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Determination of HS270, a new histone deacetylase inhibitor, in rat plasma by LC–MS/MS—Application to a preclinical pharmacokinetic study

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ABSTRACT

A rapid and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method has been developed and fully validated to determine HS270, a new histone deacetylase (HDAC) inhibitor, in rat plasma using SAHA as the internal standard (IS). After a single step liquid–liquid extraction with acetoacetate, analytes were subjected to LC–MS/MS analysis using positive electro-spray ionization (ESI^+) under selected reaction monitoring mode (SRM). The chromatographic separation was achieved on a Hypurity C_{18} column (50 mm × 2.1 mm, i.d., 5 μm). The MS/MS detection was conducted by monitoring the fragmentation of m/z 392.3 → 100.1 for HS270, m/z 265.1 → 232.1 for IS. The method had a chromatographic running time of 2.5 min and linear calibration curves over the concentrations of 0.5–1000 ng/mL. The recovery of the method was 70.8–82.5% and the lower limit of quantification (LLOQ) was 0.5 ng/mL. The intra- and inter-batch precisions were less than 15% for all quality control samples at concentrations of 1.0, 100.0, and 750.0 ng/mL. The validated LC–MS/MS method has successfully applied to a HS270 pharmacokinetic study after oral doses of 25, 50, 100, 200 mg/kg, and i.v. dose of 5 mg/kg to rats.

1. Introduction

The transcriptional activity of certain genes is modulated by the acetylation state of core histones. Histone acetyltransferases (HAT) can stimulate gene transcription by histone acetylation [1–3]. On the other hand, histone deacetylases (HDACs) are catalytic enzymes that control the structure of chromatin via removal of the acetyl inserted in lysine residues of histones [3] by HAT. The opposing activities of HDACs and HATs regulate the balance of factelation of histones.

Preclinical studies have demonstrated that the use of HDAC inhibitors (HDACis) induces cytodifferentiation, cell cycle arrest, and apoptosis in transformed cells [4] shows a promising antitumor activity both *in vivo* and *in vitro*, suggesting that the HDACis represent a new class of targeted anticancer agents. A number of structural classes of HDAC inhibitors have been developed and are in clinical trials, including short chain fatty acids (the benzamides [MS-275]) [5,6], the cyclic peptide (depsipeptide, FR901228) [7], a synthetic hydroxamic acid derivative (LAQ824) [8], and the recently commercialized suberoylanilide hydroxamic acid (SAHA) [9]. Unfortunately SAHA has low solubility and low permeability, which implies that SAHA may not be well absorbed *in vivo*. And thus potential HDACis with good absorption and pharmacokinetic properties are necessary.

A new family of HDACis was designed and synthesized by Zhejiang Hisun Pharmaceutical Co. Ltd. Among these newly synthesized HDACis, HS270 shows the most promising anti-tumor activity in preclinical studies and acts as a potent and specific HDACi in HCT116, HL-60, and COLO-205 human cancer cell lines and nude mice transplanting tumor model [data not shown]. Now, HS270 is being developed by Zhejiang Hisun Pharmaceutical as a Type-I new drug and being applied for phase I clinical trial from the State of Food and Drug Administration of China. This novel lead-compound is expected to enter Phase I trials in 1–2 years and pharmacokinetic studies are now needed to develop a dose regimen that can produce the necessary concentrations *in vivo*. So a selective, reproducible and accurate analytical method for the quantitation of HS270 was necessary.

Here, we first developed and validated a liquid chromatography/tandem mass spectrometry (LC–MS/MS) method for determination of HS270 in rat plasma using SAHA as the internal standard (IS). The validated LC–MS/MS method has successfully

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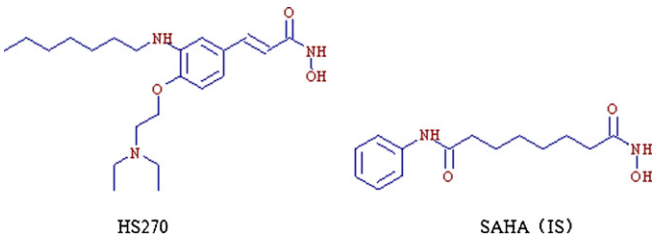

Fig. 1. Chemical structures of HS270 and SAHA. (I.S.)

applied to a HS270 pharmacokinetic study after oral doses of 25, 50, 100, 200 mg/kg, and i.v. dose of 5 mg/kg to rats.

2. Experimental

2.1. Chemical and reagents

HS270 (batch no. 270-F.B., 090730, 98.7% purity) and SAHA (as internal standard, batch no. S0901, 97.8% purity) were provided by Zhejiang Hisun Pharmaceutical Co. Ltd. (Zhejiang, China) (Fig. 1). Methanol, formic acid, acetonitrile and acetoacetate of HPLC grade were all purchased from Tedia Company Inc. (Guangzhou, China). All other reagents were of analytical grade. Ultrapure water was obtained from a Milli-Q Plus water purification system (Millipore, Bedford, MA, USA).

2.2. Preparation of standard and quality control samples

The stock standard solution of HS270 was prepared by dissolving the accurately weighted reference compounds in methanol–water (containing 1% formic acid, 90:10, v/v) to give a final concentration of 1000 ng/μL. The solution was then constantly diluted with methanol–water (containing 1% formic acid, 90:10, v/v) to obtain working solutions at concentrations over 0.005–10.0 ng/μL. A stock standard solution of SAHA (IS) at 1000 ng/μL was also prepared in methanol–water (containing 1% formic acid, 90:10, v/v) and then diluted with methanol–water (containing 1% formic acid, 90:10, v/v) to obtain a working solution of 0.1 ng/μL. All the solutions were stored at 4 °C and were brought to room temperature before use.

The analytical standard and quality control (QC) samples were prepared by spiking blank rat plasma with standard working solutions in validation and during each experimental run for preclinical pharmacokinetic study. Calibration samples were made at concentrations of 0.5, 1.0, 5.0, 25.0, 100.0, 250.0, 500.0 and 1000.0 ng/mL. Quality control samples were at concentrations of 1.0, 100.0 and 750.0 ng/mL.

2.3. Sample preparation

The sample was extracted by a simple one-step liquid–liquid extraction (LLE). 10 μL of the internal standard solution (0.1 ng/μL) was added to 100 μL rat plasma in a 1.5 mL test tube. After vortex mixing for 10 s, the extraction was carried out with 0.5 mL acetoacetate by vortexing-mixed for 60 s and standing at room temperature for 3 min. After centrifugation at 16,000 rpm for 3 min at 4 °C, 0.45 mL of the supernatant was then removed to a new 1.5 mL centrifuge tube and evaporated to dryness. The residue was reconstituted in 100 μL methanol–water (containing 1% formic acid; 90:10, v/v) and 5 μL was directly injected onto the LC–MS/MS system for analysis.

2.4. Liquid chromatographic and mass spectrometric conditions

A Finnigan Surveyor MS pump (San Jose, CA, USA) and a Finnigan Surveyor autosampler were used for solvent and sample delivery. Chromatographic separation was achieved by using a C_{18} column (Hypurity C_{18}, I.D. 2.1 mm × 50 mm, 5 μm, Thermo, USA) at 15 °C. The mobile phase consisted of methanol–acetonitrile–water (containing 1% formic acid) (90:5:5, v/v/v), pumped at a flow rate of 300 μL/min. Total running time was 2.5 min for each injection.

A Finnigan TSQ triple-quadrupole mass spectrometer equipped with an ESI source was used for mass analysis and detection. Mass spectrometric analysis was performed in the positive-ion mode (ESI^+) and set up in the selected reaction monitoring (SRM) mode. Nitrogen was used as the sheath gas (35 psi) and the auxiliary gas (10 psi). The capillary temperature was at 350 °C. The spray voltage was set at 3500 V. Collision-induced dissociation (CID) studies were performed and argon was used as the collision gas with a collision cell gas pressure of 1.0 mTorr (1 Torr D 133.3 Pa). The optimized source CID was 10 V for both HS270 and IS. The optimized collision energy was 30 V for HS270, and 10 V for IS. On the basis of the full-scan mass spectra of each analyte, the most abundant ions were selected and the mass spectrometer was set to monitor the transitions of the precursors to the product ions as follows: m/z 392.3 → 100.1 for HS270, m/z 265.1 → 232.1 for IS. The scanning time for each analyte was set to 0.3 s. Data acquisition was performed with the Finnigan Xcalibur 1.3 software, while peak integration and calibration were obtained with the Finnigan Lcquan software.

2.5. Method validation

The method was validated for selectivity, accuracy, precision, recovery, calibration curve range and reproducibility according to the FDA guideline for validation of bioanalytical methods [10]. The selectivity was investigated by preparing and analyzing six individual rat blank plasma samples at the LLOQ. The LLOQ was defined as the lowest concentration of the analyte measured with acceptable precision and accuracy [relative standard deviation (RSD) and relative error <±20%, and also the analyte response at this concentration level was >5 times the baseline noise. Linearity was assessed by analyzing HS270 standards (0.5–1000.0 ng/mL) in rat plasma. Calibration curves were analyzed by weighted linear regression ($1/x$) of assayed peak areas vs nominal concentrations.

Accuracy and precision were assessed by determining QC samples at three concentration levels (five samples each concentration) on three different validation batches. The precision was determined as the RSD (%) and the accuracy was expressed as a percentage of the nominal concentration. The criteria used to assess the suitability of precision and accuracy was as follows: the RSD should not exceed 15% and the relative error should be within ±15%. Furthermore, the recovery (extraction efficiency) of HS270 from rat plasma was determined by comparing the areas of spiked plasma samples before and after liquid extraction that represent 100% recovery.

The stability of HS270 was assessed by determining QC samples at three concentrations (five samples each concentration), exposed to different time and temperature conditions. The stability studies included: (a) stability at room temperature (22–25 °C) for 5 h; (b) stability after two freeze–thaw cycles; (c) stability of the extracted samples at 15 °C for 12 h; and (d) the long-term stability after storage at −20 °C for 32 days. During routine analysis, each analytical run included blank plasma, a set of calibration samples, a set of QC samples and unknowns.

2.6. Preclinical pharmacokinetic study

The method was applied to a preclinical pharmacokinetic study of HS270. The oral dose groups were set at 25, 50, 100 and 200 mg/kg according to the doses used in the pharmacological study, the i.v. dose group was set at 5 mg/kg, and 6 SD rats (bisexual each half) were selected for each group. HS270 was dissolved in ultrapure water at 1 mL/100 g (v/w) for oral administration before dosing. After an overnight fast (12 h), the rats were orally or intravenously administered a single dose of HS270. Blood samples (250 μL) were collected into 1.5 mL heparinized EP tubes at the following times: immediately before administration, and 0.083, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 5, 8, 12, 24, 36 and 48 h after dosing. Blood samples were centrifuged at 16,000 rpm for 3 min, and the plasma was separated and obtained. The rats should not be allowed to have water within 2 h and no food either within 4 h after administration. The plasma samples were labeled and kept frozen at −20 °C until analysis.

Calculation of pharmacokinetic parameters was done using the Pharmacokinetics Program Package (Version 2.1, Institute of Clinical Pharmacology, School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, China). The elimination rate constant (λ_z) was obtained as the slope of the linear regression of the log-transformed concentration values vs time data in the terminal phase. The elimination half-life ($t_{1/2}$) was calculated as $0.693/\lambda_z$. Time to peak plasma concentration (T_{max}) and peak plasma concentration (C_{max}) were read directly from the observed concentration vs time profiles. The area under the curve to the last measurable concentration (AUC_{0-t}) was calculated by the linear trapezoidal rule. The area under the curve to infinity ($AUC_{0\to\infty}$) was calculated as $AUC_{0\to\infty} = AUC_{0-t} + C_t/\lambda_z$, where C_t is the last measurable concentration. Absolute oral bioavailability (F) was calculated using the equation, $F(\%) = [\text{dose (i.v.)} \times AUC_{(0\to\infty)}\text{oral/dose (oral)} \_ AUC_{(0\to\infty)}\text{i.v.}] \times 100$.

3. Results and discussion

3.1. Method development

In this study, ESI was chosen as the ionization source. It was found that the signal intensity of HS270 and IS in rat plasma was high using ESI source and the regression curves were linear over 0.5–1000.0 ng/mL. By using ESI, HS270 and IS formed predominantly protonated quasi molecular ions $[M+H]^+$ in full-scan spectra, with m/z 392.3 for HS270, and m/z 265.1 for the IS. To determine these compounds using SRM mode, full-scan production spectra of HS270 and IS were investigated. The most abundant ion in the product ion mass spectrum was at m/z 100.1 for HS270, and m/z 232.1 for IS. Capillary voltage and collision energies were optimized to obtain the greatest intensity of the most abundant product ion for further MS/MS experiments. The collision behavior of the $[M+H]^+$ of these compounds was strongly dependent on the collision energy. An increase in the collision energy caused a marked increase of the fragmentation processes. After optimization of the collision energy, the collision behavior was carried out using 30 and 10 eV collision energy for HS270 and IS, respectively, to obtain the maximum intensity of product ions. Therefore, the SRM transition of m/z 392.3 → 100.1 for HS270, m/z 265.1 → 232.1 for IS were selected to obtain maximum sensitivity. Positive-ion ESI-MS/MS product-ion spectrum of these compounds are shown in Fig. 2.

Sample preparation is a critical step for accurate and reliable LC–MS/MS assay and can often be the cause of bottlenecks in a high-throughput analysis. A solid phase extraction system was used to extract SAHA from human plasma in previous report [11]. A liquid–liquid extraction (LLE) procedure with ethyl acetate, acetonitrile/n-butyl-chloride, and methyl tert-butyl ether has been used to extract other HDACi such as MS275, and Kendine 91 from human plasma in previous reports [6,12–14]. In the present study, analytes were extracted using a single step liquid–liquid extraction. The extraction efficiency of different solvents was compared during the method development, including dichloromethane, trichlormethane, acetoacetate, N-hexane, ethyl acetate, dimethylcarbinol, acetonitrile/n-butyl-chloride, tert-butyl ether, and chlorinated butane. As a result, acetoacetate was chosen as the extraction solvent in the study because of the highest extraction recovery.

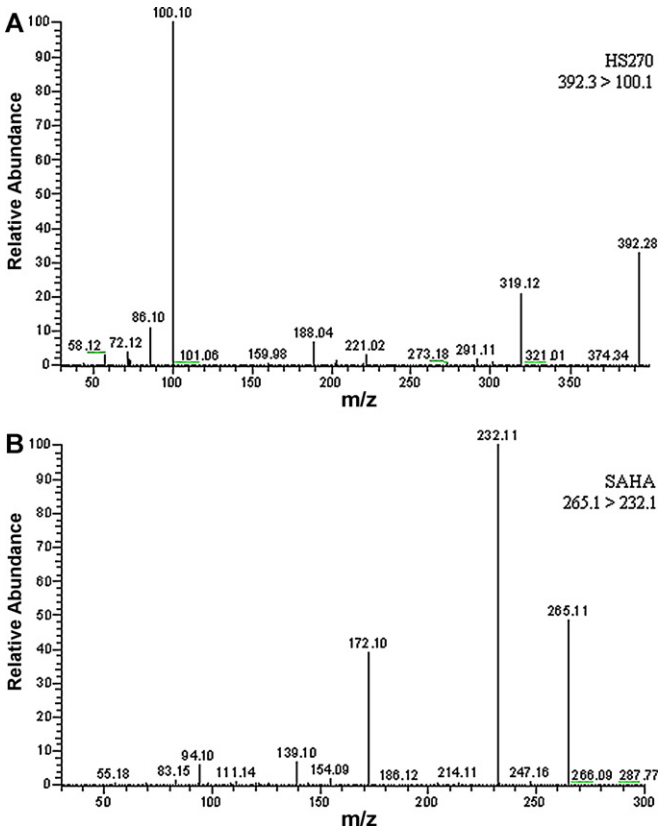

Fig. 2. MS/MS product ion spectrum of $[M+H]^+$ (A) HS270 at m/z 293.3 as the precursor ion, (B) SAHA at m/z 265.10 as the precursor ion.

LC conditions were optimized to ensure a maximal resolution and signal with a minimal run time. Different types of analytical columns including the Elite Hypersil C_{18}, Elite Hypersil C_8, Schizandzu C_{18}, Hypersil BDS C_8 column, XTerra RP C_{18}, and Zorbax SB-C_{18} were tested. All the analytes were well retained on the C_{18} column. The best results in terms of analysis time and resolution between peaks were obtained using the C_{18} column (Hypurity C_{18}, I.D. 2.1 mm × 50 mm, 5 μm, Thermo, USA) at 15 °C. To obtain the optimal mobile phase that is best compatible with MS/MS and to achieve good resolution and symmetric peak shapes for the analysis, various ratios of organic solvents and water were investigated. Various combinations of methanol, acetonitrile and water with changed content of each component were investigated and compared to identify the optimal mobile phase that produced the best sensitivity, efficiency and peak shape. Methanol as an organic solvent showed better sensitivity compared to acetonitrile. The water content was decreased to obtain a better peak shape, higher sensitivity, and shorter running time for the analytes. A mobile phase consisting of methanol–acetonitrile–water (containing 1% formic acid) was finally used and the ratio of 90:5:5 (v:v:v) was optimal. Each chromatographic run was completed within 2.5 min.

Table 1
Matrix effect data for HS270 at 1.0, 100.0 and 750.0 ng/mL in five different lots of rat plasma ($n = 5$).

Nominal con. of HS270 (ng/mL)	ME[a] (mean ± SD, %)	RSD[b] (%)
1.0	92.8 ± 4.4	4.8
100.0	86.9 ± 5.5	6.3
750.0	95.2 ± 2.9	3.1

[a] The ME (matrix effect) expressed as the ratio of the peak area of HS270 in spiked rat plasma after extraction over that of the same analyte in mobile phase multiplied by 100.
[b] RSD = relative standard deviation.

3.2. Method validation

3.2.1. Selectivity

The selectivity towards endogenous plasma matrix was tested in six different batches of rat plasma samples by analyzing blanks and samples at LLOQ level. Observing the chromatographs indicated no significant interference at the expected retention time of HS270 since HS270 was modified to elute in a region where visible interference was not observed. Chromatograms of blank rat plasma and the plasma at LLOQ levels are shown in Fig. 3.

3.2.2. Matrix effects

Since potential matrix effect is a concern with the rapid isocratic system, the co-elution effect and potential ion suppression were evaluated. Briefly, blank plasma were extracted following LLE procedure and then spiked with HS270 at QC concentrations. The corresponding peak areas of HS270 in spiked plasma post-extraction (B) were then compared with those of the aqueous standards in mobile phase (A) at equivalent concentrations. The ratio ($B/A \times 100$) is defined as the ME. An ME value of 100% indicates that the response in the mobile phase and in the plasma extracts was the same and no absolute matrix effect was observed. A value of >100% indicates ionization enhancement, and a value of <100% indicates ionization suppression.

Matrix effect data at different QC concentrations in five different lots of rat plasma are presented in Table 1. The absolute ME values were 86.9–95.2%, indicating no significant ion suppression or enhancement effect. The variability was acceptable with RSD values <15% at different concentrations. These data confirm that the relative matrix effect for HS270 was not significant. Thus, no ion suppression or enhancement effect was observed and the present analytical method was considered reliable.

In addition, the 'cross-talk' between MS/MS channels of HS270 and IS was assessed by separately injecting the plasma extract containing only one of the two analytes at LLOQ concentration and monitoring the response in the other two channels. No 'cross-talk' between HS270 and IS channels was observed.

3.2.3. Linearity and lower limit of quantification

The slope, the intercept and the correlation coefficient (r) for each standard curve from each analytical run were determined automatically by Lcquan, Thermo Finigan software program, and are presented in Table 2. The representative stand curve for HS270 was $Y = -0.0291433 + 0.0781379 \times X$. The squared correlation coefficients (r^2) for the daily calibration curves were all ≥0.991 ($n = 5$) for HS270. For each point on the calibration curves for the analyte, the concentrations back-calculated from the equation of the regression analysis were within acceptable limits for accuracy and precision of ±15%. Overall, HS270 gave linear response as a function of the concentration ranges studied and showed excellent linearity over 0.5–1000.0 ng/mL.

The lowest concentration on the calibration curve of HS270 was 0.5 ng/mL. The analyte response at these concentration levels was >50 times the baseline noise. The precision and accuracy at these concentration levels were acceptable, with 5.7% of the RSD, with 9.6% of the RE. Thus, the lowest concentration on the calibration curve was accepted as the LLOQ. And the current LLOQ was already sufficient for the determination of the preclinical pharmacokinetic study of HS270 following a single-dose administration of HS270 in rat.

Table 3
Intra- and inter-batch precision and accuracy data for assays of HS270 in rat plasma ($n = 5$).

Nominal con. of HS270 (ng/mL)	Precision		Accuracy
	Mean ± SD	RSD[a] (%)	Mean relative error[b] (%)
Intra-batch			
1.0	1.0 ± 0.1	5.4	3.3
100.0	110.3 ± 2.3	2.1	10.3
750.0	824.6 ± 12.9	1.6	9.9
Inter-batch			
1.0	1.0 ± 0.1	5.4	−0.7
100.0	103.4 ± 5.8	5.6	3.4
750.0	803.6 ± 33.1	4.1	7.2

[a] RSD = relative standard deviation.
[b] Mean relative error = [(overall mean assayed concentration − added concentration)/added concentration] × 100.

Table 4
Recovery (extraction efficiency) for HS270 in rat plasma ($n = 5$).

Nominal con. of HS270 (ng/mL)	Recovery[a] mean ± SD	RSD[b] (%)
1.0	70.8 ± 4.4	6.2
100.0	73.3 ± 0.6	0.8
750.0	82.5 ± 2.3	2.8

[a] The recovery (extraction efficiency) of HS270 from rat plasma after the extraction procedure was determined by comparing the areas of extracted HS270 with that of the non-extracted pure standards that represent 100% recovery.
[b] RSD = relative standard deviation.

3.2.4. Precision and accuracy

The intra- and inter-batch precision and accuracy data for HS270 are summarized in Table 3. All values of accuracy and precision were within recommended limits [10]. The intra-batch precision was 1.6–5.4%, and the inter-batch precision was 4.1–5.6%. The mean intra-batch error was 3.3–10.3%, and the mean inter-batch error was between −0.7% and 7.2%.

3.2.5. Recovery

Table 4 shows the recovery (extraction efficiency) of HS270, and IS from rat plasma following LLE procedure. The recovery of HS270 from rat plasma ranged over 70.8–82.5%. The recovery of IS was

Table 2
Calibration curves summary statistics for HS270 ($n = 5$).

Conc. range of HS270 (ng/mL)	Intercept[a]		Slope[a]		r^2
	Mean ± SD	RSD[b] (%)	Mean ± SD	RSD[b] (%)	
0.5–1000.0	−0.0286 ± 0.0032	−11.0555	0.0874 ± 0.0065	7.4588	≥0.991

[a] Slope and intercept were determined automatically by Finnigan Lcquan software.
[b] RSD = coefficient of variation = (mean/SD) × 100

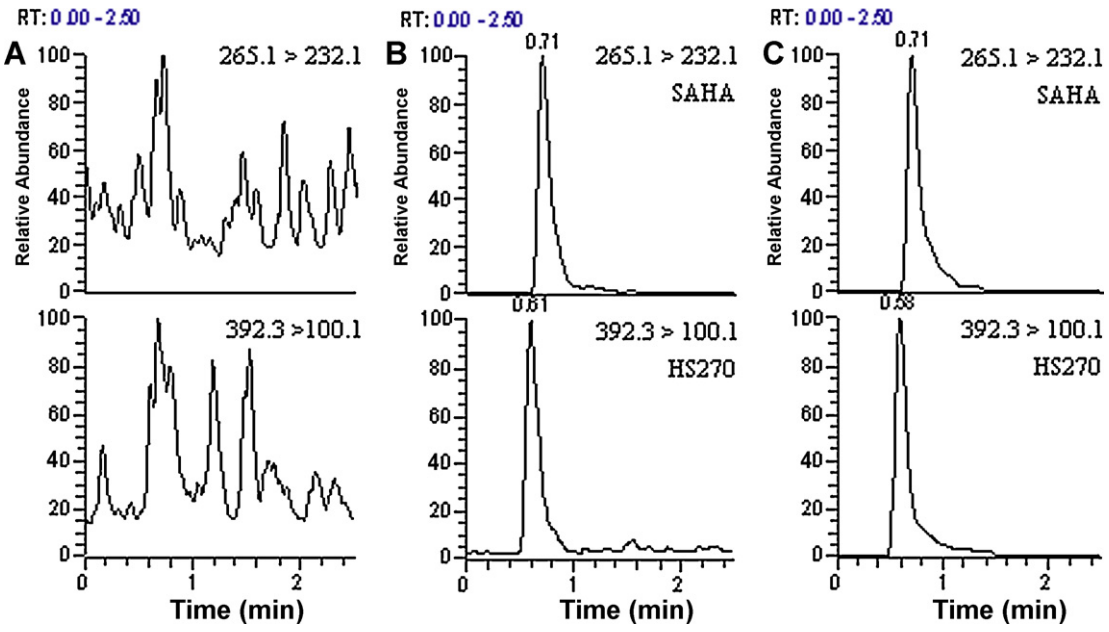

Fig. 3. Representative SRM chromatograms for HS270 and SAHA in rat plasma: (A) a blank plasma sample; (B) a blank plasma sample spiked with HS270 and SAHA at the LLOQ of 0.5 ng/mL; and (C) plasma sample from a rat after an oral administration of 100 mg/kg HS270.

72.2 ± 1.8%. These results indicated that the extraction efficiency for all analytes and IS was acceptable.

3.2.6. Stability

The analytes are considered stable in biological matrix when 85–115% of the initial concentration is detected [10]. The stability of HS270 in rat plasma under different storage conditions is presented in Table 5. There was no significant degradation under the conditions described in this study since their concentrations deviated by no more than 15% relative to the reference nominal concentrations. No degradation products were detected under the selected MS conditions. HS270 in rat plasma can therefore be stored at room temperature for 5 h, for 32 days at −20 °C and for two freeze–thaw cycles. Analysis of the QC samples following LLE procedure showed no significant degradation after 5 h at room temperature. These results indicate that HS270 was stable under routine laboratory conditions and no specific procedure is needed to stabilize the compounds.

Table 5
Stability of HS270 under various storage conditions ($n = 5$).

Storage condition	Nominal con. of HS270 (ng/mL)	Calculated con. (ng/mL)	
		Mean ± SD	RE[a] (%)
−20 °C/32 day			
	1.0	1.0 ± 0.1	−3.5
	100.0	95.2 ± 4.4	−4.8
	750.0	726.6 ± 36.6	−3.1
−20 °C/3 freeze–thaw cycles			
	1.0	1.0 ± 0.0	−4.2
	100.0	96.7 ± 3.0	−3.3
	750.0	669.5 ± 18.0	−10.7
Room temperature/5 h			
	1.0	1.1 ± 0.1	9.1
	100.0	112.7 ± 2.3	12.7
	750.0	838.0 ± 3.8	11.7
15 °C/12 h (extracted solvent)			
	1.0	1.1 ± 0.1	5.4
	100.0	109.9 ± 2.7	9.9
	750.0	805.3 ± 21.3	7.4

[a] Relative error = [(overall mean assayed − added concentration)/added concentration] × 100.

3.3. Preclinical pharmacokinetic study

In this study, we explored the pharmacokinetics of HS270 in SD rats after a single intravenous or oral administration. To our knowledge, this is the first time to evaluate its pharmacokinetic property. The plasma concentration–time profile of HS270 after intravenous administration is illustrated in Fig. 4. The compound was detectable in plasma for up to 12 h. The pharmacokinetic parameters were computed and are shown in Table 6. The elimination half-life was found to be 2.17 ± 0.38 h. The mean total body clearance was 17.27 ± 3.93 L/h/kg and the volume of distribution at steady-state was 55.0 ± 20.4 L/kg. Table 6 also shows the pharmacokinetic parameters of other HDACis (SAHA, SB639) [15]. In comparison to SAHA and SB639, HS270 shows the highest elimination half-life. Plasma clearance of HS270 was also higher than that of SAHA and SB 639.

The mean HS270 plasma concentration versus time curves obtained after a single oral dose of HS270 at 25, 50, 100 or 200 mg/kg are shown in Fig. 5 and the pharmacokinetic parameters of HS270 are presented in Tables 6 and 7. After oral gavage, HS270 was rapidly absorbed and maximal plasma concentration

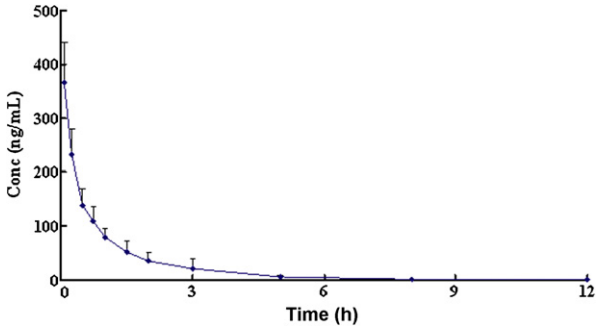

Fig. 4. Mean plasma concentration-time profiles of HS270 after a single IV administration of HS270 to rats (5 mg/kg). Each point represents the mean ± SD ($n = 6$).

Table 6
Oral and intevenous pharmacokinetic parameters of HS270, SAHA, SB639 in rats.

Parameters i.v.	HS270 5 mg/kg	SAHA [6] 2 mg/kg	SB639 [6] 2 mg/kg
C_0 (ng/mL)	930 ± 196	877 ± 745	1272 ± 412
$AUC_{0\to\infty}$ (ng h/mL)	302 ± 69	278 ± 102	526 ± 58
$t_{1/2}$ (h)	2.17 ± 0.38	0.64 ± 0.3	1.12 ± 0.1
CL (L/h/kg)	17.27 ± 3.93	8.04 ± 3.1	3.84 ± 0.5
V_d (L/kg)	55.09 ± 20.44	3.92 ± 1.4	3.67 ± 0.5

Parameters Oral	HS270 25 mg/kg	SAHA[6] 5 mg/kg	SB639[6] 5 mg/kg	Parameters Oral	HS270 25 mg/kg
C_{max} (ng/mL)	28.0 ± 18.7	83.9 ± 33.9	123 ± 34	C_{max} (ng/mL)	28.0 ± 18.7
T_{max} (h)	0.51 ± 0.37	0.17 ± 0.0	0.22 ± 0.1	T_{max} (h)	0.51 ± 0.37
$AUC_{0\to\infty}$ (ng h/mL)	109.7 ± 33.6	49.4 ± 14.9	119 ± 33	$AUC_{0\to\infty}$ (ng h/mL)	109.7 ± 33.6
$t_{1/2}$ (h)	6.4 ± 1.7	1.13 ± 0.6	2.26 ± 1.1	$t_{1/2}$ (h)	6.4 ± 1.7
F (%)	7.3	6.9	10.5	F (%)	7.3

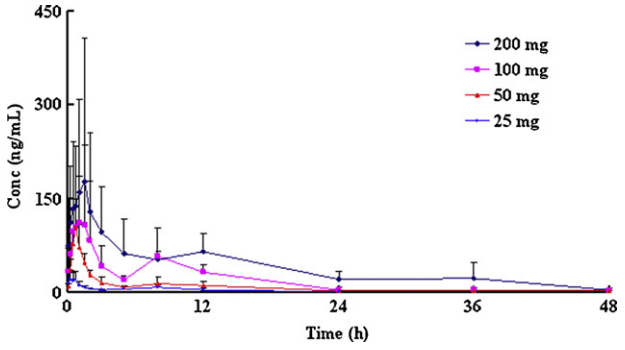

Fig. 5. Mean plasma concentration-time profiles of HS270 after a single oral administration of HS270 to rats (25, 50, 100, 200 mg/kg). Each point represents the mean ± SD (n = 6).

(C_{max}) of HS270 occurred on most occasions at 0.5–3.3 h. Following oral administration at 25, 50, 100 or 200 mg/kg, mean maximal plasma concentration of 28.0 ± 18.7 ng/mL, 105.2 ± 32.3 ng/mL, 161.7 ± 116.3 ng/mL, 222.5 ± 207.1 ng/mL was observed. Concentrations were detectable up to 24–48 h. Among these four groups, the C_{max}, AUC_{0-t} and $AUC_{0\to\infty}$ were dose-dependent. There were no significance differences in $t_{1/2}$, AUC_{0-t}/dose and $AUC_{0\to\infty}$/dose between four groups. The plasma concentration increased with the increase of dosage at the range of 25–200 mg/kg. No significant adverse reaction was observed in all the rats, which indicated HS270 was well tolerated and safe within the dosage scope of 25–200 mg/kg. Like Kendine 91 [14], a second maximum peak was observed in the plasma profile, which could be due to enterohepatic circulation. The mean oral pharmacokinetic parameters for HS270, SAHA [15] and SB639 [15] in rats are summarized in Table 6. Mean elimination half-life increased to 6.44–7.73 h compared to IV administration. The oral bioavailability of HS270 was estimated to be 7.3–15.5%, similar to SB639 (10.5%) [15], higher than that of the SAHA (6.9%) [15] and other HDAC inhibitors [16]. Antitumor activity of HS270 and this basic pharmacokinetic evaluation prompts us to believe that it is positive for further development of HS270 as an anticancer new drug.

Table 7
Pharmacokinetic parameters of HS270 in rats after a single oral administration of HS270 at 50.0, 100.0 or 200.0 mg/kg (mean ± SD, n = 6).

Parameters	Dose of HS270 (mg/kg)		
	50.0	100.0	200.0
C_{max} (ng/mL)	105.2 ± 32.3	161.7 ± 116.3	222.5 ± 207.1
T_{max} (h)	0.7 ± 0.1	0.9 ± 0.4	3.3 ± 4.6
$t_{1/2}$(h)	7.7 ± 3.2	6.5 ± 1.9	7.2 ± 2.1
$AUC_{0-48\,h}$ (ng h/mL)	314.9 ± 139.5	854.9 ± 300.1	1842.5 ± 669.0
$AUC_{0\to\infty}$(ng h/mL)	336.5 ± 146.2	873.8 ± 307.7	1878.5 ± 673.2
F (%)	11.1	14.5	15.5

4. Conclusions

A new, rapid and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) method had been developed and validated for the determination of HS270 in rat plasma using SAHA as the internal standard. The method was validated according to FDA [10] guidance and showed high sensitivity, reliability, specificity and excellent efficiency with a total running time of 2.5 min per sample and a LLOQ of 0.5 ng/mL. And this validated LC–MS/MS method was successfully applied to determine HS270 plasma concentrations in a preclinical pharmacokinetic study in rats after a simple dose of HS270. This is the first report to develop and validate a LC–MS/MS method for determination of HS270 in rat plasma and the first attempt to determine pharmacokinetics of HS270.

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