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Stability studies of vorinostat and its two metabolites in human plasma, serum and urine

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Abstract

Effects of storage time and freeze-thaw procedure on the stability of vorinostat (suberoylanilide hydroxamic acid) and its two metabolites, vorinostat *O*-glucuronide (M1) and 4-anilino-4-oxobutanoic acid (M2), in human plasma, serum and urine have been examined using high turbulence liquid chromatography (HTLC) online extraction and tandem mass spectrometry (MS/MS) [L. Du, D.G. Musson, A.Q. Wang, Rapid Commun. Mass Spectrom. 19 (2005) 1779–1787]. Vorinostat was demonstrated not to be stable in human plasma during the process of sample processing and storage. Acidifying the plasma sample to prevent possible enzymatic hydrolysis and using plasma with different anticoagulants were evaluated to increase the stability of vorinostat, but neither of these approaches improved stability. Human serum was then used as an alternative to plasma to monitor drug concentration, and vorinostat and its two metabolites maintained consistent concentrations in human serum after 3 freeze-thaw cycles and more than 1 year storage at −70 °C. By comparing the stability results of serum, EDTA plasma and heparin plasma, it was deduced that clotting proteins of plasma might be a major cause of vorinostat degradation. The stability of the three analytes during the process of serum sample collection was verified indicating that prolonged sample collection (up to 180 min) has no effect on the integrity of these analytes.

Keywords: Vorinostat stability; Human plasma; Serum; Urine; Clinical sample collection

1. Introduction

Drug stability during sample collection, processing and storage is an important factor for clinical bioanalysis. If drugs do not remain stable in a certain biofluid under the pre-specified sample processing or storage conditions, inaccurate drug concentration will be obtained, which will consequently affect drug pharmacokinetics/pharmacodynamics interpretation. Multiple factors may influence the integrity of a drug alone or synergistically prior to sample analysis and the main factors encountered are degradation and adsorption. Generally, degradation occurs naturally [2], is caused by exposure to light [3], or is the result of reaction with or catalyzation by the biofluid components [4]. Drugs may also adsorb on the surface of containers or on synthetic barriers [5], such as polymer separation gels. To reduce pre-analytical variables and provide proper sample handling conditions, drug integrity studies prior to clinical sample analysis are very important.

Another reason for drug stability evaluation is to determine whether an improperly collected sample is acceptable. Plasma or serum should be separated from red blood cells as quickly as possible to prevent ongoing degradation of a drug as well as its partition between plasma or serum and cellular components. Normally, fresh blood samples are centrifuged within 15 min for plasma collection or clotted for 30 min for serum collection. However, blood sample processing at clinical sites may not always follow the standard collection procedures; for example, samples may be left on the laboratory bench for a period of time before centrifugation or storage. The prolonged contact of plasma or serum with blood cells might cause spurious testing results. Stability studies provide evidence of how abnormal sample handling impacts the integrity of a drug in biofluids.

This paper presents a stability study for vorinostat (suberoylanilide hydroxamic acid) and its two metabolites, vorinostat

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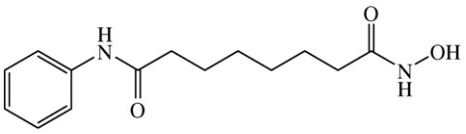
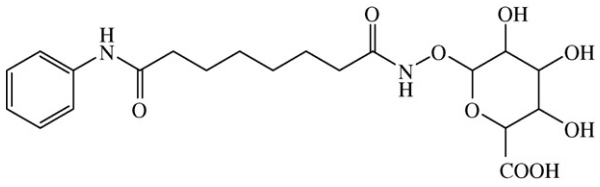
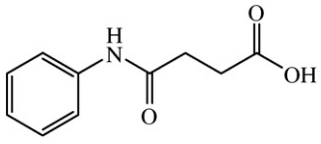


Vorinostat



M1 (vorinostat O-glucuronide)



M2 (4-anilino-4-oxobutanoic acid)

Fig. 1. Chemical structures of vorinostat, M1 (vorinostat *O*-glucuronide) and M2 (4-anilino-4-oxobutanoic acid).

O-glucuronide (M1) and 4-anilino-4-oxobutanoic acid (M2), in human plasma, serum and urine. Vorinostat is a novel agent that inhibits the enzymatic activity of histone deacetylases and is currently under development for cancer treatment. In order to obtain a reliable procedure for clinical sample collection, processing and storage, different sample matrices, EDTA plasma, heparin plasma, serum and urine, have been evaluated for drug stability. These stability studies were performed using turbulent flow online extraction coupled with tandem mass spectrometry [1].

2. Experimental

2.1. Materials

Vorinostat, M1, and M2, each with their own deuterium-labeled (d5) internal standards (IS), were obtained from Merck Research Laboratories (West Point, PA, USA); the chemical structures are shown in Fig. 1. Human control sodium EDTA plasma, sodium heparin plasma, and human control serum were purchased from Biological Specialty Corporation (Colmar, PA, USA); human control urine was obtained from our laboratory personnel. Red top (no additives) vacuum tubes (10 mL) and clot activator and gel separation serum tubes (3.5 mL) were ordered from BD Bioscience (Franklin Lakes, NJ, USA). All other chemicals such as acetonitrile (Optima), methanol (Optima) and dimethyl sulfoxide (Certified ACS) were from Fisher Scientific (Pittsburgh, PA, USA). A high turbulence liquid chromatography (HTLC) online extraction system (Cohesive Technologies Inc., Franklin, MA, USA) and a PE Sciex API 3000 mass spectrometer (Toronto, Canada) were used for sample analysis.

2.2. Standard solution preparation and storage stability

Stock standards of vorinostat, M1, and M2 were prepared separately at 10, 100, and 1000 μg/mL in methanol–water (50:50, v/v). Working standards were diluted from the stock standards and contained the three analytes (vorinostat/M1/M2) in methanol–water (15:85, v/v) at the following concentrations (ng/mL): 8/20/40, 20/40/100, 100/200/400, 200/800/1000, 400/4000/4000, 1600/6400/6400, and 2000/8000/8000, respectively. All the standards were prepared and used at room temperature.

Standard solutions were analyzed periodically and compared with fresh prepared standards to obtain the standard solution storage stability. Stock standard stability data (Table 1) indicate the stock standards can be stored for eight months when stored at −20 °C. Working standards have similar results for two months storage at 5 °C.

2.3. Preparation of EDTA plasma quality control (QC) samples and acidified EDTA plasma QC samples

QC stock standards were prepared from a different weighing as stock standards. Each analyte was prepared at 1, 10 and 100 μg/mL in methanol-water (50:50, v/v). Appropriate volumes of 1, 10 and 100 μg/mL of vorinostat standards were diluted with human control sodium EDTA plasma to obtain their corresponding plasma QC samples at 15, 100 and 400 ng/mL. QC samples of M1 and M2 were prepared in the same manner, each at three concentrations: 15, 200 and 1600 ng/mL. The preparation of acidified plasma QC was identical to plasma QC except using an acid containing plasma (0.5% acetic acid in sodium EDTA plasma).

2.4. Preparation of serum QC, urine QC and heparin plasma QC samples

Serum QC and urine QC samples contain all three analytes. Appropriate volumes of vorinostat, M1, and M2 standards at 1 μg/mL were diluted with human control serum or urine to obtain the corresponding serum or urine low QC at 6/15/30 ng/mL of vorinostat/M1/M2. The middle QC and high QC were prepared similarly using the 10 and 100 μg/mL standards to obtain vorinostat/M1/M2 in serum or urine at concentrations 100/200/200 and 400/1600/1600 ng/mL, respectively.

Heparin plasma QC samples were prepared for vorinostat only. Vorinostat standards at 1, 10 and 100 μg/mL were diluted with human control sodium heparin plasma to obtain corresponding QC samples at 6, 100 and 400 ng/mL.

Table 1
Storage stability of stock standard solutions

Analyte	Nominal conc. (ng/mL)	2-Month		4-Month		8-Month	
		Mean calc. conc. (ng/mL)	Accuracy (%)[a] [%R.S.D.][b]	Mean calc. conc. (ng/mL)	Accuracy (%)[a] [%R.S.D.][b]	Mean calc. conc. (ng/mL)	Accuracy (%)[a] [%R.S.D.][b]
Vorinostat	2.00	2.17	108.3 [3.8]	2.27	113.5 [11.5]	2.11	105.7 [1.5]
	25.0	25.7	102.7 [2.3]	26.1	104.5 [2.9]	25.5	102.0 [2.5]
	100	101	100.8 [2.4]	108	108.3 [1.5]	109	108.7 [0.4]
	500	505	101.1 [2.7]	511	102.3 [4.5]	563	112.6 [1.4]
M1	5.00	4.84	96.8 [4.3]	5.33	106.7 [1.0]	4.79	95.8 [6.5]
	50.0	54.7	109.3 [3.2]	53.1	106.2 [3.3]	53.1	106.1 [1.2]
	1000	957	95.7 [3.2]	1013	101.3 [5.0]	1053	105.3 [1.4]
	2000	1887	94.3 [2.7]	1880	94.0 [4.5]	2240	112.0 [2.3]
M2	10.0	9.36	93.6 [2.1]	8.82	88.2 [2.2]	–	–
	100	107	106.7 [2.5]	111	110.7 [1.5]	109	109.0 [1.9]
	1000	946	94.6 [3.6]	1070	107.0 [1.9]	1060	106.0 [1.0]
	2000	1783	89.2 [2.4]	1827	91.3 [0.6]	1990	99.5 [1.5]

[a] Expressed as (mean calculated concentration/nominal concentration) × 100%.
[b] Relative standard deviation was calculated using peak area ratio (analyte/IS) form ($n = 3$) analysis.

All the QC samples were divided in 400 μL aliquots into 12 × 75 mm conical polypropylene tubes with caps (Sarstedt, NC, USA) and stored under conditions identical to those used for the clinical samples at −70 °C.

2.5. QC sample analysis

Aliquots of 200 μL QC samples were transferred to a 96-well plate containing 10 μL of 15% acetic acid, 50 μL of methanol-water (15:85, v/v), and 50 μL of working IS solution (400/1600/2400 ng/mL of d5-vorinostat/d5-M1/d5-M2 in methanol-water (15:85, v/v)). A fresh calibration curve was prepared daily in the same biofluid as QC sample by mixing 10 μL of 15% acetic acid, 50 μL of working standards, 50 μL of working IS solution, and 200 μL of corresponding biofluid. The plate was then mixed thoroughly on a multi-tube vortexer and centrifuged. An aliquot of 20 μL prepared sample was injected directly onto the HTLC/LC-MS/MS system to measure the concentrations of the three analytes [1]. QC sample concentrations were calculated using peak area ratios of each analyte over its d5-IS based on the calibration curve. All the measurements were conducted in three replicates and arithmetical mean and relative standard deviation (R.S.D.) were calculated.

2.6. Serum sample collection

Fresh whole blood (∼10 mL) was drawn into a 10-mL vacutainer and immediately poured into a 15-mL polypropylene tube, which contained 10 μL of vorinostat, 20 μL of M1, and 20 μL of M2 standards at 100 μg/mL (time 0 min). The polypropylene tube was quickly vortexed and the blood was then aliquot (∼2 mL) into five 3.5-mL serum separation tubes (SST). The SSTs were inverted 5 times and placed on a laboratory bench at room temperature. The above operations were completed within 3 min before the whole blood clotted. At each time point: 15, 30, 60, 120, and 180 min, one SST was centrifuged at 3000 rpm (1450 × g),

Table 2
The effect of acids to vorinostat, M1 and M2 in human EDTA plasma

Acid	Change (%)[a] and [R.S.D. (%)][b]					
	Vorinostat		M1		M2	
	(10 ng/mL)	(500 ng/mL)	(10 ng/mL)	(2000 ng/mL)	(10 ng/mL)	(2000 ng/mL)
0% acid (pH 7.9)	100.0 [5.2]	100.0 [2.0]	100.0 [4.3]	100.0 [1.5]	100.0 [3.9]	100.0 [1.8]
0.1% HOAc (pH 6.5)	102.7 [3.5]	104.4 [3.7]	94.5 [5.3]	103.8 [3.9]	88.5 [4.6]	98.5 [3.1]
0.5% HOAc (pH 4.7)	103.7 [6.5]	104.0 [1.2]	94.8 [6.8]	102.9 [4.4]	91.1 [5.2]	97.4 [0.1]
2% HOAc (pH 3.9)	107.4 [6.2]	104.4 [2.3]	114.0 [10.9]	102.6 [3.7]	84.0 [8.6]	99.6 [1.3]
0.1% FA (pH 6.3)	109.0 [4.1]	102.5 [1.3]	93.4 [14.9]	102.1 [6.9]	92.6 [8.5]	97.7 [3.0]
0.5% FA (pH 3.9)	104.6 [3.0]	101.7 [0.4]	95.4 [7.6]	100.3 [3.3]	88.8 [12.0]	97.0 [1.8]
2% FA (pH 2.9)	103.5 [8.4]	105.4 [1.8]	99.6 [9.8]	105.6 [2.5]	95.3 [9.2]	97.3 [2.4]

[a] Calculated as (mean peak area ratio of acidified plasma/mean peak area ratio of 0% acid) × 100%.
[b] Relative standard deviation was calculated using peak area ratio from ($n = 3$) analysis.

Table 3
Intraday precision and accuracy of vorinostat, M1 and M2 in human EDTA plasma

Analyte	Nominal conc. (ng/mL)	Mean calc. conc. ($n=5$) (ng/mL)	Accuracy[a] (%)	Precision[b] (% R.S.D.)
Vorinostat	5	5.08	101.6	6.8
	10	9.66	96.6	6.5
	25	25.0	100.2	2.3
	50	50.2	100.4	4.3
	100	102	102.1	2.9
	400	405	101.4	4.2
	500	490	98.1	3.7
M1	5	4.74	94.9	9.2
	10	11.0	110.4	9.4
	50	49.2	98.4	6.0
	200	198	99.1	3.4
	1000	1000	100.0	2.1
	1600	1554	97.1	2.5
	2000	1992	99.6	2.4
M2	15	14.2	94.8	5.5
	30	30.6	102.1	4.7
	100	100	100.1	1.4
	250	255	102.0	0.8
	1000	1032	103.2	0.7
	1600	1570	98.1	2.1
	2000	1992	99.6	4.4

[a] Expressed as (mean calculated conc./nominal conc.) × 100%.
[b] R.S.D. was calculated using peak area ratio (analyte/IS).

5 °C for 15 min and stored at 5 °C until the last time point sample was prepared. All the top layers (∼1 mL serum) were then transferred from the SSTs to 12 × 75 polypropylene tubes. The serum collected at each time point was analyzed in three replicates according to the validated assay [1].

3. Results and discussion

3.1. Acid effects and assay validation

The assay for determining vorinostat and its two metabolites was initially developed with EDTA plasma. To investigate

Table 4
Intraday precision and accuracy of vorinostat, M1 and M2 in human urine

Analyte	Nominal conc. (ng/mL)	Mean calc. conc. ($n=5$) (ng/mL)	Accuracy[a] (%)	Precision[b] (% R.S.D.)
Vorinostat	2	2.00	99.8	5.0
	5	5.02	100.4	6.9
	25	24.9	99.5	6.3
	50	52.0	104.1	4.9
	100	99.1	99.1	2.4
	400	395	98.7	7.0
	500	492	98.5	4.5
M1	5	4.96	99.3	6.5
	10	10.1	100.6	2.8
	50	51.9	103.8	6.6
	200	199	99.4	2.9
	1000	1005	100.5	2.8
	1600	1576	98.5	2.8
	2000	1956	97.8	3.1
M2	10	14.2	96.7	6.9
	25	30.6	105.0	3.1
	100	100	109.2	7.1
	250	255	110.3	2.2
	1000	1032	97.0	3.9
	1600	1570	92.9	2.0
	2000	1992	88.8	3.5

[a] Expressed as (mean calculated conc./nominal conc.) × 100%.
[b] R.S.D. was calculated using peak area ratio (analyte/IS).

Table 5
Storage and freeze-thaw stability of vorinostat, M1 and M2 in human EDTA plasma and acidified EDTA plasma

Analyte	Storage		EDTA plasma QC			Acidified EDTA plasma QC		
Vorinostat		Nominal conc. (ng/mL)	15	100	400	15	100	400
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	103.1 [6.0]	92.7 [2.2]	94.5 [2.3]	111.7 [2.9]	105.0 [2.5]	104.2 [1.9]
	1-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	83.1 [6.5]	83.4 [5.0]	85.6 [2.7]	80.2 [2.6]	89.0 [5.3]	88.4 [1.5]
	2-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	94.2 [2.6]	88.7 [2.5]	85.9 [3.5]	87.1 [5.6]	85.6 [4.6]	81.5 [1.5]
	10-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	91.3 [2.0]	88.9 [3.2]	88.0 [6.9]	91.8 [4.6]	87.8 [0.7]	88.2 [6.5]
M1		Nominal conc. (ng/mL)	15	200	1600	15	200	1600
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	102.0 [6.2]	101.5 [3.4]	98.1 [3.9]	96.0 [2.8]	107.2 [3.6]	103.9 [3.8]
	1-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	94.7 [8.7]	96.8 [2.3]	94.4 [1.9]	84.2 [4.0]	96.7 [3.7]	96.0 [0.3]
	2-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	98.2 [7.7]	100.0 [4.5]	97.1 [0.7]	89.6 [1.1]	92.3 [2.9]	89.0 [4.0]
	10-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	100.9 [1.6]	99.8 [0.6]	99.0 [3.1]	100.4 [6.7]	98.8 [2.6]	102.7 [2.0]
M2		Nominal conc. (ng/mL)	15	200	1600	15	200	1600
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	103.6 [9.6]	99.1 [3.1]	99.4 [2.3]	99.3 [5.1]	99.8 [3.7]	97.0 [2.8]
	1-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	102.0 [2.2]	100.2 [4.7]	97.5 [3.8]	105.8 [3.7]	99.7 [3.2]	99.2 [0.6]
	2-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	90.7 [4.6]	106.5 [4.6]	106.5 [2.2]	99.6 [5.5]	102.0 [4.0]	101.0 [1.4]
	10-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	101.8 [4.5]	109.5 [1.9]	100.8 [9.4]	105.1 [3.6]	103.5 [1.6]	103.5 [5.2]
Vorinostat	after 3 (F/T)[c]	Nominal conc. (ng/mL)	15	100	400	15	100	400
		Accuracy[a] (%) and [%R.S.D.][b]	89.6 [2.1]	78.0 [11.6]	82.5 [11.2]	93.6 [5.5]	84.7 [4.8]	93.2 [2.9]
M1	after 3 (F/T)[c]	Nominal conc. (ng/mL)	15	200	1600	15	200	1600
		Accuracy[a] (%) and [%R.S.D.][b]	97.3 [1.6]	96.3 [1.9]	96.9 [5.1]	88.7 [3.8]	95.7 [2.1]	91.5 [0.7]
M2	after 3 (F/T)[c]	Nominal conc. (ng/mL)	15	200	1600	15	200	1600
		Accuracy[a] (%) and [%R.S.D.][b]	108.4 [0.4]	97.8 [0.4]	95.8 [4.0]	101.8 [6.7]	97.2 [0.7]	97.3 [1.3]

[a] Expressed as (mean calculated conc./nominal conc.) × 100%.
[b] R.S.D. was calculated using peak area ratio (analyte/IS).
[c] In each F/T cycle, QCs ($n=3$) were exposed at room temperature for 4 to 5 h in white light and frozen at least overnight.

Table 6
Storage stability of vorinostat, M1 and M2 in human serum

Analyte	Storage		Serum QC		
Vorinostat		Nominal conc. (ng/mL)	6	100	400
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	104.5 [5.6]	97.2 [4.0]	95.8 [6.2]
	1-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	99.1 [4.7]	99.2 [1.0]	94.2 [0.8]
	7-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	93.7 [7.1]	101.0 [6.8]	96.2 [8.6]
	57-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	96.3 [0.7]	97.6 [2.9]	96.1 [2.5]
M1		Nominal conc. (ng/mL)	15	200	1600
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	101.2 [4.6]	94.8 [0.9]	93.6 [2.9]
	1-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	105.3 [1.2]	96.5 [3.8]	94.6 [1.9]
	7-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	96.7 [3.8]	104.2 [8.4]	94.0 [4.5]
	57-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	98.9 [2.4]	98.7 [2.6]	100.2 [1.0]
M2		Nominal conc. (ng/mL)	30	200	1600
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	99.7 [5.2]	100.4 [2.7]	97.3 [3.4]
	1-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	100.3 [6.9]	102.5 [6.4]	104.0 [1.0]
	7-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	96.6 [5.5]	108.0 [5.6]	99.4 [8.4]
	57-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	103.1 [2.5]	102.9 [0.3]	99.6 [0.4]

[a] Expressed as (mean calculated conc./nominal conc.) × 100%.
[b] R.S.D. was calculated using peak area ratio (analyte/IS).

the stability of the three analytes during plasma sample processing and storage, especially the glucuronide metabolite (M1) which may potentially degrade to vorinostat, QC samples were prepared containing an individual analyte. In addition, QCs were made at three concentrations to reflect the actual drug concentrations in patients. Another concern was the possible enzymatic hydrolysis of vorinostat. Since pH has a dramatic effect on enzymatic activity, changing pH is normally used to prevent the enzymatic degradation of drugs [6]. Plasma QC samples were thus prepared in two pH conditions: neutral and acidified.

Before choosing the pH conditions, influence of acids on assay performance was evaluated. EDTA plasma standards in the following conditions: without acid, containing 0.1%, 0.5% and 2% formic acid (FA) or acetic acid (HOAc), were analyzed using HTLC/LC-MS/MS, and results were compared in Table 2. Although accuracy was acceptable, precision of M1 and M2 at low concentrations was relatively inadequate in FA containing standards. When HOAc concentration was high (2%), both precision and accuracy of M1 and M2 at low concentrations were poor. The R.S.D.s were around 10% and concentration changes were about 15%. Therefore, plasma QC samples were acidified to pH 4.7 using HOAc (0.5%, v/v).

The HTLC/LC-MS/MS assay was validated prior to QC sample analysis to ensure reliability of determination. Five sets of calibration curves prepared in five lots of human EDTA plasma were measured for assay precision and accuracy (Table 3). Human urine assay was validated similarly and results are shown in Table 4. Serum assay validation data were presented in the publication [1]. The consistent precision (R.S.D. < 10%) and accuracy (within 90–110% of nominal concentration) of the three assays indicated the reliability and ruggedness of the method.

3.2. Storage and freeze–thaw (F/T) stability of vorinostat, M1 and M2 in plasma and acidified plasma

The plasma QC samples and the acidified plasma QC samples were prepared at the same time and stored at −70 °C. They were analyzed the next day in five replicates to obtain the initial QC concentrations. After a period of storage (1-week, 2-week, and 10-week), three sets of QC samples were analyzed using the same procedure to assess the storage stability of the three analytes in plasma and in acidified plasma (Table 5). During storage, vorinostat was observed to degrade in both neutral and acidified plasma; its concentration decreased by more than 10% of nominal values after 1-week storage. Significant concentration decline was not detected with extended storage time. It appears that the degradation occurs at the beginning of storage and then this process slows down after a week. M1 shows better stability in plasma than in acidified plasma; M2 concentration is comparable under both conditions. Because acceptable intraday validation results as well as M1 and M2 stability data eliminate the possibility that the decrease of vorinostat concentration is due to assay variability, and the storage stability results of standard stock solution confirm the stability of the analyte itself, a reasonable conclusion is that vorinostat is not stable in plasma.

This conclusion was confirmed with an F/T stability study. An F/T study is used to verify the integrity of a drug during the freezing and thawing process of sample preparation. If a reassay is required, frozen samples have to be thawed again, and the drug must be stable during the repeated F/T cycles. The plasma QCs and the acidified plasma QCs were thawed at room temperature and placed on a bench for 4–5 h and then frozen at −70 °C overnight for each F/T cycle. These QC samples were analyzed after 3 F/T cycles and 1-week storage (Table 5). A comparison of F/T results with that of 1-week storage at both pH conditions indicated that the 3 F/T cycles and prolonged

exposure (12 h) to light at room temperature did not accelerate the degradation process of vorinostat; M1 and M2 concentrations were not affected by the 3 F/T cycles.

When the plasma QCs and the acidified plasma QCs were analyzed, mass transitions of the three analytes were all monitored to examine the possible conversions of analytes during storage and processing. If an M1 or M2 peak were observed in a vorinostat QC sample, that peak might indicate the occurrence of vorinostat conversion. After 10 weeks of storage, the only conversion found was M1 to vorinostat. Less than 0.2% of M1 was converted to vorinostat based on the peak height ratio for both neutral and acidified plasma QCs.

Since the storage stability of vorinostat in plasma was poor and acidifying the plasma did not improve its stability, the assay for measuring vorinostat and its two metabolites was then switched to human serum and corresponding stability studies of the analytes in serum were also conducted. Because the inter-conversion among analytes in human plasma is negligible, serum QC samples were prepared containing the three analytes.

3.3. Storage stability of vorinostat, M1 and M2 in serum

Using the same assay procedure for plasma, storage stability of the three analytes in human serum was assessed at 1-week, 7-week and 57-week intervals (Table 6). Throughout storage, stability data were all within 90–110% of nominal concentrations for the three analytes. These results indicate that vorinostat, M1 and M2 are stable in human serum when stored at −70 °C. The long-term storage data not only verify the analyte stability but also allow ample time for sample shipment and analysis. Analyte F/T stability in serum was also conducted using the same procedure as plasma QC, and the results were comparable to the storage stability [1].

3.4. Analyte stability during serum collection

Serum collection time is longer than that of plasma due to a clot formation step. Since vorinostat degrades in human plasma at the early stage of storage, stability of the analytes during serum sample collection was investigated using the procedure described in Section 2.6. Analyte concentrations were measured up to 180 min after blood collection at room temperature (Fig. 2). The concentration at the initial time point was set as 100%, and concentrations at other time points were compared to the initial value to calculate concentration changes. Variability of relative concentrations over 180 min was less than 7%, which indicates that the three analytes are stable during serum sample collection. In addition, up to 180 min of blood clotting at room temperature

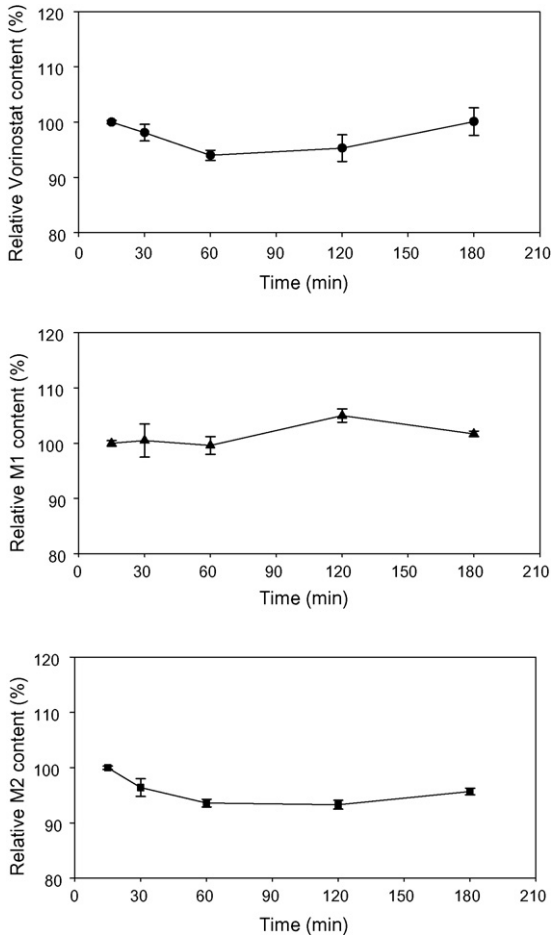


Fig. 2. Stability of vorinostat, M1 and M2 during serum sample collection.

Table 7
Storage and freeze-thaw stability of vorinostat in human heparin plasma

Analyte	Storage		Heparin plasma QC		
			6[a]	100[a]	400[a]
Vorinostat	Initial ($n=5$)	Accuracy[b] (%) and [%R.S.D.][c]	102.7 [4.0]	100.5 [4.7]	101.6 [1.1]
	1-week ($n=3$)	Accuracy[b] (%) and [%R.S.D.][c]	100.2 [0.8]	95.3 [2.7]	97.4 [1.3]
	5-week ($n=3$)	Accuracy[b] (%) and [%R.S.D.][c]	99.5 [0.7]	96.9 [1.7]	93.2 [3.1]
	after 3 F/T[d]	Accuracy[b] (%) and [%R.S.D.][c]	92.5 [1.0]	88.9 [2.2]	88.5 [2.8]

[a] Nominal concentration (ng/mL).
[b] Expressed as (mean calculated conc./nominal conc.) × 100%.
[c] R.S.D. was calculated using peak area ratio (analyte/IS).
[d] In each F/T cycle, QCs ($n=3$) were exposed at room temperature for 4 to 5 h in white light and frozen at least overnight.

Table 8
Storage and freeze-thaw stability of vorinostat, M1 and M2 in human urine

Analyte	Storage		Urine QC		
Vorinostat		Nominal conc. (ng/mL)	6	100	400
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	101.8 [3.8]	102.6 [2.7]	101.5 [2.9]
	9-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	101.4 [8.9]	96.1 [5.1]	93.3 [3.9]
	58-week ($n=2$)	Accuracy[a] (%) and [%R.S.D.][b]	102.7 [1.8]	100.6 [0.2]	99.8 [0.8]
M1		Nominal conc. (ng/mL)	15	200	1600
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	100.8 [7.5]	102.5 [2.7]	98.6 [2.3]
	9-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	99.1 [5.1]	104.0 [1.3]	93.3 [5.1]
	58-week ($n=2$)	Accuracy[a] (%) and [%R.S.D.][b]	102.8 [2.8]	103.7 [1.0]	97.2 [3.7]
M2		Nominal conc. (ng/mL)	30	200	1600
	Initial ($n=5$)	Accuracy[a] (%) and [%R.S.D.][b]	110.8 [5.4]	105.8 [1.1]	104.1 [2.2]
	9-week ($n=3$)	Accuracy[a] (%) and [%R.S.D.][b]	107.8 [6.0]	107.1 [1.4]	94.2 [2.1]
	58-week ($n=2$)	Accuracy[a] (%) and [%R.S.D.][b]	104.5 [1.0]	113.8 [0.7]	111.1 [0.9]
Vorinostat	after 3 F/T[c]	Nominal conc. (ng/mL)	6	100	400
		Accuracy[a] (%) and [%R.S.D.][b]	102.3 [8.6]	96.2 [1.7]	103.3 [1.8]
M1	after 3 F/T[c]	Nominal conc. (ng/mL)	15	200	1600
		Accuracy[a] (%) and [%R.S.D.][b]	94.9 [3.6]	94.3 [4.1]	92.3 [4.1]
M2	after 3 F/T[c]	Nominal conc. (ng/mL)	30	200	1600
		Accuracy[a] (%) and [%R.S.D.][b]	107.1 [6.1]	107.2 [2.6]	106.0 [3.7]

[a] Expressed as (mean calculated conc./nominal conc.) × 100%.
[b] R.S.D. was calculated using peak area ratio (analyte/IS).
[c] In each F/T cycle, QCs ($n=3$) were exposed at room temperature for 4 to 5 h in white light and frozen at least overnight.

verified the integrity of the analytes under prolonged sample handling conditions and facilitated the sample collection process at clinical sites.

3.5. Storage and F/T stability of vorinostat in heparin plasma

Since vorinostat is prone to chelate metal ions through the –CO and –OH groups at its active site [7,8], the binding activity of vorinostat might be reduced in EDTA plasma due to competition for metal ions from the EDTA. To determine if EDTA is the cause of vorinostat degradation in plasma, vorinostat QC samples in sodium heparin plasma were prepared. EDTA plasma is obtained by chelating calcium with EDTA to prevent blood coagulation; in contrast, heparin prevents blood coagulation by inactivating clotting substances needed to convert fibrinogen to fibrin. Fibrinogen is a clotting protein and the clot is formed from fibrin. The difference between serum and plasma is that serum does not contain clotting proteins. Studies of vorinostat in human EDTA plasma, heparin plasma, and serum were conducted to clarify the reasons for the observed stability differences. Human heparin plasma QC samples containing vorinostat were analyzed using the same procedure as for the EDTA plasma QC samples (Table 7). After 5 weeks of storage, vorinostat did not demonstrate an obvious degradation in heparin plasma, but its F/T results showed a decrease of about 10% of nominal concentrations. In these stability studies, vorinostat concentration did not remain consistent in EDTA plasma nor in heparin plasma, consequently, it is suspected that clotting proteins other than EDTA might be a major cause of vorinostat degradation in human plasma. Therefore, serum is the proper media for measuring vorinostat concentration from blood.

3.6. Storage and F/T stability of vorinostat, M1 and M2 in urine

The three analytes are stable in human urine as verified by the 58-week storage and F/T stability studies. The results of these two studies are listed in Table 8.

4. Conclusions

The processing and storage conditions of clinical samples need to maintain the integrity of a drug or at least keep the variation of pre-analysis as minimal as possible. Otherwise, the measured drug concentrations will not reflect the real concentrations in human biofluids and will result in inaccurate pharmacokinetics/pharmacodynamics interpretations. The stability studies initiated for vorinostat, M1 and M2 ensure clinical sample integrity. The problem of discordant vorinostat results in human plasma was resolved by using human serum as an alternative monitoring media. In addition, these studies indicate that some drugs have different stabilities in plasma and in serum, therefore without a stability test, sample collection and storage conditions should not be changed.

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