Original article

# Influence of traditional Chinese medicines on the *in vivo* metabolism of lopinavir/ritonavir based on UHPLC-MS/MS analysis

Linlin Li, Xinxiang Yu, Dongmin Xie, Ningning Peng, Weilin Wang, Decai Wang[*], Binglong Li[**]

*Department of Clinical Pharmacy, School of Pharmacy, Shandong First Medical University & Shandong Academy of Medical Sciences, Tai'an, Shandong, 271016, China*

A B S T R A C T

A fast, reliable, and cost-effective liquid chromatography-tandem mass spectrometry method was established to determine the effects of the traditional Chinese medicine employed to treat coronavirus disease 2019, namely, Lianhua Qingwen granules, Huoxiang Zhengqi capsules, Jinhua Qinggan granules, Shufeng Jiedu capsules, and Angong Niuhuang pills, on the pharmacokinetics of lopinavir/ritonavir in rats. Blood samples were prepared using the protein precipitation method and atazanavir was selected as the internal standard (IS). Separation was performed on an Agilent ZORBAX eclipse plus $C_{18}$ (2.1 mm × 50 mm, 1.8 μm) column using acetonitrile and water containing 0.1% formic acid as the mobile phase for gradient elution. The flow rate was 0.4 mL/min and the injection volume was 2 μL. Agilent Jet Stream electrospray ionization was used for mass spectrometry detection under positive ion multiple reaction monitoring mode at a transition of $m/z$ 629.3→447.3 for lopinavir, $m/z$ 721.3→296.1 for ritonavir, and $m/z$ 705.4→168.1 for the IS. The method showed good linearity in the concentration range of 25−2500 ng/mL ($r$=0.9981) for lopinavir and 5−500 ng/mL ($r$=0.9984) for ritonavir. The intra-day and inter-day precision and accuracy were both within ±15%. Items, such as dilution reliability and residual effect, were also within the acceptable limits. The method was used to determine the effects of five types of traditional Chinese medicines on the pharmacokinetics of lopinavir/ritonavir in rats. The pharmacokinetic results showed that the half-life of ritonavir in the groups administered Lianhua Qingwen granules and Huoxiang Zhengqi capsules combined with lopinavir/ritonavir was prolonged by approximately 1.5- to 2-fold relative to that in the control group. Similarly, the pharmacokinetic parameters of lopinavir were altered. Overall, the results of this study offer important theoretical parameters for the effective clinical use of five types of traditional Chinese medicines combined with lopinavir/ritonavir to reduce the occurrence of clinical adverse reactions.

## 1. Introduction

In December 2019, a type of pneumonia of unknown origin suddenly emerged and proliferated at an extremely fast rate throughout the world, resulting in a global pandemic [1,2]. This disease-causing virus was named "2019 new coronavirus" by the World Health Organization (WHO) on January 12, 2020 [3]; however, it was later named coronavirus disease 2019 (COVID-19) [4]. To date, 156,696,833 cases of COVID-19 have been diagnosed globally, and 3,269,661 deaths have been reported [5]. Currently, the global epidemic is still in a very severe state.

Coronavirus is a type of enveloped single-stranded ribonucleic acid (RNA) virus [6] with a high mutation rate and fast recombination speed [7]. Coronavirus, which belongs to the *Coronavirus* subfamily of the *Coronaviridae* family, consists of four genera: α, β, γ, and δ. All coronaviruses have similar characteristics in the structure and expression of their genomes [8]. COVID-19 is the seventh type of coronavirus that can lead to infection [9]. This oval or round virus particle has a diameter of 60−140 nm [10] and can cause lower respiratory tract infection and lead to pneumonia. The virus genome encodes spike protein [11−14] and some nonstructural proteins, such as RNA-dependent RNA polymerase, 3C-like protease ($3CL^{pro}$), and papain, to complete the cycle of virus replication [15,16].

Currently, there is no targeted therapeutic drug for COVID-19. As a result, people generally use classic medicine administered in clinics and adopt a combination of Chinese and Western medicine [17]. Lopinavir/ritonavir (LPV/r), also known as Kaletra, is a compound preparation composed of lopinavir and low-dose ritonavir. LPV/r is an antiretroviral drug that is used to treat human immunodeficiency virus (HIV) infection [18]. Lopinavir is mainly metabolized by the cytochrome P450 (CYP450) isoenzyme, CYP3A4. Ritonavir is not only a substrate of CYP3A4 and CYP2D6, but also a strong inhibitor of CYP3A4 [19]. Ritonavir is used to inhibit the metabolism of lopinavir, thereby increasing the concentration of lopinavir in the plasma to achieve a therapeutic effect. Ritonavir is also a strong inhibitor of organic anion transporting polypeptide 1B1 (OATP1B1), organic anion transporting polypeptide 1B3 (OATP1B3), and P-glycoprotein (P-gp) transporter [20,21]. LPV/r has been reported to have a certain effect on patients with Middle East respiratory syndrome coronavirus and severe acute respiratory syndrome coronavirus infection [22,23]. Currently, there is no definite theory regarding the mechanism of LPV/r against COVID-19. According to the literature, both lopinavir and ritonavir can inhibit 3CL$^{pro}$ and reverse transcription of the virus [24]. However, lopinavir is the main drug that can combine with viral proteases to produce immature and non-infectious virus particles. Ritonavir can restrain the liver metabolism of lopinavir and increase the blood concentration of lopinavir, thereby exerting a synergistic antiviral effect [25]. Lin et al. [26] predicted the interaction of LPV/r with COVID-19 protease and indicated that the therapeutic effect of lopinavir and ritonavir on new coronary pneumonia may be derived from its inhibitory effect on coronavirus endopeptidase C30. In addition to Western medicines, some Chinese medicines also play a crucial role in combating COVID-19 due to their anti-inflammatory and anti-viral effects. For example, Lianhua Qingwen granules, Jinhua Qinggan granules, Huoxiang Zhengqi capsules, Angong Niuhuang pills, and Shufeng Jiedu capsules are the drugs recommended in the New Coronavirus Pneumonia Diagnosis and Treatment Plan (trial seventh edition) [27]. Based on the results of related research, the combination of Chinese and Western medicines can significantly relieve the clinical pathologies of patients and shorten the patients' treatment cycle [28]; however, the mechanism of interaction between them and the possible adverse reactions are still unknown.

The aim of this study was to develop a simple, fast, and cost-effective UHPLC-MS/MS method that can be employed to determine the pharmacokinetic effects of Lianhua Qingwen granules, Jinhua Qinggan granules, Shufeng Jiedu capsules, Angong Niuhuang pills, and Huoxiang Zhengqi capsules on LPV/r in rats. We also sought to provide a rationale for the clinical use of these therapies to reduce adverse reactions.

## 2. Experimental

### 2.1. Materials and reagents

The lopinavir standard (192725-17-0, purity > 99%) was manufactured by Dalian Meilun Biotechnology Co., Ltd. (Dalian, China). The ritonavir standard (155213-67-5, purity: 99%) was purchased from Shanghai Xianding Biotechnology Co., Ltd. (Shanghai, China). The internal standard (IS), atazanavir (198904-31-3, purity: 98%), was provided by Tianjin Xiensi Biochemical Technology Co., Ltd. (Tianjin, China). Kaletra was obtained from Abbvie Co., Ltd. (North Chicago, IL, USA). Acetonitrile, methanol, and formic acid were all of LC-MS grade and were obtained from Fisher Scientific (Waltham, MA, USA). Distilled water was obtained from Wahaha Co., Ltd. (Hangzhou, China). Other chemicals

used in this study were of analytical grade and were obtained from the market (Tianjin Yongda Chemical Reagent Co., Ltd., Tianjin, China).

### 2.2. Instruments and conditions

The UHPLC-MS/MS system consisted of an Agilent 1290 Infinity II liquid chromatography system (containing a binary pump, an automatic injector, and a diode array detector, Agilent, Waldbronn, Baden-Württemberg, Germany) and a triple quadrupole tandem mass spectrometer (G6470A, Agilent, Singapore). An Agilent ZORBAX Eclipse Plus C$_{18}$ column (2.1 mm × 50 mm, 1.8 μm) was employed and the column temperature was maintained at 35 °C. An aqueous solution containing 0.1% formic acid (A) and acetonitrile (B) was used as the mobile phase and a linear gradient elution was applied. The gradient elution procedure applied was as follows: 0–1.5 min, 20% B; 1.5–2.0 min, 20%–60% B; 2.0–3.0 min, 60%–50% B; 3.0–6.0 min, 50% B; 6.0–7.0 min, 50%–20% B; 7.0–8.0 min, 20% B. The flow rate of mobile phase was set to 0.4 mL/min and the injection volume was 2 μL.

Mass spectra were obtained using an Agilent Jet Stream Electrospray ionization (AJS ESI) source in the positive mode at a temperature of 350 °C; the main parameters were: capillary voltage, 4000 V; nozzle voltage, 2000 V; dry gas temperature, 300 °C; sheath gas temperature, 350 °C; dry gas flow, 10 L/min; sheath gas flow, 11 L/min; and nebulizer pressure, 40 psi. MS scanning was performed in multiple reaction monitoring (MRM) mode. The reaction parameters of the precursor and product ions are shown in Table 1.

### 2.3. Preparation of the calibration standards and quality control (QC) samples

Stock solutions of lopinavir and ritonavir (500 μg/mL) were prepared with pure methanol and stored at 4 °C. The stock solutions of lopinavir and ritonavir were diluted with methanol and mixed with lopinavir concentrations of 125, 250, 625, 1250, 2500, 6250, and 12500 ng/mL and ritonavir concentrations of 25, 50, 125, 250, 500, 1250, and 2500 ng/mL to create a series of standard solutions. Blank plasma (50 μL) was added to an Eppendorf tube. Thereafter, 10 μL of the above-mentioned series of standard solution was added, vortexed, and mixed for 30 s to obtain calibration standard samples with lopinavir concentrations of 25, 50, 125, 250, 500, 1250, and 2500 ng/mL and ritonavir concentrations of 5, 10, 25, 50, 100, 250, and 500 ng/mL. Plasma samples with concentrations of 75, 625, and 1875 ng/mL of lopinavir and 15, 125, and 375 ng/mL of ritonavir were prepared as QC samples. The IS working solution was diluted with methanol to a concentration of 400 ng/mL and stored at 4 °C.

**Table 1**
The retention time ($t_R$), optimized MS/MS transitions, fragmentor and collision energy for lopinavir, ritonavir, and the internal standard (IS).

| Parameters | Lopinavir | Ritonavir | Atazanavir (IS) |
|---|---|---|---|
| Retention time ($t_R$, min) | 3.89 | 3.53 | 2.83 |
| Precursor ion (m/z) | 629.3 | 721.3 | 705.4 |
| Product ion (m/z) | 447.3 | 296.1 | 168.1 |
| Fragmentor (V) | 140 | 160 | 185 |
| Collision energy (eV) | 10 | 16 | 36 |

## 2.4. Sample preparation

The rat plasma sample (50 μL) was transferred to a 1.5 mL plug centrifuge tube. The IS solution (10 μL) and acetonitrile (150 μL) were subsequently added into the tube. The mixture was swirled for 3 min and centrifuged at 13,000 r/min for 10 min (20 °C). After the supernatant was removed and filtered through a 0.22 μm filter membrane, 2 μL of sample was injected into the system for LC-MS/MS detection.

## 2.5. Method validation

The "9012 Guidelines for the Validation of Biological Sample Quantitative Analysis Methods" (Pharmacopoeia Commission of the People's Republic of China) [29] and "Technical Guidelines for Non-clinical Pharmacokinetic Research of Drugs" (National Medical Products Administration, China) [30] were used for method verification.

### 2.5.1. Specificity and selectivity

Six different sources of blank plasma from rats were used to verify the specificity and selectivity of the method. Lopinavir and ritonavir were added into blank plasma at the lower limit of quantification (LLOQ). Plasma samples were collected from rats after gavage administration of LPV/r for 1 h. Endogenous substances did not interfere with the retention time of lopinavir, ritonavir, or IS.

### 2.5.2. Linearity and LLOQ

Standard curves were established with a concentration range of 25–2500 ng/mL lopinavir and 5–500 ng/mL ritonavir, and comprised 7 points. The ratio of the peak area of lopinavir and ritonavir to IS was used as the ordinate $y$, and the nominal concentrations of lopinavir and ritonavir were used as the abscissa $x$. A linear least squares regression model ($w = 1/x^2$) was used for analysis. The lowest point of the standard curve was the LLOQ for lopinavir and ritonavir. The precision should be less than 20% of the relative standard deviation (RSD), and the accuracy should be 80%–120% of the theoretical concentration.

### 2.5.3. Accuracy and precision

The precision and accuracy were estimated by analyzing QC samples at LLOQ, low, medium, and high concentration levels in six replicates on three successive days. The results were assessed using the calibration curve of the day, and the intra-day and inter-day precisions of the method were explored according to the results of the QC samples. Precision was expressed as RSD while accuracy was expressed as relative error (RE). When the RSD was <15% (<20% at the LLOQ) and RE was within ±15% (±20% at the LLOQ), the precision and accuracy were considered acceptable.

### 2.5.4. Extraction recovery and matrix effect

QC samples of low, medium, and high concentrations were used to determine the extraction recovery ($n$=6). The responses of the extracted QC samples at the above concentrations were compared to those of the analytes at the corresponding concentrations added into the post-extraction blank rat samples to obtain the recovery rates of lopinavir, ritonavir, and IS.

The peak areas of lopinavir, ritonavir, and the IS added into the post-extraction supernatants of six blank plasma samples (obtained from six different rats, respectively) were recorded as A, and the responses of lopinavir, ritonavir, and IS at equivalent concentrations spiked into water were recorded as B. The value of A/B was the matrix effect ($n$=3). The matrix factor ratio between the analytes and the IS was used to express the IS-normalized matrix effect factor.

### 2.5.5. Residual effects and dilution reliability

The residual effect was evaluated by six alternate injections of the upper limit of quantification (ULOQ) and blank plasma samples. If the measured peak area was less than 20% of the LLOQ, it was considered negligible. In addition, if the acquired peak area was less than 20% of the LLOQ response, it was considered negligible.

Plasma samples ($n$=6) with five times the ULOQ ($n$=6) were diluted with blank plasma 5 times and 10 times, respectively, and then measured to evaluate the reliability of dilution. The accuracy and precision of the samples after dilution were within ±15%.

### 2.5.6. Stability

The stability of lopinavir and ritonavir in rat blood was estimated by analyzing replicates ($n$=3) at two QC concentrations (the concentrations of lopinavir were 75 ng/mL and 1875 ng/mL, the concentrations of ritonavir were 15 ng/mL and 375 ng/mL, respectively) under different conditions and procedures. The short-term stability of the sample was investigated after being placed at room temperature for 8 h and the long-term stability was assessed by evaluating the QC samples frozen at −80 °C for 14 days. The samples were thawed at room temperature after being frozen at −80 °C for 24 h; this was repeated three times to assess the freeze-thaw stability. To evaluate the stability of the processed samples, the samples were stored in an autosampler for 12 h at 20 °C. If the concentration variance was within 15%, the sample was regarded as stable in the biological matrix.

## 2.6. Pharmacokinetic study

Thirty-six healthy Wistar rats (250–300 g, SPF level, license number: SCXK 20190003) were randomly divided into six groups: one control group and five experimental groups. The control group was administered normal saline + Kaletra (0.52 g/kg), while the experimental groups were administered Lianhua Qingwen granules (2 g/kg) + Kaletra (0.52 g/kg), Huoxiang Zhengqi capsules (0.25 g/kg) + Kaletra (0.52 g/kg), Jinhua Qinggan granules (1.6 g/kg) + Kaletra (0.52 g/kg), Shufeng Jiedu capsules (0.65 g/kg) + Kaletra (0.52 g/kg), and Angong Niuhuang pills (0.3 g/kg) + Kaletra (0.52 g/kg), respectively. The rats were treated with traditional Chinese medicines (TCM) or normal saline via gavage for 7 days; however, they were allowed to eat and drink freely for the first six days. On the seventh day, rats were fasted for 12 h but allowed to drink water freely. At 1 h after the last intragastric administration, Kaletra was administered by gavage. Whole blood (0.2 mL) was collected from the tail vein at 10 min, 30 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 6 h, 8 h, 10 h, 13 h, and 16 h after administration, placed into heparinized tubes, and centrifuged at 4,000 r/min for 10 min (4 °C). The obtained plasma was placed into 1.5 mL centrifuge tubes and stored at −20 °C until analysis. The DAS3.2 pharmacokinetic program and the non-compartmental model were used to analyze and calculate the pharmacokinetic parameters. The pharmacokinetic parameter values of the two analytes are presented as mean ± standard deviation (SD).

## 3. Results and discussion

### 3.1. Mass spectrometric conditions

The MS conditions were optimized using AJS ESI as the ionization source with the operation conducted in the MRM mode. The AJS ESI conditions for the analytes and IS were optimized in both positive and negative ion modes; however, more stable and intense ions were obtained in positive ion mode, giving rise to the [M+H]$^+$

ion peak at $m/z$ 629.3 for lopinavir, $m/z$ 721.3 for ritonavir, and $m/z$ 705.4 for the IS in the Q1 full scan spectra. In the full scan spectrum of the product ion, $m/z$ 447.3, 296.1, and 168.1 for lopinavir, ritonavir, and IS, respectively, showed highly abundant fragment ions (Fig. 1). Therefore, the transitions of $m/z$ 629.3→447.3 for lopinavir, $m/z$ 721.3→296.1 for ritonavir, and $m/z$ 705.4→168.1 for the IS were selected for quantitative analysis. Parameters, such as gas temperature, sheath gas temperature, capillary and nozzle voltage, and flow rate of the sheath gas, were optimized to obtain the highest intensity of protonated molecules of lopinavir, ritonavir, and the IS.

## 3.2. Chromatographic conditions

Water, 0.1% formic acid, and acetonitrile were used as the mobile phase in this experiment. The detection time was only 8 min after optimization of the elution gradient, which was lower than 12 min [31] and 16.3 min [32] reported in the literature. Three chromatographic columns, namely, Agilent ZORBAX Eclipse Plus $C_{18}$ column (2.1 mm × 50 mm, 1.8 μm; Santa Clara, CA, USA), Shimadzu Shim-pack GIST-HP $C_{18}$ column (2.1 mm × 100 mm, 3.0 μm; Kyoto, Japan), and Waters ACQUITY UPLC® BEH $C_{18}$ column (2.1 mm × 100 mm, 1.7 μm; Milford, MA, USA) were compared successively to determine their resolution, peak shape, column pressure, and column efficiency. Ultimately, the Agilent ZORBAX Eclipse Plus $C_{18}$ column (2.1 mm × 50 mm, 1.8 μm) was selected for analysis.

## 3.3. Sample preparation

Several studies have reported the use of LC-MS/MS to determine the drug concentrations of lopinavir and ritonavir in biological samples after administration. Sample pretreatment methods include liquid-liquid extraction [33], solid phase extraction [34], and alkaline protein precipitation combined with liquid-liquid extraction [31]. In this study, the protein precipitation method, liquid-liquid extraction method (ethyl acetate as extraction solvent), and liquid-liquid extraction after alkaline protein precipitation (NaOH alkalization, ethyl acetate as solvent) were compared. The results revealed that good peak shape and low residue were obtained using the three treatment methods. However, the recovery rate of liquid-liquid extraction and liquid-liquid extraction after alkaline precipitation of the protein was low. Furthermore, reproducibility was found to be poor. The liquid—liquid extraction method after alkaline protein precipitation also had a strong matrix effect, which made it difficult to meet the requirements of pharmacokinetic analysis. After a comprehensive comparison, the

protein precipitation method was identified to be convenient, simple, and associated with low cost, low matrix effect, and high recovery. Therefore, this method was selected for sample treatment.

## 3.4. Method validation

### 3.4.1. Specificity and selectivity

Fig. 2 displays the represented chromatograms of lopinavir, ritonavir, and the IS in a blank plasma sample, blank plasma containing the analytes at the LLOQ and the IS, and a plasma sample from a normal rat at 1 h after gavage administration of Kaletra. The analytes were demonstrated to be well separated with high resolution and displayed good peak shapes. The retention time of lopinavir, ritonavir, and the IS was 3.89, 3.53, and 2.83 min, respectively. There was no endogenous interference in the IS; however, a minor interference was found in lopinavir and ritonavir, and the peak area was far less than 20% of LLOQ, which could not affect the subsequent determination. Several efforts were thus made to eliminate this part of the interference. However, due to their strong volatility and their high capability to contamiante the instrument system, this interference could not be completely removed. Thus, all attempts were made to minimize the interference as much as possible.

### 3.4.2. Linearity and LLOQ

The standard curves demonstrated great linearity within the concentration range of 25—2500 ng/mL for lopinavir and 5—500 ng/mL for ritonavir. The representative regression equation of lopinavir was $y = 3.525 \times 10^{-1}x + 3.720 \times 10^{-2}$ ($r=0.9981$), and that of ritonavir was $y = 3.584 \times 10^{-1}x + 3.002 \times 10^{-3}$ ($r=0.9984$), where $y$ represents the peak area ratio of lopinavir and ritonavir to IS, and $x$ indicates the concentration of lopinavir and ritonavir in blood.

The LLOQ was 25 ng/mL for lopinavir, which was markedly lower than 62.5 ng/mL [31] and 100 ng/mL [35] reported in the literature; and 5 ng/mL for ritonavir, which was lower than 12.5 ng/mL [31] and 99 ng/mL [36] reported in the literature. The RE values of lopinavir and ritonavir at the LLOQ were 3.45% and 1.46%, and the RSDs were 4.03% and 4.32%, respectively. These results align with the principle of biological sample determination.

### 3.4.3. Accuracy and precision

The intra-day and inter-day precision (RSD) and accuracy (RE) of the analytes under this method at four different concentrations (LLOQ, low, medium, and high) are shown in Table 2. Both RSD and RE were within ±15%, indicating that the accuracy and precision of the established method were acceptable.
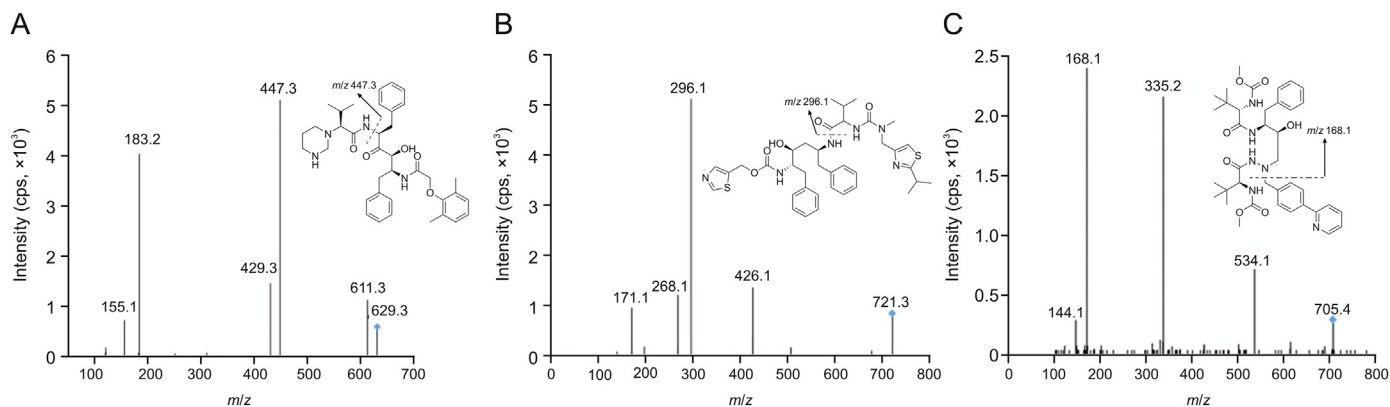


**Fig. 1.** Product ion spectra (MS/MS) of (A) lopinavir, (B) ritonavir, and (C) the internal standard (IS).
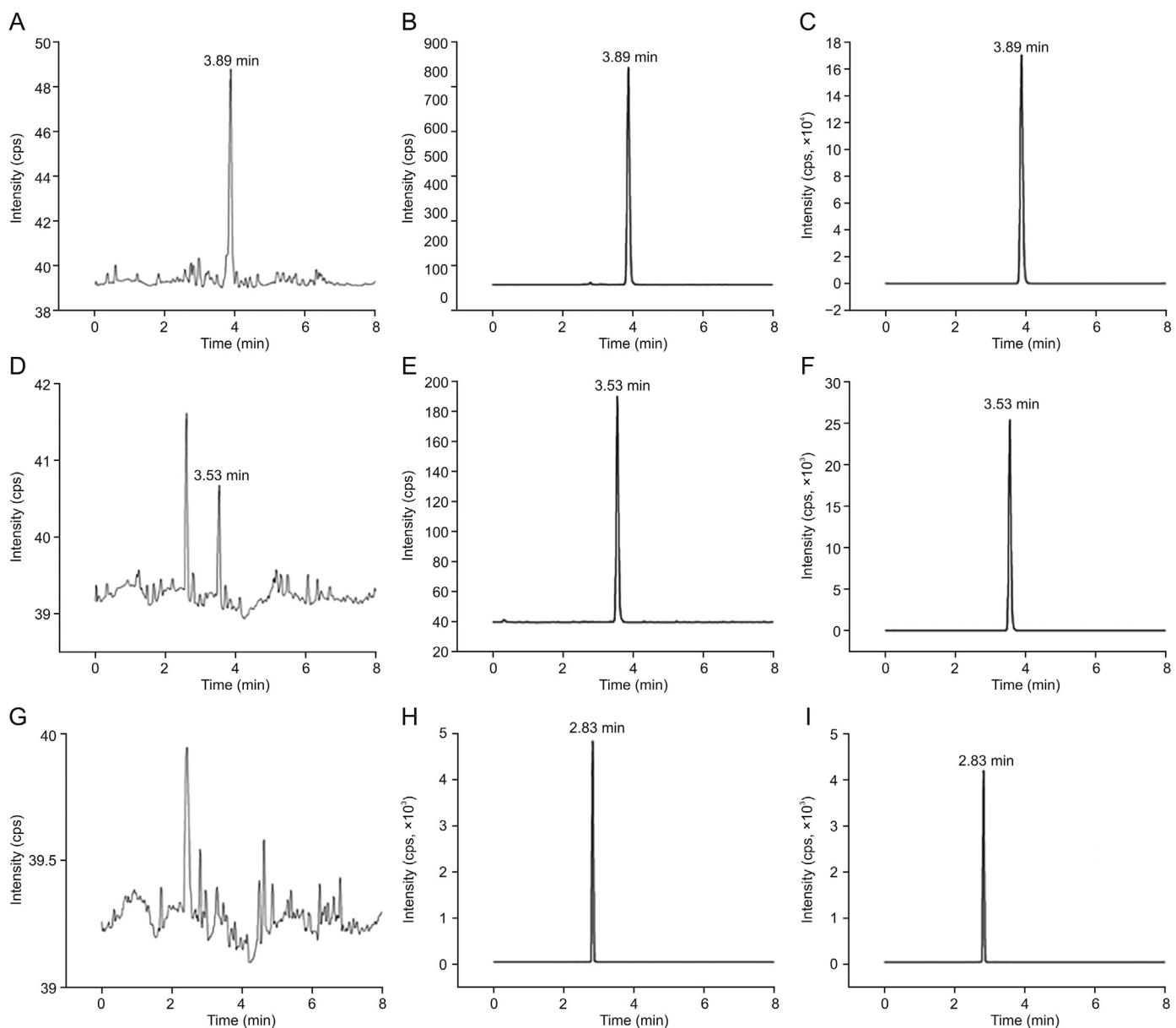
**Fig. 2.** MRM chromatograms of (A–C) lopinavir, (D–F) ritonavir, and (G–I) the IS in (A, D, and G) a blank plasma sample, (B, E, and H) blank plasma containing the analytes at the lower limit of quantification and the IS, and (C, F, and I) a plasma sample from a normal rat at 1 h after gavage administration of Kaletra.

**Table 2**
Intra-day and inter-day accuracy and precision of lopinavir and ritonavir in rat plasma ($n$=6).

| Analyte | Concentration (ng/mL) | Intra-day | | Inter-day | |
|---|---|---|---|---|---|
| | | RSD (%) | RE (%) | RSD (%) | RE (%) |
| Lopinavir | 25 | 2.6 | 1.54 | 5.1 | 1.9 |
| | 75 | 2 | 2.32 | 6.8 | 3.5 |
| | 625 | 3.3 | −4.43 | 13.6 | 2.7 |
| | 1875 | 5.8 | −0.17 | 3.8 | 1.6 |
| Ritonavir | 5 | 3.6 | 0.59 | 2.6 | 1.8 |
| | 15 | 3.6 | 7.56 | 10.2 | 2.8 |
| | 125 | 3.2 | −0.57 | 4.8 | 0.1 |
| | 375 | 3.8 | 6.76 | 10.2 | 2.0 |

RSD: relative standard deviation; RE: relative error.

**Table 3**
Summary of the recovery of lopinavir and ritonavir in rat plasma ($n$=6).

| Analyte | Concentration (ng/mL) | Recovery (%, mean ± SD) | RSD (%) |
|---|---|---|---|
| Lopinavir | 75 | 98.4 ± 3.4 | 3.4 |
| | 625 | 95.7 ± 2.1 | 2.2 |
| | 1875 | 97.4 ± 3.7 | 3.8 |
| | 15 | 96.8 ± 4.6 | 4.8 |
| Ritonavir | 125 | 96.8 ± 1.6 | 1.6 |
| | 375 | 98.4 ± 2.2 | 2.2 |
| IS | 80 | 101.1 ± 4.1 | 4.1 |

### 3.4.4. Extraction recovery and matrix effect

The extraction recovery of lopinavir and ritonavir in rat plasma at low, medium, and high concentrations and the IS is shown in

Table 3. The recoveries of lopinavir and ritonavir in QC samples and the IS were in the range of 95.7%−101.1%, with an RSD of less than 4.8%. Such finding indicates that the established method was accurate, accordant, and reusable.

**Table 4**
Summary of the matrix effect of lopinavir and ritonavir in rat plasma (*n*=6).

| Analyte | Concentration (ng/mL) | Matrix effect (%) | | IS-normalized matrix effect (%) | |
|---|---|---|---|---|---|
| | | Mean ± SD | RSD | Mean ± SD | RSD |
| Lopinavir | 75 | 101.9 ± 5.7 | 5.6 | 103.3 ± 6.0 | 5.8 |
| | 1875 | 100.6 ± 4.4 | 4.4 | 101.3 ± 3.0 | 3 |
| Ritonavir | 15 | 98.8 ± 1.2 | 1.3 | 100.3 ± 1.8 | 1.7 |
| | 375 | 94.6 ± 5.7 | 6.1 | 95.2 ± 2.9 | 3.1 |
| IS | 80 | 99.0 ± 2.9 | 2.9 | – | – |

The results of the matrix effects are shown in Table 4. The matrix effects of lopinavir and ritonavir at the low and high concentration levels were found to be within the range of 94.6%–101.9%, with an RSD of less than 6.1%. The IS-normalized matrix effect was within the range of 95.2%–103.3%, with an RSD of less than 5.8%. Such findings indicate that there was no apparent influence of the rat plasma matrix on the determination of lopinavir and ritonavir in plasma.

### 3.4.5. Residual effects and dilution reliability

The residual effect was determined as the ratio of the analyte peak area in blank plasma to the LLOQ peak area after 6 alternate injections. The peak areas of the analytes of lopinavir and ritonavir after six alternate injections of a blank blood sample and the ULOQ samples were less than 4.4% and 2.1% of the LLOQ while that of the IS was less than 0.08%, demonstrating that the residual effects can be ignored.

The differences between the measured concentration and the theoretical concentration was employed as the definition for accuracy (RE) to investigate the reliability of dilution. The results are listed in Table 5. The precision and accuracy of each analyte were within ±15%, and the reliability of the dilution was consistent with the requirements of biological sample determination.
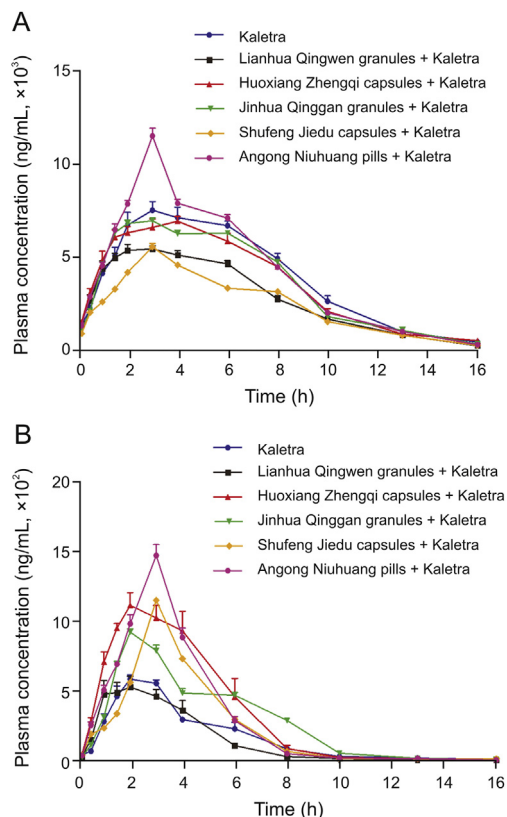
### 3.4.6. Stability

The results for the stability of lopinavir and ritonavir in rat plasma under various conditions are shown in Table 6. Lopinavir and ritonavir displayed sufficient stability when samples were placed at 20 °C for 8 h, stored at −80 °C for 14 days, gone through three freeze-thaw cycles, and placed in an autosampler at 20 °C for 12 h.



**Fig. 3.** Mean blood concentration curves of (A) lopinavir and (B) ritonavir in rats after oral administration (*n*=6).

### 3.5. Pharmacokinetic study

The above LC-MS/MS assay was employed in the pharmacokinetic study of lopinavir and ritonavir after intragastric administration of LPV/r (0.52 g/kg) combined with five TCMs to rats. The mean plasma concentration-time curves of lopinavir and ritonavir are illustrated in Fig. 3, and the major pharmacokinetic parameters of lopinavir and ritonavir from the non-compartment model analysis are shown in Tables 7 and 8. The pharmacokinetic

**Table 5**
Summary of the dilution effect of lopinavir and ritonavir in rat plasma (*n*=6).

| Analyte | Dilution factor | Concentration (ng/mL) | Measured concentration (ng/mL) | RSD (%) | RE (%) |
|---|---|---|---|---|---|
| Lopinavir | 10 | 1250 | 1308.0 ± 106.2 | 8.1 | 4.6 |
| | 5 | 2500 | 2340.6 ± 69.9 | 3.0 | −6.4 |
| Ritonavir | 10 | 250 | 263.6 ± 15.7 | 6.0 | 5.4 |
| | 5 | 500 | 484.0 ± 18.6 | 3.9 | −3.2 |

**Table 6**
Stability of lopinavir and ritonavir in rat plasma under various conditions based on LC-MS/MS (*n*=3).

| Analyte | Concentration (ng/mL) | 8 h, 20 °C | | 14 days, −80 °C | | Three freeze-thaw cycles | | 12 h, 20 °C | |
|---|---|---|---|---|---|---|---|---|---|
| | | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) | RE (%) | RSD (%) |
| Lopinavir | 75 | −0.9 | 3.8 | −1.6 | 2.8 | −3.3 | 4.4 | −3 | 3.1 |
| | 1875 | 8.4 | 2.3 | 9.1 | 3 | 1.2 | 1.5 | 0.4 | 4.6 |
| Ritonavir | 15 | 1.7 | 5.6 | 7 | 2.9 | −1.9 | 2.9 | −0.1 | 3.7 |
| | 375 | 4.9 | 1.5 | 4.6 | 1.9 | 1.3 | 1.8 | 3.7 | 4.2 |

**Table 7**
The pharmacokinetic parameters of lopinavir in the experimental groups and the control group (mean ± SD, $n=6$).

| Pharmacokinetic parameters | Lianhua Qingwen granules + Kaletra | Huoxiang Zhengqi capsules + Kaletra | Jinhua Qinggan granules + Kaletra | Shufeng Jiedu capsules + Kaletra | Angong Niuhuang pills + Kaletra | Control group |
|---|---|---|---|---|---|---|
| $AUC_{0-t}$ (mg/LF0D7h) | 45.038 ± 4.197** | 58.028 ± 4.761 | 58.282 ± 1.836 | 39.154 ± 1.473** | 67.826 ± 4.300 | 62.445 ± 5.974 |
| $AUC_{0-\infty}$ (mg/LF0D7h) | 46.247 ± 4.172** | 59.708 ± 4.379 | 59.336 ± 1.823 | 40.061 ± 1.799** | 68.774 ± 4.803 | 63.692 ± 6.018 |
| $MRT_{0-t}$ (h) | 5.357 ± 0.161* | 5.483 ± 0.359 | 5.525 ± 0.282 | 5.647 ± 0.091 | 5.221 ± 0.154** | 5.694 ± 0.23 |
| $MRT_{0-\infty}$ (h) | 5.741 ± 0.288 | 5.904 ± 0.519 | 5.768 ± 0.335 | 5.955 ± 0.218 | 5.408 ± 0.243** | 5.960 ± 0.302 |
| $t_{1/2}$ (h) | 2.511 ± 0.566 | 2.610 ± 0.812 | 2.127 ± 0.295 | 2.274 ± 0.322 | 2.051 ± 0.332 | 2.190 ± 0.337 |
| $t_{max}$ (h) | 2.500 ± 0.548* | 3.833 ± 1.169 | 2.667 ± 0.516* | 3.000 ± 0 | 3.000 ± 0 | 3.833 ± 1.169 |
| V/F (L/kg) | 41.073 ± 10.788* | 33.200 ± 11.124 | 26.904 ± 3.649 | 42.507 ± 4.884** | 22.292 ± 2.453 | 25.948 ± 4.279 |
| CL/F (L/h/kg) | 11.320 ± 1.020** | 8.751 ± 0.687 | 8.771 ± 0.268 | 13.002 ± 0.575** | 7.592 ± 0.532 | 8.221 ± 0.715 |
| $c_{max}$ (mg/L) | 5.765 ± 0.595** | 7.234 ± 0.494 | 7.047 ± 0.360 | 5.547 ± 0.412** | 11.484 ± 0.996** | 7.631 ± 1.051 |

*$P<0.05$, **$P<0.01$ vs. control group. $AUC_{0-t}$: area under the plasma concentration-time curve from zero to time; $AUC_{0-\infty}$: area under the plasma concentration-time curve from zero to infinite; $MRT_{0-t}$: mean retention time from zero to time; $MRT_{0-\infty}$: mean retention time from zero to infinite; $t_{1/2}$: the half time; $t_{max}$: peak time of drug concentration in plasma; V/F: the apparent volume of distribution; CL/F: the total body clearance; $c_{max}$: the maximum blood concentration.

**Table 8**
The pharmacokinetic parameters of ritonavir in the experimental groups and the control group (mean ± SD, $n=6$).

| Pharmacokinetic parameters | Control group | Lianhua Qingwen granules + Kaletra | Huoxiang Zhengqi capsules + Kaletra | Jinhua Qinggan granules + Kaletra | Shufeng Jiedu capsules + Kaletra | Angong Niuhuang pills + Kaletra |
|---|---|---|---|---|---|---|
| $AUC_{0-t}$ (mg/L·h) | 2.628 ± 0.319 | 2.308 ± 0.848 | 5.439 ± 1.070** | 4.486 ± 0.367** | 3.888 ± 0.093** | 5.033 ± 0.663** |
| $AUC_{0-\infty}$ (mg/L·h) | 2.656 ± 0.325 | 2.344 ± 0.851 | 5.505 ± 1.048** | 4.512 ± 0.362** | 3.911 ± 0.112** | 5.065 ± 0.661** |
| $MRT_{0-t}$ (h) | 4.057 ± 0.397 | 3.364 ± 0.180** | 3.613 ± 0.348 | 4.436 ± 0.186 | 3.882 ± 0.027 | 3.477 ± 0.037* |
| $MRT_{0-\infty}$ (h) | 4.220 ± 0.444 | 3.682 ± 0.242* | 3.897 ± 0.338 | 4.525 ± 0.336 | 3.976 ± 0.117 | 3.588 ± 0.035* |
| $t_{1/2}$ (h) | 2.340 ± 0.607 | 4.354 ± 0.713** | 3.420 ± 0.479** | 1.678 ± 0.975 | 2.319 ± 0.698 | 3.313 ± 0.791* |
| $t_{max}$ (h) | 2.083 ± 0.492 | 1.833 ± 0.753 | 2.667 ± 0.816 | 2.000 ± 0 | 3.000 ± 0** | 3.000 ± 0** |
| V/F (L/kg) | 664.0 ± 159.1 | 1532 ± 498.4** | 659.7 ± 117.4 | 280.6 ± 165.7** | 442.6 ± 122.2* | 488.1 ± 83.409* |
| CL/F (L/h/kg) | 198.3 ± 24.088 | 245.5 ± 80.601 | 97.387 ± 18.733** | 115.9 ± 10.16** | 133.0 ± 3.787** | 104.0 ± 12.509** |
| $c_{max}$ (mg/L) | 0.604 ± 0.082 | 0.560 ± 0.185 | 1.214 ± 0.205** | 0.923 ± 0.059** | 1.152 ± 0.046** | 1.474 ± 0.192** |

*$P<0.05$, **$P<0.01$ vs. control group.

parameters of the group administered Lianhua Qingwen granules were significantly altered compared to those of control group administered normal saline and Kaletra. Furthermore, the area under the plasma concentration-time curve from zero to time ($AUC_{0-t}$), the area under the plasma concentration-time curve from zero to infinite ($AUC_{0-\infty}$), and the maximum blood concentration ($c_{max}$) of lopinavir displayed a 1.5-fold decrease while the total body clearance (CL/F) displayed a 1.5-fold increase. The half time ($t_{1/2}$) and the apparent volume of distribution (V/F) of ritonavir were found to increase. Lianhua Qingwen granules mainly contain *Forsythia*, *Honeysuckle*, *Radix isatidis*, and *Ephedra sinica*. Previous studies have reported that *Forsythia* can induce the expression of P-gp and drug metabolism enzymes, such as CYP3A4, CYP2C9, and CYP2C19 in the intestine and liver [37]. Therefore, the change in the pharmacokinetic parameters of this group was speculated to be caused by the induction of CYP3A4 expression by *Forsythia*, which resulted in a decrease in the $AUC_{0-t}$, $AUC_{0-\infty}$, and $c_{max}$ of lopinavir, and an increase in CL/F. The $AUC_{0-t}$, $AUC_{0-\infty}$, and $c_{max}$ of ritonavir in the group administered both Huoxiang Zhengqi capsules and Kaletra were increased by 2-fold, $t_{1/2}$ was prolonged by approximately 1.5-fold, and CL/F was significantly decreased. However, the pharmacokinetic parameters of lopinavir did not change. This may be due to the complicated composition of this TCM or the influence of the microenvironment in rats on the action of the CYP450 enzyme. The pharmacokinetic parameters of lopinavir in the group administered both Jinhua Qinggan granules and Kaletra were basically unchanged; the $AUC_{0-t}$ and $AUC_{0-\infty}$ of ritonavir increased by 1.7-fold, the $c_{max}$ of ritonavir increased by 1.5-fold, while CL/F and V/F decreased. The components of TCM are complex. Furthermore, TCMs contain too many unknown components. Drug-drug interactions cause changes in drug transporters and CYP450 enzymes in rats, which will lead to changes in the metabolism of lopinavir/ritonavir. In the group administered both Shufeng Jiedu

capsules and Kaletra, the $AUC_{0-t}$, $AUC_{0-\infty}$, and $c_{max}$ of lopinavir were decreased by 1.5-fold, and CL/F and V/F were significantly increased. Moreover, the $AUC_{0-t}$ and $AUC_{0-\infty}$ of ritonavir increased by approximately 1.5-fold, $c_{max}$ increased 2-fold, and CL/F was apparently decreased. Shufeng Jiedu capsules contain *Polygonum cuspidatum*, *Forsythia suspensa*, and *Radix isatidis*. Therefore, the change in pharmacokinetic parameters in this group was speculated to be caused by the induction of CYP3A4 by *Forsythia*. Although the $AUC_{0-t}$, $AUC_{0-\infty}$, and $c_{max}$ of ritonavir was found to increase, it was not sufficient to inhibit the metabolism of lopinavir by CYP3A4. The mean retention time from zero to time ($MRT_{0-t}$) and mean retention time from zero to infinite ($MRT_{0-\infty}$) of lopinavir in the group administered both Angong Niuhuang pills and Kaletra were significantly reduced, and $c_{max}$ was evidently increased. Furthermore, the $AUC_{0-t}$ and $AUC_{0-\infty}$ of ritonavir increased by two-fold, $t_{1/2}$ was prolonged, and CL/F decreased significantly. Angong Niuhuang pills mainly contain bezoar, musk, buffalo horn, and other ingredients. The interaction between these ingredients slows down the metabolism of ritonavir, prolongs its half-life, and reduces its clearance rate.

## 4. Conclusions

In this study, a UHPLC-MS/MS method was developed to determine the effects of five TCMs on the pharmacokinetic parameters of lopinavir and ritonavir in rat plasma. The developed method had the following advantages: short sample processing time, strong selectivity, high sensitivity, good repeatability, and convenient operation. Pretreating biological samples is a key step in drug analysis. As a result, the protein precipitation method was used to treat biological samples in this study. This method had good repeatability, resulted in a high recovery rate, and involved a simple operation. Further, the solvent used for protein precipitation was consistent with the mobile

phase, and there was no interference of other components, which provided a good condition for detection. Altogether, this method can provide analytical technical support for determining the concentrations of lopinavir and ritonavir in the blood. Lianhua Qingwen granules, Huoxiang Zhengqi capsules, Jinhua qinggan granules, Shufeng Jiedu capsules, and Angong Niuhuang pills have a certain degree of influence on the pharmacokinetic parameters of lopinavir and ritonavir. Accordingly, they can serve as a reference for administering clinical drugs, reducing the occurrence of adverse reactions, and ensuring the safety of medications.

## CRediT author statement

**Linlin Li:** Data curation, Writing - Original draft preparation; **Xinxiang Yu:** Validation, Visualization; **Dongmin Xie:** Investigation, Visualization; **Ningning Peng:** Visualization, Writing - Reviewing and Editing; **Weilin Wang:** Supervision, Writing - Reviewing and Editing; **Decai Wang** and **Binglong Li:** Conceptualization, Methodology, Project administration.

## Declaration of competing interest

The authors declare that there are no conflicts of interest.

## Acknowledgments

## References

[1] Y.-C. Li, W.-Z. Bai, T. Hashikawa, Response to Commentary on: The neuro-invasive potential of SARS-CoV-2 may play a role in the respiratory failure of COVID-19 patients, J. Med. Virol. 92 (2020) 707—709.

[2] L. Setti, F. Passarini, G. de Gennaro, et al., Airborne transmission route of COVID-19: why 2 meters/6 feet of inter-personal distance could not be enough, Int. J. Environ. Res. Public Health 17 (2020), 2932.

[3] F. Yu, L. Du, D.M. Ojcius, et al., Measures for diagnosing and treating infections by a novel coronavirus responsible for a pneumonia outbreak originating in Wuhan, China, Microbes Infect. 22 (2020) 74—79.

[4] L. Ni, L. Zhou, M. Zhou, et al., Combination of western medicine and Chinese traditional patent medicine in treating a family case of COVID-19, Front. Med. 14 (2020) 210—214.

[5] COVID live update: 156,696,833 cases and 3,269,661 deaths. https://www.worldometers.info/coronavirus/. (Accessed 6 May 2021).

[6] F. Wu, S. Zhao, B. Yu, et al., A new coronavirus associated with human respiratory disease in China, Nature 579 (2020) 265—269.

[7] J.F.W. Chan, S.F. Yuan, K.H. Kok, et al., A familial cluster of pneumonia associated with the 2019 novel coronavirus indicating person-to-person transmission: a study of a family cluster, Lancet 395 (2020) 514—523.

[8] J. Cui, F. Li, Z.L. Shi, Origin and evolution of pathogenic coronaviruses, Nat. Rev. Microbiol. 17 (2019) 181—192.

[9] H.A. Rothan, S.N. Byrareddy, The epidemiology and pathogenesis of coronavirus disease (COVID-19) outbreak, J. Autoimmun. 109 (2020), 102433.

[10] N. Zhu, D. Zhang, W. Wang, et al., A novel coronavirus from patients with pneumonia in China, 2019, N. Engl. J. Med. 382 (2020) 727—733.

[11] S.Q. Deng, H.J. Peng, Characteristics of and public health responses to the coronavirus disease 2019 outbreak in China, J. Clin. Med. 9 (2020), 575.

[12] K. Knoops, M. Kikkert, S.H.E. van den Worm, et al., SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum, PLoS Biol. 6 (2008), e226.

[13] D. Wrapp, N. Wang, K.S. Corbett, et al., Cryo-EM structure of the 2019-nCoV spike in the prefusion conformation, Science 367 (2020) 1260—1263.

[14] M. Hoffmann, H. Kleine-Weber, S. Schroeder, et al., SARS-CoV-2 cell entry depends on ACE2 and TMPRSS2 and is blocked by a clinically proven protease inhibitor, Cell 181 (2020) 271—280.

[15] Y.M. Báez-Santos, S.E. St. John, A.D. Mesecar, The SARS-coronavirus papain-like protease: structure, function and inhibition by designed antiviral compounds, Antiviral Res. 115 (2015) 21—38.

[16] J. Deval, Z. Jin, Y.C. Chuang, et al., Structure(s), function(s), and inhibition of the RNA-dependent RNA polymerase of noroviruses, Virus Res. 234 (2017) 21—33.

[17] C. del Rio, P.N. Malani, COVID-19-New insights on a rapidly changing epidemic, JAMA 323 (2020) 1339—1340.

[18] T. Wada, K. Shimode, T. Hoshiyama, et al., Three novel COVID-19 pneumonia cases successfully treated with lopinavir/ritonavir, Front. Med. (Lausanne) 7 (2020), 241.

[19] J.B. Mah Ming, M.J. Gill, Drug-induced rhabdomyolysis after concomitant use of clarithromycin, atorvastatin, and lopinavir/ritonavir in a patient with HIV, AIDS Patient Care STDS 17 (2003) 207—210.

[20] U.M. Zanger, M. Schwab, Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation, Pharmacol. Therapeut. 138 (2013) 103—141.

[21] P. Kaufmann, S. Niglis, S. Bruderer, et al., Effect of lopinavir/ritonavir on the pharmacokinetics of selexipag an oral prostacyclin receptor agonist and its active metabolite in healthy subjects: lopinavir/ritonavir effects on selexipag pharmacokinetics, Br. J. Clin. Pharmacol. 80 (2015) 670—677.

[22] J.F.W. Chan, Y. Yao, M.L. Yeung, et al., Treatment with lopinavir/ritonavir or interferon-β1b improves outcome of MERS-CoV infection in a nonhuman primate model of common marmoset, J. Infect. Dis. 212 (2015) 1904—1913.

[23] S.K. Singh, Middle East respiratory syndrome virus pathogenesis, Semin. Respir. Crit. Care Med. 37 (2016) 572—577.

[24] C. Boulanger, V. Rolla, M.H. Al-Shaer, et al., Evaluation of super-boosted lopinavir/ritonavir in combination with rifampicin in HIV-1-infected patients with tuberculosis, Int. J. Antimicrob. Agents 55 (2020), 105840.

[25] T. Lancet, Emerging understandings of 2019-nCoV, Lancet 395 (2020), 311.

[26] S. Lin, R. Shen, J. He, et al., Molecular modeling evaluation of the binding effect of ritonavir, lopinavir and darunavir to severe acute respiratory syndrome coronavirus 2 proteases, bioRxiv. https://www.biorxiv.org/content/10.1101/2020.01.31.929695v2. (Accessed 31 January 2020).

[27] Diagnosis and treatment of corona virus disease-19 (7th trial edition). http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfe4cef80dc7f5912eb1989.shtml. (Accessed 4 March 2020).

[28] Z. Wang, X. Chen, Y. Lu, et al., Clinical characteristics and therapeutic procedure for four cases with 2019 novel coronavirus pneumonia receiving combined Chinese and Western medicine treatment, Biosci. Trends 14 (2020) 64—68.

[29] National Pharmacopoeia Commission, Pharmacopoeia of the People's Republic of China, Chemical Industry Press, Beijing, 2015, pp. 363—368.

[30] Center for Drug Evaluation, Guidance for Nonclinical Pharmacokinetics of Medicinal Products. https://www.cde.org.cn/zdyz/domesticinfopage?zdyzIdCODE=3e1a118fa1599529d3406fe6ee5821a5. (Accessed 13 May 2014).

[31] Y.-M. Yao, J.-J. Sun, J. Chen, et al., LC-MS/MS method for simultaneous quantification of lopinavir and ritonavir in human plasma, Acta Pharm. Sin. 45 (2010) 279—282.

[32] R. Verbesselt, E. Wijngaerden, J. Hoon, Simultaneous determination of 8 HIV protease inhibitors in human plasma by isocratic high-performance liquid chromatography with combined use of UV and fluorescence detection: amprenavir, indinavir, atazanavir, ritonavir, lopinavir, saquinavir, nelfinavir and M8-nelfinavir metabolite, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 845 (2007) 51—60.

[33] P.G. Wang, J.S. Wei, G. Kim, M. Chang, et al., Validation and application of a high-performance liquid chromatography—tandem mass spectrometric method for simultaneous quantification of lopinavir and ritonavir in human plasma using semi-automated 96-well liquid—liquid extraction, J. Chromatogr. A 1130 (2006) 302—307.

[34] M. Yadav, R. Rao, H. Kurani, et al., Application of a rapid and selective method for the simultaneous determination of protease inhibitors, lopinavir and ritonavir in human plasma by UPLC-ESI-MS/MS for bioequivalence study in Indian subjects, J. Pharm. Biomed. Anal. 49 (2009) 1115—1122.

[35] S. Chachad, A. Lulla, G. Malhotra, et al., Bioequivalence study of two fixed dose combination tablet formulations of lopinavir and ritonavir in healthy volunteers, Arzneimittelforschung 59 (2009) 263—268.

[36] S.K. Tippabhotla, N.R. Thudi, R. Raghuvanshi, et al., A bioequivalence study comparing two formulations of lopinavir/ritonavir capsules, Int. J. Clin. Pharmacol. Ther. 46 (2008) 204—210.

[37] R. Rahimi, M. Abdollahi, An update on the ability of St. John's wort to affect the metabolism of other drugs, Expert Opin. Drug Metab. Toxicol. 8 (2012) 691—708.