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Determination of the cyclic depsipeptide FK228, a histone deacetylase inhibitor, by liquid chromatography–mass spectrometry

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Abstract

An analytical method was developed for the quantitative determination of the novel histone deacetylase inhibitor, depsipeptide FK228 (formerly FR901228; NSC 630176), in human plasma. Calibration curves were constructed in the range of 0.5–100 ng/ml, and were analyzed using a weight factor proportional to the nominal concentration. Sample pretreatment involved a liquid–liquid extraction with ethyl acetate using 500 μl aliquots of plasma. The analyte was separated on a column (50 mm × 4.6 mm i.d.) packed with 3.5 μm C8 material, and eluted with methanol—10 mM ammonium formate (55:45; v/v; pH 8). The column effluent was monitored by mass spectrometry with electrospray ionization. The values for precision and accuracy were always ≤7.88% and <3.33% relative error, respectively. The method was successfully applied to examine the pharmacokinetics of FK228 in a cancer patient.

Keywords: Cyclic depsipeptide; FK228

1. Introduction

Acetylation and deacetylation of histones plays a major role in the regulation of gene transcription and in the modulation of chromatin structure [1]. The state of acetylation of these nucleosome core proteins is determined by two classes of enzymes with opposing activity, which are referred to as histone acetyl transferases and histone deacetylases (HDACs). During the last decade, various agents have been identified that inhibit HDAC activity and induce cell growth arrest, differentiation and/or apoptotic cell death [2]. These agents belong to diverse structural classes and include short-chain fatty acids, hydroxamic acids, synthetic benzamides, and certain cyclic tetrapeptides. In the latter group, cyclic depsipeptide FK228 (formerly FR901228, NSC 630176; (*E*)-(1*S*,4*S*,10*S*,21*R*)-7-[(*Z*)-ethylidene]4,21-diisopropyl-2-oxa-12,13-dithia-5,8,20,23-tetraazabicyclo[8,7,6]tricos-16-ene-3,6,9,19,22-pentanone; Fig. 1) is a novel, highly potent histone deacetylase inhibitor [3], which was first isolated from the fermentation broth of *Chromobacterium violaceum* [4]. This agent induces expression of the cyclin-dependent kinase inhibitor $p21^{WAF1}$ [5–7] through ataxia telangiectasia-mutated-related protein kinase [8], and has also shown antiproliferative activity in various in vitro and in vivo models for human solid tumors [9,10] and chronic lymphocytic leukemia [11,12].

Clinical trials of FK228 in patients with refractory solid tumors and hematological malignancies are currently ongoing [13,14]. Preliminary data indicate that FK228 is a potentially effective agent for the treatment of peripheral and cutaneous T-cell lymphoma [15] as well as renal cell carcinoma [14]. It has been suggested that, in vivo, FK228 may act as a prodrug that requires glutathione-mediated intracellular activation [16,17]. As part of a project to further assess the disposition and the pharmacodynamic profile of FK228, we report here on the development and validation of an analytical method that allows the determination of the drug at low concentrations in human plasma samples.

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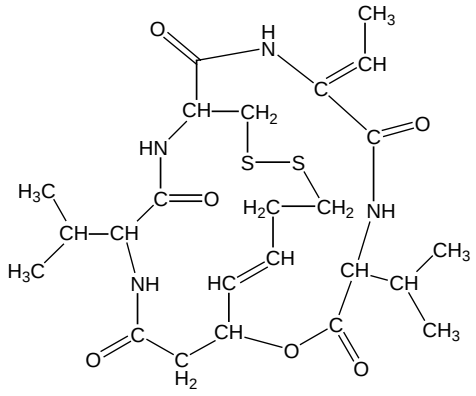

Fig. 1. Chemical structure of the cyclic depsipeptide, FK228.

2. Experimental

2.1. Chemicals and materials

Depsipeptide (HPLC purity, 98.56%) was supplied as a crystalline white powder by the Pharmaceutical Management Branch, Cancer Therapy Evaluation Program, National Cancer Institute (Bethesda, MD, USA). HPLC-grade methanol was obtained from J.T. Baker (Phillipsburg, NJ, USA). Ammonium formate and ammonium hydroxide were purchased from Sigma (St. Louis, MO, USA). Deionized water was generated with a hydro-reverse osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Malbourough, MA, USA). Drug-free heparinized human plasma was obtained from the National Institutes of Health Clinical Center Blood Bank (Bethesda, MD, USA).

2.2. Equipment

The experiments were carried out with a HP1100 system (Agilent Technology, Palo Alto, CA, USA). The system consisted of a G1312A binary pump, a mobile phase vacuum degassing unit, a G1329A autosampler, a temperature-controlled column compartment, and a HP1100 single-quadrupole mass spectrometric (MS) detector equipped with an electrospray source. The autosampler seat and needle sets consisted of a polyether–ether–ketone-based needle seat and assembly, and a Tefzel seal (Agilent Technology) was used in the injector valve to avoid carry-over. Data were acquired and integrated by the ChemStation software run on a HP Vectra 150/PC with a Windows NT operating system. The stationary phase was composed of C8 material (Agilent Technology) packed in a stainless steel column (50 mm × 4.6 mm i.d. with 3.5 μm particle size), and a C8 guard column (20 mm × 3.9 mm i.d. with 3.5 μm particle size) attached to a column-inlet filter (3 mm × 0.5 μm; Varian, Walnut Creek, CA, USA). PEEK tubing of 0.127 mm i.d. (Upchurch Scientific, Oak Harbor, WA, USA) was used to connect the column to the pump and the MS detector with minimal tubing length to avoid an extensive post-column volume.

2.3. Chromatographic and MS conditions

Chromatographic separations were achieved using a mobile phase consisting of methanol and 10 mM ammonium formate (pH 8, adjusted with ammonium hydroxide) (55:45, v/v), with a flow rate set at 0.6 ml/min. The analytical column was kept at 40 °C. The column effluent was connected to an electrospray ionization MS interface without splitting. The MS detector was operated in the positive ion mode, with single-ion monitoring at a fragmentor setting of 80 V and a multiplier gain of two. Nitrogen was used as the nebulizer gas at a pressure of 45 PSI and as the drying gas at a flow rate of 10 l/min and a temperature of 300 °C. The capillary voltage was set at 2000 V, and selected-ion monitoring was accomplished at *m/z* 541 for the protonated molecular ion of depsipeptide. Monitoring was performed using a dwell time of 578 ms and was monitored in the high-resolution mode. Simultaneously, UV detection was performed at 215 to detect possible metabolites in clinical samples. After data acquisition, the selected-ion monitoring chromatograms were integrated using the HP ChemStation software and used for quantitation.

2.4. Preparations of standards

Stock solutions were prepared in triplicate by accurately weighing, after correction for purity, an appropriate amount of depsipeptide and dissolving in DMSO. The final concentration of depsipeptide in the stock solutions was 1 mg/ml, and these were stored at −20 °C. Working standard solutions were prepared over a range of 0.01–40 μg/ml by serial dilution of the stock solution with methanol, and then stored at −20 °C. Plasma calibration standards of 0.5, 2, 5, 10, 20, 50 and 100 ng/ml were prepared by mixing the working standard solution with blank human plasma. Quality control (QC) samples were prepared from an independent stock solution at concentrations of 1.5, 40, and 80 ng/ml by dilution of the working stock solution with blank human plasma. These QC samples were subdivided into 0.6 ml aliquots, and stored at −20 °C.

2.5. Sample preparation

Standards, QC samples, and patient samples were allowed to thaw at room temperature. To each polypropylene tube (Greiner Bio-One, Frickenhausen, Germany) was added a 0.5 ml aliquot of sample plasma, 2 ml of 10 mM ammonium formate (pH 8), and 6 ml of ethyl acetate. The mixture was vortex-mixed for 30 s, and then centrifuged for 10 min at 3000 rpm. The clear supernatant was transferred to a glass tube and evaporated to dryness under desiccated air in a water bath at 40 °C in a Zymark TurboVap LV (Hopkinton, MA, USA). The residue was reconstituted in 200 μl of a

mixture of methanol and 10 mM ammonium formate (50:50, v/v), followed by vortex-mixing and centrifugation for 5 min at 13,000 rpm. A 50 μl volume of the clear supernatant was injected into the chromatographic system.

2.6. Validation procedure

To evaluate the specificity of the analytical procedure, blank human plasma samples obtained from six different individuals were extracted and analyzed for the presence of interfering endogenous substances. In addition, plasma samples containing mixtures of several commonly used drugs were tested for potential chromatographic interference with FK228.

Calibration curves were constructed by plotting the peak area of the analyte versus the nominal concentration (x) of the calibration standards. The regression parameters of slope, intercept and correlation coefficient were calculated by least-squares linear regression analysis using a weight factor of $1/x^2$. The linearity was evaluated by comparing the correlation coefficient (r^2), residuals and errors between theoretical and back calculated concentrations of calibration standard samples.The accuracy and precision were assessed by analyzing QC samples prepared at three different concentrations equally distributed over the tested range (i.e., spiked at 1.5, 40, and 80 ng/ml) in six replicates on three different days. The accuracy of the assay was evaluated by the percentage deviation (DEV) from the theoretical concentration (TC) using the formula

$$\mathrm{DEV} = 100\left\{\frac{[\mathrm{analyte}]_{\mathrm{mean}} - [\mathrm{analyte}]_{\mathrm{nominal}}}{[\mathrm{analyte}]_{\mathrm{nominal}}}\right\}$$

Estimates of the between-run precision were obtained by one-way analysis of variance (ANOVA) using the run day as the classification variable. The between-groups mean square ($\mathrm{MS_{bet}}$), the within-groups mean square ($\mathrm{MS_{wit}}$), and the grand mean (GM) of the observed concentrations across runs were calculated using the software package NCSS 2001 (J. Hintze, Number Cruncher Statistical Systems, Kaysville, UT, USA). The between-run precision (BRP), expressed as a percentage relative standard deviation, was defined as

$$\mathrm{BRP} = 100\left(\sqrt{\frac{(\mathrm{MSbet} - \mathrm{MSwit})/n)}{\mathrm{GM}}}\right)$$

where n represents the number of replicate observations within each run. For each concentration, the estimate of the within-run precision (WRP) was calculated as

$$\mathrm{WRP} = 100\left(\sqrt{\frac{\mathrm{MSwit}}{\mathrm{GM}}}\right)$$

The extraction recovery for FK228 in human plasma was determined at three concentration levels in triplicate using samples spiked to contain 1.5, 40, and 80 ng/ml, using comparison with samples prepared in 50% (v/v) methanol in 10 mM ammonium formate (pH 8) injected without extraction.

The stability of FK228 in human plasma was assessed during three freeze-thaw cycles and at room temperature for up to 24 h. Four aliquots of QC samples of three different concentrations were thawed at room temperature, and kept at this temperature for 0, 12, and 24 h, and immediately analyzed. For the freeze-thaw stability study, QC samples at three different concentrations in quadruplicate, were stored at −80 °C for 24 h. Next, the samples were thawed at room temperature, and refrozen for 12 h under the same conditions. The freeze-thaw cycle was repeated two more times, and then analyzed on the third cycle.

2.7. Clinical experiment

Blood samples were obtained from a female patient with cancer who was treated with FK228 administered as a 4 h intravenous infusion at a dose of 13.3 mg/m^2. The protocol was approved by the Institutional Review Board of the National Cancer Institute (Bethesda, MD, USA), and the patient provided written informed consent.

The samples were collected in heparin-containing tubes before drug administration at 1, 2, and 3 h after the start of infusion, immediately before the end of infusion (approximately 4 h after start), and at 15 and 30 min, and 1, 3, 6, 12, 20, and 44 h after the end of infusion. Samples were processed immediately by centrifugation for 10 min at 3000 × g at ambient temperature. The plasma supernatant was collected and stored frozen at −70 °C until the time of analysis.

For quantitation of FK228 in patient plasma samples, QC samples at a low, medium, and high concentration were assayed in duplicate and were distributed among the calibrators and unknown samples in the analytical run; no more than 33% of the QC samples were greater than ±15% of the nominal concentration. Samples with anticipated FK228 concentrations greater than the assay upper limit of quantitation of 100 ng/ml, depending on the time point of blood collection relative to the end of infusion, were pre-diluted with blank human plasma prior to extraction and quantitation at volume ratios between 1:5 and 1:10. Plasma concentration–time data of FK228 were analyzed by non-compartmental methods using the software package WinNonlin Version 4.0 (Pharsight Corporation, Mountain View, CA).

3. Results and discussion

In recent years, several analytical methods based on reversed-phase HPLC have been reported for the quantitative determination of FK228 in human plasma either based on UV detection [18] or triple-quadrupole MS detection [19,20]. These methods seem to either lack in sensitivity to support clinical pharmacological investigations or require specialized equipment that is currently unavailable in most laboratories for routine analyses, such as HPLC linked to

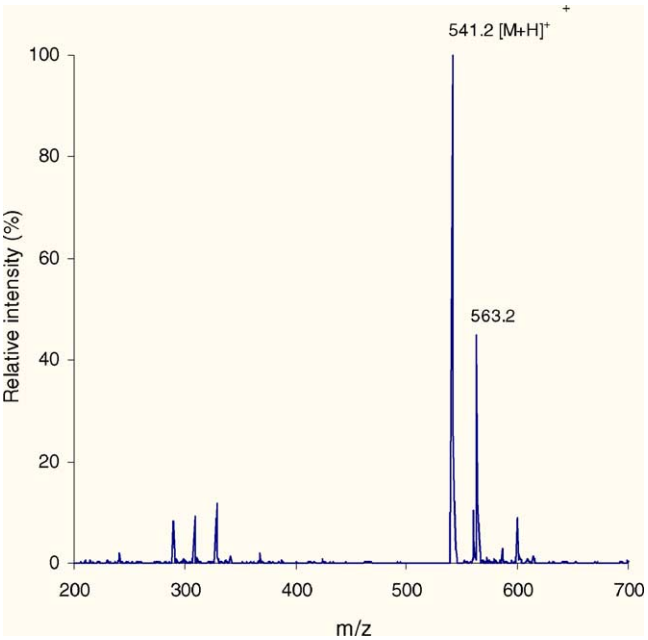

Fig. 2. Liquid chromatographic-electrospray mass spectrum of FK228.

tandem mass spectrometry. The present paper describes an alternative procedure, which uses a simple liquid–liquid extraction step followed by reversed-phase HPLC and single-quadrupole MS detection. The method is sufficiently sensitive to be applied to samples from patients receiving low doses of FK228, and yet avoids the use of complex extraction procedures for sample clean-up.

3.1. Detection and chromatography

The mass spectrum of FK228 showed a protonated molecule $[M + H^+]$ at *m*/*z* 541.2 (Fig. 2), in accordance with the NTP chemical repository database. The only other prominent ion in the spectrum was observed at *m*/*z* 563.2, and likely represents a sodium adduct resulting from sodium ions present in the HPLC tubing and/or the glass bottles containing the mobile phase. The ratio of the relative intensity for the ions at *m*/*z* 563.2 to *m*/*z* 541.2 was constant and independent of the spiked FK228 concentration, and hence the adduct did not negatively affect the overall performance of the assay. During the development of this method for FK228, various potential internal standards were evaluated other than the previously used peptide *N*-t-Boc-Met-Leu-Phe [19], which is currently not commercially available. These agents included the cyclosporin D analogue valspodar (PSC-833; Novartis Pharmaceutical Corp., East Hanover, NJ, USA), t-Boc-D-glutamic acid benzylester, and the tetrapeptides *N*-t-Boc-Met-Asp-Phe, *N*-t-Boc-Nle-Leu-Phe and *N*-t-Boc-Trp-Met-Phe (all from Sigma, St. Louis, MO, USA). The relative intensity of these molecules was consistently very low (not shown), and it was therefore decided not to use an internal standard for the current analytical procedure.

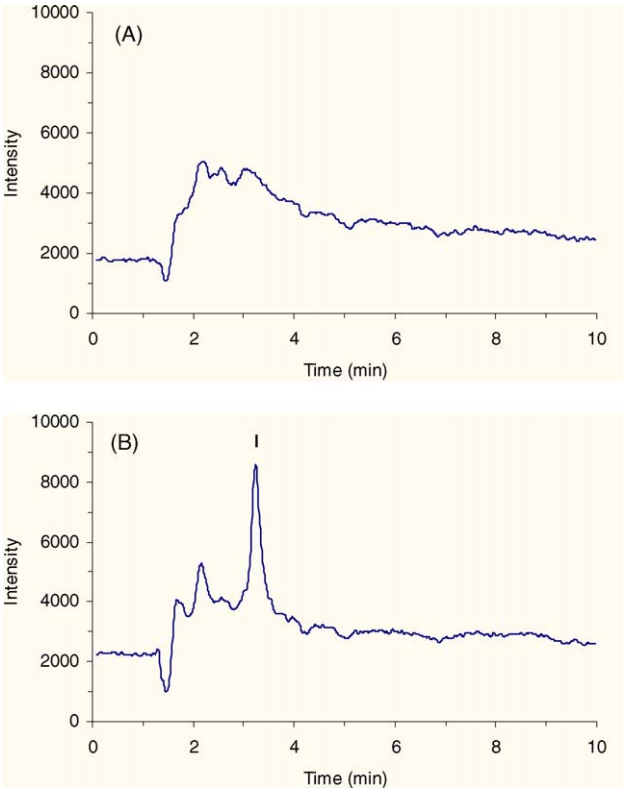

Fig. 3. Reversed-phase liquid chromatographic analysis of a blank human plasma sample (A), and a human plasma sample spiked with FK228 at a concentration of 0.5 ng/ml (B). The labeled chromatographic peak indicates FK228 (I).

Representative chromatograms of blank and spiked human plasma samples containing FK228 at a concentration of 0.5 ng/ml are shown in Fig. 3. The mean (±standard deviation) retention time for FK228 under the optimal conditions was 3.34 ± 0.017 min, with an overall chromatographic run time of 10 min. The selectivity for the analysis is shown by symmetrical resolution of the peaks, with no significant chromatographic interference around the retention time of the analyte in drug-free specimens obtained from a total of six different individuals. There was also no chromatographic interference observed from 35 drugs that are commonly co-administered with anticancer drugs (Table 1).

3.2. Linearity of detector responses

The calculated detector response of FK228 versus the nominal concentration of the analyte displayed a linear relationship in the tested range (i.e., 0.5–100 ng/ml). The measurement variance over this range increased proportionally with the drug concentration, as detected by a one-sided *F*-test at an α-value of 5%. Because of this heteroscedasticity a weighting factor was used, which is inversely proportional to the variance at the given concentration level (x), as proposed by Hartmann et al. [21]. After applying the peak area ratio in combination with a weighting factor of $1/x^2$, a mean least-squares linear-regression correlation coefficient

Table 1
Commonly administered drugs tested for interference

Amlodipine besylate	Loperamide
Atenolol	Metronidazole
Ciprofloxacin	Morphine sulfate
Clotrimazole	Omeprazole
Cyanocobalamine	Ondansetron
Dexamethasone	Oxycodone
Diazepam	Pamidronate disodium
Diphenhydramine	Phenytoin
Docusate sodium	Pseudoephedrine
Epoietin alpha	Pyridoxine hydrochloride
Fluticasone proprionate	Raloxifene
Folic acid	Ranitidin
Glucosamine sulfate	Rofecoxib
Hydromorphone	Sertraline hydrochloride
Hydroxyzine	Verapamil
Ketoconazole	Warfarin
Levofloxacin	Zolpidem tartrate
Levothyroxine	–

of greater than 0.995 was obtained in all analytical runs. The statistical evaluation of the coefficients of the ordinary least-squares line indicated small bias in the slope and in the intercept further indicating minor matrix effects and blank problems, respectively.

For each point on the calibration curves for FK228, the concentrations back-calculated from the equation of the regression analysis were always within acceptable limits for accuracy and precision of ±20% (Table 2). A linear regression of the back-calculated concentrations versus the nominal values provided a unit slope and an intercept not significantly different from zero. The distribution of the residuals showed random variation, was normally distributed and centered on zero. The bias was not statistically different from zero, and the 95% confidence intervals included zero (not shown).

The lower limit of quantitation (LLOQ) for the determination of FK228 in human plasma was established at 0.5 ng/ml, which concentration was associated with a mean (±standard deviation) signal-to-noise ratio of 5.50 ± 1.75. At this concentration, the values for precision did not exceed 15%, and the percentage deviation from the nominal concentration was on average less than 3% (Table 2). This quantitation limit for FK228 represents a 100-fold increase in sensitivity compared to a published analytical assay based on HPLC with UV detection [18], and is similar in sensitivity compared to recent procedures based on HPLC with triple-quadrupole MS detection using a multiple reaction monitoring mode [19,20]. The present LC–MS assay is based on a single-step liquid–liquid extraction, and has the advantage of being less expensive relative to the earlier method using triple-quadrupole MS, without severely compromising assay sensitivity.

Table 2
Back-calculated concentrations from calibration curves run

Nominal (ng/ml)	GM (ng/ml)	S.D. (ng/ml)	DEV (%)	R.S.D. (%)
0.5	0.51	0.01	+2.4	1.96
2	1.79	0.09	−10.4	5.03
5	5.22	0.47	+4.3	9.00
10	9.38	0.33	−6.2	3.52
20	19.9	0.36	−0.6	1.81
50	54.2	5.32	+8.4	9.82
100	109	2.71	+8.8	2.49

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; R.S.D., relative standard deviation.

Table 3
Accuracy and precision for human plasma samples spiked with FK228

Parameter	Nominal concentration (ng/ml)		
	1.5	40	80
Accuracy			
Mean observed (ng/ml)	1.55	40.40	79.96
Deviation (%, $n = 18$)	+3.33	+1.00	−0.05
Precision			
Intraday (%, $n = 6$)	7.88	6.12	4.30
Interday (%, $n = 18$)	7.42	6.05	5.71

Abbreviation: *n*, number of replicates analyzed.

3.3. Accuracy, precision, and recovery

For QC samples spiked with FK228, the within-run and between-run variability (precision), expressed as the percentage relative standard deviations, were always less than 8%, whereas the mean predicted concentration (accuracy) was within 4% of the nominal value at the various concentrations analyzed in replicates of six on three separate occasions (Table 3). The extraction efficiency of FK228 from human plasma, determined at 1.5, 40, and 80 ng/ml was concentration-independent and averaged 80.0%.

QC samples undergoing three freeze-thaw cycles showed no concentration-dependent degradation for FK228, and a loss of drug of less than 2% was observed during the second cycle. This most likely represents just analytical variability, and not drug instability, and suggest that multiple analytical measurements or re-analyses can be made on the same frozen material. In extracts, FK228 could last at least 24 h on the autosampler without any significant degradation (Table 4), allowing for more than 100 samples to be analyzed simultaneously and overnight within a single chromatographic run.

3.4. Plasma concentration–time profiles

The suitability of the developed method for clinical use was demonstrated by the determination of FK228 in plasma samples from a patient with cancer treated with FK228 as a 4 h intravenous infusion given at a dose level of 13.3 mg/m^2 (Fig. 4). The assay allowed plasma concentrations of FK228 to be detectable for more prolonged time periods (up to 48 h after the start of treatment) compared to less sensitive methods based on HPLC with UV detection, which

Table 4
Short-term temperature stability of FK228 in plasma

Time (h)	Nominal (ng/ml)	Recovered (ng/ml)[a]	Deviation (%)[b]
0	1.5	1.5 ± 0.08	1.57
	40	41.2 ± 1.87	−3.05
	80	79.7 ± 3.10	0.43
12	1.5	1.3 ± 0.07	10.72
	40	36.4 ± 1.53	9.03
	80	72.3 ± 3.03	9.64
24	1.5	1.4 ± 0.07	7.31
	40	35.7 ± 1.49	10.65
	80	72.4 ± 2.59	9.53

[a] Data expressed as mean ± standard deviation.
[b] Percent deviation from the nominal value.

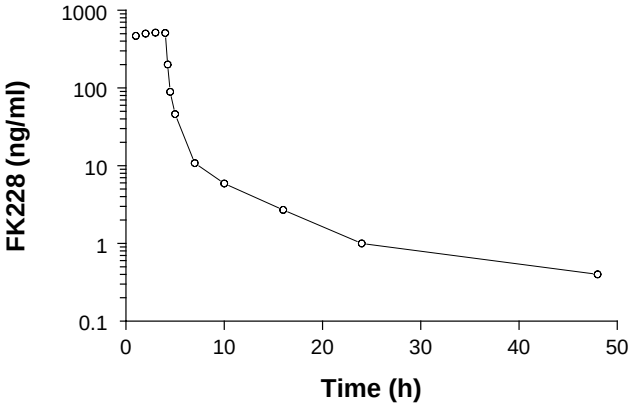

Fig. 4. Plasma concentration–time profile FK228 in a patient with cancer after a single 4 h intravenous infusion of the drug at a dose of 13.3 mg/m^2.

typically only allow detection of circulating levels up to 4 h at a lower limit of quantitation of 50 ng/ml [18]. In this patient, the peak concentration of FK228 and area under plasma concentration–time curve were 516 ng/ml and 2040 ng h/ml, respectively. The terminal half-life of FK228 was 9.07 h and the systemic clearance was 6.52 L/h/m^2, which is consistent with data reported previously for FK228 given as a 4 h intravenous infusion at similar dose levels [14]. UV detection was also carried out on all samples, but no additional peaks that might represent metabolites of FK228 were detected.

4. Conclusion

A novel assay for the measurement of a novel histone deacetylase inhibitor, the cyclic depsipeptide FK228, was developed. The method was validated according to the US Food and Drug Administration bioanalytical guidance, and met the pre-defined acceptance criteria for precision and accuracy [21]. The described method permits the analysis of patient samples to concentrations of FK228 as low as 0.5 ng/ml, which is sufficiently sensitive to allow pharmacokinetic monitoring after intravenous administration of the drug, even at low doses that are currently being used clinically. The method is currently being used to further study the clinical pharmacology of FK228 in patients with cancer.

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