WILEY

# A Virtual Event Designed For The Masses

## Now Available On-Demand!

Scale up your research and translate your results more rapidly and simply than ever before. Welcome to vLC-MS.com - the event for Orbitrap Exploris mass spectrometers and much more!

**Tune in to:**

- Explore the LC-MS portfolio and meet the expanded Orbitrap Exploris MS system in our staffed Exhibit Hall.
- Learn from mass spectrometry experts, such as Professor Alexander Makarov himself, about Orbitrap mass spectrometry technology and the applications it enables.
- Browse posters and short presentations in our application area.

**Event Highlights:**

**Prof. Alexander Makarov**

**Dr. Christian Münch**

**Thomas Moehring**

vLC-MS.com

**REGISTER NOW**

**Thermo Fisher**
SCIENTIFIC

**Biomedical Chromatography** WILEY

# Development and validation of a cost-effective and sensitive bioanalytical HPLC-UV method for determination of lopinavir in rat and human plasma

Chaolong Qin[1]  |  Wanshan Feng[1]  |  YenJu Chu[1]  |  Jong Bong Lee[1]  |
Mattia Berton[1,2]  |  Sara Bettonte[1,2]  |  Yeong Yeu Teo[1]  |  Michael J. Stocks[1]  |
Peter M. Fischer[1]  |  Pavel Gershkovich[1]

[1]School of Pharmacy, University of Nottingham, Nottingham, UK

[2]Department of Pharmaceutical and Pharmacological Science, University of Padova, Padova, Italy

**Correspondence**
Pavel Gershkovich, School of Pharmacy, Biodiscovery Institute (BDI), University of Nottingham, University Park, Nottingham NG7 2RD, UK.
Email: pavel.gershkovich@nottingham.ac.uk

**Abstract**

A simple, sensitive and cost-effective HPLC-UV bioanalytical method for determination of lopinavir (LPV) in rat and human plasma was developed and validated. The plasma sample preparation procedure includes a combination of protein precipitation using cold acetonitrile and liquid–liquid extraction with *n*-hexane–ethyl acetate (7:3, *v/v*). A good chromatographic separation was achieved with a Phenomenex Gemini column ($C_{18}$, 150 mm × 2.0 mm, 5 μm) at 40°C with gradient elution, at 211 nm. Calibration curves were linear in the range 10–10,000 ng/mL, with a lower limit of quantification of 10 ng/mL using 100 μL of plasma. The accuracy and precision in all validation experiments were within the criteria range set by the guidelines of the Food and Drug Administration. This method was successfully applied to a preliminary pharmacokinetic study in rats following an intravenous bolus administration of LPV. Moreover, the method was subsequently fully validated for human plasma, allowing its use in therapeutic drug monitoring (TDM). In conclusion, this novel, simple and cost-efficient bioanalytical method for determination of LPV is useful for pharmacokinetic and drug delivery studies in rats, as well as TDM in human patients.

**KEYWORDS**

HIV, lopinavir, preclinical pharmacokinetics, SARS-CoV-2, therapeutic drug monitoring

## 1  |  INTRODUCTION

Lopinavir (LPV) is an HIV (human immunodeficiency virus) protease inhibitor (PI) coadministered with a low dose of ritonavir (RTV) under the brand name Kaletra (LPV/r) as part of antiretroviral treatment (ART) in people affected by HIV. The combination was approved by the U.S. Food and Drug Administration (FDA) two decades ago (Oldfield & Plosker, 2006). Since 2006, the World Health Organization (WHO) guidelines have consistently

recommended LPV/r as one of PIs in second-line regimens (World Health Organization, 2006). In the latest WHO guidelines (2019), LPV/r is still recommended as the preferred PI therapy for second-line ART regimen, alternative first-line ART regimen in children and in special circumstances in neonates (World Health Organization, 2019). Most recently, due to the global outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection leading to COVID-19 disease, LPV/r is being considered as one of the potential candidates, with multiple clinical trials initiated to test its

efficacy (ChiCTR2000029496, ChiCTR2000029539, NCT04307693, NCT04252885).

By the end of 2018, an estimated 37.9 million people globally were living with HIV (including 1.7 million children). Moreover, 1.7 million in total were newly infected with HIV (including 0.16 million children) in 2018 alone (UNAIDS, 2019). However, only US$ 19 billion was available for HIV/AIDS (acquired immunodeficiency syndrome) response in low- and middle-income countries (end 2018), about US$ 1 billion less than that was available in 2017 (US$ 19.9 billion; UNAIDS, 2019). The relatively low funding, as well as the stagnation in the rate of new HIV infections in recent years, is a challenge for efficient and rapid diagnostics and monitoring for people in resource-limited settings. More than 70% of patients from low- to middle-income countries undergoing second-line regimens are receiving LPV/r (World Health Organization, 2018). Therefore, cost-effective and sensitive bioanalytical methods for therapeutic drug monitoring (TDM) of LPV are needed, mostly in developing countries. To be relevant for TDM, these methods need to cover the range of clinically relevant plasma concentrations of LPV in HIV-infected individuals receiving LPV/r regimen (Eron et al., 2004; Ribera et al., 2004). In addition, the bitter taste reported with various formulations of LPV/r combination (Phelps & Rakhmanina, 2011) is likely to affect adherence, which increases the need for TDM in HIV-infected children. Moreover, application of the new bioanalytical method for the preclinical studies in rats also allows more cost-effective preclinical research, and, importantly, inclusion of resource-limited developing countries in the research activities.

To date, a large number of bioanalytical methods were developed for the determination of LPV with other antiretroviral drugs in plasma using HPLC with UV detection (Faux, Venisse, Olivier, & Bouquet, 2001; Justesen, Pedersen, & Klitgaard, 2003; Notari et al., 2006; Poirier, Robidou, & Jaillon, 2005; Ray, Pang, & Carey, 2002; Rezk, Tidwell, & Kashuba, 2004; Weller, Brundage, Balfour, & Vezina, 2007) or LC–tandem mass spectrometry (LC–MS/MS; Else et al., 2010; Estrela, Ribeiro, Seixas, & Suarez-Kurtz, 2008; Martin et al., 2009; Temghare, Shetye, & Joshi, 2009). However, previously reported bioanalytical methods for determination of LPV utilizing the HPLC-UV methodology are not sensitive enough when low-volume samples are used (Vats, Murthy, & Ravi, 2011). By contrast, the LC–MS/MS methodology does provide sufficient sensitivity, but is too expensive for efficient TDM or preclinical research in resource-limited countries. One published bioanalytical method achieved good sensitivity with lower limit of quantification (LLOQ) of 5 ng/mL using HPLC-UV for determination of LPV (Notari et al., 2006), while another published method utilized LC–MS/MS to obtain LLOQ as low as 1 ng/mL (Estrela et al., 2008). However, a relatively high volume of plasma (600 and 200 μL, respectively) was needed to achieve these high sensitivities in both methods. The relatively high volume of plasma on the scale of 600 μL is problematic for TDM in children, while the scale of 200 μL is still quite high for pharmacokinetic studies in small laboratory animals, such as rats or mice. In addition, due to the fact that safe handling of HIV-positive biological samples in clinics is a priority, drug

stability in heat-inactivated conditions [56°C, 30 mins (Tjøtta, Hungnes, & Grinde, 1991)] has been mostly validated in published methods (Avolio et al., 2011; Egge-Jacobsen et al., 2004; Else et al., 2010; Poirier et al., 2005). As a result, the proposed validation of our bioanalytical method also covers drug stability in heat-inactivated conditions, as well as a full validation recommended by FDA guidelines (Food and Drug Administration, 2018).

Therefore, in this work, a simple, sensitive, cost-efficient and low sample volume bioanalytical method for determination of LPV in rat plasma was developed and fully validated using HPLC with UV detection. This method was successfully implemented in a pharmacokinetic study following intravenous administration of LPV in rats. Moreover, the method has been fully validated for human plasma to provide a cost-effective option for clinical research and TDM.

## 2 | MATERIAL AND METHODS

### 2.1 | Chemicals and reagents

LPV (CAS: 192725–17-0) was purchased from Fisher Scientific (Leicestershire, UK), RTV (CAS: 155213–67-5) from Sigma-Aldrich (Gillingham, UK) and cannabidiol (CAS: 13956–29-1) from THC Pharm (Germany). HPLC-grade ethyl acetate, n-hexane and acetonitrile were purchased from Fisher Scientific (Leicestershire, UK). HPLC-grade water was obtained from PURELAB Ultra system (ELGA LabWater, UK). Rat plasma was purchased from Sera Laboratories (West Sussex, UK) and human plasma from TCS Bioscience (Buckingham, UK). All other research reagents used were of HPLC grade or higher.

### 2.2 | Chromatographic system

The HPLC-UV system consisted of a Waters Alliance 2695 separations module equipped with a Waters 996 photodiode array detector. Separation was accomplished at 40°C using an analytical $C_{18}$ Gemini column (150 mm × 2.0 mm internal diameter) with a particle size of 5 μm, coupled with a guard column (2 mm × 4 mm) with a particle size of 3 μm (Phenomenex, Macclesfield, UK). All compounds were monitored at 211 nm. The final composition of the mobile phase consisted of

**TABLE 1** Gradient conditions

| Time (min) | Flow (mL/min) | Water (%) | Acetonitrile (%) |
| --- | --- | --- | --- |
| 0 | 0.30 | 50 | 50 |
| 10 | 0.30 | 50 | 50 |
| 11 | 0.30 | 30 | 70 |
| 19 | 0.30 | 30 | 70 |
| 20 | 0.30 | 50 | 50 |
| 25 | 0.30 | 50 | 50 |

water and acetonitrile mixture with gradient program (Table 1) at the flow rate of 0.3 mL/min. The injection volume was 40 μL. The temperature of autosampler was maintained at 4°C. Empower 2 software was used for data acquisition and analysis throughout the method development and validation.

## 2.3 | Calibration curves and quality control samples

Stock standard solutions of LPV and internal standard (IS) cannabidiol were prepared by dissolving 10 mg of each compound in 1.0 mL of acetonitrile (10 mg/mL final concentration) and stored at −20°C. Working standard solutions of LPV for calibration curves were diluted from stock standard solution using acetonitrile to yield concentrations of 0.1, 0.2, 0.5, 1, 5, 10, 50 and 100 μg/mL. Working standard solution of IS was prepared in a same procedure to yield a concentration of 10 μg/mL. Calibration curves were established by spiking 100 μL of rat plasma with 10 μL of corresponding working standard solutions of LPV to yield concentrations of 10, 20, 50, 100, 500, 1000, 5000 and 10,000 ng/mL.

Working standard solutions of LPV for quality control (QC) samples were independently prepared in acetonitrile at concentrations of 0.1, 0.25, 4 and 80 μg/mL. A volume of 10 μL of working standard solutions was spiked into 100 μL rat plasma to obtain LLOQ, low quality control (LQC), medium quality control (MQC) and high quality control (HQC) samples at concentrations of 10, 25, 400 and 8000 ng/mL, respectively.

## 2.4 | Sample preparation procedure

All samples went through the same process of combination of protein precipitation and liquid–liquid extraction for HPLC-UV analysis. IS (10 μL) was added to 100 μL of rat plasma in a 16 mm × 150 mm glass tube. Then, 300 μL of cold acetonitrile (−20°C) was added, followed by vortex mixing for 1 min. HPLC-grade water (300 μL) was then added and vortex mixed again for 1 min (Zgair et al., 2015). A volume of 3 mL of extraction solvent, consisting of a mixture *n*-hexane and ethyl estate (7:3, *v/v*), was added and the samples were vortex mixed for 3 min. Following centrifugation (1160*g*, 10°C, 10 min), the upper organic layers were transferred to fresh tubes using a Pasteur pipette and evaporated under nitrogen at 40°C (Techne DRI-Block type DB-3D, Cambridge, UK). Dry residues were reconstituted in 100 μL of mobile phase (50% acetonitrile in water), vortex mixed for 10 min and then transferred to HPLC vials.

## 2.5 | Method validation

A full validation of the new method was carried out in accordance with the FDA guidance for Bioanalytical Method Validation (Food and Drug Administration, 2018).

### 2.5.1 | Selectivity

The selectivity of this new HPLC-UV analytical method was assessed by comparing the chromatograms of pooled blank rat plasma samples with the chromatograms of six replicates of plasma samples spiked with LPV at LLOQ standard solution. The selectivity was further assessed in the samples from intravenous bolus administration of LPV to rats.

### 2.5.2 | Accuracy and Precision

Accuracy was expressed as relative error (RE) and precision as relative standard deviation (RSD). The intra-day accuracy and precision were validated using six-replicate analysis of QC (LQC, MQC and HQC) and LLOQ samples on the same day. The inter-day accuracy and precision were also assessed at the same concentrations on 6 separate days. Based on FDA guidelines, the mean value of accuracy should be within ±15% of the nominal concentration except for the LLOQ which should be within ±20%. The percentage of precision should not exceed 15% except for the LLOQ in which 20% deviation is accepted (Food and Drug Administration, 2018).

### 2.5.3 | Sensitivity

The LLOQ was defined as the lowest tested concentration of the drug with the RE within ±20% and RSD ≤ 20%, for both intra- and inter-day runs (Food and Drug Administration, 2018).

### 2.5.4 | Linearity

Calibration curves (the ratio of LPV peak to IS peak area versus nominal concentration) were fitted by least squares linear regression analysis using a weighted factor (1/*X*). To validate linearity, correlation coefficient ($r^2$) should be over 0.99 and accuracy should be within ±15% (expect for LLOQ in which variability within ±20% is allowed; Food and Drug Administration, 2018).

### 2.5.5 | Recovery

Percentage (%) recovery for LPV was determined by comparing the chromatographic peak areas of QC samples following extraction with peak areas obtained from equivalent levels dissolved directly in reconstitution solvent. The recovery of IS was determined in the same way.

### 2.5.6 | Stability

Stability assays were designed to mimic the likely conditions in which samples will be stored, transferred, processed and analyzed.

QC Samples (LQC, MQC and HQC) were prepared by spiking LPV solution into blank rat plasma. Stability during thermization (heat-inactivation of HIV) was validated by incubating six replicates of QC samples (LQC, MQC and HQC) at 56°C for 30 min (Tjøtta et al., 1991). Freeze–thaw stability was assessed by three cycles of freezing the samples at −80°C for 24 hr and then thawing at room temperature. Benchtop stability was assessed for 6 hr at room temperature. Short-term stability was studied for 24 hr at −20°C, and long-term stability for 4 and 8 weeks at −80°C. Autosampler stability was also assessed by storing six replicates of processed QC samples (LQC, MQC and HQC) at 4°C for 24 hr. Working standard solutions (0.25, 4 and 80 μg/mL) were prepared and stored at room temperature for 6 hr, and then used to prepare six replicates of QC samples (LQC, MQC and HQC) for assessing stock solution stability. Analytes were considered to be stable in plasma when accuracy and precision were within acceptable limits (RE within ±15% and RSD ≤ 15%, respectively; Food and Drug Administration, 2018).

## 2.6 | Method application and validation in human plasma

The method of sample preparation procedure, chromatography conditions and method validation (excluding long-term stability conditions) of LPV in human plasma were identical to those reported for rat plasma above.

## 2.7 | Pharmacokinetic study in rats

Animal welfare and all experimental procedures were reviewed and approved by the University of Nottingham Ethical Review Committee under the Animals [Scientific Procedures] Act 1986. Three male Sprague Dawley rats (300–330 g) were obtained from Charles River Laboratories (UK) and housed at Bio Support Unit, University of Nottingham. They were kept in an environmentally controlled room (12:12-hr light–dark cycle) with free access to food and water for at least six days before starting experiment. Surgical anesthesia was induced by inhalation of 2.5% isoflurane in oxygen (World Precision Instruments, 2019). Cannulation surgery was performed by implantation of silastic-polyethylene (PE-50) tubing into the right external jugular vein as previously described (Lee et al., 2017; Thrivikraman, Huot, & Plotsky, 2002; Zgair et al., 2015). Rats were allowed to recover for two nights before pharmacokinetic experiment.

A solution of LPV was prepared at a concentration of 4 mg/mL in a mixture of propylene glycol–sterile water–ethanol (70:20:10, v/v/v). The drug was administered as an intravenous bolus through the jugular vein cannula at a dose of 4 mg/kg. Blood samples (0.2 mL) were withdrawn from catheter before administration, and at 5, 15, 30, 60, 120, 180, 240 and 360 min following administration. Plasma samples were separated immediately by centrifugation

(3000*g*, 5 min) and stored at −80°C until analysis. Pharmacokinetic parameters were obtained by non-compartment analysis using Phoenix WinNonlin 6.3 software (Pharsight, Mountain View, CA, USA).

## 3 | RESULTS AND DISCUSSION

## 3.1 | Method development

Because LPV is not a highly lipophilic compound (Log P = 1.7, experimental result; Ford, Khoo, & Back, 2004), n-hexane (non-polar organic solvent) was not efficient in extraction of LPV from plasma (the absolute recovery was zero). Adding 30% ethyl acetate to n-hexane enhanced extraction efficiency dramatically and the mixture of n-hexane–ethyl acetate 7:3 (v/v) was selected as the optimum extraction solvent. In addition to the extracting solvent composition, the duration of the extraction was also optimized in this assay. It was observed that LPV was extracted very rapidly (within 3-min vortex mixing) into the organic phase during the liquid–liquid extraction procedure. Further increase in the duration of the extraction did not improve the recovery. Similar to a previously reported work from our group for bioanalysis of phytocannabinoids, addition of water following plasma protein precipitation before the liquid–liquid extraction improved the extraction efficiency and the chromatography baseline (Zgair et al., 2015). Cannabidiol was selected as an IS because it had similar extraction procedure and efficiency to LPV in our previous work (Zgair et al., 2015), as well as longer retention time than LPV in chromatography conditions developed in this work.

A simple buffer-free gradient mobile phase, starting from a mixture of acetonitrile–water (50:50, v/v), gave optimal separation of LPV from background peaks. A higher percentage of acetonitrile was gradually blended in mobile phase to elute IS (Table 1). Both LPV and IS were monitored at 211 nm, as LPV had the highest UV absorbance and chromatography baseline was clean at LPV retention time at this wavelength.

## 3.2 | Method validation for rat plasma

## 3.2.1 | Selectivity

This analytical method showed good selectivity and the peak of LPV was well-separated from endogenous peaks in blank rat plasma (Figure 1a–c), indicating minimum matrix effect and absence of carryover. In addition, this method was applied to evaluate drug concentrations in rat plasma following intravenous administration, and in these samples there was also no interference between the LPV, IS and endogenous peaks (Figure 1d). Therefore, this method is selective for determination of LPV in rat plasma and suitable for pharmacokinetic experiments in these species.
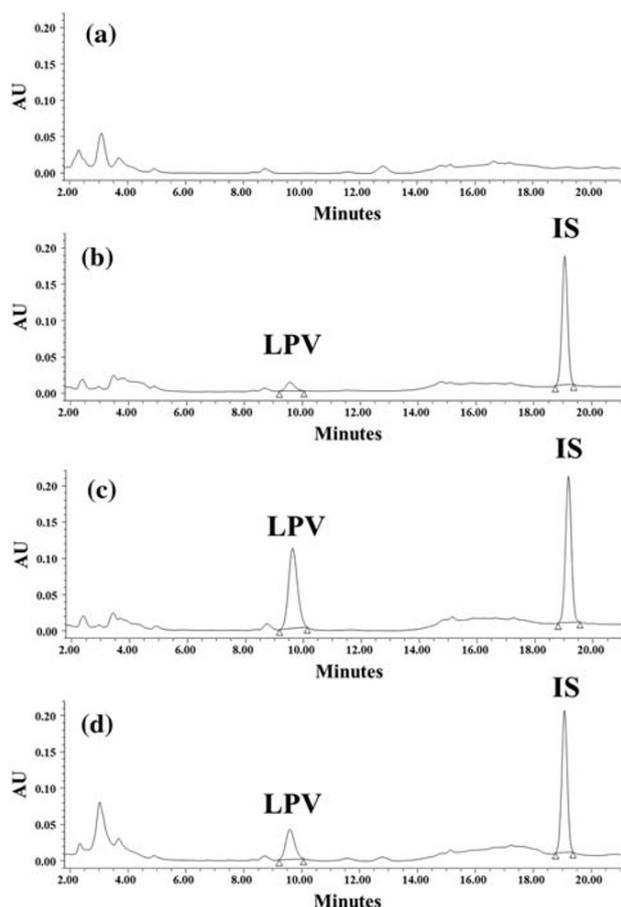
**FIGURE 1** Representative chromatograms of (a) rat plasma blank sample; (b) rat plasma spiked with 1000 ng/mL LPV; (c) rat plasma spiked with 8000 ng/mL LPV and (d) plasma obtained at 5 minutes following intravenous administration of LPV in rat (4 mg/kg). All chromatograms are observed at $\lambda = 211$ nm. IS, internal standard; LPV, lopinavir

## 3.2.2 | Accuracy and precision

Samples were assessed at four different concentrations in six replicates to evaluate accuracy (RE) and precision (RSD), respectively. Results for intra- and inter-day validation of LLOQ and QC samples are listed in Table 2. For accuracy and precision validation, the mean values of all QC samples (LQC, MQC and HQC) were within acceptable range (RE within ±15% and RSD ≤ 15%). For the LLOQ, RE was within ±20% and RSD ≤ 20%. These results indicate that this analytical method is suitable for determination of LPV levels in rat plasma in an accurate and precise manner (Food and Drug Administration, 2018).

## 3.2.3 | Sensitivity and linearity

In this method, 10 ng/mL was determined as LLOQ, because intra- and inter-day accuracy and precision validations for LLOQ samples were within acceptable range (RE within ±20% and RSD ≤ 20%; Table 2). The method was linear for LPV with correlation coefficient ($r^2$) greater than 0.99 in all calibration curves in the range of

10–10,000 ng/mL, indicating dilution integrity in this range under the conditions of this assay. To note, a low volume of plasma (100 μL) was needed to achieve the LLOQ of 10 ng/mL which makes this assay sufficiently sensitive to perform preclinical pharmacokinetic studies in small laboratory animals or other situations when sample volume is limited. In previously reported studies, some bioanalytical methods achieved better sensitivities (LLOQ < 10 ng/mL), but required higher volume of plasma and/or more expensive analytic equipment such as an LC–MS/MS system (Estrela et al., 2008; Notari et al., 2006).

## 3.2.4 | Recovery

Mean absolute recoveries of LPV in LQC, MQC and HQC samples were 88.7% ± 2.1%, 96.5% ± 1.0% and 96.4% ± 2.1%, respectively (mean ± SD, $n = 6$). The absolute recovery of IS was 70.6% ± 2.5% (mean ± SD, $n = 3$). The optimized liquid–liquid extraction step contributed to excellent recovery of LPV from rat plasma, thereby achieving good sensitivity of this method.

## 3.2.5 | Stability

The results of all stability experiments are presented in Table 3. All QC samples (LQC, MQC and HQC) were considered to be stable under different realistic storage conditions, with the values of accuracy (RE) and precision (RSD) in the acceptable range (RE within ±15%, RSD ≤ 15%). Very limited LPV stability data in rat plasma have been reported in previous studies and only included freeze–thaw, short-term and autosampler stability experiments (Vats et al., 2011). To the best of our knowledge, this is the first report that covers full validation and stability of LPV in rat plasma according to FDA guidelines (Food and Drug Administration, 2018). Moreover, the stability of LPV in rat plasma was originally validated at heat-inactivated conditions only (Tjøtta et al., 1991).

**TABLE 2** Intra- and inter-day validation results for determination of lopinavir in rat plasma

| Nominal concentration levels | Intra-day ($n = 6$) | | Inter-day ($n = 6$) | |
| --- | --- | --- | --- | --- |
| | Accuracy (RE, %) | Precision (RSD, %) | Accuracy (RE, %) | Precision (RSD, %) |
| LLOQ (10 ng/mL) | 5.59 | 15.80 | 15.36 | 2.93 |
| LQC (25 ng/mL) | −7.02 | 1.64 | −2.09 | 10.90 |
| MQC (400 ng/mL) | −5.67 | 2.26 | −2.91 | 8.61 |
| HQC (8000 ng/mL) | −1.78 | 3.69 | 1.22 | 5.68 |

HQC, high quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; RE, relative error; RSD, relative standard deviation.

**TABLE 3** Stability results of lopinavir in rat plasma under various storage conditions (n = 6)

| Concentration levels | Inactivation stability (56°C, 30 min) | | Benchtop stability (25°C, 6 hr) | | Stock solution stability (25°C, 6 hr) | | Autosampler stability (4°C, 24 hr) | | Short-term stability (−20°C, 24 hr) | | Freeze–thaw stability (−80°C, 3 cycles) | | Long-term stability (−80°C, 4 weeks) | | Long-term stability (−80°C, 8 weeks) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) |
| LQC (25 ng/mL) | 3.91 | 8.02 | 0.92 | 10.89 | 0.23 | 2.68 | 6.57 | 4.19 | 8.03 | 4.03 | −0.16 | 3.94 | 4.61 | 4.90 | 12.76 | 1.68 |
| MQC (400 ng/mL) | −3.89 | 6.26 | 1.39 | 1.65 | −5.96 | 4.20 | −11.18 | 1.34 | −8.99 | 6.07 | −0.20 | 3.94 | −2.03 | 4.00 | −9.73 | 1.35 |
| HQC (8000 ng/mL) | −7.42 | 6.33 | 7.42 | 2.59 | −3.22 | 2.82 | −3.23 | 3.30 | −1.29 | 2.92 | −0.94 | 3.51 | 5.23 | 2.88 | −0.91 | 3.38 |

HQC, high quality control; LQC, low quality control; MQC, medium quality control; RE, relative error; RSD, relative standard deviation.

## 3.3 | Pharmacokinetic study in rats

The developed bioanalytical method was applied to a preclinical pharmacokinetic study in rats following single intravenous bolus administration. The individual plasma concentration–time profiles of LPV in three rats are shown in Figure 2, and mean plasma pharmacokinetic parameters obtained using non-compartmental analysis are presented in Table 4. Limited number of preclinical pharmacokinetic studies following single intravenous bolus of LPV in rats have been reported (Kumar et al., 2004; Vats et al., 2011). The elimination $t_{1/2}$ (0.49 ± 0.01 hr) obtained in this study is shorter than the $t_{1/2}$ previously reported (0.82 ± 0.03 hr) using the HPLC-UV method for the detection of LPV (Vats et al., 2011). The lower sensitivity of the previously reported method (LLOQ = 250 ng/mL) limited sampling up to 3 hr, which resulted in sampling duration of less than four times the elimination $t_{1/2}$. However, to better characterize a pharmacokinetic profile, sampling should preferably be four to five times the elimination $t_{1/2}$ (Dunnington et al., 2018). In the current study, a high sensitivity (LLOQ = 10 ng/mL) allows sampling up to 4 hr (more than eight times the elimination $t_{1/2}$), thereby accurately characterizing the pharmacokinetic profile of LPV. Kumar et al. (2004) reported a significantly longer elimination $t_{1/2}$ (mean value of 2.07 hr) compared with other studies. This prolonged elimination $t_{1/2}$ is probably a result of the analytical assay which utilized LPV. This trend has been previously observed for other compounds in which radioactivity-based bioanalytical methods were used (Isin, Elmore, Nilsson, Thompson, & Weidolf, 2012).

## 3.4 | Assessment and validation of the developed bioanalytical method in human plasma for clinical therapeutic drug monitoring

Following the development and validation of the bioanalytical method for determination of LPV in rat plasma, the suitability of this method for clinical research and clinical TDM of HIV-infected
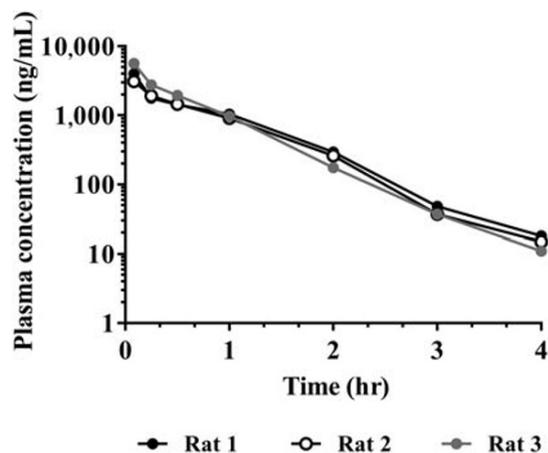
**TABLE 4** Pharmacokinetic parameters of lopinavir following a single intravenous administration of 4 mg/kg to rats ($n = 3$)

| Parameters | Mean | SD |
|---|---|---|
| $AUC_{inf}$ (h·ng/mL) | 2709 | 371 |
| $AUC_{0 \to t}$ (h·ng/mL) | 2699 | 372 |
| $C_0$ (ng/mL) | 5992 | 2033 |
| $t_{1/2}$ (h) | 0.49 | 0.01 |
| $V_{ss}$ (mL/kg) | 1053 | 271 |
| CL (mL/h/kg) | 1495 | 200 |

$AUC_{0 \to t}$, area under the curve from time zero to the last sampling time point; $AUC_{inf}$, area under the curve from time zero to infinity; $C_0$, concentration extrapolated to time zero; CL, clearance; SD, standard deviation; $t_{1/2}$, elimination half-life; $V_{ss}$, volume of distribution at steady state.

patients receiving LPV/r therapy was assessed. A routine TDM plays an important role in ART to assist clinicians in assessing adherence and optimizing the regimens for HIV-infected patients, and therefore to reduce the risks of insufficient therapeutic response, drug resistance or toxicity. This is particularly important in relation to PIs, as the pharmacokinetics of PIs is known to have high inter-individual variability (Barry et al., 1998; Regazzi et al., 1999; van der Leur, Burger, Porte, & Koopmans, 2006). Very high concentration of LPV can lead to adverse effects such as hyperlipidemia (Gutiérrez et al., 2003; Limsreng, Marcy, Ly, Ouk, & Chanroeurn, 2016; Montes et al., 2005) and gastrointestinal disorders (Boffito et al., 2005; Hill & Balkin, 2009), whereas concentration below the therapeutic window would lead to therapeutic failure and drug resistance (Breilh, Pellegrin, Berthoin, & Xuereb, 2004; Masquelier et al., 2002). Moreover, the poor palatability of LPV/r liquid formulations (Phelps & Rakhmanina, 2011) increases the risk of suboptimal antiretroviral efficiency in children due to poor adherence. Therefore, this cost-effective and sensitive bioanalytical method, involving low volume samples of plasma, could be useful for monitoring plasma concentrations of LPV in adults and children in both developed and resource-limited countries.

The same calibration curve range (10–10,000 ng/mL) was used in the assessment of the newly developed bioanalytical method for human plasma, because the mean $C_{max}$, $C_{min}$ and $C_{trough}$ of LPV were reported to be within this range during LPV/r regimen (400/100 mg b. i.d.) in HIV-infected adults (Eron et al., 2004; Ribera et al., 2004). The reported mean $C_{trough}$ levels of LPV following LPV/r regimens based on body weight in HIV-infected children were also within this range (Puthanakit et al., 2010). The same sample preparation procedure and chromatography conditions used for rat plasma were also applied to human plasma. Good selectivity was observed by comparison of chromatograms between LPV/RTV-spiked samples and blank human plasma samples (Figure 3). Intra- and inter-day accuracy (RE) and precision (RSD) were assessed using six spiked human plasma samples at four different concentrations (LLOQ and QCs) for LPV (Table 5). The same sensitivity (LLOQ = 10 ng/mL) and linearity range ($r^2 > 0.99$) as for rat plasma were achieved when the method was applied to human plasma. The extraction recovery of human plasma LQC, MQC and



**FIGURE 2** Plasma concentration–time profiles of LPV in rats following intravenous administration at 4 mg/kg ($n = 3$). LPV, lopinavir

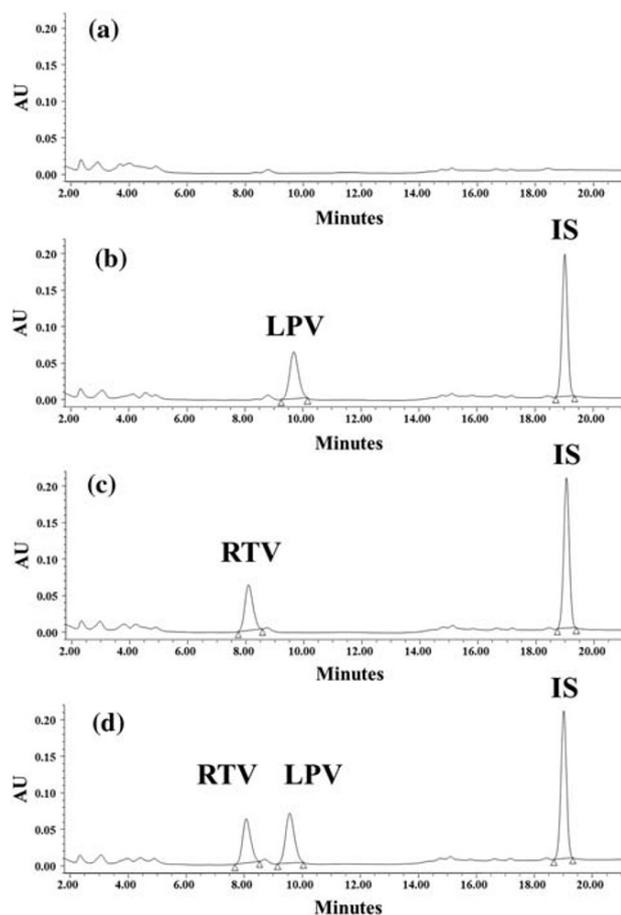**FIGURE 3** Representative chromatograms of (a) blank human plasma; (b) and (c) human plasma spiked with 5000 ng/mL of LPV and 5000 ng/mL of RTV, respectively; (d) human plasma sample spiked with 5000 ng/mL of LPV and RTV simultaneously. All chromatograms are observed at $\lambda$ = 211 nm. IS, internal standard; LPV, lopinavir; RTV, ritonavir

HQC samples were 94.3% ± 4.9%, 99.6% ± 2.2% and 99.5% ± 3.5%, respectively (mean ± SD, n = 6).

The stability of LPV at three concentrations (QCs) in human plasma under different realistic storage conditions is shown in Table 6. The LPV was stable in human plasma samples under all tested conditions, because the values of RE and RSD are within acceptable range (RE within ±15%, RSD ≤ 15%; Food and Drug Administration, 2018).

**TABLE 5** Intra- and inter-day validation results for determination of lopinavir in human plasma

| Nominal concentration levels | Intra-day (n = 6) | | Inter-day (n = 6) | |
|---|---|---|---|---|
| | Accuracy (RE, %) | Precision (RSD, %) | Accuracy (RE, %) | Precision (RSD, %) |
| LLOQ (10 ng/mL) | 5.61 | 5.60 | 6.53 | 10.44 |
| LQC (25 ng/mL) | 3.54 | 4.52 | 2.11 | 5.20 |
| MQC (400 ng/mL) | −1.79 | 4.35 | −1.92 | 2.77 |
| HQC (8000 ng/mL) | −5.57 | 2.95 | 2.40 | 3.56 |

HQC, high quality control; LLOQ, lower limit of quantification; LQC, low quality control; MQC, medium quality control; RE, relative error; RSD, relative standard deviation.

The heat-inactivation stability has demonstrated that the samples are stable during the patient plasma HIV inactivation procedure prior to sample preparation. The benchtop stability and stock solution stability show that samples and stock solutions are stable during the sample preparation procedure. The processed samples are also stable at 4°C for 24 hr, mimicking the condition in autosampler. Freeze–thaw stability indicated that samples are stable after undergoing three cycles of freeze and thaw, which should allow TDM even after the samples have been previously used for other tests, such as viral load monitoring. Plasma samples are also stable under short-term storage conditions with the acceptable values of RE and RSD. Therefore, a low plasma volume, good extraction efficiency, high sensitivity and good stability in human plasma at different storage situations make this versatile and robust bioanalytical method suitable for clinical research and clinical TDM of HIV-infected adult and pediatric patients. This new method of determination of LPV using HPLC-UV detection maintains similar sensitivity to that previously achieved by the LC–MS/MS methodology (Estrela et al., 2008), and requires much lower volume of plasma compared with the previously reported HPLC-UV method (Notari et al., 2006). Importantly, the excellent cost-efficacy of this method compared with the much more expensive LC–MS/MS methodology allows TDM and clinical research activity in resources-limited countries in the developing world.

**TABLE 6** Stability results of lopinavir in human plasma under various storage conditions (n = 6)

| Concentration levels | Inactivation stability (56°C, 30 min) | | Benchtop stability (25°C, 6 hr) | | Stock solution stability (25°C, 6 hr) | | Autosampler stability (4°C, 24 hr) | | Short-term stability (−20°C, 24 hr) | | Freeze–thaw stability (−80°C, 3 cycles) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) |
| LQC (25 ng/mL) | −1.69 | 5.78 | −7.72 | 2.73 | −3.71 | 6.46 | 7.43 | 4.87 | −10.10 | 4.39 | 7.50 | 4.45 |
| MQC (400 ng/mL) | −2.14 | 5.28 | −3.27 | 3.12 | −5.19 | 4.21 | −7.82 | 3.76 | 7.04 | 3.56 | 4.28 | 3.39 |
| HQC (8000 ng/mL) | 9.80 | 3.06 | 12.27 | 1.91 | 2.63 | 5.59 | −2.05 | 4.64 | 8.04 | 4.38 | 6.29 | 4.88 |

HQC, high quality control; LQC, low quality control; MQC, medium quality control; RE, relative error; RSD, relative standard deviation.

## 4 | CONCLUSION

A simple, sensitive and cost-efficient HPLC-UV method for the determination of LPV in rat plasma was developed and fully validated. This assay achieved higher sensitivity using lower volume of plasma compared with previously reported HPLC-UV methods. This method will allow preclinical pharmacokinetic and drug delivery studies in rats in the developed and developing countries in a cost-efficient and reliable manner. Moreover, the newly developed bioanalytical method was applied and validated for human plasma with similar good selectivity, sensitivity, linearity and stability under different storage conditions. Therefore, this method can be used for clinical research and clinical TDM of HIV-infected and potentially SARS-CoV-2-infected patients undergoing LPV/r treatment in a cost-effective manner in the resource-rich and resource-limited settings.

### ORCID

*Chaolong Qin* https://orcid.org/0000-0003-1161-5580
*Jong Bong Lee* https://orcid.org/0000-0002-8404-0581
*Pavel Gershkovich* https://orcid.org/0000-0003-3136-0933

### REFERENCES

Avolio, A. D., Simiele, M., Siccardi, M., Baietto, L., Sciandra, M., Oddone, V., ... Perri, G. D. (2011). A HPLC – MS method for the simultaneous quantification of fourteen antiretroviral agents in peripheral blood mononuclear cell of HIV infected patients optimized using medium corpuscular volume evaluation. *Journal of Pharmaceutical and Biomedical Analysis*, *54*(4), 779–788. https://doi.org/10.1016/j.jpba.2010.10.011

Barry, M. G., Merry, C., Lloyd, J., Halifax, K., Carey, P., Mulcahy, F., & Back, D. J. (1998). Variability in trough plasma saquinavir concentrations in HIV patients—a case for therapeutic drug monitoring. *British Journal of Clinical Pharmacology*, *45*, 501–502. https://doi.org/10.1046/j.1365-2125.1998.00714.x

Boffito, M., Acosta, E., Burger, D., Fletcher, C. V., Flexner, C., & Garaffo, R. (2005). Current status and future prospects of therapeutic drug monitoring and applied clinical pharmacology in antiretroviral therapy. *Antiviral Therapy*, *10*(3), 375–392.

Breilh, D., Pellegrin, I., Berthoin, K., & Xuereb, F. (2004). Virological, intracellular and plasma pharmacological parameters predicting response to lopinavir/ritonavir (KALEPHAR Study). *Aids*, *18*(9), 1305–1310. https://doi.org/10.1097/01.aids.0000125993.32646.b1

Dunnington, K., Benrimoh, N., Brandquist, C., Cardillo-marricco, N., Di Spirito, M., & Grenier, J. (2018). Application of Pharmacokinetics in Early Drug Development Development-Chapter 4. https://doi.org/10.5772/intechopen.74189

Egge-Jacobsen, W., Unger, M., Niemann, C. U., Baluom, M., Hirai, S., Benet, L. Z., & Christians, U. (2004). Automated, fast, and sensitive quantification of drugs in human plasma by LC/LC-MS: Quantification of 6 protease inhibitors and 3 nonnucleoside transcriptase inhibitors.

*Therapeutic Drug Monitoring*, *26*(5), 546–562. https://doi.org/10.1097/00007691-200410000-00014

Else, L., Watson, V., Tjia, J., Hughes, A., Siccardi, M., Khoo, S., & Back, D. (2010). Validation of a rapid and sensitive high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assay for the simultaneous determination of existing and new antiretroviral compounds. *Journal of Chromatography B*, *878*(19), 1455–1465. https://doi.org/10.1016/j.jchromb.2010.03.036

Eron, J. J., Feinberg, J., Kessler, H. A., Horowitz, H. W., Witt, M. D., Carpio, F. F., ... Sun, E. (2004). Once-Daily versus Twice-Daily Lopinavir/Ritonavir in Antiretroviral-Naive HIV-Positive Patients: A 48-Week Randomized Clinical Trial. *The Journal of Infectious Diseases*, *189*(2), 265–272. https://doi.org/10.1086/380799

Estrela, R. C. E., Ribeiro, F. S., Seixas, B. V., & Suarez-Kurtz, G. (2008). Determination of lopinavir and ritonavir in blood plasma, seminal plasma, saliva and plasma ultra-filtrate by liquid chromatography/-tandem mass spectrometry detection. *Rapid Communications in Mass Spectrometry*, *22*(5), 657–664. https://doi.org/10.1002/rcm.3411

Faux, J., Venisse, N., Olivier, J. C., & Bouquet, S. (2001). Rapid high-performance liquid chromatography determination of lopinavir, a novel HIV-1 protease inhibitor, in human plasma. *Chromatographia*, *54*(7–8), 469–473. https://doi.org/10.1007/BF02491201

Food and Drug Administration. (2018). Bioanalytical Method Validation Guidance for Industry. *U.S. Department of Health and Human Services*, (May), 1–41.

Ford, J., Khoo, S. H., & Back, D. J. (2004). The intracellular pharmacology of antiretroviral protease inhibitors. *Journal of Antimicrobial Chemotherapy*, *54*(6), 982–990. https://doi.org/10.1093/jac/dkh487

Gutiérrez, F., Padilla, S., Navarro, A., Masiá, M., Hernández, I., Ramos, J., ... Martin-Hidalgo, A. (2003). Lopinavir Plasma Concentrations and Changes in Lipid Levels During Salvage Therapy with Lopinavir/-Ritonavir-Containing Regimens. *Journal of Acquired Immune Deficiency Syndromes*, *33*(5), 594–600. https://doi.org/10.1097/00126334-200308150-00007

Hill, A., & Balkin, A. (2009). Risk Factors for Gastrointestinal Adverse Events in HIV Treated and Untreated Patients. *AIDS Reviews*, *11*(1), 30–38.

Isin, E. M., Elmore, C. S., Nilsson, G. N., Thompson, R. A., & Weidolf, L. (2012). Use of Radiolabeled Compounds in Drug Metabolism and Pharmacokinetic Studies. *Chemical Research in Toxicology*, *25*(3), 532–542. https://doi.org/10.1021/tx2005212

Justesen, U. S., Pedersen, C., & Klitgaard, N. A. (2003). Simultaneous quantitative determination of the HIV protease inhibitors indinavir, amprenavir, ritonavir, lopinavir, saquinavir, nelfinavir and the nelfinavir active metabolite M8 in plasma by liquid chromatography. *Journal of Chromatography B*, *783*(2), 491–500. https://doi.org/10.1016/S1570-0232(02)00728-6

Kumar, G. N., Jayanti, V. K., Johnson, M. K., Uchic, J., Thomas, S., Lee, R. D., ... Roberts, S. A. (2004). Metabolism and Disposition of the HIV-1 Protease Inhibitor Lopinavir (ABT-378) Given in Combination with Ritonavir in Rats, Dogs, and Humans. *Pharmaceutical Research*, *21*(9), 1622–1630. https://doi.org/10.1023/b:pham.0000041457.64638.8d

Lee, J. B., Zgair, A., Kim, T. H., Kim, M. G., Yoo, S. D., & Fischer, P. M. (2017). Simple and sensitive HPLC-UV method for determination of bexarotene in rat plasma. *Journal of Chromatography B*, *1040*, 73–80. https://doi.org/10.1111/j.1095-8649.2005.00727.x

Limsreng, S., Marcy, O., Ly, S., Ouk, V., & Chanroeurn, H. (2016). Dyslipidemias and Elevated Cardiovascular Risk on Lopinavir-Based Antiretroviral Therapy in Cambodia. *PLoS ONE*, *11*(8), e0160306, 1–13. https://doi.org/10.1371/journal.pone.0160306

Martin, J., Deslandes, G., Dailly, E., Renaud, C., Reliquet, V., Raffi, F., & Jolliet, P. (2009). A liquid chromatography-tandem mass spectrometry assay for quantification of nevirapine, indinavir, atazanavir,

amprenavir, saquinavir, ritonavir, lopinavir, efavirenz, tipranavir, darunavir and maraviroc in the plasma of patients infected with HIV. *Journal of Chromatography B*, 877(27), 3072–3082. https://doi.org/10.1016/j.jchromb.2009.07.031

Masquelier, B., Breilh, D., Neau, D., Lawson-ayayi, S., Ragnaud, J., Dupon, M., ... Ame, P. (2002). Human Immunodeficiency Virus Type 1 Genotypic and Pharmacokinetic Determinants of the Virological Response to Lopinavir-Ritonavir-Containing Therapy in Protease Inhibitor-Experienced Patients. *Antimicrobial Agents and Chemotherapy*, 46(9), 2926–2932. https://doi.org/10.1128/AAC.46.9.2926

Montes, M. L., Pulido, F., Barros, C., Condes, E., Rubio, R., Dronda, F., ... Pen, J. M. (2005). Lipid disorders in antiretroviral-naive patients treated with lopinavir/ritonavir-based HAART: frequency, characterization and risk factors. *Journal of Antimicrobial Chemotherapy*, 55(5), 800–804. https://doi.org/10.1093/jac/dki063

Notari, S., Bocedi, A., Ippolito, G., Narciso, P., Pucillo, L. P., Tossini, G., ... Ascenzi, P. (2006). Simultaneous determination of 16 anti-HIV drugs in human plasma by high-performance liquid chromatography. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 831(1–2), 258–266. https://doi.org/10.1016/j.jchromb.2005.12.016

Oldfield, V., & Plosker, G. L. (2006). Lopinavir/ritonavir: A review of its use in the management of HIV infection. *Drugs*, 66(9), 1275–1299. https://doi.org/10.2165/00003495-200666090-00012

Phelps, B. R., & Rakhmanina, N. (2011). Antiretroviral Drugs in Pediatric HIV-Infected Patients Pharmacokinetic and Practical Challenges. *Pediatric Drugs*, 13(3), 175–192. https://doi.org/10.2165/11587300-000000000-00000

Poirier, J., Robidou, P., & Jaillon, P. (2005). Simple and Simultaneous Determination of the Nelfinavir Metabolite and the Nonnucleoside Reverse Transcriptase Inhibitors Efavirenz and Nevirapine in Human Plasma by Reversed-Phase Liquid Chromatography. *Therapeutic Drug Monitoring*, 27(2), 186–192. https://doi.org/10.1097/01.ftd.0000152680.36517.5d

Puthanakit, T., Chokephaibulkit, K., Suntarattiwong, P., Gorowara, M., Vanprapar, N., Leawsrisuk, P., ... Ruxrungtham, K. (2010). Therapeutic drug monitoring of lopinavir in HIV infected children receiving adult tablets. *The Pediatric Infectious Disease Journal*, 29(1), 79–82. https://doi.org/10.1097/inf.0b013e3181b21040

Ray, J., Pang, E., & Carey, D. (2002). Simultaneous determination of indinavir, ritonavir and lopinavir (ABT 378) in human plasma by high-performance liquid chromatography. *Journal of Chromatography B*, 775(2), 225–230. https://doi.org/10.1016/S1570-0232(02)00295-7

Regazzi, M. B., Villani, P., Maserati, R., Cocchi, L., Giacchino, R., Burroni, D., & Rettani, M. (1999). Pharmacokinetic variability and strategy for therapeutic drug monitoring of saquinavir (SQV) in HIV-1 infected individuals. *British Journal of Clinical Pharmacology*, 47(4), 379–382. https://doi.org/10.1046/j.1365-2125.1999.00919.x

Rezk, N. L., Tidwell, R. R., & Kashuba, A. D. M. (2004). High-performance liquid chromatography assay for the quantification of HIV protease inhibitors and non-nucleoside reverse transcriptase inhibitors in human plasma. *Journal of Chromatography B*, 805(2), 241–247. https://doi.org/10.1016/j.jchromb.2004.03.002

Ribera, E., Lopez, R. M., Diaz, M., Pou, L., Ruiz, L., Crespo, M., ... Ruiz, I. (2004). Steady-State Pharmacokinetics of a Double-Boosting Regimen of Saquinavir Soft Gel plus Lopinavir plus Minidose Ritonavir in Human Immunodeficiency Virus-Infected Adults. *Antimicrobial Agents and Chemotherapy*, 48(11), 4256–4262. https://doi.org/10.1128/AAC.48.11.4256

Temghare, G. A., Shetye, S. S., & Joshi, S. S. (2009). Rapid and sensitive method for quantitative determination of lopinavir and ritonavir in human plasma by liquid chromatography-tandem mass specrtometry. *E-Journal of Chemistry*, 6(1), 223–230. https://doi.org/10.1155/2009/709478

Thrivikraman, K. V., Huot, R. L., & Plotsky, P. M. (2002). Jugular vein catheterization for repeated blood sampling in the unrestrained conscious rat. *Brain Research Protocols*, 10(2), 84–94. https://doi.org/10.1016/S1385-299X(02)00185-X

Tjøtta, E., Hungnes, O., & Grinde, B. (1991). Survival of HIV-1 Activity After Disinfection, Temperature and pH Changes, or Drying. *Journal of Medical Virology*, 35(4), 223–227. https://doi.org/10.1002/jmv.1890350402

UNAIDS. (2019). Global HIV and Aids Statistics Fact Sheet– World Aids Day 2019. Retrieved from http://www.unaids.org/en/resources/fact-sheet

van der Leur, M. R., Burger, D. M., Porte, C. J. L., & Koopmans, P. P. (2006). A Retrospective TDM Database Analysis of Interpatient Variability in the Pharmacokinetics of Lopinavir in HIV-infected Adults. *Therapeutic Drug Monitoring*, 28(5), 650–653. https://doi.org/10.1097/01.ftd.0000245681.12092.d6

Vats, R., Murthy, A. N., & Ravi, P. R. (2011). Simple, rapid and validated LC determination of lopinavir in rat plasma and its application in pharmacokinetic studies. *Scientia Pharmaceutica*, 79(4), 849–863. https://doi.org/10.3797/scipharm.1107-24

Weller, D. R., Brundage, R. C., Balfour, H. H., & Vezina, H. E. (2007). An isocratic liquid chromatography method for determining HIV nonnucleoside reverse transcriptase inhibitor and protease inhibitor concentrations in human plasma. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 848(2), 369–373. https://doi.org/10.1016/j.jchromb.2006.10.022

World Health Organization. (2006). Antiretroviral therapy for HIV infection in adults and adolescents: Recommendations for a public health approach.

World Health Organization. (2018). Updated recommendations on first-line and second-line antiretroviral regimens and post-exposure prophylaxis and recommendations on early infant diagnosis of HIV: Interim guidelines.

World Health Organization. (2019). Update of FIRST- and second-line antiretroviral regimens.

World Precision Instruments. (2019). EZ_anesthesia_isoflurane. Retrieved from https://www.Wpiinc.Com/Media/Wysiwyg/Pdf/EZ_IM.Pdf, 8.

Zgair, A., Wong, J. C. M., Sabri, A., Fischer, P. M., Barrett, D. A., Constantinescu, C. S., & Gershkovich, P. (2015). Development of a simple and sensitive HPLC-UV method for the simultaneous determination of cannabidiol and $\delta^9$-tetrahydrocannabinol in rat plasma. *Journal of Pharmaceutical and Biomedical Analysis*, 114, 145–151. https://doi.org/10.1016/j.jpba.2015.05.019