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A subnanogram API LC/MS/MS quantitation method for depsipeptide FR901228 and its preclinical pharmacokinetics

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Abstract

A highly sensitive and specific atmospheric pressure ionization (API) liquid chromatographic-tandem mass spectrometric (LC/MS/MS) method for the quantitation of depsipeptide FR901228 (NSC-630176, FR), a naturally occurring antitumor agent, was developed and validated. FR was extracted from human or rat plasma along with the internal standard, *t*-Boc-Met-Leu-Phe (BMLP) with ethyl acetate. Components in the extract were separated on a 5-μm C8 Spherisorb 50 × 4.6 mm i.d. column by isocratic elution with methanol/acetonitrile/12 mM ammonium acetate (60:10:30, v/v/v). The liquid flow was passed through a presource splitter and 5% of the eluate was introduced into the API source. The components were analyzed in the multiple-reaction monitoring (MRM) mode to enhance specificity. Linear calibration curves were obtained in the range of 0.1–100.0 ng/ml with 0.5 ml human plasma and 0.5–100.0 ng/ml with 0.1 ml rat plasma. The limit of quantitation (LOQ) was 0.1 ng/ml using 0.5 ml human plasma and 0.5 ng/ml using 0.1 ml rat plasma. The overall within-day precision was below 12% in human plasma and below 7% in rat plasma; and the between-day precision was below 10.2% in human plasma and 7.2% in rat plasma. The accuracy at low, medium and high levels ranged from 99.3 to 111.7% in human plasma and 96.2–107.3% in rat plasma. The high sensitivity permitted pharmacokinetic study of FR in the rat at a single i.v. dose as low as 1 mg/kg. At this dose, plasma FR levels declined biexponentially with a mean terminal $t_{1/2}$ of 187.7 min ($n = 6$) and were detectable up to 24 h. After an oral dose at 5 mg/kg, plasma FR levels were highly erratic and yielded a mean bioavailability of 1.6% ($n = 6$). At a higher oral dose of 50 mg/kg, a mean bioavailability of 10.6% was obtained, both being estimated by a non-crossover method.

Keywords: Depsipeptide; FR; API LC/MS/MS; Quantitation; Pharmacokinetics

1. Introduction

Depsipeptide FR901228 (NSC-630176, FR), (*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-pentan-

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one (Fig. 1 inset), was first isolated from *Chromobacterium violaceum* No. 968 in 1993 [1,2]. FR possesses potent antitumor activities against human tumor cell lines and significant inhibition effects on the growth of human solid tumors implanted in mice, including A549, MCF-7, M5076 and Colon 38. FR was also found to exhibit significant selectivity to tumor cells. Its IC_{50} value against P388 tumor cells is about 0.2 ng/ml and against human normal fibroblast cells is >1000 ng/ml [3]. A recent study in our laboratory has shown that growth-inhibition and apoptosis on oncogene-transformed cells induced by FR may involve blockage of the mitogen-activated signaling pathway, stimulation of a p53-independent expression of p21Cip, and phosphorylation of Bcl-2 [4]. Recently, FR has been found to be a potent histone deacetylase inhibitor [5].

Initially, a HPLC-UV assay with a detection limit of 100 ng/ml [6] was developed and this assay was adequate to support FR pharmacokinetics in mice at a high dose of 10 mg/kg [6]. However, since this compound is very potent and rather toxic, the eventual clinical dose in humans will be significantly lower than 10 mg/kg. Recently, an API LC/MS/MS method for quantitation of FR with a good sensitivity of 1.0 ng/ml was reported [7]. However, the sensitivity of 1.0 ng/ml was still considered inadequate for the

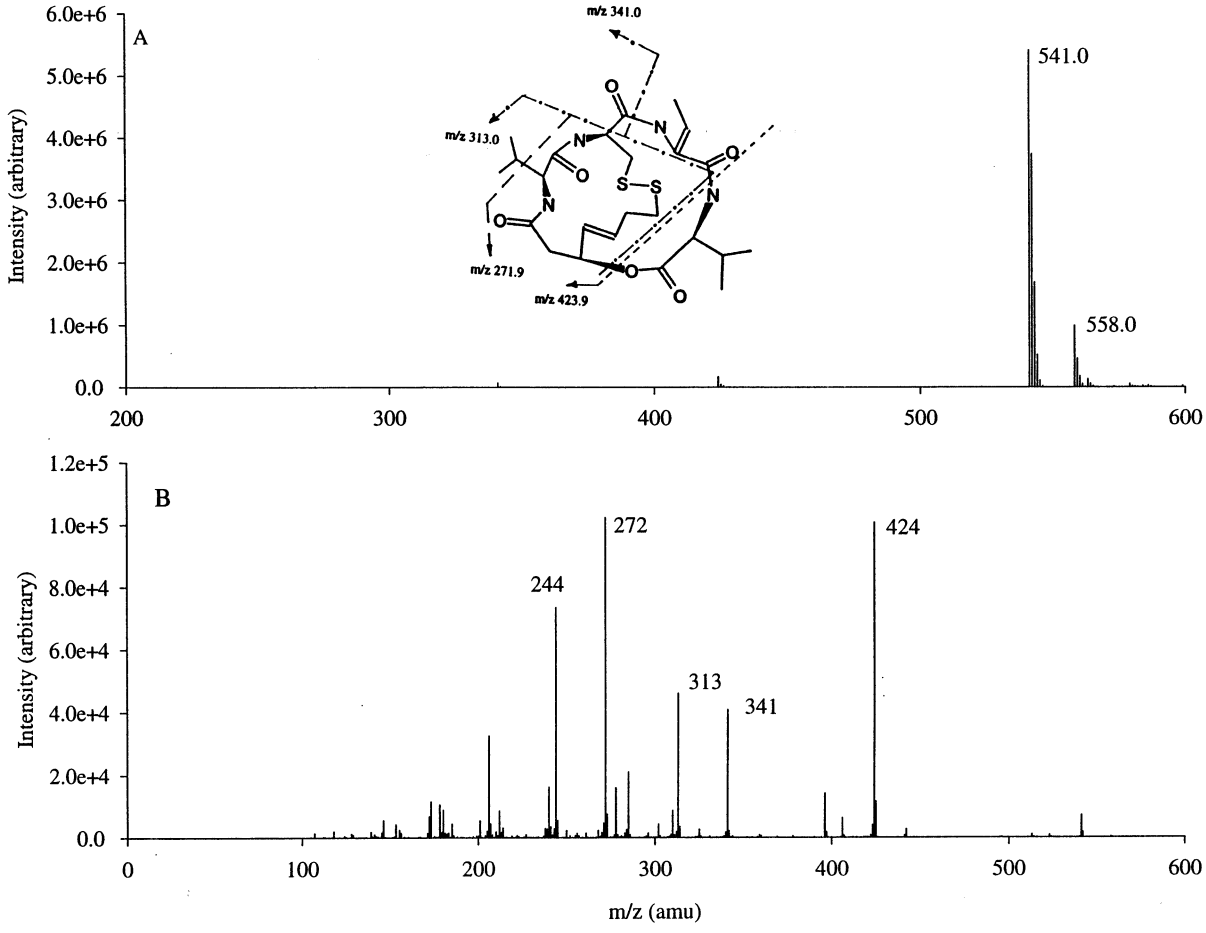

Fig. 1. (A) LC/electrospray mass spectrum of FR. The ions are: m/z 541.0, $(MH)^+$ and m/z 558.0, $(MNH_4)^+$. (B) Collision-induced dissociation mass spectrum of FR. The parent ion is at m/z 541.0. Inset: the proposed fragmentation pathway.

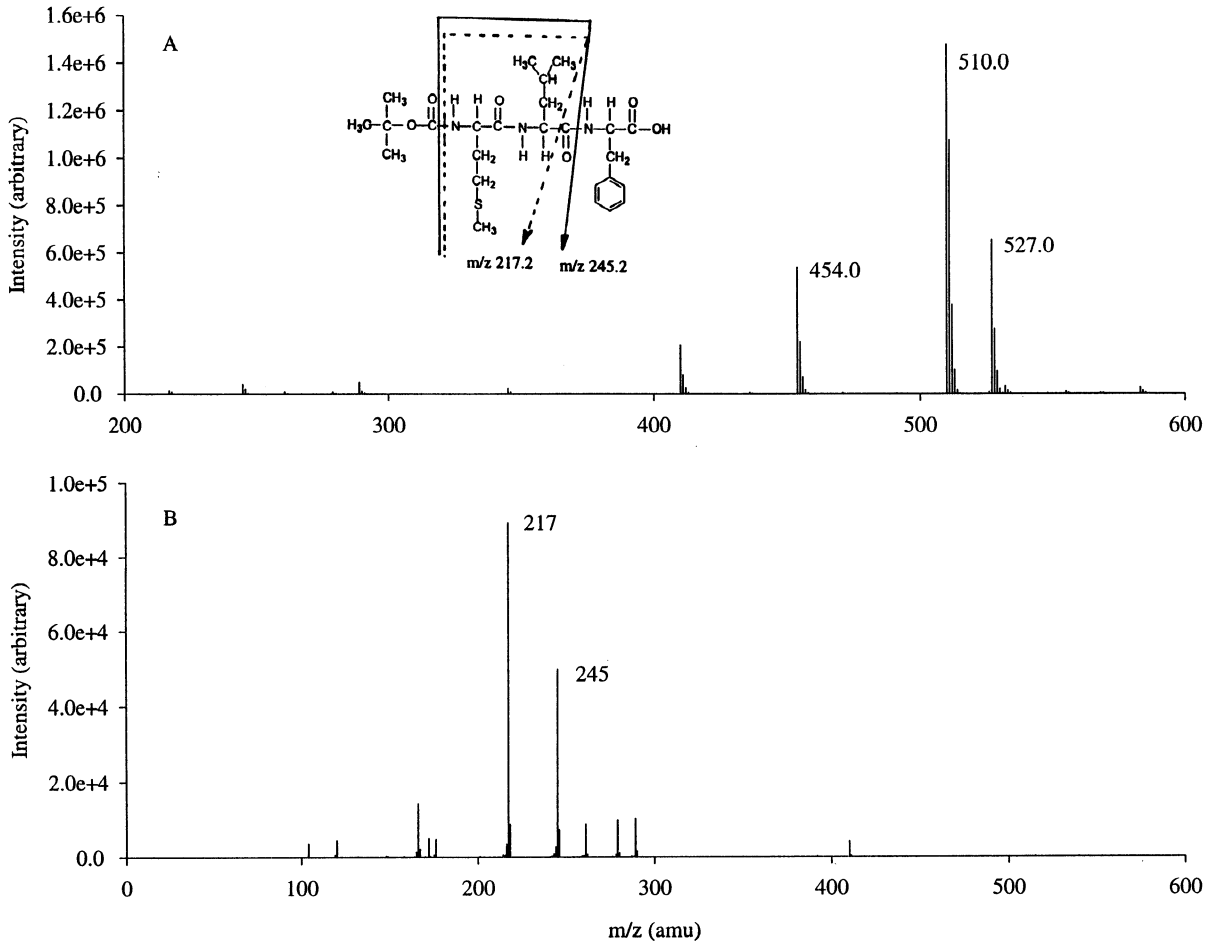

Fig. 2. (A) LC/electrospray mass spectrum of BMLP. The ions are: m/z 510.0, $(MH)^+$ and m/z 527, $(MNH_4)^+$. (B) Collision-induced dissociation mass spectrum of BMLP. The parent ion is at m/z 510.0. Inset: the proposed fragmentation pathway.

monitoring of this compound in the ensuing Phase I clinical pharmacokinetic studies. In addition, the coefficients of variation of the LC/MS method were rather large possibly due to the instability of the internal standard used.

In this report, we present an improved API LC/MS/MS method which provides a routine sensitivity limit of 0.1 ng/ml using 0.5 ml human plasma and 0.5 ng/ml using 0.1 ml rat plasma. Using this method, pharmacokinetic studies of FR in the rat using clinically applicable doses by i.v. and p.o. routes were revisited.

2. Experimental

2.1. Materials

FR (purity > 99% by LC/MS) was provided by the Drug Synthesis and Chemistry Branch, the National Cancer Institute (Bethesda, MD) and used without further purification. The internal standard, *N*-*t*-Boc-Met-Leu-Phe (Fig. 2 inset, BMLP, purity > 97%), was obtained from Sigma (St Louis, MO) and used without further purification. Potassium phthalate buffer (pH 4, 50 mM)

was obtained from Van Water and Roger Scientific (Chicago, IL). All organic solvents were obtained from Fisher Scientific (Pittsburgh, PA) and were of HPLC grade. The HPLC-grade water (>18 mΩ) was obtained from an E-pure water purification system (Barnstead, Dubuque, IA).

2.2. LC/MS/MS assay

The LC/MS system used consisted of a Perkin-Elmer Sciex API 300 triple-quadruple mass spectrometer (Thornhill, Ontario, Canada) coupled to a Shimadzu HPLC system (Shimadzu, Columbia, MD). The HPLC system was equipped with an SCL-10A system controller, an LC-10AD pump, a GT-104 degasser and an SIL-10A autosampler. The components were separated on a 50×4.6 mm Spherisorb 5-μm C8 stainless steel column (Keystone, Bellefonte, PA) which was coupled to a 2-μm precolumn filter (Upchurch, Oak Harbor, WA) and a 20×4.6 mm Spherisorb 5-μm C8 guard column (Keystone, Bellefonte, PA). The components were eluted with a mobile phase consisting of 60% methanol, 10% acetonitrile and 30% 12 mM ammonium acetate at a flow rate of 0.55 ml/min. The run-time was 4 min. The LC eluate was introduced into the API source at 27.5 μl/min after a 95:5 (LC/MS) split.

The mass spectrometer was operated using an atmospheric pressure ionization (API) with an ionspray voltage of $+5000$ V. The positive ion multiple reaction monitoring (MRM) mode was performed using nitrogen as the collision gas. The curtain gas (nitrogen) flow and the Ionspray flow were set at 0.95 and 1.02 l/min, respectively. The pressure in the collision cell was set at 2.37 mTorr. The orifice voltage and ring voltages were set to $+45$ and $+400$ V, respectively. A dwell time of 800 ms and a pause time of 5 ms between scans were used to monitor the following precursor/product ion pairs: m/z 541.0/272.0 for FR and m/z 510.0/217.0 for BMLP. The mass spectrometer was tuned to its optimum sensitivity and mass accuracy by infusion of a standard calibration solution of polypropylene glycol (PPG) on a daily basis. This tuning was further adjusted by injection of a fresh standard solution of FR at 1.0 ng/ml in the HPLC mobile phase as described above. Data acquisition was performed using the PE Sciex software Sample Control 1.2 and the data were analyzed by PE Sciex software MacQuan 1.4.

2.3. Sample extraction

FR and BMLP in plasma were extracted by ethyl acetate. To each polypropylene centrifuge tube was added 0.5 ml of human sample plasma (or 0.1 ml of rat sample plasma plus 0.4 ml blank rat plasma), 0.1 ml of the internal standard solution at 10 ng/ml in acetonitrile, and 2.0 ml of potassium phthalate buffer (pH 4.0; 50 mM). The content was allowed to stand at room temperature for 1 min. The solution was then extracted with 6 ml ethyl acetate and the mixture was centrifuged at $1000 \times g$ for 5 min. The organic layer was transferred to a clean polypropylene tube and evaporated to dryness under a stream of nitrogen. The residues were dissolved in 80 μl of the HPLC mobile phase and a 40-μl aliquot was introduced into the LC/MS system for analysis.

2.4. Assay validation

Plasma samples for the standard curves were prepared by spiking 0.5 ml human plasma or 0.1 ml rat plasma each with various amounts of FR and a constant amount of BMLP. The standard curves were obtained in the concentration range of 0.1–100 ng/ml in human plasma and 0.5–100 ng/ml in rat plasma. The within-day precision values were determined in six replicates at each concentration of 0.1, 0.2, 0.5, 5.0 and 100.0 ng/ml of FR in human plasma and 0.5, 1.0, 5.0 and 100.0 ng/ml of FR in rat plasma. The between-day precision was determined across six concentrations at different days and the mean concentrations and the coefficients of variation were calculated. The accuracy of the assay was determined by comparing the nominal concentrations with the corresponding calculated concentrations via linear regression. The specificity of the assay was established by simultaneously monitoring more than one major product ions from the molecular ion of FR in blank plasma; no trace of interference substance was found at the same

retention time as FR.

The recovery of FR and BMLP was also evaluated. The recovery study for FR was accomplished at three concentration levels (0.5, 5.0, 100.0 ng/ml) in human plasma and at one concentration level (20.0 ng/ml) in rat plasma. The recovery of BMLP was evaluated at one concentration level (10.0 ng/ml) in human or rat plasma.

2.5. Pharmacokinetic study of FR in rats

Sprague–Dawley male rats weighing about 300 g were used in the pharmacokinetic studies. For single i.v. dose, the right femoral and left jugular veins of six rats (rat no. 1–6) were cannulated. The rats were allowed to recover for about 1 h prior to drug administration. A 0.4-ml aliquot of blank blood was collected through the jugular vein cannula. FR, formulated in 40% ethanol, 5% PEG 400, and 55% normal saline, was given at 1 mg/kg as an i.v. bolus dose through the femoral vein cannula. Approximately 0.4 ml each of heparinized blood was withdrawn according to a typical schedule of 0 (predose), 2, 5, 10, 20, 30, 60, 120, 180, 240, 360, 600 and 1440 min after dosing, and the loss of fluid was replaced by flushing the cannula with an equal volume of normal saline. The blood samples were centrifuged at $2000 \times g$ for 10 min and the supernatants of each were collected and kept frozen at -80°C until analysis.

For single p.o. dose, only the left jugular vein was cannulated. The oral pharmacokinetics was studied at two dose levels, 5 and 50 mg/kg. Six rats (rat no. 7–12) for the low dose and four rats (no. 13–16) for the high dose were fasted overnight and cannulated. Formulated FR was given at each dose by gavage. Following a typical schedule of 0 (predose) 5, 10, 15, 25, 40, 60, 90, 120, 150, 180, 210, 240 and 360 min for the low dose and 0, 2, 5, 10, 15, 20, 30, 60, 120, 180, 240, 360 and 600 min for the high dose after dosing, approximately 0.4 ml each of heparinized blood was withdrawn at each time and processed using a similar procedure as described for the i.v. dose study.

3. Results

3.1. LC/MS/MS assay

Under the API conditions, FR gave two ions, a MH^+ ion at m/z 541 and a $M(NH_4)^+$ ion at m/z 558 (Fig. 1A). The MH^+ ion was selected for collision-induced dissociation (CID) experiment which generated five major product ions at m/z 424, 341, 313, 272, and 244 (Fig. 1B). The amide bonds and the two 3° carbon atoms linked by the disulfide bridge of FR were possibly the primary sites for fragmentation (Fig. 1 inset). The precursor/product ion pair at m/z 541/272 was selected in the MRM mode for quantitation of FR. Similarly, the BMLP generated MH^+ ion at m/z 510 and $M(NH_4)^+$ at m/z 527 (Fig. 2A). Under the CID condition, the product ion scan of BMLP generated two major ions at m/z 217 and 245 (Fig. 2B). The cleavage of the ester bond for the t-boc group and loss of the phenylalanine at the C-terminal gave the most intense fragment ion of m/z 217 for BMLP. The loss of the entire t-boc group and the phenylalanine resulted in the other major fragment ion at m/z 245 (Fig. 2 inset). Since the ion at m/z 217 was the most abundant ion, the precursor/product ion pair at m/z 510/217 was selected for the assay.

3.2. Assay validations

The assay was validated in both human plasma and rat plasma. The MRM chromatograms of plasma spiked with FR and blank human plasma are shown in Fig. 3. As shown, FR and the internal standard were baseline separated (Fig. 3A). The absence of chromatographic components at the retention times of FR and the internal standard in the blank established the specificity of the assay (Fig. 3B). Similar results were found in rat plasma (data not shown). The limit of quantitation (LOQ) was set at 0.1 ng/ml in human plasma and 0.5 ng/ml in rat plasma, both of which gave a signal-to-noise level above 10:1 (Fig. 3C,D).

The assay was linear from 0.1 to 100.0 ng/ml using 0.5 ml of human plasma and 0.5 to 100.0 ng/ml using 0.1 ml of rat plasma. The within-day

precision, expressed as the coefficients of variation (% C.V.), for human plasma are shown in Table 1. As shown, the values ranged from 3.5 to 11.9%. The within-day C.V.s for rat plasma are shown in Table 2. As shown, the values ranged from 3.7 to 6.8%. The between-day precision values ranged

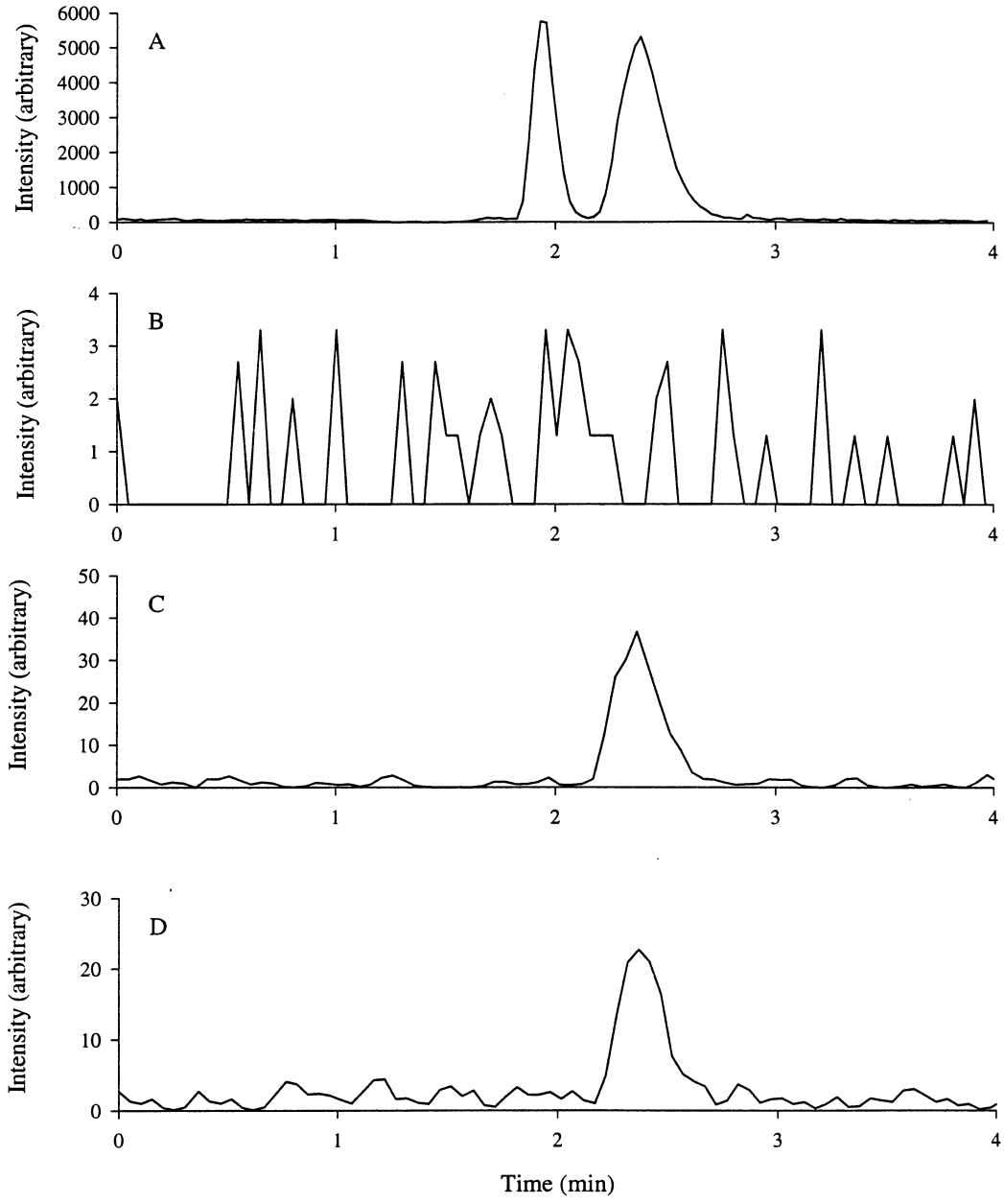

Fig. 3. (A) The ion chromatogram of human plasma spiked with FR (20 ng/ml) and BMLP (10 ng/ml); (B) The extracted ion chromatogram of FR in blank human plasma; absence of a peak at the corresponding retention time indicated that no significant chemical interference existed. (C) The extracted ion chromatogram of FR in human plasma spiked with 0.1 ng/ml FR. (D) The extracted ion chromatogram of FR in rat plasma spiked with 0.5 ng/ml FR.

Table 1
Assay validation characteristics of FR in rat plasma by API LC/MS/MS

	Concentration (ng/ml plasma)						
	0.1	0.2	0.5	1	5	20	100
Within-day							
Calculated	0.10	0.19	0.53		5.34		118.3
	0.12	0.20	0.47		5.34		103.8
	0.085	0.21	0.45		5.53		106.8
	0.11	0.23	0.47		5.76		105.6
	0.093	0.19	0.54		5.69		123.1
	0.10	0.20	0.53				103.8
Average ± S.D.	0.10 ± 0.012	0.20 ± 0.015	0.50 ± 0.041		5.53 ± 0.195		110.2 ± 8.34
C.V. (%)	11.9	7.5	8.2		3.5		7.6
Accuracy	101.0	102.4	99.3		110.7		110.2
Between-day							
Day 1	0.12		0.52	0.87	4.82	18.4	102.2
Day 2	0.096		0.49	0.95	5.24	21.4	101.2
Day 3	0.11		0.49	1.02	4.79	19.2	95.5
Day 4	0.12		0.53	1.06	4.91	19.7	97.8
Day 5	0.10		0.59	1.03	5.16	20.5	100.7
Day 6	0.098		0.47	1.04	5.20	19.5	103.5
Average ± S.D.	0.11 ± 0.011		0.52 ± 0.043	1.0 ± 0.072	5.02 ± 0.203	19.8 ± 1.05	100.2 ± 2.97
C.V. (%)	10.2		8.3	7.2	4.0	5.3	3.0

Table 2
Assay validation characteristics of FR in rat plasma by API LC/MS/MS

	Concentration (ng/ml plasma)					
	0.5	1	2	5	20	100
Within-run						
Calculated	0.50	0.99		5.38		103.1
	0.53	0.92		5.31		102.3
	0.45	0.93		5.57		101.4
	0.48	0.99		5.08		89.9
	0.45	1.01		5.20		101.2
	–	0.97		5.65		102.1
Average ± S.D.	0.48 ± 0.033	0.97 ± 0.036		5.37 ± 0.219		100.0 ± 5.01
C.V. (%)	6.8	3.7		4.1		5.0
Accuracy	96.2	97.0		107.3		100.0
Between-day						
Day 1	0.45	0.91	2.18	5.10	21.2	103.5
Day 2	0.54	1.01	2.03	4.76	20.5	96.4
Day 3	0.51	0.96	1.96	5.15	18.6	97.6
Day 4	0.47	0.92	1.94	5.24	19.4	107.2
Day 5	0.53	1.05	2.07	4.81	20.2	112.4
Day 6	0.48	0.97	1.89	4.89	22.1	99.7
Average ± S.D.	0.50 ± 0.036	0.97 ± 0.053	2.01 ± 0.105	4.99 ± 0.198	20.3 ± 1.25	102.8 ± 6.16
C.V. (%)	7.2	5.5	5.2	4.0	6.1	6.0

from 3.0 to 10.2% for human plasma and from 4.0 to 7.2% for rat plasma. The accuracy of the assay varied in the range from 99.3 to 110.7% in human plasma and from 96.2 to 107.3% in rat plasma. The recovery values of FR for the entire procedure were found to be 86, 89 and 85% at 100.0, 5.0, and 0.5 ng/ml, respectively, in human plasma and 79% at 20.0 ng/ml in rat plasma (all $n = 6$). The recovery value for the internal standard at 10 ng/ml was 78% in human plasma and 75% in rat plasma.

3.3. Pharmacokinetics of FR in the rat at low i.v. and oral dose

Using the LC-MS-MS method, pharmacokinetics of FR after i.v. dose and p.o. dose in S–D male rats were studied. Following i.v. dosing at 1 mg/kg in the rat, plasma concentration–time profiles of FR showed a biexponential decay and the concentrations were detectable up to 24 h (Fig. 4). The concentration–time profiles were fitted to a two-compartment model (WinNonlin) and the relevant pharmacokinetic parameters were computed (Table 3). As shown, the initial half-life was about 18.4 min and the terminal half-life was about 3 h. The total clearance was found to be 824 ml/min per kg.

Pharmacokinetics after p.o. dosing at 5 mg/kg were assessed using the model-independent method. Because the plasma FR levels showed irregular patterns (Fig. 5), the bioavailability of FR was determined using the AUC value from the p.o.versus that from i.v. to the last collected datapoint (360 min) and found to be 1.6% ($n = 6$) (Table 3).

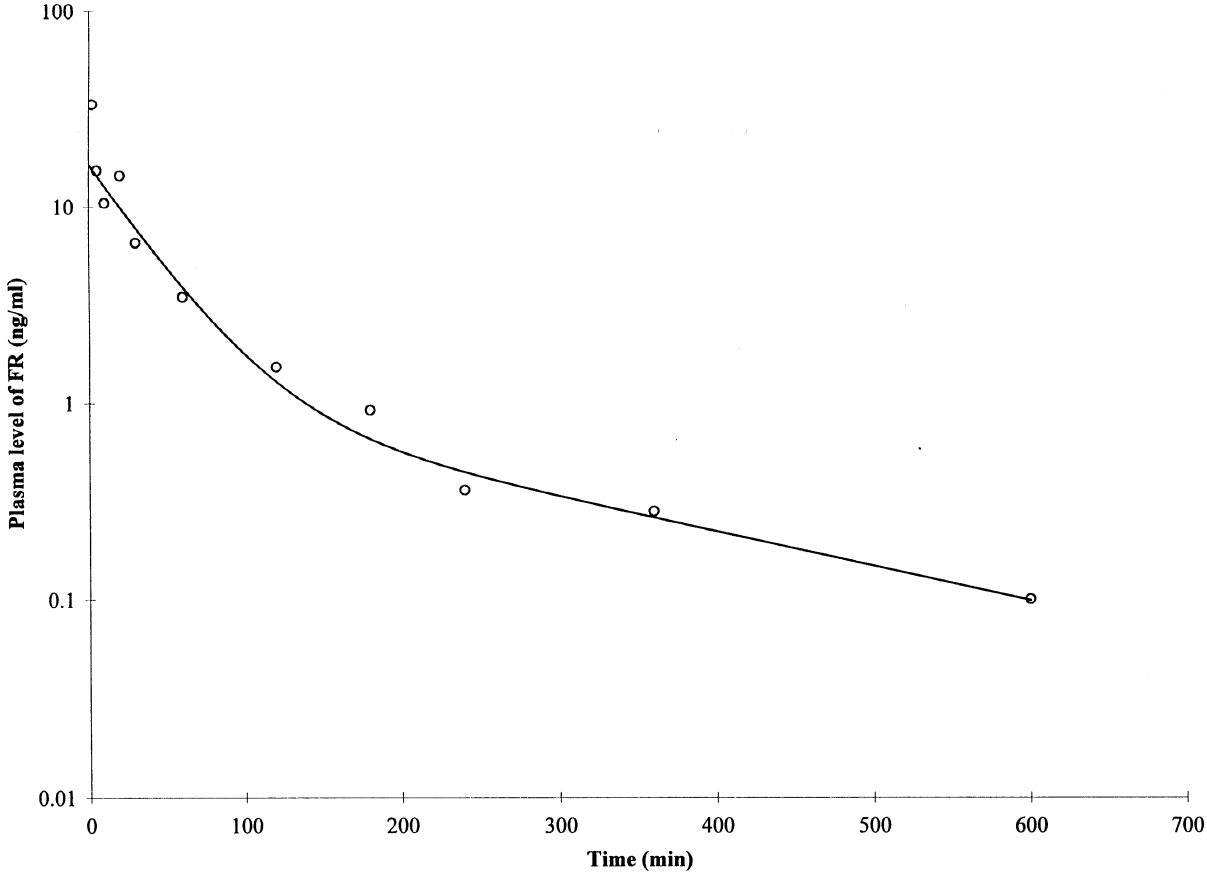

Fig. 4. A representative plasma concentration–time profile of FR in the rat following its i.v. administration at 1 mg/kg; (○) measured concentrations, (—) line fitted to a two-compartmental model.

Table 3
Relevant pharmacokinetic parameters of FR in rats receiving the drug i.v. at 1 mg/kg or p.o. at 5 mg/kg or p.o. at 50 mg/kg

PK parameters	Average ± S.D. ($n = 6$)
A. i.v. at 1 mg/kg	
C_0 (ng/ml)	42.9 ± 26.2
A (ng/ml)	40.7 ± 25.4
α (min^{-1})	0.046 ± 0.023
$t_{1/2\alpha}$ (min)	18.4 ± 8.6
B (ng/ml)	2.2 ± 2.2
β (min^{-1})	0.004 ± 0.0028
$t_{1/2\beta}$ (min)	187.7 ± 87.8
MRT (min)	117.2 ± 80.8
Cl_t (ml/min per kg)	824 ± 247
Vd_{ss} (l/kg)	100 ± 80.7
AUC_∞ (ng min per ml)	1625 ± 562.3
AUC_{360} (ng/ml per min)	1406 ± 462.6
B. p.o. at 5 mg/kg	
$AUC_{p.o.}$ (ng min per ml)	110.0 ± 49.8
F ($AUC_{p.o.}/AUC_{i.v.}$) (%)	1.6 ± 0.71
C. p.o. at 50 mg/kg	
C_{max}	141.2 ± 65.6
T_{max}	12.5 ± 11.9
β (min^{-1})	0.0025 ± 0.0014
$t_{1/2\beta}$ (min)	333 ± 129
$MRT_{p.o.}$ (min)	942 ± 867
AUC_∞ (ng min per ml)	8632 ± 6866
AUC_{last} (ng/ml per min)	3974 ± 2013
F_1 (%)[a]	5.7 ± 8.7
F_2 (%)[b]	10.6 ± 24.4

[a] F_1 is calculated using the AUC_{last}.
[b] F_2 is calculated using the AUC_∞.

Following a high single p.o. dose at 50 mg/kg, plasma FR concentrations showed a biexponential decay after reaching the C_{max} (Fig. 5B). The mean peak concentration of 141.2 ng/ml occurred at about 13 min (Table 3). Due to the fast absorption behavior of this drug and the lack of data points during the absorption phase, only the apparent terminal phase was estimable. The mean terminal half-life was 333 min. The bioavailability of FR was estimated to be 5.7% up to the last time point monitored and 10.6% up to time infinity by the non-crossover method.

4. Discussion

When the clinical trial of FR was initiated recently, previously developed methods [6,7] were found not sensitive enough for the monitoring of this agent in plasma in the clinical pharmacokinetic studies. For this reason, we developed a more sensitive LC-MS-MS assay using electrospray MS.

The LC/MS/MS assay using atmospheric pressure chemical ionization (APCI) has been widely used for quantitation of drugs [7–16]. It has been reported that the use of APCI can reduce the matrix effect from biological fluids and to increase sensitivity due to its compatibility with a higher flow rate than that used in atmospheric pressure ionization (API) [16]. During the early stage of method development, we examined the effects of these two different ionization sources, API and APCI, on sensitivity. No significant gain in sensitivity by the use of APCI was found. The API source was therefore selected due to its simplicity in use and the better response of the internal standard under a milder ionization condition of API.

Initially, the selected ion monitoring mode (SIM) was used and significant interference was found at the retention times of interest (data not shown). In contrast, using the MRM mode, the background interference was completely eliminated and it was therefore selected for the present assay.

The current method has achieved a significantly higher sensitivity when compared with the previously developed LC/MS/MS assay [7]. Several factors contributed to this improvement. The major factor was the modification of the extraction condition, which yielded a higher extraction recovery and a lower background. The pH control by the phthalate buffer solution increased the extraction recovery to over 79% with concomitant lowering of the plasma background. The matrix components coeluting with the analytes, although precluded from MRM detection, may still suppress the ionization of the analytes and thereby decrease the sensitivity [16]. By reducing the plasma background via pH control, more efficient ionization and thus higher sensitivity could be

achieved. In the current extraction procedure, we have found that the control matrix (blank plasma extract) did not significantly suppress the FR signal. Additionally, we have found that the use of a different internal standard from the previous assay also contributed to the improved sensitivity. The previously used internal standard with *t*-boc-D-glutamic acid alpha benzyl ester underwent degradation in certain biological matrices and this instability increased the coefficients of variation of the assay. No degradation was observed for the new internal standard BMLP after overnight incubation in plasma at 37°C (data not shown).

Another advantage of this LC/MS/MS method is the capacity to accommodate a higher mobile phase flow rate, which enabled a very short run-time for each sample. Since sensitivity is proportional to the concentration instead of the mass flow, the use of post-column splitting did not cause any loss of sensitivity. This split, in fact, helped to improve the liquid spray which is very critical to achieve the high sensitivity. The run-time for each sample on LC-MS-MS was 4 min only, allowing high sample throughout.

Using the present method, pharmacokinetics of FR was investigated in the rat following a low i.v. dose of 1 mg/kg. Plasma FR levels were detectable for at least 10 h and in some cases for 24 h. In contrast, the previous LC/MS/MS method detected FR levels for only up to 3–5 h following a higher i.v. dose at 10 mg/kg [7]. The drug showed a rather high total clearance, which sug-

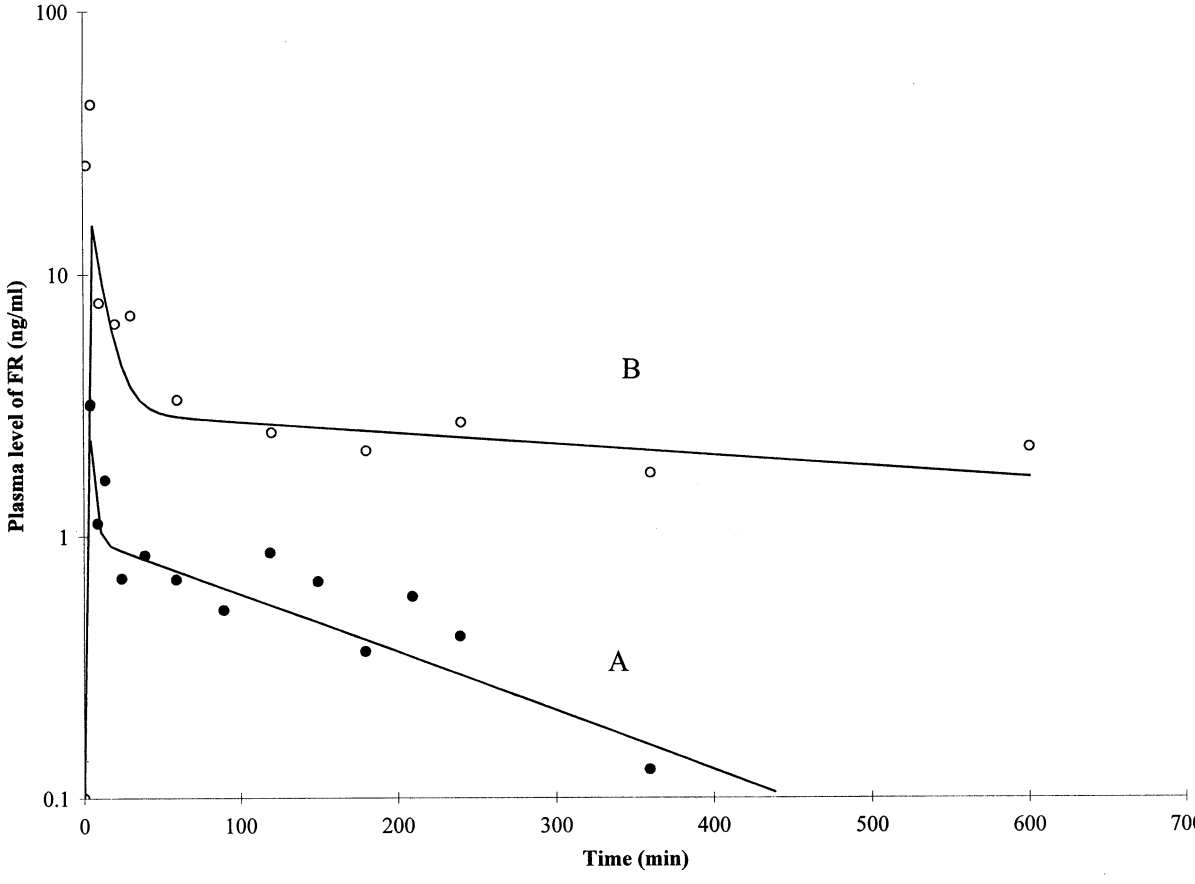

Fig. 5. Representative plasma concentration–time profiles of FR in rats following p.o. administration at 5 mg/kg (A) and 50 mg/kg (B); symbols represent measured concentrations and lines are fitted to a two-compartmental model with first order input.

gests that the hepatic clearance might not be the only pathway. Other clearance mechanisms, such as renal and uptake by the red blood cells and clearance from the blood vessel walls, may be involved as well.

The bioavailability of FR in this study was evaluated by a non-crossover method. The reason for the selection of this approach was twofold. First, even with this highly sensitive method, the potentially low absorption of this drug may require the use of a relatively high oral dose in order to produce measurable drug levels. Secondly, since FR is rather toxic, the rats would not survive multiple experiments in a crossover study. Previously, based on the limited number of animals and short time duration of drug monitor (5 h), Chan et al. [7] found that the oral bioavailability of FR was 15.6% following an oral dose of 50 mg/kg as compared to an i.v. dose of 10 mg/kg. Using this new assay method, the oral bioavailability of FR was re-evaluated at 5 and 50 mg/kg in a larger number of animals also in reference to an i.v. dose of 1 mg/kg. The oral bioavailability of FR as estimated up to the last measured time point of 6 h was 1.6%, considerably lower than the previously reported value. At the higher oral dose (50 mg/kg), although the high drug toxicity precluded sampling beyond 10 h in all animals, the plasma FR concentrations appeared to be less erratic allowing assessment of the terminal half-life by curve-fitting. Therefore, the oral bioavailability value was estimated based on the AUC values calculated to time infinity. This yielded the oral bioavailability value of 10.6%. This value is also lower than the previously reported value at the same dose [7]. This discrepancy might be due to the fluctuation of the previous data and to the better measurement of AUC in the current case. We think that the quality of oral data and the method of calculation (AUC to time infinity) made the bioavailability value of 10.6% more reliable. This bioavailability is also higher than that of 1.6% for the lower dose. It is possible that the oral clearance may be higher at a lower dose, resulting in a lower AUC at the lower oral dose.

In a recent Phase I clinical trial of FR at the National Cancer Institute, we employed this assay method and have detected sub- to low ng/ml levels of FR in patients treated with the drug 4-h infusion at 1 mg/m^2 (W. Figg, pers. commun.).

5. Conclusion

A highly sensitive and specific LC/MS/MS method for the quantitation of FR has been developed. The method has been validated with a routine sensitivity limit of 0.1 ng/ml in 0.5 ml human plasma and 0.5 ng/ml in 0.1 ml rat plasma. Using this method, pharmacokinetics of FR at 1 mg/kg following the i.v. route in the rat was investigated. The plasma levels of the drug have been detected at lower levels and for longer duration than previously documented. The oral pharmacokinetics of FR at a low dose of 5 mg/kg and a high dose of 50 mg/kg were also studied. FR showed a bioavailability of 10.6%, although it is considered high for a peptide drug. The assay has been successfully applied to preliminary clinical studies. The assay will provide a valuable tool to generate more information and reliable pharmacokinetic parameters for clinical studies of FR in the near future.

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