

Validation of a RP-HPLC Method for the Quantitation of Vorinostat in Rat Plasma and its Application to a Pharmacokinetic Study

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Abstract

A simple, specific and reproducible high-performance liquid chromatography (HPLC) assay method has been developed and validated for the estimation of vorinostat in rat plasma. The bioanalytical procedure involves extraction of vorinostat and phenacetin (internal standard, IS) from rat plasma with simple liquid-liquid extraction process. The chromatographic analysis was performed on a Waters Alliance system using a gradient mobile phase conditions at a flow rate of 1.0 mL/min and Symmetry Shield C₁₈ column maintained at 35 ± 1°C. The eluate was monitored using an UV detector set at 245 nm. Vorinostat and IS eluted at 5.3 and 6.3 min, respectively and the total run time was 10 min. Method validation was performed as per FDA guidelines and the results met the acceptance criteria. The calibration curve was linear over a concentration range of 255-5566 ng/mL ($r^2 = 0.995$). The intra- and inter-day precisions were in the range of 2.60-7.93 and 3.99-8.64%, respectively. The validated HPLC method was successfully applied to a pharmacokinetic study in rats.

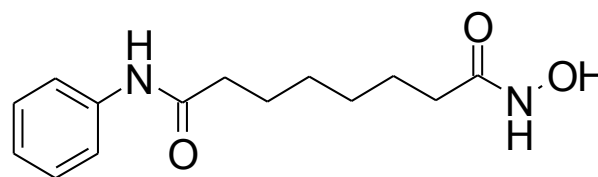
Keywords: Vorinostat; HPLC; Method Validation; Rat Plasma; Pharmacokinetics

Introduction

Histone deacetylases (HDAC) are a family of enzymes that play an important role in regulation of gene transcription. Abnormal activities/alterations in the expression and/or activity of HDAC have been linked to the pathogenesis of cancer [1]. HDAC inhibitors selectively induce cellular differentiation, growth arrest (including drug resistant subtypes) and apoptosis in a broad spectrum of tumor cells. The biological consequences of HDAC inhibition include reversion of transformed cell morphology and inhibition of cell proliferation by induction of G1/S and G2/M phase cell cycle arrest, differentiation, and/or apoptosis [2]. Vorinostat (Figure 1, CAS no: 149647-78-9) or suberanilohydroxamic acid (SAHA) is a novel and first HDAC inhibitor approved by US FDA for treatment of cutaneous T cell lymphoma (CTCL) in 2006. Vorinostat inhibits the enzymatic activity of HDAC1, HDAC2, and HDAC3 (Class I) and HDAC6 (Class II) at nanomolar concentrations [3]. Vorinostat inhibits HDAC by binding to a zinc ion in the catalytic domain of the enzyme [4]. Vorinostat demonstrated activity in murine xenograft models and it was additive or synergistic when combined with chemotherapy drugs in induction of differentiation and apoptosis of various cancer cell lines [5]. Commercially it is available as 100 mg capsules. In cancer patients vorinostat has shown potential anticancer activity against variety of hematological and solid tumors, particularly in combination with other anticancer agents, as well as in monotherapy. Clinically, vorinostat is well tolerated and showed dose-linear pharmacokinetics (200-600 mg) with good (43%) oral bioavailability [6]. Following oral administration of vorinostat (400 mg) to humans in fasted-state, the maximum plasma concentration (C_{max} : 1.2 ± 0.35 μM) of vorinostat attained at ~1.5 h (T_{max}). High-fat meal increase extent (33%) and rate of absorption (2.5 h delay of T_{max}) of vorinostat. Vorinostat is ~71% bound to plasma proteins. Vorinostat is not a substrate for P-gp and does not inhibit P-gp transport system. Vorinostat undergoes extensive metabolized principally by glucuronidation and hydrolysis followed by β-oxidation. Cytochrome P 450 enzymes has no role in vorinostat metabolism. Both metabolites (vorinostat-O-glucuronide and 4-anilino-4-oxobutanoic

acid) are pharmacologically inactive and at steady state the exposure in humans was 4- and 13-fold, respectively higher than vorinostat. The terminal half-life of vorinostat and vorinostat-O-glucuronide was ~2 h, while that of 4-anilino-4-oxobutanoic acid was ~11 h. The primary route of elimination for vorinostat and its metabolite is urine. The most drug-related adverse events are diarrhea, fatigue, nausea and anorexia [6].

Only two LC-MS/MS bioanalytical methods were reported in literature for quantification of vorinostat [7,8]. LC-MS/MS is an expensive technique, which requires high investment in both equipment purchase, maintenance and it may be not available in all analytical laboratories. To date there is no HPLC method reported



Vorinostat

Figure 1: Structural representation of vorinostat.

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for quantification of vorinostat in any preclinical species and human plasma. Hence, we felt that there is a great need to develop and validate a HPLC method for quantification of vorinostat in plasma, which will be a valuable tool to support pre-clinical pharmacokinetic studies. In this paper, we report the development and validation of a simple, specific and reproducible HPLC method for quantitation of vorinostat in rat plasma. The method was successfully applied to quantitate levels of vorinostat in a rat pharmacokinetic study.

Materials and Methods

Chemicals and reagents

Vorinostat was procured from Cayman Chemicals, Ann Arbor, Michigan, USA. Itraconazole was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade acetonitrile and methanol were purchased from Rankem, Ranbaxy Fine Chemicals Limited, New Delhi, India. Ortho phosphoric acid was purchased from Rankem, Ranbaxy Fine Chemicals Limited, New Delhi, India. Blank rat plasma (with Na₂EDTA as an anti-coagulant) was procured from Jubilant Biosys Animal House, Bangalore, India. All other chemicals/reagents were of research grade and used without further purification.

HPLC operating conditions

The HPLC system consisted of Waters 2695 Alliance system (Waters, Milford, USA) equipped with performance PLUS inline degasser along with an auto-sampler and ultra-violet (UV) detector set at 245 nm for vorinostat and IS. The chromatographic separation vorinostat and IS in processed samples was achieved on a Symmetry Shield RP₁₈ column (250 x 4.6 mm, 5 µm; Waters Corporation, Milford, USA), which was maintained at 35 ± 1°C. The binary mobile phase system consisted of A (0.5% ortho phosphoric acid in Milli Q water) and B (methanol: acetonitrile :: 50:50, v/v) was run as per the gradient program (0-4.9 min: 60% A and 40% B; 5.0-5.59 min: 40% A and 60% B and 6.0-10.0 min: 60% A and 40% B) for the chromatographic resolution of vorinostat and IS.

Preparation of stock and standard solutions

Vorinostat and IS were accurately weighed into volumetric flasks using an analytical micro balance. They were then diluted with methanol. Vorinostat stock solutions for spiking the calibration standards and quality control (QC) samples were prepared from separate weighing at 580 µg/mL. The IS stock solution of 200 µg/mL was prepared in methanol. The stock solutions of vorinostat and IS were stored at 4°C, which were found to be stable for one month (data not shown) and successively diluted with methanol:water (50:50, v/v) to prepare working solutions to prepare calibration curve (CC). Another set of working stock solutions of vorinostat were made in methanol:water (50:50, v/v) (from different stock) for preparation of QC samples. Appropriate dilutions of vorinostat stock solution were made in methanol:water (50:50, v/v) to produce working stock solutions for CC and QC spiking. A working IS solution (2000 ng/mL) was prepared in methanol:water (50:50, v/v). Calibration samples were prepared by spiking 180 µL of blank rat plasma with the appropriate working solution of vorinostat (20 µL) on the day of analysis. Samples for the determination of precision and accuracy were prepared by spiking blank rat plasma in bulk with vorinostat at appropriate concentrations (255, 765, 2782 and 4404 ng/mL) and 200 µL aliquots were distributed into different tubes. All the samples were stored together at -80 ± 10°C until analysis.

Sample preparation

Liquid-liquid extraction method was followed for extraction of

vorinostat from rat plasma. To an aliquot of 200 µL plasma sample 1.5 mL of ethyl acetate was added and vortex mixed for 3 min; followed by centrifugation for 10 min at 14,000 rpm on a Centrifuge 5430R (Eppendorff, Germany) at 4°C. The organic layer (1.3 mL) was separated and evaporated to dryness at 50°C using a gentle stream of nitrogen (Turbovap®, Zymark® Kopkinton, MA, USA). The residue was reconstituted in 200 µL of acetonitrile containing IS and 30 µL was injected onto HPLC system.

Validation procedures

A full validation according to the FDA guidelines [9] was performed for the assay in rat plasma.

Specificity and selectivity

The specificity of the method was evaluated by analyzing rat plasma samples from ten different lots (six normal lots, two hemolyzed lots and two lipemic lots) to investigate the potential interferences at the LC peak region for analyte and IS. These samples were processed using the proposed extraction protocol and analyzed with the set chromatographic conditions at LLOQ level. The peak area of the co-eluting components or interferences in blank samples should be less than 20 and 5% from those of the analyte and IS, respectively.

Recovery

The efficiency of vorinostat and IS extraction from rat plasma was determined by comparing the responses of the analyte extracted from replicate QC samples (n=6) with the response of analyte from neat standards at equivalent concentrations by liquid-liquid extraction process. Recovery of vorinostat was determined at QC low, QC medium and QC high concentrations viz., 765, 2782 and 4404 ng/mL, whereas the recovery of the IS was determined at a single concentration of 2000 ng/mL.

Calibration curve

The eight point calibration curve (255, 510, 1147, 1970, 2550, 3941, 4637 and 5565 ng/mL) was constructed by plotting the peak area ratio of vorinostat:IS against the nominal concentration of calibration standards in blank rat plasma. Following the evaluation of different weighing factors, the results were fitted to linear regression analysis with the use of 1/X² (X=concentration) weighting factor. The calibration curve had to have a correlation coefficient (r) of 0.99 or better. The acceptance criteria for each back-calculated standard concentration were ± 15% deviation from the nominal value except at LLOQ, which was set at ± 20% [9].

Precision and accuracy

The intra-assay precision and accuracy were estimated by analyzing six replicates containing vorinostat at four different QC levels i.e., 255, 765, 2782 and 4404 ng/mL. The inter-assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within 85-115% from the nominal values and a precision of within ± 15% relative standard deviation (RSD) except for LLOQ, where it should be within 80-120% for accuracy and less than 20% of RSD [9].

Stability experiments

Stability tests were conducted to evaluate the stability of vorinostat in rat plasma samples under different conditions. Bench top stability (7 h), processed samples stability (auto-sampler stability for 26 h at 10°C), freeze thaw stability (three cycles), long term stability (30 days at -80 ±

10°C) were performed at LQC (765 ng/mL) and HQC (4404 ng/mL) levels using six replicates at each level. Samples were considered stable if assay values were within the acceptable limits of accuracy (i.e., 85-115% from fresh samples) and precision (i.e., $\pm 15\%$ RSD) [9].

Dilution integrity

Dilution integrity was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. Dilution integrity experiment was performed for study sample concentrations greater than the upper limit of quantitation (ULOQ). Vorinostat spiked rat plasma samples prepared at 5- and 10-fold above the HQC (5600 ng/mL) concentration and diluted by 5- and 10-fold with rat blank plasma to obtain the final test concentrations of 1120 and 560 ng/mL, respectively (n=6). The back-calculated standard concentrations had to comply to have both precision of $\leq 15\%$ and accuracy of $100 \pm 15\%$ similar to other experiments [9].

Pharmacokinetic study in rats

All the animal experiments were approved by Institutional Animal Ethical Committee. Male Sprague-Dawley rats (n = 6) were procured from Bionees, Bangalore, India. The animals were housed in Jubilant Biosys animal care facility in a temperature and humidity controlled room with a 12:12 h light:dark cycles, had free access to food (Provimin, Bangalore, India) and water for one week before using for experimental purpose. Animals (224-243 g) received vorinostat intravenously [solution formulation prepared using 5% DMSO, 10% Solutol: ethanol (1:1, v/v) and 85% HP- β -CD (40% hydroxypropyl- β -cyclodextrin in Milli-Q water) (dose volume: 2 mL/kg) at 10 mg/kg dose. Post-dosing blood samples (400 μ L) were collected into polypropylene tubes containing Na₂EDTA solution as an anti-coagulant at 0.12, 0.25, 0.5, 1, 2, 4, 8 and 24 h (sparse sampling protocol was adopted during blood collection and at each time point blood was collected from three animals). Plasma was harvested by centrifuging the blood using Biofuge (Hereaus, Germany) at 1760 g for 5 min and stored frozen at $-80 \pm 10^\circ\text{C}$ until analysis. Animals were allowed access to feed 2 h post-dose of formulations.

Thawed plasma sample were processed as mentioned in sample preparation section. Along with plasma samples, QC samples at low, medium and high concentration (made in blank plasma) were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) 67% of the QC samples accuracy must be within 85-115% of the nominal concentration (ii) not less than 50% at each QC concentration level must meet the acceptance criteria (US DHHS, FDA and CDER, 2001). Plasma concentrations of vorinostat were determined.

Results and Discussion

Chromatographic conditions

Selection of chromatographic conditions for the proposed method was optimized to suit the preclinical pharmacokinetic studies. Different mobile phases comprising several combinations of buffers (eg: phosphate buffer and ammonium acetate buffer) and organic solvents (acetonitrile and methanol) along with altered flow-rates (in the range of 0.80-1.50 mL/min) were tested to optimize for an effective chromatographic resolution of vorinostat and IS (data not shown). The resolution of peaks was best achieved with a binary mobile phase system consisted of A (0.5% ortho phosphoric acid in Milli Q water)

and B (methanol:acetonitrile :: 50:50, v/v) was run as per the gradient program at a flow rate of 1 mL/min on a Symmetry Shield C₁₈ column.

Method validation parameters

Specificity and selectivity: The specificity of the proposed method was investigated using blank rat plasma samples from ten different lots including lipemic and hemolyzed, in order to check if components from the biological origin could interfere significantly with the assay. The mean interference observed at the retention time of the analyte and IS with different lots of human plasma including hemolyzed and lipemic plasma was calculated as 6.4 and 0.9% (data not shown). Figure 2 shows a typical overlaid HPLC chromatogram for the blank rat plasma (free of analyte and IS), blank rat plasma spiked with IS, blank rat plasma spiked with vorinostat at LLOQ and IS and an in vivo plasma sample obtained at 0.25 h after i.v administration of vorinostat to rats. No interfering peaks from endogenous compounds are observed at the retention times of analyte and IS, thus the method is considered selective. The retention time of vorinostat and IS was ~ 5.3 and 6.5 min, respectively. The total chromatographic run time was 10 min.

Recovery: One step liquid-liquid extraction technique gave best results in terms of extraction efficiency, reproducibility, cleaner samples and no interference with vorinostat and IS quantification. The results of the comparison of neat standards versus rat plasma-extracted standards were estimated for vorinostat 765, 2782 and 4404 ng/mL and the mean recovery was found to be 92.2 ± 0.74 , 92.7 ± 1.07 and $94.8 \pm 1.23\%$, respectively. The recovery of IS at 2000 ng/mL was $91.3 \pm 1.95\%$.

Calibration curve: The plasma calibration curve was constructed using eight calibration standards (viz., 255-5565 ng/mL) in rat plasma. The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. Calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) versus concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression (n = 4) was found to be ≥ 0.995 . The accuracy observed for the mean of back-calculated concentrations for four calibration curves for odanacatib was within 93.9-106%; while the precision (% CV) values ranged from 1.49-6.91%.

Accuracy and precision: Accuracy and precision data for intra- and inter-day rat plasma samples are presented in (Table 1). The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits. The data show that the method possesses adequate accuracy and repeatability for analyzing vorinostat in rat plasma samples.

Stability: Table 2 summarizes the results of stability studies conducted for vorinostat in rat plasma. The predicted concentrations for vorinostat at 765 and 4404 ng/mL samples deviated within $\pm 15\%$ of the nominal concentrations in a battery of stability tests viz., in-injector (26 h), bench-top (7 h), repeated three freeze/thaw cycles and freezer stability at $-80 \pm 10^\circ\text{C}$ for at least for 30 days. The results were found to be within the assay variability limits during the entire process and demonstrated that vorinostat can be stored under tested conditions without compromising the integrity of samples.

Dilution effect: The dilution integrity was confirmed for QC samples that exceeded the upper limit of standard calibration curve. The results showed that the precision and accuracy for 5 and 10x diluted test samples were within the acceptance range (data not shown).

Pharmacokinetic study: The sensitivity and specificity of the assay were found to be sufficient to characterize the plasma pharmacokinetics

Theoretical concentration (ng/mL)	1. Run	Measured concentration (ng/mL)		
		1. Mean \pm SD	RSD	Accuracy (%)
Intra-day variation (Six replicates at each concentration)				
255	1	270 \pm 9.08	3.36	106
	2	278 \pm 7.23	2.60	109
	3	282 \pm 14.0	4.97	110
	4	258 \pm 30.9	1.09	101
765	1	782 \pm 21.1	2.70	102
	2	778 \pm 25.3	3.25	102
	3	866 \pm 34.3	3.96	113
	4	704 \pm 32.3	4.58	92.0
2782	1	2842 \pm 142	5.00	102
	2	2787 \pm 102	3.66	100
	3	3180 \pm 212	6.66	114
	4	2715 \pm 171	6.31	97.6
4404	1	4528 \pm 279	6.15	103
	2	4389 \pm 204	4.65	99.7
	3	5028 \pm 399	7.93	114
	4	4309 \pm 332	7.70	97.8
Inter-day variation (twenty four replicates at each concentration)				
255		272 \pm 19.1	7.01	107
765		782 \pm 64.5	8.24	102
2782		2881 \pm 237	8.21	104
4404		4563 \pm 407	8.92	104

RSD: Relative standard deviation (SD x 100/Mean)

Table 1: Intra- and inter-day precision of determination of vorinostat in rat plasma.

Nominal concentration (ng/mL)	Stability	Mean \pm SD ^a n = 6 (ng/mL)	Accuracy (%) ^b	Precision (% CV)
765	0 h	782 \pm 21.1	102	2.70
	7 h (bench-top)	772 \pm 21.9	101	2.80
	26 h (in-injector)	785 \pm 30.0	103	3.82
	3 rd F/T cycle	848 \pm 112	111	13.2
	30 day at -80°C	704 \pm 32.3	90.0	4.58
4404	0 h	4528 \pm 279	103	6.15
	7 h (bench-top)	4550 \pm 270	103	5.93
	26 h (in-injector)	4494 \pm 218	102	4.84
	3 rd F/T cycle	4998 \pm 335	113	6.71
	30 day at -80°C	4309 \pm 332	95.2	7.70

^aBack-calculated plasma concentrations; ^b(Mean assayed concentration / mean assayed concentration at 0 h) x 100; F/T: freeze-thaw

Table 2: Stability data vorinostat quality controls in rat plasma.

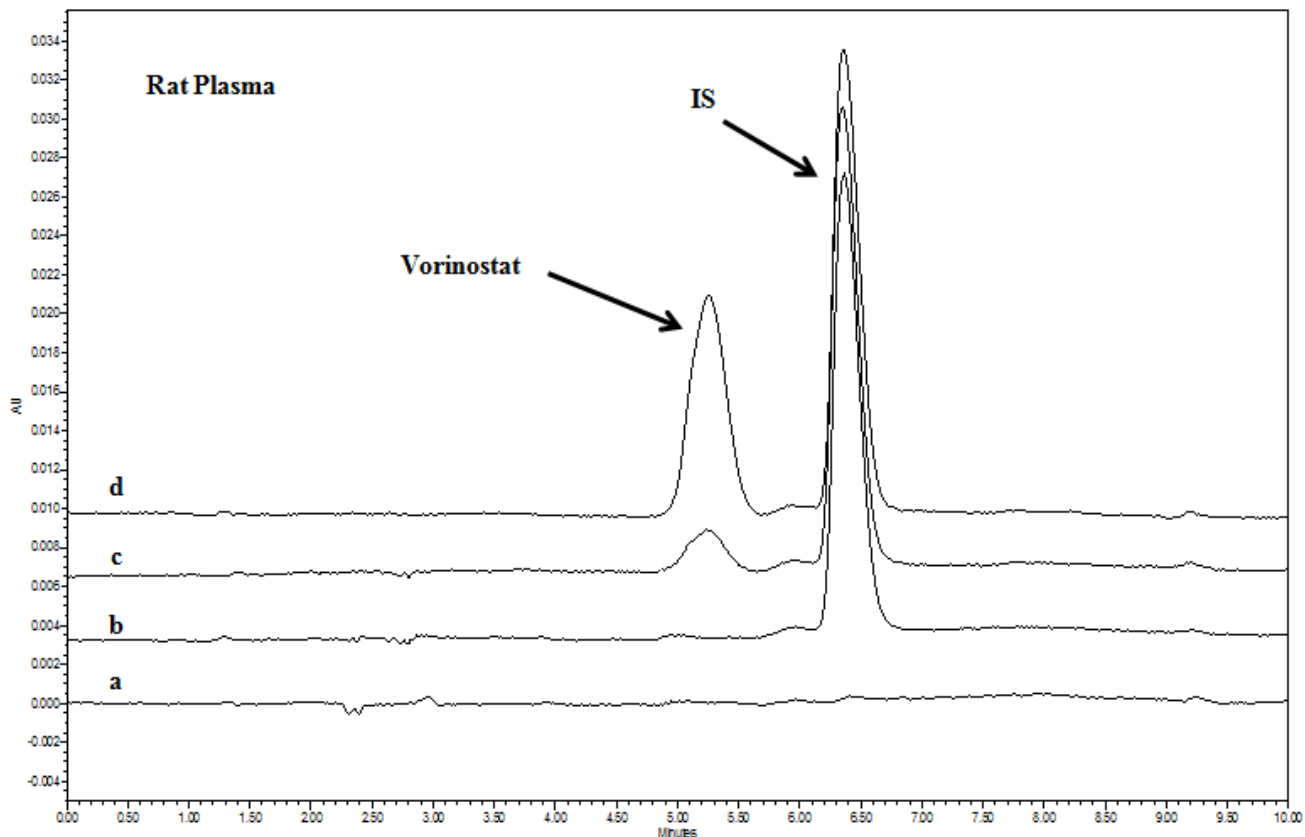


Figure 2: HPLC chromatograms of a 30 μ L injection of (a) rat blank plasma (b) blank rat plasma spiked with IS (c) blank rat plasma spiked with vorinostat at LLOQ (255 ng/mL) and IS (d) an in vivo plasma sample collected from a rat at 0.25 h (1333 ng/mL) time point following intravenous administration of vorinostat along with IS.

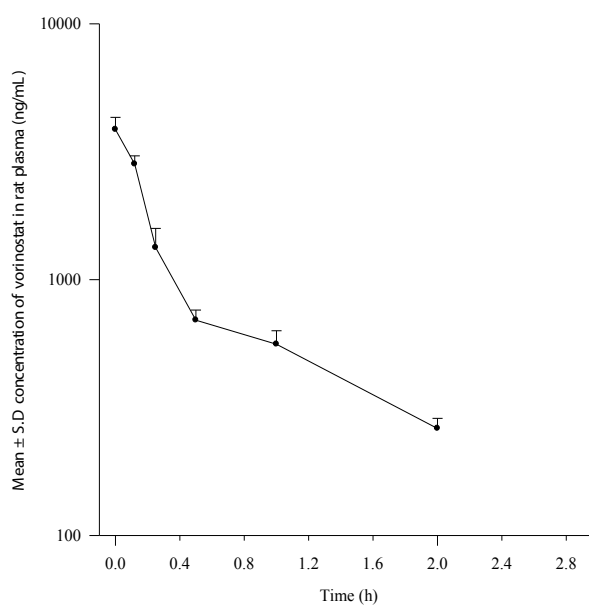


Figure 3: Mean plasma concentration versus time profile of vorinostat in rat plasma following intravenous and oral administration of vorinostat to rats (n=6).

of vorinostat in rats following i.v administration. Profile of the mean plasma concentration versus time was shown in (Figure 3). Vorinostat was quantifiable up to 2 h post-dosing followed by i.v administration. Following i.v administration the clearance (Cl) and volume of distribution (Vd) were found to be 81.3 ± 14.4 mL/min/kg and 3.25 ± 0.58 L/kg, respectively. The $AUC_{0-\infty}$ (area under the plasma concentration-time curve from time zero to time infinite) was found to be 2041 ± 171 ng.h/mL. The terminal half-life ($t_{1/2}$) was 1.04 ± 0.24 h. **6.**

Conclusion

A simple reversed-phase HPLC method for determination of vorinostat in rat plasma has been developed and validated. The proposed method is highly specific, accurate, precise and reproducible. The method requires less plasma sample for analysis and involves simple sample preparation. All the validation parameters were within the acceptable limits for bioanalytical method. This method has been successfully applied to a pharmacokinetic study in rats. We believe that the current method with little or no modifications and by increasing the plasma volume for analysis (which will improve the sensitivity of the present method) can be extended to other pre-clinical species and human plasma matrix. The method can provide a lot of potential information to assist the researchers in deciding their approach for quantitation strategy towards pharmacokinetics and/or toxicokinetics in pre-clinical species.

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