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ORIGINAL RESEARCH

Development and Validation of a UHPLC-MS/MS Method for Ciprofol Detection in Plasma: Application in Clinical Pharmacokinetic Studies

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Objective: This study aimed to develop and validate an ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method for the quantification of ciprofol in human plasma, with the goal of applying this method to pharmacokinetic studies in patients undergoing elective surgery under general anesthesia.

Methods: A methanol-based protein precipitation method was employed for sample preparation, using ciprofol-d6 as the internal standard. Chromatographic separation was achieved on a Shimadzu Shim-pack GIST-HP C18 column (3 μm, 2.1×150 mm) with a mobile phase consisting of 5 mmol·L^{-1} ammonium acetate (A) and methanol (B). The flow rate was maintained at 0.4 mL·min^{-1}, and the column temperature was set at 40°C. Detection was performed using electrospray ionization (ESI) in negative ion mode with multiple reaction monitoring (MRM). The quantification ion pairs were m/z 203.100→175.000 for ciprofol and m/z 209.100→181.100 for the internal standard.

Results: Ciprofol exhibited excellent linearity across the concentration range of 5 to 5000 ng·mL^{-1} ($r > 0.999$). The intra-batch and inter-batch precision values were within 4.30% to 8.28%, and the relative deviation ranged from −2.15% to 6.03%. The extraction recovery rate was 87.24% to 97.77%, and the matrix effect relative standard deviation (RSD) was less than 15%.

Conclusion: The developed UHPLC-MS/MS method is simple, rapid, accurate, and highly specific, making it suitable for the determination of ciprofol plasma concentrations and pharmacokinetic studies in clinical settings. This method provides a reliable analytical tool for future research on ciprofol in complex biological matrices.

Keywords: ciprofol, UHPLC-MS/MS, pharmacokinetics

Introduction

Ciprofol (HSK3486) is a novel intravenous anesthetic developed in recent years. It is a 2,6-disubstituted phenol derivative, with a chemical structure similar to propofol, thus possessing similar pharmacological properties. Its mechanism of action is similar to propofol, as it is a γ-aminobutyric acid (GABA) receptor agonist. It can bind to the GABAA receptor, enhancing the Cl^- influx mediated by the GABA receptor, leading to hyperpolarization of the neuronal membrane, resulting in central nervous system depression, thereby achieving sedative or anesthetic effects.[1–6] However, compared to propofol, ciprofol has a stronger affinity for the GABAA receptor and possesses higher lipophilicity, hydrophobicity, and a more suitable spatial volume, with a potency 4 to 5 times that of propofol.[7,8] Preclinical and clinical studies have shown that ciprofol, similar to propofol, has rapid sedative effects, is well tolerated by subjects, and has good efficacy and safety; additionally, it offers advantages such as rapid onset, quick recovery, high clearance rate, minimal residual effects, mild respiratory depression, minimal hemodynamic impact, and mild injection pain.[8–10] Its indications include painless gastroscopy and induction and maintenance of general anesthesia.

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The concentration of anesthetic drugs in the human body is closely related to the depth of anesthesia, perioperative safety and recovery, and the occurrence of anesthesia-related complications.[11] Various methods for measuciprofol concentrations in human plasma have been established for clinical pharmacokinetic studies and drug concentration monitoring.[12–16] However, as a newly launched sedative-hypnotic agent, ciprofol currently has no established blood concentration detection methods domestically or internationally, which has become a technical challenge in human pharmacokinetic research and blood concentration monitoring. UHPLC-MS/MS technology can separate the analyte through ultra-performance liquid chromatography and detect the quasi-molecular ions and characteristic fragment ions of the analyte using a mass spectrometer, characterized by fast analysis speed, good selectivity, and high sensitivity.[17] Therefore, it is very necessary to establish a detection method for ciprofol for drug concentration determination and pharmacokinetic studies. This article establishes a UHPLC-MS/MS analysis method for ciprofol in human plasma, providing a reference for the pharmacological monitoring of ciprofol and the implementation of individualized perioperative medication.

Materials and Methods

Instruments

The main instruments used in this study include the LC-20ADXR high-performance liquid chromatograph (Shimadzu Corporation, Japan), the Triple Quad 4500MD mass spectrometer (AB Sciex, USA), the XPE105DR balance (Shanghai Mettler-Toledo Company), the Vortex-5 vortex mixer (Qilinbei Instrument Manufacturing Co., Ltd., Haimen City), the Milli-Q Integral 5 water purification system (Millipore Corporation, USA), and the Milli-Q IQ7003 ultrapure water instrument (Merck, Germany).

Reagents

Ciprofol standard (Haisco Pharmaceutical Group, batch number: 12CN0210701); Ciprofol-d6 reference substance (Haisco Pharmaceutical Group, batch number: LL-HSK23287-20200506-001); Methanol, formic acid, ammonium formate (Fisher Scientific, USA, chromatographic purity). Blank plasma was provided by the hospital's blood transfusion department.

Chromatographic Conditions

Column: Shimadzu, Shim-pack GIST-HP C18, 3μm, 2.1×150 mm (HSS); Guard column: Shimadzu, 3μm, 2.1×100 mm. Mobile phase A is a 5 mmol·L^{-1} ammonium acetate aqueous solution, and mobile phase B is methanol. Gradient elution is performed with ammonium acetate (A) – methanol (B) as the mobile phase (0 ~ 0.1 min, 25% B; 0.1 ~ 0.5 min, 25% ~ 95% B; 0.5 ~ 2.9 min, 95% B; 2.9 ~ 2.95 min, 95% ~ 25% B; 2.95 ~ 4.0 min, 25% B). The flow rate is 0.4 mL·min^{-1}. Column temperature: 40°C. Injection volume: 5μL. Sample compartment temperature is 8°C.

Mass Spectrometry Conditions

ESI ion source, negative ion mode (Positive), multiple reaction monitoring mode (MRM); nebulizer and drying gases are nitrogen, collision gas is high-purity nitrogen, pressure 0.1 MPa; the optimal MRM parameters for ciprofol and ciprofol-d6 are shown in Supplementary Table 1; the mass spectrometry maps generated by the measured components and internal standards are shown in Supplementary Figure 1; other optimized mass spectrometry parameters are as follows: Turbo Ion Spray Temperature 450°C, Dry gas flow 10 L·min^{-1}, Nebulizer gas 50 psi, Auxiliary Gas (Gas 2) 45 psi, Sheath gas temperature 300°C, Sheath gas flow 12 L·min^{-1}, Capillary voltage 4500 V, Dwell time 100 ms, the resolution of MS1 and MS2 is Unit.

Solution Preparation

Reference Substance Reserve Solution and Quality Control Reserve Solution

Accurately weigh 10.00 mg of ciprofol reference substance, dissolve it in 1 mL of methanol, mix well to obtain a reserve solution with a final concentration of about 10 mg·mL^{-1}. Prepare a quality control reserve solution with a mass concentration of 10 mg·mL^{-1} in the same way. The above solutions are stored sealed in a refrigerator at 4°C for future use.

Internal Standard Solution

Accurately weigh 5.00 mg of ciprofol-d6 internal standard, dissolve it in 1 mL of methanol, and mix well to obtain a ciprofol-d6 internal standard solution with a concentration of 5.00 mg·mL^{-1}. Take 0.5 mL of the above solution, place it in a 500 mL volumetric flask, and dilute it to the mark with 50% methanol to obtain an internal standard solution with a mass concentration of 5000 ng·mL^{-1}. The above solutions are stored sealed in a refrigerator at 4°C for future use.

Standard Curve Solution

Take 100 μL of the reference substance reserve solution from item "2.3.1", add 900 μL of methanol-water solution, vortex mix for 1 min to obtain a solution with a mass concentration of 100 μg·mL^{-1}. Take the above solution and dilute it with methanol-water solution to obtain standard curve working solutions with mass concentrations of 50000, 200000, 5000, 2000, 500, 250, 100, 50 ng·mL^{-1}. Take 15 μL of each standard curve working solution, add 135 μL of blank plasma, vortex mix for 1 min to obtain standard curve solutions with mass concentrations of 5–5000 ng·mL^{-1}.

Quality Control Sample Solution

Take 100 μL of the quality control reserve solution from item "2.3.1", add 900 μL of methanol-water solution, vortex mix for 1 min to obtain a solution with a mass concentration of 100 μg·mL^{-1}. Take the above solution and dilute it with methanol-water solution to obtain quality control working solutions with mass concentrations of 50, 125, 3750, 18750, 37500, 60000, 100000 μg·mL^{-1}. Take 15 μL of each quality control working solution, add 135 μL of blank plasma, vortex mix for 1 min to obtain quality control sample solutions with mass concentrations of 5, 12.5, 375, 1875, 3750, 6000, 10000 ng·mL^{-1}.

Plasma Sample Pretreatment

Using a pipette, add 135 μL of blank plasma (blank samples, zero concentration samples, standard curve samples, quality control samples) to a 1.5 mL EP tube, then add 15μL of standard curve working solution/quality control working solution. For blank samples and zero concentration samples, add 15μL of 50% methanol-water, vortex mix evenly. Then add 10μL of internal standard working solution to each sample. For blank samples, add 10μL of 50% methanol-water, then add 300μL methanol for protein precipitation, vortex mix vigorously for 3 min. Centrifuge at 4°C, 14000 r·min^{-1} for 10 min, take the supernatant for UHPLC-MS/MS analysis, injection volume 5 μL.

Methodological Investigation

The method was investigated according to the US Food and Drug Administration (FDA) Bioanalytical Method Validation Guidance,[18] and the relevant method validation guidelines successively examined the selectivity, standard curve, lower limit of quantification (LLOQ), accuracy, precision, matrix effect, extraction recovery, and stability of samples and stock solutions for the UHPLC-MS/MS quantitative method. Potential error sources and quality control standards are detailed in Supplemental Data I. The specific experimental operations are as follows:

Specificity

The purpose of specificity is to investigate whether there is interference between the endogenous components (such as phospholipids, proteins, etc) in blank plasma, the analytes, and the internal standard. This experiment examined six blank plasmas from different individuals, blank plasma with ciprofol (5 ng·mL^{-1}) reference substance, blank plasma with ciprofol-d6 (500 ng·mL^{-1}) reference substance, blank plasma with ciprofol (5 ng·mL^{-1}) and ciprofol-d6 (500 ng·mL^{-1}) reference substances, and clinical blood samples after medication. After processing the samples according to the method in "2.4" above, UHPLC-MS/MS analysis was performed under the conditions in "2.1" and "2.2". The MRM mode chromatograms of blank plasma, each standard-added sample, and clinical blood samples after medication were compared. It is required that there should be no interference at the corresponding peak positions of ciprofol and ciprofol-d6.

Linear Range and Lower Limit of Quantification (LLOQ)

Take the standard curve solution from "2.3.3", process it according to the method in "2.4", and then inject and measure according to the chromatographic and mass spectrometry conditions in "2.1" and "2.2". The mass concentration of ciprofol is

taken as the abscissa (x), and the ratio of the response value of ciprofol to the internal standard is taken as the ordinate (y). Weighted least squares method (weighting factor $1/x^2$) is used for regression equation fitting. The linear regression coefficient (r) of the standard curve should be greater than 0.990. On the same day, five standard curves at eight concentration levels were completed. Except for the lower limit of quantification, the recalculated concentration of the analyte in the correction standard at other concentration levels should be within ±15% of the labeled value.

The lower limit of quantification (LLOQ) is the lowest concentration of the analyte in the sample that can be reliably quantified, which is the lowest concentration point on the standard curve. The response value of the analyte should be more than 10 times (S/N ≥10) the response value of the blank biological matrix interference, and the accuracy of the analyte RE (%) ≤ ±20%, and the precision RSD (%) ≤ 20%.

Precision and Accuracy

Prepare quality control samples at five concentrations of LLOQ (5 $ng \cdot mL^{-1}$), LQC (12.5 $ng \cdot mL^{-1}$), MQC1 (375 $ng \cdot mL^{-1}$), MQC2 (1875 $ng \cdot mL^{-1}$), and HQC (3750 $ng \cdot mL^{-1}$) according to the method in "2.3.4". Each concentration is prepared in parallel with six samples. After processing according to the method in "2.4", inject and measure according to the chromatographic and mass spectrometry conditions in "2.1" and "2.2" to investigate the intra-batch precision. Measure continuously for three days to investigate the inter-batch precision, and take the deviation of the measured mass concentration from the theoretical mass concentration as the accuracy. It is required that at least four samples in each analytical batch have an accuracy RE (%) ≤ 20%, the inter-batch accuracy is within ±20%, and the intra-batch and inter-batch precision RED (%) ≤ 20%.

Extraction Recovery and Matrix Effect

Extraction recovery (ER) refers to the percentage of the MS/MS response value of the analyte before and after extraction of the biological sample. Matrix effect (ME) is mainly evaluated by comparing the percentage of the MS/MS response value of the analyte obtained from the biological sample and the pure solvent (or mobile phase) after the same method treatment, to evaluate whether the influence of endogenous substances on the response value of the analyte is an enhancement effect or an inhibition effect. In this experiment, quality control samples at four concentrations of LQC (12.5 $ng \cdot mL^{-1}$), MQC1 (375 $ng \cdot mL^{-1}$), MQC2 (1875 $ng \cdot mL^{-1}$), and HQC (3750 $ng \cdot mL^{-1}$) were prepared according to the method in "2.3.4". Each concentration was prepared with five parallel samples. After processing according to the method in "2.4", dilute with 50% methanol and inject for analysis to obtain the response value A. Blank plasma was extracted according to the method in "2.4", diluted with 50% methanol containing LQC, MQC1, MQC2, and HQC four concentrations of analytes, and injected for analysis to obtain the response value B. Ultrapure water was extracted according to the method in "2.4", diluted with 20% methanol containing LQC, MQC1, MQC2, and HQC four concentrations of analytes, and injected for analysis to obtain the response value C. Each concentration of the above samples was operated in parallel with three samples. Extraction recovery and matrix effect were calculated according to the following formulas: ER (%) = A/B × 100%; ME (%) = B/C × 100%.

Stability Investigation

The stability of QC standard plasma samples at high, medium, and low concentrations under different conditions was investigated. This includes freeze-thaw stability, that is, the sample was subjected to three freeze-thaw cycles; short-term stability, that is, the sample was placed at room temperature 25°C for 12 hours; long-term stability, that is, the sample was stored at −20°C for 4 weeks; and post-sample processing stability, that is, the sample was placed in the autosampler (4°C) for 12 hours. Each concentration of the samples was operated in parallel with five samples. The results of the injection analysis were substituted into the standard curve equation of the same day to obtain the actual concentration of the analyte, and RE (%) and RSD (%) were calculated to evaluate the stability. It is required that the RE (%) of each concentration correction standard should be within ±15%, and RSD (%) should be ≤15%.

Hemolysis Effect

Take two portions of blank fresh whole blood from different sources into clean centrifuge tubes, freeze at −20°C for 24 hours, and thaw to prepare hemolyzed whole blood. Add an appropriate amount of hemolyzed whole blood to blank plasma to prepare a 2%

hemolyzed plasma experimental group according to the volume ratio. Replace the human blank plasma in "2.4" with plasma containing 2% whole blood cell lysate to prepare low and high concentration quality control samples with concentrations of 12.50 ng·mL^{-1} and 3750.00 ng·mL^{-1}, respectively. Three parallel samples were prepared for each concentration. Operate according to the method in "2.4". The above samples were analyzed by UPLC-MS/MS to evaluate the matrix effect of hemolyzed plasma.

Dilution Reliability

After the method was established, if the clinical sample exceeded the linear range during the measurement process, the sample should be diluted by a certain multiple to bring its concentration within the linear range before measurement and analysis. In this experiment, correction standards with concentrations higher than the upper limit of quantification were prepared and diluted with blank plasma. The dilution multiples should cover the dilution multiples used for clinical samples to investigate the reliability of dilution. It is required that the accuracy of the diluted sample RE (%) $\leq \pm 15\%$, and the precision RSD (%) $\leq 15\%$.

Clinical Application

This study has been approved by the Ethics Committee of the General Hospital of the Southern Theater Command of the Chinese People's Liberation Army (NZLLKZ2024150) and registered with the Chinese Clinical Trial Registration Center (ChiCTR2400094359). A total of six patients undergoing scheduled surgery were recruited in this study. All subjects provided informed consent and signed the informed consent form before the start of the trial. Patients with severe cardiovascular, pulmonary, hepatic, or renal dysfunction; allergy to the test drug; history of drug abuse, smoking, alcoholism, or infectious diseases; participation in other drug clinical trials within the past six months; and patients with language communication barriers were excluded. All patients were fasting, water-restricted, or restricted from any other food for 8 hours before the first blood collection. During the operation, patients were intravenously infused with ciprofol emulsion injection (Liaoning Haisco Pharmaceutical Co., Ltd., specification 20 mL: 50 mg, registration number H20200013), with an induction dose of 0.4 mg·kg^{-1} and a maintenance dose of 0.8 mg·kg·h^{-1}. Arterial blood samples (1 mL) were collected at preset intervals: before administration of ciprofol (0 minutes); 1, 3, and 5 minutes after bolus injection; 5, 10, 20, 30, 45, and 60 minutes after continuous infusion of ciprofol; and 3, 5, 10, 20, 30, 60, 90, and 120 minutes after cessation of ciprofol infusion. The collected blood was placed in tubes containing EDTA-K_2 anticoagulant. The obtained samples were then centrifuged (4°C, 14,000 r·min^{-1} for 10 minutes), and the upper plasma was separated. After processing according to the method in "2.4", the samples were injected and measured according to the chromatographic and mass spectrometry conditions in "2.1" and "2.2" (plasma samples exceeding the upper limit of detection needed to be diluted before measurement).

Statistical Analysis

The sample size was determined in accordance with the National Medical Products Administration (NMPA) guidelines for pharmacokinetic (PK) studies. A cohort of 8–12 subjects was deemed adequate for evaluating the PK properties of the investigational drug.[5] The peak area data of ciprofol and ciprofol-d_6 were quantitatively calculated using the Analyst 1.7.2 data processing system, and the obtained plasma concentration data were used to calculate pharmacokinetic parameters with Phoenix WinNonlin (version 8.3). GraphPad Prism® (version 10.1.2) was used to plot the plasma concentration–time curves. Continuous normally distributed variables were expressed as mean ± standard deviation, and SPSS software (version 26.0) used Student's *t*-test to compare pharmacokinetic parameters between groups.

Results

Selectivity

Chromatograms of blank plasma, blank plasma with Ciprofol, blank plasma with Ciprofol-d6, and Patient plasma samples are shown in Figure 1. The retention times for ciprofol and ciprofol-d6 were 2.461 min and 2.455 min, respectively, which meet the analytical requirements for rapid and accurate quantification. The experimental results indicate that endogenous substances in plasma do not affect the determination of ciprofol and ciprofol-d6, and there is no interference between ciprofol and ciprofol-d6. This method has high specificity.

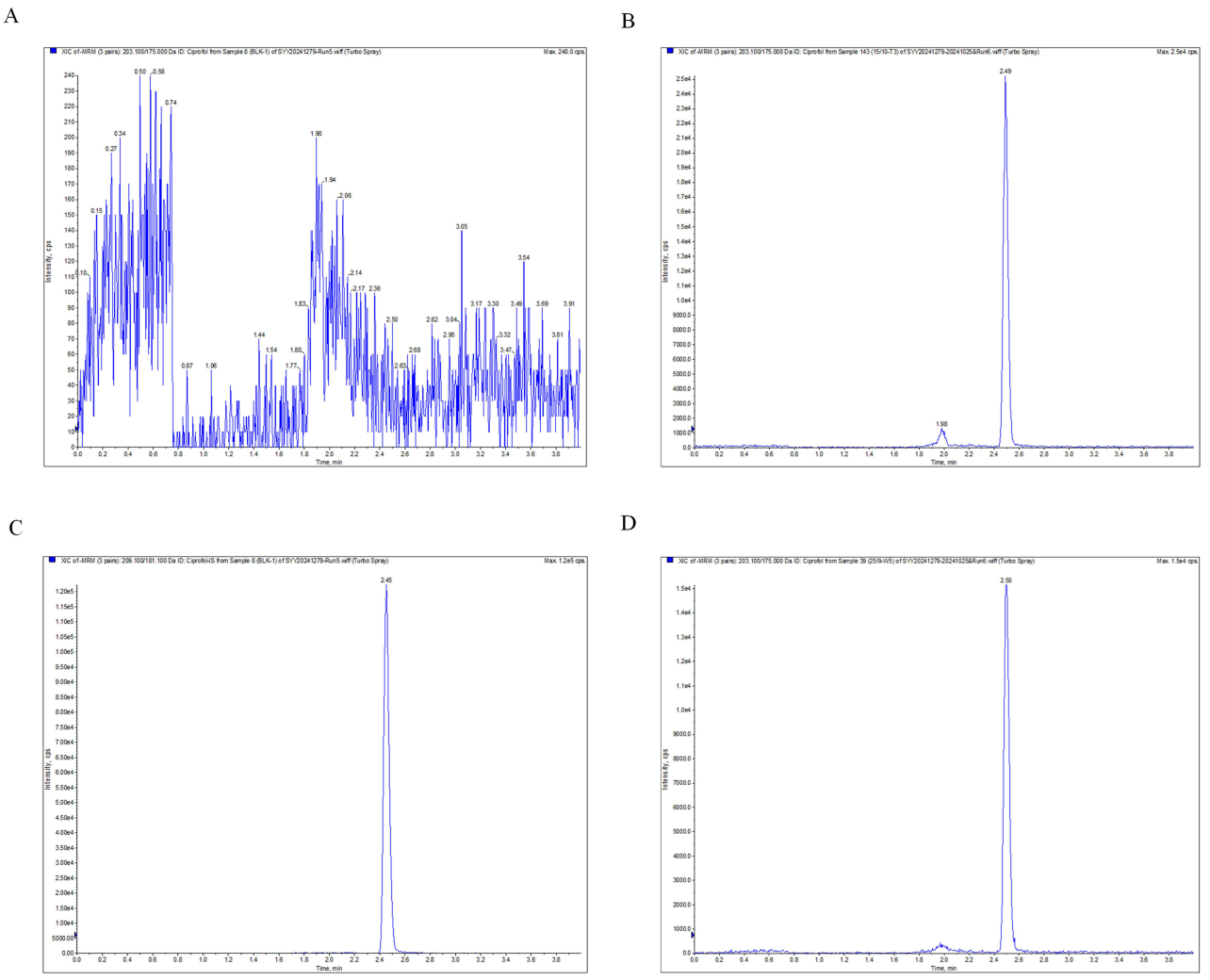

Figure 1 The chromatogram of MRM pattern: (**A**) Blank plasma; (**B**) Blank plasma spiked with Ciprofol; (**C**) Blank plasma spiked with Ciprofol-d6; (**D**) Patient plasma samples.

Standard Linearity and Lower Limit of Quantification

Five parallel sets of correction standards at eight concentration levels and six parallel LLOQ samples were prepared within the same day. The samples were analyzed according to the procedure in "2.2". Linear regression was performed using the $1/x^2$ weighted least squares method to obtain the standard curve equation for ciprofol (Figure 2). The linear range, standard curve equation, and LLOQ for ciprofol are detailed in Supplementary Table 2. The experimental results show that the five parallel standard curves within the same day exhibit a good linear relationship within the concentration range of ciprofol. The precision RSD (%) at the LLOQ concentration level for ciprofol was 4.04% and 10.99%, and the accuracy RE (%) was 4.43%, which meet the requirements for method validation.

Extraction Recovery and Matrix Effect

Table 1 lists the results of extraction recovery and matrix effect for QC samples at high, medium, and low concentration levels. The data in the table indicate that after processing the plasma samples according to the method in "2.2", the extraction recovery of ciprofol was between 97.03% and 101.1%. The results show that the extraction recovery obtained by the protein precipitation method for extracting the analytes from biological samples meets the requirements for quantitative analysis. It has been reported in the literature that a matrix effect between 85% and 115% is generally considered negligible. When the matrix effect is >115%, there is an ion enhancement effect, and when it is <85%, there is

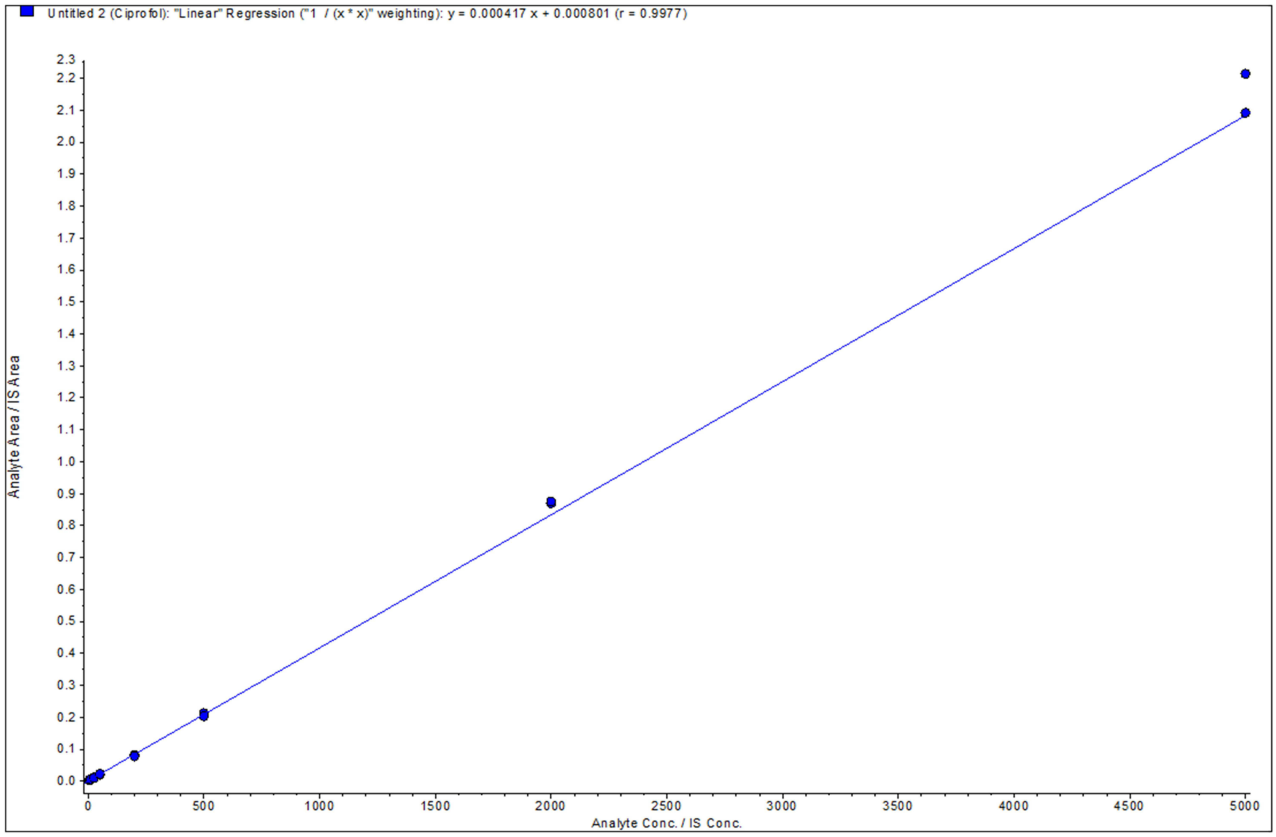

Figure 2 Linearity of Ciprofol.

an ion suppression effect. The results of this experiment show that the matrix effect of ciprofol was between 116.07% and 117.60%, indicating that endogenous substances in plasma have a weak ion enhancement effect on ciprofol. Although the use of an internal standard did not eliminate the influence of endogenous substances in plasma on ciprofol, it can reduce the impact of the matrix effect on the determination results of ciprofol.

Precision and Accuracy

The intra-day and inter-day precision RSD (%) for QC samples of ciprofol at high, medium, and low concentration levels were between 3.05% and 15.79% and 1.09% and 7.82%, respectively. The intra-day and inter-day accuracy, expressed as RE (%), were between 2.41% and 6.4% and −1.23% and 2.76%, respectively (Table 2). The experimental results show that the precision and accuracy of ciprofol meet the requirements for quantitative analysis.

Table 1 Extraction Recovery and Matrix Effect for the Analytes in Human Plasma (n = 3)

Analyte	Nominal Concentration (ng/mL)	Intra-Day (n = 5)			Inter-Day (n = 5)		
		Measured Concentration (ng/mL)	Precision RSD (%)	Accuracy RE (%)	Measured Concentration (ng/mL)	Precision RSD (%)	Accuracy RE (%)
Ciprofol	5	5.32±0.84	15.79	6.4	4.94±0.39	7.82	−1.23
	12.5	12.99±1.22	9.38	3.95	12.78±0.23	1.81	2.21
	375	390.93±14.95	3.83	4.25	392.56±14.03	3.57	2.68
	1875	1898.02±73.03	3.85	1.23	1926.67±35.21	1.83	2.76
	3750	3840.53±117.06	3.05	2.41	3830.48±41.85	1.09	2.15

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Table 2 Intra-Day and Inter-Day Precision and Accuracy of Ciprofol and Ciprofol-d_6 in Human Plasma (n = 5)

Analyte	Nominal Concentration (ng/mL)	Extraction Recovery		Matrix Effect	
		Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Ciprofol	12.5	101.1 ± 15.68	15.51	103.12 ± 6.82	6.61
	375	97.03 ± 7.44	7.67	106.57 ± 4.62	4.34
	1875	97.90 ± 4.92	5.03	101.28 ± 6.27	6.19
	3750	98.91 ± 3.48	3.52	98.1 ± 6.53	6.66
Ciprofol-d_6	12.5	104.25 ± 6.51	6.24	101.15 ± 2.63	4.32
	375	107.91 ± 5.21	4.83	102.18 ± 5.21	5.01
	1875	108.1 ± 2.89	2.67	105.25 ± 6.52	6.19
	3750	107.83 ± 4.97	4.61	102.59 ± 5.63	5.49

Sample Stability

The stability of QC samples at high, medium, and low concentration levels under different conditions, ie, freeze-thaw stability (5-freeze thaw cycles stability), short-term stability (6 hours), long-term stability (30 days), and post-preparative stability, is expressed as RE (%) (Table 3). The results show that the RE (%) for ciprofol was between −0.03% and 8.22% and −0.91% and −12.91%, which meet the requirements for quantitative analysis of biological samples. The experimental results show that clinical samples can be subjected to no more than three freeze-thaw cycles, placed at room temperature (25°C) for 12 hours, stored at −20°C for 4 weeks, and placed in the autosampler (4°C) for 24 hours after pre-treatment without affecting the accuracy of the determination results.

Dilution Effect

To meet the requirements for clinical sample determination, the linear range established in this experiment differs by 1000 times, ie, the linear range for ciprofol is 5 to 5000 ng·mL^{-1}. This linear range can meet the determination of most clinical samples, but there are still a few samples whose determination results are higher than the highest quantification concentration. Therefore, clinical samples should be diluted with blank plasma by a certain multiple, and then processed according to the method in "2.2" before analysis. Six parallel standard addition samples containing 20000 ng·mL^{-1} of ciprofol were prepared and diluted with blank plasma to 2 and 5 times, respectively. The diluted standard addition samples were then processed according to the method in "2.2" and injected for analysis. The accuracy of the diluted standard addition samples, ciprofol, was between 85% and 115% (Table 4), which meets the requirements. The experimental results show that the dilution of clinical samples with blank plasma does not affect the accuracy of the determination results of ciprofol.

Table 3 Stability of Analytes in Human Plasma (n=5)

Condition	Nominal Concentration (ng/mL)	Mean ± SD	RSD (%)	RE (%)
Room Temperature, 12 h	12.5	12.59±0.77	6.08	0.75
	3750	3702.9±124.5	3.36	−1.26
Autosampler 4 °C, 12 h	12.5	12.58±2.12	16.88	0.64
	3750	3374.82±184.64	5.47	−10
5 Freeze Thaw Cycles	12.5	13.61±1.18	8.68	8.89
	3750	3593.82±132.93	3.7	−4.16
−20°C, 4 Weeks	12.5	13.32±1.37	10.29	6.56
	3750	3643.25±152.16	4.18	−2.85

Table 4 Stability of Analytes in Human Plasma

Analyte	Nominal Concentration (ng/mL)	Measured Concentration (ng/mL)	Precision RSD (%)	Accuracy RE (%)
Ciprofol	6000	6115.65±208.99	3.42	1.93
	10000	9630.22±195.6	2.03	−3.7

Hemolysis Effect

A 2% hemolyzed sample from the same batch was selected and analyzed at low and high concentration levels, with five parallels for each concentration, to investigate accuracy and precision. The results show that for hemolyzed samples with a hemolysis degree of less than 2%, the presence of the hemolysis effect does not affect the accuracy of the determination results of ciprofol (Supplementary Table 3).

Pharmacokinetic

According to the method in "1.3", plasma from six patients undergoing elective surgery under general anesthesia was collected for monitoring. The patients were ASA class I–II, with an average age of 48.36 ± 22.16 years and a BMI of 24.25 ± 2.48 $kg \cdot m^{-2}$. After pre-processing according to the method in "2.5" and injection analysis, the average plasma concentration–time curve of ciprofol at each time point is shown in Figure 3. The plasma concentration data were processed with WinNonlin software to obtain the pharmacokinetic parameters listed in Table 5.

Discussion

Optimization of UHPLC-MS/MS Conditions

Buffer substances are often added in mass spectrometry analysis, which can not only adjust the pH of the solution but also improve the peak shape of the analyte. For example, adding formic acid, ammonium acetate, and ammonia water to the mobile phase can significantly improve the chromatographic retention time and peak shape of the analyte, and enhance the ionization efficiency to increase the response value of the analyte.[19] Studies have shown that the most commonly used mobile phase for measuciprofol concentration by UHPLC-MS/MS is formic acid water/methanol.[12,20] In this study, by comparing the extraction recovery rate and matrix effect of methanol: plasma (3:1, 4:1) and acetonitrile: plasma (2:1, 3:1), methanol: plasma (3:1) was selected for sample pretreatment. Considering that a high proportion of water in the sample for injection analysis has a better ionization effect, the supernatant after protein precipitation was diluted. By comparing the extraction

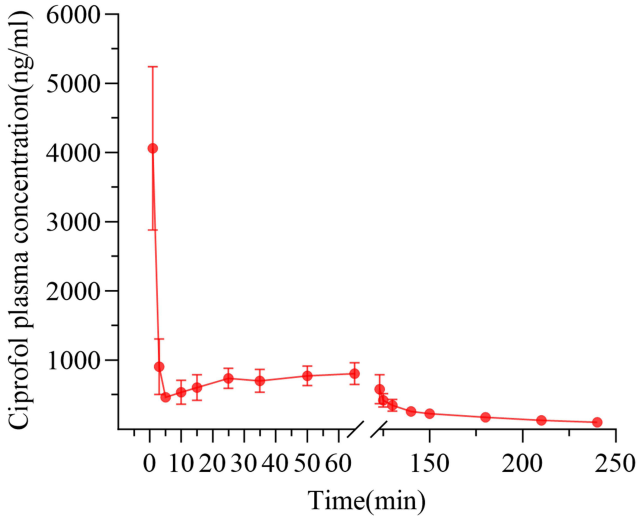

Figure 3 Plasma concentration versus time curves of ciprofol for eight patients.

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Table 5 Pharmacokinetic Parameters of Different Administration Methods

Parameters	Induction (Ciprofol 0.4mg/kg)	Maintenance (Ciprofol 0.8mg/kg/h)
$AUC_{0\rightarrow t}$ (h·ng/mL)	219.39 ± 54.03	1164.34 ± 121.12
$AUC_{0\rightarrow\infty}$ (h·ng/mL)	226.84 ± 54.81	1374.62 ± 147.46
$T_{1/2}$ (h)	0.22±0.003	1.29 ± 0.32
C_{max} (ng/mL)	4143.63 ± 1191.3	895.57 ± 109.55
T_{max} (h)	0.02±0.00	0.67± 0.26
CL (L/h)	121.49 ± 30.01	43.13 ± 6.13
V_{ss} (L)	3.12 ± 1.14	44.46 ± 11.42
$MRT_{0\rightarrow t}$ (h)	0.019±0.002	0.49 ± 0.06
$MRT_{0\rightarrow\infty}$ (h)	0.023±0.005	1.07 ± 0.30

recovery rate and matrix effect of different dilution solvents, mobile phases, 20% methanol, 50% methanol, 70% methanol, and water, 50% methanol was chosen as the dilution solvent. Furthermore, the dilution ratio of the supernatant with 50% methanol was compared, including 1:1, 1:2, 1:3, 1:4, and 1:5. The results showed that after protein precipitation with methanol: plasma (3:1), the supernatant diluted 5 times with 50% methanol-water resulted in good peak shapes, appropriate retention times, and signal-to-noise ratios for both ciprofol and the internal standard. In this study, the retention time of ciprofol was 2.461 min, and the detection could be completed within 2.455 min.

In addition, considering that ciprofol is a weakly acidic compound, this study investigated the addition of different concentrations of formic acid (0.05%, 0.1%, 0.2%, 0.5%), glacial acetic acid (0.1%), and ammonium acetate (5 mM, 10 mM) to the aqueous phase. Therefore, ammonia water was added to the aqueous phase to provide a weakly alkaline environment to enhance the response. After further comparison and optimization, it was found that adding 5 mM ammonium acetate to the aqueous phase could provide sufficient ammonium ions, improve the tailing of the chromatographic peak, and increase the response by about 50%, while also avoiding the impact on the column life due to the addition of excessive alkali. The chromatographic columns Agilent Poroshell 120 SB-C18 (2.1 mm×75 mm, 2.7 μm), Shimadzu, Shim-pack GIST-HP C18, 3μm, 2.1×150 mm (HSS), and Inertsil ODS-3 (2.1 mm×50 mm, 5 μm) were examined. The results showed that the Shimadzu, Shim-pack GIST-HP C18, 3μm, 2.1×150 mm (HSS) had higher column efficiency and better peak shape, response value, and reproducibility of the analytes.

Optimization of Desolvation Voltage and Collision Energy

The desolvation voltage is a certain voltage applied at the capillary outlet, aimed at allowing the maximum amount of the analyte ions to pass through the nitrogen barrier. Different ions correspond to different desolvation voltages. If the desolvation voltage is too small, too few target ions will pass through, while if it is too large, the target ions are prone to fragmentation, indirectly leading to a reduced throughput. The optimal collision energy is the energy at which the selected parent ion can produce the most offspring ions when the parent and offspring ions are determined. In this study, the desolvation voltage and collision energy for the ion pairs formed by the two substances were optimized. The final desolvation voltage and collision energy for ciprofol and its internal standard were determined to be 110 V, 14V and 86 V, 13 V, respectively.

Optimization of Ion Source Parameters

Since the structure of ciprofol and the internal standard contains phenolic hydroxyl groups, which are prone to losing hydrogen ions and carrying negative charges, ESI was selected in the negative ion mode. Higher signal intensity tests were conducted for ciprofol and the internal standard. The mass spectrometry parameters for each analyte were optimized, and the nebulizer pressure, drying gas flow rate and temperature, and capillary voltage were finally determined to be 30 psi, 10 $L \cdot min^{-1}$, 350°C, and 4500 V, respectively, to further enhance the sensitivity of the detection.

Selection of Internal Standard Compounds

Compared with the use of thymol or carbamazepine as the internal standard for measuciprofol in previous studies, this experiment selected a deuterated reagent with a chemical structure more similar to the analyte as the internal standard compound, which has essentially the same physicochemical properties, chromatographic behavior, and response characteristics as ciprofol.[21] Therefore, it is more conducive to accurate quantification. In addition, ciprofol-d6 is 6 mass units higher than ciprofol, which can effectively avoid isotope interference.[22]

Optimization of Hemolysis Effect

Hemolysis often occurs during sample collection and processing. Hemolysis can lead to the release of hemoglobin, enzymes, lipids, and sugars into the plasma, and these substances can cause matrix effects and stability issues for compounds. Therefore, we examined the addition of 2% whole blood cell lysate to the plasma to evaluate whether hemolyzed samples had stability and matrix effect issues. The results showed that even with the addition of 2% whole blood cell lysate, the stability and matrix effect of the compounds were negligible.

Pharmacokinetic Study

This trial involved the injection of ciprofol in 8 patients scheduled for general anesthesia. Plasma samples were monitored for drug concentration at the following time points: before administration (0 minutes); 1, 3, and 5 minutes after bolus injection; 5, 10, 20, 30, 45, and 60 minutes after continuous infusion of ciprofol; and 3, 5, 10, 20, 30, 60, 90, and 120 minutes after cessation of ciprofol infusion. The results showed that the maximum plasma concentration was reached around 0.02 hours after induction administration of ciprofol, followed by slow and stable metabolism and elimination, which was basically consistent with the report by Li et al.[3] The pharmacokinetic parameters after constant-rate infusion of ciprofol showed: T_{max} (0.67 ± 0.26) h, C_{max} (895.57 ± 109.55) ng·mL^{-1}, and CL (43.13 ± 6.13) L·h^{-1}. Hu et al[5] investigated the pharmacokinetic characteristics of initial infusion or bolus dose and subsequent continuous maintenance infusion of ciprofol in healthy subjects, and found that C_{max} was 550.7 (13.4) ng·mL^{-1} and T_{max} was 0.92 h (0.50, 12.00). Therefore, we speculate that the reasons for the pharmacokinetic differences of ciprofol among patients under general anesthesia may be related to the population, dosage, and method of administration.

Limitation

Although this study established a reliable UHPLC-MS/MS method and revealed the pharmacokinetic profile of ciprofol, the following limitations need to be addressed: first, the study was limited to a single-center and small-sample cohort, which may affect the generalizability of the results to a broader population; second, the effects of individualized factors, such as age and differences in hepatic and renal function, on the metabolism of ciprofol were not adequately included; and lastly, the study lacked data on metabolism under long-term dosing metabolism data in a long-term dosing scenario. Future studies should further validate the clinical monitoring efficacy of the method through multicenter and prospective studies and develop individualized dose prediction models by combining real-time monitoring data with artificial intelligence technology.

Conclusion

This study established a rapid, simple, accurate, reliable, specific, and highly sensitive UHPLC-MS/MS method, which was successfully applied to the detection of ciprofol concentration in human plasma and the determination of ciprofol in plasma samples from patients undergoing general anesthesia surgery, and reported the corresponding pharmacokinetic parameters. Our current method demonstrates excellent performance, such as a small amount of plasma required (5 μL), a short runtime for each sample (2.5 minutes), and a highly sensitive LLOQ (5 ng·mL^{-1}). It provides a convenient, fast, accurate, and sensitive detection method for the pharmacokinetic study of ciprofol during the perioperative period and for monitoring drug concentration in clinical surgery, which is conducive to safe and rational clinical drug use and reducing or alleviating adverse reactions.

Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the General Hospital of the Southern Theater Command (NZLLKZ2024150) and registered with the Chinese Clinical Trial Registration Center (ChiCTR2400094359).

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare that they have no conflicts of interest.

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