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A sensitive and specific liquid chromatography–tandem mass spectrometric method for determination of belinostat in plasma from liver cancer patients

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ABSTRACT

A novel, sensitive and reliable liquid chromatography–tandem mass spectrometric (LC–MS/MS) method was developed and validated for the determination of belinostat (PXD101) in human plasma. Oxamflatin was used as the internal standard. Liquid–liquid extraction of the plasma sample was performed using *tert*-butyl methyl ether as the organic solvent. Chromatographic separation was achieved on a BDS Hypersil C18 column (2.1 mm × 100 mm, 5 μm) using gradient elution mode using 0.05% formic acid in water and 0.05% formic acid in acetonitrile as solvents A and B, respectively, 60/40. The run time was 6 min. The mass spectrometer was operated under a positive electrospray ionization condition and a multiple reaction monitoring mode. An excellent linear calibration was achieved in the range of 0.5–1000 ng/mL. An average recovery of belinostat for four quality controls was 72.6% and the recovery of the internal standard at 1000 ng/mL was 67.8%. The intra-day and inter-day precisions for belinostat were ≤8.0 and ≤10.3%, respectively, and their accuracy ranged from 100.2 to 106.7%. No significant matrix effect was identified. In analysis of patient samples, belinostat glucuronide was identified and baseline separated from belinostat. This well-validated assay has been applied for quantification of belinostat in plasma samples within 24 h after the start of infusion for Asian hepatocellular carcinoma patients in a dose escalation study.

1. Introduction

In recent years, epigenetic alterations approaches have been widely applied to preclinical cancer research and clinical trials in several types of cancers to enhance the efficacy of current agents and reverse *de novo* or acquired drug resistance [1]. Low expression of either tumor suppressor or pro-apoptotic genes caused by a hypo-acetylated state of the chromatin can be up-regulated by histone deacetylase inhibitors (HDACi) which are a new and promising class of anticancer agents. Among a panel of novel HDACi, belinostat has shown very promising anticancer effects in preclinical models [2–5] and tolerable toxicities in phase I clinical trial [6]. Like trichostatin A, belinostat belongs to the family of hydroxymic acid derivatives (Fig. 1A) which have been demonstrated to induce an increase in acetylation of both histone and non-histone proteins. It kills cancer cells through regulation of genes controlling cell cycle arrest, pro-apoptosis and inhibition in cell proliferation, etc. [7]. Currently, several phase II studies of belinostat are ongoing, in various malignancies. In this study, we described the use of a sensitive and specific LC–MS/MS method for determining plasma concentrations of belinostat in patients with hepatocellular carcinoma.

Initially, we developed and validated a HPLC–UV assay with the lower limit of quantification (LLOQ) of 10 ng/mL for the determination of belinostat in human plasma [8]. However, concentrations in the plasma samples taken at 24 h after the start of infusion are usually too low to be quantified by the current HPLC–UV assay due to fast disposition of belinostat in the human body. Although a short description on liquid chromatographic–tandem mass spectrometric assay was reported recently for determination of belinostat in monkey plasma using protein precipitation, but its LLOQ is only 8 ng/mL quite similar to our published HPLC–UV method [9]. Furthermore, protein precipitation would not remove conjugated metabolites of belinostat, which may affect the accuracy of this method. However, liquid–liquid extraction can be used to concen-

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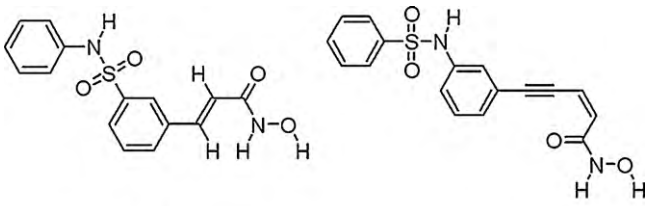

Fig. 1. The chemical structures of belinostat (A) and oxamflatin (B).

trate the analyte and reduce matrix effect caused by endogenous substances and proteins in human plasma. In addition, more than 99% of belinostat glucuronide would be removed from the analyte through LLE with *tert*-butyl methyl ether as the organic solvent based on our preliminary test. We therefore sought to establish a more sensitive and specific method for the determination of belinostat to more fully characterize the pharmacokinetics of belinostat in an ongoing phases I–II study.

2. Materials and methods

2.1. Chemicals and reagents

Belinostat was kindly provided by the National Cancer Institute (Bethesda, MD 20892-7448, USA). Belinostat glucuronide was chromatographically separated in HPLC–UV and isolated from human plasma using a fraction collector. The internal standard, oxamflatin, was purchased from Sigma–Aldrich (Steinheim, Germany). Acetonitrile (HPLC grade), methanol (HPLC grade), ethanol (analytical grade), formic acid (analytical grade) and *tert*-butyl methyl ether (analytical grade) were obtained from Merck (Darmstadt, Germany). Direct-Q^{TM} water (Millipore Milford, MA, USA) was used for the mobile phase preparation.

2.2. Sample collection and preparation

This phase I clinical trial received approval by the institutional ethics review board, and all patients provided written informed consent. Blood specimens were collected from patients before initiation of the *i.v.* infusion (baseline) and at 15, 30, 45 min, 1, 1.5, 2, 3, 5 and 24 h following the start of 30 min *i.v.* infusion in a clinical trial of single agent belinostat in Asian hepatocellular carcinoma patients. Blood samples were centrifuged for 5 min at approximately 1500 × *g* at 4 °C. The plasma samples were collected and stored at −80 °C prior to bio-analysis.

Liquid–liquid extraction (LLE) of 100 μL human plasma was performed with 1 mL of *tert*-butyl methyl ether (TBME) using oxamflatin as the internal standard. Briefly, 100 μL of human plasma was transferred into a 1.5 mL Eppendorf tube. After addition of the internal standard (50 μL of 2 μg/ml oxamflatin solution in methanol), 1 mL of TBME was added. Extraction was performed by vortex mixing the tube for 30 s, followed by centrifugation for 5 min at 10,000 × *g*. The upper organic layer was collected and transferred into another Eppendorf tube and dried under a stream of nitrogen. The residue was reconstituted in 80 μL of the mobile phase and transferred into class inserts (250 μL) of the auto-sampler vials prior to analyses by LC–MS/MS.

2.3. HPLC–MS/MS instrumentation

HPLC system consisted of an Agilent 1100 Binary pump equipped with an Agilent 1100 auto-sampler injector with a 100 μL loop and 1100 column oven (Agilent Technologies, Waldbronn, Germany) set at 20 °C. Chromatographic separations were achieved on a BDS Hypersil C18 column (Thermo Scientific, 2.1 mm × 100 mm, 5 μm) with gradient elution mode. Mobile phase solvent A was water containing 0.05% formic acid and solvent B was acetonitrile containing 0.05% formic acid. The initial mobile phase composition of 60% solvent A and 40% solvent B was maintained for 0.5 min. From 0.5 to 2 min, the percentage of solvent B was increased linearly to 95% which was maintained till 3.5 min. Between 3.5 and 3.6 min, the percentage of solvent B was decreased linearly to 40% which was maintained to the end of run at 6 min. The flow rate was consistently set at 0.5 mL/min. 20 μL of reconstituted supernatant was injected into the HPLC column and the elutant was directed to the mass spectrometer turbo-ionspray source without splitting. In order to avoid contaminating the ion source detector, the solvent front eluting in the first 0.8 min was switched to the waste container. LC–MS/MS analyses were performed using an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, MDS SCIEX, Ontario, Canada). The instrument was operated in positive ion mode calibrated by polypropylene glycol. The plasma samples were analyzed by tandem MS using the ionspray needle at +5500 V and the cluster breaking orifice voltage at 30 V. The ions of belinostat at *m*/*z* 319 and internal standard at *m*/*z* 343 were passed through the first quadrupole (Q1) and into the collision cell (Q2). The product ions for belinostat (*m*/*z* 93) and internal standard (*m*/*z* 185) were monitored through the third quadrupole (Q3). The dwell time per channel was 200 ms for data collection. The optimum temperature was set at 400 °C. Nitrogen was used as the nebulizer gas and auxiliary gas and set to back-pressures of 60 and 70 psi, respectively; the curtain gas was set to 40 psi.

2.4. Construction of standard curve

Standard stock solutions of belinostat and oxamflatin were prepared in ethanol as 1 mg/mL and were stored at −20 °C. Standard working solutions containing belinostat at concentrations of 10,000, 5000, 2000, 1000, 500, 100, 20, 5 ng/mL were prepared by serial dilution of the stock solution with methanol. Quality control (QC) working solutions were prepared at concentrations of 8000, 1500, 200 and 15 ng/mL. The internal standard working solution (2000 ng/mL) was prepared by 500-fold dilution of the stock solution of oxamflatin with methanol. 10 μL of standard/QC working solution and 50 μL of internal standard working solution were spiked into 100 μL of blank plasma for establishing standard curves and evaluating precision and accuracy, respectively. Concentrations of belinostat were back-calculated from the weighted (1/*x*) linear least-squares fitted line of peak area ratio of belinostat to the internal standard versus standard concentrations. The resultant plasma standards of belinostat were at the concentrations of 1000, 500, 200, 100, 50, 10, 2, 0.5 ng/mL and the QCs of belinostat were at the concentrations of 800, 150, 20, 1.5 ng/mL. In the process of quantifying the concentrations of belinostat in the human plasma samples, some plasma samples that exceeded the upper limit of quantitation (1000 ng/mL) were diluted 10 or 100 times with blank human plasma, prior to extraction. In order to verify this dilution integrity, a plasma test sample spiked at 8000 ng/mL was analyzed with different dilution factors (1/10 and 1/100).

The lower limit of detection (LLOD) was defined as the lowest concentration at which the analytical assay can reliably differentiate signal of the analyte peak from background noise ($S/N > 3$). The lower limit of quantification (LLOQ) was defined as the lowest calibrator with an inter-day coefficient of variation <20% [10]. The specificity of the method was evaluated by checking chromatograms of all blank plasma samples from the clinical trial subjects co-administered with antiemetic drug, granisetron.

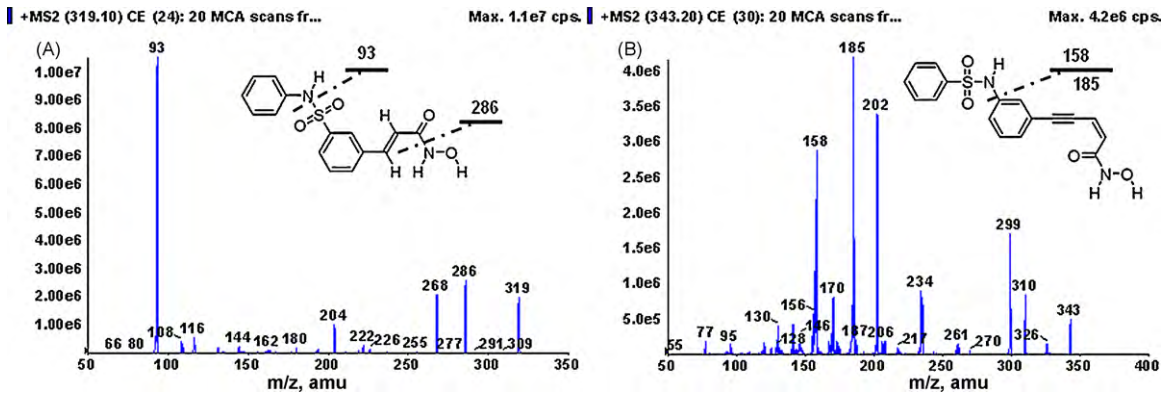

Fig. 2. MS/MS product ion scan of (A) belinostat (precursor ion *m/z* 319); (B) oxamflatin (precursor ion *m/z* 343).

2.5. Validation description

Validation was performed through establishing intra-day and inter-day precision and accuracy of the method on quality control samples (QCs). The calibration curves were daily constructed using nine different calibrator concentrations of belinostat. Intra-day variability was determined by analyzing 4 times the QCs using the same calibration curve. Inter-day variability was determined by analyzing the QCs on four different days using calibration curves obtained daily. The precision of the method at each QC concentration was expressed as a coefficient of variation (% CV) by calculating the standard deviation as a percentage of the mean calculated concentration, while the accuracy of the assay was determined by expressing the percentage of the mean from the true value of plasma QC samples.

For any rapid analysis, the possibility of conjugated metabolites co-eluting with the parent compound could exist. To elucidate this, a series of post-dosed patient samples were re-analyzed. The likely metabolic conjugates for belinostat alcohol groups would be *O*-glucuronide (*m/z* 495) or *O*-sulfate (*m/z* 399). For these analyses, MRM for possible masses of the glucuronide and expected transitions (*m/z* 495 [M+H+176] > 319 [M+H], *m/z* 495 [M+H+176] > 93 [product ion]), and of sulfate and the expected transition (*m/z* 399 [M+H+80] > 319 [M+H], *m/z* 399 [M+H+80] > 93 [product ion]), were monitored [11].

2.6. Matrix effect and recovery

Matrix effect, a common and detrimental phenomenon in LC–MS or LC–MS/MS procedures was evaluated according to the following formula [12]:

$$\text{ME}\ (\%) = \left[\frac{\text{Peak area in control matrix}}{\text{Peak area in neat standard}}\right] \times 100 \quad (1)$$

When ME (%) is equal to 100, this indicates no matrix effect for current LC–MS/MS conditions and sample preparation procedures. Deviations of ME (%) below or above 100 indicate ion suppression or ion enhancement, respectively. In our current validation procedure, the concentration levels evaluated were at 1.5, 20, 150, 800 ng/mL for belinostat and 1000 ng/mL for the internal standard. Three separate complete sets were prepared. The first set (Set A) was to determine the MS/MS response for neat standard belinostat and internal standard. The second set (Set B) consisted of the analytes spiked into plasma from four different donors after extraction. By comparing the absolute area of Set B against those of Set A, the matrix effect (ion suppression or enhancement) associated with a given lot of plasma can be measured. The third set (Set C) was prepared using the same four plasma sources as in Set B, but the analytes were spiked into plasma before the extraction process. Absolute recovery was determined by comparing the peak area of set C with those of set B.

3. Results and discussion

3.1. Method development

Belinostat and oxamflatin were optimized for their mass precursor ions and MS/MS product ions. Both compounds formed predominantly protonated molecules ($[M+H]^+$) in the mobile phase containing formic acid (0.1%). The following mass transitions were identified to be optimal for quantitative analysis due to their relative abundance – belinostat: *m/z* 319 > 93 and the internal standard: *m/z* 343 > 185 (Fig. 2).

Liquid chromatography–tandem mass spectrometry has been recommended for its excellence in specificity in biopharmaceutical analysis [13]. However, chromatographic separation is critical for those compounds, which are able to form conjugated metabolites. If these conjugated metabolites are eluted together with the parent compound, a potential interference could occur during quantitative analysis. This was observed when patient plasma samples were analyzed. Belinostat glucuronide was identified by MRM at two transitions (*m/z* 495 [M+H+176] > 319 [M+H], *m/z* 495 [M+H+176] > 93 [product ion]. This metabolite was isolated from patients' plasma using a fraction collector. After purification, it was verified through mass chromatographic comparison between belinostat (2.5 ng), belinostat glucuronide (2.5 ng), belinostat (2.5 ng) plus belinostat glucuronide (2.5 ng) and a patient sample with 10-fold dilution at 1.5 h (Fig. 3). No sulfated belinostat metabolite was identified by monitoring mass transitions of *m/z* 399 [M+H+80] > 319 [M+H], *m/z* 399 [M+H+80] > 93 [product ion]. The potential interference from glucuronide conjugate can be removed efficiently through good chromatographic separation by the choice of an appropriate column. Belinostat possesses a hydroxymic acid structure which would potentially be affected by the unreacted silanol residue on the surface of reversed phased chromatographic packing materials (C8/C18) that would lead to severe tailing effect. Hence, a BDS column with such minimal interaction was used for chromatographic separation. On the other hand, the composition of mobile phase was optimized to achieve a baseline separation of belinostat from belinostat glucuronide. Acetonitrile was used as organic conditioning solvent because it can reduce the total run time with satisfactory peak shape for belinostat and internal standard. In addition, the method of sample preparation could reduce the interference of belinostat glucuronide on the quantitative analysis of belinostat. This is because the two compounds have different polarity. With BTME liquid–liquid extraction, the recovery of belinostat was about 73% (Table 1) while that of belinostat glucuronide was less than 0.9% in a neutral pH environment. In the

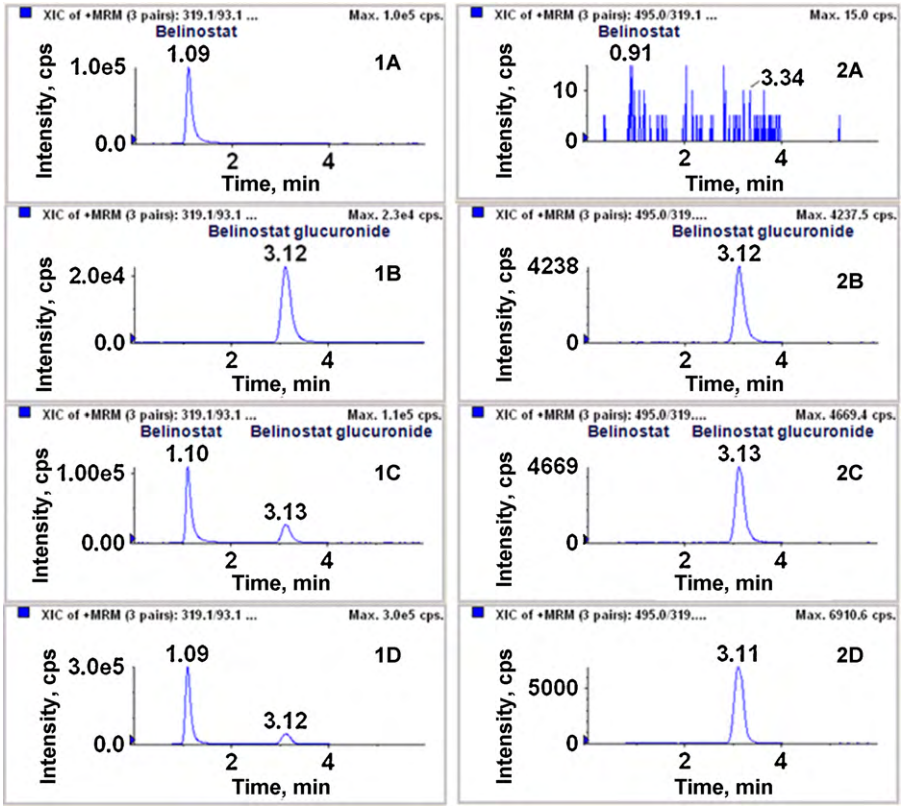

Fig. 3. Verification of belinostat glucuronide isolated from human plasma. (A) Belinostat (2.5 ng) only; (B) belinostat glucuronide (2.5 ng) only; (C) belinostat (2.5 ng) plus belinostat glucuronide (2.5 ng); (D) a patient sample at 1.5 h. Number represents mass transitions as follows – 1: 319 > 93 *m/z*; 2: 495 > 319 *m/z*.

Table 1
Matrix effect tested in patient control plasma at four concentration levels ($n = 4$).

Nominal conc. (ng/mL)	Mean peak area ($\times 10^4$)			Recovery (%)	ME (%)
	Set A	Set B	Set C		
Belinostat					
1.5	3.02 (3.50%)[a]	3.07 (2.27%)	2.21 (9.31%)	73.2	101.5
20	39.3 (1.22%)	41.3 (2.42%)	27.6 (7.20%)	70.2	105.1
150	295 (1.96%)	309 (2.45%)	209 (4.66%)	71.1	104.7
800	1357 (1.84%)	1430 (1.51%)	1030 (6.18%)	75.9	105.4
Oxamflatin					
1000	276 (3.22%)	280 (3.43%)	187 (7.28%)	67.8	101.4

[a] Coefficient of variation. Neat standard belinostat and oxamflatin (Set A); belinostat and oxamflatin spiked into plasma from four different donors after extraction (Set B) and before extraction (Set C).

final optimized conditions, belinostat eluted at 1.1 min well separated from its glucuronide with a retention time of 3.1 min (Fig. 4). Retention time for the internal standard was 1.3 min.

3.2. Method validation

With 100 μL of plasma, a satisfactory sensitivity was achieved with a low limit of detection (LLOD) at 0.1 ng/mL and LLOQ at 0.5 ng/mL. The precision and accuracy at LLOQ were 8.3% and 98.0%. The ME (%) values were in the range of 101.4–105.4 indicating very minor matrix effects that can be ignored during analyses (Table 1). Similar recoveries for belinostat (72.6%) and internal standard (67.8%) were obtained and the assay yielded an excellent linearity on its standard curve in the tested range of 0.5–1000 ng/mL with a mean least-squares linear-regression correlation coefficient (r^2) of greater than 0.999. A typical equation of the calibration curves was as follow: $y = 0.00715x + 0.00969$, $r^2 = 0.9996$, where y represents the ratios of belinostat peak area to that of IS and x represents the plasma concentrations of belinostat. A weighting factor which is inversely proportional to the variance at the given concentration level (x) was used. When a plasma sample contained a concentration higher than 1000 ng/mL, the analysis of this sample

Table 2
Dilution effect on the quantification of high concentration plasma samples ($n = 3$).

Spiked conc. (ng/mL)	Dilution factor	Expected conc. (ng/mL)	Back calc. conc. (ng/mL)	Accuracy (%)
8000	1	8000	4396.7 ± 221.9	55.0
	10	800	801.3 ± 70.9	100.2
	100	80	83.4 ± 3.8	104.3

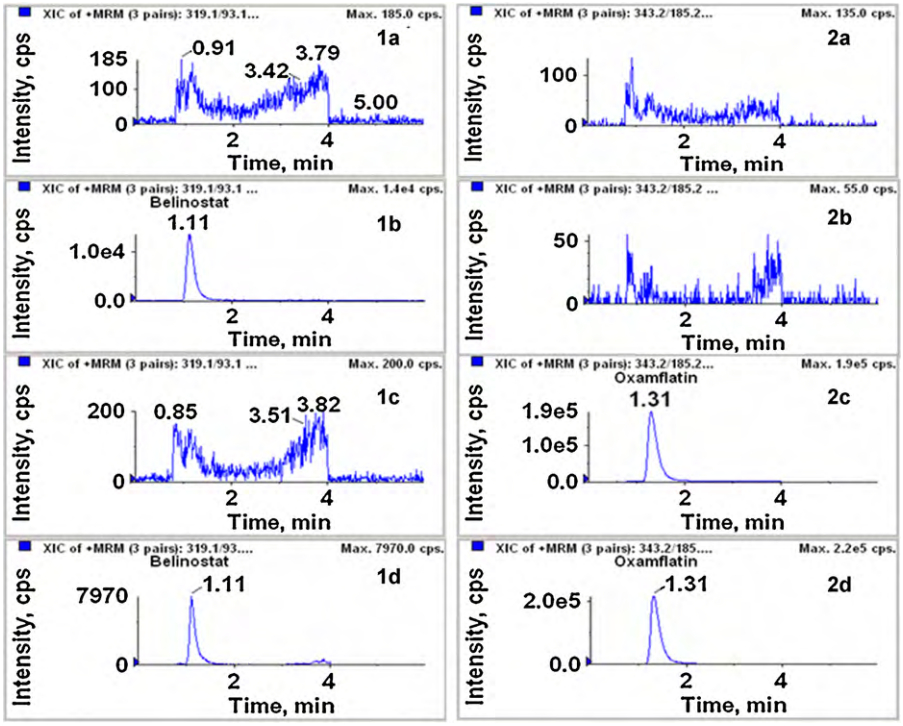

Fig. 4. Representative chromatograms of belinostat (1.1 min) and IS-oxamflatin (1.3 min). (a) Blank plasma; (b) spiked 1.5 ng/mL of belinostat only in blank plasma; (c) spiked internal standard only in blank plasma; (d) 24 h plasma sample after start of *i.v.* infusion of belinostat (1400 mg/m^2) for a subject in study cohort. Number represents mass transitions as follows – 1: 319 > 93 *m/z*; 2: 343 > 185 *m/z*.

can be processed only after it was diluted with blank plasma 10- or 100-fold. For instance, a sample with 8000 ng/mL of belinostat was quantified as 4396.7 ng/mL without dilution. After dilution, the back-calculated concentrations were 8013 and 8340 ng/mL with the accuracy as 100.2 and 104.3%, respectively (Table 2).

The accuracy and precision of this method were evaluated using the four QC samples. The precision and accuracy of belinostat for QCs are listed in Table 3. The intra-day and inter-day precisions for belinostat were ≤8.0 and ≤10.3%, respectively, and their accuracy ranged from 100.2 to 106.7%. Belinostat in plasma sample was previously shown to be very stable according to our previous HPLC–UV assay. [10] This well-validated bioanalytical method has a LLOQ of 0.5 ng/ml and is sensitive enough to quantify belinostat in the 24 h samples from all the subjects that our previous HPLC–UV method could not. The specificity was evaluated through monitoring the patient samples before *i.v.* infusion. No significant peaks co-eluted with belinostat and internal standard in the chromatograms. This sensitive and specific method has been successfully utilized in a phases I and II clinical trials of belinostat at an escalation dosage range of 600–1500 mg/m^2 in 30-min *i.v.* infusion in hepatocellular carcinoma patients in Chinese University of Hong Kong and Singapore National University Hospital. The area under the plasma concentration–time curve following administration of single *i.v.* infusion of belinostat 600 mg/m^2 was shown in Fig. 5. This well-validated bioanalytical method provides us a platform to do a fully pharmacokinetic description of belinostat in cancer patients.

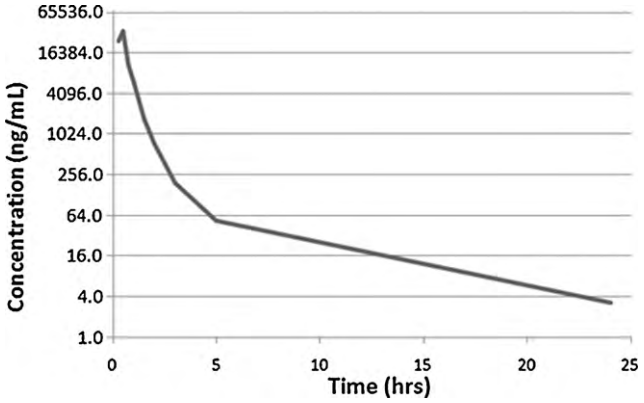

Fig. 5. The area under the plasma concentration–time curve following administration of single *i.v.* infusion of belinostat 600 mg/m^2.

Table 3
Intra-day and inter-day precision and accuracy for belinostat (n = 4).

Interval	Concentration (ng/mL)		Precision (%)	Accuracy (%)
	Nominal	Found		
Intra-day	1.5	1.6	5.1	106.7
	20	20.8	8.0	104.0
	150	155.8	5.2	103.8
	800	812.7	6.1	101.6
Inter-day	1.5	1.5	7.0	100.8
	20	20.5	10.3	102.8
	150	153.1	7.8	102.1
	800	801.8	8.3	100.2

4. Conclusions

We developed and validated a sensitive and specific LC–MS/MS method for quantifying belinostat in human plasma. This is the first method which is capable of determining belinostat concentrations in all plasma samples at 24 h post infusion from 17 patients in this phase I clinical trial with a LLOQ of 0.5 ng/mL. This will greatly facilitate pharmacokinetic descriptions of trials involving belinostat.

Acknowledgments

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