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Development and validation of a liquid chromatography–tandem mass spectrometry for the determination of Kendine 91, a novel histone deacetylase inhibitor, in mice plasma and tissues: Application to a pharmacokinetic study

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ABSTRACT

A liquid chromatography–tandem mass spectrometric method (LC–MS/MS) has been developed and validated to determine the concentration of Kendine 91 in mice plasma and tissues. Simvastatin was employed as the internal standard. Separation was performed on a C_8 column, with a mobile phase consisting of methanol and aqueous 10 mM formic acid (73:27 v/v). Both analyte and internal standard were determined using electrospray ionization and the MS data acquisition was via multiple-reaction monitoring (MRM) in positive scanning mode. Quantification was performed using the transitions m/z 444 → 169 and 441 → 325 for Kendine 91 and simvastatin, respectively. The method was validated with respect to linearity, accuracy, precision, recovery and stability. This assay has been successfully applied to a pharmacokinetic study after intravenous injection of Kendine 91 in mice in a dose of 10 mg/kg.

1. Introduction

Histone deacetylase inhibitors (HDACIs) are one of the most promising small molecules for the treatment of different diseases [1–3]. These compounds have different biochemical and biologic properties but share a common activity: induction of acetylation in histones. Histone acetylation has a crucial role in the control of transcription of many genes, including tumor suppressor genes. The dynamic degree of core histone acetylation is maintained in vivo by the balance between the activities of histone acetylases (histone acetyl transferases, HATs) and histone deacetylases (HDACs) [4]. Hypoacetylated histones have been associated with transcriptionally silent genes.

HDACIs have the capacity to induce cytodifferentiation, cell cycle arrest, and apoptosis in transformed cells [5]. Preclinical studies of HDACIs have demonstrated antitumor activity both in vivo and in vitro, suggesting that the HDACIs may be potentially important novel anticancer therapeutics [6].

Trichostatin A (TSA) was the first potent HDAC inhibitor (HDACI) discovered [7]. Although the synthesis [8] of the compound itself is quite complex and its metabolism is too rapid [9], it was an interesting lead compound for the development of HDACIs as it features a high affinity for HDAC [10] and a small size in comparison with other natural HDACIs like Trapoxin B [11]. Following the discovery of the HDAC inhibitory activity of TSA, a wide variety of HDACIs have been identified, synthesized and tested [12]. Some of these products are already in clinical trials [13], such as MS-275 [14], which is in phase II, and suberoylanilide hydroxamic acid (SAHA) [15], which has recently been registered. Within this context, we have designed and synthesized a family of new HDACIs [16], being Kendine 91 the most promising candidate. Its chemical structure is presented in Fig. 1.

Pharmacokinetic studies constitute an important phase in the process of development of new medicines, being their objectives to know the disposition process (absorption, distribution and elimination) of the new drug candidate that helps to select the most appropriate route of administration and the best dose regimen. Analytical methods employed for the quantitative determination of drugs and their main metabolites in biological samples are the key determinants in generating reproducible and reliable data which in turn are used in the evaluation and interpretation of pharmacokinetic finding. It is essential to employ well characterized and fully validated analytical methods to yield reliable results which can be satisfactory interpreted. It is recognised that analytical methods and techniques are constantly undergoing changes and improve-

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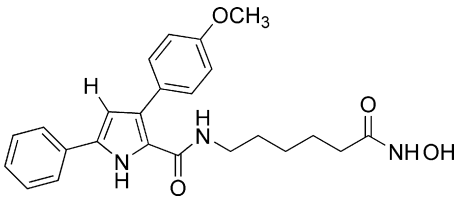

Fig. 1. Chemical structure of Kendine 91.

ments; and in many instances, they are at the cutting edge of the technology. It is also important to emphasize that each analytical technique has its own characteristics, which will vary from analyte to analyte. Moreover, that appropriateness of the technique may also be influenced by the ultimate objective of the study [17].

The objective of the present work was to develop and validate a LC–MS/MS methodology for the determination and quantification of Kendine 91 in mice plasma and liver, heart, kidney and lung tissues. The HPLC method was established and validated according to FDA's "Guidance for Industry" – Bioanalytical Method Validation [18] by determining its accuracy, precision, sensitivity, recovery, specificity and stability. The method has been successfully applied to the determination of Kendine 91 time–concentration profiles in plasma, liver and lung from mice following single intravenous administration.

2. Experimental

2.1. Chemicals and reagents

Kendine 91 was synthesized by an organic chemistry group of EHU-UPV (San Sebastian, Spain) and was characterized using spectroscopic techniques (^{1}H NMR, ^{13}C NMR, IR, melting point and combustion microanalysis). Simvastatin, used as internal standard was purchased from Sigma (St. Louis, MO, USA). Methanol (gradient HPLC grade) was obtained from Scharlau (Barcelona, Spain). Formic acid, ortho-phosphoric acid 85%, sodium di-hydrogen phosphate 1-hydrate and *tert*-butyl methyl ether (MTBE) were purchased from Panreac Química (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q® Plus apparatus (Millipore). Other chemicals were all analytical grade. Plasma was obtained from Centro Vasco de Transfusiones (Galdakao, Spain).

Female balb/c nude mice weighting 18–22 g (5 weeks of age) were purchased from the Harlam Interfauna Ibérica S.L. (Barcelona, Spain).

2.2. Instrumentation

The liquid chromatography system consisted of a Waters HT Alliance 2795 with a temperature control autosampler. The detector was a Micromass (Waters Corporation) Quattro Micro mass spectrometer. The system was controlled by Masslynx 4.1 software (Waters, UK).

2.3. Chromatographic and mass spectrometric conditions

Samples were separated on a Symmetry®C_8, 5 μm (3.9 mm × 150 mm) column (Waters, Milford, MA) which was maintained at room temperature. The mobile phase consisted of 73% methanol and 27% (v/v) of aqueous formic acid solution (10 mM). The flow rate was 1 mL/min in isocratic elution and it was split, with 0.48 mL entering the mass spectrometer. The sample volume injected was 50 μL and the autosampler was set at 4 °C.

MS/MS analysis was performed on a triple–quadrupole mass spectrometer operated in positive mode. Electrospray ionization in the sample introduction and detection was operated in the multiple-reaction monitoring (MRM) mode. Nitrogen was used at flow rates of 600 L/h. Argon was used as collision gas at a pressure of 0.35 mL/h. The cone voltage and the collision energy were optimized for the MRM transitions. The chosen values were 25 eV for the collision energy and 30 V for the cone voltage. The optimum source and desolvation temperature were set at 120 and 350 °C, respectively. Transition ions m/z 444 → 169 and 441 → 325 were selected for Kendine 91 and simvastatin (used as internal standard), respectively.

2.4. Preparation of stock solutions

Stock solutions of Kendine 91 (500 μg/mL) were prepared in methanol and simvastatin (IS, 100 μg/mL) in a mixture of methanol–water (1:1, v/v). Working solutions for calibration and quality control (QC) were prepared by appropriate dilution in methanol–water (60:40, v/v).

Calibration solutions were prepared at concentrations of 20, 50, 100, 500, 1000, 5000 and 10,000 ng/mL and QC at 60, 750 and 7500 ng/mL. Simvastatin was diluted with methanol–water (60:40, v/v) to obtain a concentration of 50 ng/mL. All solutions were freshly prepared every day prior to use.

2.5. Calibration standards and quality control samples

Calibration curves were prepared by mixing 100 μL of one of the working solutions mentioned above and 900 μL of plasma to produce the standard curve point's equivalent to 2, 5, 10, 50, 100, 500 and 1000 ng/mL of Kendine 91. Blank plasma samples (without IS) were also analyzed.

Quality control samples were prepared as described above to produce a final concentration equivalent to 6 ng/mL (low quality level), 75 ng/mL (middle quality level) and 750 ng/mL (high quality level). The considered limit of quantification was the lowest level included in the calibration curve.

Standards and QC in liver, lung, heart and kidney homogenates were prepared at the same levels as in plasma. The homogenate samples were spiked with the working solutions of Kendine 91. Blank plasma samples (without IS) were also analyzed.

2.6. Sample preparation

Liquid–liquid extraction was employed for the sample preparation. To a 50 μL aliquot of plasma sample, 100 μL of IS (50 ng/mL simvastatin in methanol:water 1:1, v/v) and 1000 μL of pH 3 and 0.1 M phosphate buffer solution were added. The samples were mixed and 2 ml of MTBE (*tert*-butyl methyl ether) were added. The samples were stirred for 10 min and centrifuged at 3000 rpm for 10 min. After centrifugation, the upper organic layer was removed and evaporated to dryness at 40 °C under a stream of nitrogen. The resulting residue was then reconstituted in 120 μL methanol–water (60:40 v/v) and 50 μL of the solution obtained was injected into the LC–MS/MS system.

Tissue samples were accurately weighed and 500 μL PBS (phosphate buffer solution) (pH 7.4) was added per 250 mg of tissue and homogenized. Tissues homogenates were processed similarly as that of plasma samples and analyzed by HPLC.

2.7. Method of validation

Calibration curves were generated by using the ratios of the analyte peak area to the IS peak area versus concentration and were fitted to the equation $y = bx + a$. Weighted $(1/x)$ least squares regression was used. Linearity was determined in three correlative

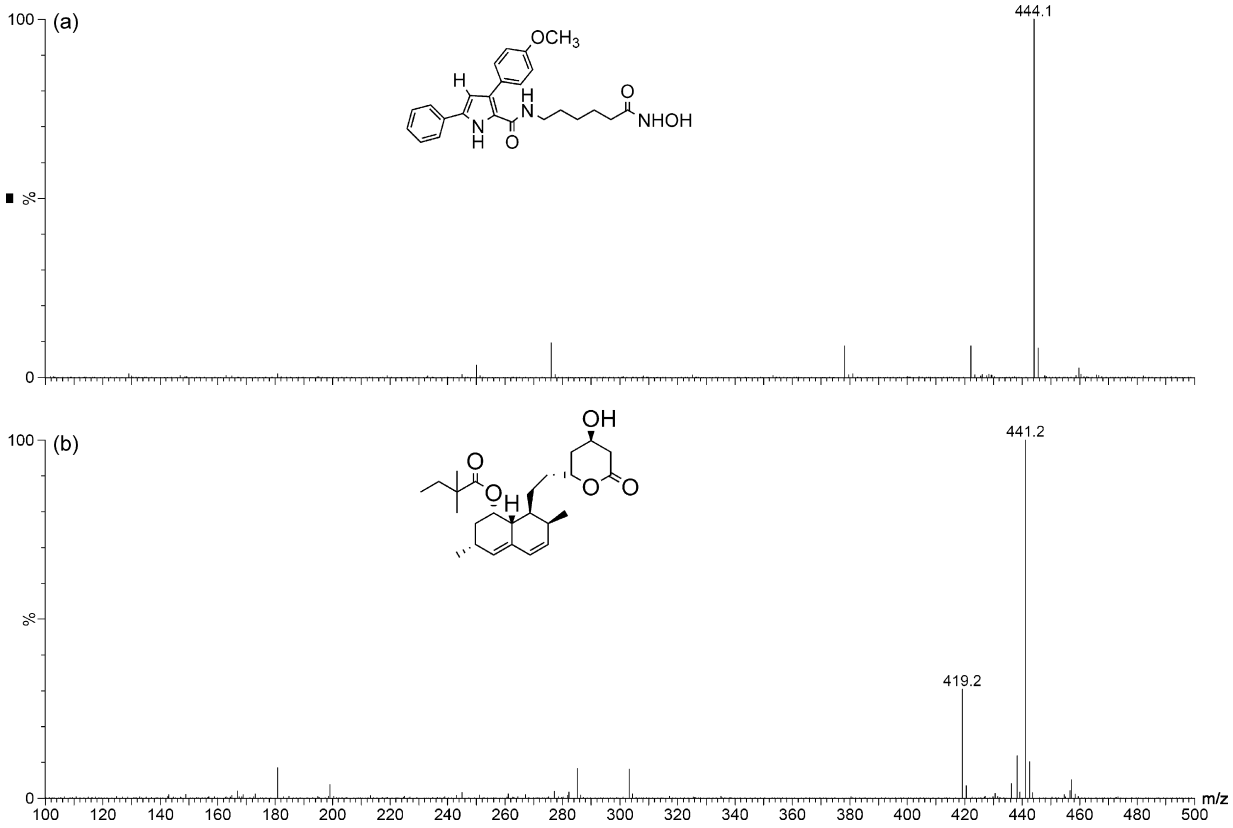

Fig. 2. Full scan ESI (+) ion spectra of (a) Kendine 91 and (b) simvastatin (IS).

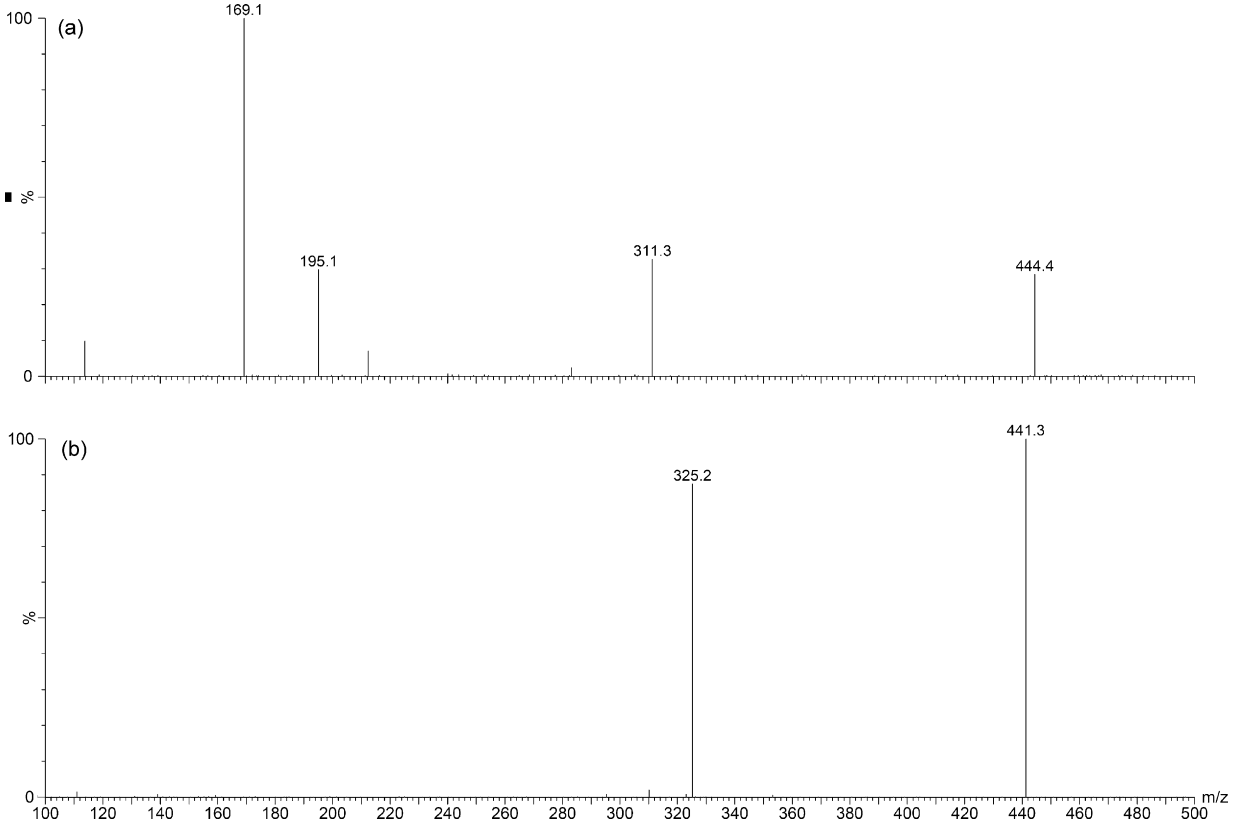

Fig. 3. Daughter spectra of (a) Kendine 91 and (b) simvastatin (IS).

days, and coefficients of determination (r^2) and relative standard deviation (R.S.D. %) of the response factors of each standard were calculated each day.

The intra-batch precision and accuracy were determined by analyzing five plasma samples of Kendine 91 at each QC level (6, 75 and 750 ng/mL) in three consecutive days. The inter-batch precision and accuracy were determined by analyzing the fifteen plasma samples of Kendine 91 at each QC level (6, 75 and 750 ng/mL) in a day.

Intra- and inter-assay precision and accuracy of the limit of quantification (LQ), which was considered the lowest level included in the calibration curve, were determined by analyzing five plasma samples of Kendine 91 at 2 ng/mL in three consecutive days. The concentration of samples was calculated using the curve prepared and analyzed the same day.

To test the method specificity, the following samples were prepared and processed using the proposed extraction procedure and chromatographic and spectroscopic conditions: six blank plasma samples obtained from six different sources, zero sample, lower limit of quantification, the higher standard concentration without internal standard and the internal standard sample without analyte.

The extraction recovery of Kendine 91 and simvastatin were assessed by comparing the peak areas obtained from extracted plasma samples with those of post extraction plasma blanks spiked with corresponding concentration. The recovery studies were assessed at least five replicates at three concentration levels (6, 75 and 750 ng/mL).

Matrix effect was evaluated by comparing the absolute peak areas of post-extraction spiked samples with corresponding absolute peak areas of neat standards prepared in the reconstitution solution. This study was assessed with four replicates at three concentration levels (25, 312 and 3125 ng/mL for Kendine 91 and 417 ng/mL for simvastatin). Moreover, the effect of ion suppression on the MS/MS signals of the analyte, Kendine 91 and the internal standard, simvastatin was assessed by continuous post-column infusion (50 μL/min) of both compounds into the mobile phase and injection of an extracted blank plasma sample. Any ion suppression would be observed as a depression of the MS signal.

In order to quantify plasma levels of Kendine 91 higher that the upper limit of the standard curve, dilution effect was evaluated. A spiked plasma sample with Kendine 91 at a concentration level of 5000 ng/mL was prepared and later diluted with plasma to a final concentration of 500 ng/mL. Five replicates were analyzed on three days. Accuracy and precision were calculated.

The stability of Kendine 91 under storage conditions was evaluated. QC samples at the same concentration levels as the QC used in accuracy and precision study were prepared in plasma, aliquoted and stored at −80 °C until the day of the assay, when three samples of each concentration were analyzed. Stability of the analytes was also determined after three freeze and thaw cycles. Three samples of low and high QC underwent three freeze and thaw cycles. Samples were thawed at room temperature. The autosampler stability was measured by determining QC samples kept under the autosampler conditions (4 °C) for 24 h. To evaluate the short-term stability, three aliquots of the low and high QC were frozen, thawed and maintained at room temperature for 8 h, which exceeds the expected duration that samples could be maintained at room temperature after thawing until they are analyzed.

Recovery of Kendine 91 from liver, lung, heart and kidney samples relative to plasma samples was obtained by comparing the chromatographic response of Kendine 91 in the tissue samples with the chromatographic response of Kendine 91 in the plasma samples. This factor was later used to calculate the Kendine 91 concentration in unknown tissue samples from calibration curves obtained with plasma samples.

2.8. Application of the assay

The method was applied to a mice pharmacokinetic study. Kendine 91 was dissolved in a mixture of PEG, ethanol and water. Mice were grouped 5 per point and administered a single i.v. dose (10 mg/kg) of compound via the tail vein. At predefined time points (5, 15, 30 min and 1, 2, 3, 6, 8 and 12 h) mice were sacrificed by CO_2 overdose, and blood samples were collected by cardiac puncture. The blood samples were centrifuged for 5 min at 8000 rpm to separate plasma, and the plasma was kept frozen at −80 °C until analysis by LC–MS/MS. Moreover, liver and lung were also removed from the animals.

2.9. Pharmacokinetic analysis

Pharmacokinetic parameters were calculated by a non-compartmental method using WinNonlin 4.1 Pro (Pharsight, Mountain View, CA, USA). The area under the plasma concentration versus time curve up to the last quantifiable time point, AUC_{0-t}, was obtained by the linear and log–linear trapezoidal summation. The AUC_{0-t} was extrapolated to infinity ($AUC_{0-\infty}$) by adding the quotient C_{last}/k_{el}, where C_{last} represents the apparent terminal rate constant. k_{el} was calculated by the linear regression of the log-transformed concentrations of the drug in the terminal phase. The half-life of the terminal elimination phase was obtained using the relationship $t_{1/2} = 0.693/k_{el}$. Systemic clearance was calculated by the relationship $CL = (D_{iv})/(AUC_{0-\infty,iv})$ where D_{iv} is the dose of the compound. The apparent volume of distribution was obtained from the equation $Vd_z = D/(AUC_{0-\infty}k_{el})$.

3. Results and discussion

3.1. Mass spectrometry

The MS/MS parameters were optimized to the maximum response for Kendine 91 and simvastatin. Both, the positive and negative ionization modes were investigated and the response of positive ions was stronger than negative ions; therefore, the positive ionization mode was chosen for LC–MS/MS analysis. The MS/MS conditions were optimized and the full product spectra of the two compounds, Kendine 91 and the internal standard, simvastatin, were acquired. Fig. 2 shows the full scan ESI (+) precursor ion mass spectra of Kendine 91 and simvastatin. Full scan ESI (+) product ion mass spectra of both compounds are gathered in Fig. 3. The MS/MS transition of m/z 444 → 169 for Kendine 91 and 441 → 325 for simvastatin were selected.

3.2. Chromatography

A number of C_{18} columns (XTerra, Symmetry C_8 and Symmetry Shield C_8) were evaluated and the Symmetry C_8, (5 μm, 3.9 mm × 150 mm i.d.) gave the best chromatography with a flow rate of 1 mL/min. The chromatographic conditions, especially by the composition of the mobile phase, were optimized through several trials to achieve good resolution and symmetric peak shapes of Kendine 91.

3.3. Sample preparation

Liquid–liquid extraction was employed for the sample preparation. Different organic solvents and different pH conditions

Table 1
Mean parameters of three calibration curves for Kendine 91 in plasma

$y = a + bx$	Assay		
	1	2	3
a	0.00102	0.00090	0.00122
b	0.00192	0.00187	0.00185
R^2	0.9947	0.9943	0.9953
Response factor (R.S.D., %)	13.16	5.50	11.23

were assayed. Best results were obtained when plasma samples were buffered with 0.1 M phosphate buffer solution (pH 3) and later extracted with *tert*-butyl methyl ether. After extraction, several reconstitution media were assayed; being the mixture methanol–water (60:40 v/v) the solution that provided best results.

3.4. Method validation

3.4.1. Assay selectivity

Plasma samples from six different drug-free donors were tested for presence of endogenous components which might interfere with Kendine 91 or the internal standard. These samples were prepared in accordance with the sample–preparation procedure. Chromatograms of six blank plasma, a plasma sample with Kendine 91 (2 ng/mL) and simvastatin (50 ng/mL) were compared to analyze the specificity of the procedure. The retention time for Kendine 91 and simvastatin were 3.48 and 13.60 min, respectively. No interfering peaks were observed. Fig. 4 shows the representative HPLC chromatograms of a drug-free plasma sample indicating that no endogenous peaks are present at the retention times of Kendine 91 and simvastatin. This figure also shows the chromatograms of a blank mice plasma spiked with Kendine 91 (100 ng/mL) and internal standard (50 ng/mL) and the chromatograms of the LQ sample (2 ng/mL). Fig. 5 shows the chromatograms of liver, lung and plasma samples after a single intravenous dose (10 mg/kg) of Kendine 91 obtained at 0.08 h.

3.4.2. Linearity of calibration curves and specificity

A linear least-squares regression with a weighting index of $1/x$ was carried out on the peak area ratios of Kendine 91 and simvastatin versus Kendine 91 concentrations over the range of 2–1000 ng/mL in plasma. The coefficients of determination were always $\geq$0.99 and deviation of each standard was <15%. The residuals (difference between observation and prediction) were normally distributed and around zero. Results of three representative standard curves for LC–MS/MS determination of Kendine 91 are given in Table 1.

Table 2
Precision (R.S.D., %) and accuracy (R.E., %) of the HPLC assay for Kendine 91 in plasma

Nominal conc. (ng/mL)	Measured conc. (mean ± S.D., ng/mL)	Intra-batch ($n = 5$)	
		R.S.D. (%)	Accuracy (%)
2 (LQ)	2.08 ± 0,16	6.54	103.9
6	6.38 ± 0.62	10.33	106.33
75	73.89 ± 1.71	2.28	98.52
750	746.63 ± 36.4	4.85	99.47
Nominal conc. (ng/mL)	Measured conc. (mean ± S.D., ng/mL)	Inter-batch ($n = 15$)	
		R.S.D. (%)	Accuracy (%)
2 (LQ)	1.94 ± 0,16	8.32	97.00
6	6.20 ± 0.57	9.50	103.33
75	76.86 ± 4.41	5.88	102.48
750	788.31 ± 74.48	9.93	105.11

Table 3
Extraction recovery and assessment of matrix effect on ionization

Spiked plasma concentration (ng/mL)	Extraction recovery (%)[a]	Spiked plasma concentration (ng/mL)	Matrix effect (%)[b]
6	78	25	104
75	69	312	98
750	83	3125	99
50 (simvastatin)	88	417 (simvastatin)	98

[a] Extraction recovery was calculated using the following formula: recovery (%) = [(mean raw peak area) pre-ext. spike/(mean raw peak area) post-ext. spike] × 100.

[b] Matrix effect was calculated using the following formula: matrix effect (%) = [(mean raw peak area) post-ext. spike/(mean raw peak area) neat] × 100.

3.4.3. Precision and accuracy

The accuracy and precision of intra- and inter-batch assay data for Kendine 91 were determined at concentration of 2, 6, 75 and 750 ng/mL, respectively. Table 2 shows a summery of intra- and inter-batch accuracy and precision for Kendine 91. Accuracy was the percentage of the concentration found compared with the theoretical concentration. Precision was based on calculation of the R.S.D. The intra-batch accuracy (R.E., %) ranged from 98.52% to 106.33% throughout the four concentrations. The intra-batch precision of Kendine 91 for the four concentrations examined was 6.54%, 10.33%, 2.28% and 4.85%, respectively. The inter-batch accuracy and precision was studied over 3 days. The accuracy ranged from 97.00% to 105.11% and the precision ranged from 5.88% to 9.93% throughout the four concentrations studied. The accuracy and precision were in agreement with the FDA [18] acceptance criteria ($\leq$15%).

Table 4
Stability of Kendine 91 in plasma under different conditions

Nominal conc. (ng/mL)	6 ng/mL			75 ng/mL			750 ng/mL		
	Measured conc. (ng/mL)	R.S.D. (%)	Accuracy deviation (%)	Measured conc. (ng/mL)	R.S.D. (%)	Accuracy deviation (%)	Measured conc. (ng/mL)	R.S.D. (%)	Accuracy deviation (%)
Stability in storage (−80 °C, 2 months)	5.74 ± 0.82	13.67	4.23	–	–	–	789.16 ± 70.55	9.40	5.22
Stability after 3 freeze/thaw cycles	6.24 ± 0.53	8.85	4.05	–	–	–	765.10 ± 92.09	12.28	2.01
Short-term stability	5.36 ± 0.17	3.21	10.62	–	–	–	759.07 ± 64.80	8.50	1.2
Autosampler stability (4 °C, 24 h)	5.43 ± 0.42	7.81	9.5	71.01 ± 2.83	3.99	5.32	683.37 ± 5.37	0.78	8.89

Results are presented as mean ± standard deviation.

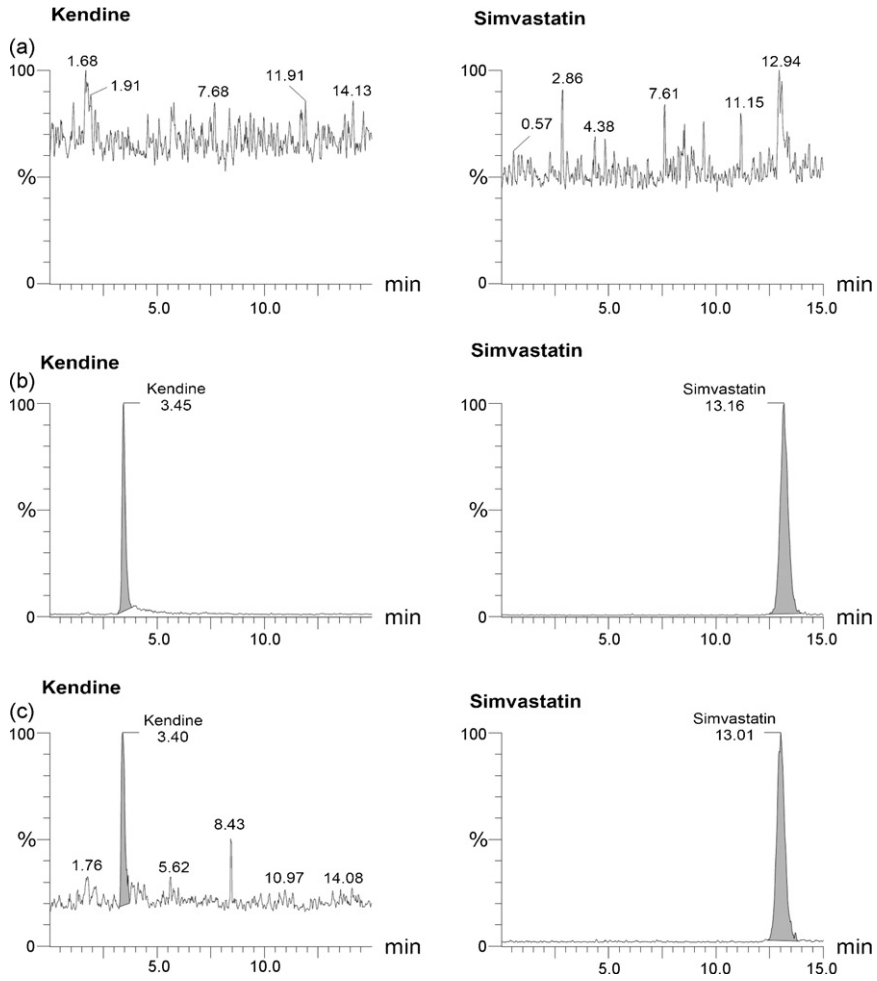

Fig. 4. Representative chromatograms for Kendine 91 and the IS (a) resulting from analysis of blank plasma (Kendine 91 and IS free), (b) standard curve sample (100 ng/mL) and (c) LQ (2 ng/mL) sample.

3.4.4. Extraction efficiency and matrix effect

Results of extraction efficiency are presented in Table 3. The data of extraction efficiency measured for Kendine 91 and the IS in plasma were consistent, precise and reproducible. Matrix effect results are also in Table 3. No significant (less than 4%) matrix effect was observed. These results are confirmed by the ion suppression study, since no significant ion suppression in the region where the analyte and internal standard eluted was detected.

3.4.5. Dilution effect

In order to quantify plasma levels of Kendine 91 higher that the upper limit of the standard curve, dilution effect was evaluated. After dilution of the plasma sample, intra-batch ($n = 5$) and inter-batch precisions were 7.86% and 8.15%, respectively. Intra- and inter-batch accuracies were 100.84% and 99.08%, respectively. These results demonstrated that it is adequate to dilute plasma samples to ensure plasma levels measurement within the standard curve range.

3.4.6. Analyte stability

Stock solutions stability was analyzed at 1 day, 2 days and 1 week at room temperature and at 4 °C. Samples were stable for 1 day at room temperature and 2 days at 4 °C. Stability of the plasma samples was followed at −80 °C during 2 months. Table 3 summarizes the results of the long-term stability, through freeze thawing and in autosampler. Table 4 also shows the results of the short-term stability study. The accuracy deviation values were always <15% as FDA recommends [18].

3.4.7. Tissue recovery

Recovery of Kendine 91 from liver, heart, kidney and lung samples relative to plasma samples was obtained by comparing the chromatographic response of Kendine 91 in the tissue samples with the chromatographic response of Kendine 91 in the plasma samples. Tissue recoveries were 0.70, 0.74, 0.67 and 0.77 for liver, lung, heart and kidney, respectively.

Table 5
Pharmacokinetic parameters of Kendine 91 in mice after a single dose of 10 mg/kg intravenous dose

Parameter	Units	Kendine 91
AUC_{0-t}	ng h/mL	1276.65
$AUC_{0-\infty}$	ng h/mL	1295.03
$t_{1/2}$	h	5.87
C_{max}	ng/mL	3715.15
CL	L/h/kg	7.72
Vd_z	L/kg	65.39

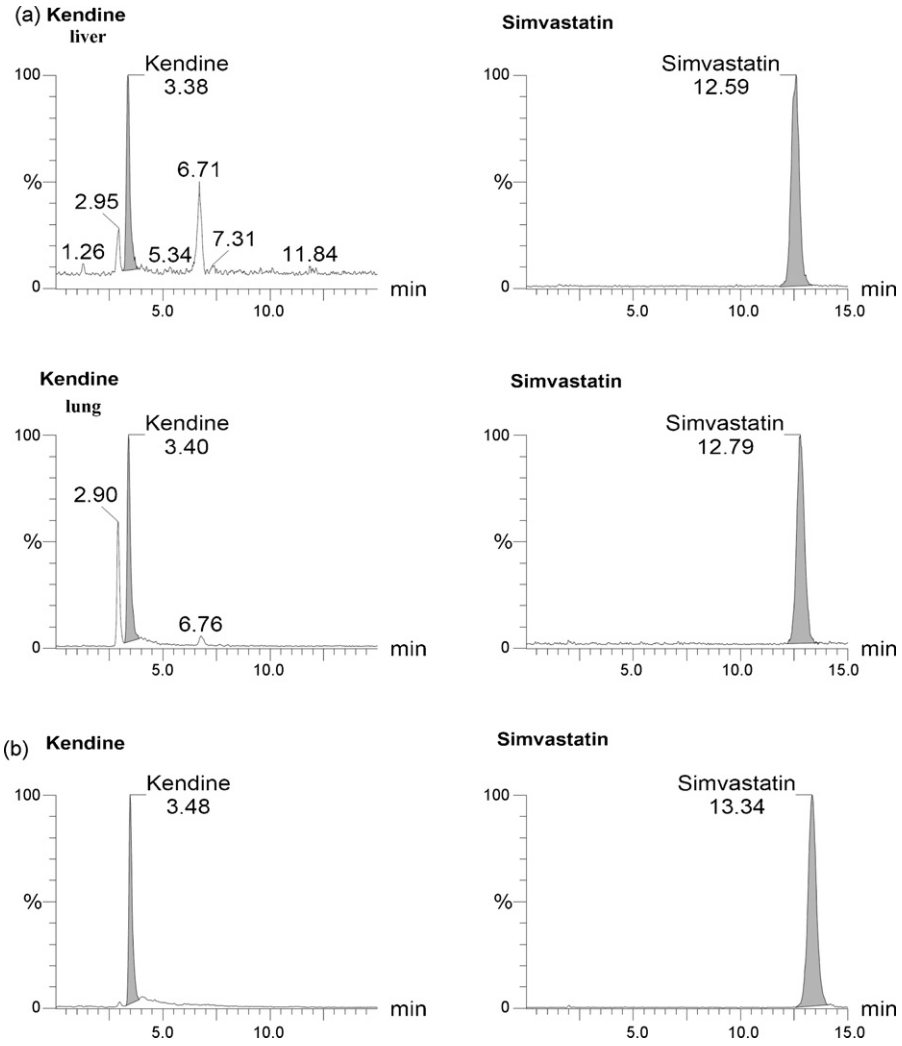

Fig. 5. Representative chromatograms for Kendine 91 in (a) liver and lung samples and (b) plasma sample after a single intravenous dose (10 mg/kg) of Kendine 91 obtained at 0.08 h post-dose.

3.5. Application of the analytical method to a pharmacokinetic study

The developed and validated method was applied to a pharmacokinetic study after single 10 mg/kg intravenous dose of Kendine 91 to mice. Fig. 6 shows the concentration–time profile of Kendine 91 in plasma, liver and lung tissue. The concentration of Kendine 91 in plasma samples could be detected up to 12 h after drug administration. In liver and lung tissues, Kendine 91 was only detected up to 8 h. The pharmacokinetic parameters of Kendine 91 were calculated and they are presented in Table 5. The apparent elimination half-life ($t_{1/2}$) was 5.87 h and the volume of distribution 65.39 L/kg.

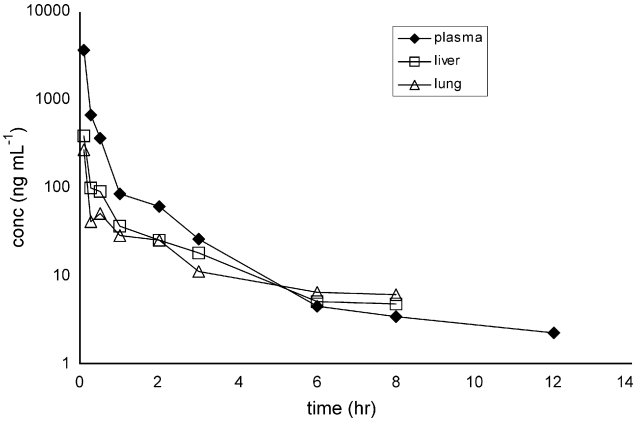

Fig. 6. Mean Kendine 91 plasma, liver and lung concentration-time curve in mice after intravenous administration (10 mg/kg).

4. Conclusions

A LC–MS/MS method was developed and validated for the quantification of Kendine 91, a novel HDACI, in mice plasma and tissues. The method is selective and highly sensitive with a detection limit of 2 ng/mL and offers a wide range of linearity and allows quantification over the range 2–1000 ng/mL. The procedure was successfully applied to evaluate the pharmacokinetics of Kendine 91 after administration of an i.v. dose of 10 mg/kg to healthy mice.

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