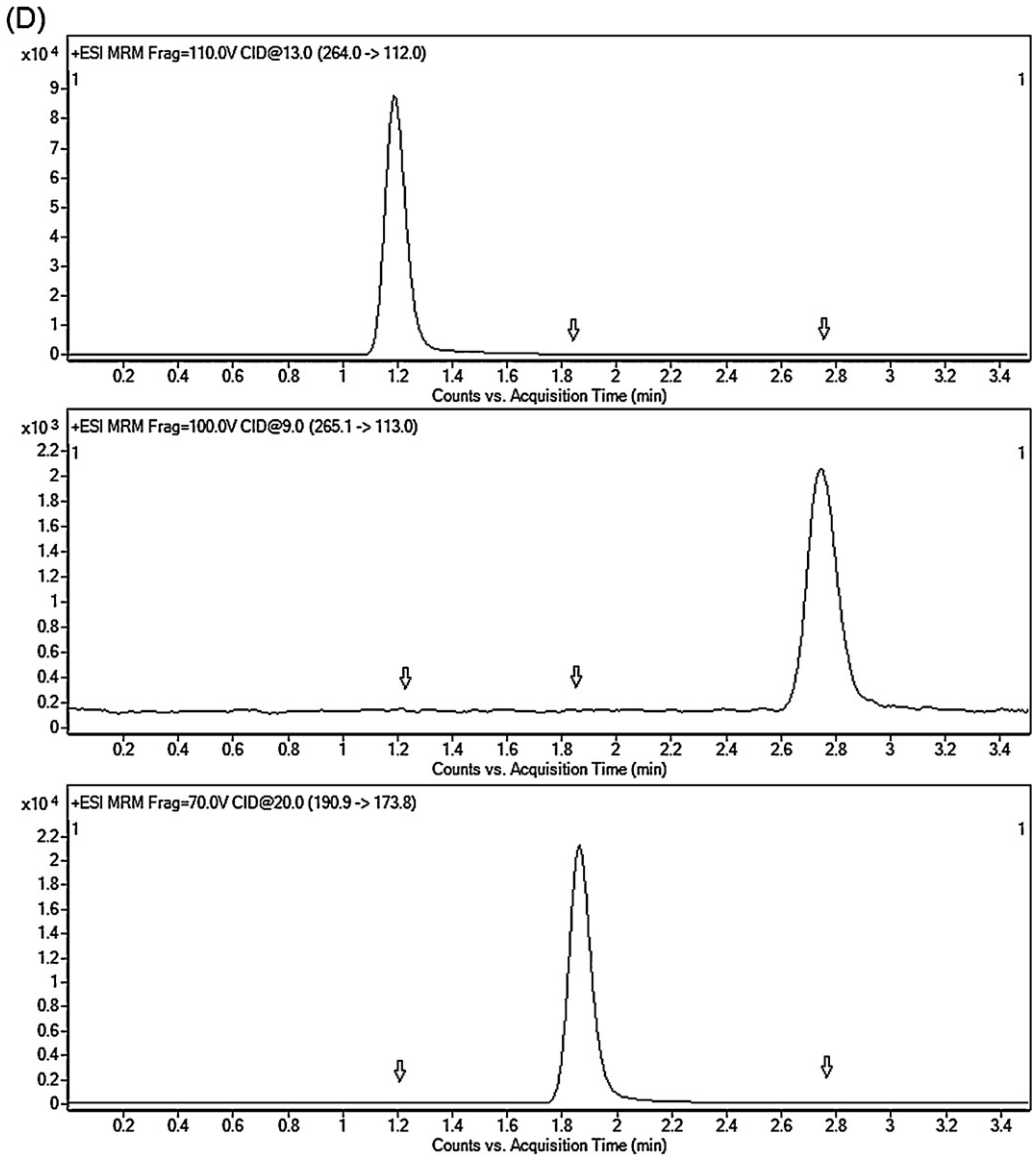

Fig. 3. (*Continued*).

Table 2
Stability of gemcitabine and dFdU in microdialysate at different QC levels ($n = 5$).

Storage conditions	Gemcitabine				dFdU			
	Added concentration (ng/mL)	Detected concentration (ng/mL)	Accuracy (%)	RSD (%)	Added concentration (ng/mL)	Measured concentration (ng/mL)	Accuracy (%)	RSD (%)
Freeze-thaw (3 cycles)	1	0.92 ± 0.11	92.30 ± 10.86	11.8	0.5	0.47 ± 0.05	94.40 ± 10.66	11.3
	50	47.94 ± 4.51	95.89 ± 9.02	9.4	25	24.38 ± 2.00	97.52 ± 8.01	8.2
	500	483.20 ± 45.31	96.64 ± 9.06	9.4	250	251.39 ± 20.65	100.56 ± 8.26	8.2
Short-term (4 h, 25 °C)	1	0.95 ± 0.07	94.71 ± 7.45	7.9	0.5	0.50 ± 0.05	100.9 ± 9.18	9.1
	50	49.31 ± 3.76	98.63 ± 7.51	7.6	25	25.55 ± 1.73	102.2 ± 6.91	6.8
	500	494.33 ± 33.52	98.87 ± 6.50	6.6	250	249.04 ± 7.83	99.62 ± 3.13	3.2
Long-term (20 d, −20 °C)	1	0.93 ± 0.09	92.79 ± 8.53	9.2	0.5	0.96 ± 0.06	95.99 ± 5.52	5.8
	50	48.69 ± 4.52	97.38 ± 9.03	9.3	25	49.72 ± 2.73	99.44 ± 5.47	5.5
	500	483.77 ± 23.01	96.75 ± 4.60	4.8	250	493.57 ± 9.33	98.71 ± 1.87	1.9
Auto-sampler (24 h, 25 °C)	1	0.50 ± 0.03	99.16 ± 6.85	6.9	0.5	0.50 ± 0.03	100.5 ± 6.37	6.4
	50	25.07 ± 1.37	100.3 ± 5.47	5.5	25	25.30 ± 0.90	101.1 ± 3.62	3.6
	500	243.96 ± 13.72	97.58 ± 5.49	5.6	250	250.55 ± 9.38	100.2 ± 3.75	3.8

three freeze–thaw cycles. Due to the need for occasional delayed injection or reinjection of extraction samples, the stability of reconstituted samples in auto sampler vials was assessed at ambient temperature for 24 h. Five replicates of all the QC samples were analyzed for stability. Table 2 summarized the freeze and thaw stability, short-term stability, long-term stability and post-preparative samples in auto sampler vials stability data of gemcitabine and dFdU. All the results showed the stabilities during these tests and there were no stability-related problems during the samples' routine analysis.

3.3. *In vivo* relative recoveries of microdialysis probe

During the study of cerebral microdialysis, the determination of microdialysis probe recovery is essential for accurate quantification of substance in extracellular fluid and keeping the stability of microdialysis probe recovery. The ratio between the dialysate and the interstitial concentrations of the substance studied is defined as relative recovery. There are two methods to determine the relative recovery of a microdialysis probe, namely *in vivo* and *in vitro* relative recovery determination. Determination of *in vitro* relative recovery usually neglects the effects of physiological factors on the microdialysis probe. The *in vivo* relative recovery estimation minimizing the effect of variability is usually adopted.

Evaluation of *in vivo* recovery includes the point of no net-flux, retrodialysis on gain, and retrodialysis on loss. The point of no net-flux is more accurate than retrodialysis and has been considered as a 'gold standard'. However, this method requires a number of repeated samples at steady state and is not suitable for dynamic studies [31]. Therefore, retrodialysis on loss was selected to determine the *in vivo* relative recovery of the microdialysis probe in our experiment.

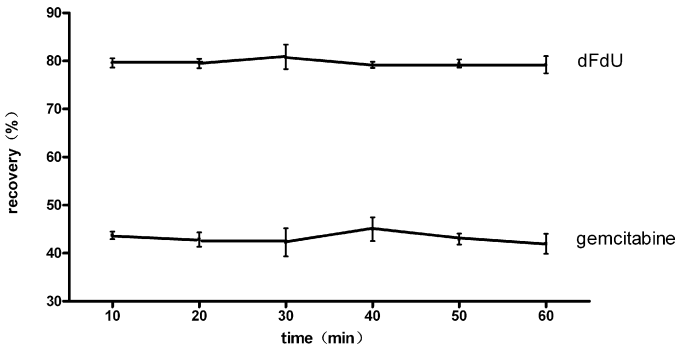

Fig. 4. Recovery-time profiles of gemcitabine and dFdU in microdialysis samples from rat tumor brain. Data were presented as means ± SD for six probes.

Based on the delivery experiments, the relative recoveries of gemcitabine and dFdU were determined to be 43.56 ± 2.2% and 79.50 ± 0.4%, respectively. The results (Fig. 4) indicated that the performance of the microdialysis system was stable during a 6 h study.

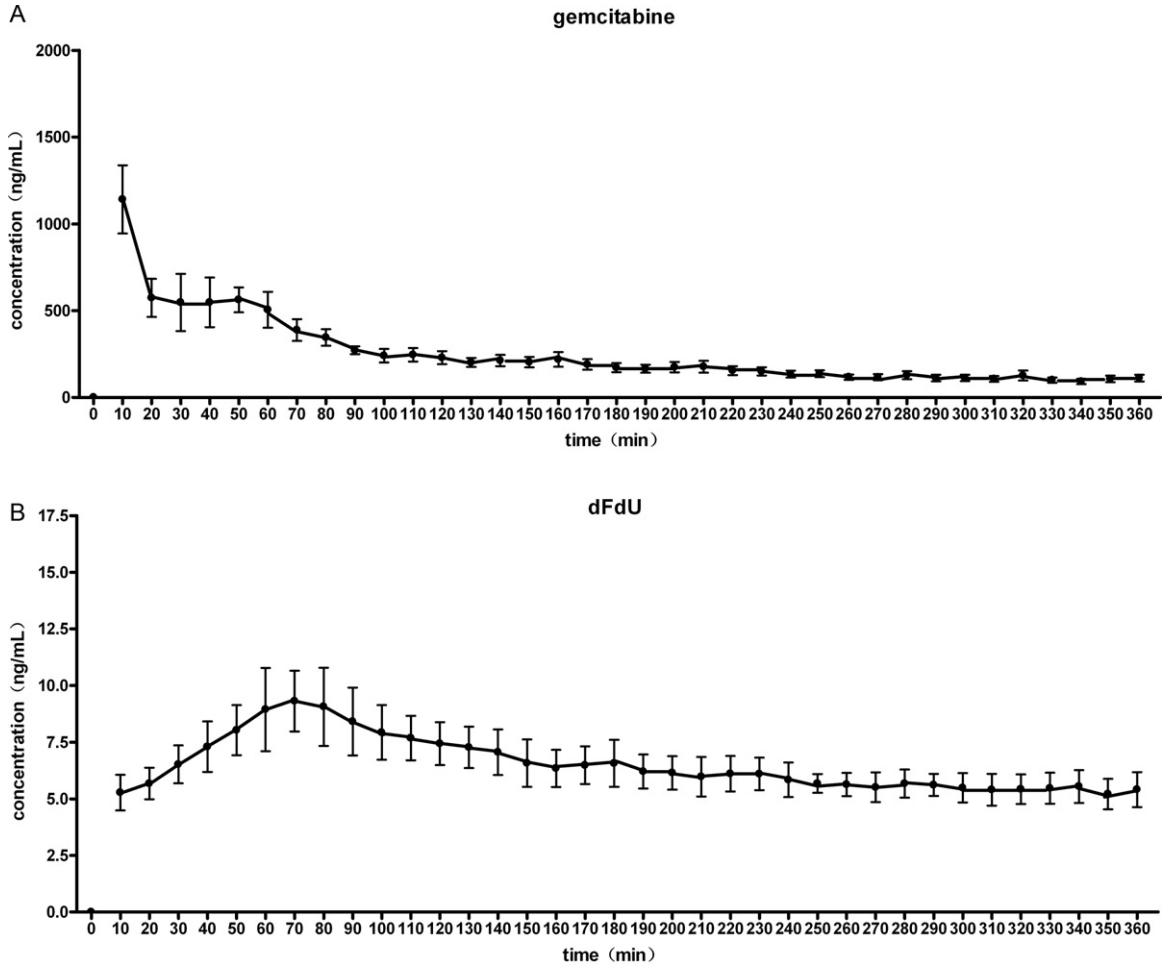

Fig. 5. The concentration-time profile ($n = 6$, mean ± SD). Gemcitabine (A) and dFdU (B) in tumor-region of glioma-implanted rat treated with gemcitabine (0.64 mg/kg) using intralesional chemotherapy.

3.4. Application of the assay

The concentrations of gemcitabine and dFdU in dialysate from rat brain tumor were calculated from the calibration curves. The mean concentrations–time profiles of gemcitabine and dFdU after administration were shown in Fig. 5. The concentrations of gemcitabine and dFdU determined in physiological samples were corrected for the relative recovery of the probe used.

4. Conclusion

In conclusion, a rapid and sensitive UPLC–MS/MS method was developed for the determination of gemcitabine and dFdU from *in vivo* microdialysis sampling of rat brain tumor-region. The method allowed the quantification of gemcitabine and dFdU with a small volume of microdialysis samples and in a short run time of 3.5 min. Good linearity, stability, precision and accuracy were achieved. All validated parameters met the criteria set in FDA guidelines for bioanalytical methods. The validated method had been successfully applied to the determination of gemcitabine and dFdU in microdialysis samples obtained from extracellular fluid of rat brain tumor after intracranial injection.

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