Original Research Paper

Development and validation of a UPLC–MS/MS assay for the determination of gemcitabine and its L-carnitine ester derivative in rat plasma and its application in oral pharmacokinetics

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A R T I C L E   I N F O

Article history:
Received 1 October 2016
Accepted 5 January 2017
Available online 9 January 2017

Keywords:
Gemcitabine
L-carnitine
Prodrug
Pharmacokinetics
UPLC/MS/MS

A B S T R A C T

A simple and rapid UPLC–MS/MS method to simultaneously determine gemcitabine and its L-carnitine ester derivative (2’-deoxy-2’, 2’-difluoro-N-((4-amino-4-oxobutanoyl)oxy)-4-(trimethyl amm-onio) butanoate-cytidine, JDR) in rat plasma was developed and validated. The conventional plasma sample preparation method of nucleoside analogues is solid-phase extraction (SPE) which is time-consuming and cost-expensive. In this study, gradient elution with small particles size solid phase was applied to effectively separate gemcitabine and JDR, and protein precipitation pretreatment was adopted to remove plasma protein and extract the analytes with high recovery (> 81%). Method validation was performed as per the FDA guidelines, and the standard curves were found to be linear in the range of 5–4000 ng/ml for JDR and 4–4000 ng/ml for gemcitabine, respectively. The lower limit of quantitation (LLOQ) of gemcitabine and JDR was 4 and 5 ng/ml, respectively. The intra-day and inter-day precision and accuracy results were within the acceptable limits. Finally, the developed method was successfully applied to investigate the pharmacokinetic studies of JDR and gemcitabine after oral administration to rats.

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1. Introduction

Gemcitabine is a water-soluble pyrimidine nucleoside analogue with significant anticancer activity for several types of cancers, such as pancreatic adenocarcinoma, bladder cancers and breast cancers [1,2]. Unfortunately, due to high hydrophilicity and poor membrane permeability, it is difficult for gemcitabine to enter the blood circulation after oral administration. Therefore, gemcitabine is administered by intravenous administration. Therefore, gemcitabine is administered by intravenous injection in clinic due to its low oral bioavailability [3]. Although many efforts have been made to design advanced intravenous drug delivery system, oral route is still the most preferred route due to its patient compliance, therapeutic efficacy and convenience [4].

Prodrug strategies based on various gastrointestinal nutrient transporters have been utilized to overcome undesirable and pharmacokinetic properties of drug, since these transporters play an important role in the oral absorption of nutrients and therapeutic drugs. It has been reported that validanosine and valdeacetabine could be transported across intestinal epithelium by oligopeptide transporter 1 [5–7]. In order to further improve the oral bioavailability of nucleoside drugs, some new attempts are required. For example, prednisolone–carnitine conjugate was studied for nasal delivery mediated by organic cation/carnitine transporters 2 (OCTN2) [8]. This high-affinity carnitine transporter is highly expressed in kidney, trachea and intestine and is a promising target protein because it can transport organic cations and nutrients, such as carnitine and ergothioneine [9–13]. OCTN2 is also crucial for the β-oxidation and energy metabolism of fatty acids, and responsible for a primary systemic carnitine deficit. Following this idea, we synthesized the L-carnitine ester derivative of gemcitabine as shown in Fig. 1, namely JDR, to enhance the oral bioavailability of gemcitabine. To compare oral pharmacokinetics of JDR and gemcitabine, it was necessary to develop a sensitive method for simultaneous quantification of both drugs.

Various quantitative methods have been developed for the determination of gemcitabine and its derivatives. Wickremsinhe et al. developed a method for the determination of LY2334737 (gemcitabine prodrug), gemcitabine and its metabolite (dFdU) by LC–MS/MS and column switching chromatography [14]. Bowen et al. had validated a method for the analysis of gemcitabine and dFdU using solid phase extraction (SPE) [15]. However, these methods have some notable limitations, such as time-consuming and cost-expensive features of SPE sample preparation, unconventional column switching chromatography.

There are several challenges in the quantification of gemcitabine and its analogues. The similar chemical structure requires highly selective methods for their quantification. What is more, it is difficult to be simultaneously retained in RP system for the compounds with different polarity. Last, the current available quantification methods have been developed based on SPE of a large-volume sample and long analytical time. However, a simple and efficient protein precipitation method with short analytical time was rarely used in nucleoside analogue pretreatment due to low extraction recovery and matrix effect. Therefore, developing a simple and reliable method to simultaneously determine gemcitabine and its analogue is critical to the pre-clinical study of gemcitabine prodrugs.

In the present study, a rapid, sensitive UPLC–MS/MS method was developed to simultaneously determine JDR and gemcitabine. Compared with the previous assay methods of plasma volume of 100 μl and single run time of 5 min, this method provided shorter analysis time (3 min), reduced volume requirements (50 μl) and simplified plasma sample pretreatment. The lower limit of quantification (LLOQ) of gemcitabine was 4 ng/ml, which was sensitive enough to detect relatively low concentration of gemcitabine in rat plasma. This method was successfully applied to characterize the pharmacokinetic profiles of JDR and gemcitabine after a single dose of oral administration.

2. Materials and methods

2.1. Chemicals and reagents

Gemcitabine (98.8% purity) was purchased from Nanjing Chemlin Chemical Industry Co., Ltd (Jiangsu, Nanjing, PR China). L-carnitine was obtained from Kaiyuan Hengtai Chemical Co., Ltd (Liaoning, Shenyang, P R China). IS (99% purity, didanosine) was synthesized in Shenyang Pharmaceutical University (Shenyang, China). Ammonium acetate (HPLC grade) was obtained from Tianjin Kemiu Chemical Reagent Co. (Tianjin, China). Ultra pure water was prepared by EASYPURE®II RF/UV system (Boston, MA, USA). Tetrahydrouridine (THU), the cytidine deaminase inhibitor, was purchased from J&K Scientific (HPLC grade). HPLC-grade methanol was purchased from Fisher Scientific (Fairlawn, NJ, USA). All other chemicals were of analytical grade.

![Fig. 1 – Structure of JDR and gemcitabine. (A) gemcitabine, (B) JDR.](image-url)
2.2. Synthesis of JDR

2.2.1. Synthesis of benzyl carnitine

L-carnitine (0.322 g, 2 mmol) was dissolved in 10 ml DMF and heated to 140 °C. Benzyl bromide (0.342 g, 2 mmol) was added dropwise, and the reaction was allowed to reflux 2 h. The solvent was concentrated under reduced pressure and the residue purified by recrystallization from acetone. The product was concentrated under reduced pressure, and dried under high vacuum at room temperature until the weight was constant. Yield: 85% of a white powder. MS (ESI): m/z = 535.2 [M + H]+.

2.2.2. Synthesis of 5-(benzyloxy)-3-(3-carboxypropanoyloxy)-N, N, N-trimethyl-5-oxopentan-1-a minium

Succinic acid (0.2 g, 2 mmol) was dissolved in 10 ml 1, 4-dioxane and heated to reflux. Sulfoxide chloride (0.238 g, 2 mmol) was added dropwise, and the reaction was allowed to reflux 4 h. The solvent was concentrated under reduced pressure and the residue was dissolved in 10 ml DMF. Benzyl carnitine (0.504 g, 2 mmol) and triethylamine (0.202 g, 2 mmol) were then filled in the reaction flask and heated to 60 °C overnight. The solvent was evaporated, and the residue was purified by column chromatography on a silica gel, eluting with methanol in dichloromethane (gradient 5–10%). Yield: 53% of a yellow powder. MS (ESI): m/z = 353.2 [M + H]+.

2.2.3. Synthesis of JDR

5-(benzyloxy)-3-(3-carboxypropanoyloxy)-N, N, N-trimethyl-5-oxopentan-1-aminium

JDR, gemcitabine and IS were prepared by dissolving compounds in water at the concentrations of 8 μg/ml, 4 μg/ml and 150 μg/ml, respectively. A series of working standard solutions of JDR, gemcitabine and IS were prepared by diluting standard stock solution with methanol at appropriate concentrations. All solutions were stored in a 4 °C freezer and brought to room temperature before use.

2.3. Instrumentation

An ACQUITY triple-quadrupole tandem mass spectrometer equipped with an electrospray ionization (ESI) interface (Waters Corp., Milford, MA, USA) was used. BEH C18 column (50 mm x 2.1 mm, 1.7 μm, Waters) was used for chromatographic separation. Data acquisition was performed by Masslynx 4.1 software with Quanlynx program (Waters Corp., Milford, MA, USA).

2.4. UPLC-MS/MS condition

The mobile phase was composed of methanol (A) and water (B) (containing 2 mM ammonium acetate) at a flow rate of 0.2 ml/min. A gradient elution was performed as described below, 0 - 0.2 min, 5% A; 0.21 - 1.2 min, 70% A; 1.21 - 3 min, 5% A. The column and auto-sampler temperatures were set at 40 °C and 10 °C, respectively. The injected volume was 5 μl.

For three compounds, the positive ion mode of electrospray ionization (ESI) was chosen for JDR, gemcitabine and negative ion mode for IS. Quantification was achieved by multiple reactions monitoring (MRM) mode. Conditions of the ESI source were optimized as follows: the capillary was set at 4.0 kV, desolvation gas temperature was 350 °C, and cone voltage was 40, 30 and 30V for JDR, gemcitabine and IS. Collision Energies (CE) was 25, 15 and 20 eV for JDR, gemcitabine and IS. The most sensitive ion transitions were selected for the monitoring of JDR m/z 507.0→162.1 for JDR, m/z 264.0→112.0 for gemcitabine, and m/z 235.1→134.9 for IS.

2.5. Sample preparation

An aliquot of 50 μl rat plasma sample in 1.5 ml Eppendorf tube was added with 25 μl IS solution (1500 ng/ml) and 225 μl of methanol. Then the samples were vortex-mixed for 3 min. The sample was centrifuged at 13,000 x g for 5 min. An aliquot of 5 μl of the supernatant was injected for the LC-MS/MS analysis.

2.6. Preparation of standard and quality control (QC) samples

Standard stock solutions of JDR, gemcitabine and IS were prepared by dissolving the accurately weighed reference compounds in water at the concentrations of 8 μg/ml, 4 μg/ml and 150 μg/ml, respectively. A series of working standard solutions of JDR, gemcitabine and IS were prepared by diluting standard stock solution with methanol at appropriate concentrations. All solutions were stored in a 4 °C freezer and brought to room temperature before use.

Calibration samples and QC samples were prepared according to sample preparation item. Methanol (75 μl) was replaced by 25 μl JDR and 50 μl gemcitabine standard solutions in THU-pretreated rat plasma. The final concentrations of blood sample were 5, 15, 125, 500, 3200, and 4000 ng/ml for JDR, 4, 12, 125, 500, 3200 and 4000 ng/ml for gemcitabine and 750 ng/ml for IS. QC samples were obtained in the same manner with three levels of 15, 125 and 3200 ng/ml for JDR and 12, 125 and 3200 ng/ml for gemcitabine.

2.7. Method validation

The selectivity of this method was evaluated by comparing chromatograms of six different batches of blank rat plasma with the corresponding spiked rat plasma at LLOQ for interferences between analytes, IS and endogenous substances. Linearity was measured by linear regression with a weighted (1/x²) least-squares analysis. Intra- and inter-day precision (the relative standard deviation, RSD %) and accuracy (the relative error, RE %) were verified by analysis of three levels of QC samples (n = 6) on 3 different days. The matrix effect on the
ionization of the analytes was tested by comparing the peak areas between post extraction sample and standard solutions at different concentrations. Recovery was calculated by comparing the mean peak areas of a pre- and post-extraction spiked sample at low, middle and high QC concentrations. The stability was assessed by determining QC samples \( n = 3 \) in three freeze/thaw cycles (−80 to 20 °C), long-term sample storage (−80 °C for 2 months) and short-term sample storage (20 °C for 1 h). The stability of extracted samples in the autosampler was also assessed at 10 °C for 8 h.

### 2.8. Pharmacokinetic study in rats

Twelve Wistar male rats weighting 180 to 220 g were supplied by the Animal center of the Shenyang Pharmaceutical University (Shenyang, China). All protocols for animal experiments were approved by Shenyang Pharmaceutical University Animal Care and Use Committee. The validated analytical method was then applied to the pharmacokinetic study after oral administration of JDR (equivalent to 50 mg/kg gemcitabine) or gemcitabine (50 mg/kg) to rats. The rats were randomly divided into two groups and fasted for 12 h until the administration of the drug. Serial blood samples were obtained at 0.083, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 10, 12, 24, 48 h for JDR and 0.25, 0.5, 2, 4, 8, 12, 24 h for gemcitabine, respectively. Blood samples were collected into THU-pretreated heparinized tubes (10 mg/ml) and immediately centrifuged at 3000 \( \times g \) for 15 min and supernatant (plasma) was stored at −80 °C prior to analysis.

### 2.9. Statistical analysis

Descriptive statistics were showed as mean ± standard deviation. The pharmacokinetic parameters (including \( C_{\text{max}} \), \( T_{\text{max}} \), AUC, \( T_{\frac{1}{2}} \)) of gemcitabine in control group and prodrug group were estimated using a one-tailed Student t test at the \( P < 0.05 \) level.

### 3. Results and discussions

#### 3.1. Optimization of chromatographic and mass conditions

UPLC-MS/MS operation parameters were optimized for the determination of analytes. The standard solutions of analyte were infused with the mobile phase into the mass spectrometer with ESI as the ionization source. The response of analyte to ESI were evaluated by recording the full-scan mass spectrum in both positive and negative ionization modes, and they had a good mass spectrum response in positive mode for gemcitabine and JDR, and negative mode for IS. Therefore, the most sensitive molecular ion transitions were with ESI for the MRM determination of \( m/z \) 264.0→112.0 for gemcitabine, \( m/z \) 507.0→162.1 for JDR and \( m/z \) 235.1→134.9 for IS as shown in Fig. 2. Other main mass spectrometry parameters, such as capillary voltage, cone voltage, collision energy and desolvation temperature were optimized to obtain the optimal response of analytes.

The separation and ionization of analytes are affected by compositions of mobile phase. Therefore, the selection of mobile phase is important for improving peak shape, sensitivity and short run time. Acetonitrile and methanol were both tested as organic modifier of mobile phase. A lower MS response was caused by addition of acetonitrile. Therefore, methanol was chosen as the organic modifier. It was known that the additives of mobile phases can affect the LC resolution and MS response of analytes. In the present work, the role of added formic acid and ammonium formate at various concentrations in the mobile phases were investigated. The results showed that the additive of ammonium formate improved the ionization of analytes. Particularly, the addition of 2 mM ammonium acetate not only enhanced the MS response of analytes but also improved the peak shapes of analytes. As a result, the addition of 2 mM ammonium acetate in the mobile phase was used in this study. The use of small particles of stationary phase allowed UPLC to strengthen the limits of both peak capacity and speed of analysis without compromising resolution. In the
present work, the column and gradient method were optimized. Several of reversed-phase columns, namely phenomenon Ultracarb C18 (50 mm × 4.6 mm, 2.1 μm), Waters SunFire C18 (50 mm × 4.6 mm, 2.1 μm) and Waters BEH C18 (50 mm × 2.1 mm, 2.1 μm) were studied. Compared with the other two columns, the sensitivity for analytes increased significantly with the use of BEH C18 column. To avoid undesirable matrix effect, gradient elution was used to provide a better MS response and peak shape. Various different gradient conditions were investigated and optimized. The peaks were efficiently separated from the interference with a signal-to-noise ratio of more than 10 for all analytes of interest.

### 3.2. Sample preparation

In order to inhibit the transformation of gemcitabine during sample preparation, the inhibitor were screened at three different concentrations (2, 5 and 10 ng/ml in rat plasma) under room temperature. THU at 10 ng/ml was the most effective inhibitor for stabilizing gemcitabine in rat plasma.

In recent years, solid-phase extraction (SPE) is often applied in the plasma pretreatment for nucleoside drugs, due to clean sample and high sensitivity. However, high cost and tedious-operation is inevitable. In this study, the simple protein precipitation using methanol or acetonitrile was applied and the optimal organic solvent was methanol, because the MS response of analytes is not good in acetonitrile. More important, the extraction recovery was greater than 80% for all analytes and there were no matrix effects. Because of the adequate sensitivity of optimized UPLC–MS/MS conditions, the plasma volume was reduced to 50 μl, much lower than the previous methods (100 μl of plasma).

Compared with the previous assay methods of the LLOQ values of gemcitabine was 0.25 ng/ml, this method provided higher LLOQ values (4 ng/ml) [15]. In this study, the supernatant of sample dried under nitrogen cannot be completed since prodrug could be hydrolyzed by a small amount of esterase remaining in biological samples. The result showed that the sensitivity, recovery and matrix effect of the method could meet the needs of pharmacokinetic study.

### 3.3. Method validation

The establishment and validation of this method were performed according to the FDA bioanalytical method validation guidelines.

For specificity, analyses of blank samples of plasma were obtained from six different rats, blank samples spiked with JDR (5 ng/ml), gemcitabine (4 ng/ml), and IS (750 ng/ml) and plasma samples after oral administration of JDR. Fig. 3 represented the chromatogram of LLOQ. Any interference from plasma could not be detected around the analytes or the internal standard.

Linear regression was achieved with 1/x² as weighing factor and standard curve were established. All coefficients (r) were greater than 0.99, which showed a good linearity over the studied concentration range. The LLOQ was 5 ng/ml for JDR and 4 ng/ml for gemcitabine.

The inter-day and intra-day precision and accuracy for all analytes from three QC levels were summarized in Table 1. The precision were fitted and the RSD % were over the range of 0.8% to 3.7% for JDR and 0.9% to 5.3% for gemcitabine, respectively, while the RE% ranged from −9.9% to 4.0%. These results indicated that this method was accurate and reliable.

The recovery and matrix effect of all the QC samples were summarized in Table 2. The mean recovery ranged from 88.5% to 110.8% for JDR, 81.4% to 100.8% for gemcitabine and 102.2% to 111.7% for IS. The matrix effect of plasma for three analytes was more than 90%, indicating that it was negligible.

### Table 1 – Inter-run accuracy and precision following a 3-batch validation of JDR and gemcitabine in rat plasma (data were mean ± SD, n = 18).

| Analytes | Nominal conc. (ng/ml) | Measured concentration (ng/ml) | Precision (RSD%) | Accuracy (RE%) | Measured concentration (ng/ml) | Precision (RSD%) | Accuracy (RE%) |
|----------|-----------------------|-------------------------------|------------------|---------------|-------------------------------|------------------|---------------|
| JDR      | 15.00                 | 15.35 ± 1.4                  | 9.4              | −1.6          | 15.12 ± 0.5                  | 3.4              | −3.1          |
|          | 125.00                | 116.13 ± 3.8                 | 3.2              | −7.1          | 112.60 ± 4.2                 | 3.7              | −9.9          |
|          | 3200.00               | 3573.15 ± 61.5               | 1.7              | 11.6          | 3083.75 ± 25.9               | 0.8              | 1.8           |
| Gemcitabine | 12.00                 | 11.26 ± 1.2                  | 10.4             | 4.3           | 11.02 ± 0.3                  | 2.3              | 2.0           |
|          | 125.00                | 124.66 ± 3.9                 | 3.1              | −0.3          | 129.96 ± 6.9                 | 5.3              | 4.0           |
|          | 3200.00               | 2912.59 ± 148.1              | 5.1              | −9.0          | 2931.04 ± 26.2               | 0.9              | −8.4          |

### Table 2 – Extraction efficiency and matrix effect for JDR, gemcitabine in rat plasma (data were mean ± SD, n = 6).

| Analytes | JDR | Gem | IS | Matrix effect (%) (mean ± SD) |
|----------|-----|-----|----|-------------------------------|
| JDR      | Low | 88.5 ± 0.06 | 81.4 ± 0.04 | 102.2 ± 0.03 | Low | 111.3 ± 0.09 | 94.2 ± 0.03 | 99.9 ± 0.01 |
|          | Mid | 101.4 ± 0.03 | 100.8 ± 0.07 | 106.5 ± 0.04 | Mid | 96.0 ± 0.02 | 102.8 ± 0.01 | 94.1 ± 0.01 |
|          | High| 110.8 ± 0.08 | 97.3 ± 0.05 | 111.7 ± 0.05 | High| 98.4 ± 0.02 | 96.8 ± 0.03 | 100.2 ± 0.01 |
Table 3 showed the results of the stability under four different conditions. The percent deviations for all analytes were less than 8% from theoretical value. The results showed that pre-preparative sample was stable when stored at room temperature for 1 h, −80 °C for 2 months, three freeze-thaw cycles, and kept in the auto-sampler at 10 °C for 8 h.

3.4. Application to pharmacokinetic

This validated method was successfully applied to a pharmacokinetic study after oral administration of JDR or gemcitabine to the Wistar rats at a dose of 50 mg/kg (calculated as gemcitabine). The mean plasma concentration-time curves of JDR and gemcitabine were displayed in Fig. 4. The pharmacokinetic parameters of JDR and gemcitabine were calculated using noncompartmental method and were summarized in Table 4. With a short peak time $T_{\text{max}}$ (JDR) of 1 h, JDR demonstrated a faster absorption rate than gemcitabine. Furthermore, the plasma elimination half-time $t_{1/2}$ of gemcitabine released from JDR was significantly longer than the parent drug gemcitabine, suggesting that JDR was more stable in plasma than gemcitabine and the bioactivation of JDR to gemcitabine
was slowly performed by the esterase in plasma. Finally, the area under the curve of gemcitabine following oral administration of JDR was 16132.95 mg · h/l, approximate 3-fold increase compared to that of gemcitabine (5031.61 mg · h/l). Hence, the pharmacokinetic results indicated that L-carnitine ester derivative of gemcitabine can improve the oral bioavailability of gemcitabine.

### 4. Conclusions

A simple, highly sensitive and reliable UPLC–MS/MS analytical method that can simultaneously determine JDR and gemcitabine in rat plasma has been developed and validated. The method required relatively simply sample pretreatment and accomplished high sensitivity using as small as 50 μl volume of plasma. The established method provided the LLOQs of 5 ng/ml for JDR and 4 ng/ml for gemcitabine with a single run time of 3 min. An effective method to select cytidine deaminase inhibitors to stabilize gemcitabine in rat plasma was developed. This method was successfully implemented to pharmacokinetic study of JDR and gemcitabine to rats after oral administration.

### Acknowledgments

We thank the financial support from the National Natural Science Foundation of China (No. 81173009) and Technology Bureau in Shenyang (No. ZCJJ2013402). We also thank the financial support from Project for New Century Excellent Talents of Ministry of Education (No.NCET-12-1015), Specific Science Foundation of Shenyang Pharmaceutical University (No. ZCJJ2014409) and National Undergraduate Training Program for Innovation and Entrepreneurship (2016).

### Table 3 – Stability of JDR and gemcitabine in rat plasma exposed to various storage conditions (data were mean ± SD, n = 3).

| Concentration (ng/ml) | RSD% | RE% |
|-----------------------|------|-----|
| **Added**             |      |     |
| JDR                   |      |     |
| Low                   | 15.00| 15.46 ± 1.3 | 8.6 | −0.9 |
| Mid                   | 125.00| 113.58 ± 1.3 | 4.3 | −9.1 |
| High                  | 3200.00| 3169.38 ± 150.5 | 4.7 | −1.0 |
| Gemcitabine           |      |     |
| Low                   | 12.00| 10.29 ± 1.0 | 10.0 | −4.8 |
| Mid                   | 125.00| 134.78 ± 1.6 | 1.2 | 7.8 |
| High                  | 3200.00| 2915.15 ± 95.7 | 3.3 | −8.9 |
| **Founded**           |      |     |
| JDR                   |      |     |
| Low                   | 15.00| 16.78 ± 0.3 | 1.5 | 7.5 |
| Mid                   | 125.00| 113.35 ± 5.6 | 5.0 | −10.1 |
| High                  | 3200.00| 3129.46 ± 137.9 | 4.4 | −2.2 |
| Gemcitabine           |      |     |
| Low                   | 12.00| 10.90 ± 1.3 | 12.0 | 0.9 |
| Mid                   | 125.00| 136.30 ± 1.8 | 1.4 | 9.1 |
| High                  | 3200.00| 2996.32 ± 151.9 | 5.1 | −6.4 |
| **Autosampler rack at 10 °C for 8 h** |      |     |
| JDR                   |      |     |
| Low                   | 15.00| 16.39 ± 0.5 | 1.3 | 4.1 |
| Mid                   | 125.00| 113.35 ± 1.6 | 2.2 | −12.7 |
| High                  | 3200.00| 3129.46 ± 137.9 | 4.4 | −2.2 |
| Gemcitabine           |      |     |
| Low                   | 12.00| 10.77 ± 0.1 | 12.0 | 0.9 |
| Mid                   | 125.00| 135.30 ± 1.8 | 1.4 | 9.1 |
| High                  | 3200.00| 2996.32 ± 151.9 | 5.1 | −6.4 |

### Table 4 – Pharmacokinetic parameters of JDR and gemcitabine in rat after oral administration JDR and gemcitabine (data were mean ± SD, n = 6).

| Ingredients | Cmax (ng/ml) | Tmax (h) | AUC0–24 (mg · h/l) | T1/2 (h) |
|-------------|--------------|----------|-------------------|----------|
| JDR-JDR     | 4153.18 ± 1927.40 | 1.08 ± 0.9 | 12828.59 ± 2204.5 | 6.31 ± 3.9 |
| JDR-gemcitabine | 2658.04 ± 1212.6** | 1.00 ± 0.0 | 16132.95 ± 5128.0** | 6.36 ± 2.1** |
| Gemcitabine | 1181.17 ± 47.1 | 1.50 ± 0.9 | 5031.61 ± 627.4 | 2.61 ± 1.1 |

* P < 0.01 compared with gemcitabine.
Conflicts of interest

The authors declare that there is no conflicts of interest.

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