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ORIGINAL RESEARCH

UPLC-MS/MS Method for Givinostat in Rat Plasma: Development, Validation, in vivo Pharmacokinetics Study and in vitro Metabolic Stability Research

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Background: Givinostat, a potent histone deacetylase (HDAC) inhibitor, is promising for the treatment of relapsed leukemia and myeloma.

Purpose: This study aimed to develop and verify a quick assay for the measurement of givinostat concentration using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) with eliglustat as the internal standard (IS), establishing a basic pharmacokinetic profile for its pre-clinical application and metabolic stability in vitro.

Methods: Sample preparation was performed via protein precipitation using acetonitrile. The analyte (givinostat) and IS were gradient eluted on a Waters ACQUITY UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm) with 0.1% formic acid (A) and acetonitrile (B) as the mobile-phase system. The multiple reaction monitoring (MRM) in positive ion mode was used to detect the mass transition pairs for givinostat and IS as follows: *m/z* 422.01→186.11 for givinostat, and *m/z* 405.40→84.10 for IS, respectively.

Results: In the bioanalytical method, good linearity was observed between 2 and 4000 ng/mL (r^2=0.998). The intra- and inter-day precisions (RSD%) were lower than 15%, with an accuracy (RE%) of 95.8%–108.6%. The recovery exceeded 90%, and the matrix effect was within the range of 98.2%–107.6%. Additionally, this method was successful in evaluating pharmacokinetics in rats after an oral dose of 10 mg/kg givinostat. Finally, in vitro results showed that givinostat had a slow intrinsic clearance (CLint) value of 14.92 μL/min/mg protein with a half-life ($t_{1/2}$) value of 92.87 min.

Conclusion: Givinostat was rapidly absorbed and cleared slowly in vivo, and it was confirmed by in vitro experiments. This study provides a potential reference for givinostat in clinical studies.

Keywords: givinostat, UPLC-MS/MS, rat plasma, pharmacokinetic study, in vitro metabolic stability

Introduction

Inhibition of histone deacetylase (HDAC) is an effective strategy for the treatment of hematologic malignancies and solid tumors. The expression of many genes is regulated by HDACs via the acetylation status of nucleosomal histones, which alters chromatin structure.[1] HDAC inhibitors (HDACi) enhance histone acetylation. It can promote the binding of transcription factors to DNA chains and initiate the expression of specific genes such as tumor suppressor genes, thereby inhibiting tumor cell growth and inducing apoptosis in tumor cells.[2] Currently, four HDAC inhibitors (HDACi) have been authorized by the FDA for the treatment of multiple hematological and solid neoplasms.[3–6] Furthermore, several new HDAC inhibitors have been tested in clinical trials at different stages or in preclinical research and have shown impressive inhibitory properties.

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As a potent, orally bioavailable HDAC depressant, givinostat contains the hydroxamate radical -CO-NH(OH).[7] The zinc-reliance family I and II HDACs are inhibited by givinostat.[8] The high inhibitory efficacy of givinostat is due to the hydroxamate moiety chelating zinc inside the hydrophobic catalytic combining domain of HDAC.[9–11] Givinostat inhibits HDACs with constants (Ki) ranging from 0.004 to 0.39 μM (HDAC1, HDAC2, and HDAC6 are strongly binded to givinostat).[12] It was developed by Italfarmaco, Italy. Givinostat was efficacy in II clinical studies on the treatment of patients with myeloproliferative neoplasms (MPNs) (NCT01761968 and EudraCT# 2012–003499-37).[13] Similar to other HDACi, givinostat has inhibitory effects on multiple myeloma and plays an anti-inflammatory role in autoimmune diseases.[14] In 2021, an international stage III clinical test estimated the efficiency and security of givinostat versus hydroxycarbamide in high-hazard polycythemia vera (PV) people with JAK2V617F+ mutations.[15] Italfarmaco has also completed Phase 3 clinical trials of givinostat for the treatment of patients with Duchenne Muscular Dystrophy (DMD) (ClinicalTrials.gov: NCT02851797),[16] based on which givinostat was received its first approval on 21 March 2024, in the USA, for the treatment of DMD in patients 6 years of age and older.[17] In an animal model of DMD, givinostat was found to be able to improve muscle function and histological parameters in two DMD murine models expressing different haplotypes of the LTBP4 gene.[18]

Till now, few studies are available on the pharmacokinetic studies of givinostat. Silvia established a population pharmacokinetic approach to determine the exposure parameters (C_{max} and daily AUC) in blood after repeated doses in mdx male mice.[19] Antonio reported a Phase I safety and pharmacokinetics trial in healthy males administered 50, 100, 200, 400 or 600 mg orally. After 100 mg, C_{max} reached 199 nmol/L at 2.1 h with a half-life of 6.0 h.[20] A population pharmacokinetic (PK) model was developed to simulate pediatric dosing recommendations for boys with DMD.[21] Another population pharmacokinetic/pharmacodynamic model for PV patients was developed and integrated with a control algorithm implementing the adaptive dosing protocol.[22] Recently, people have found the efficacy of givinostat in various nervous system diseases, including Alzheimer's disease[23] and Parkinson's Disease.[24] The process of exploring, developing, and validating both specific and multitargeted inhibitors as therapeutic agents is still underway. However, to the best of our knowledge, its pharmacokinetic characteristics in rats have not yet been characterized. To quantify givinostat in biological fluids, a stable, sensitive, and optimized method must be developed, optimized, and fully validated to evaluate its pharmacokinetic characteristics. However, to date, no bioanalytical methods have been developed for measuring the plasma concentration of givinostat in rats using UPLC-MS/MS.

In this article, the newly developed method for the measurement of givinostat by UPLC-MS/MS was described with detailed method development and validation. This methodology was also employed successfully in pharmacokinetic research of givinostat (10 mg/kg) administered orally to rats and metabolic stability studies in vitro.

Materials and Methods

Drugs and Reagents

Givinostat was purchased from Shanghai Macklin Biochemical Co. Ltd. (Shanghai, China). Eliglustat was used as an internal standard (IS) and acquired from Sigma-Aldrich (St. Louis, MO, United States). The chemical structures of givinostat and eliglustat were shown in Figure 1. Rat liver microsomes (RLMs) were purchased from iPhase Pharmaceutical Services (Beijing, China). HPLC-level methanol and acetonitrile were purchased from Merck KGaA (Darmstadt, Germany). Formic acid was also at the HPLC level and provided by Anaqua Chemicals Supply (ACS, USA). A Milli-Q Water Purification System (Millipore, Bedford, USA) was used to prepare ultrapure water.

Experimental Animals

Six Male Sprague-Dawley (SD) rats (200–220 g) were supplied by the Experimental Animal Center at the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). A 12 h light/dark cycle, controlled temperature, relative humidity (45–65%), and ad libitum food were provided to the rats. The experimental protocol met the criteria of the Ethical Council of The First Affiliated Hospital of Wenzhou Medical University (WYYY-IACUC-AEC-2023-056) and followed the general code of animal welfare of GBT42011-2022 for laboratory animals.

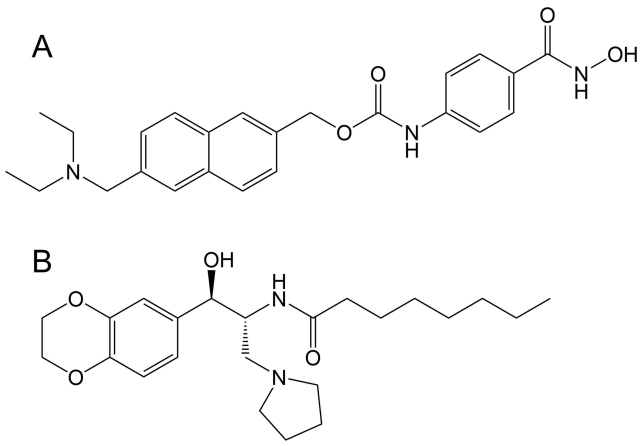

Figure 1 Chemical structure of givinostat (**A**) and eliglustat (IS) (**B**).

LC-MS/MS Instrument and Conditions

A XEVO TQS triple quadrupole tandem mass spectrometer coupled with an ACQUITY I-Class UPLC (Waters Corp., Milford, MA, USA) was used for the analysis.

Separation by liquid chromatography was conducted on an Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 50 mm), and the column temperature was maintained at 40°C. About 0.1% formic acid (aqueous phase) and acetonitrile (organic phase) were used as the mobile-phase systems with a flow velocity at 0.3 mL/min. The gradient elution was as follows: 0–0.5 min maintained at 10% acetonitrile, 0.5–1.0 min added to 90% acetonitrile in linearity, 1.0–1.4 min held at 90% acetonitrile, 1.4–1.5 min acetonitrile was rapidly changed back to 10%, and 1.5–2.0 min sustained at 10% acetonitrile for re-equilibration (1.0 min). The injected sample volume was 1.0 μL.

MS/MS detection was performed using multiple reaction monitoring (MRM) in ESI positive ionization mode. The monitored ion transition pairs of givinostat were *m/z* 422.01→186.11 (collision voltage 25 eV, cone voltage 30 V) and *m/z* 405.40→84.10 for IS (collision voltage, 20 eV; cone voltage, 30 V), respectively. The ion source temperature was set to 150°C, with a capillary voltage of 2.0 kV. The desolvent and cone gases were both nitrogen, whereas the collision gas was argon. The desolvent gas (nitrogen) temperature was 600°C, the desolvent gas flow rate was 1000 L/h, and the cone gas flow rate was 150 L/h. Peak matching, alignment, and normalization were performed using the Waters Corporation Masslynx V4.1 (Waters Corporation, USA).

Samples for Calibration and Quality Control (QC)

Dimethyl sulfoxide was used to dissolve givinostat and IS to obtain a standard stock solution (1.00 mg/mL). Both givinostat and 200 ng/mL IS working solutions were prepared by diluting the corresponding stock solutions with methanol. The calibration standards and QC samples were prepared by mixing 10 μL of the givinostat or QC working solution with 90 μL of blank rat plasma. The final calibration curve concentrations were 2, 5, 10, 20, 50, 200, 500, 1000, 2000, and 4000 ng/mL, whereas the QC samples for givinostat were 5, 800, and 3200 ng/mL.

Preparation of Plasma Samples

The samples were prepared by protein precipitation using acetonitrile as the sample preparation solvent. This meets the demand for high-throughput testing, as reported previously.[25] We blended 100 μL of plasma sample with 10 μL IS working solution (200 ng/mL) and added 300 μL acetonitrile to precipitate the protein. The mixture was vortexed for 1.0 min and centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant (100 μL) was transferred to a sample vial for UPLC-MS/MS detection.

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UPLC-MS/MS Method Verification

In light of the FDA's Bioanalytical Method Validation Guidance for Industry,[26] this method was fully validated for the determination of givinostat in rat plasma, including selectivity, linearity, lower limit of quantification (LLOQ), accuracy, precision, extraction recovery, matrix effect, and stability. The chromatograms of blank rat plasma, spiked plasma, and real rat plasma containing givinostat were evaluated to observe whether any chemical substances interfere with givinostat and the IS, in order to verify the selectivity of the method. A weighted ($1/x^2$) least squares regression model was used to plot the ratio of peak area of analyte to peak area of IS against the nominal concentrations of the analyte in order to evaluate the calibration curves. The sensitivity of this method was performed in terms of LLOQ. The measurement of QC samples and LLOQ (n = 5) was conducted continuously over three days to validate precision and accuracy. The matrix effect could be calculated using the formula: Matrix effect (%) = (Peak areas of spiked sample after extraction) /(Peak areas of pure solution). By comparing the peak areas of givinostat before and after extraction, the recovery rate could be obtained. Stability was assessed under four different conditions: at room temperature for 3 h, 10°C for 4 h, at −80°C for 21 days, and after three freeze-thaw cycles.

Pharmacokinetic Study

In this study, givinostat was suspended in 0.5% sodium carboxymethyl cellulose (CMC-Na) solution. Givinostat (10 mg/kg) was administered orally to the rats (n = 6) after fasting for 12 h with free access to water. We collected 0.3 mL of caudal vein blood after givinostat dosing at 0.333, 0.667, 1, 1.5, 2, 3, 4, 6, 8, 12, 24, and 48 h, and then centrifuged and freezed at −80°C for further operation and analysis.

The main kinetic parameters, including the area under the concentration–time curve (AUC), half-life ($t_{1/2}$), peak time (T_{max}), clearance ($CL_{z/F}$), and peak plasma concentration (C_{max}), were calculated using DAS 2.0 (Drug and Statistics, Shanghai University of Traditional Chinese Medicine, China). The mean plasma givinostat concentration versus time was explored using Origin 8.0 (Originlab Company, Northampton, MA, USA).

Metabolic Stability of Givinostat

Metabolic stability studies of givinostat were performed by assessing the reduction in the givinostat concentration after incubation with RLMs. First, buffer (pH 7.4, 0.1 M) was mixed with RLMs (0.5 mg/mL) and incubated in triplicates. Second, the mixture was pre-incubated in a 37 °C water bath for 5 min, and NADPH (1 mm) was added to initiate the metabolic reaction. Finally, at specific time intervals (0, 10, 15, 30, 45, 60, and 90 min), parallel tubes were removed and stored at −80°C to terminate the reaction. Moreover, givinostat was extracted from the RLMs incubation using protein precipitation. The specific post-treatment method involved adding 2 times the volume of acetonitrile (400 μL) and 20 μL of the IS working solution to the incubation mixture. The subsequent processing steps were identical to those used for plasma sample preparation. The content of givinostat was analyzed using UPLC-MS/MS. The metabolic stability of the drug was plotted, and the half-life ($t_{1/2}$) of in vitro incubation and intrinsic clearance (CLint) were calculated. The calculation was performed using the following formula: $t_{1/2}$=0.693/k, V (μL/mg) = incubation volume (μL)/protein in the incubation (mg), CLint (μL/min/mg protein) = V × 0.693/$t_{1/2}$.

Outcomes and Discussion

Method Exploit and Optimization

The samples were prepared by protein precipitation using acetonitrile as the sample preparation solvent. This could meet the demand for efficacious tests, as previously reported.[25] To obtain a favorable peak shape, high assay sensitivity, and short retention time, we made an optimization of the liquid chromatographic parameters. Compared with UPLC BEH C18 column (2.1 × 100 mm, 1.7 mm) and HSS T3 column (2.1 × 100 mm, 1.7 mm), the UPLC BEH C18 column (2.1 × 50 mm, 1.7 mm) proved satisfactory separation and sharper peaks. Diverse flow units such as acetonitrile, methanol, and 0.1% formic acid were also evaluated. The results showed that a flow rate of 0.3 mL/min with acetonitrile and 0.1% formic acid yielded a high assay sensitivity and short runtime. Furthermore, a better resolution ratio and higher

responsivity were achieved by optimizing mass parameters. The quantitative ion pairs at *m/z* of 422.01→186.11 and *m/z* 405.40→84.10 were selected for givinostat and IS, respectively.

Selectivity

Blank plasma samples (Figure 2A), blank plasma mixed with givinostat (Figure 2B), and rat plasma after givinostat administration (Figure 2C) were analyzed to assess the selectivity of the method. In contrast to blank plasma, no endogenous compounds interfered with givinostat or IS.

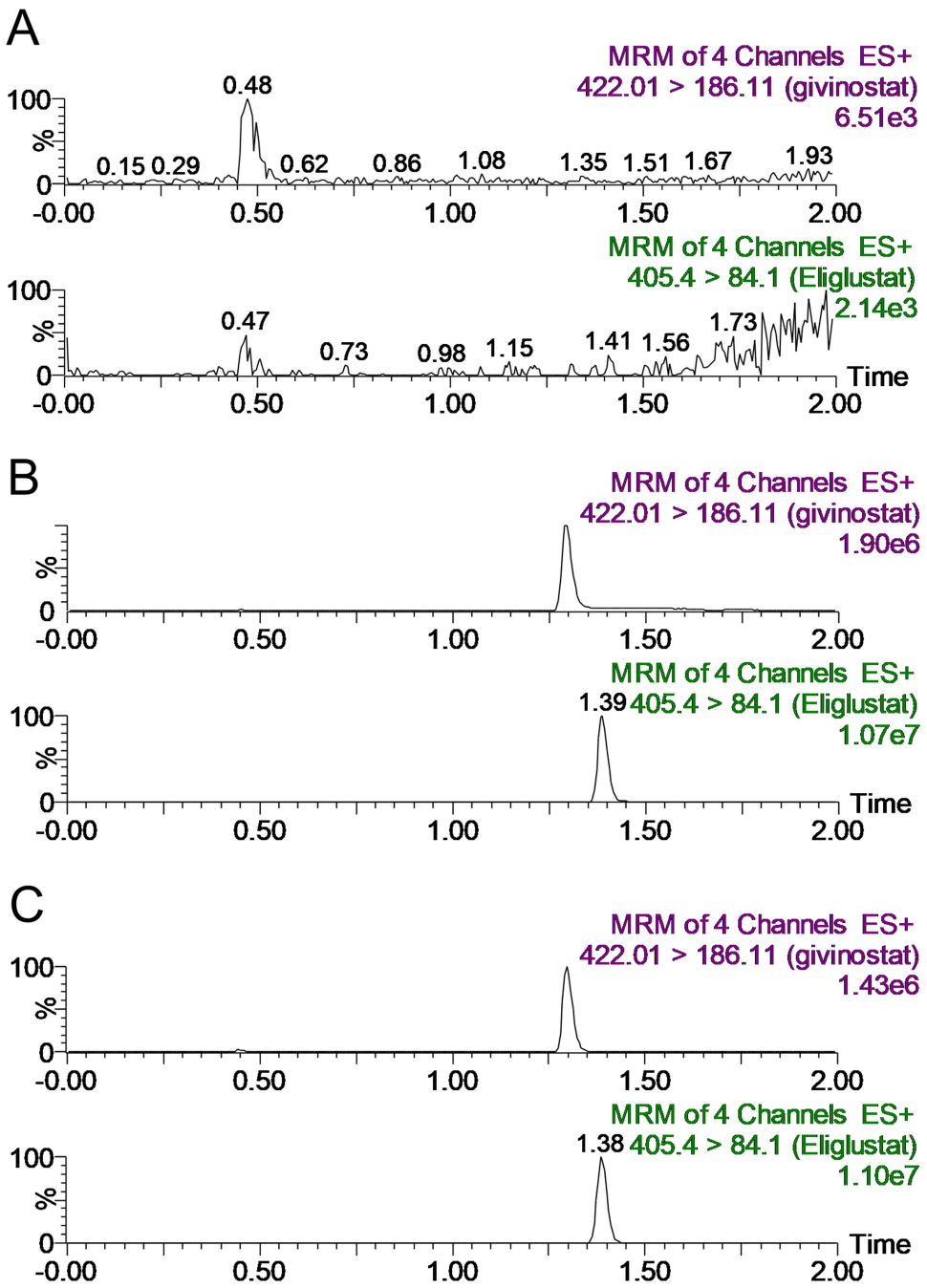

Figure 2 Representative givinostat and IS chromatograms. (**A**) a blank plasma sample; (**B**) a blank plasma sample blended with givinostat and IS; (**C**) a rat plasma sample after oral dose of 10 mg/kg givinostat.

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Linearity and LLOQ

The standard curve equation (2–4000 ng/mL) for givinostat was Y = 0.000382639*X+0.0044672 (r^2=0.998). The relative peak area of givinostat to the IS was represented by Y, and the concentration of givinostat was represented by X. LLOQ was set at 2 ng/mL with an acceptable RE% and RSD% of 103.0% and 4.5%, respectively. The linearity was good and met the methodological requirements.

Accuracy and Precision

Within three consecutive days, we used five duplicates of QC samples at four concentration levels: LLOQ (2 ng/mL), LOQ (5 ng/mL), MOQ (800 ng/mL), and HOQ (3200 ng/mL) to calculate inter- and intraday accuracy and precision. As shown in Table 1, the inter- and intra-day accuracies (RE%) ranged from 95.8% to 108.6%. The deviation of the QC samples was less than ±15% and the deviation of the LLOQ within ±20%, which were acceptable limits for precision and accuracy. Our results were all within this range, indicating that the method could be used accurately for the determination of givinostat in rat plasma.

Extraction Recovery and Matrix Effect

Extraction recovery and matrix effects were appraised using QC samples (5, 800, and 3200 ng/mL) in five replicates, as shown in Table 2. The recovery was in the extent of 102.9%–107.8%, and the matrix effect was within the range of 98.2%–107.6%. The method demonstrated an acceptable recovery rate, and the matrix effect had little effect on the ionization of the analyte, which did not affect the optimization accuracy of UPLC-MS/MS and could be ignored.

Stability

The results of the stability tests for givinostat in rat plasma for each concentration (5, 800, 3200 ng/mL) under various preservation and processing conditions were listed in Table 3. According to the data, givinostat was stable in the automatic sampler at 10°C for 4 h and after three freeze–thaw cycles (from −80°C to room temperature). Furthermore, short- and long-term stabilities were examined after storage at room temperature for 3 h and at 80°C for 21 days. The accuracy (RE%) was between 88.0% and 108.6%. The RSD% values were within an acceptable range of 15%. This indicated that givinostat was stable in rat plasma under various storage conditions.

Table 1 The Precision and Accuracy of Givinostat in Rat Plasma (n = 5)

Analyte	Concentration (ng/mL)	Precision (RSD%)		Accuracy (RE%)	
		Intra-day	Inter-day	Intra-day	Inter-day
Givinostat	2	4.5	5.3	103.0	99.4
	5	7.8	13.8	107.8	103.6
	800	1.6	7.1	101.5	95.8
	3200	4.3	5.0	108.6	104.1

Abbreviations: RSD, Relative Standard Deviation; RE, Relative Error.

Table 2 Recovery and Matrix Effect of Givinostat in Rat Plasma (n = 5)

Analyte	Concentration (ng/mL)	Recovery (%)		Matrix Effect (%)	
		Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Givinostat	5	107.8 ± 11.2	10.4	107.6 ± 4.4	4.1
	800	102.9 ± 4.0	3.9	98.2 ± 5.2	5.3
	3200	105.9 ± 6.2	5.8	103.1 ± 8.5	8.3

Abbreviations: SD, Standard Deviation; RSD, Relative Standard Deviation.

Table 3 Stability Results of Givinostat in Rat Plasma Under Different Conditions (n = 5)

Analyte	Concentration (ng/mL)	Room Temperature, 3 h		Autosampler 10 °C, 4 h		Three Freeze-Thaw		−80°C, 21 Days	
		RSD%	Accuracy (RE%)	RSD%	Accuracy (RE%)	RSD%	Accuracy (RE%)	RSD%	Accuracy (RE%)
Givinostat	5	10.1	96.7	10.5	99.1	4.4	88.0	11.0	102.1
	800	6.6	91.4	1.3	96.2	3.3	90.3	2.9	97.5
	3200	7.2	104.6	6.7	100.6	7.1	108.6	2.1	101.3

Abbreviations: RSD, Relative Standard Deviation; RE, Relative Error.

Pharmacokinetics

The technique was verified and used to quantify the plasma profiles of givinostat in rats after the administration of 10 mg/kg givinostat. The dose used in the experiment was selected based on previous studies: in a phase I safety and pharmacokinetic trial in healthy men, doses of 50 or 100 mg/d were safe and effective. To maintain the same effect, we chose a reference dose of 100 mg/d. According to the transformation of human and rat body surface, the oral dose of rats is 10mg/kg.[20] Plasma samples after oral administration of givinostat were detectable immediately after dosing (0.333 h for oral administration until 48 h post-administration). The mean plasma givinostat concentration in rats over time after an oral dose of 10 mg/kg was shown in Figure 3. A non-compartment model was fitted to the major pharmacokinetic parameters of givinostat according to the pharmacokinetic results (Table 4). They reached peak levels at 2651.24 ng/mL (C_{max}) approximately 1.72 h (T_{max}) after dosing and presented biphasic elimination profiles, with a mean apparent terminal elimination phase ($t_{1/2}$) 19.69 h. The mean residence time (MRT) and clearance rate of givinostat were 7.76 ± 4.80 h and 1.21 ± 0.32 L/h/kg, respectively. The results demonstrated that givinostat was rapidly absorbed and cleared slowly in rats, and this result agreed with previous studies using mdx mouse model of DMD after repeated oral dose of givinostat at 1, 5 and 10 mg/kg/d.[19]

Metabolic Stability Study

The givinostat concentration in the RLMs matrix was calculated using a standard curve regression equation. Metabolic stability was determined by measuring the incubation time versus the percentage of the remaining givinostat (Figure 4). Based on the constructed curve, the concentrations that showed linear distributions (0–90 min) were selected to plot an additional graph of time versus the natural logarithm (ln) of the remaining givinostat percentage, which was used to calculate $t_{1/2}$ in vitro (Figure 5). The linear regression equation was $Y = -0.007462X + 4.592$ ($r^2 = 0.9663$). Using the

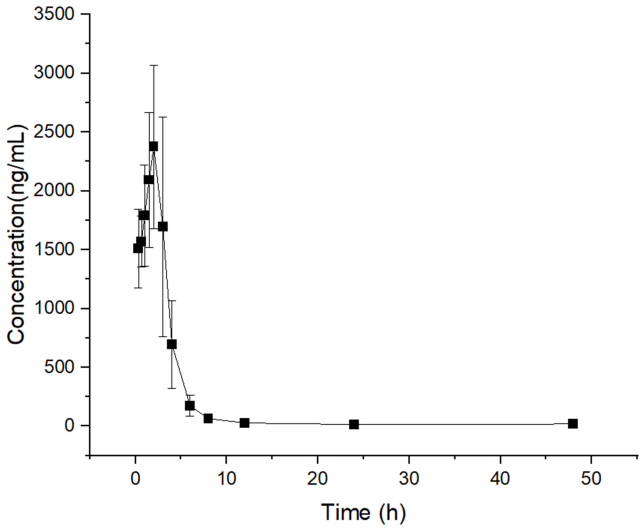

Figure 3 Pharmacokinetic lineament of givinostat in rats after the oral dose of givinostat (10 mg/kg).

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Table 4 The Pharmacokinetic Parameters of Givinostat After Oral Administration of 10 Mg/Kg (n = 6)

Parameters	Unit	Po (10 mg/kg)
$AUC_{(0-t)}$	ng/mL×h	8489.50 ± 2233.35
$AUC_{(0-\infty)}$	ng/mL×h	8729.89 ± 1968.38
$MRT_{(0-\infty)}$	h	7.76 ±4.80
$t_{1/2}$	h	19.69 ± 15.96
T_{max}	h	1.72 ± 0.87
$CL_{z/F}$	L/h/kg	1.21 ± 0.32
C_{max}	ng/mL	2651.24 ± 594.55

Notes: Measurement Data: Presented as mean ± SD.
Abbreviations: Po, per os; AUC, Area Under the Curve; MRT, Mean Residence Time; $t_{1/2,}$ half life; T_{max}, peak time; $CL_{z/F}$ clearance rate; C_{max}, maximum plasma concentration.

formula $t_{1/2}$=0.693/k, the in vitro $t_{1/2}$ was calculated to be 92.87 min based on the slope k = 0.007462. According to the in vitro $t_{1/2}$, givinostat CLint was calculated as 14.92 μL/min/mg protein. The results suggested that the metabolism of givinostat had a slow CLint in vitro, which verified the results of pharmacokinetic experiments.

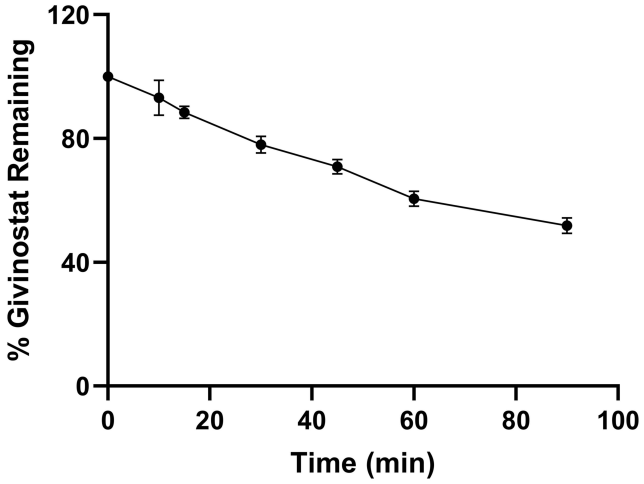

Figure 4 The metabolic stability datagram of givinostat after incubation with RLMs.

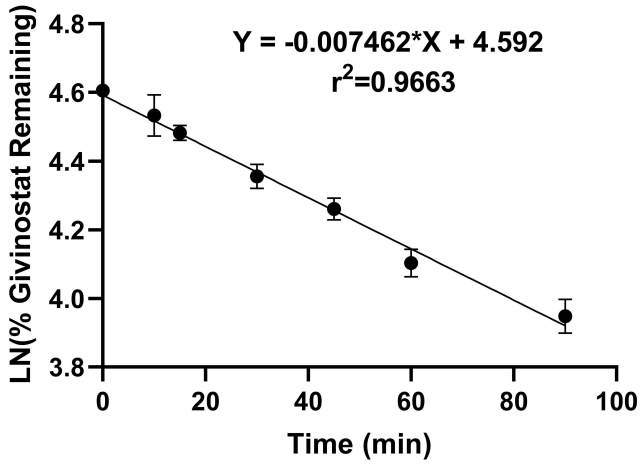

Figure 5 The linear part of the metabolic stability curve of givinostat treated with RLMs.

Conclusions

Currently, the use of HDAC inhibitors in clinical settings is mainly directed at cancer and DMD, with drug development for other diseases such as neurological conditions still in preclinical and Phase II phases. HDAC specificity will be a key area of focus in future research. The process of exploring, developing, and validating both specific and multitargeted inhibitors as therapeutic agents is still underway. In our research, a sensitive, specific, rapid, and reliable UPLC-MS/MS method for the quantification of givinostat in rat plasma samples was developed and verified. This new method has been utilized in animal studies of rat pharmacokinetic profiles and metabolic stability in RLMs. The in vivo $t_{1/2}$ after oral dose was 19.69 h and in vitro $t_{1/2}$ was calculated to be 92.87 min. The research may provide valuable data for subsequent analysis of pharmacokinetic characteristics and can provide conditions for drug–drug interaction exploration.

Disclosure

The authors report no conflicts of interest in this work.

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