Development and validation of an LC-MS/MS Method for determination of vorinostat in beagle dog plasma and its application to a pharmacokinetic study

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Abstract

Aim An LC-MS/MS method for determination of vorinostat (SAHA) was developed and validated in order to study the pharmacokinetics of SAHA in beagle dogs. Methods SAHA and chlorpropamide (internal standard, IS) were extracted by liquid-liquid extraction and separated on a Shimadzu Shim-pack VP-ODS column, with a gradient mobile phase composed of methanol and water containing 0.1% acetic acid as the mobile phase. Detection was performed on a triple quadrupole tandem mass spectrometer equipped with electrospray ionization source operated in positive ion selected reaction monitoring mode. After validating, the developed method was used for evaluating pharmacokinetic of SAHA in 8 beagle dogs following oral administration of 100 mg of SAHA. Results The method was linear in the concentration range 0.975–125.0 ng·mL⁻¹. Intra- and inter-day precisions (as relative standard deviation) in all samples were both within 15%. SAHA was proved to be stable during sample storage, preparation and analysis procedure. After oral administration of 100mg of SAHA to beagle dogs, the main pharmacokinetic parameters $C_{\text{max}}$, $t_{\text{max}}$, $AUC_{0-T}$ and $t_{1/2}$ were 94.69±50.05 μg·L⁻¹, 1.31±0.77 h, 207.27±124.26 μg·h·L⁻¹ and 2.48±0.50 h, respectively. Conclusions This LC-MS/MS method for determination of SAHA proved to have sufficient selectivity, sensitivity and reproducibility and successfully applied to a pharmacokinetic study of SAHA in beagle dogs.

Key words Beagle dog; plasma; LC-MS/MS; pharmacokinetics; vorinostat

Introduction

Epigenetic modification of chromatin has received increasing recognition as an important factor in gene expression and as a target for antineoplastic drug development [1, 2]. One
epigenetic modification that has received much attention is histone acetylation, a post-translational alteration of lysine-rich proteins that are part of the densely packed structure of chromatin\[^3,4\]. Histone acetyltransferases add acetyl groups to lysine residues in histones, thereby relaxing chromatin and allowing transcription to occur\[^5\]. Conversely, histone deacetylases remove acetyl groups from lysine residues in histones, thereby tightening and condensing chromatin structure and preventing transcription\[^6,7\]. Dysregulation of histone deacetylases is present in a variety of cancers, and histone deacetylase inhibitors have been shown to induce apoptosis and cell differentiation in solid tumor and hematopoietic cancer cells. This has made histone deacetylase an attractive target for anticancer agent development\[^8,9\].

Vorinostat (suberoylanilide hydroxamic acid, SAHA) (Fig. 1) is a potent histone deacetylase inhibitor\[^10\] that has demonstrated antineoplastic activity in vitro against a variety of cell lines and in vivo against several human tumor xenograft models\[^11\]. SAHA suppress the activities of multiple histone deacetylases, leading to an increase in histone acetylation. The histone acetylation induces an enhancement of the expression of specific genes that elicit extensive cellular morphologic and metabolic changes, such as growth arrest, differentiation and apoptosis\[^12,13\]. This encouraging preclinical activity has prompted extensive clinical evaluation of SAHA, initially as a single agent and more recently as a component of a variety of combination chemotherapy regimens\[^14,15\].

Several publications reported the methods for measuring plasma concentration of SAHA including HPLC-UV, LC-MS and LC-MS/MS. However, HPLC-UV or LC-MS assay showed less than ideal sensitivity\[^16,17\]. And LC–MS/MS methods previously described were developed using high turbulence liquid chromatography coupled with column switching technology\[^18\] or isotope-labeled internal standard\[^19,20\]. All the indicated that the reported methods seemed not to be suitable for rational clinic investigation or pharmacokinetics of SAHA. The aim of the study was to develop one sensitive, rapid and simple method for the determination of SAHA in bio-samples of both experimental animals and clinical patients. In addition, the well validated LC/MS/MS method was successfully applied for a pharmacokinetic study after oral administration of SAHA to beagle dogs.

**Materials and Methods**

**Chemicals and reagents**

Reference standard of SAHA and chlorpropamide (used as an internal standard, IS) (Fig. 1) were purchased from J&K Chemical Co., Ltd. (Shanghai, China) and Sigma-Aldrich, Inc. (Shanghai, China), respectively. SAHA capsules (one capsule contains 100 mg of SAHA) were supplied by Patheon Inc. (Mississauga, Ontario,
Canada). Methanol of HPLC grade was purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was distilled and purified using a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). Other chemicals were all of analytical grade.

Stock solutions
The stock solution of SAHA was prepared by dissolving 10 mg accurately weighed authentic sample in 10 mL of methanol. The stock solution was stored at 4°C and it was proved stable for at least for one months. A set of working solution were obtained by serial dilution of the stock solution with the methanol. The stock solution of Chlorpropamide (IS, 1.0 mg·mL−1) was prepared by dissolving 10 mg of accurately weighed sample in 10 mL of methanol. A working solution of the IS (2.0 μg·mL−1) was prepared by diluting the stock solution with methanol. All solutions were stored at 4 ºC and used within one month after preparation.

LC/MS/MS conditions
A Finnigan Surveyor™ HPLC system (Thermo Electron, San Jose, CA, USA) coupled to a Finnigan TSQ Quantum Ultra tandem mass spectrometer (ThermoElectron, San Jose, CA, USA) via an electrospray ionization (ESI) source was used for analysis. Data acquisition was performed with Xcalibur 1.1 software (Thermo-Finnigan, USA). Peak integration and calibration were performed using LCQuan software (Thermo-Finnigan, USA). SAHA and I.S. were separated on a Shim-pack VP-ODS column (5 μm, 150mm×4.6mm i.d., Shimadzu, Kyoto, Japan) maintained at 25 ºC. The mobile phase consisted of A: water (containing 0.1% acetic acid) and B: methanol and was delivered at a flow rate of 1 ml/min. The gradient was as follows: 0 min 25% B, 1.5 min 95% B, 4.5min 95% B, 4.6min 25% B, 7 min stop.

Mass spectrometer was operated in positive ion mode. The tuning parameters were optimized for SAHA and IS in a flow injection system by infusing a standard solution, which contained 5 μg·mL−1 of each analyte, at a flow rate of 20 μL/min. SAHA and IS were separately scanned under Q1 MS full-scan mode and Q1/Q3 (MS/MS) product ion scan mode at different collision energies to determine the quasi-molecular ion and the corresponding product ions (Fig. 2). Selected reaction monitoring (SRM) of the precursor ion to the most abundant product ion under optimized CID energy was applied to determine the analytes of interest. Quantification was thus performed using SRM of the transitions of m/z 265 → 232 for SAHA, m/z 276 → 111 for IS with a scan time of 0.2 s per transition. The collision energies for SAHA and IS were 20 and 46 eV, respectively. The optimized MS parameters were as follows: sheath and auxiliary gases flow rates were 40 and 5 psi, respectively; spray voltage was 5000 V; capillary temperature was 350 ºC; collision gas (argon) pressure was 0.7mTorr.

Sample Preparation
After thawing at room temperature for about 30 min, plasma samples were vortexed. A 200 μL aliquot plasma sample was placed in a 2.0 mL tube followed by adding 10 μL IS solution. The mixture was vortexed for 30 s. Then 1 mL of acetic ether was added followed by vortexing vigorously for 3 min on an automated multi-tube vortex mixer (Scientific Industries In c., NY, USA). The tubes were then centrifuged at 8,000 g for 5 min, and 800 μL of the upper organic layer was transferred to a new tube and evaporated to dryness in a Thermo Savant SPD 2010 SpeedVac System (ThermoElectron, San Jose, CA, USA) at 45ºC. The residues were reconstituted in 0.2 mL of methanol and centrifuged at 20,000 g at 4 ºC for 10 min. The supernatant (150 μL) was transferred to a polypropylene autosampler vial and 10 μL was injected for LC/MS/MS analysis. Serial standard and QC samples were prepared following above method.

Assay validation
Blank beagle dog plasma samples were spiked with standard solutions of SAHA to get the final concentration ranges (0.975, 1.95, 3.90, 7.80, 15.60, 31.20, 62.50 and 125.00 ng·mL−1). Linearity was assessed by weighted linear regression of analyte–internal standard peak area ratios based on three independent calibration curves prepared on each of three separate days. The lower limit of quantitation (LLOQ) was evaluated by analyzing
six plasma samples spiked with the analyte at a final concentration of 0.975 ng·mL$^{-1}$ at which the signal-to-noise (S/N) ratio should be greater than 10, the relative deviation of precision should be less than 20% and the accuracy should be within 80–120%.

![Fig 2. MS$^2$ product ions spectra of the deprotonated molecular ions of SAHA and chlorpropamide (IS).](image)
Quality control samples (QC samples, 1.95, 7.80 and 62.5 ng/mL) were prepared to evaluate the accuracy, precision, recovery of the assay. Accuracy and intra- and inter-day precisions (expressed as relative standard deviation, RSD) were assessed by assay of six replicate QC samples on three different days. Recovery of SAHA was determined by comparing peak areas of extracted QC samples with peak areas of post-extraction plasma blanks spiked at corresponding concentrations. Matrix effects for SAHA were evaluated by comparing peak areas of post-extraction blank plasma spiked at concentrations of QC samples with the areas obtained by direct injection of corresponding standard solutions. Stability of SAHA was evaluated using QC samples subjected to three freeze–thaw cycles, stored at -20 °C for one month and at room temperature for 6 h. Stability in stock solutions (4 °C) and mobile phase on storage in plastic autosampler vials under autosampler conditions for 12 h were also assessed.

Application to Pharmacokinetic Study of SAHA

Eight beagle dogs (four males and four females), weighing 11.0 ± 2.1 kg (mean±S.D.), were purchased from Nanjing Anlimo Biotechnology Co., Ltd. (Nanjing, China) and housed with free access to food and water. Animals were housed under controlled conditions (temperature, 20 ± 2 °C and relative humidity, 50 ± 10%) with a natural light–dark cycle. They were acclimated in the animal facility for at least 1 week prior to study.

The dogs were fasted for 12 h and had free access to water before dosing. After oral administration of SAHA (100mg) to dogs through gavage, serial blood samples (0.3 mL) were collected in 1.5mL heparinized polythene tubes at 0, 20, 30, 40, 50, 60, 80, 100, 120, 150, 180, 240, 480 and 600 min. Then each collected blood sample was centrifuged at 8,000 g for 5 min, immediately. All plasma samples were stored at -20 °C until analysis.

Results and discussion

Method development

Selection of IS

It was difficult to find a compound that could ideally mirror the analyte to serve as a suitable IS. Several compounds were investigated such as acetaminophen, phenylpropanolamine, berberine and diclofenac but they had poor peak shape under the condition and finally chlorpropamide (Fig. 1) was found to be the most appropriate for the present purpose. The behavior of chlorpropamide’s retention time was similar to that of SAHA. Chromatograms were obtained and no significant direct interference in the MRM channels at the relevant retention times was observed.

Sample pre-treatment

Due to the complex nature of plasma, a sample pre-treatment is often needed to remove protein and potential interferences prior to LC–MS/MS analysis. Currently, the most widely employed biological sample preparation methodologies are protein precipitation (PPT), solid phase extraction (SPE), and liquid-liquid extraction (LLE). As the PPT procedure has the advantages of simplicity and universality for drug molecules in plasma, our initial approach of developing an assay for SAHA in plasma was based on PPT with methanol and acetonitrile. However, this technique resulted in strong interferences and high baseline from the sample matrix. LLE was adopted in the end because this technique can not only purify but also concentrate the sample. Acetic ether, n-hexane and diethyl ether were all tested, and finally acetic ether was adopted because of its high extraction efficiency and less interference.

Chromatographic and Mass Spectrometric Conditions

Optimal HPLC conditions of SAHA required chromatographic separation and in-source dissociation. Based on the analytes’ structures and the intensity of the precursor ions, acidified water and methanol were used in the mobile phases. Several HPLC columns, a Shimadzu Shim-pack VP-ODS C18s, a Waters Atlantis HILIC C18s, a Phenomenex Luna C18s, and an Agilent ZORBAX Eclipse XDB-C8 were evaluated with respect to
separation, peak shape, and mass spectral response. The best results were achieved with the Shim-pack VP-ODS C18. Formic acid and acetic acid were compared at three concentrations, 0.02, 0.05, and 0.1% (v/v), in water for optimal mobile phases. The assay performance in acetic acid was better than in formic acid. 0.1% acetic acid in water was chosen as mobile phases because the peak shapes of the analytes tended to become broader and tailed more with decreasing acid concentrations.

In order to develop a method with high sensitivity, MS/MS detection was used instead of UV detection because the latter does not reach this limit. Another benefit of using MS/MS detection is its inherent selectivity. The MS parameters and collision energies were optimized by auto-tuning to obtain the highest sensitivity and the optimal parameters were mentioned above. SAHA and IS were separately scanned under the Q1 MS full-scan mode to determine the parent ion, and under the Q1/Q3 (MS/MS) product ion scan mode to locate the most abundant production. [M+H]⁺ was the predominant ion in the Q1 spectrum, and was used as the parent ion to obtain the product ion spectra. The most sensitive mass transition was from m/z 265.1 to 232.1 for SAHA and from m/z 277.0 to 111.0 for IS, respectively. The negative ion [M−H]⁻ was also tested. The result showed that the response intensity of [M−H] for SAHA was unstable.

**Method Validation**

**Selectivity**

Under the current optimized HPLC and MS/MS conditions, SAHA and the IS eluted at retention times of 4.5 and 5.1 min, respectively. Six sources of blank rat plasma were analyzed and no interference was observed due to the high selectivity of the selected reaction monitoring mode in MS/MS (Fig. 3).

![Fig 3A Chromatograms by selected reaction monitoring (SRM) scan mode: (A) blank plasma (drug and IS free);](image-url)
Fig 3B. Chromatograms by selected reaction monitoring (SRM) scan mode: (B) blank plasma spiked with 156 ng/mL SAHA and 2 μg/mL IS.

Fig 3C. Chromatograms by selected reaction monitoring (SRM) scan mode: (C) plasma sample of a beagle dog 80 min post-oral administration of SAHA 100 mg.
Calibration curve and LLOQ

Standard solutions for calibration were prepared by spiking 0.2 mL of blank beagle dog plasma with 10 μL of standard working solutions, resulting in SAHA concentrations of 0.975, 1.95, 3.90, 7.80, 15.60, 31.20, 62.50 and 125.00 ng·mL⁻¹ in plasma. The representative regression equation was \( y = 0.0969x - 0.0824 \), with a \( 1/x \) weighting factor, where \( y \) and \( x \) were the peak area ratios of analyte to IS and the plasma SAHA concentrations, respectively. Good linearity with a correlation coefficient \( (r^2) \) exceeding 0.9986 was observed for the analyte. The slopes of the regression equations were consistent for the calibration curves prepared on three different days. The LLOQ under the optimized conditions was judged as 0.975 ng·mL⁻¹ for SAHA from the fact that the signal to noise ratio was larger than 10, the deviation of precision (RSD) was 7.2% and the accuracy was 107.4±3.1%.

Precision and accuracy

The intra- and inter-day precision and accuracy were assessed from the results of QC samples. Intra-day precision and accuracy were assessed from five consecutive analyses of the QCs at three different concentrations in plasma; inter-day precision and accuracy was obtained from consecutive analyses of the same batch of QC samples on three separate occasions. Precision was expressed as the relative standard deviation (RSD). Accuracy of the method was expressed by (mean measured concentration)/(nominal concentration) × 100%. The mean values and RSD for QC samples at three concentration levels were calculated using a one-way analysis of variance (ANOVA). Table 1 shows a summary of intra- and inter-day precision and accuracy of SAHA. The intra-day accuracy for SAHA ranged from 101.7 to 103.6% at the tested concentrations with the precision (RSD) between 3.6 and 6.1%. The inter-day accuracy for SAHA ranged from 103.3 to 106.7% at three different concentrations with the precision (RSD) between 2.8 and 3.4%. These results demonstrated that the present method has satisfactory accuracy, precision and reproducibility.

Extraction recovery and matrix effect

The best recovery was obtained with acetic ether. Extraction recovery of SAHA from spiked plasma was determined at the concentrations of 1.95, 7.80 and 62.50 ng·mL⁻¹ five times. The extraction method described above proved to be simple, rapid and successful, with an average recovery ratio of over 90%. Recoveries of SAHA at 1.95, 7.80 and 62.50 ng·mL⁻¹ were 92.6%, 99.8% and 101.9%, respectively. Extraction recovery of the IS was determined to be 82.7% at the spiked concentration (50 ng·mL⁻¹). Matrix effect, caused by competing ionization between the analyte and the endogenous co-elutants, was evaluated at 1.95, 7.80 and 62.50 five times. The observed matrix effects ranged from 96.6 to 109.7%, and were within an acceptable range.

Table 1. Intra- and inter-batch precision and accuracy of SAHA in beagle dog plasma

<table>
<thead>
<tr>
<th>Concentration (ng·mL⁻¹)</th>
<th>Spiked</th>
<th>Intra-batch assay (n=5)</th>
<th>Inter-batch assay (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Precision (%)</td>
<td>Accuracy (%)</td>
</tr>
<tr>
<td></td>
<td>(ng·mL⁻¹)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>1.95</td>
<td>2.02</td>
<td>6.10</td>
<td>103.6</td>
</tr>
<tr>
<td>7.80</td>
<td>8.04</td>
<td>5.80</td>
<td>103.1</td>
</tr>
<tr>
<td>62.50</td>
<td>63.57</td>
<td>3.60</td>
<td>101.7</td>
</tr>
</tbody>
</table>

Stability

The freeze–thaw, short-term and long-term storage stabilities were determined using QC samples at low, medium and high concentration levels for five times. The stability of SAHA in drug-free plasma was satisfactory and no significant degradation had been found under all the conditions examined. The results of the stability...
assessment are shown in detail in Table 2. SAHA in stock solutions kept at 4 °C was also stable (>99% drug remaining).

Table 2. Stability of SAHA in beagle dog plasma during the storing and preparing procedures (n=5)

<table>
<thead>
<tr>
<th>Spiked Concentration (ng·mL⁻¹)</th>
<th>Freeze-thaw (three cycles)</th>
<th>Long-term stability at -20 °C for 30 days</th>
<th>Short-term stability at 20 °C for 6 h</th>
<th>Autosampler tray at 4 °C for 12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.95</td>
<td>109.0</td>
<td>106.3</td>
<td>105.7</td>
<td>104.7</td>
</tr>
<tr>
<td>7.80</td>
<td>94.7</td>
<td>95.1</td>
<td>97.0</td>
<td>100.0</td>
</tr>
<tr>
<td>62.50</td>
<td>101.4</td>
<td>96.4</td>
<td>91.8</td>
<td>99.1</td>
</tr>
</tbody>
</table>

Pharmacokinetic Study

The fully validated method was successfully applied to the pharmacokinetic study of SAHA after an oral dose (100 mg) to 8 beagle dogs. The chromatograms of plasma obtained from pre- and post-dosed beagle dogs showed that no significant interfering peak was detected at the retention times of SAHA and the internal standard, indicating the method was specific enough for the intended pharmacokinetic study. The plasma concentrations measured were shown(Fig 4). The pharmacokinetic parameters (Table 3) of SAHA were estimated using BioAailability Program Package (BAPP, Version 3.0, China Pharmaceutical University, China). The $C_{\text{max}}$ and $T_{\text{max}}$ were directly obtained from the plasma concentration–time profiles. It was found that maximum concentration in beagle dog plasma ($C_{\text{max}}$ 94.7 ng·mL⁻¹) occurred at about 1.3 h ($t_{\text{max}}$). The estimated half-life ($t_{1/2}$) and AUC$_{0-T}$ were 2.5 h and 207.3 μg h·L⁻¹, respectively. A report showed that the $t_{1/2}$ and the peak time were 1.7 h and 1.5 h, respectively, in advanced cancer patients after a single oral dose of 400 mg of SAHA[21]. This indicated that the oral pharmacokinetic parameters of SAHA were similar in beagle dogs and humans. The large individuality in the plasma concentrations was also observed, which may come from the different weights of the experimental beagle dogs and several notable influencing factors such as sex, metabolic enzyme and the drug itself. Real mechanism was unclear.

Fig 4. Mean plasma concentration-time profile after a single oral administration of 100 mg of SAHA to beagle dogs. Each point represents the mean±SD (n=8).
Table 3. Pharmacokinetic parameters of SAHA after an intragastric administration (100 mg) to beagle dogs (n=8, Mean ±SD)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Values</th>
<th>Parameters</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (µg.L⁻¹)</td>
<td>94.695±0.05</td>
<td>T₁/₂ (h)</td>
<td>2.48±0.50</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>1.31±0.77</td>
<td>MRT (h)</td>
<td>3.940.48</td>
</tr>
<tr>
<td>AUC₀→₄ (µg.h.L⁻¹)</td>
<td>207.27±124.26</td>
<td>CL (L.h⁻¹)</td>
<td>668.62±379.16</td>
</tr>
<tr>
<td>AUC₀→∞ (µg.h.L⁻¹)</td>
<td>221.71±126.01</td>
<td>Vd (L)</td>
<td>2543.02±1685.65</td>
</tr>
</tbody>
</table>

Conclusion

An LC/MS/MS method combined with the liquid-liquid extraction for the quantification of SAHA in beagle dog plasma was firstly developed. The new assay is rapid, sensitive, accurate and reproducible. This method was successfully applied to a pharmacokinetic study of SAHA in beagle dogs.

References


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