

# Determination of Intracellular Darunavir by Liquid Chromatography Coupled with Fluorescence Detection

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**The concentration of darunavir (DRV) in peripheral blood mononuclear cells (PBMCs) was reported to affect the clinical symptoms of patients and the development of drug-resistant viruses. We developed a simple and highly sensitive method to quantify the concentration of DRV in human PBMCs using high-performance liquid chromatography with fluorescence detection. PBMC samples were collected using commercially available tubes for density-gradient centrifugation. To disrupt the cells, we used a heating fragmentation method. To compensate for the disadvantages of the heating fragmentation method, liquid–liquid partitioning was used to destroy the unbroken cells by the degeneration of proteins using an organic solvent. As an analytical column, we used an ODS column. The mobile phase consisted of 20 mmol/L potassium phosphate buffer (pH 4.3)/acetonitrile (57/43, v/v) and was pumped at 1.0 mL/min. The lower limit of quantification was 5 ng/10<sup>6</sup> cells. Good linearity was obtained with 5–100 ng/10<sup>6</sup> cells. The intra- and inter-assay precision and accuracy were <15%. Because our method makes possible the measurement of DRV concentration in PBMCs at medical facilities or laboratories without tandem mass spectrometry systems, we believe that it will contribute to clinical studies and will improve the medical treatment of human immunodeficiency virus infections with DRV.**

## Introduction

Several new types of drugs have recently been introduced for the treatment of drug-resistant human immunodeficiency virus 1 (HIV-1) infection. Darunavir (DRV), a protease inhibitor, is one such drug and has become crucial for antiretroviral therapy (ART) because it potently suppresses the replication of wild-type and drug-resistant HIV-1. Thus, DRV has been focused on as one of the main drugs for the treatment of patients infected with multidrug-resistant HIV-1, and therapeutic drug monitoring (TDM) of DRV has been considered to be important to obtain the expected clinical efficacy as an alternative to counting CD4<sup>+</sup> cells or viral load (1, 2). Moreover, some recent studies reported that the concentration of DRV in peripheral blood mononuclear cells (PBMCs), the site of action for DRV, affected the clinical symptoms of patients and the development of drug-resistant viruses, and monitoring the concentration of DRV in PBMCs has been argued to be more important than its level in plasma (3). In the field of transplantation, the impact of intracellular concentration of a drug on its clinical efficacy has been reported (4).

To determine the blood level of DRV, several methods using high-performance liquid chromatography (HPLC) with an

ultraviolet (UV) detector or with tandem mass spectrometry (LC–MS/MS) have been developed. Goldwirt *et al.* and Takahashi *et al.* reported HPLC–UV methods with lower limits of quantification (LOQs) of 250 and 130 ng/mL, respectively (5, 6). However, because of its low sensitivity and specificity, HPLC–UV requires a huge volume of blood and complex sample preparation procedures to detect DRV at a low concentration such as in PBMCs. D’Avolio *et al.* reported an HPLC–photodiode array method with a sensitivity of 200 ng/mL using 500  $\mu$ L of plasma; however, this method also has insufficient sensitivity to determine the concentration of DRV in PBMCs (7). Recently, some highly sensitive methods using LC–MS/MS were reported for the simultaneous determination of DRV and other ART drugs in plasma (8–12), dried blood spots (13, 14), and PBMCs (15, 16). In 2011, Gupta *et al.* developed an LC–MS/MS method to detect DRV in plasma with an LOQ of 1 ng/mL (17). Thus, LC–MS/MS methods are highly sensitive and useful for determining the concentration of DRV in PBMCs. However, LC–MS/MS methods are not considered suitable for routine clinical use because many medical facilities and laboratories do not have access to an MS/MS system due to its high cost and complexity of use, at least at the present time.

Conversely, a method using HPLC with fluorescence detection (FLR), which is more sensitive than HPLC–UV and simpler and more widely used than LC–MS/MS, has not been reported for the detection of DRV. Thus, in this study, we established a simple and highly sensitive method to determine the concentration of DRV in PBMCs using HPLC coupled with fluorescence detection (HPLC–FLR), which is suitable for routine clinical use as an additional tool to detect DRV in PBMCs, and for use in medical facilities and laboratories that cannot use LC–MS/MS systems.

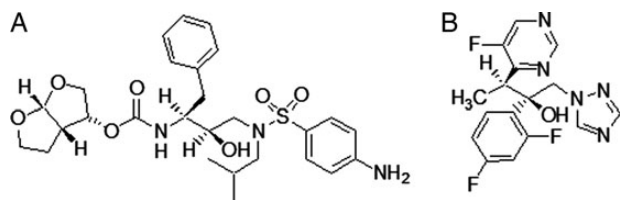
## Experimental

### Chemicals and reagents

DRV and voriconazole (VCZ) were purchased from Toronto Research Chemicals Inc. (North York, ON, Canada) and Sigma Aldrich Inc. (St. Louis, MO, USA), respectively (Figure 1). All other reagents were obtained from commercial sources and were graded for HPLC or analytical use.

### Chromatography conditions

The HPLC analysis was performed using a Waters Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with a



**Figure 1.** Chemical structures of DRV (A) and VCZ (B).

2475 scanning fluorescence detector (Waters). The appropriate wavelength for the detection of DRV was determined by 3D spectrum analysis using 100 ng/mL DRV solution and set at 235 nm for excitation and 337 nm for emission, which was found to be the maximum wavelength for DRV. We used a YMC-Pack Pro C<sub>18</sub> column (250 × 4.6 mm, 5 μm particle size; YMC, Tokyo, Japan) that was maintained at 40°C. The mobile phase was 20 mmol/L potassium phosphate buffer (pH 4.3)/acetonitrile (57/43, v/v) and was pumped at a flow rate of 1.0 mL/min. Empower 2.0 was used for data collection and integration.

#### Collection of blood and separation of PBMCs

BD Vacutainer Mononuclear Cell Preparation Tubes (Becton Dickinson and Company, Franklin Lakes, NJ, USA) were used as blood collection tubes to collect PBMCs by the density-gradient centrifugation method. Blood samples were centrifuged at 1600 × g for 20 min, and the PBMC layer was separated. PBMC samples were washed twice with 10 mL phosphate-buffered saline (PBS), and suspended in PBS to obtain a concentration of 1.0 × 10<sup>6</sup> cells/mL. PBMC samples were transferred to a polyethylene tube and treated immediately.

#### Preparation of standard stock solutions and calibration standard samples

The DRV stock solution was prepared in 50% methanol at a concentration of 500 μg/mL. This stock solution was diluted with 50% methanol to obtain the concentrations required for the standard working solutions. Calibration standard samples were prepared by spiking a suspended PBMC solution (4% total volume of PBMC suspension) with the standard working solutions. Calibration curve standards to determine the DRV concentrations were made at 5, 10, 20, 50, and 100 ng/10<sup>6</sup> cells. The VCZ stock solution (1 mg/mL), as an internal standard (IS), was dissolved in 50% methanol. This stock solution was diluted with 50% methanol to the working concentration of 50 μg/mL. All samples were stored at -20°C until use.

#### Sample preparation from PBMCs

Two hundred microliters of PBMC suspension and 20 μL of calibration standard samples or 50% methanol were mixed by vortexing for 1 min, and heated at 95°C for 5 min to destroy the cell and nuclear membranes. Then, 20 μL of VCZ (5.0 μg/mL), 500 μL of 500 mmol/L sodium carbonate buffer (pH 9.0), and chilled samples were placed into glass centrifuge tubes. Two milliliters of ethyl acetate were added and the samples were shaken vigorously for 10 min with a mechanical shaker, followed

by centrifugation at 1500 × g for 5 min. The upper organic layer was transferred to another disposable glass tube, and evaporated to dryness under reduced pressure. The residue was dissolved in 200 μL of 50% methanol and 25 μL of the solution was injected into the HPLC system.

#### Assay validation

Specificity was checked using five different PBMC samples. The extraction ratio for DRV and the IS was calculated by comparing spiked PBMC samples and stock solutions diluted to the same DRV concentration. The extraction ratio were calculated at five different concentrations, 5.0, 10, 20, 50, and 100 ng/10<sup>6</sup> cells, by comparing the peak areas of the samples after the clean-up procedure with the areas obtained by direct injection of the aqueous solutions.

The lowest concentration of DRV with acceptable precision and accuracy (< 20%) was defined as the lower limit of quantification (LOQ). The lower limit of detection (LOD) was defined as the concentration at which the height of the peak was three times greater than the baseline. For the other concentrations, precision and accuracy should be within 15%. Precision was expressed as the percentage coefficient of variation (CV), while accuracy was expressed as the relative error of the nominal versus measured concentration (RE). The intra-assay variability was tested on five different PBMC samples. The inter-assay variability was tested on five different days and a new calibration curve was constructed each day.

#### Sample stability

To confirm the stability of DRV, PBMC suspensions with 100 ng/10<sup>6</sup> cells spiked with 1.0 μg/mL of DRV were stored at -20°C. After storage for 4 weeks, the stability of DRV in the samples was evaluated by comparing their peak areas with those that were measured immediately after collection.

#### Clinical samples

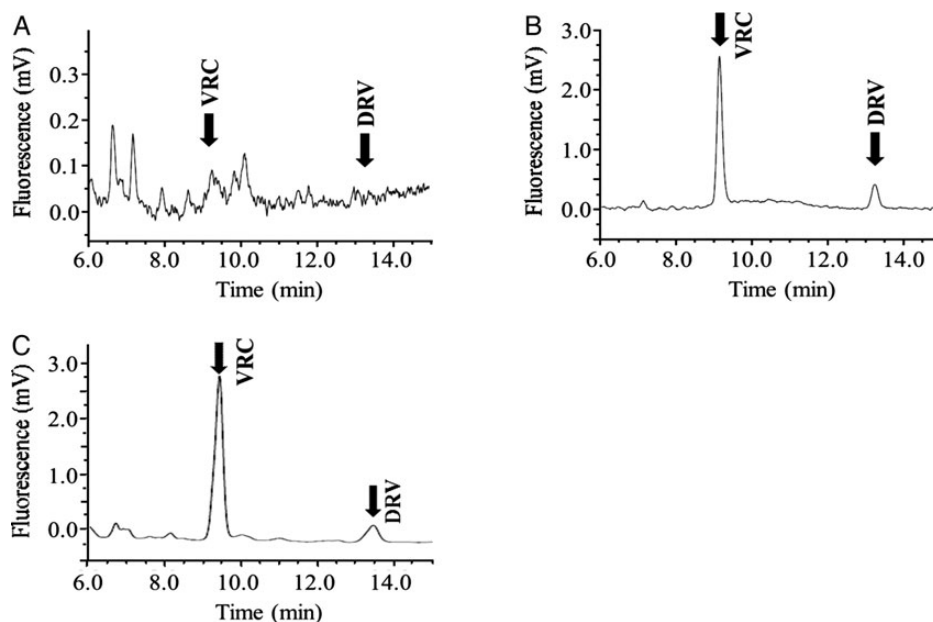
Eight milliliters of blood, which was the minimum amount required to separate the PBMC layer and the same volume used in a previous report (3), were obtained from patients with HIV receiving an 800-mg daily dose of DRV before oral administration of DRV. For the collection of PBMC samples, BD Vacutainer Mononuclear Cell Preparation Tubes were used as described above, and PBMC sample were separated within 2 h after blood sampling. PBMC samples were stored at -80°C until extraction of DRV, which was performed as described above within 1 day. The treated sample was assayed into an HPLC-FLR system immediately.

Approval for this study was obtained from the Institutional Review Board of Gunma University Hospital, and written consent was obtained from all patients after they had been informed of the experimental procedure and the purpose of the study.

## Results

#### Selectivity and linearity

Typical chromatograms for the detection of DRV in PBMCs are shown in Figure 2. The peaks for DRV and VCZ were well



**Figure 2.** Typical chromatograms of blank human PBMC (A), human PBMC spiked with DRV (5 ng/10<sup>6</sup> cells) and VCZ (5 μg/10<sup>6</sup> cells) (B) and PBMC of a patient treated with DRV (C).

separated, with retention times of 13.8 and 9.8 min, respectively. The resolution of these peaks was 8.0. The theoretical plates and the symmetry coefficient value of the DRV peak were  $1.2 \times 10^4$  and 6.0, respectively, and those of the IS peak were  $6.1 \times 10^3$  and 4.0, respectively.

The mean equation describing the calibration curve to determine the concentration of DRV in PBMCs 5–100 ng/10<sup>6</sup> cells was  $y = 0.0222x + 0.0456$  ( $R^2 = 0.999$ ), where  $y$  is the peak area ratio of DRV against the IS and  $x$  is the nominal concentration of DRV.

#### **Precision, accuracy and recovery**

The intra- and inter-assay precision and accuracy values are shown in Table I. The intra- and inter-assay precision and accuracy were <15%.

The extraction ratio of DRV and VRC varied from 84.5 to 93.2% and 82.5 to 88.5%, respectively.

#### **Lower limit of detection and lower limit of quantification**

The LOD and LOQ for DRV in PBMCs were 1.0 and 5.0 ng/10<sup>6</sup> cells, respectively. The quantification range for of DRV in PBMCs was 5.0–100 ng/10<sup>6</sup> cells.

#### **Sample stability**

The peak area of DRV after storage for 4 weeks was  $95.3 \pm 0.4\%$  ( $n = 5$ ) compared with samples that were measured immediately. Therefore, DRV was stable in PBMCs at  $-20^\circ\text{C}$  for at least 4 weeks.

**Table I**

Precision and Accuracy of the Assay for DRV in PBMCs

Nominal concentration (ng/10 <sup>6</sup> cells)	Measured concentration mean $\pm$ SD (ng/10 <sup>6</sup> cells)	Accuracy (RE %)	Precision (CV %)
Intra-assay ( $n = 5$ )			
5	$4.59 \pm 0.33$	-8.16	7.19
10	$9.01 \pm 0.79$	-9.94	8.77
20	$22.4 \pm 1.43$	12.0	6.37
50	$52.2 \pm 3.56$	4.49	6.82
100	$99.1 \pm 5.32$	-0.90	5.37
Inter-assay ( $n = 5$ )			
5	$4.48 \pm 0.25$	-10.3	5.52
10	$9.11 \pm 0.69$	-8.82	7.54
20	$22.2 \pm 1.02$	10.8	4.58
50	$55.5 \pm 3.05$	11.9	5.50
100	$97.3 \pm 3.84$	-2.74	3.96

CV, coefficient of variation; RE, relative error of the nominal versus the measured concentration.

#### **Application of the method**

The established method was applied to obtain the trough concentration of DRV in PBMC samples from nine subjects. We detected PBMC DRV concentrations of  $20.05 \pm 6.06$  ng/10<sup>6</sup> cells using 8 mL of blood, which was the minimum amount where it was possible to separate the PBMC layer from the whole blood sample and was the same volume used in a previous report (3) (Table II). These data were found to be similar to previously published data (3).

#### **Discussion**

In the treatment of HIV-1 infection, maintaining the blood concentration of DRV within appropriate levels is considered to be essential to prevent the proliferation of drug-resistant viruses. Recently, the importance of measuring the concentration of

**Table II**  
DRV Concentration in PBMC and Plasma Obtained from Nine Subjects

Pt.	DRV in PBMC (ng/10 <sup>6</sup> cells)
1	12.85
2	24.31
3	16.29
4	16.89
5	16.03
6	19.84
7	29.62
8	15.95
9	28.69
Mean	20.05
SD	6.06

DRV, especially that in PBMCs, has been focused upon and discussed (18). Clinically, the trough plasma concentration of DRV was found to be 3.8 µg/mL (2), which is sufficiently high to be detected by conventional HPLC-UV methods; however, HPLC-UV methods are not suitable for the detection of DRV in PBMCs in the clinical setting because a huge volume of blood is required and easily confounded by the presence of foreign substances. In our preliminary test using a UV detector to detect intracellular concentration of DRV, some interfering foreign substance peaks were found at the same retention time with DRV; however, when using an FLR detector, we could eliminate these contaminated peaks and obtain a clear base line due to FLR's higher specificity. Regarding the sensitivity, Glodwirt *et al.* reported that they could detect 250 ng/mL of DRV with an injection volume of 50 µL using HPLC-UV without condensation from plasma, and here, we detected 500 ng/mL of DRV with an injection volume of 25 µL using HPLC-FLR with 10 times dilution from original plasma. These data clearly show the advantages of FLR, namely higher selectivity and sensitivity. Recently, some even more sensitive methods to detect DRV using LC-MS/MS have been developed (8–17); however, they are not considered to be suitable for routine clinical use because of cost and complexity. Thus, we established here a simple and highly sensitive method to determine the concentration of DRV in PBMCs by HPLC-FLR as an additional tool for the measurement of DRV concentration in PBMCs for use in medical facilities and laboratories that cannot use MS/MS systems.

In the first step of sample preparation, we used Vacutainer Cell Preparation Tubes to collect PBMCs because of their simplicity of use. Using these tubes, PBMCs can be isolated simply and quickly without complex manipulations as compared with density-gradient centrifugation using Ficoll. However, in our preliminary examination, we found that the recovery ratio of PBMCs from whole blood samples using Vacutainer Cell Preparation Tubes was 70–80% of that when using conventional density-gradient centrifugation. At this point, because 8 mL of whole blood was required as a minimum volume to separate the PBMC layer due to limitations in the methodology, ~6 mL of PBMC suspension with a concentration of 1.0 × 10<sup>6</sup> cells/mL was collected as a minimum amount of PBMC sample, and therefore the 70–80% recovery ratio of PBMCs is not a critical disadvantage.

The disruption of cell membranes by the addition of an acid or base, heating or ultrasonic fragmentation is usually performed before the extraction of chemical compounds. According to

D'Avolio *et al.* (13), we initially used ultrasonic fragmentation to destroy the cell and nuclear membranes; however, because we did not achieve a sufficient extraction ratio (near 57%) or sufficient reproducibility, the extraction ratio varied between 34.8 and 72.8% in a preliminary study. This low sufficient extraction ratio and reproducibility might have been caused by low capability or unstable performance of the ultrasonication systems and might have been resolved by use of a high-performance system. In this study, we assessed a heating fragmentation method using a commonly used heating system, which is rarely affected by technical fluctuations because of its simplicity. Additionally, to address the disadvantages of the heating fragmentation method (i.e., the low efficacy for the destruction of cell membranes), we used liquid-phase extraction to disrupt the unbroken cells by protein degeneration using an organic solvent, which is necessary for liquid-phase extraction, and could successfully extract DRV from PBMCs at a high extraction ratio (~91%) and reproducibility (the extraction ratio varied between 84.5 and 93.2%).

Using the above process, we have established a simple and highly sensitive method to detect DRV by HPLC-FLR with an LOQ of 5.0 ng/10<sup>6</sup> cells. In a past study, Heine *et al.* established the LC-MS/MS method to measure the concentration of DRV in PBMCs with a LOQ of 1.0 ng/mL using a lysate containing 5 × 10<sup>6</sup> cells per 200 µL prepared from 8 mL of blood. This indicates that their LOQ was 0.4 ng/10<sup>6</sup> cells, which is ~10 times more sensitive than our method reported here (16). However, this difference in sensitivity is not presently a critical problem for clinical use since we can obtain 6 mL of 1.0 × 10<sup>6</sup> cells/mL PBMC suspension as a minimum volume, as described above. We successfully detected DRV in the PBMCs of patients receiving an 800 mg daily dose of DRV with a blood sampling volume of 8 mL, which is the same volume reported for LC-MS/MS by Heine *et al.* (16), and found that our data were similar to previously reported data (16). Although the recovery ratio might be a key factor in reducing sampling volume when a new method to separate PBMCs from a lower volume of whole blood is developed, our method requires only ~0.2 × 10<sup>6</sup> cells for the measurement of DRV concentration in PBMC, which could theoretically be collected from as little as 0.5 mL of whole blood. In other words, following the development of a method to separate the PBMC layer from a lower volume of whole blood, our method will allow for blood sampling of under 0.5 mL—a clinically sufficient albeit low volume—to reduce the burden on patients.

Using an FLR detector, we could obtain high sensitivity and a clear baseline by eliminating peaks derived from foreign substances, which sometimes interrupt the measurement process when using a UV detector. Additionally, using the above processes such as heating and liquid-phase extraction, we could obtain high accuracy, precision and reproducibility. We believe that our study provides an additional tool to measure the concentration of DRV in PBMC using HPLC-FLR for medical facilities and laboratories, and believe this method will be useful and suitable for clinical use due to its simplicity and high sensitivity. In conclusion, we have established a simple, rapid and highly sensitive quantification method for DRV in PBMCs. The present method will contribute to the progress of pharmacokinetic studies of DRV or the TDM system and improve the medical treatment of HIV infections with DRV.

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