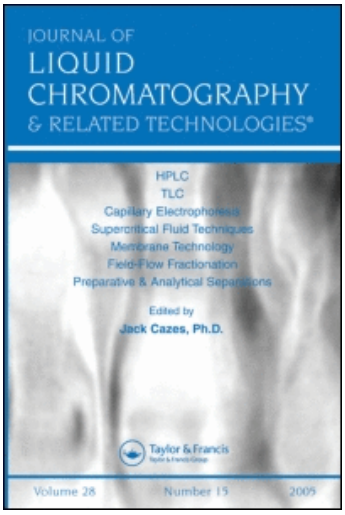

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Determination of Depsipeptide (FR901228) in Human Plasma by Liquid Chromatography–Tandem Mass Spectrometry

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ABSTRACT

A sensitive method for the determination of depsipeptide (FR901228) in plasma was developed using high performance liquid chromatographic (HPLC) separation with tandem mass spectrometric detection. FR901228 was acidified by potassium acid phthalate (0.05 M, pH 4.0) and extracted with ethyl acetate (5 : 12, v/v); the supernatant of ethyl acetate extract was evaporated, reconstituted in 250 μL mobile phase,

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and then separated on a Keystone spherisorb C8, 5μ 2.1 × 100 mm^2 column with a mobile phase consisting of methanol–12 mM ammonium acetate (85 : 15, v/v) at a flow rate of 0.2 mL/min. Detection was achieved by a PE SCIEX API365 LC/MS/MS System at unit (Q1) and low (Q3) resolution in positive multiple reaction monitoring (MRM) mode, monitoring the transition of the FR901228 molecular ion m/z 541.0 to the product ion m/z 272.0, and of the internal standard (IS) (*t*-Boc-D-glutamic acid 1-benzyl ester, BGBE) molecular ion m/z 338.0 to the product ion m/z 91.0. The mean recovery for FR901228 was 60% with a lower limit of quantification (LLOQ) of 0.2 ng/mL using 0.5 mL plasma for extraction. This method was validated over a linear range of 1.0–1000 ng/mL, using BGBE as the IS. Results from a 5-day validation study demonstrated good within-day and between-day precision (CV% values were ≤3.5% and ≤5.5%, respectively) and accuracy (range from 99.7% to 112.5%) across the calibration range of 1.0–1000 ng/mL.

Key Words: Depsipeptide; Liquid chromatography; Tandem mass spectrometry; Histone deacetylase inhibitor.

INTRODUCTION

Depsipeptide (FR901228) is a bicyclic peptide isolated from *Chromobacterium violaceum* that demonstrates anti-neoplastic activity.[1] It has been shown to be active as a histone deacetylase inhibitor, which causes the arrest of cell cycle transition at the G_1 and G_2M phases and leads to internucleosomal breakdown of chromatin.[2] FR901228 causes a G_1 arrest in H-ras transformed cells and down regulates c-myc in activated T lymphocytes.[3] FR901228 also inhibits tumor angiogenesis and suppresses the expression of angiogenic stimulating factors or the kinase insert domain receptor, suggesting that FR901228 may suppress tumor expansion, at least in part, by inhibition of neovascularization.[4] In vitro studies have shown that FR901228 has potent antitumor activity against a number of human tumor cell lines, including breast cancer cell lines MCF-7 and MDA-MB231.[5] Activity of FR901228 has also been shown in vivo in mouse xenograft models of murine ascitic tumors, murine solid tumors, and human solid tumors (lung carcinomas and stomach adenocarcinoma) (Fig. 1).[6]

Two phase I trials of FR901228 have been conducted in adults with advanced cancer, and these have shown that biologically active FR901228 plasma concentrations can be achieved with minimal toxicity.[7] A phase II trial in adults with lung cancer is currently underway at the time of writing. Early phase I clinical trials of FR901228 are planned in children with cancer, and extensive clinical pharmacokinetic studies of this compound

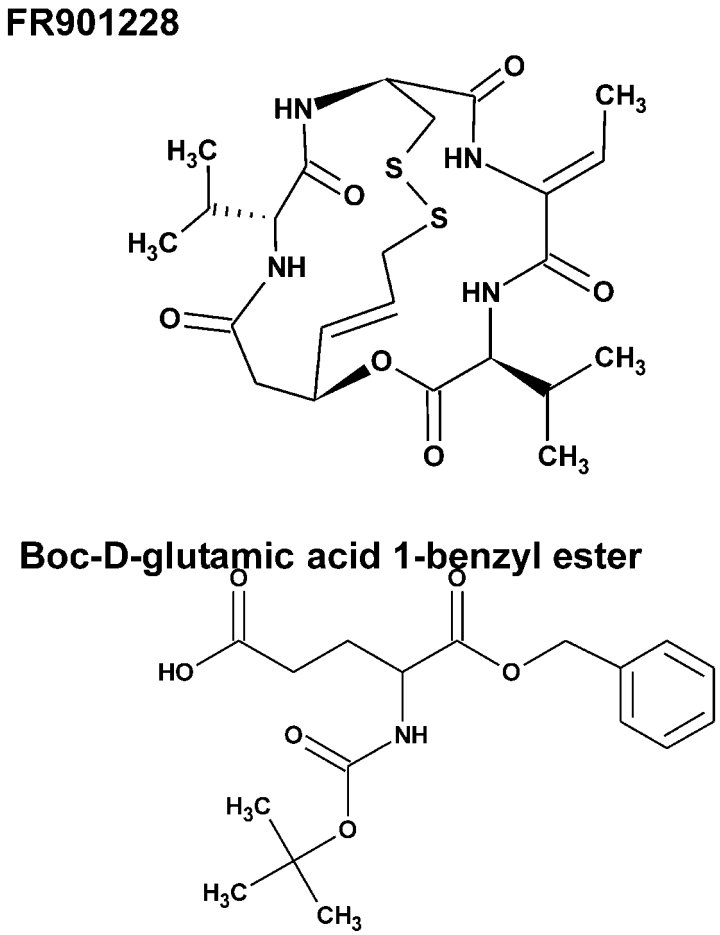

Figure 1. Chemical structure of FR901228 and BGBE.

will be performed in this population. Only two high performance liquid chromatography (HPLC) methods for the bioanalysis and quantification of FR901228 in human plasma have been published. The first used UV detection[8] with a lower limit of quantification (LLOQ) of 50 ng/mL; the second method explored the LC–tandem mass spectrometry technique with a Met–Leu–Phe as internal standard (IS) for a highly selective analysis of plasma FR901228 with a analytical range 0.1–100 ng/mL.[9]

Here, we describe an LC–MS/MS method with *t*-Boc-D-glutamic acid 1-benzyl ester (BGBE) as IS for quantification of FR901228 in plasma over

a wide concentration range that permits a very sensitive, reproducible, and highly selective analysis.

EXPERIMENTAL

Chemicals

FR901228, reference standard used for preparation of standards and quality control samples, was supplied by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan; FR901228 Lot No.: 100118G, 99.6% purity, Date 8/2001). BGBE, as the IS, was purchased from Fluka (Packed in Switzerland; *P/N*: 15106, 98% purity). The following chemicals were used: methanol, HPLC grade (Burdick & Jackson, Muskegon, MI); ammonium acetate, minimum 97.5% (Sigma, St. Louis, MO); ethyl acetate, 99.9% HPLC grade (Fisher Scientific, Fair Lawn, NJ); and potassium acid phthalate, 0.05 N, pH 4.0 (LabChem Inc, Pittsburgh, PA). Blank human plasma was obtained from Lifeblood (Memphis, TN). All water was distilled, deionized, and further purified via a Millipore Milli-QUV plus Ultra-Pure Water System (Tokyo, Japan) (resistance: 18.2 MΩ).

Chromatographic Conditions

The HPLC system consisted of a Shimadzu (Kyoto, Japan) system controller (SCL-10AVP), pump (LC-10ADVP), autosampler (SIL-10ADVP), and on-line degasser (DGU-14A). Reconstituted plasma extract of 30 μL was injected onto a Hypersil-Keystone (Bellefonte, PA) Waters Spherisorb C8, 5μ analytical column (100 × 2.1 mm^2), preceded by a guard column of the same material (20 × 4.6 mm^2) with a Upchurch 2μ pre-column filter. The flow rate was 0.20 mL/min with a mobile phase that consisted of methanol–12 mM ammonium acetate (85 : 15, v/v). The column was maintained at room temperature (20–25°C). Under these conditions, typical retention time was 3.30 min for FR901228 and 2.65 min for BGBE, and backpressure values of approximately 42–45 bar were observed between the columns. The total run time for each sample was 5.0 min.

Mass Spectrometric Conditions

Detection was performed with a PE SCIEX API 365 triple quadrupole mass spectrometer (Toronto, Canada) equipped with a thermally and

pneumatically assisted electrospray source, which was run at the unit-resolution of Q1 and the low-resolution of Q3, in positive mode with multiple reaction monitoring (MRM). Scan positive-ion mass spectra showed the parent molecular ion for FR901228 at m/z 541.0 to the predominant ion m/z 272.0, and for BGBE at m/z 338.0 to m/z 91.0, respectively (Fig. 2). The optimized conditions of MS/MS with the turbo ion spray source were as follows: 400°C ion spray temperature, 8 psi nebulizer (NEB) gas pressure, 6 psi curtain (CUR) gas pressure, 5 L/min turbo gas flow, 4500 V ionspray voltage (IS), and 6.0 units collision gas (CAD); 40 V declustering potential (DP), 210 V focusing potential (FP), −8 V entrance potential (EP), 26 V collision cell entrance potential (CEP), 28 V collision energy (CE), and 6.0 V collision cell exit potential (CXP). The mass spectrometer was interfaced to a computer workstation running Analyst software (Version 1.0; Applied Biosystems, Foster City, CA) for data acquisition and processing.

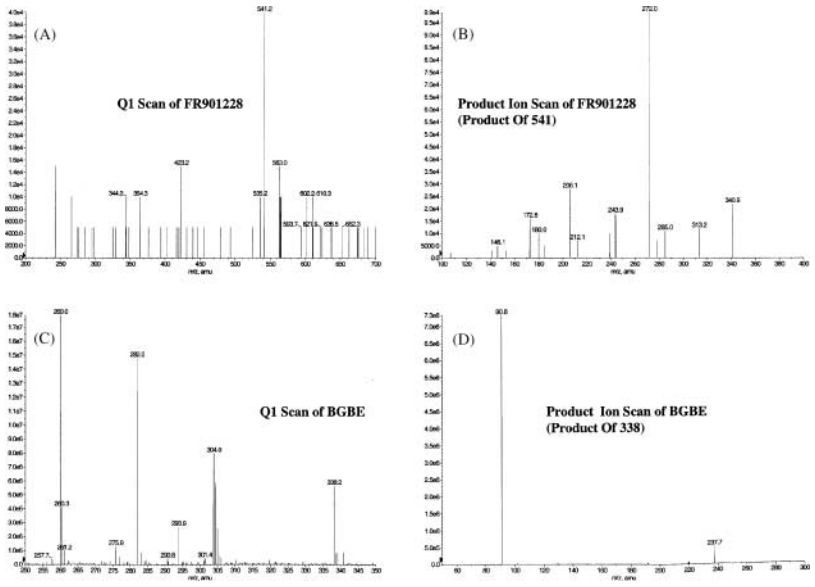

Figure 2. (A) Scan positive-ion mass spectra for parent molecular ion for FR901228 at m/z 541.0; (B) collision induced dissociation spectra for FR901228 to the predominant ion m/z 272.0; (C) scan positive-ion mass spectra for parent molecular ion for BGBE at m/z 338.0; (D) collision induced dissociation spectra for BGBE to the predominant ion m/z 91.0.

Sample Preparation

Standard Solutions

Stock solutions were prepared by separately dissolving either FR901228 or BGBE in 80% methanol to yield a concentration of 1.0 mg/mL. The stock solutions were stored at -80°C and lost less than 5% of their nominal values over 3 months. The working solutions (0.01, 0.1, 1.0, and 10.0 μg/mL) were prepared at the time of assay from the 1.0 mg/mL stock solutions by making dilutions with cold methanol.

Calibration Standards and Quality Controls

Calibration standards were made by adding FR901228 working solutions to plasma to give final concentrations of 1.0, 5.0, 30, 100, 300, 500, 700, and 1000 ng/mL. Quality controls were prepared to give final concentrations of 3.0, 60, and 800 ng/mL.

Plasma Sample Preparation

Spiked FR901228 plasma sample of 500 μL, or patient plasma, was added into a 15-mL amber glass tube at ambient temperature, then 16 μL of 1.0 μg/mL BGBE, 2.0 mL 0.5 N potassium acid phthalate (pH 4.0), and 6.0 mL ethyl acetate were added into each tube. The samples were vortexed at high speed for at least 5 min, and then centrifuged at $4000 \times g$ for 10 min at 4°C. The organic layer was transferred to a clean 15-mL amber glass tube and evaporated to dryness under a stream of nitrogen. The residues were immediately dissolved in 250 μL of mobile phase and a 50 μL aliquot was injected onto the LC-ISP-MS/MS system by autosampler.

Method Validation

The method developed for FR901228 quantitation in human plasma was validated over 5 days by analysis of quality control samples to determine within-day and between-day precision and accuracy. Two calibration curves were analyzed during this validation. The linear regression of the ratio of FR901228/BGBE peak area was weighted by $1/x^2$. The coefficient of determination (R^2) was used to evaluate the linearity of the calibration curve.

The limit of detection (LOD) and LLOQ were defined as the minimum value at which the ratio of S/N was ≥ 3 and 10, respectively. These were

determined by triplicate analysis of an extensive calibration curve in the low concentration range (0.01–1.0 ng/mL).

The stability of FR901228 in plasma at −80°C was evaluated in duplicate, at two concentrations (3.0 and 800 ng/mL) over 1 month. For FR901228 in reconstituted extracted plasma, we evaluated stability at two concentrations (3.0 and 800 ng/mL) and two different storage conditions (25°C and 4°C) for up to 2 days.

Patient Sample Collection

Patient blood samples (2 mL) were collected in green-top (heparin sodium) Vacutainer tubes (Franklin Lakes, NJ), aliquots were transferred to microcentrifuge tubes, and centrifuged at 7000 × *g* for 2 min to separate the plasma. The plasma samples were then processed as described for calibrators and controls.

RESULTS

Chromatography and Mass Detection

We used reversed-phase chromatography (Hypersil-Keystone Waters Spherisorb C8, 5 μ (100 × 2.1 mm^2 column) with a mobile phase consisting of 85% methanol in 12 mM ammonium acetate for best results. The MRM chromatograms of blank plasma and the plasma spiked with FR901228 and BGBE are shown in Fig. 3. As shown, FR901228 (541.0/272.0) and IS (338.0/91.0) were eluted at 3.29 and 2.65 min, respectively. The absence of chromatographic components at the retention time of both FR901228 and IS, in the blank plasma samples from six different sources established the selectivity of the assay (blank intensity ≤30 cps).

Method Validation

To assess within-day and between-day precision, and accuracy, we evaluated validation parameters for FR901228 (Table 1). The 10 injections of low, medium, and high quality control samples as described previously, were made on day 1 and day 2 to assess within-day variability, and again on day 3, 4, and 5 to evaluate between-day variability. The LOD and LLOQ in plasma for this method were 0.20 ng/mL ($S/N = 6.3$, $n = 3$) and 0.50 ng/mL ($S/N = 13.3$, $n = 3$, CV ≤ 11%), respectively. The extraction recovery was ∼75% at the concentrations of 3.0 and 800 ng/mL in triplicate.

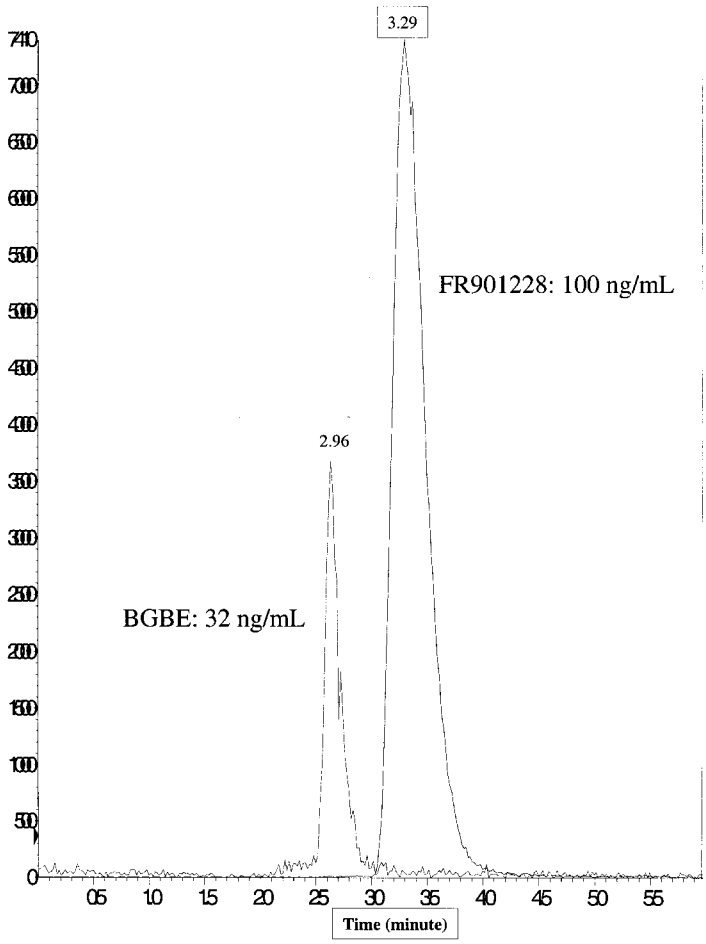

Figure 3. MRM chromatogram of plasma sample spiked with FR901228 and BGBE (IS), which eluted at 3.29 and 2.65 min, respectively.

The calibration curves for human plasma were linear from 1.0 to 1000 ng/mL, with correlation coefficients (R^2) greater than 0.997.

Stability

To test the stability of FR901228 in plasma at −80°C, we evaluated two concentrations (3.0 and 800 ng/mL) over 1 month. As depicted in

Table 1. Validation parameters of FR901228 in human plasma.

Quality control FR901228 (ng/mL)	Within-day ($n = 10$)		Between-day ($n = 12$)	
	RSD (%)	Error (%)	RSD (%)	Error (%)
3.0	2.7	12	3.4	6.8
60	4.7	2.0	4.8	1.6
800	3.5	0.3	5.5	1.9

Note: The 10 injections of low, medium, and high quality control samples were run within 1 day to assess within-day variability and 12 injections were run within 3 days to evaluate between-day variability. Variability reported as relative standard deviation (%RSD) and percentage error (%Error).

Table 2, for FR901228 in plasma at -80°C, the decrease of peak area ratio ($\triangle$FR901228 : BGBE) remained less than 10% within 1 month. Thus, FR901228 is stable in plasma at -80°C for at least 1 month. To assess the stability of FR901228 in reconstituted extracted plasma, we evaluated two concentrations (3.0 and 800 ng/mL) and two different storage conditions (25°C and 4°C) for up to 24 hr. As depicted in Table 3, for FR901228 in extracted plasma samples, we observed less than 10% decrease in peak area within 6 hr at 25°C, and a 10% decrease within 24 hr at 4°C. Thus, depsipeptide is considered stable in extracted plasma samples for 6 hr at 25°C and for 24 hr at 4°C.

Application of Method to Patient Samples

To show the applicability of this method, we analyzed plasma samples from a child enrolled on a phase I trial of FR901228. Serial plasma samples

Table 2. Stability of FR901228 in plasma at -80°C.

Plasma FR901228 (ng/mL)	1 hr	24 hr	5 days	9 days	16 days	22 days	30 days
3.0	100	96	92	93	90	94	93
800	100	100	94	94	90	94	91

Note: Spiked plasma samples with high and low FR901228 concentrations were aliquotted and stored at -80°C. The samples were then assayed over a month. Stability was assessed by the ratio of peak area ($\triangle$FR901228 : BGBE). Data presented is mean percent of initial peak ratio of two samples tested; ($n = 2$).

Table 3. Stability of extracted plasma FR901228 in mobile phase.

Extracted FR901228 (ng/mL)	25°C			4°C		
	1 hr	6 hr	24 hr	1 hr	6 hr	24 hr
3	100	90	88	100	91	93
800	100	91	87	100	92	93

Note: The reconstituted FR901228 samples were separately stored (in duplicate) at 25°C and 4°C and analyzed over 24 hr. Stability was assessed by the ratio of peak area (△FR901228 : BGBE). Data is expressed as mean percent of initial peak ratio of two samples tested; $n = 2$.

were collected in green-top (heparin sodium) Vacutainer tubes and centrifuged at $7000 \times g$ for 2 min to separate the plasma, then followed by liquid–liquid extraction. The reconstituted samples were analyzed by the method described in this report. A representative plasma concentration–time profile for FR901228 after intravenous administration is depicted in Fig. 4. All plasma samples were stored at −80°C until analysis.

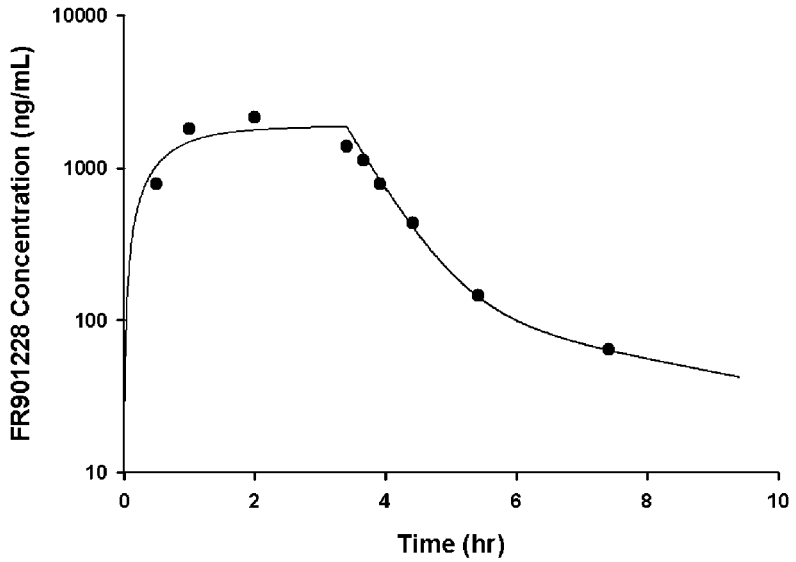

Figure 4. Concentration–time plot from a patient receiving FR901228 after intravenous administration of 10 mg/m^2. Individual time points in plasma (/) are plotted and the solid line represents the best-fit line from the pharmacokinetic analysis.

DISCUSSION

FR901228 does not have a strong chromophore amenable for UV detection at relatively long wavelengths; therefore, sensitivity for FR901228 detected in that manner is limited. Moreover, it is difficult to adequately resolve FR901228 and BGBE from other compounds in plasma matrix within 5 min with conventional detection methods.[8] This is especially a problem with plasma from children, in which complete chromatographic resolution is necessary to obtain precise quantitative results. To overcome these problems, we chose HPLC with mass spectrometry for this analysis. Since the manufacturer stopped producing the compound that had been used as IS (Met–Leu–Phe) in the previous publication,[9] we chose the compound BGBE as the IS in this method.

A 2.1 mm i.d., narrow-bore column instead of the 4.6 i.d. column used in a previous report[9] helped to achieve a flow rate of 0.2 mL/min, making it most compatible with the ESI interfaces of LC-MS without a flow split, and allowing the solvent to be saved for better routine practice. The 100 mm length has a very high plate count, which allows for a quick and extensive separation of FR901228 and BGBE for MS/MS quantitation. Furthermore, in comparison to a 4.6 mm id column, the 2.1 mm id narrow-bore column increases sensitivity by $4.6^2/2.1^2 = 4.8$ in theory[10,11] and reduces band broadening effects.[12] This allows for use of a larger volume of reconstituted solution (e.g., 250 μL) compared with 80 μL, which has been used previously[9] while maintaining a similar LLOQ and better peak shape.

The mean absolute recoveries of FR901228 determined in triplicate at 3.0 and 800 ng/mL were 73% and 75%, respectively. These values are close to the values reported by Li and Chan ($> 79\%$).[9] Although we did not find that potassium acid phthalate buffer (0.05 N, pH 4.0) helped recovery of FR901228 (68% without the buffer) from plasma, it significantly increased the recovery of BGBE, from 26% to 76%. Li's group also chose *N*-*t*-Boc-Met–Leu–Phe instead of BGBE as an IS for the reason of compound stability. We chose BGBE since the *N*-*t*-Boc-Met–Leu–Phe was no longer available commercially. We also determined the recovery of FR901228 by using *t*-butyl methyl ether instead of ethyl acetate, and found that recovery was 10% less with ethyl acetate (data not reported).

Mobile phases consisting of 45–85% methanol, acetonitrile, and mixture of both organics with 12 mM ammonium acetate were evaluated. Because a 2.1 mm id column was used at a flow rate of 0.2 mL/min, a higher organic percentage in mobile phase is needed in order to obtain a shorter eluting time. The results of methanol with ammonium acetate showed a better resolution and peak shape for FR901228 and BGBE than acetonitrile or acetonitrile with methanol in 12 mM ammonium acetate. Finally, 85%

methanol in 12 mM ammonium acetate as mobile phase, at an ambient temperature (22–25°C), produced the best resolution with a good peak shape and a favorable retention time (Fig. 2).

In the present method, we chose to use thermally and pneumatically assisted ESI-MS/MS with 2.1 id mm narrow-bore column and a simple mobile phase. We expected the FR901228 plasma concentrations in our clinical trials to extend over a rather broad range, which may be important when the method is applied to clinical pharmacokinetic studies in the pediatric population. According to a maximum of three orders range for a good linear fit,[13,14] the representative calibration curve was set from 1.0 to 1000 ng/mL. The least squares linear regression of the peak area ratio of FR901228/BGBE was weighted by $1/x^2$, which gives a better fit to points on the low end of the curve.[14–17]

The mass spectrometer conditions were optimized stepwise (from compound, ion source/gas, and resolution to detector) to produce the maximum m/z signal for both FR901228 and BGBE. The LLOQ ($S/N \geq 10$) for FR901228 is 0.5 ng/mL ($S/N = 13.3$). The validated calibration curves for plasma FR901228 were linear from 1.0–1000 ng/mL. The retention time of both FR901228 and BGBE was within 4 min. The accuracy of the method is reflected in our validation results, in which within-day and between-day measurements did not exceed 12% and 6.8% for FR901228, respectively (Table 1).

In summary, we have developed and validated an LC-ESI-MS/MS method for the rapid and precise quantitation of FR901228 in human plasma. The method described is both sensitive and specific, which makes it useful in performing clinical pharmacology studies of FR901228. Moreover, with the small sample requirement and sensitivity of this method ($S/N \geq 6.9$ at 0.2 ng/mL) it may also be useful for in vitro studies of FR901228, where low concentrations or small sample volumes may be expected. In addition, by using a narrow-bore column instead of 4.6 id column with lower flow rate, this assay saves solvent costs and reduces the environmental impact of the toxic solvent. Finally, we have successfully applied this HPLC-thermally ESI-MS/MS system with MRM mode method, by measuring FR901228 in human plasma from clinical pharmacokinetic studies in children treated with FR901228.

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