Optimization of Analytical Procedure for In-hospital Rapid Quantification of Serum Level of Favipiravir in the Pharmacological Treatment of COVID-19

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An in-hospital rapid method for quantifying the serum level of favipiravir (FPV) in the pharmacological treatment of COVID-19 was developed by an appropriate combination of a solid-phase extraction treatment and a reversed-phase HPLC/UV detection system. The quantification method was well-validated and applied to measuring the serum FPV level in a clinical practice at a general hospital that accepts COVID-19 patients. Furthermore, an analysis of data from our preliminary interaction analysis revealed, for the first time, that FPV selectively forms complexes with ferric (Fe³⁺) and cupric (Cu²⁺) ions.

Keywords In-hospital quantification, solid-phase extraction, HPLC, serum level, favipiravir, COVID-19, ferric ion

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Introduction

The current pandemic of coronavirus disease 2019 (COVID-19) has spread rapidly worldwide. Several drugs are currently being tested in clinical trials for their efficacy and safety in the treatment of COVID-19. An orally administered agent (tablet) that contains favipiravir (FPV) as the active substance is one of the candidate drugs for the treatment of COVID-19 in Japan and various other countries.1–3 FPV is an RNA-dependent RNA polymerase inhibitor that has been approved in Japan as a treatment for a new type of influenza.1 FPV exhibits non-linear pharmacokinetics, and a blood level of FPV (i.e., a FPV concentration in plasma or serum) following the first and second doses of a tablet containing FPV increases considerably before gradually decreasing with time.4,5 FPV inhibits replication by the target virus depending on the FPV’s plasma concentration,5 and the dosage of FPV varies depending on the disease state of the patient. Furthermore, FPV has been reported to interact with other medicinal drugs.6 Therefore, to provide pharmacological treatments for COVID-19 by the administration of FPV, therapeutic monitoring of the blood level of FPV is very important, and a rapid and accurate analytical method for the quantification of FPV in human plasma (or serum) is urgently needed.

In this report, we propose a simple and rapid method for the quantification of FPV in serum by an appropriate combination of a solid-phase extraction (SPE) treatment and a reversed-phase (RP) HPLC/UV detection system. The procedure of this method was well-optimized and simplified, and then applied for use by medical staff (such as a clinical laboratory technician or a pharmacist) in a general hospital that accepts COVID-19 patients.

Experimental

Reagents and chemicals

Acetonitrile (CH₃CN; HPLC grade), phosphoric acid (special grade), and 5 mol/L hydrochloric acid were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Water was purified using a Milli-Q system (Merck Millipore, Darmstadt, Germany). FPV was purchased from Medchemexpress Co., Ltd. (New York, USA). Normal human serum was obtained from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan).

UV-VIS spectroscopic analysis

A stock solution (1 mg mL⁻¹) of FPV was prepared by dissolving 1 mg of FPV in 1 mL of water. A test solution of FPV (20 μg mL⁻¹) for UV-VIS spectroscopy was prepared by diluting the stock solution 50-fold with an aqueous solution (0.1% phosphate buffer, pH 2 or 7) containing 5 v/v% CH₃CN.

A UV-VIS absorption spectrum of the test solution was recorded on a UV-1800 spectrophotometer (Shimadzu, Tokyo, Japan) in the 200 to 450 nm range.

Preparation of FPV test solutions for SPE and HPLC analyses

For use in the optimization of SPE and HPLC conditions, a FPV test solution (50 μg mL⁻¹) was prepared by dissolving the stock solution (1 mg mL⁻¹) 20-fold with an arbitrary aqueous solution or a normal human serum. In addition, solutions of FPV-spiked serum (100 μg mL⁻¹; for preparing a calibration curve) and of three other concentrations of FPV-spiked sera (5, 20, and 80 μg mL⁻¹; QC samples for validation of the present quantification method) were prepared by diluting the stock solution into normal human serum.
SPE cartridge and optimized protocol

A monolithic C18-silica disk built-in centrifugal spin-cartridge, MonoSpin C18 (GL Sciences, Inc., Tokyo, Japan), was chosen for the SPE treatment prior to HPLC analysis of FPV. The MonoSpin cartridge was pretreated by sequential the passage of 500 μL, each, of CH3CN and water before use. Each solution was passed through the cartridge by centrifugation at 5000 rpm (2300 x g) for 1 min using a Centrifuge 5415R (Eppendorf, Hamburg, Germany). Details of the SPE procedure using the pretreated cartridge were as follows. First, an aliquot (6 μL) of 5 M HCl was added to a FPV test solution or FPV spiked serum (150 μL) in a microfuge tube. After vortex mixing for 5 s, one-third of the volume (52 μL) of the mixture was loaded onto a MonoSpin cartridge. Next, aliquots of water (500 μL; wash solution), and aqueous 10% CH3CN (150 μL; eluting solution for FPV) were sequentially passed through the cartridge, and the final eluate containing FPV was collected in a test tube for subsequent HPLC analysis.

HPLC apparatus and optimized conditions

All HPLC experiments were carried out with a Chromaster system (Hitachi High-Tech Science Corp., Tokyo, Japan) equipped with a 5310-column oven, a 5210 autosampler, a 5110 pump, and a 5430 diode-array detector. RP HPLC separation of FPV was performed at 30°C on a Chromolith HighResolution RP-18 column (100 × 4.6 mm i.d., Merck, Darmstadt, Germany) equipped with a guard column (5 × 4.6 mm i.d.). An analyte containing FPV (injection volume: 20 μL) was eluted with a mobile-phase solvent (0.1% phosphoric acid and CH3CN, 95:5, v/v) at a flow rate of 2 mL/min, and FPV in the eluate was detected by UV absorption at 325 nm.

Validation method

A one-point calibration curve for HPLC quantitation of the serum level of FPV was prepared from HPLC data of the SPE eluate of the FPV-spiked serum (100 μg mL−1) without the use of an internal standard (I.S.) compound. The limit of detection (LOD) and the limit of quantitation (LOQ) of FPV were estimated from a chromatogram of the SPE eluate of the FPV-spiked serum (5 μg mL−1) at a signal-to-noise ratio of 3:1 and 10:1, respectively.

An intra-day study (repeatability) was performed by analyzing the QC samples (serum spiked with FPV at 5, 20, and 80 μg mL−1) 4 times during the same day, while an inter-day study (intermediate precision) was performed by analyzing the QC samples once-per-day on 3 separate days. The accuracy was reported by calculating the bias, expressed as ((measured concentration)/nominal concentration)) × 100%; the precision was reported as the coefficient of variation (CV), expressed as (SD/mean of measured concentration)) × 100%. The specificity was measured by analyzing blank serum and comparing its chromatograms with the corresponding FPV-spiked serum samples.

Medical application

To evaluate the applicability of the present method, human serum samples from four different patients with COVID-19 who had been treated with FPV were obtained from Hokushin General Hospital (Nagano, Japan). The study protocol was approved by the Ethics Committees of Hokushin General Hospital (Receipt No. 2020005). The patient sera were filtered with DISMIC 13HP syringe filters (0.45 μm, ADVANTEC), and each filtered sample (after addition of 5 mol/L HCl) was loaded onto a MonoSpin C18 cartridge by centrifugation for 3 min. The subsequent SPE protocol was the same as that described above.

Screening method for complexation between metal ions and FPV

For identifying metal ions capable of forming complexes with FPV, our recently developed screening method using MonoSpin cartridge' was applied for analyzing the complexation between FPV and each of twelve different metal ions. The general procedure of the method is described in Supporting Information.

Results and Discussion

Over the last decade, nine different papers bearing descriptions of procedures for HPLC quantification of plasma/serum levels of FPV have been published.4–6,8,10–14 However, as listed in Table S1 (in Supporting Information), detailed descriptions of the analytical procedures and chromatograms were lacking from most of these previous papers. Therefore, we performed our own research to develop a method for the rapid quantification of the serum FPV level in specimens from patients with a COVID-19 administered pharmacological treatment with FPV.

In our preliminary HPLC experiment (see Fig. S1 in SI), FPV was well-retained on an octadecl-silica (ODS; C18) column under acidic pH conditions below FPV’s pH value (5.1) conditions under which the compound exists in a hydrophobic non-ionized form. As shown in the UV spectra in Fig. 1, the λmax of FPV was 325 nm in an acidic (pH 2) mobile-phase solvent containing 5% CH3CN. Therefore, the UV detection wavelength of our LC system was fixed at the 325 nm. Under these conditions, a good RP-HPLC profile of FPV (eluted within 3 min) was obtained, as shown in Fig. S1-a (in SI).

We next sought to optimize the SPE conditions for FPV-spiked serum, prior to quantitation of the compound by HPLC. A SPE technique is commonly used for the pretreatment of a serum specimen before a drug analysis.15–17 Among the tested MonoSpin cartridges (C18, C18-AX, and C18-CX), the C18 cartridge had the highest adsorptive capacity for FPV under acidic pH conditions, and the optimum volume of sample loading to the cartridge was 50 μL. Furthermore, when FPV adsorbed on the C18 cartridge was eluted directly with the eluting solution (aq. 10% CH3CN; 150 μL) by omitting a washing-up step with d.HCl, the SPE recovery rate (%) of FPV from serum was highest (see the results of Test Nos. 1 - 5 in Table S2 in SI). Based on these data, we devised an optimized protocol for SPE of FPV-spiked serum, as described above in the Experimental section.

Ultimately, the analytical procedure for the quantification of
FPV in serum was established by using an appropriate combination with the SPE treatment and subsequent RP-HPLC/UV analysis, as described above; the procedure of the proposed method is shown schematically in Fig. 2. Specifically, the HPLC chromatogram of the SPE eluate of the FPV-spiked serum (50 μg mL⁻¹) is shown in Fig. 3b. Compared to the chromatogram of blank (FPV-free) serum (Fig. 3c), the FPV-spiked serum exhibited no interfering peaks derived from the serum constituents at retention times corresponding to those of FPV (Fig. 3b). Furthermore, a comparison of the FPV peak areas in Figs. 3b and 3a (FPV test solution (50 μg mL⁻¹)) indicated a SPE recovery rate of FPV from serum of approximately 83% (average value (n = 4), CV: 2.2%). The LOD and LOQ values of FPV were 0.773 and 2.58 μg/mL, respectively.

Based on these results, a quantification of FPV in serum was performed by the above method using a one-point calibration curve that was prepared from the FPV peak area of the SPE eluate of the FPV-spiked serum (100 μg mL⁻¹) without the use of an I.S. compound. As shown by the results of the method validation using three levels of QC samples (sera spiked with FPV at 5, 20, and 80 μg mL⁻¹), the accuracy values ranged from 97.9 to 105.0% for all QC samples (Table 1). Intra-day and inter-day precision (CV%) values were below 6% for the three tested concentrations. The acceptance criteria for the accuracy and precision were met in all QC samples.

As a final step, we implemented a quantification of the FPV levels in sera from four patients with COVID-19, who had been treated by administration of FPV; this experiment was intended to verify the utility of our method in clinical practice at a general hospital. The FPV concentrations in the serum specimens from the four patients were quantified as 2.7, 33.1, 77.6, and 93.5 μg mL⁻¹; the HPLC chromatograms of the quantification assays are shown in Fig. S2 in SI. Interestingly, although all four patients had been administered FPV at the same dose level, their serum FPV levels (trough levels) varied over a greater-than-30-fold range. Although the actual cause of the observed variation remains unknown, we expected that each patient’s serum contains distinct levels of certain intrinsic factors that may interact with FPV. Therefore, we additionally examined the sera for the presence of metal ions capable of forming a complex with FPV, given the fact that FPV has a typical multidentate ligand structure (see the chemical structure in Fig. 1). For this purpose, we used a metal ion screening method that we have recently developed. As shown in Fig. 4, the
Table 1  Accuracy and precision of the present quantification method for FPV

| Conc in serum/μg mL⁻¹ | Accuracy, % | Repeatability, CV % | Intermediate precision, CV % |
|-----------------------|-------------|---------------------|-----------------------------|
| 5                     | 105.0       | 1.0                 | 2.4                         |
| 20                    | 103.4       | 4.0                 | 2.4                         |
| 80                    | 97.9        | 5.5                 | 1.8                         |

The accuracy (%) was calculated from the data of quadruplicate samples (n = 4) at a given concentration. The repeatability (CV %) was also from quadruplicate samples (n = 4). The intermediate precision (CV %) was calculated over a series of three analyses on different days (3 days).

Fig. 4  Comparison of the adsorption capacities (%) of FPV of each of twelve different metal ions chelated onto a MonoSpin ME cartridge that had been modified with iminodiacetic acid. Further details of the experimental procedure are described in the caption of Fig. S3 in SI.

The screening data revealed that FPV selectively formed complexes with ferric (Fe³⁺) and cupric (Cu²⁺) ions among the twelve tested types of metal ions; more detailed data also are shown in Fig. S3 in SI. To the best of our knowledge, these results represent the first demonstration of FPV complex formation with metal ions. Based on these data, we speculate that the serum FPV levels may be sensitive to differences in the serum ferritin levels of various patients; we are planning further experiments to evaluate the correlation between the levels of FPV and ferritin in the sera of patients with COVID-19.

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Supporting Information

This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/.