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A liquid chromatography–electrospray ionization tandem mass spectrometric assay for quantitation of the histone deacetylase inhibitor, vorinostat (suberoylanilide hydroxamicacid, SAHA), and its metabolites in human serum

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Abstract

Vorinostat (suberoylanilide hydroxamic acid, SAHA) is undergoing evaluation as an antineoplastic agent. We developed a liquid chromatography–tandem mass spectrometry (LC–MS/MS) assay for quantitating vorinostat and its major metabolites, vorinostat glucuronide and 4-anilino-4-oxobutanoic acid, in human serum. The assay uses: deuterated internal standards; acetonitrile protein precipitation; a BDS Hypersil C18 (3 μm, 100 mm × 3 mm) column; a gradient mobile phase of 0.5% acetic acid in acetonitrile and water; and electrospray positive-mode ionization with selected reaction monitoring (SRM) detection. The lower limit of quantitation was 3.0 ng/ml for each analyte. The assay is being employed in at least 12 clinical studies of vorinostat-containing regimens.

Keywords: Vorinostat; Suberoylanilide hydroxamic acid; SAHA; Histone deacetylase inhibitor; LC–MS/MS

1. Introduction

Epigenetic modification of chromatin has received increasing recognition as an important factor in gene expression and as a target for antineoplastic drug development [1–5]. 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 [1–3]. Histone acetyltransferases add acetyl groups to lysine residues in histones, thereby relaxing chromatin and allowing transcription to occur [1,3,6–8]. Conversely, histone deacetylases remove acetyl groups from lysine residues in histones, thereby tightening and condensing chromatin structure and preventing transcription [1,3,9–12]. 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 [1–3,5,9,11–13]. This has made histone deacetylase an attractive target for anticancer agent development [1–3,5,9,11–13].

Vorinostat (suberoylanilide hydroxamic acid, SAHA, *N*-hydroxy-*N*′-phenyloctanediamide, M.W. 264.32) (Fig. 1) is a potent histone deacetylase inhibitor [14] that has demonstrated antineoplastic activity in vitro against a variety of cell lines and in vivo against several human tumor xenograft models [13,15,16]. This encouraging preclinical activity has prompted extensive clinical evaluation of vorinostat, initially as a single agent [17–19] and more recently as a component of a variety of combination chemotherapy regimens. Evaluation of the pharmacokinetics of vorinostat and, ideally, its known metabolites is an essential component of many of these clinical studies. Two major metabolites of vorinostat have been identified in serum and urine of patients treated on single-agent vorinos-

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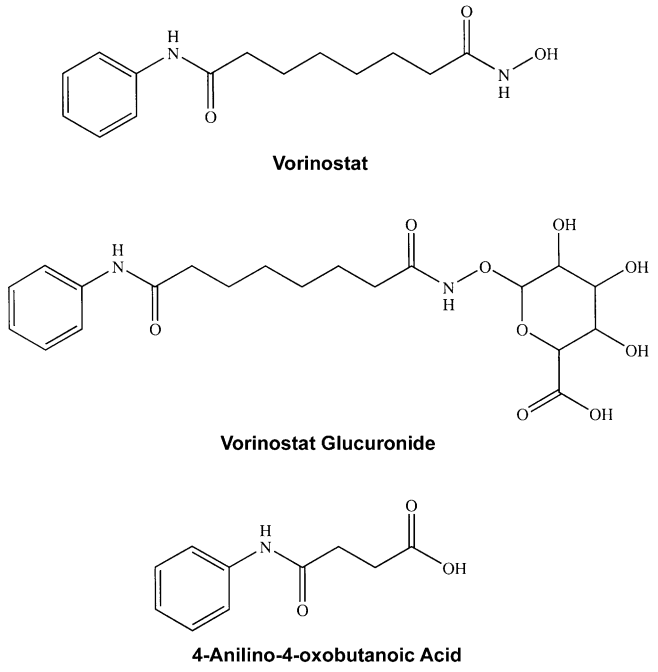

Fig. 1. Structures of vorinostat, vorinostat glucuronide, and 4-anilino-4-oxobutanoic acid.

tat protocols. These metabolites are vorinostat glucuronide and 4-anilino-4-oxobutanoic acid (Fig. 1). Initial clinical pharmacokinetic studies of intravenously administered vorinostat used an HPLC assay with absorbance detection that had less than ideal sensitivity and did not quantitate either vorinostat metabolite [18]. A subsequent clinical pharmacokinetic study of orally administered vorinostat [19] used an LC–MS assay that was more sensitive than the HPLC assay with absorbance detection but still did not quantitate either vorinostat metabolite. A recent publication [20] described an LC–MS/MS assay for quantitation of vorinostat and its two major metabolites; however, that assay uses high turbulence liquid chromatography coupled with column switching technology for online removal of analytes from serum. Because we recognized that the chromatography instrumentation necessary for this assay is not available at institutions performing clinical studies of vorinostat, we developed an LC–MS/MS assay that employs a simple acetonitrile precipitation procedure for analyte recovery and chromatographic and mass spectrometric conditions that allow sensitive and facile quantitation of vorinostat and its major metabolites in serum. This method is currently being used in at least 12 clinical pharmacokinetic studies of vorinostat.

2. Experimental

2.1. Reagents and materials

All solvents and glacial acetic acid were of HPLC grade and were purchased from Fisher Scientific (Fairlawn, NJ, USA). Control human serum was purchased from Cambrex (Baltimore, MD, USA). Each batch of serum is a pool from 50 to 100 people. Nitrogen (99.99% pure), argon (grade 5.0), and liquid nitrogen were purchased from Valley National Gases Inc. (Pittsburgh, PA, USA). Vorinostat, vorinostat glucuronide, 4-anilino-4-oxobutanoic acid, and deuterium-labeled (d5) internal standards were graciously provided by Merck Research Laboratories (West Point, PA, USA). Serum samples used for evaluating potential interference with the assay were obtained from the University of Pittsburgh Medical Center clinical chemistry laboratory under a protocol approved by the University of Pittsburgh Institutional Review Board.

2.2. Sample preparation

Stock solutions containing 1 mg/ml vorinostat, vorinostat glucuronide, or 4-anilino-4-oxobutanoic acid were prepared by dissolving each respective compound in methanol:water (50:50, v/v). These solutions were stored at 4 °C until further dilutions were made for standard curves and quality control samples. Individual 1 mg/ml stock solutions of d5-vorinostat, d5-vorinostat glucuronide, and d5-4-anilino-4-oxobutanoic acid internal standards were prepared by dissolving each deuterated internal standard in methanol:water (50:50, v/v). These solutions were diluted further and combined in order to obtain a single solution that had a final concentration of 100 ng/ml d5-vorinostat, 1000 ng/ml d5-vorinostat glucuronide and 1000 ng/ml d5-4-anilino-4-oxobutanoic acid in methanol:water (50:50, v/v). On three successive days, three triplicate standard curves were prepared by placing 0.2 ml samples of control serum containing 3, 10, 30, 100, 300, or 1000 ng/ml vorinostat, vorinostat glucuronide and 4-anilino-4-oxobutanoic acid into 1.5-ml microcentrifuge tubes. Quality control samples were prepared at concentrations of 5, 50 and 500 ng/ml in serum. Ten microliters of deuterated internal standard was added to make a final concentration of 5 ng/ml d5-vorinostat, 50 ng/ml d5-vorinostat glucuronide and 50 ng/ml d5-4-anilino-4-oxobutanoic acid. Next, 1 ml of acetonitrile was added to each tube. The samples were vortexed for 1 min at a setting of 8 on a Vortex Genie (Model G-560 Scientific Industries, Bohemia, NY, USA) and then centrifuged at 12,000 × *g* at room temperature for 6 min. The resulting supernatants were transferred to 12 mm × 75 mm borosilicate glass tubes and evaporated to dryness under a stream of nitrogen at 27 °C. Each dried residue was re-dissolved in 100 μl of acetonitrile:water (25:75, v/v), vortexed briefly, transferred to HPLC autosampler vials, and 10 μl of the redissolved material was injected into the LC–MS/MS system.

2.3. Chromatography

The LC system consisted of an Agilent (Palo Alto, CA, USA) 1100 autosampler and binary pump, a ThermoElectron (Bellefonte, PA, USA) BDS Hypersil C18 (3 μm, 3 mm × 100 mm) column, and a gradient mobile phase. Mobile phase solvent A was acetonitrile: 0.5% acetic acid (99.5:0.5, v/v), and mobile phase solvent B was water:0.5% acetic acid (99.5:0.5, v/v). The initial mobile phase composition of 25% solvent A and 75% solvent B was maintained for 2 min at a flow rate of 0.2 ml/min. Between 2 and 8 min, the percentage of solvent A was increased linearly to 55%. Between 8 and 9 min, the percentage of sol-

vent A was increased linearly to 90%, and the flow rate was increased to 0.4 ml/min. These conditions were maintained for 4 min. Between 13 and 14 min, the percentage of solvent A was decreased linearly to 25%, and at 19.6 min the flow rate was decreased to 0.2 ml/min. The overall run time was 20 min.

2.4. Mass spectrometer

Mass detection was carried out using a Waters (Milford, MA, USA) Quattromicro triple-stage, benchtop quadrupole mass spectrometer with electrospray ionization in positive-ion, selected reaction monitoring (SRM) mode. For both full scan MS and MS/MS, the settings of the mass spectrometer were as follows: capillary voltage 4.0 kV; cone voltage 20 V; source temperature 120 °C; and desolvation temperature 350 °C. The cone and desolvation gas flows were 200 and 550 l/h, respectively. The collision voltage used SRM scans was 13 V. Quadrupoles 1 and 3 each had low mass and high mass resolution set at 12.0. The dwell time was 0.25 s, and the interscan delay was 0.1 s. The span was set at 0 amu. The SRM *m/z* transitions monitored for each analyte are shown in Table 1. The LC system and mass spectrometer were controlled by Waters MassLynx software (version 4.0), and data were collected with the same software. The analyte-to-internal standard ratio was calculated for each standard by dividing the area of each analyte peak by the area of the respective internal standard peak for that analyte. Standard curves of the analytes were constructed by plotting the analyte-to-internal standard ratio versus the known concentration of analyte in each sample. Standard curves were fit by linear regression with weighting by $1/y^2$, followed by the back calculation of concentrations.

2.5. Analysis of patient samples

Blood samples were obtained from a 74-year-old male patient before and at 0.5, 1, 1.5, 2, 3, 4, and 6 h after his ingestion of a 200 mg dose of vorinostat. Blood was allowed to coagulate for 20–30 min at 4 °C before being centrifuged for 15 min at 2000 × *g* and 4 °C. The resulting serum was transferred to polypropylene tubes and stored at −70 °C until analysis. Vorinostat concentration versus time data were analyzed non-compartmentally with the Lagrange function [21] as implemented by the LAGRAN computer program [22]. The patient who provided samples was enrolled in a protocol approved by the University of Pittsburgh Institutional Review Board and gave written, informed consent before he participated in the study.

Table 1
Ion transitions monitored for detection of vorinostat, vorinostat metabolites, and their respective deuterated internal standards

Compound	Transition
Vorinostat	*m/z* 265.2 → 232.2
d5-Vorinostat	*m/z* 270.2 → 237.2
Vorinostat glucuronide	*m/z* 441.2 → 265.2 and 441.2 → 232.2
d5-Vorinostat glucuronide	*m/z* 446.2 → 270.2
4-Anilino-4-oxobutanoic acid	*m/z* 194.0 → 176.2
d5-4-anilino-4-oxobutanoic acid	*m/z* 199.0 → 181.0

3. Results

3.1. Mass spectrometry and chromatography

Each analyte and internal standard was continuously infused into the mass spectrometer in order to obtain a Q1 scan of the parent compound. Specifically, a 1 μg/ml solution of each compound in acetonitrile:water (50:50, v/v) was infused at a rate of 10 μl/min into the HPLC flow, which was an isocratic mobile phase of acetonitrile:water:acetic acid (50:50:0.5, v/v/v) pumped at 0.2 ml/min The tuning parameters of the mass spectrometer were adjusted to maximize the intensity of the parent ion of each analyte. The resulting spectrum of the each compound consisted of $[M+H]^+$ and $[M+Na]^+$ ions. The mass spectrometer parameters were adjusted to maximize the intensity of the $[M+H]^+$ ion, which was selected as the precursor ion for SRM detection. Each compound was again infused as the collision cell voltage and argon gas were adjusted to maximize the intensity of the product ions (Fig. 2). Based on the Q1 and Q3 scans, the *m/z* transitions shown in Table 1 were chosen for SRM detection of each analyte and internal standard. With the settings used, unit mass resolution was achieved with each analyte. The structures of the monitored fragments have been described previously [20]. With the chromatographic conditions described, vorinostat, vorinostat glucuronide and 4-anilino-4-oxobutanoic acid eluted at approximately 7.9, 6.4, and 5.1 min, respectively (Fig. 3), and each deuterated internal standard co-eluted with its respective analyte. There was baseline separation of all analytes. To test for interference at the different SRM transitions, serum samples from 12 different human sources were analyzed as described above. None of the sera tested showed interference at any SRM transition monitored (Fig. 4). Isotopic purity of the deuterated internal standards was determined by injecting 10 μl of a 1000 ng/ml solution of each standard. The area of internal standard was compared to the area of the respective non-deuterated analyte. Vorinostat glucuronide and d-5-4-anilino-4-oxobutanoic acid internal standards were determined to be greater than 99.9% pure and vorinostat internal standard was determined to greater than 99.6% pure.

3.2. Linearity

Triplicate standard curves were performed in serum on three sequential days. The assay proved to be linear and acceptable, as the regression coefficients were >0.99 for each of the three standard curves (Table 2) with $1/y^2$ weighting. The line was not forced through the origin. The lower limit of quantitation (LLQ) for all three analytes was 3.0 ng/ml, as the signal/noise ratio was determined to be above 10 for all analytes at this concentration. The slopes of the lines describing the three standard curves in serum are shown in Table 2. The individual values for mean and standard deviations of back-calculated values at each nominal concentration used in the standard curves are displayed in Table 3, as is the accuracy calculated from those values.

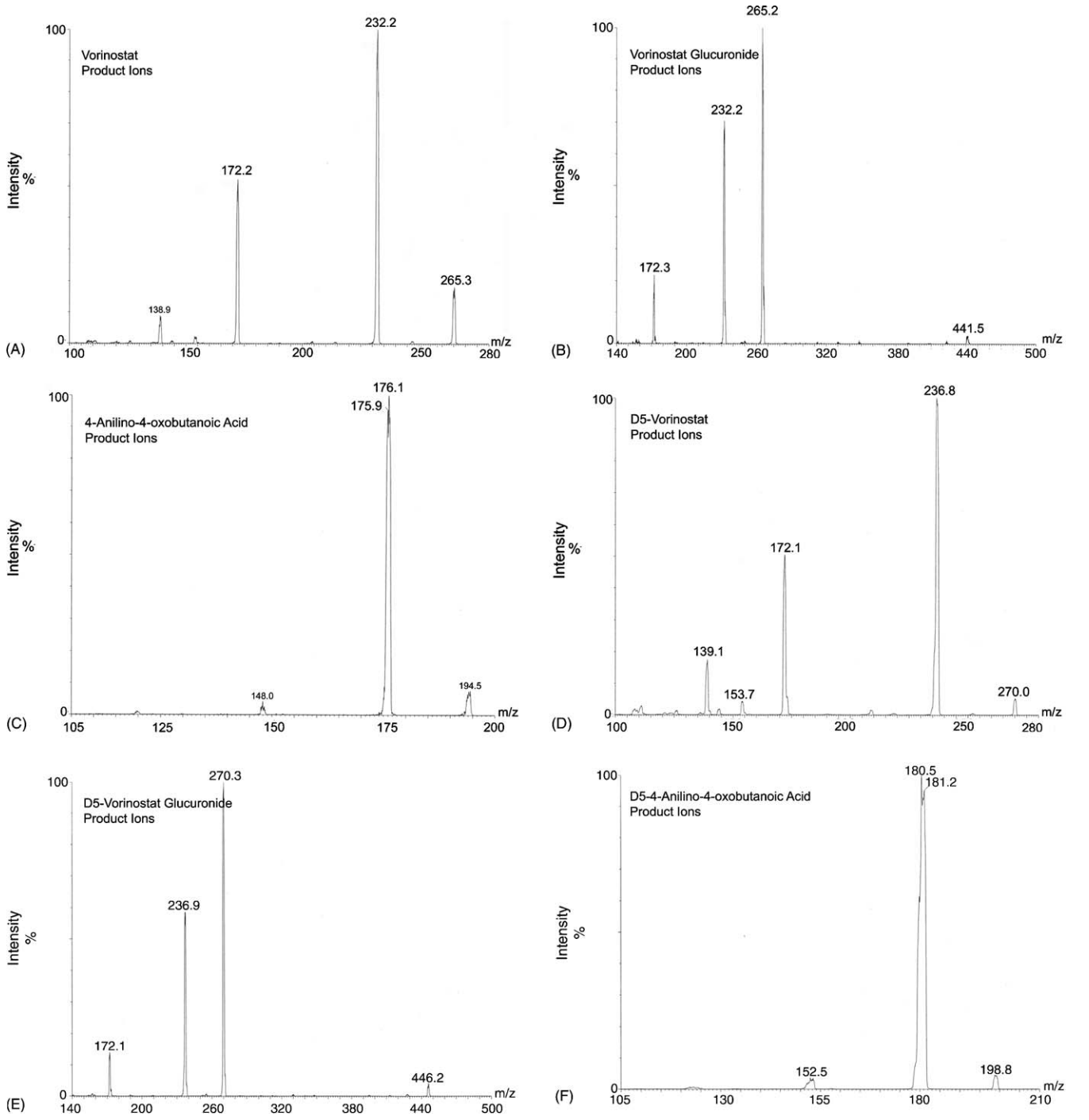

Fig. 2. CID product ion spectra from (A) *m/z* 265 of vorinostat, (B) *m/z* 441 of vorinostat glucuronide, (C) *m/z* 194 of 4-anilino-4-oxobutanoic acid, (D) *m/z* 270 of d5-vorinostat, (E) *m/z* 446 of d5-vorinostat glucuronide and (F) *m/z* 199 of d5-4-anilino-4-oxobutanoic acid.

3.3. Accuracy and precision

In order to assess the accuracy and precision of the assay in serum, quality control samples at concentrations of 5, 50, and 500 ng/ml (six samples at each concentration) were analyzed on three sequential days. These concentrations were chosen because they represented the range of concentrations reported in patients treated with vorinostat [18,19]. The inter- and intra-day back-calculated concentrations with standard deviations, coefficients of variation, and accuracies resulting from these analyses are displayed in Table 4. The coefficients of variation for both inter- and intra-day variability were less than 15% at each concentration studied. The mean accuracy for both the intra- and inter-day evaluations was between 94 and 98% over the 3 days on which quality control samples were tested.

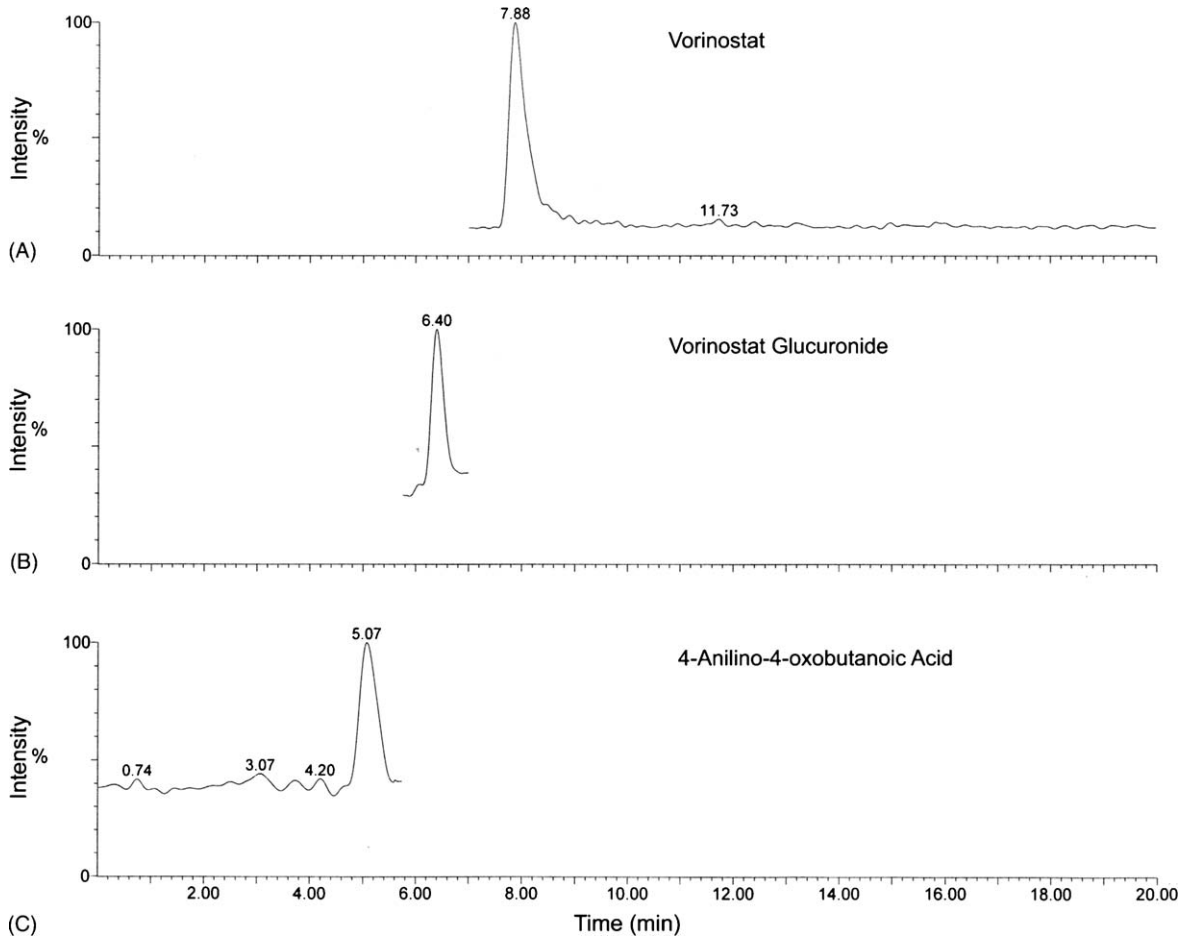

Fig. 3. LC–MS/MS chromatogram of 3 ng/ml (A) vorinostat, (B) vorinostat glucuronide and (C) 4-anilino-4-oxobutanoic acid isolated from human serum.

3.4. Recovery and stability

The recovery of vorinostat, vorinostat glucuronide, and 4-anilino-4-oxobutanoic acid was tested using the LC–MS/MS assay. This was done by calculating the ratio of absolute responses of analyte that was precipitated from serum to the analyte of the same concentration that was added to blank serum after the reconstitution phase. This was done in triplicate using concentrations of 5, 50, and 500 ng/ml for each of the three analytes. The mean percentage recovery for vorinostat at 5 ng/ml was 72.8 ± 1.2%, while the mean recoveries at 50 and 500 ng/ml were 78.6 ± 7.1 and 77.5 ± 6.0%, respectively. The mean percentage recovery for vorinostat glucuronide at 5 ng/ml was 84.1 ± 4.3%, while the mean recoveries at 50 and 500 ng/ml were 76.0 ± 7.1 and 82.3 ± 4.4%, respectively. The mean percentage recovery for 4-anilino-4-oxobutanoic acid at 5 ng/ml was 90.3 ± 9.6%, while the mean recoveries at 50 and 500 ng/ml were 85.6 ± 11.5 and 76 ± 4.0%, respectively. Although the recovery varied from 72 to 90%, the accuracy and precision were within reasonable limits.

Ion suppression was assessed in a similar fashion. Analyte and internal standard for each of the three compounds were

Table 2
Linear regression parameters of standard curves for vorinostat and metabolites

Analyte	Day	Curve	r^2	Slope	*y*-axis	Mean	S.D.	%C.V.
Vorinostat	1	1–3	0.996	0.34	0.0022	0.37	0.030	8.1
	2	4–6	0.997	0.40	−0.0046			
	3	7–9	0.991	0.38	0.0088			
Vorinostat glucuronide	1	1–3	0.998	0.079	−0.019	0.075	0.004	5.3
	2	4–6	0.999	0.072	0.00090			
	3	7–9	0.998	0.073	−0.013			
4-Anilino-4-oxobutanoic Acid	1	1–3	0.996	0.06	−0.028	0.059	0.002	2.6
	2	4–6	0.998	0.057	0.0059			
	3	7–9	0.998	0.059	0.00091			

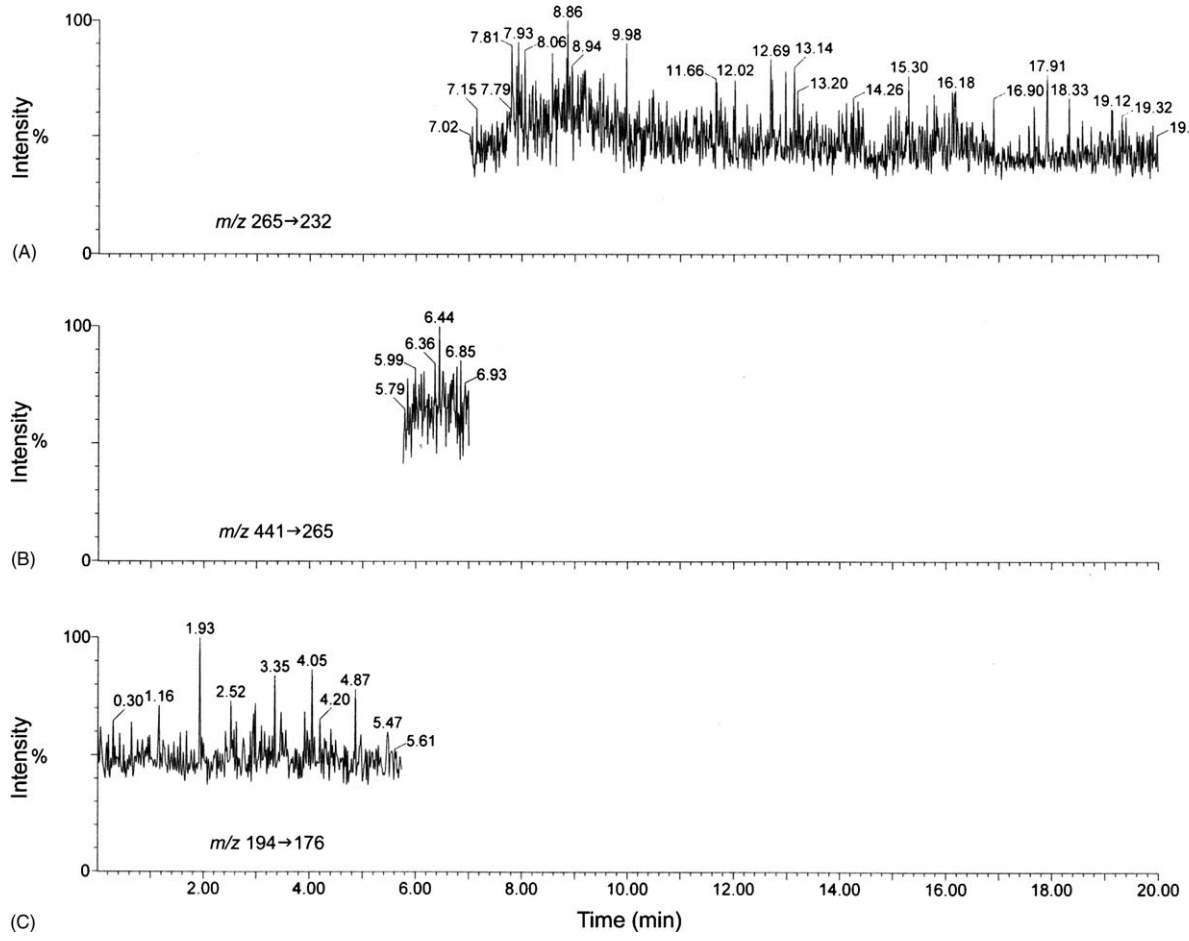

Fig. 4. LC–MS/MS chromatogram of *m/z* transitions (A) 265 → 232, (B) 441 → 265 and (C) 194 → 176 from blank human serum.

added to the dried residues of precipitated blank serum to achieve concentrations of 5, 50 and 500 ng/ml. These ratios were compared to the ratios obtained by adding the same concentration of analytes in mobile phase. No ion suppression was observed.

The freeze-thaw and long term stability of the analytes in serum was performed previously [20] and did not report any degradation or stability problems. Samples stored at −70 °C for 4 weeks have been shown to be stable.

Table 3
Accuracy and precision of back-calculated concentrations of calibration samples of vorinostat and metabolites in human serum

Analyte	Nominal concentration (ng/ml)	Mean ($n=9$)	S.D.	C.V. (%)	Accuracy (%)
Vorinostat	3	3.1	0.2	7.7	103.9
	10	9.9	0.4	4.5	99.0
	30	31.2	2.9	9.2	104.0
	100	101.2	4.9	4.9	101.2
	300	311.4	32.1	10.3	103.7
	1000	969.8	66.0	6.8	96.9
Vorinostat glucuronide	3	3.1	0.1	4.4	102.6
	10	9.8	0.3	3.1	98.0
	30	29.9	1.1	3.6	99.8
	100	100.1	3.8	3.8	100.1
	300	304.3	9.0	3.0	101.4
	1000	1000.5	24.9	2.5	100.0
4-Anilino-4-oxobutanoic acid	3	3.0	0.1	4.9	99.5
	10	10.1	0.4	3.9	101.4
	30	30.5	1.2	3.8	101.8
	100	100.3	3.5	3.5	100.3
	300	301.9	12.0	4.0	100.6
	1000	985.3	45.7	4.6	98.5

Table 4
Accuracy and precision of QC samples of vorinostat and metabolites in human serum

Analyte	Nominal concentration (ng/ml)		Intra-day			Inter-day mean
Vorinostat	5	Mean accuracy (%)	94.7	93.9	97.4	95.3
		S.D. (%)	2.5	5.9	1.3	4.0
		C.V. (%)	2.6	6.3	1.3	4.2
	50	Mean accuracy (%)	97.6	96.8	95.1	96.5
		S.D. (%)	1.6	3.2	2.9	2.7
		C.V. (%)	1.6	3.3	3.0	2.8
	500	Mean accuracy (%)	95.5	97.8	93.5	95.6
		S.D. (%)	2.8	1.4	6.2	3.9
		C.V. (%)	3.0	1.4	6.7	4.1
Vorinostat glucuronide	5	Mean accuracy (%)	96.4	97.3	96.4	96.7
		S.D. (%)	2.5	1.6	1.6	2.0
		C.V. (%)	2.6	1.6	1.7	2.1
	50	Mean accuracy (%)	96.5	97.1	97.4	97.0
		S.D. (%)	3.1	3.0	1.8	2.6
		C.V. (%)	3.2	3.1	1.9	2.6
	500	Mean accuracy (%)	97.6	95.4	97.2	96.7
		S.D. (%)	2.6	2.2	2.8	2.5
		C.V. (%)	2.6	2.3	2.9	2.6
4-Anilino-4-oxobutanoic acid	5	Mean accuracy (%)	95.2[a]	96.1	96.2	95.9[b]
		S.D. (%)	2.0	3.2	2.4	2.4
		C.V. (%)	2.1	3.3	2.5	2.5
	50	Mean accuracy (%)	95.9	95.6	96.3	96.0
		S.D. (%)	3.7	2.9	2.1	2.8
		C.V. (%)	3.9	3.1	2.1	2.9
	500	Mean accuracy (%)	98.2	96.1	95.6	96.7
		S.D. (%)	1.2	3.4	3.3	2.5
		C.V. (%)	1.2	3.5	3.4	2.6

[a] $n = 5$, for all other intra-day means $n = 6$.
[b] $n = 17$, for all other inter-day means $n = 18$.

In order to assess the stability of the analytes in the autosampler, each analyte was added to serum to produce a final concentration of 100 ng/ml, and the samples were analyzed as described above. The reconstituted samples were transferred to an autosampler vial that was placed into the autosampler, which was maintained at 4 °C, and serial injections were made for 24 h. There was less than 1% loss of any of the three analytes over that time period.

3.5. Analysis of patient samples

When applied to clinical samples, the assay proved suitable for quantitating the concentrations of vorinostat and both metabolites in the serum of a patient treated with his first oral dose of vorinostat (Fig. 5). The maximum serum concentration of vorinostat measured was 1.45 μM (384 ng/ml), and the half-life of vorinostat disappearance from serum was 112 min. Each of these values is consistent with values previously described using earlier vorinostat assays [18,19]. The concentration versus time data for vorinostat glucuronide and 4-anilino-4-oxobutanoic acid in human serum represent the first description of such information. Of note, at 6 h after ingestion of vorinostat, serum concentrations of vorinostat (0.052 μM, 14 ng/ml), vorinostat glucuronide (0.341 μM, 150 ng/ml) and 4-anilino-4-oxobutanoic acid (2.04 μM, 396 ng/ml) were still in excess of the LLQ for each of these analytes.

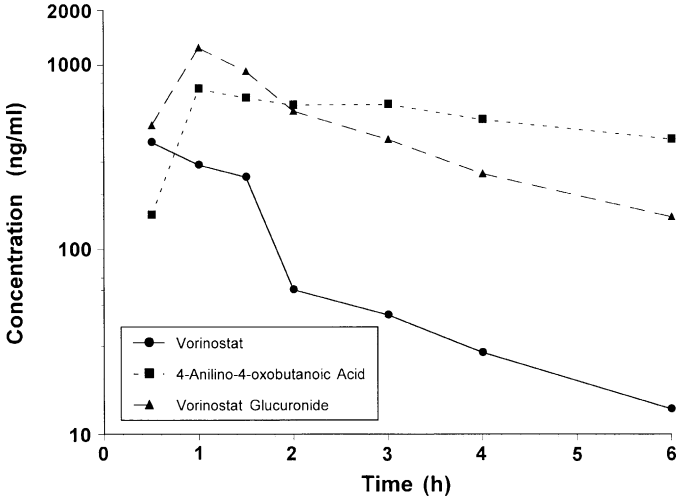

Fig. 5. Concentrations of vorinostat (●), vorinostat glucuronide (■), and 4-anilino-4-oxobutanoic acid (▲) in serum of a patient after ingestion of his first oral 200 mg dose of vorinostat.

4. Discussion

Histone deacetylase inhibitors represent a class of agents that have undergone, and continue to undergo, development and evaluation as potential anticancer agents. Inhibitors of histone deacetylase come from a variety of classes of com-

pounds, including: short chain fatty acids such as butyric acid, 4-phenylbutyric acid, and valproic acid; hydroxamic acids, such as vorinostat and trichostatin; cyclic tetrapeptides, such as depsipeptide; and benzamides, such as MS-275 [13]. Clinical development of some agents such as butyrate, tributyrin, and 4-phenylbutyrate [23–25] was frustrated by logistics associated with prolonged drug delivery or toxicities that precluded the ability to achieve or sustain drug concentrations in patients comparable to those associated with in vitro activity. However, these early clinical candidates have been followed by agents, such as vorinostat, depsipeptide [26], and MS-275 [27], that have more desirable pharmaceutical, pharmacokinetic, and toxicological profiles and that continue to undergo clinical evaluation as single agents and as components of combination chemotherapy regimens. With each agent, information regarding the concentration versus time profile of parent compound and its relevant metabolites in plasma, and ideally in normal and tumor tissue, can be used to formulate the most appropriate dose, route, and frequency of administration for that agent.

The LC–MS/MS assay described in this paper is well-suited to address a number of pharmacologic issues involved in the clinical development of vorinostat. The sample preparation is simple and no expensive reagents are required. Furthermore, the assay is applicable to the numbers of samples routinely obtained in clinical pharmacology studies. The sensitivity of the assay is suitable for defining unbound as well as total vorinostat and metabolites in serum and assessing how vorinostat concentrations in patient serum compare with those required for activity in vitro and, potentially, in vivo preclinical tumor models. The assay is also suitable for investigating potential drug–drug interactions in the numerous vorinostat-containing combination chemotherapy regimens being evaluated clinically. The ability of the assay to quantitate vorinostat glucuronide and 4-anilino-4-oxobutanoic acid allows its use in investigations of the potentially important clinical implications of UDP-glucuronosyltransferase polymorphisms in vorinostat pharmacology. In addition, the persistence in serum of 4-anilino-4-oxobutanoic acid means that it might be monitored as a measure of adherence by patients to their daily or twice-daily oral dosing of vorinostat. In fact, the assay is currently being utilized to address each of these issues, as it is being employed by the laboratories responsible for the pharmacokinetic aspects of all of the vorinostat clinical studies sponsored by the National Cancer Institute.

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