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Nirmatrelvir increases blood tacrolimus concentration in COVID-19 patients as determined by UHPLC-MS/MS method

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Abstract. – OBJECTIVE: Transplant recipients have a higher risk of SARS-CoV-2 infection owing to the use of immunosuppressive drugs like tacrolimus (FK506). FK506 and nirmatrelvir (NMV) (an anti-SARS-CoV-2 drug) are metabolized by cytochrome P450 3A4 and may have potential drug-drug interactions. It is important to determine the effect of NMV on FK506 concentrations.

PATIENTS AND METHODS: Following protein precipitation from blood, FK506 and its internal standard (FK506-^{13}C,^{2}d4) were detected by ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC-MS/MS). Total 22 blood samples (valley concentrations) from two coronavirus disease 2019 (COVID-19) patients were collected and analyzed for FK506 concentrations.

RESULTS: Blood levels of FK506 (0.5-100 ng/mL) showed good linearity. The UHPLC-MS/MS method was validated with intra- and inter-batch accuracies of 104.55-107.85%, and 99.52-108.01%, respectively, and precisions of < 15%. Mean blood FK506 concentration was 12.01 ng/mL (range, 3.15-33.1 ng/mL). Five-day co-administration with NMV increased the FK506 concentrations from 3.15 ng/mL to 33.1 ng/mL, returning to 3.36 ng/mL after a 9-day-washout.

CONCLUSIONS: We developed a simple quantification method for therapeutic drug monitoring of FK506 in patients with COVID-19 using UHPLC-MS/MS with protein precipitation. We found that NMV increased FK506 blood concentration 10-fold. Therefore, it is necessary to re-consider co-administration of FK506 with NMV.

Key Words:

COVID-19, Tacrolimus, Nirmatrelvir, Therapeutic drug monitoring, UHPLC-MS/MS, immunosuppressive.

Introduction

Since December 2019, the coronavirus disease 2019 (COVID-19) pandemic caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has posed an extraordinary threat to public health worldwide. In the second quarter of 2022, there was a COVID-19 outbreak in Shanghai, China, causing 610,000 people to get infected with nearly 600 deaths. The main risk factors for death were old age and pre-existing comorbidities, including lung transplantation[1-3], kidney transplantations[4,5], cancer[6,7] and cardiovascular disease[8].

Given their immunosuppression, transplant recipients are expected to be at higher risk of SARS-CoV-2 infection, and have higher mortality from COVID-19[4,5,9]. According to a previous report[1], kidney transplant recipients who acquire COVID-19 had a 23% risk of mortality, and 50% of them developed acute kidney injury. For lung transplant recipients with COVID-19, the mortality rate was 14.3% within a follow-up of 50 days[3].

Tacrolimus (FK506), a first-line immunosuppressive agent, has been widely used in patients with kidney, heart, lung, intestinal, and bone marrow transplants[10]. However, FK506 has a narrow therapeutic index, high toxicity, and significant individual differences in pharmacokinetics (PK) and pharmacodynamics (PD)[11-13]. Furthermore, in COVID-19 patients, the liver function and immune response may alter due to the infection[14], which may change the PK characteristics of FK506, resulting in a higher risk of graft rejection and/or toxicity of FK506. However, data on PK of FK506 in patients with COVID-19 and transplant

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comorbidities are limited. Therefore, therapeutic drug monitoring (TDM), a recommended method to guide therapy for several immunosuppressive drugs[15,16], is necessary in patients with COVID-19 and transplant comorbidities.

Herein, we developed an ultra-high performance liquid chromatography/tandem mass spectrometry (UHPLC-MS/MS) method for quantifying the blood concentration of FK506 and performing TDM of FK506 in transplant patients with COVID-19, which can assist clinicians in achieving individualized FK506 treatment.

Patients and Methods

Patient Enrollment and Sample Collection

This study was performed at Shanghai Public Health Clinical Center, which is a certified hospital for patients with COVID-19. The project was reviewed and approved by the Ethics Commission of Shanghai Public Health Clinical Center (No. 2022-S070-02). All procedures were performed in accordance with the recommendations of the Declaration of Helsinki on biomedical research involving human participants. Patients with COVID-19, comorbid organ transplantation, and those taking FK506 were enrolled in this study. Informed consent was waived because the TDM of FK506 was a routine clinical test. Patients were administered 0.75, 0.5, or 0.25 mg/day of FK506. Following medical advice, blood (2 mL) was collected at trough times after continuous dosing for at least 3 days, or follow-up time intervals after discontinuation of dosage, and was drawn into an EDTA-K2-anticoagulant tube. In this study, we collected only the clinical data and analyzed the effect of nirmatrelvir on the FK506 concentration.

Measurement of FK506 Concentration

Chemicals and reagents

FK506 (98% purity) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada); the internal standard (IS) FK506-^{13}C,^{2}d4 (98.8% purity, 99.2% ^{13}C, 98.0% ^{2}H) was obtained from ALSACHIM (Illkirch Graffenstaden, France). High-performance liquid chromatography (HPLC)-grade methanol and acetonitrile were supplied by Fisher Chemical (Shanghai, China), and formic acid (FA) was obtained from ANPEL Laboratory Technologies (Shanghai, China). HPLC-grade ammonium formate was purchased from FLUKA (Shanghai, China). Purified water was prepared using a MilliQ system (Millipore, Billerica, Massachusetts, USA).

Liquid Chromatography

HPLC analysis was performed using a Waters ACQUITY UPLC system (Waters company, Massachusetts, USA). Chromatographic separation was carried out on a C18 column (Luna® 5 µm C18 (2) 100 Å, LC Column 100 x 50 mm) (Phenomenex Company, Tianjin, China) at a flow rate of 0.35 mL/min. The mobile phase included: (1) 10 mmol/L (mM) ammonium formate with 0.1% FA in water and (2) 10 mM ammonium formate with 0.1% FA in methanol: acetonitrile in a gradient elution. Column temperature was 40°C, and sampler was at 4°C. The sample injection volume was 5 µL and the total running time was 3.5 min per sample.

Mass spectrometry

A QTRAP 5500 tandem mass spectrometer (Applied Biosystems/AB SCIEX, Boston, USA) equipped with Turbo Ionspray source was used for quantitative analysis in a multiple reaction monitoring (MRM) mode. The compounds were detected by positive electrospray ionization with a source temperature of 550°C, capillary voltage of 5.5 kV, sources GS1 and GS2 of 60 psi, curtain gas of 20 psi, collision energy (CE) of 25 eV, and declustering potential (DP) of 82 eV for FK506 and 70 eV for FK506-^{13}C,^{2}d4.

Standard solutions

The standard stock solution of FK506 was 5.0 mg/mL in 90% methanol and stored at -20°C. The working solutions were prepared in methanol: water (50:50 v/v) to obtain serial concentrations of 1,000, 500, 250, 100, 50, 12.5, and 5 ng/mL, and quality control of 800, 100, 15, and 5 ng/mL. The standard solutions were spiked with the appropriate amounts of FK506 working solutions in blood at 10 dilutions. The precipitate solutions containing the IS were prepared at a concentration of 2.5 ng/mL in methanol: acetonitrile (1:1, v/v).

Sample Treatment

Protein precipitation of blood samples was performed as follows: blood (50 µL) was mixed with 50 µL 0.1 M $ZnSO_4$ and 150 µL precipitate solution, and centrifuged at 4°C, 12,000 rpm for 10 min. The supernatant (5 µL) was then injected into the UHPLC-MS/MS system.

Method Validation

Validation of the UHPLC-MS/MS was performed according to the guidelines of the Food and Drug Administration (FDA), that included selectivity, linearity, precision and accuracy, matrix effect, extraction recovery, and stability (at 4°C, 25°C, and 56°C).

Statistical Analysis

All the statistical analyses were carried out with Microsoft Excel 2016 (Washington, USA). Results were expressed as mean plus relative standard deviation. Data were considered statistically significant at $p < 0.05$.

Results

Validation of the UHPLC-MS/MS Method

A molecular ion with the addition of ammonium $[M+NH_4]^+$ (m/z 821.7) and its product ion (m/z 768.4) were detected from FK506 using an Electron Spray Ionization (ESI) source (Figure 1A1), while 826.7/773.4 was detected from IS (FK506-$^{13}C,^2d4$) (Figure 1A2). No endogenous interference was observed for FK506 and FK506-$^{13}C,^2d4$ as depicted by the double blanks (Figure 1B1). The retention time for both FK506 and IS was 1.37 min on the C18 column (Figure 1B2 and B3).

The limit of detection [signal to noise (S/N) ratio of 3] and limit of quantification (S/N of 10) were 0.125 and 0.5 ng/mL, respectively. The quantification range was 0.5-100 ng/mL, with determination coefficients (r^2) higher than 0.99 for all calibration baths. The intra- and inter-batch accuracies were 104.55-107.85%, and 99.52-108.01%, respectively, with a precision of < 15% (Table I).

FK506 was stable at the clinical sample treatment conditions [(room temperature, approximately 25°C) for 6 h, 4°C for 24 h, and 30 min at 56°C (required to inactivate the virus)]. Furthermore, FK506 was also stable in general laboratory experimental conditions, comprising 24 h at 4°C in a sampler, three freeze and thaw cycles, and frozen at -80°C for three months. Their precisions were all less than 15%, and their accuracies ranged from 85.00 to 115.00% (Table II).

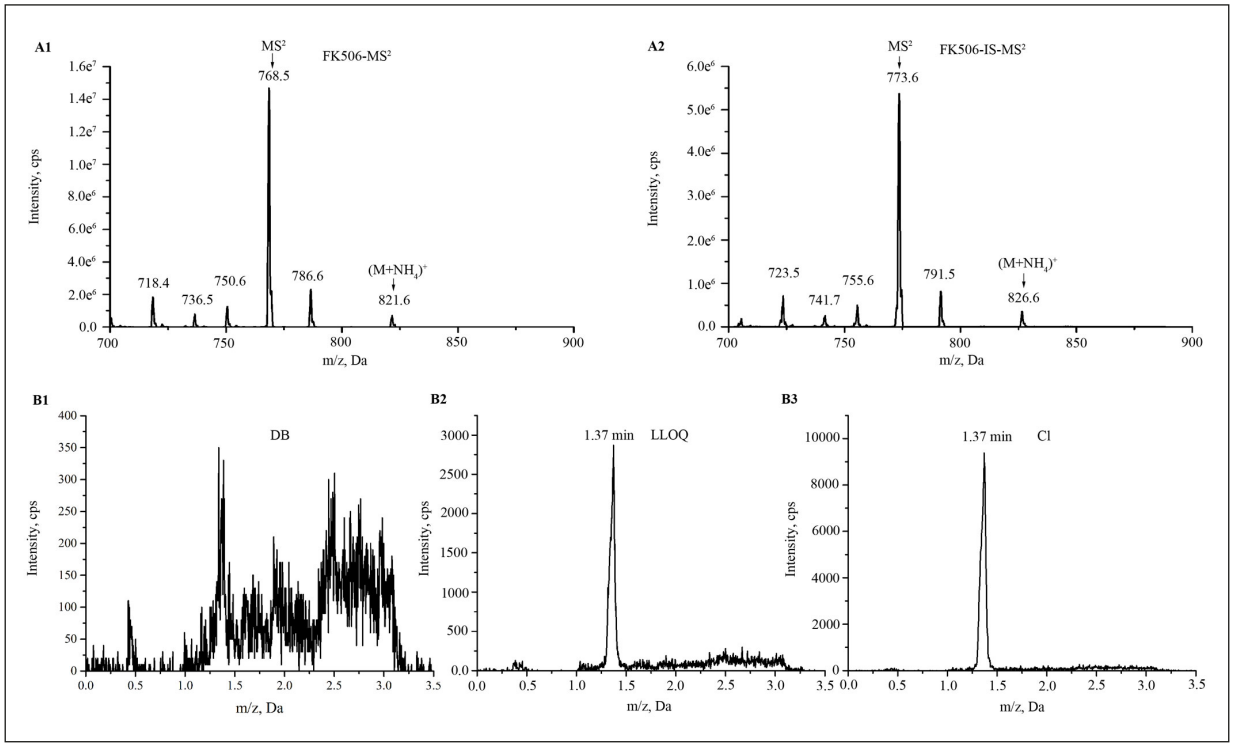

Figure 1. The UHPLC-MS/MS profiles of FK506 and FK506-$^{13}C,^2d4$. A1-A2, the molecular ion with the addition of ammonium, and the product ion mass spectra profile of FK506, FK506-$^{13}C,^2d4$, respectively. B1-B3, the chromatogram profiles of FK506 from blank blood, spiked blood at the concentration of lower limit of quantification (LLOQ) (0.5 ng/mL), and a present clinical sample, respectively. FK506, tacrolimus; LC-MS, liquid chromatography-mass spectrometry.

Table I. Intra- and inter-batch precisions and accuracies of FK506 in human blood.

Compounds	Concentration (ng/mL)	Intra-batch (n = 6)			Inter-batch (n = 18)		
		Measured (ng/mL)	Precision (%)	Accuracy (%)	Measured (ng/mL)	Precision (%)	Accuracy (%)
FK506	8 0	80.15	2.50	100.19	79.62	3.64	99.52
	10	10.75	1.55	107.52	10.81	2.08	108.01
	1.5	1.62	3.48	107.85	1.63	3.73	107.33
	0.5	0.52	3.84	104.55	0.52	3.54	103.40

FK506, tacrolimus.

Table II. The stabilities of FK506 at different temperatures.

Compound (FK506)	Concentration (ng/mL)	80	1.5
6 h at 25°C	Measured (ng/mL)	88.78	1.61
	Precision (%)	3.64	5.40
	Accuracy (%)	111.08	107.31
24 h at 4°C	Measured (ng/mL)	85.28	1.57
	Precision (%)	1.76	4.44
	Accuracy (%)	106.62	104.82
30 min at 56°C	Measured (ng/mL)	81.46	1.55
	Precision (%)	4.77	6.21
	Accuracy (%)	101.83	103.46
24 h at 4°C sampler	Measured (ng/mL)	77.08	1.54
	Precision (%)	2.97	3.70
	Accuracy (%)	96.34	102.70
Frozen and thaw	Measured (ng/mL)	71.25	1.44
	Precision (%)	0.98	3.87
	Accuracy (%)	89.06	95.66
Three months at -80°C	Measured (ng/mL)	73.41	1.34
	Precision (%)	3.31	4.84
	Accuracy Å (%)	92.61	91.78

FK506, tacrolimus.

After normalization by IS, the extraction recoveries and matrix effects in the low (1.5 ng/mL) and high (80 ng/mL) quality control samples were 11.63 and 111.70% and 101.79 and 94.89%, respectively. The relative standard deviations for extraction recoveries and matrix effects were all < 6.06%.

Application to Therapeutic Drug Monitoring

Twenty-two blood samples from two patients were included in this study. As shown in Table III and Supplementary Table I, the two patients who received TDM were men, aged 64 and 52 years, respectively. Patient No. 1 had dysfunctional liver condition on the first day of FK506 monitoring but was normal in the later stages of treatment (Table III). Patient No. 2 had no obvious changes in liver and kidney functions during TDM (**Table SI**). The FK506 dosages in the two patients were 0.25 to 0.75 mg/day. For patient No. 1, FK506 had been administered for 114 days prior to his admission to our hospital on April 11 and was continued with 0.75 mg/day (QD) until May 13. Simultaneously, the patient received NMV (150 mg) and ritonavir (RTV) (100 mg) from April 12 to 16. FK506 detection was initiated from May 3, with a blood concentration of 13.9 ng/mL, which reduced to 12.4 ng/mL on May 14, before the 0.5 mg/day dosage was administered. Then FK506 concentrations were maintained at 5.42 to 3.15 ng/mL un-

Table III. The clinical characters of patient No. 1.

Patient No.1, Male 64 years old																					
Date	**May 3**	**May 14**	**May 20**	**May 31**	**June 6**	**June 13**	**June 17**	**June 18**	**June 19**	**June 20**	**June 21**	**June 22**	**June 23**	**June 24**	**June 25**	**June 26**	**June 27**	**June 28**	**June 30**	**July 3**	**July 7**
Date	May 3	May 14	May 20	May 31	June 6	June 13	June 17	June 18	June 19	June 20	June 21	June 22	June 23	June 24	June 25	June 26	June 27	June 28	June 30	July 3	July 7
RTV (mg)	/	/	/	/	/	100/100	100/100	/	/	/	/	/	/	/	/	/	/	/	/	/	/
NMV (mg)	/	/	/	/	/	300/300	300/300	/	/	/	/	/	/	/	/	/	/	/	/	/	/
FK506 (mg)	0.75/0.75	0.50/0.50	0.50/0.50	0.50/0.50	0.50/0.50	0.50/0.50	/	/	/	/	/	/	/	/	/	0.25/0.25	0.25/0.25	0.25/0.25	0.25/0.25	0.25/0.25	0.25/0.25
FK506 concentration (ng/mL)	13.90	12.40	5.42	4.23	4.72	3.15	33.10	31.30	29.40	28.3	25.20	14.90	12.80	6.25	5.19	3.36	3.84	3.90	4.39	4.94	3.77
C-reactive protein (mg/L)	11.26	NA	NA	NA	2.11	26.3	3.71	2.16	1.96	2.06	1.38	6.64	13.94	NA	NA	NA	3.97	NA	NA	NA	NA
CD3+T cell number (cell/μL)	NA	NA	NA	NA	NA	778.01	833.72	1,223.5	1,182.81	1,042.95	967.48	NA	828.39	NA	NA	NA	NA	NA	NA	NA	NA
CD4+T cell number (cell/μL)	NA	NA	NA	NA	NA	319.89	429.12	616.77	596.05	484.67	450.59	NA	392.99	NA	NA	NA	NA	NA	NA	NA	NA
Alanine aminotransferase (U/L)	109	NA	NA	NA	31	NA	23	27	29	30	NA	NA	20	NA	NA	NA	19	NA	NA	31	57
Aspartate aminotransferase (U/L)	56	NA	NA	NA	24	NA	23	25	24	24	NA	NA	20	NA	NA	NA	19	NA	NA	35	49
Alkaline phosphatase (U/L)	209	NA	NA	NA	99	NA	67	64	60	48	NA	NA	57	NA	NA	NA	54	NA	NA	83	142
L-ɤ-glutamyl transferase (U/L)	427	NA	NA	NA	149	NA	89	79	73	68	NA	NA	56	NA	NA	NA	53	NA	NA	163	425
Lactate dehydrogeNAse (U/L)	265	NA	NA	NA	229	NA	216	222	233	235	NA	NA	271	NA	NA	NA	256	NA	NA	236	258
Creatinine (μmol/L)	70.4	NA	NA	NA	80.2	NA	58.1	60.3	55.8	59	NA	NA	75.5	NA	NA	NA	93.4	NA	NA	74.6	71
Glucose (mmol/L)	20.93	NA	NA	NA	12.71	NA	3.19	4.65	4.65	4.8	NA	NA	4.22	NA	NA	NA	4.04	NA	NA	NA	NA
eGFR [mL/(min*1.73m^2)]	103.997	NA	NA	NA	89.475	NA	129.796	124.347	135.989	127.514	NA	NA	95.934	NA	NA	NA	75.048	NA	NA	97.27	102.984

†The patient was lung transplanted on 29th December 2021, then given FK506 every day. The patient entered Shanghai Public Health Clinical Center on 11th April 2022. The detection values higher than upper limit were highlighted by red color, and the detection values lower than lower limit were highlighted by blue. RTV, ritonavir; NMV, nirmatrelvir; FK506, tacrolimus. 0.75/0.75 represents 0.75 mg every time, and once every day. /, no drug. NA represents no data.

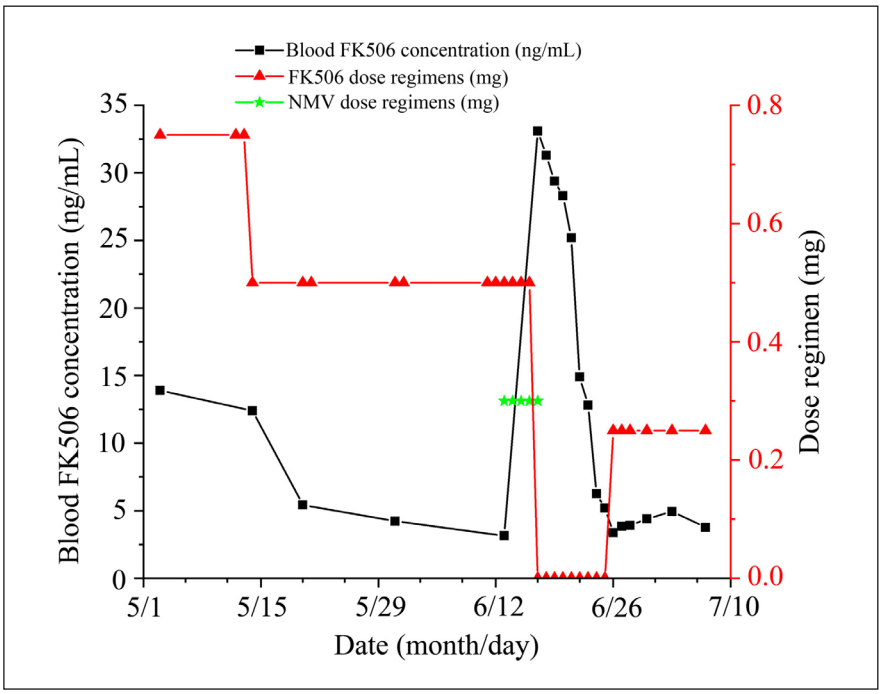

Figure 2. The curve of FK506 concentrations and drug dose regimens. FK506 concentrations were collected between May 3 and July 7, 2022. The horizontal ordinate represents the data collection date. FK506 doses were changed from 0.75, 0.5, 0, to 0.25 mg/day, as shown in red triangles. NMV was administered for five days as shown by the green stars. Blood FK506 concentrations were significantly increased after its co-administration with NMV, remaining at elevated levels even after withdrawal of FK506 administration, as shown by the black squares. FK506, tacrolimus; NMV, nirmatrelvir.

der the dosage of 0.5 mg/day. However, FK506 concentration increased to 33.1 ng/mL after its co-administration with NMV for 4 days. Subsequently, FK506 was withdrawn with immediate effect, and its concentrations were decreased slowly from 31.3, 29.4, 28.3 to 25.2 ng/mL within the following four days and were further decreased from 14.9 to 5.19 ng/mL in another four days. Next, FK506 was administered at a dosage of 0.25 mg/day, and the concentration was maintained at 3.36 to 4.94 ng/mL. As shown in Figure 2, FK506 concentrations were increased with its co-administration with NMV. No correlation between FK506 concentrations and clinical characteristics of patients was found (Table III).

Discussion

Organ transplant patients have a high risk of getting infected with COVID-19 owing to the use of immunosuppressive drugs, and are expected to have higher mortality from COVID-19[4,5,9]. FK506, a first-line immunosuppressive drug, has been widely used in organ transplant patients[10,16]. Subclinical tacrolimus dosage may lead to immune activation, followed by organ lesions, and its overexposure may result in drug toxicity associated with adverse events. TDM has long been used to monitor tacrolimus[17]. To decrease the toxicity and improve the therapeutic effect of FK506, we performed TDM in two COVID-19 patients. Comparing the clinical test indicators, no significant toxicity was observed for the co-administration of FK506 and other antiviral drugs.

Tacrolimus is primarily metabolized by Cytochrome p450 3A4 (CYP3A4)[18]. NMV is a CYP3A4 substrate[19]. Since RTV, an anti-HIV drug is used as a pharmacokinetic booster of NMV, NMV/RTV has a high potential to cause clinically important drug-drug interactions with FK506. In this study, we found that NMV can significantly increase the blood concentration of FK506 with a 10-fold rise after a four-day administration, and this effect could persist up to 9 days. This may be due to the inhibition of CYP3A4 by RTV[20]. Similarly, anti-HIV drugs (lopinavir and RTV have been shown to significantly increase FK506 blood concentration in transplantation patients[21,22]. Therefore, it is necessary to monitor the concomitant use of FK506 and NMV and decrease the drug dose of FK506 or stop its administration till the FK506 levels return to normal.

Limitations

This study has some limitations: 1) only two patients were involved, 2) only one sample from patient No. 2 was detected, and 3) we did not completely monitor the two cycles of concomitant use of FK506 and NMV in the patients.

Conclusions

In this study, we developed a UHPLC-MS method to quantify blood FK506 concentrations and found that FK506 could be quantified after virus inactivation at 56°C for 30 min. TDM of FK506 in COVID-19 patients was performed, and significant drug-drug interactions were detected between FK506 and NMV. Therefore, it is necessary to decrease the dosage of FK506 when co-administered with NMV/RTV.

Conflict of Interest

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Ethics Approval

The ethical approval was obtained from the Ethics Committee of Shanghai Public Health Clinical Center (No. 2021-S070-02). All human research procedures followed were in accordance with the standards set forth in the Declaration of Helsinki Principles of 1975, as revised in 2013.

Informed Consent

Informed consent was waived because the TDM of FK506 was a routine clinical test.

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