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Liquid chromatographic determination of FP-21399 in plasma of patients with HIV infection

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Abstract

A high-performance liquid chromatographic (HPLC) analysis method for the novel anti-HIV drug FP-21399 in human plasma was developed. The method employed the combination of organic solvent extraction and solid phase extraction. Analysis of FP 21399 and two major metabolites was achieved within 18 min using a reverse phase Puresil Cl 8 analytical column (4.6 × 150 mm, 5 μm, Waters) with a mobile phase of water-acetonitrile containing 20 mM triethylamine acetate (apparent pH 7.0). Linear gradient of mobile phase was applied as water-acetonitrile from 78:22 (v/v) to 55:45 over 8 min, and held at this ratio for the next 4 min. An ultraviolet-visible detector was operated at 265 mn from 0 to 8 min and at 600 nm from 8 min and after. The retention time of FP-21399 was 8.8 min and a linear response was observed over the concentration range 0.01–100 μg ml^{-1} ($r = 0.994$). Lower limit of quantitation was found to be 0.01 μg ml^{-1}. Intra- and inter-assay precision varied in the range of 0.2 to 8% and 1–12%, respectively. The bias ranged from −17–3% for all analyses. A series of clinical plasma specimens were successfully analyzed using this method. The strategies for the method optimization on HIPLC separation and extraction procedure are discussed as well.

Keywords: FP-21399; High-performance liquid chromatography; Solid phase extraction; Human plasma; HIV; Fusion inhibitor

1. Introduction

FP-21399 (1), a newly developed anti-HIV drug, targets the entry step of the HIV-1 replication cycle. It inhibits HIV envelope protein (gp120/41)-mediated cell fusion and appears to be interacting with the V3 loop of gp120 [1,2]. This interaction seems to be important for the action of the drug. This unique feature explores a new class of anti-HIV medicine and brings an additional candidate for combination therapy [3]. Recently the research of fusion inhibitors, which is targeted to the interaction of HIV gp120/41 and the coreceptors on the host cell surface, is becoming a hot field [4–7].

In the pharmacokinetic studies using rodents and dogs, the drug tended to accumulate in lymph nodes selectively compare to other major organs

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[1]. This tendency is another special characteristic making this drug more interesting because lymph nodes are the primary place where the battles of HIV versus defense system take place [3]. For the pharmacokinetic studies in animals, Nemori et al. of Fuji Photo Film have developed the methods for quantitation of FP-21399 and two primary metabolites in plasma and organs (unpublished). Our study was based on their method and was designed to make a new method applicable for human plasma samples potentially containing HIV and other biohazardous agents.

The determination of plasma concentration of metabolites would be also important for examining the detailed pharmacokinetic profile of the drug. This study was intended to develop a detection method for the metabolites as well as the parent compound.

2. Experimental

2.1. Chemicals

FP-21399 was supplied by Fuji Photo Film. HPLC grade acetone, acetonitrile and water were obtained from EM science (Gibbstown, NJ) with certification documents. Dimethylsulfoxide (DMSO), triethylamine, acetic acid and trifluoroacetic acid were all analytical grade and purchased from EM science. Triethylamine acetate butter was prepared by the following method. Aqueous solution of acetic acid was prepared as 2.5 M. Then the solution was slowly neutralized with triethylamine until pH became 6.8–7.0. The solution was adjusted to 2.0 M of acetate then filtered though 0.22 μm filter and stored at room temperature. The reagent containing tetrabuthylammonium phosphate (Pic A Low UV) was a premixed product of Waters (Milford, MA). The LC isocratic standard sample was obtained from Hewlett Packard.

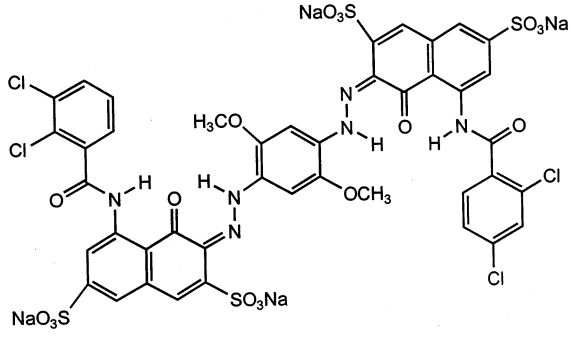

Fig. 1. Chemical structure of FP-21399.

2.2. Instrumentation

A model LC1050 liquid chrornatograph (Hewlett Packard, Wilmington, DE) was equipped with quaternary pump, autosampler and variable wavelength detector. The Chemstation LC2D software (version 2.4) operated the system and processed the data. The chromatographic separation was performed on a Puresil C 18 analytical column (4.6 × 150 mm, 5 μm, Waters) with guard cartridge (2.1 × 20 mm, 5 μm), which was set in a column heater maintained at 30°C. Water–acetonitrile ratio was shifted in a linear manner from 78:22 to 55:45 (v/v) in the initial 8 min after injection, and this ratio sustained at 55:45 for the following 4 min. The flow rate was 0.6 ml min^{-1} and 20 mM of triethylamine acetate buffer was added to maintain pH at 7.0. Detector was set at 600 nm.

A dual beam spectrophotometer (Lambda 6, Perkin Ehner, Norwalk, CT) received manufacturer's service maintenance every six months. Wavelength calibration test was done every month and at the occasion of changing lamps.

2.3. Preparation of standards

Standard stock solution was prepared from formulated FP-21399. The powder was reconstituted and diluted with DMSO to give 1.0 mg ml^{-1}. The accurate concentration was determined by a spectrophotometric method ($\varepsilon = 87\ 700\ cm^{-1}\ M^{-1}$ at 717 nm). The stock solution was diluted with DMSO to give a series of working standards.

Human plasma standards ranging from 0.01 to 100 μg ml^{-1} were prepared by adding the working standard into normal human plasma. For the calculation of the recovery, the working standard was diluted with water–acetonitrile 78:22 (v/v), 20 mM triethylamine acetate.

Large volumes of plasma standards of 0.1, 1.0 and 10 μg ml^{-1} were prepared for the use of quality control and the aliquots were kept at −80°C.

2.4. Preparation of samples

Prior to the extraction, all samples received 0.5% (v/v, final) of Triton X-100 and were heated at 57°C for 30 min. Then, 0.2 ml of 2.0 M triethylamine acetate was spiked to 1.0 ml aliquot of each sample. To extract drugs and metabolites, 2.0 ml of DMSO and 2.5 ml of acetone were added to the sample. After the centrifugation ($2000 \times g$, for 10 min), the supernatant was divided in three (1.25 ml each). These samples were diluted 1/8 with water and loaded onto solid phase extraction (SPE) cartridges (SepPak light C 18, Waters). Fractions containing FP-21399 were obtained by eluting with 0.3 ml of 0.2 M triethylamine acetate buffered water-acetonitrile 50:50 followed by 0.2 ml of 0.2 M triethylamine acetate buffer. For the analysis, 0.1 ml of the eluate was injected to HPLC.

2.5. Analysis and validation

All data were processed by Chemstation software (Hewlett Packard). Peak area and height were used for testing accuracy, precision and sensitivity. The system performance test function of the software was applied to get the values of symmetry factor, tailing factor, plate number and selectivity. These factors were useful to evaluate the suitability of the method. In order to evaluate the low limitation of detection, noise level and signal to noise ratio were determined by the software.

Standard operation procedures (SOP) provided by Hewlett Packard were employed to verify the system. Detecting wavelength was calibrated using built-in holmium standard. Chromatographic performance was tested with LC isocratic standard samples and Hypersil ODS analytical column (4.6×100 mm, 5 μm, Hewlett Packard). Injection precision and carry over rate were also tested with the same sample and column.

Plasma standard samples were prepared and analyzed to obtain the standard curve. The same procedure was repeated with different donor's plasma on different day. The intraday precision was evaluated from relative standard deviation (RSD) data of the same day. And inter-day precision was determined from the standard curves obtained on three different days with different plasma. Extraction recoveries were calculated based on the comparison of the peak areas from extracted sample and unextracted standard.

3. Results and discussion

3.1. Extraction efficiency

In order to inactivate viruses, every human plasma sample received 0.5% (v/v) of Triton X-100. Then all samples were heated at 57°C for 30 min. These procedures were designed to be done at clinical sites prior to shipping for analysis. All plasma standards also went through the same procedure.

Acetone, acetonitrile and methanol were tested to extract drugs and to eliminate plasma proteins. Acetone was most effective among them, but it was not good enough by itself. The reason seemed that FP-21399 had poor solubility in the most of organic solvents. Since DMSO was known as a good organic solvent to dissolve this drug, DMSO was added to the extraction procedure. The combination of acetone and DMSO gave the highest recovery rate.

The solid phase extraction (SPE) technique was applied to concentrate the sample and to reduce the materials that interfere with the analysis. Several reverse phase SPE columns were tested including SPE C8 and C18 from Hewlett Packard, SepPak tC2, C8, C18 and tC18 from Waters. HP's C18 and SepPak C18 and tC18 had strong retaining ability, but HP's C18 and SepPak tC18 required extra volume of organic solvent and higher ionstrength to elute the compound. Overall, SepPak C18 showed the highest recovery rate.

Table 1
Recovery of FP-21399 from human plasma

Concentration		Recovery (%)	RSD (%)	Bias (%)
Added (μg ml^{-1})	Observed (μg ml^{-1})			
0.01	0.0091	91.0	7.5	−9.0
0.02	0.0171	85.5	11.9	−15.4
0.05	0.0427	85.4	12.9	−15.6
0.1	0.0832	83.2	5.5	−16.8
1	0.985	98.5	2.8	−1.5
2	2.02	100.9	0.2	0.9
5	5.03	100.6	3.8	0.6
10	9.84	98.4	2.8	−1.6
100	93.3	93.3	0.7	−6.7

We tested the direct loading method for the samples onto SPE columns under several conditions, but we could not get high yield. The compound showed a low retention rate on SPE columns and it was deemed the result of the high protein binding nature of FP21399 in plasma.

Because triethylamine acetate buffer was found an effective enhancer to improve the chromatographic performance on reverse phase HPLC analysis of FP-21399, we added this buffer during the extraction procedure. It increased the recovery and reduced the variation of the data.

Recovery results are shown in Table 1. From 0.01 to 0.1 μg ml^{-1} range, over 80% of test compound was detected after extraction and RSD was about 10%. In the range of 1 to 100 μg ml^{-1}, 90% or higher yield was observed with 10% or better RSD.

The period of heating prior to extraction was extended from 30 to 60 min to test the stability of samples. The recovery rate was the same as the samples treated for 30 min.

One of the key steps in achieving this high recovery rate was the combination of liquid extraction and solid phase extraction. Another key was the presence of triethylamine acetate buffer during the extraction. Since the test compound has negatively charged moieties, we expect these ions to disrupt the ionic interaction of test compound and proteins as well as remaining silanols on the surface of the SPE column.

3.2. Chromatographic performance

Initial evaluation of chromatographic performance of analytical columns was carried out under the following conditions. The mobile phase was water–acetonitrile–triethylamine–acetic acid, 70:30:0.2:0.2 (v/v) pH 4. FP-21399 prepared in the mobile phase (0.1 mg ml^{-1}) was loaded (20 μl) to the analytical column set at ambient temperature with a flow rate of 1.0 ml min^{-1}. Signal was detected at 254 run and the column's performance was evaluated based on the tailing factor, symmetry factor and retention time. We obtained several commercially available analytical columns, such as Delta-pack C18, Nova-pack C18, Puresil C18, Symmetry C18 from Waters, Capcell-pack C18 from Shiseido and YMC-pack ODS-AQ from YMC. These column's dimensions were i.d. 4.6 or 3.9 × 150 mm. Nova-pack did not retain the compound under these conditions and Symmetry showed moderate tailing factor and relatively short retention time. Delta-pack, Puresil, Capcell-pack and YMC-AQ performed with a good retention and a fine peak shape for FP-21399. Among them, Puresil C18 (4.6 × 150 mm, 5 μm) scored the best performance and the colurim was selected for further optimization.

Considering the acidic nature of the FP-21399, an ion pairing reagent was tested. PIC-A Low-UV (Waters) containing tetrabuthylammonium phosphate improved the retention and peak shape in the case of separation with Nova-pack and Sym-

metry. However, with Puresil, the change of peak profile was minimal since it had been quite high already. In the mean time, the retention time was considerably increased and it required a much higher concentration of acetonitrile to elute the compound. Considering the cost and speed of assay, PIC-A use was considered suboptimal. Triethylamine appears to act as a weak ion-pairing reagent as well as a buffer.

The effect of pH was also investigated. The apparent pH of mobile phase was adjusted to 2 (0.2% TFA), 4, 5.5, 7 (20 mM acetic acid with desired amount of triethylamine). FP21399 and four related compounds (found as metabolites in plasma samples from animal studies) were prepared in DMSO (0.1 mg ml^{-1}) and loaded onto HPLC (10 μl). Regarding the peak shape and separation of the five compounds, pH 7 showed the best performance.

In order to make the method practical, we tried to reduce the cycle time to under 20 min. A gradient method was employed to enhance the speed of separation. We spiked FP-21399 and the four related compounds to normal human plasma and prepared an extracted sample mixture. The sample was loaded to HPLC (50 μl) and separated under many different gradient methods (the range from 15 to 60% of acetonitrile) to get optimized conditions. Finally we reached the gradient which separated compounds well with a relatively short analysis cycle time. The mobile phase was started as water acetonitrile 78:22 (v/v) and changed to 55:45 in 8 min and held at this ratio for 4 min. Injection volume was set as 100 μl and flow rate was adjusted to 0.6 ml min^{-1} to earn higher peak area without sacrificing speed and efficiency of analysis.

Peak separation was not affected by the column temperature in the range of ambient to 50°C, but the retention time was sengitive to the temperature change. A column heater was included and the temperature was set at 30°C.

Table 2
Inter-patient and inter-day reproducibility

Standard curve	r	Slope	RSD at 1 μg ml^{-1}
Day 1, Donor A	0.996	75.3	5.5
Day 2, Donor B	0.999	74.7	2.8
Day 3, Donor C	0.999	76.6	2.2

3.3. Linearity

Using the analyzed data of standard samples, peak area value was plotted against concentration in the range from 0.1 to 10 μg ml − 1. The plot showed a linear relationship ($r = 0.996 - 0.999$) and the intercept was not significantly different from zero (within 95% confident area). The standard curve was also drawn in the range of 0.01 to 100 μg ml^{-1}, and it still showed linear relationship ($r = 0.994$).

3.4. Accuracy and precision

The observed concentrations of standard samples were found to be in good agreement with the nominal concentrations; the bias ranged from − 17 to 3% throughout the concentration of 0.01 to 100 μg ml^{-1}.

The three independent standard curves were generated with different donors' plasma on different days. Within a day, the RSD values ranged between 0.2 and 8% ($n = 5$) in the concentration range of 0.1 to 100 μg ml^{-1}. The values were slightly increased at lower concentration range (2–13% at 0.01–0.1 μg ml^{-1}). On the inter-assay study, the data varied between 1 and 12% ($n = 15$) for all analyses. Slopes of three standard curves were also compared to evaluate inter-day variation (Table 2). The values were found to be reproducible with RSD 1.1% ($n = 3$).

3.5. Specificity

Specificity of the method is demonstrated in the representative chromatogram (Fig. 2). The data are from the spiked human plasma sample. Detecting wavelength was set at 265 nm between 0 and 8 min, then switched to 600 nm from 8 min on. The retention time of FP-21399 was 8.8 min. Two peaks observed at 10.4 and 10.8 were the metabolites of FP-21399. No interference was observed near these peaks at 600 nm. There was only one set of peaks observed at 9.6 and 9.9 min

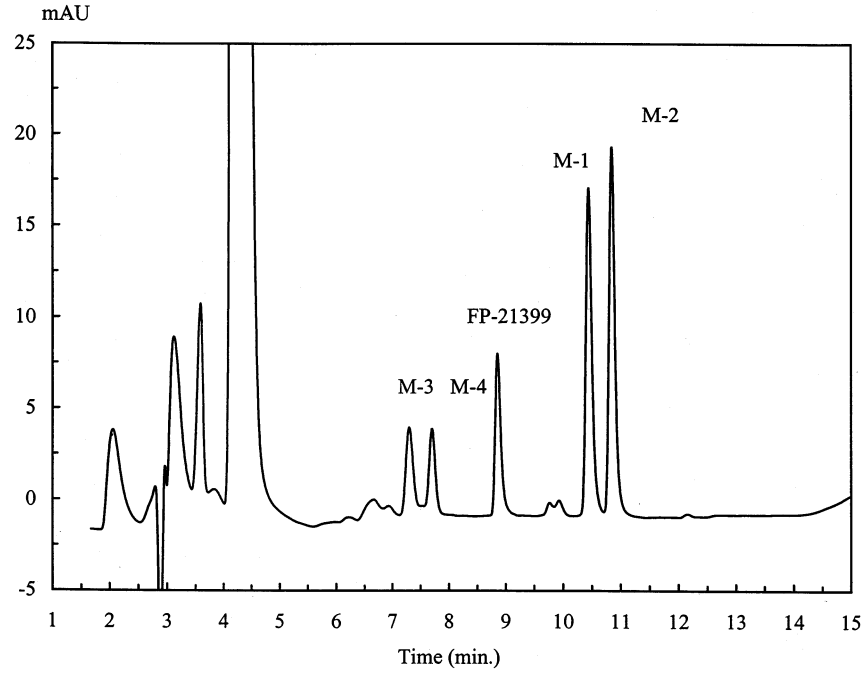

Fig. 2. Chromatogram after extraction of a human plasma sample fortified with 1 μg ml^{-1} each of FP-21399 and four metabolites.

that originated from blank human plasma. The peak intensity of this interference varied from donor to donor, but the peak area was relatively small (comparable to the area of 0.02–0.1 μg ml^{-1} of FP21399). These substances did not conflict with ether peak of the target compound nor the two metabolites.

These well separated peaks and reproducible retention times (Table 4) clearly demonstrate good specificity. This feature of detection method underlies the suitability and robustness of the whole assay.

3.6. Detection limit

The precision and accuracy values were acceptable (within 20% RSD) at the concentration of 0.01 μg ml^{-1} and above (Table 1). At the same time, the signal to noise (S/N) ratio was evaluated using the low concentration standard samples. S/N ratio was 5.8 ± 0.5 ($n = 3$) at the concentration of 0.01 μg ml^{-1}. On the study of clinical samples, the pre-dose sample for each donor was analyzed and it was confirmed that the background was flat from 8.0 to 9.5 min.

3.7. Robustness and applicability

Durability of the column was examined by checking the chromatographic performance at the 5th, 60th and 160th injections of a new column. As shown in Tables 3 and 4, the performance was excellent and reproducible. On the clinical sample analysis, each set of specimens was processed with a quality control sample and the peak profile was evaluated. The method was tolerant to a large number of injections. The guard column was changed at every 180 to 200 injections.

HIV viral replication assay was performed in order to check the safety of the procedure. No viral activity was detected from HIV-spiked plasma samples after adding Triton X100. All human plasma specimens were treated as biohazards and these materials were restrictively controlled under the guidelines.

As intended, this method was applied to the clinical samples. The concentration of FP21399 in all samples (from low to high dosage and from post to 168 h) landed within the quantitative range of the method. Excellent reproducibility

Table 3
Chromatographic performance results of FP-21399

Injection number (concentration)	5th (8.0 μg ml^{-1})	60th (10 μg ml^{-1})	160th (10 μg ml^{-1})
Observed concentration (μg ml^{-1})	8.44	10.04	10.25
Retention time (min.)	8.81	8.86	8.82
Peak height (mAU)	86.2	103	107
Peak area (mAU*s)	672	749	765
Symmetry factor	0.66	0.67	0.68
Tailing factor (UPS method)	1.45	1.45	1.45
Plate number/column	38505	39998	39592
Selectivity to preceding peak	1.25	1.24	1.23

Table 4
Reproducibility of HPLC analysis at 0.1 μg ml^{-1} ($n = 6$)

Compound	Retention time (min)	RSD (%)	Peak area (mAU*s)	RSD (%)	Peak height (mAU)	RSD (%)
FP-21399	8.80	0.096	6.26	5.5	0.68	6.5
M-1	10.40	0.095	11.8	6.5	1.50	6.7
M-2	10.79	0.094	12.8	8.2	1.73	6.6

and robustness of the method was proved by processing real specimen (RSD of all data were less than 10% typically within 5%, $n = 3$). Taking triplicate samples proved necessary since it was later discovered that plasma variation from patients was greater than expected based on standard plasma samples. Since the method is simple and quick, two sets (72 injection) of samples are able to be processed by one person and one HPLC. So far, this through put number has been high enough.

Recently, this method was applied to analyze urine and tissue specimens.

4. Conclusions

Quick and simple analytical method for the determination of FP-21399 was developed for human plasma samples potentially containing HIV. The quantitative range was from 0.01 to 100 μg ml^{-1}. This procedure is applicable to urine and tissue specimens as well. The method is also able to determine the two primary metabolites simultaneously.

Regardless of the high protein binding nature of the compound, good recovery rate was obtained even at low concentration. The combination of organic solvent extraction and solid phase extraction contributed to the high yield and the high sensitivity.

Distinct advantages of this method are suitability and robustness as well as simplicity, rapidity and safety. The procedure will be easily automated.

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