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ORIGINAL RESEARCH

Development and Validation of an HPLC-MS/MS Method for Determining I-BET151 in Rat Plasma and Its application to Pharmacokinetic Studies

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Propose: A bromodomain and extra-terminal domain inhibitor (I-BET151) is effective in treating chronic graft-versus-host disease and has been extensively studied in recent years. However, there is limited research on the pharmacokinetics of I-BET151, especially the lack of methods for determining the concentration of I-BET151 in vivo. Therefore, the purpose of this study is to establish an HPLC-MS/MS method for determining the plasma concentration of I-BET151 and use it for pharmacokinetic study in rats.

Methods: The chromatographic column is a Poroshell 120EC-C18 column (50 mm × 4.6 mm, 2.7 μm). The mobile phase consists of water containing 20 mmol ammonium acetate and 0.1% formic acid, and methanol containing 0.1% formic acid, with a flow rate of 0.6 mL/min. The extraction of I-BET151 is liquid–liquid extraction, and the extraction solvent is ether:dichloromethane=2:3. The HPLC-MS/MS method was validated based on the guidelines of quantitative methods for biological samples in the Chinese Pharmacopoeia, including specificity, standard curve, lower limit of quantification, residual effects, precision, recovery rate, matrix effects, stability, etc.

Results: The results showed that the established method met the requirements of methodological validation standards and could be used for pharmacokinetic studies of I-BET151. The pharmacokinetic results displayed that the half-life of oral and intravenous administration of I-BET151 was 4.3 h and 3.1 h, respectively. The oral bioavailability was about 60%, indicating that I-BET151 had a high oral bioavailability and appropriate half-life, demonstrating good clinical application prospects.

Keywords: HPLC-MS/MS, method validation, I-BET151, pharmacokinetics, bioavailability

Introduction

Allogeneic hematopoietic stem cell transplantation, as an important means of treating various blood diseases, has been increasingly widely used in clinical practice.[1,2] However, the complications after transplantation, especially the occurrence of chronic graft-versus-host disease, have caused concern for many patients and their families.[2,3] Researches show that the incidence rate of chronic graft-versus-host disease in transplant recipients is as high as 30%-70%, especially in patients after systemic radiotherapy or compulsory immunosuppression treatment, the risk of chronic graft-vers-host disease is significantly increased.[4,5] Chronic graft-versus-host disease not only seriously affects the patient's life, but may also lead to more serious complications, making it one of the most difficult problems after transplantation. Therefore, it is particularly important to have a deep understanding of the diagnosis and treatment of chronic graft-versus-host disease. The pathogenesis of chronic graft-versus-host disease is relatively complex, mainly related to the attack of donor immune cells on recipient tissues, including immune response activation, cytokine release, and damage and fibrosis of recipient tissues.[6,7] Unlike acute graft-versus-host disease, chronic graft-versus-host disease usually does not appear until more than 3 months after transplantation. Currently, it is recommended in clinical practice to use glucocorticoids, alone or in combination with calcineurin inhibitors, which are used as first-line treatment options.[8] If first-line treatment is effective and the symptoms of chronic graft-versus-host disease are effectively controlled, corticosteroids should be gradually

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reduced. However, researches have found that about half of patients lack sensitivity to first-line treatment options, and long-term use of hormones may result in side effects such as infection, hypertension, osteoporosis, and may reduce the anti-tumor effect of transplants, leading to malignant disease recurrence and secondary tumors.[9] It is recommended to initiate second-line treatment in the face of this situation, mainly including low-dose methotrexate, ruxolitinib, belumosudil, sirolimus, imatinib, rituximab, ibrutinib and other drugs.[10] Therefore, it is necessary to develop new methods or drugs for the treatment of chronic graft-versus-host disease.

Bromodomain and extra-terminal domain (BET) is protein domains that can recognize acetylated lysine residues and play an important role in regulating cell cycle and mediating gene transcription.[11,12] They are closely related to various diseases and are one of the main targets of epigenetics. The BET family consists of four members, BRD2, BRD3, BRD4, and BRDT, each containing two N-terminal bromine domains (BRD1, BRD2), which are associated with the occurrence and development of various diseases such as obesity, pulmonary fibrosis, chronic obstructive pneumonia, epilepsy, tumors, heart failure, and chronic graft-versus-host disease.[12] BET protein is a key regulatory factor that controls specific transcription by mediating protein interactions in enhancers. At present, many BET bromine domain inhibitors with different structures have been reported, and multiple inhibitors have entered the clinical trial stage, especially the BRD4 inhibitor based on tumor control development (with up to 30 clinical trials, of which 2 have entered the Phase III clinical trial stage).[13] I-BET151 is a novel small molecule inhibitor targeting BET protein, which has high affinity for BRD2, BRD3, and BRD4.[14] It exerts multiple pharmacological effects by selectively acting on various proteins such as COX-2, P450, GSK3 β, PI3K, etc., and has inhibitory effects on the malignant phenotype of various tumors. For example, research has shown that I-BET151 has a significant inhibitory effect on the proliferation of mixed lineage leukemia positive leukemia cell lines, and I-BET151 treatment can benefit the survival of mixed lineage leukemia positive mice.[15] It was also found that I-BET151 can inhibit the progression of stem cell carcinoma by blocking the transcription of the oncogene Yap1 regulated by DOT1L mediated H3K79me2 positive histone methylation modification.[16] Other studies have shown that I-BET151 can significantly inhibit dendritic cell and T cell function. In the allo HSCT mouse model, early administration of short-term I-BET151 can significantly reduce the severity of chronic graft-versus-host disease.[17,18] Therefore, it is extremely necessary to conduct in-depth research on the feasibility of I-BET151 in the treatment of chronic graft-versus-host disease.

At present, researchers have conducted comprehensive studies on the mechanism, efficacy, and potential toxicity of I-BET151 in the treatment of chronic graft-versus-host disease. However, there is a lack of corresponding data and reports on the pharmacokinetic behavior of I-BET151 in vivo, which directly affects the subsequent development of preparations, drug interaction studies, and formulation of dosing regimens. Therefore, it is necessary to conduct systematic pharmacokinetic studies on I-BET151. In terms of in vivo drug concentration determination, HPLC-MS/MS is the most commonly used detection method. Compared to HPLC, UV spectrophotometry and immunoassay, HPLC-MS/MS method has many advantages, such as high specificity, high sensitivity, high throughput, and the ability to simultaneously detect multiple drugs and metabolites, making it the gold standard for in vivo analysis of small molecule drugs.[19–21] Based on this, this study aims to establish a rapid HPLC-MS/MS method for determining the plasma drug concentration of I-BET151 in rats and conduct systematic methodological validation, including specificity, standard curve and linear range, residual effects, precision, recovery rate, matrix effects, stability, dilution reliability, etc. The established HPLC-MS/MS method will be applied to study the pharmacokinetics of I-BET151 in rats, aiming to provide theoretical support for further research on I-BET151. The results showed that the established HPLC-MS/MS method has high sensitivity, wide linear range, qualified recovery rate, matrix effect, and good precision. Finally, it was successfully used to study the pharmacokinetic behavior of I-BET151 in rats. The pharmacokinetic results showed that the oral half-life of I-BET151 is about 4 hours, and the relative bioavailability of oral administration exceeds 60%, which has further research value.

Methods

Experimental Reagents

I-BET151 (purity 99.5%) and diazepam (internal standard, IS, purity 99.9%) were purchased from Selleck & bimake (Texas, USA) and Sigma-Aldrich, respectively (St. Louis, USA). Methanol and acetonitrile were obtained from Fisher

Scientific (Pittsburgh, USA). Water was prepared using a Milli-Q water purification system procured from Millipore (Millipore Corp., USA). Formic acid and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, USA). All solvents and chemicals were HPLC-grade and did not require further purification before application. Figure 1 showed structure diagram of I-BET151 and diazepam.

Equipment and Conditions

The Jasper™ HPLC system (Shimazu, Japan), containing SCIEX Dx Pump (x2), SCIEX Dx Sampler, SCIEX Dx Oven, SCIEX Dx Controller and SCIEX Dx Degasser. I-BET151 and diazepam were separated from rat plasma via a Poroshell 120 EC-C_{18} column (50 mm × 4.6 mm, 2.7 μm) at 35 °C. Water containing 20 mmoL ammonium acetate and 0.1% formic acid and methanol containing 0.1% formic acid were used as eluents A and B, respectively. The gradient elution conditions were showed in Table 1.

The MS spectrometric detection of I-BET151 and diazepam was carried out on an AB SCIEX Triple Quad (TM) AB Sciex 4500MD equipped with electrospray ionization (ESI) detector. The ionization mode of I-BET151 and diazepam was positive. The MRM transition of I-BET151 and diazepam was m/z 416.0/311.1 and m/z 285.1/193.0. The main MS parameters were displayed in Table 2. Other optimized MS parameters were source temperature (TEM) 500 °C, entrance Potential (EP) 25, curtain Gas (CUR) 45, Turbo Gas 2 (GS2) 40, Turbo Gas 1 (GS1) 40. Analyst software 1.6.2 was used for the acquisition of data.

Stock Solutions, Quality Control Samples and Calibration Standards

Two milligrams of I-BET151 reference substance was weighed precisely and placed in a 10 mL volumetric flask. Methanol was added to dissolved I-BET151 and the volume was made up to the mark, preparing a stock solution of 200.0 μg/mL for later use. Additionally, 1.00 mg of diazepam reference substance was precisely weighed and put into a 10 mL volumetric flask, methanol was added to dissolve it and the volume was adjusted to the mark, obtaining a 100.0 μg/mL stock solution. Then, gradually diluted the I-BET151 stock solution with methanol to obtain a series of I-BET151 standard solutions with concentrations of 0.2, 0.5, 2.0, 5.0, 20.0, 50.0 and 100.0 μg/mL; 200 ng/mL diazepam solution was obtained by diluting the diazepam stock solution.

Took 100.0 μL of rat blank plasma, and added 10.0 μL of the I-BET151 series standard solutions and 10.0 μL of 200 ng/mL diazepam solution, respectively, to prepare plasma samples with I-BET151 concentrations of 20.0, 50.0, 200.0, 500.0, 2000.0, 5000.0, and 10000.0 ng/mL. According to the procedures described as "Treatment Method of Plasma Samples", took 2.0 μL for analysis and recorded the chromatogram. The peak area ratio of I-BET151 to diazepam was plotted on the y-axis, and the concentration of I-BET151 was plotted on the x-axis. We can use the least squares method to obtain the best fitting curve, which is the standard curve.

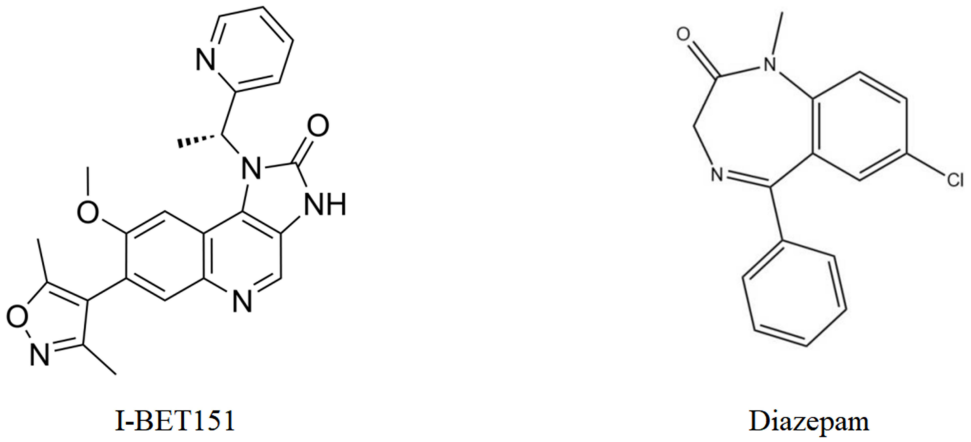

Figure 1 The structure diagram of I-BET151 and diazepam.

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Table 1 Gradient Condition of HPLC

Time(min)	A(%)[a]	B(%)[b]	Flow Rate (mL/min)
Initial	90	10	0.6
0.50	90	10	0.6
0.51	50	50	0.6
1.50	25	75	0.6
1.60	10	90	0.6
2.50	10	90	0.6
2.51	90	10	0.6
3.00	90	10	0.6

Notes: [a]water containing 20 mmol ammonium acetate and 0.1% formic acid; [b]Methanol containing 0.1% formic acid.

Table 2 MS Parameters of I-BET151 and Diazepam

	Ionization Mode	Transition (m/z)	Collision Energy (v)	Declustering Potential (v)	Spray Voltage (v)	Cell exit potential (v)
I-BET151	Positive	416.0→311.1	29	110	4500	10
Diazepam	Positive	285.1→193.0	44	80	4500	6

The quality control (QC) samples were used to evaluate the method stability, including high-concentration QC, medium-concentration QC, low-concentration QC and the lower limit of quantification (LLOQ). Their concentrations were 8000.0 ng/mL, 500.0 ng/mL, 40.0 ng/mL, and 20.0 ng/mL, respectively.

Plasma Sample Processing Method

A 100.0 μL sample of plasma was taken and placed in a 7.0 mL plastic round-bottom EP tube with a stopper. 10.0 μL of the diazepam solution and 3 mL of the mixed extraction solvent (ether:dichloromethane = 2:3) were added. It was vortexed for 6 min, and then centrifuged at 3000 rpm for 8 min. The supernatant was taken and transferred to a 10 mL pointed-bottom EP tube. The solvent was evaporated to dryness under a nitrogen stream at 40 °C. Then it was redissolved with 300 μL of methanol, vortexed for 6 min and centrifuged at 15000 rpm for 8 min. The supernatant was taken for injection analysis.

Method Validation

All validation procedures of the developed HPLC-MS/MS method were performed following the Chinese pharmacopoeia guidelines for bioanalytical method validation.[22]

Specificity

A sample of 100.0 μL blank plasma obtained by mixing the blood from 6 rats was taken. Without adding the internal standard, the operations were carried out based on the procedures "Plasma Sample Processing Method" section, and 3.0 μL of the sample was injected to obtain the chromatogram. A suitable concentration of I-BET151 solution was added into the blank rat plasma to prepare a simulated plasma sample. The operations were carried out based on the procedures "Plasma Sample Processing Method" section, and 3.0 μL of the sample was injected to obtain the chromatogram. The plasma samples collected from rats after administration, following the procedures in the "Processing Methods for Plasma Samples" section, and 3.0 μL of the supernatant was injected to obtain the chromatogram. It was required that the response of the interfering components should be lower than 20% of the response of the lower limit of quantification of I-BET151 and lower than 5% of the response of diazepam.

Linearity, LLOQ and Carryover

According to the results of the preliminary experiment, the linear range of I-BET151 was set to be 20.0–10,000.0 ng/mL. It was required that the recalculated concentration of the calibration standard should be less than ±15% of the labeled value, and

the LLOQ should be within ±20%. The LLOQ refered to the lowest concentration of the analyte in a sample that can be reliably quantified, and it should have acceptable accuracy and precision. In this study, the LLOQ was the lowest point of the standard curve. The carryover effect would affect the accuracy of the determination, and the carryover should be investigated and minimized. After injecting the sample with the highest concentration of the standard curve, a blank plasma sample was injected to evaluate the carryover effect. It was required that the carryover in the blank plasma sample after the high-concentration sample should be within 20% of the LLOQ and should not exceed 5% of diazepam.

Accuracy and Precision

Accuracy described the degree of closeness between the detected concentration and the labeled concentration of the analyte, while precision described the degree of closeness of the concentrations of the analyte obtained from repeated determinations and was defined as the relative standard deviation of the concentration determination.

Took 100.0 μL of blank plasma and carried out the procedures described as "Preparation of Standard Curve". Quality control (QC) samples of I-BET151 were prepared at three concentrations of low, medium and high (40.0, 500.0, 8000.0 ng/mL) respectively. Six samples for each concentration were prepared according to the procedures described as "Plasma Sample Processing Method" and conducted continuous determinations for 3 d. Then, the concentrations of the QC samples were calculated according to the standard curve. The accuracy and precision were calculated according to the results of the QC samples. It was required that both the relative standard deviation (RSD) of precision and the relative error (RE) of accuracy should be lower than 15.0%.

Extraction Recovery and Matrix Effect

A sample of 100.0 μL blank plasma was taken and carried out the procedures described as "Preparation of Standard Curve" to prepare QC samples of I-BET151 at three concentrations of low, medium and high (40.0, 500.0, 8000.0 ng/mL), respectively. Six samples for each concentration were prepared according to the procedures described as "Plasma Sample Processing Method" to obtain the peak area A.

Additionally, another 100.0 μL of blank plasma was taken and mixed with 3 mL of the extraction solvent, it was vortexed for 6.0 min and centrifuged at 3000.0 rpm for 8.0 min. Then, the supernatant was taken into a 10.0 mL plastic tube, 10.0 μL of I-BET151 solution and 10.0 μL of diazepam solution were added, respectively. The processing steps were carried out based on the procedures "Plasma Sample Processing Method", the sample was injected to obtain the peak area B. The extraction recovery rate was calculated according to the ratio A/B of the peak areas of the two treatment methods for each concentration.

A sample of 100.0 μL blank plasma was taken and mixed with 3 mL of the extraction solvent, it was vortexed for 6.0 min and centrifuged at 3000.0 rpm for 8.0 min. Then, the supernatant was taken into a 10.0 mL plastic tube, 10.0 μL of I-BET151 solution and 10.0 μL of diazepam solution were added, respectively. The processing steps were carried out based on the procedures "Plasma Sample Processing Method", the sample was injected to obtain the peak area A. Next, 100.0 μL of water was taken and mixed with 3 mL of the extraction solvent, it was vortexed for 6.0 min and centrifuged at 3000.0 rpm for 8.0 min. Then, the supernatant was taken into a 10.0 mL plastic tube, 10.0 μL of I-BET151 solution and 10.0 μL of diazepam solution were added, respectively. The processing steps were carried out based on the procedures "Plasma Sample Processing Method", the sample was injected to obtain the peak area B. The matrix effect was calculated according to the ratio A/B of the peak areas of the two treatment methods for each concentration.

Stability

The stability of I-BET151 in rat plasma was assessed using QC samples under following conditions, including short-term stability (room temperature, 12 h), long-term stability (−20 °C, 30 d), freeze-thaw stability (from −20.0 °C to room temperature, three cycles) and autosampler stability (4 °C, 24 h). The RE should be less than 15%.

Dilution Integrity

A plasma sample of I-BET151 with a concentration of 40000.0 ng/mL was prepared, and it was diluted with blank plasma to prepare plasma samples with concentrations of 500.0 ng/mL and 8000.0 ng/mL, respectively. Three samples for each concentration were prepared according to the procedures described as "Plasma Sample Processing Method". It

was required that the RE% between the detected concentration and the theoretical concentration of all dilution tests should be less than 15.0%.

Application

Animal Experimental Protocol

Male Sprague-Dawley (SD) rats weighing approximately 200 g were randomly divided into 2 groups, with 10 rats in each group. The rats were fasted for 12 h before the experiment but had free access to water. All rats use procedures were in accordance with the regulation for animal experimentation issued by the State Committee of Science and Technology of the People's Republic of China. In addition, this experiment was approved by the Medical Ethics Committee of Cancer Hospital of China Medical University, ethical number KT20240319. In the first group, the rats were administered I-BET151 suspension by gavage, and the dosage was 20 mg/kg. In the second group, I-BET151 solution was administered via the tail vein at a dosage of 20 mg/kg. Blood samples of approximately 0.3 mL were collected from the rat eye sockets using heparinized capillary glass tubes (inner diameter 0.9–1.1 mm) at 0.25, 0.5, 0.75, 1, 2, 4, 6, 8, 12, 24, and 48 h after administration. The blood samples were placed in 1.5 mL heparinized and dried centrifuge tubes. The samples were then centrifuged at a speed of 3000 rpm for 6 min to separate the plasma. The extracted plasma was processed and analyzed according to the "Plasma Sample Processing Method".

Processing and Analysis of Pharmacokinetic Parameters

After completing the pharmacokinetic experiment on rats and detecting the plasma concentrations of each group at various time points, the Drug and Statistics Software (DAS) 3.0 software was used to process and analyze the data. The software was utilized to calculate important pharmacokinetic parameters such as the maximum blood drug concentration (C_{max}), the time to reach the peak concentration (T_{max}), the area under the concentration–time curve from 0 h to 48 h ($AUC_{0\rightarrow t}$), the elimination half-life ($t_{1/2}$), the area under the concentration–time curve from 0 h to infinity ($AUC_{0\rightarrow\infty}$), the clearance rate (CL), and the apparent volume of distribution (V_d).

Results and Discussion

Method Optimization

Although the MRM mode of mass spectrometry did not require the analytes to meet the baseline separation standard, it was required that the chromatographic peaks of the analytes were sharp and symmetrical, and the retention time should be moderate, which was closely related to the composition of the mobile phase, the selection of the chromatographic column, and the column temperature, etc. Through systematic optimization, it was found that when the chromatographic column was Poroshell 120 EC-C_{18} column (50 mm × 4.6 mm, 2.7 μm particle size), the mobile phase consisted of water containing 20 mmol ammonium acetate and 0.1% formic acid and methanol containing 0.1% formic acid, and the column temperature was set at 35 °C, satisfactory chromatographic peaks could be obtained. Moreover, ammonium acetate and formic acid significantly improved the peak shape and sensitivity. In addition, gradient elution helped to obtain more appropriate retention times, and the gradient elution conditions were shown in Table 1. Under these chromatographic conditions, the retention times of I-BET151 and diazepam were 2.03 min and 2.34 min, respectively.

In order to obtain highly sensitive and stable analyte signals, parameters such as the ionization mode, collision energy, declustering voltage, ion source temperature, and curtain gas were optimized. Through optimization, it was found that the response of I-BET151 and diazepam were higher and more stable in the positive ion ionization mode, and the noise was lower as well. The MS parameters, chromatograms and the final ion pairs were shown in Table 2 and Figure 2. The remaining mass spectrometry parameters were as follows: CUR: 40, TEM: 500, GS1: 40, GS2: 50, CAD: Medium, IS: 5500, EP: 18, CXP: 10.

There were mainly three extraction methods for biological samples, namely solid-phase extraction, protein precipitation, and liquid–liquid extraction. Among them, the protein precipitation method was the simplest, but it was generally very difficult to completely precipitate the matrix, resulting in poor sample cleanliness, which was likely to cause the contamination of the mass spectrometry ion source and the occurrence of the matrix effect. The samples processed by the liquid–liquid extraction method and the solid-phase extraction method had higher cleanliness, but the processing

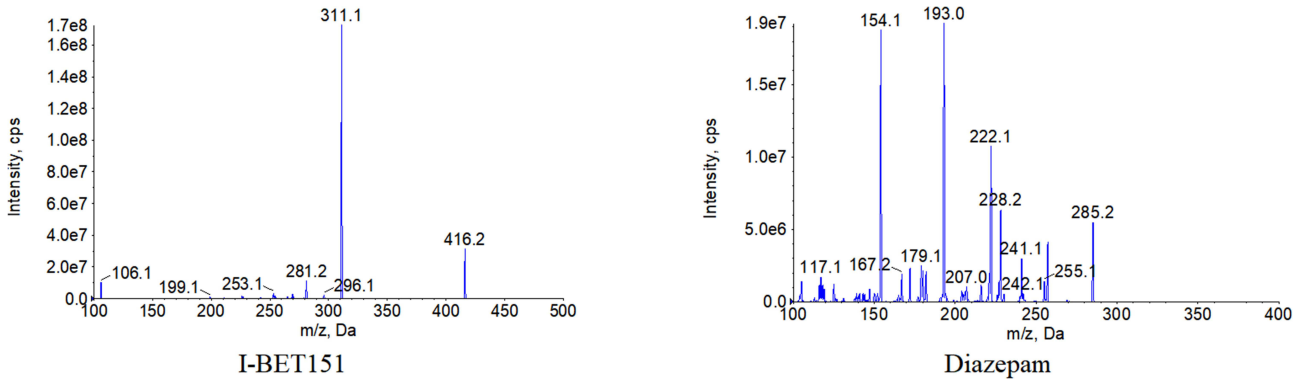

Figure 2 The diagrams of daughter scan of I-BET151 and diazepam.

procedures were more complicated than the protein precipitation method and required the support of other equipment. In this study, taking the extraction recovery as the measurement standard, I investigated diethyl ether, dichloromethane, ethyl acetate, a mixed solvent of diethyl ether and dichloromethane (diethyl ether:dichloromethane=1:1), and a mixed solvent of diethyl ether and dichloromethane (diethyl ether:dichloromethane=2:3), respectively. The results showed that when the mixed solvent of diethyl ether and dichloromethane (diethyl ether:dichloromethane=2:3) was used as the extraction solvent, I-BET151 and diazepam could obtain higher and more stable recovery.

Method Validation

Specificity

The results of the specificity demonstrated that the endogenous substances in the plasma did not interfere with the determination of I-BET151 and diazepam. It indicated that the matrix in the plasma could be largely removed thoroughly by liquid–liquid extraction. The typical chromatograms of the blank plasma, I-BET151, and diazepam were shown in Figure 3.

Linearity and LLOQ

In different rat plasma, eligible linearity was obtained at 20.0–20,000.0 ng/mL for I-BET151, and the typical equation of the calibration curve was as follows: y=0.4793x+1.1085 (r=0.9998). The LLOQ of I-BET151 was 20.0 ng/mL.

Precision and Accuracy

The results of the accuracy and precision were displayed in Table 3. The intra-day precision, inter-day precision, and accuracy of I-BET151 were all less than 15.0%. These results met the requirements of methodological validation. This indicated that the established HPLC-MS/MS method had good precision and accuracy and could ensure the accuracy of the in-vivo drug determination results.

Recovery and Matrix Effect

The results of the recovery and matrix effect were shown in Table 3. The extraction recovery of I-BET151 and diazepam were 93.7–103.6% and 96.2–99.2%, respectively. The matrix effects of I-BET151 and diazepam ranged from 86.5% to 112.4%, which met the requirements of the methodological validation. It also exhibited that the established HPLC-MS /MS method and the drug extraction method were quite appropriate, and the matrix such as phospholipids and proteins in rat plasma did not interfere with the ionization and accurate determination of the analyte and the internal standard.

Stability

The results of the stability were displayed in Table 4. The results indicated that the freeze-thaw stability, the autosampler stability, and the long-term stability of the plasma sample all met the requirements, which revealed that both I-BET151 and diazepam had good stability.

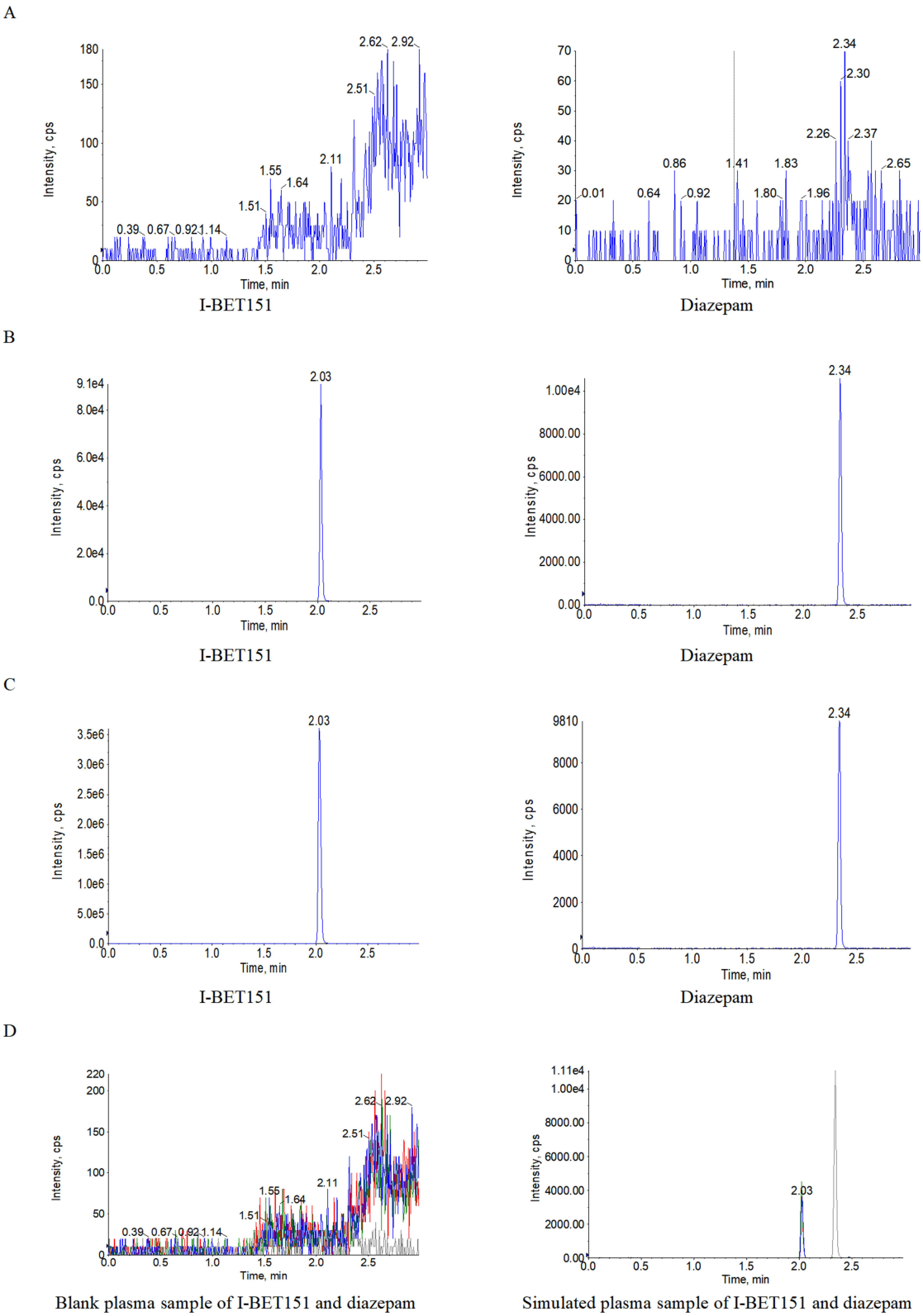

Figure 3 Representative HPLC-MS/MS chromatograms for I-BET151 and diazepam in rats plasma samples: (**A**) a blank plasma sample; (**B**) a blank plasma sample spiked with I-BET151 and diazepam, (**C**) a rat plasma sample, and (**D**) total chromatogram of I-BET151 and diazepam.

Table 3 Methodology Verification Results of Precision, Accuracy, Recovery and Matrix Effect

Drug	QC Concentration (ng/mL)	Inter-Day Precision (RSD %)	Intra-Day Precision (RSD %)	Accuracy (RE %)	Recovery (mean ± SD %)	Matrix Effect (mean ± SD %)
I-BET151	20.0	6.89	5.81, 8.90, 7.22	−2.31	95.3±3.86	97.1±3.05
	40.0	5.93	6.43, 5.37, 6.69	4.74	97.8±2.94	98.5±2.10
	500.0	8.16	4.73, 11.5, 6.08	6.58	94.9±3.53	103.6±4.28
	8000.0	4.47	3.71, 6.25, 5.11	−5.46	103.4±3.05	100.3±3.44
Diazepam	500.0	3.55	4.26, 3.18, 4.32	2.69	96.4±2.38	98.9±1.89

Table 4 Stability of I-BET151 and Diazepam Under Various Storage Conditions (Mean ± SD, n=4)

Drug	QC Concentration (ng/mL)	Room Temperature	−20 °C for 30 d	Freeze-thaw Cycles	Autosampler Stability	Dilution Integrity	
						5-fold	20-fold
I-BET151	20.0	21.3±1.63	19.3±1.84	20.5±1.97	21.8±2.11	——	——
	40.0	38.9±2.58	39.6±2.46	41.5±3.33	38.1±2.10	——	——
	500.0	521.2±30.4	484.2±27.8	508.5±32.6	512.9±22.4	495.2±27.5	516.4±38.7
	8000.0	7846.1±436.8	7952.7±378.5	8067.3±578.4	7902.6±422.7	——	——
Diazepam	500.0	487.9±27.1	495.1±30.7	518.3±39.9	505.4±27.5	——	——

Dilution Integrity

The results of the dilution integrity was displayed in Table 4. The results represented that the RE% between the detected concentration and the theoretical concentration in all the dilution tests was less than 15.0%, which indicated that dilution did not affect the accurate determination of the sample by this method.

Application

The samples were processed according to the "Plasma Sample Processing Method", and then the established HPLC-MS /MS method was applied to determine the plasma concentration, and the pharmacokinetic parameters were calculated. The results were shown in the Table 5 and Figure 4.

It can be seen from Figure 4 and Table 5 that the bioavailability of I-BET151 by gavage was relatively high, about 60% of that by tail vein administration. Meanwhile, we found that there was no significant individual difference among rats. Due to the absorption process involved in gavage administration, it showed lower peak concentrations and longer peak times compared to tail vein administration. In addition, there was no significant difference in clearance rate and half-life between the two groups. It can be seen from Figure 4 that there are obvious absorption phase, distribution phase and elimination phases in the drug time curve of oral administration, which is consistent with the characteristics of the two-compartment model. In summary, the research findings revealed the in vivo behavior of I-BET151 in rats, which would contribute to the development of I-BET151 related formulations and further pharmacological studies.

Table 5 Pharmacokinetic Parameters of I-BET151 in Rats (Means ± SD, n = 6)

	Oral Administration	Tail Vein Administration
$AUC_{(0-t)}$(μg/Lh)	66.43±15.37	103.7±20.85
$AUC_{(0-\infty)}$(μg/Lh)	66.50±14.88	104.7±21.49
CL (L/h)	208.8±32.45	225.6±42.73
C_{max} (μg/L)	12.64±2.56	22.81±4.61
$t_{(1/2)}$ (h)	4.30±0.76	3.11±0.85
T_{max} (h)	1.57±0.73	0.25±0.00

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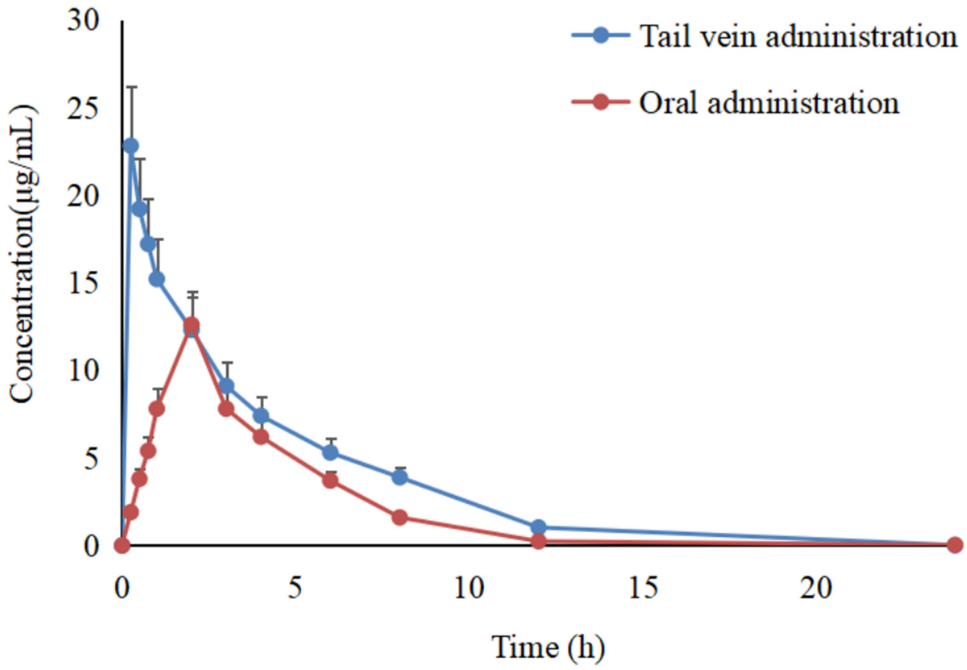

Figure 4 Mean plasma concentration profiles of I-BET151 in rats plasma after oral and tail vein administration of I-BET151 (means ± SD, n = 6).

Conclusion

In this paper, a rapid and specific HPLC-MS/MS assay method was developed and validated for the determination of I-BET151 in rat plasma, and successfully applied it to the pharmacokinetic study of I-BET151 in SD rats. The pharmacokinetic results showed that I-BET151 had good oral bioavailability, up to 60% of intravenous administration, and exhibited regular pharmacokinetic behavior and appropriate half-life. In summary, the research findings provided a reference for further studying the dose–response relationship and mechanism of action of I-BET151.

Disclosure

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this manuscript.

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