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Short communication

Determination of the cyclic depsipeptide FK228 in human and mouse plasma by liquid chromatography with mass-spectrometric detection

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Abstract

An analytical method was developed and validated for the quantitative determination of the cyclic depsipeptide FK228 (romidepsin, formerly FR901228; NSC 630176), a histone deacetylase inhibitor, in human and mouse plasma. Calibration curves were linear in the concentration range of 2–1000 ng/mL. Sample pretreatment involved a liquid–liquid extraction of 0.1 mL aliquots of plasma with ethyl acetate. FK228 and the internal standard, harmine, were separated on a Zorbax SB C18 column (75 mm × 2.1 mm, 3.5 μm), using a mobile phase composed of methanol and 0.2% formic acid. The column eluent was monitored by mass spectrometry with electrospray ionization. Accuracy and precision of four concentrations of quality control samples ranged from 101.5 to 106.4% and 0.7 to 3.5% in human plasma and 93.6 to 100.6% and 0.6 to 6.5%, in mouse plasma, respectively. This method represents a significant improvement over our previously published analytical assay for this agent, decreasing the sample volume requirements, increasing the accuracy and precision (through addition of a suitable internal standard), expanding the analytical range and validating in additional biological matrices. The developed method was applied to study the pharmacokinetics of FK228 in over 1000 clinical and preclinical samples.

Keywords: Depsipeptide; Human plasma; Mouse plasma; LC/MS

1. Introduction

FK228 (romidepsin, formerly FR 901228, 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 naturally occurring bicyclic tetrapeptide that was first isolated from *Chromobacterium violaceum* by Fujisawa Company [1,2]. This agent is a novel histone deacetylase inhibitor that has demonstrated potent cytotoxic activity against human tumor cell lines and *in vivo* efficacy against both human tumor xenografts and murine tumors [3,4]. Multiple phase I and phase II clinical trials of FK228 have been initiated at the National Cancer Institute [5–7].

An analytical method was previously developed and validated in our laboratory for the quantitation of FK228 in human plasma, in support of multiple clinical trials [8]. However, the lack of internal standard became a major concern in daily practice, since the assay was being used long-term to analyze large numbers of samples. Furthermore, the original assay was not suitable for analysis of preclinical mouse samples. These animal experiments require a reliable analytical method that only uses 100 μL plasma or less, as compared to the 500 μL that was required in the previous method. In addition, the original assay was not suitable for the analysis of samples following higher doses of the drug. As exposure increased, due to the rapid dose escalation design of the study, the original upper limit of quantitation (ULOQ), 100 ng/mL, was no longer sufficient. Attempts at extending the range failed due to signal fluctuation without the application of an internal standard. Due to these major limitations, we report here an improved analytical method for determination of FK228 concentration in human and mouse plasma based on liquid chromatography coupled with single-quadrupole mass-spectrometric detection.

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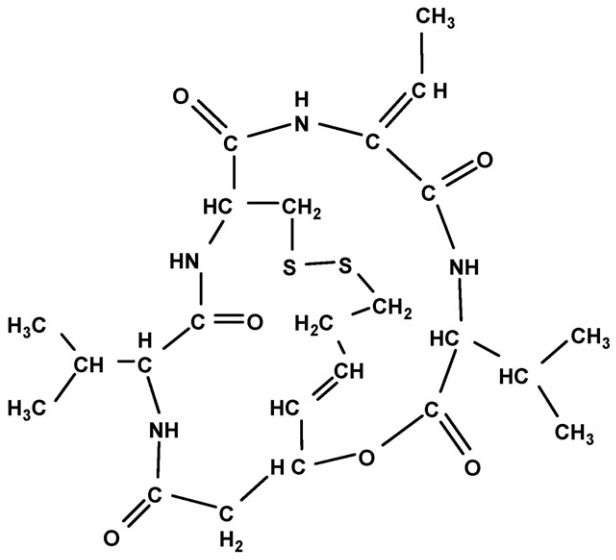
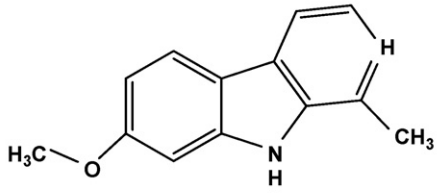


A. Structure of FK228



B. Structure of harmine, internal standard

Fig. 1. Structure of FK228 (A), and internal standard, harmine (B).

2. Experimental

2.1. Chemicals

FK228 was supplied by Pharmaceutical Management Branch, Cancer Therapy Evaluation Program, Division of Cancer Treatment and Diagnosis, NCI (Bethesda, MD, USA). Internal standard, harmine, was purchased from Sigma–Aldrich (St. Louis, MO, USA). Formic acid (98%) was obtained from Fluka (through Sigma–Aldrich, St. Louis, MO, USA). Ethyl acetate (Fisher Scientific, Fairlawn, NJ, USA) and methanol (J.T. Baker, Phillipsburg, NJ, USA) are of HPLC grade. Deionized water was generated with a Hydro-Reverse Osmosis system (Durham, NC, USA) connected to a Milli-Q UV Plus purifying system (Billerica, MA, USA). Drug-free heparinized human plasma was obtained from the National Institutes of Health Clinical Center Blood Bank (Bethesda, MD, USA).

2.2. Preparation of stock solutions and standards

Master stock solutions of FK228 were prepared by dissolving the drug in absolute ethanol at a concentration of 1 mg/mL and stored in glass tubes at −20 °C. From the master stock solution, a working solution containing 40 μg/mL of drug in methanol was prepared each week and stored at −20 °C between uses. Serial dilutions were prepared from this working solution for the preparation of calibration and quality control (QC) samples. A master stock of the internal standard, harmine, was prepared at a concentration of 1 mg/mL in absolute ethanol. From the master stock, a working solution of the internal standard was prepared by dilution to 40 ng/mL in ethyl acetate, the extraction solvent. Both the master and working internal standard solutions were stored at −20 °C.

With each analytical run, calibration standards in drug-free human heparinized plasma were freshly prepared in duplicate at FK228 concentrations of 2, 5, 10, 25, 100, 500 and 1000 ng/mL, such that the total amount of methanol added was identical in each sample (4%). QC samples were prepared in batch at concentrations of 6, 75, 800 and 2000 ng/mL, by adding plasma to the required amount of working solution in a volumetric flask. After vortexing to ensure complete mixing, these QC samples were subdivided into 0.6 mL aliquots (100 μL for 2000 ng/mL QC samples) in cryovials and stored at −20 °C.

2.3. Sample preparation

Samples were prepared by spiking 240 μL of blank human plasma in a 2 mL cryovial with 10 μL of the appropriate FK228 working solution for a total volume of 250 μL. After vortexing for 15 s, 100 μL was transferred to each of two 5 mL disposable glass centrifuge tubes (Kimble, Vineland, NJ, USA) per concentration. Patient samples were allowed to thaw at room temperature, vortex-mixed for 20 s to ensure uniformity, and a volume of 100 μL of each sample was transferred into a glass tube. QC samples were also thawed at room temperature, vortex-mixed and then aliquotted out into each glass tube. To each tube, 600 μL of ethyl acetate containing 40 ng/mL internal standard was added, followed by an additional 1 mL of ethyl acetate. Tubes were then capped and vortex-mixed for 5 min, followed by centrifugation for 10 min at 1303 × *g*. The clear supernatant was transferred to a clean glass drying 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 50 μL of a mixture of methanol/0.2% formic acid (55:45, v/v), and vortex-mixed for 15 s. Finally, each solution was transferred to a glass vial for injection. A volume of 15 μL of this solution was then injected into the chromatographic system.

2.4. Equipment

Experiments were conducted on an Agilent 1100 system (Agilent Technology, Palo Alto, CA, USA) which included a G1312 binary pump, a G1329 refrigerated autosampler, a mobile phase vacuum degassing unit, and a temperature-controlled column compartment, coupled with a single-quadrupole mass-spectrometric (MS) detector (Agilent 1100 MSD) equipped with an electrospray ionization source. The autosampler was maintained at 4 °C and the column was at 40 °C. An Agilent ZORBAX SB-C18 column (75 mm × 2.1 mm I.D.) packed with 3.5-μm packing material was employed. Samples were eluted using a step-wise gradient at a flow rate of 300 μL/min, comprised of 0.2% formic acid (A) and methanol (B). After 1 min at 60% A and 40% B, the methanol was increased to 80%

at 1.01 min which was maintained for 4.5 min. From 5.51 min until the end of the 9 min run time, 60:40 A:B was run to achieve re-equilibration. MS conditions were as follows: fragmentor, 80 V; gain, 2; drying gas flow, 11 L/min; nebulizing gas pressure 45 psi; drying gas temperature, 300 °C; and capillary voltage 2000 V. Selected-ion monitoring was accomplished at *m/z* 541.2 for FK228, and *m/z* 213.1 for the internal standard. The chromatographic data were acquired and analyzed using the Chemstation software package (Agilent).

2.5. Validation procedures

Validation of the method with respect to accuracy and precision was carried out according to procedures reported in detail previously by FDA [9]. Calibration standards and pools of QC samples were prepared as stated previous. QC samples at each concentration were thawed at room temperature and 100 μL aliquots were analyzed daily in quintuplicate. Each of the 2000 ng/mL QC samples was diluted 10-fold by adding 90 μL blank plasma to 10 μL of the spiked plasma, prior to addition of the IS or extraction, to test the accuracy and reproducibility of diluting samples which upon initial analysis are found to exceed the ULOQ. Validation runs included blank (zero concentration) and internal standard only samples, along with calibration and QC samples.

Calibration curves were calculated by least-squares linear regression analysis of the peak area ratio of FK228 and the internal standard versus the drug concentration of the nominal standard. The regression parameters of slope, intercept and correlation coefficient were calculated using a weighting factor of $1/x$ for the human plasma and $1/x^2$ for mouse plasma. The linearity was evaluated by comparing the correlation coefficient (r^2) and residuals between nominal and back-calculated concentrations of calibration standard samples. The zero concentration (blank), and internal standard only sample were used to visually verify the purity of the reagents and the lack of other potentially interfering substances, but were not considered for the regression analysis of standards. This calibration curve was then used to calculate measured QC concentrations, and that of unknown samples, by interpolation.

The lower limit of quantitation (LLOQ) of the assay was assessed by determining the concentration of FK228 at which the values for precision and accuracy were less than 20%. At least five different lots of plasma were used and resulted in accurate and reproducible measurement at the concentration of LLOQ, based on the deviation from nominal concentration.

The accuracy and precision of the assay were assessed by the mean relative percentage deviation (DEV) from the nominal concentrations and the within-run and between-run precision, calculated according to previously published equations [10]. 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 (MS_{bet}), the within-groups mean square (MS_{wit}), and the grand mean (GM) of the observed concentrations across runs were calculated using Microsoft Excel 2003 (Redmond, WA, USA).

The extraction efficiency and matrix effect of FK228 in human plasma, expressed as percentages, were determined at 10 and 800 ng/mL, in four replicates, by comparing samples spiked into plasma, into extracted blank plasma or mobile phase. Different lots of plasma were used to prepare QC samples and calibration curves that were analyzed on the same day, in order to assess relative matrix effect. Ion suppression or crossover of drug and IS was examined by comparing samples spiked with only IS, only FK228 at the ULOD (1000 ng/mL), or both.

Stability of the drug in the reconstitution solution was assessed by reinjection of calibrator and QC samples after remaining in the autosampler for 24 h following initial injection. The stability of FK228 in human plasma was evaluated following three freeze–thaw cycles, using QC samples at concentrations of 6 and 800 ng/mL, in triplicate.

2.6. Animal studies

All experiments were performed on female FVB mice obtained from Charles River Laboratories (Wilmington, MA). Blank mouse plasma was obtained from Innovative Research (Novi, MI). NCI and NCI-Frederick are accredited by AAALAC International and follow the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal care was provided in accordance with the procedures outlined in the "Guide for Care and Use of Laboratory Animals" (National Research Council; 1996; National Academy Press; Washington, DC). The study protocol was approved by the NCI Animal Care and Use Committee (Bethesda, MD). All animals were administered with 3.6 mg/kg FK228 as an IV bolus. Destructive sampling was employed. At each timepoint, three mice were exsanguinated by cardiac puncture and blood was transferred into heparinized tubes. Following centrifugation, the plasma layer was transferred to a cryovial and stored at −80 °C until analysis.

3. Results and discussion

3.1. Specificity

Due to the previously reported difficulty in finding a suitable internal standard with similar chemical structure and properties [8], a wider variety of different compounds were tested, including Boc-Met-Leu-Phe-OH and 6-methylprednisolone. Finally, harmine (Fig. 1) was chosen. Fig. 2 displays typical chromatograms of an extract of a blank human plasma sample (A), and an extract of a plasma sample spiked with IS and FK228 at a concentration of 2 ng/mL (B). The selectivity of the analysis is shown by symmetrical resolution of the peaks, with no interference around the retention time of the analyte in drug-free plasma obtained from six different individuals. Overall chromatographic run time was established at 9 min with FK228 eluting at $t_R = 5.67$ min and internal standard, harmine, at $t_R = 1.93$ min.

3.2. Validation characteristics

The calculated detector response of the FK228/IS ratio versus the nominal concentration displayed a linear relationship in

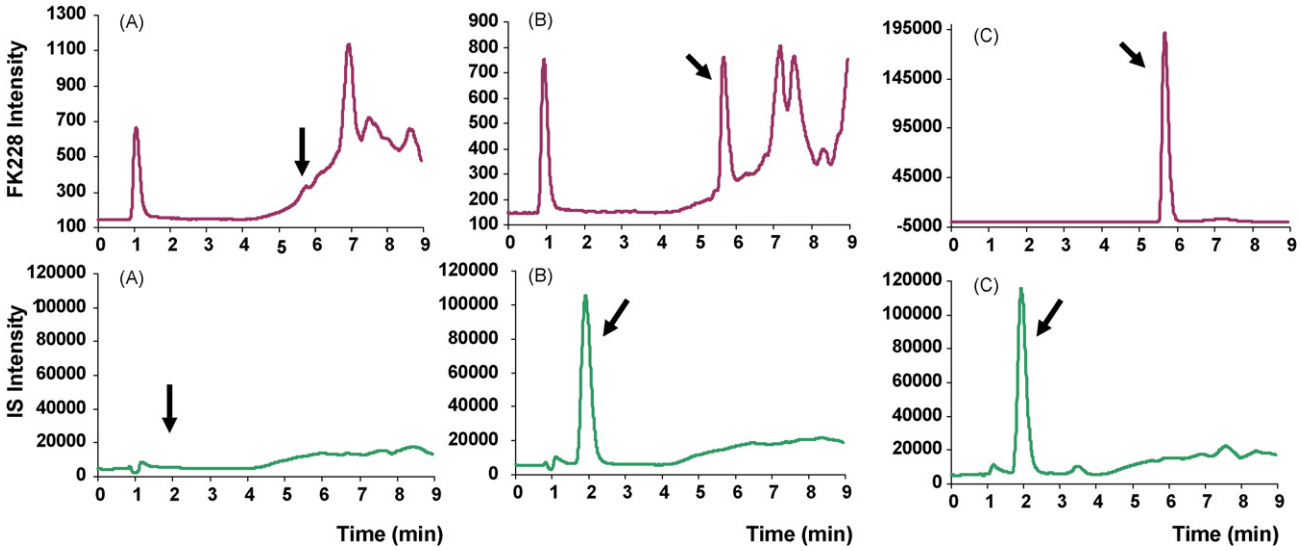


Fig. 2. Chromatograms from reversed-phase HPLC of blank human plasma (A); a human plasma spiked with harmine (IS) and 2 ng/mL (LLOQ) FK228, (B); and a patient sample at the end of infusion with 777.72 ng/mL of FK228 spiked with IS, (C).

the tested range of 2–1000 ng/mL. However, variance increased proportionally with drug concentration. A weighting factor was applied, inversely proportional to the variance at the given concentration level $1/x$ for human plasma and $1/x^2$ for mouse plasma, x being the nominal FK228 concentration. Using least-squares linear-regression, a mean (±S.D.) correlation coefficient of 0.9996 ± 0.00039 (range, 0.9990–0.9999) was obtained for human plasma and 0.9980 ± 0.00143 (range, 0.9959–0.9992) was obtained for mouse plasma.

In blank human plasma spiked with FK228 at a concentration of 2 ng/mL, all of the eight calibration samples run on four separate days were within ±20% deviation of the nominal value. The mean percentage deviation from nominal value for these eight samples was −5.80% (Table 1, A), together with within and between-run variability of 8.66 and 5.67% (not shown in the table), respectively. Samples at 2 ng/mL prepared on the same day from different lots of human plasma were back calculated from the same calibration curve which gave the following results: 1.97, 2.12, 2.05, 1.98, 2.04 and 1.88 ng/mL, all within 6% deviation of the nominal value. In mouse plasma, all of the eight calibration samples run on four separate days were within ±10% deviation of the nominal value. Based on these results, the lower limit of quantitation for FK228 was established at 2 ng/mL.

Validation data for the analytical method in terms of accuracy and precision are summarized in Tables 1 and 2 for both of the human plasma and mouse plasma. Table 1 displays the data calculated from duplicate calibration curves on four separate days. Shown in Table 2 is data from the QC samples that were run in quintuplicate at each concentration, on each of these 4 days. Values were back calculated using the calibration curve from the same run. The assay was found to be accurate, within 6.4% (−6.4% for mouse plasma) at all four concentrations, and precise with within-run and between-run precision error of less than 3.5% (6.5% for mouse plasma).

The mean overall extraction efficiency for FK228, estimated by comparing the MS response of FK228 spiked into plasma and extracted plasma blank, was approximately 96.8%, independent of the spiked concentration. The mean overall recovery of FK228 was determined to be 95.4%, also independent of the spiked concentration. The matrix effect was estimated to be 1.4%, which was well within the range of experimental error, leading to the conclusion that no significant matrix effect is present. QC samples prepared using different lot of human plasma from the calibration curve did not show additional plasma to plasma variation. This result is also consistent with the slopes of calibration curves obtained from different lots of human plasma with a mean (±S.D.) of 0.001866 ± 0.000094 (range, 0.001763–0.001975),

Table 1
Back-calculated concentrations from calibration curves run in duplicate on four occasions

Nominal (ng/mL)	GM (ng/mL)	S.D. (ng/mL)	DEV (%)	R.S.D. (%)	*n*
A. Human plasma					
2	1.9	0.19	−5.80	9.82	8
5	5.0	0.19	−0.81	3.77	8
10	10.0	0.34	0.00	3.39	8
25	25.4	0.82	1.51	3.21	8
100	104.3	2.72	4.29	2.61	8
500	512.6	10.57	2.52	2.06	8
1000	982.9	11.71	−1.71	1.19	8
B. Mouse plasma					
2	2.0	0.12	−1.85	5.91	8
5	5.2	0.29	2.98	5.68	8
10	10.3	0.40	2.51	3.92	8
25	25.5	0.57	1.91	2.23	8
100	100.9	4.72	0.92	4.68	8
500	490.2	22.65	−1.96	4.62	8
1000	954.8	44.75	−4.53	4.69	8

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; R.S.D., relative standard deviation; *n*, total number of replicate observations within the validation runs.

Table 2
Assessment of accuracy and precision from quality control samples[a]

Nominal (ng/mL)	*M* (ng/mL)	S.D. (ng/mL)	DEV (%)	BRP (%)	WRP (%)	*n*
A. Human plasma						
6	6.2	0.23	3.00	1.60	3.51	20
75	79.8	0.95	6.39	0.66	1.04	20
800	811.8	19.52	1.48	2.07	1.55	20
2000	2029.2	64.01	1.46	1.19	2.97	20
B. Mouse plasma						
6	6.0	0.41	−0.72	6.50	3.62	20
75	74.5	4.30	−0.66	5.36	3.26	20
800	748.4	35.80	−6.45	2.60	4.19	20
2000	2012.2	82.55	0.61	0.62	4.07	20

Abbreviations: GM, grand mean; S.D., standard deviation; DEV, percent deviation from nominal value; WRP, within-run precision; BRP, between-run precision; *n*, total number of replicate observations within the validation runs.
[a] Five samples at each concentration were run on four different occasions.

the interception at *y* axis of 0.000669 ± 0.000123 (range, 0.000560–0.000801).

Possible crossover interference was also evaluated by comparing the direct instrument responses to the following three sets of experiments in triplicate: samples spiked with IS only, 1000 ng/mL FK228 only, or both. No interference between FK228 and the IS was found, as the response of each analyte did not vary with the addition of the other.

3.3. Stability

The reinjection measurements were consistent with the initial run, allowing samples to be reanalyzed on the following day when necessary (for example, in the case of machine failure). For the freeze–thaw stability test, back-calculated values at both 6 and 800 ng/mL after each freeze–thaw cycle were within 10% of the nominal values, indicating no degradation.

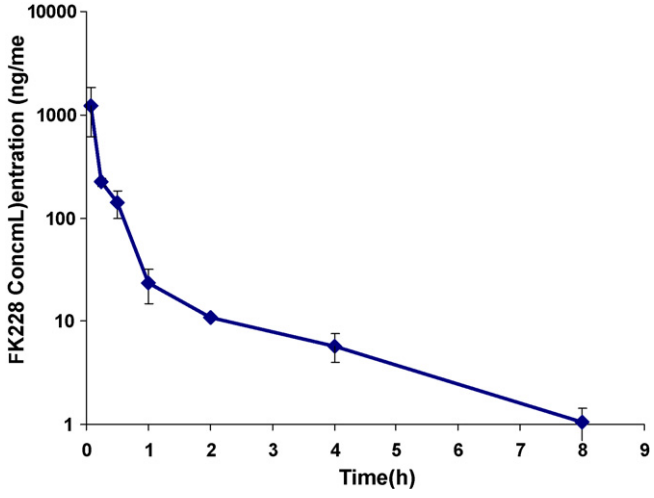


Fig. 3. Concentration–time profile for FVB mice that received 3.6 mg/kg of FK228 as an I.V. bolus. Each timepoint represents the mean (±S.D.) of three individual mice. Destructive sampling was employed, and each mouse was only sampled once.

4. Application

The validated method was then applied to study the pharmacokinetics of FK228 in patients on NCI initiated phase I and phase II clinical trials, as well as on additional preclinical mouse studies. Fig. 3 shows the observed plasma concentration time profile of FVB mice that received 3.6 mg/kg of FK228 as an I.V. bolus. Each data point was the average from three individual mice. A typical chromatogram of a human patient sample is presented in Fig. 2(C) with a calculated concentration of 777.72 ng/mL.

5. Conclusion

In conclusion, a novel chromatographic method with mass-spectrometric detection has been developed and validated for the quantitative determination of FK228 in human and mouse plasma. This method is specific, accurate and precise, and can be easily implemented into routine practice. Compared to our previously published LC/MS method [8], the addition of IS greatly reduced assay variability, allowing for the reduction of plasma volume from 500 to 100 μL, which made it possible to apply this method to preclinical samples in addition to reducing patient sample volume requirements. Additionally, validation of sample dilution extended the assay range up to 10,000 ng/mL, as necessary for measurement of drug following administration of higher doses.

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