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ORIGINAL RESEARCH

Simultaneous Determination of Fexinidazole and Its Major Metabolites in Rat Plasma by UPLC-MS/MS: Method Validation and Application to Pharmacokinetic Study

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Introduction: Fexinidazole is an oral antitrypanosomal drug approved for the treatment of human African trypanosomiasis (HAT). Its therapeutic efficacy primarily depends on the in vivo formation of its active metabolites, sulfoxide fexinidazole (M1) and sulfone fexinidazole (M2). Accurate and sensitive quantification of fexinidazole and its major metabolites is essential for pharmacokinetic evaluation and preclinical research. Therefore, a reliable bioanalytical method for their simultaneous determination in plasma is required.

Methods: An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC–MS/MS) method was developed and validated for the simultaneous determination of fexinidazole, M1, and M2 in rat plasma, using fluconazole as the internal standard (IS). Chromatographic separation was achieved on an Acquity UPLC BEH C18 column with gradient elution consisting of 0.1% formic acid in water and acetonitrile. The method was validated in accordance with bioanalytical guidelines, including assessments of selectivity, linearity, lower limit of quantification (LLOQ), precision, accuracy, recovery, matrix effects, and stability. The validated method was subsequently applied to a pharmacokinetic study in rats.

Results: The method demonstrated good linearity within the investigated concentration ranges for all analytes. The LLOQs were 1 ng/mL for fexinidazole, 10 ng/mL for M1, and 50 ng/mL for M2. Intra-day and inter-day precision (RSD%) and accuracy (RE%) were within ±15%. Recovery was consistent and reproducible, matrix effects were acceptable, and stability under various conditions met bioanalytical requirements. The method was successfully applied to the pharmacokinetic evaluation of fexinidazole and its metabolites in rats.

Discussion: The developed UPLC–MS/MS method exhibited satisfactory sensitivity, accuracy, and reproducibility for the quantification of fexinidazole and its active metabolites in rat plasma. This method provides reliable analytical support for pharmacokinetic studies and contributes to the preclinical investigation of fexinidazole.

Keywords: fexinidazole, pharmacokinetics, UPLC–MS/MS, rat

Introduction

Neglected tropical protozoan infections continue to pose a significant global health burden, particularly in resource-limited regions.[1,2] Human African trypanosomiasis (HAT), caused by Trypanosoma brucei, can be fatal if untreated.[3] The disease progresses from haemo-lymphatic stage to meningo-encephalitic stage, characterized by neuroinflammation, cognitive deficits, and sleep disorders.[4,5] Conventional therapies (such as melarsoprol and nifurtimox-eflornithine combination regimens) require parenteral administration, prolonged hospitalization, and involve complex treatment

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protocols.[6,7] Thus, the development of safe, effective and orally available antitrypanosomal agents has long been a critical clinical objective.

Fexinidazole is an orally administered 5-nitroimidazole prodrug that represents a major advance in the pharmacotherapy of HAT.[8] Following administration, fexinidazole undergoes metabolic activation to two principal metabolites, fexinidazole sulfoxide (M1) and fexinidazole sulfone (M2), which are primarily responsible for systemic pharmacodynamic activity.[8] Its antiparasitic mechanism involves enzymatic reduction by trypanosome-specific type I nitroreductase, producing active intermediates that inhibit DNA synthesis.[9] Unlike prior regimens that require intravenous infusion or combination therapy, fexinidazole can be administered as a fully oral regimen and is suitable for both first-stage and second-stage HAT.[10,11] The drug was developed through a collaboration between the Drugs for Neglected Diseases initiative (DNDi) and Sanofi, and it was approved for marketing by the European Medicines Agency in 2018, establishing its status as the first all-oral treatment for HAT.[12,13]

Although fexinidazole has been introduced into clinical use, its detailed pharmacokinetic profile, particularly across different animal models, remains to be fully elucidated. A Phase II/III clinical study demonstrated that a 10-day oral regimen of fexinidazole for patients with HAT was non-inferior to nifurtimox-eflornithine combination therapy in terms of long-term cure rates, while also reducing hospitalization requirements and logistical burdens on healthcare systems.[10] Another study employing population pharmacokinetic modeling has investigated the exposure of fexinidazole and its primary metabolite in healthy volunteers, providing a basis for dosing regimens.[14] Pharmacokinetic studies have also been conducted in mouse models of acute and chronic HAT to evaluate the efficacy of fexinidazole at different stages of the disease.[15] However, to our knowledge, a systematic characterization of its pharmacokinetic properties in commonly used animal models, such as rats, has not yet been completed. Pharmacokinetic studies in different models not only reveal the impact of interspecies differences on drug efficacy and toxicity, but also provide a basis for predicting exposure levels and potential safety in humans. To address these limitations, a highly sensitive and stable bioanalytical method capable of accurately quantifying fexinidazole and its major metabolites is required. Nevertheless, to date, no bioanalytical method utilizing ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) for determining fexinidazole concentrations in rat plasma has been reported.

In this study, an optimized UPLC-MS/MS method was established and fully validated for determining the concentrations of fexinidazole, M1 and M2 in rat plasma. This method was subsequently applied to evaluate the pharmacokinetic characteristics of fexinidazole (20 mg/kg) following oral administration in rats. These results provide crucial analytical support for future preclinical studies, optimization of dosing strategies, and investigations into the mechanism of action of fexinidazole.

Materials and Methods

Drugs and Reagents

Chemical structures and mass spectra of fexinidazole, M1 and M2 were displayed in Figure 1, and they were all purchased from Shanghai Canspec Scientific Instruments Co., Ltd. (Shanghai, China), and fluconazole was supplied as the internal standard (IS) from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). All reagents used in this study were of analytical grade or higher. Methanol and acetonitrile were HPLC grade and purchased from Merck (Darmstadt, Germany). Formic acid was obtained from Anaqua Chemicals Supply (ACS, USA). Ultrapure water was prepared through the Milli-Q Water purification system manufactured by Millipore (Bedford, USA).

Analytical Conditions of the Instrumentation

Chromatographic analysis was performed using a Waters Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm) maintained at 40°C, with a mobile phase comprising 0.1% aqueous formic acid (A) and acetonitrile (B), which was delivered at a flow rate of 0.40 mL/min. The gradient program was optimized as follows: 90% A (0–0.5 min), 90% to 10% A (0.5–1.0 min), 10% A (1.0–1.4 min), 10% to 90% A (1.4–1.5 min), and 90% A (1.5–2.0 min). Mass spectrometric detection was carried out on a Waters Xevo TQ-S triple quadrupole mass spectrometer (Milford, MA, USA), operated in positive electrospray ionization mode with multiple reaction monitoring (MRM). The monitored transitions were as

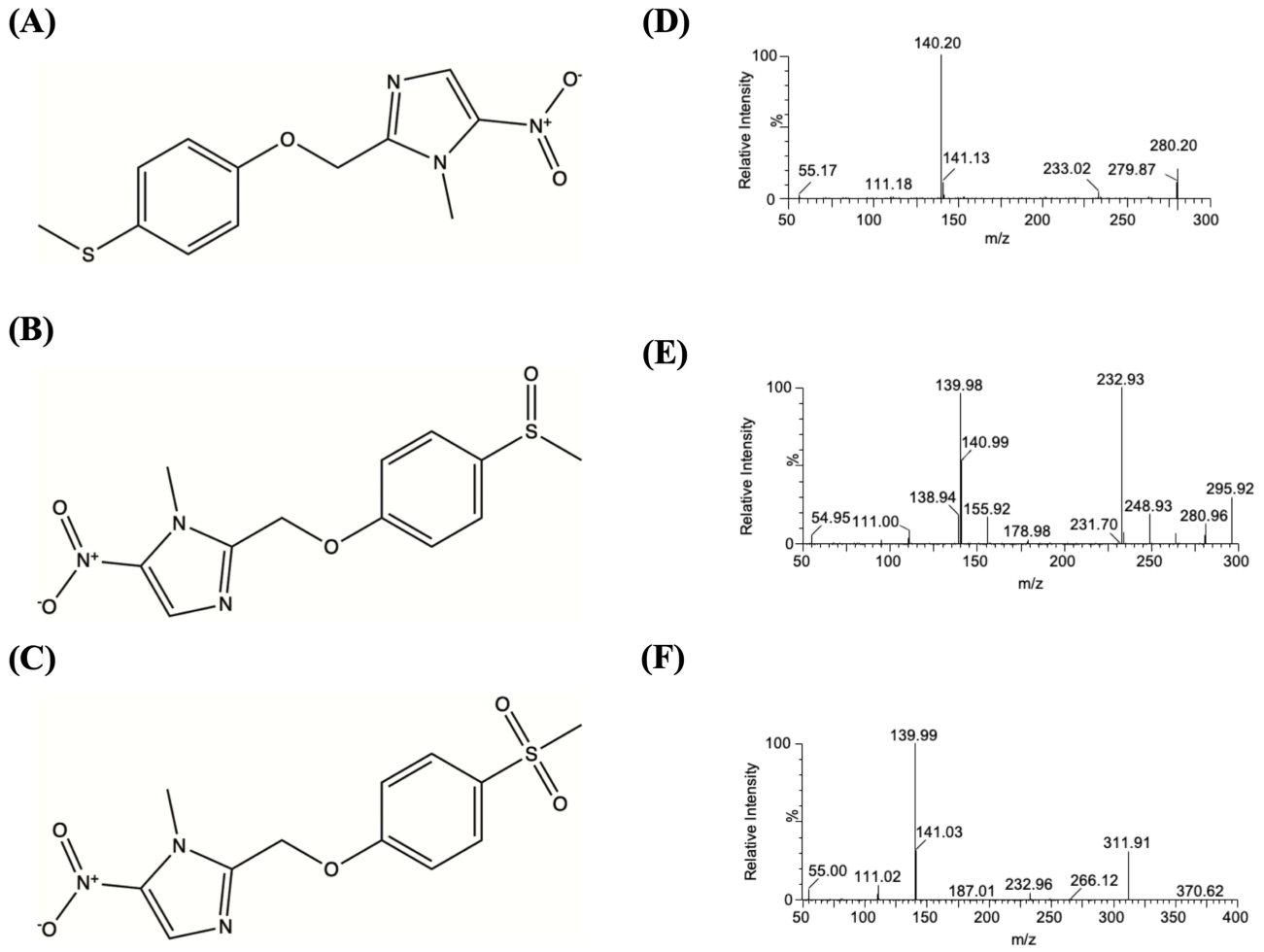

Figure 1 Chemical structures and mass spectra of fexinidazole and its metabolites. (**A**–**C**) Chemical structures of fexinidazole, metabolite M1, and metabolite M2, respectively. (**D**–**F**) Corresponding mass spectra of the above compounds.

follows: *m/z* 280.20→140.20 for fexinidazole (collision voltage 10 eV, cone voltage 25 V), *m/z* 295.92→232.93 for M1 (collision voltage 10 eV, cone voltage 20 V), *m/z* 311.91→139.99 for M2 (collision voltage 20 eV, cone voltage 15 V), and *m/z* 307.13→220.11 for IS (collision voltage 30 eV, cone voltage 20 V), respectively. The injection volume was 0.1 μL with a total run time of 2.0 min, and data acquisition was controlled by MassLynx 4.1 software (Milford, MA, USA).

Preparation of Calibration Standards and Quality Control (QC) Samples

Primary stock solutions of fexinidazole, M1 and M2 (1.00 mg/mL) were prepared in methanol from accurately weighed amounts, followed by serial dilution with methanol to produce standard working solutions. The calibration standards were prepared by spiking 10 μL of each working solution into 70 μL of blank rat plasma. The final concentration ranges of the calibration standards were 1–500 ng/mL (1, 3, 5, 10, 50, 100, 400, 500 ng/mL) for fexinidazole, 10–20,000 ng/mL (10, 50, 500, 1000, 2000, 8000, 16,000, 20,000 ng/mL) for M1, and 50–18,000 ng/mL (50, 100, 500, 1000, 3000, 6000, 12,000, 18,000 ng/mL) for M2, respectively. In the same manner, QC samples were prepared at final concentrations of 400 ng/mL (high quality control, HQC), 200 ng/mL (medium quality control, MQC), 3 ng/mL (low quality control, LQC), and 1 ng/mL (lower limit of quantification, LLOQ) for fexinidazole. The corresponding concentrations for M1 were set at 16,000 ng/mL, 8000 ng/mL, 30 ng/mL, and 10 ng/mL, respectively, while the corresponding concentrations for M2 were 14,400 ng/mL, 7200 ng/mL, 100 ng/mL, and 50 ng/mL. All materials were stored at −80°C for subsequent use.

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Preparation of Samples

The samples were prepared by protein precipitation. 10 μL of IS working solution (200 ng/mL) was added to 100 μL of plasma sample, followed by the addition of 300 μL of acetonitrile. After vortexing for 2.0 min, the tubes were centrifuged at 13,000 rpm for 10 min. Finally, 100 μL of the supernatant was aspirated and analyzed by UPLC-MS /MS system.

Method Validation

A comprehensive validation of the developed bioanalytical method was conducted in accordance with the FDA guidelines. Key parameters assessed included selectivity, linearity, LLOQ, precision, accuracy, extraction recovery, matrix effect, and stability. The selectivity of the method was rigorously investigated by analyzing blank matrix samples (without analytes or IS) and experimental samples. Results confirmed that no endogenous interference was detected within the retention time ranges of fexinidazole, M1, M2, and IS. Calibration curves for fexinidazole (1–500 ng/mL), M1 (10–20,000 ng/mL), and M2 (50–18,000 ng/mL) were established using weighted (1/x2) least squares regression analysis, where the nominal concentration was plotted on the x-axis and the peak area ratio of the analyte to the IS was plotted on the y-axis. The LLOQ was defined as the lowest concentration on the calibration curve and was required to demonstrate both precision and accuracy within ±20%. Precision was evaluated to assess random error, reflecting the repeatability and reproducibility of the results, while accuracy was evaluated to reveal systematic error, representing the deviation between the measured mean and the true value. Intra-day and inter-day precision and accuracy were determined by analyzing QC samples at four concentration levels over three consecutive days, with at least five replicates per concentration. Precision and accuracy were expressed as relative standard deviation (RSD) and relative error (RE), respectively. Typically, at the LLOQ level, both RSD and RE were required not to exceed 20%, while at low-medium-high QC samples, RSD and RE were controlled within 15%. Furthermore, extraction recovery and matrix effect were evaluated at three QC concentration levels to ensure that ionization suppression or enhancement effects, as well as sample preparation efficiency, were within acceptable limits. The recovery (%) was calculated as (concentration of fexinidazole, M1 or M2 spiked before plasma extraction)/ (concentration of fexinidazole, M1 or M2 spiked after plasma extraction) × 100%. The matrix effect (%) was calculated as (concentration of fexinidazole, M1 or M2 spiked after plasma extraction)/ (concentration of fexinidazole, M1 or M2 in methanol solvent) × 100%. Finally, the stability of fexinidazole, M1, and M2 in plasma was comprehensively demonstrated, including short-term stability at room temperature, long-term stability at −80°C, stability after three freeze-thaw cycles, and sample stability in the autosampler at 10°C.

Pharmacokinetic Study

Six male Sprague–Dawley rats, weighing between 200 and 220 g, were provided by the Experimental Animal Center of the First Affiliated Hospital of Wenzhou Medical University (Zhejiang, China). The animals were housed under controlled environmental conditions, including a 12 h light/dark cycle, regulated temperature, and relative humidity maintained at 45–65%, with free access to standard laboratory feed and water. All procedures were conducted in compliance with the guidelines approved by the Ethical Council of the institution (WYYY-IACUC-AEC-2025-082), the national standard GBT 42011–2022 on the welfare of laboratory animals, as well as in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 80–23, Revised 1996) and the International Association for the Study of Pain Ethical Guidelines for Studies Involving Conscious Animals.

In the pharmacokinetic study, fexinidazole was dissolved in a 0.5% sodium carboxymethyl cellulose (CMC-Na) solution for oral administration. After a 12 h fasting period during which water remained freely accessible, a single oral dose of fexinidazole (20 mg/kg) was administered to the rats (n = 6). Blood samples were collected from the tail vein at predetermined time intervals: 0, 0.33, 1, 2, 3, 4, 5, 6, 8, 12 and 24 h post-dosing. The collected blood was subsequently centrifuged, and the supernatant was stored at −80°C until further processing and analysis.

Statistical Analysis

The mean plasma concentration-time curves were generated using GraphPad Prism 9.5 software. The pharmacokinetic parameters of fexinidazole, M1 and M2 including the area under the concentration-time curve (AUC), half-life ($t_{1/2}$), peak time (T_{max}), clearance (CL_z/F) and peak plasma concentration (C_{max}), were derived through non-compartmental analysis performed with DAS 2.0 software (Chinese Committee of Mathematical Pharmacology, Shanghai, China).

Results

Method Validation

Selectivity

Representative chromatograms of blank and analyte-spiked samples were presented in Figure 2. Good selectivity of the method was confirmed under the established conditions by the absence of significant interfering peaks at the respective retention times of 1.43 min for fexinidazole, 1.23 min for M1, 1.28 min for M2 and 1.19 min for IS, respectively.

Linearity and LLOQ

A linear calibration range from 1 to 500 ng/mL was established for fexinidazole, characterized by the regression equation Y = 0.00414922X + 0.000865997 (r^2 = 0.996). The calibration for M1 was linear over the range of 10–20,000 ng/mL, with a regression of Y = 0.00311631X - 0.0206928 (r^2 = 0.999). The calibration for M2 was linear over the range of 50–18,000 ng/mL, with a regression of Y = 0.00844807X - 0.0279086 (r^2 = 0.998). The LLOQ of the three analytes were determined to be 1 ng/mL, 10 ng/mL and 50 ng/mL, respectively.

Precision and Accuracy

Through three consecutive days of analysis of QC samples, the precision and accuracy of the method were comprehensively evaluated. As shown in Table 1, the intra-day RSD% ranges for fexinidazole, M1 and M2 were 7.8%-12.6%, 5.3%-6.8%, and 2.4%-5.6%, respectively, while the inter-day RSD% ranges were 7.0–13.7%, 5.3–7.2% and 2.1%-6.8%, respectively. In terms of accuracy, the RE% for all analytes was observed to range from −5.4% to 10.3%. These results clearly demonstrated that the established UPLC-MS/MS method exhibited excellent precision and reliable accuracy, fully meeting the requirements for the analysis of biological samples.

Extraction Recovery and Matrix Effect

The extraction recovery and matrix effects of the method were quantitatively evaluated by comparing the concentrations of the analytes in spiked samples before and after plasma processing, as well as in pure solution (Table 2). The extraction recoveries of fexinidazole, M1 and M2 were consistently within the ranges of 94.4–96.7%, 90.9–98.6% and 98.5%-102.2%, respectively, with RSD% below 13%. This indicated that the sample pretreatment process employed in this method was highly efficient and reproducible. Regarding matrix effects, the ranges of fexinidazole, M1 and M2 were 97.3–102.4%, 99.3–103.7% and 99.6%-110.6%, respectively. This indicated that endogenous substances in rat plasma did not significantly inhibit or enhance the ionization processes of fexinidazole, M1 and M2. In summary, this method exhibited stable and controllable recovery rates and was not affected by significant matrix interference.

Stability

To ensure the reliability of this analytical approach, the stability of fexinidazole, M1 and M2 in rat plasma was investigated under various conditions. Results (Table 3) confirmed that the RSD% and RE% of all QC samples were within ±12% after being placed at room temperature for 3 h, subjected to three complete freeze-thaw cycles, stored long-term at −80°C for 21 days, and kept in the autosampler (10°C) for 4 h. These results of the stability clearly indicated that fexinidazole, M1, and M2 remained stable under various storage and analytical conditions, thereby effectively ensuring the accuracy of quantitative analysis.

Animal Study

Based on the established analytical method, the pharmacokinetic profiles of fexinidazole, M1 and M2 were systematically characterized in rats. As shown in Figure 3 and Table 4, the parent drug fexinidazole was rapidly absorbed,

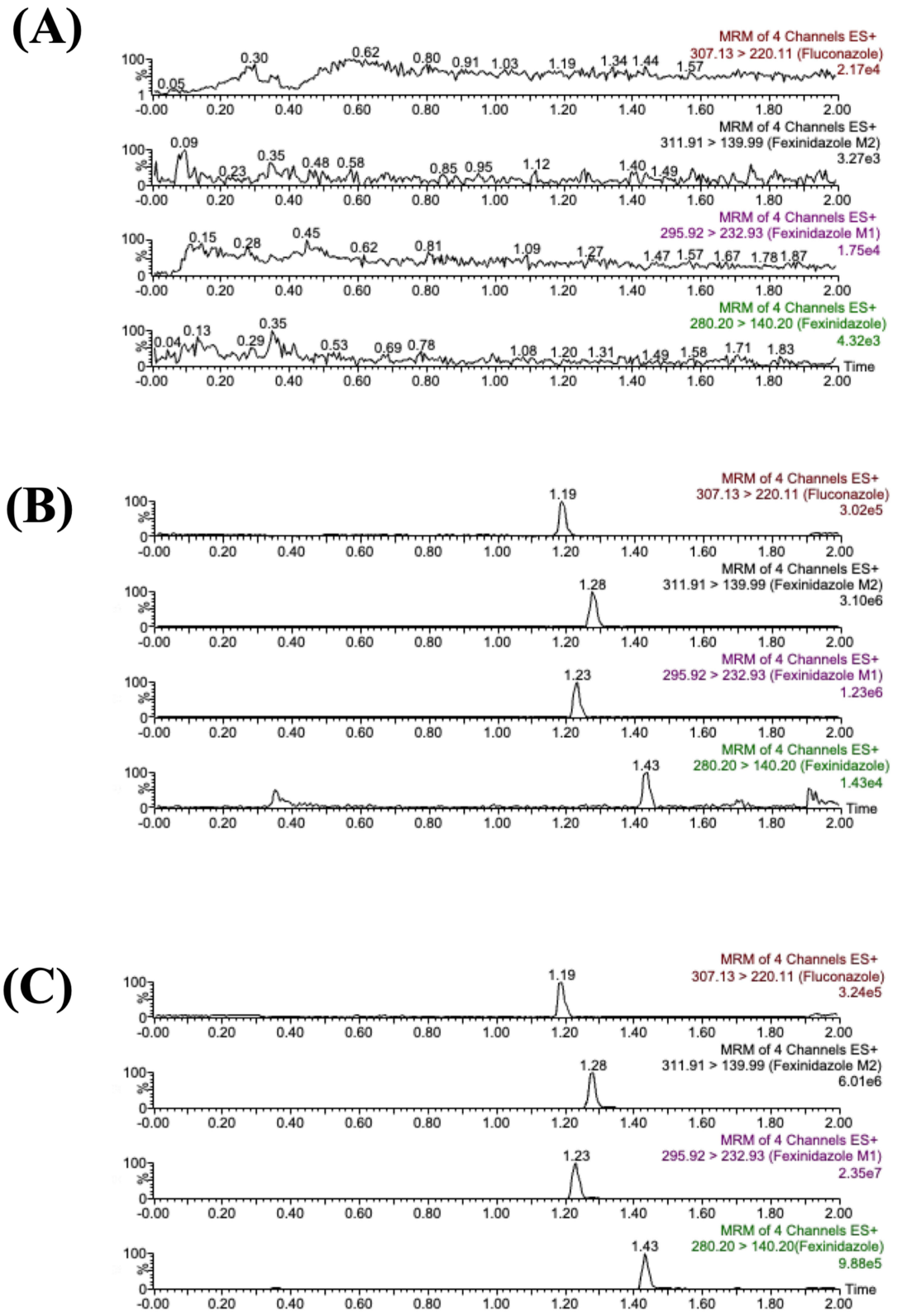

Figure 2 Representative chromatograms of fexinidazole, M1, M2 and fluconazole (IS) in rat plasma: blank plasma (**A**), blank plasma with analytes at LLOQ and IS (**B**), and plasma sample from a rat after oral administration of 20 mg/kg fexinidazole (**C**).

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Table 1 Precision and Accuracy of Fexinidazole and Its Metabolite M1 and M2 in Rat Plasma (n = 5)

Analytes	Concentration (ng/mL)	Intra-Day		Inter-Day	
		RSD%	RE%	RSD%	RE%
Fexinidazole	1	12.6	9.8	13.7	2.0
	3	10.2	−5.4	9.6	−3.9
	200	8.8	10.3	8.8	5.6
	400	7.8	7.3	7.0	4.2
M1	10	6.8	7.1	5.6	8.2
	30	6.0	−5.1	5.3	−4.0
	8000	5.3	8.2	7.2	4.8
	16,000	6.1	9.7	5.6	4.6
M2	50	2.4	−0.9	2.1	−1.1
	100	2.7	4.1	6.8	4.7
	7200	4.2	4.4	3.4	5.1
	14,400	5.6	5.4	4.8	1.4

Table 2 Recovery and Matrix Effect of Fexinidazole and Its Metabolite M1 and M2 in Rat Plasma (n = 5)

Analytes	Concentration (ng/mL)	Recovery (%)		Matrix Effect (%)	
		Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
Fexinidazole	3	94.7 ± 11.5	12.2	97.3 ± 6.2	6.4
	200	94.4 ± 5.8	6.2	97.3 ± 3.5	3.6
	400	96.7 ± 5.3	5.5	102.4 ± 10.0	9.7
M1	30	98.6 ± 8.9	9.1	99.3 ± 5.5	5.6
	80	90.9 ± 4.7	5.2	103.3 ± 2.3	2.2
	16,000	93.0 ± 3.4	3.7	103.7 ± 5.5	5.3
M2	100	98.5 ± 4.8	4.9	99.6 ± 3.7	3.7
	7200	102.2 ± 6.2	6.1	100.0 ± 2.9	2.9
	14,400	99.3 ± 4.1	4.2	110.6 ± 6.4	5.8

Table 3 Stability Results of Fexinidazole and Its Metabolite M1 and M2 in Plasma (n = 5)

Analytes	Concentration (ng/mL)	Room Temperature, 3 h		Autosampler 10°C, 4 h		Three Freeze-Thaw		−80°C, 3 Weeks	
		RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)	RSD (%)	RE (%)
Fexinidazole	3	10.4	−6.0	9.0	−1.6	7.7	6.2	9.5	3.9
	200	2.5	4.8	6.1	2.9	2.8	−0.7	3.0	8.0
	400	10.3	0.1	5.1	0.3	7.2	−0.2	3.1	11.9
M1	30	1.6	−1.5	3.0	−5.3	5.4	0.4	7.5	1.7
	8000	3.4	7.5	5.0	10.7	5.0	2.6	3.8	8.0
	16,000	8.6	0.4	7.4	7.7	7.5	1.1	3.0	1.8
M2	100	3.0	12.2	5.9	3.2	3.1	2.0	2.8	11.2
	7200	6.0	2.0	5.9	9.2	2.0	0.4	3.2	4.3
	14,400	7.0	3.4	7.5	11.5	4.6	2.5	2.5	4.1

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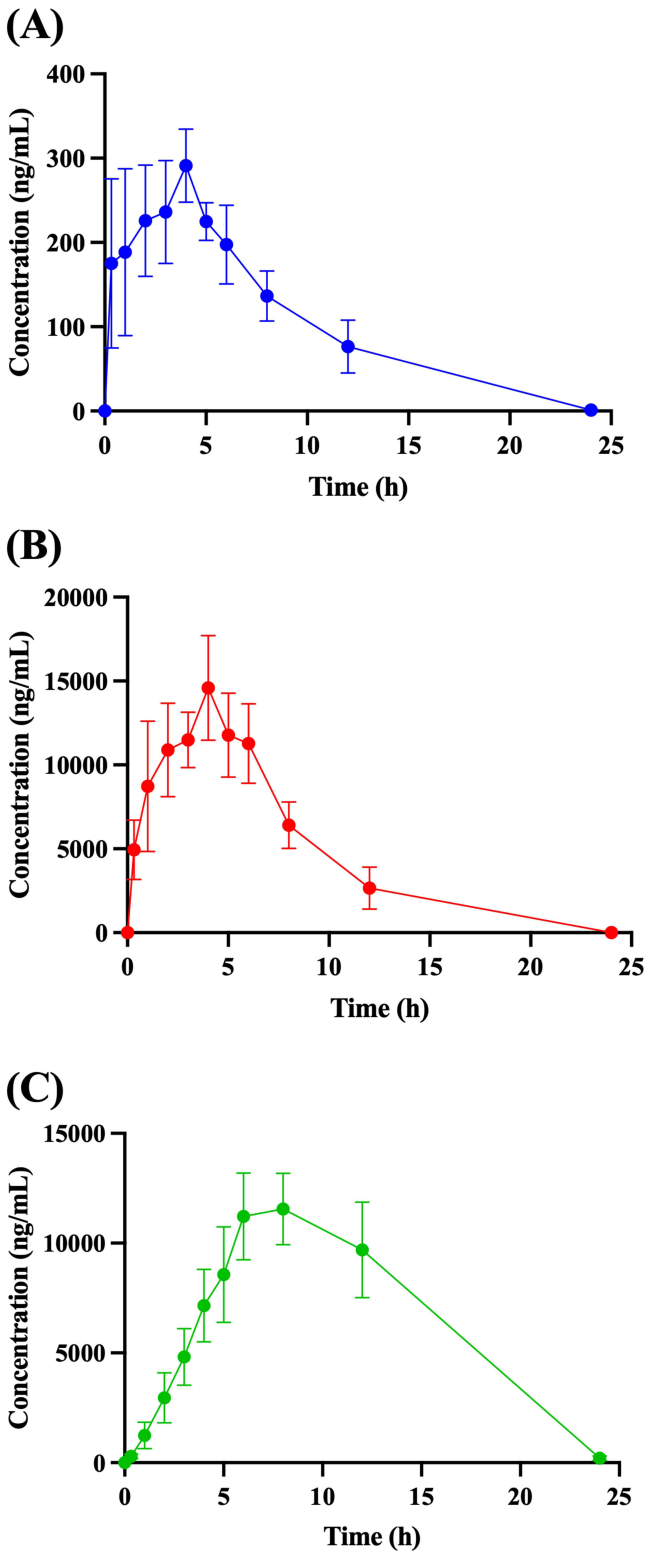

Figure 3 Mean plasma concentration-time curves of fexinidazole (**A**), M1 (**B**) and M2 (**C**) in rats. Data are presented as the means ± SD, n = 6.

achieving a C_{max} of 310.21 ± 35.56 ng/mL at approximately 2.38 h (T_{max}) post-administration. In contrast, both metabolites demonstrated significantly greater systemic exposure compared to the parent drug. Metabolite M1 reached its peak concentration at 4.50 h, which was later than that of fexinidazole. The $t_{1/2}$ of M1 was 2.20 ± 0.79 h, with a CL_Z/F

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Table 4 The Main Pharmacokinetic Parameters of Fexinidazole, M1 and M2 in Sprague-Dawley Rats (n = 6)

Parameters	Fexinidazole	M1	M2
$AUC_{(0-t)}$ (μg/L*h)	2549.11 ± 251.22	116,034.13 ± 16,680.01	154,953.67 ± 21,047.95
$AUC_{(0-\infty)}$ (μg/L*h)	2552.97 ± 252.33	116,349.55 ± 16,560.38	155,959.98 ± 21,240.63
$t_{1/2}$ (h)	2.38 ± 0.24	2.20 ± 0.79	3.07 ± 0.52
T_{max} (h)	3.83 ± 0.41	4.50 ± 0.84	8.00 ± 2.19
CL_z/F (L/h/kg)	7.90 ± 0.85	0.18 ± 0.03	0.13 ± 0.02
C_{max} (μg/ L)	310.21 ± 35.56	15,033.66 ± 2725.48	12,555.25 ± 1351.06

of 0.18 ± 0.03 L/h/kg. Similarly, metabolite M2 displayed the highest overall exposure among the three analytes, with an $AUC_{(0-\infty)}$ of 155,959.98 ± 21,240.63 μg/L*h. The C_{max} was 125,55.25 ± 1351.06 ng/mL, but T_{max} was markedly delayed, indicating a slower formation or absorption phase. Collectively, these pharmacokinetic parameters revealed that fexinidazole was rapidly absorbed and metabolized into M1 and M2. Both metabolites exhibited substantially higher plasma concentrations and greater systemic exposure than the parent drug, highlighting their extensive formation and potentially important roles in overall pharmacological activity.

Discussion

Fexinidazole, a 5-nitroimidazole derivative, has emerged as a pivotal therapeutic agent for the treatment of HAT, offering the first all-oral regimen effective against both the haemolymphatic and meningoencephalitic stages of the disease.[16,17] The pharmacological efficacy of this prodrug is intrinsically linked to its extensive in vivo bioactivation, wherein it is metabolized into two biologically active derivatives: M1 and M2.[18,19] Although the drug has been clinically approved and is currently in use, its pharmacokinetic profile in animal models remains insufficientlydocumented. Currently, few bioanalytical methods for the determination of fexinidazole and its metabolites have been reported. To date, only one method has been documented in the literature, primarily focusing on human plasma and dried blood spot analysis to support clinical trials.[20] However, this method was not directly applicable to rat plasma matrices. Therefore, the primary objective of this study was to develop and validate a stable, sensitive and high-throughput UPLC-MS/MS assay to simultaneously determine the concentrations of fexinidazole, M1 and M2 in rat plasma, thereby providing data support for clinical research.

Although liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for fexinidazole and its metabolites have been previously reported using human DBS samples, the present study established and validated a matrix-specific analytical method for rat plasma. DBS (whole blood) and plasma differ substantially in matrix composition, extraction characteristics, and ionization behavior, which may significantly influence analytical performance. Therefore, a dedicated validation in rat plasma is essential for accurate and reliable quantification in preclinical pharmacokinetic investigations. Furthermore, the previously reported DBS method was primarily developed for clinical exposure monitoring, whereas the current method was optimized to support systematic pharmacokinetic evaluation in rats. The analytical range and sensitivity were tailored to adequately characterize the complete concentration–profiles of fexinidazole and its major metabolites, enabling accurate estimation of pharmacokinetic parameters.

This study developed a bioanalytical method using UPLC-MS/MS for the quantitative determination of fexinidazole, M1 and M2. The method demonstrated several significant advantages over LC-MS/MS, particularly in terms of chromatographic efficiency and instrument throughput. Compared to the 5–10 min required by Filali-Ansary et al,[20] the 2-min run time substantially reduced time costs, making it highly suitable for processing large-scale experimental samples. The Waters Acquity BEH C18 column (2.1 mm × 50 mm, 1.7 μm) used in this study provided exceptional peak resolution and theoretical plate number. Higher theoretical plate numbers enabled superior baseline separation, while the sharp and high chromatographic peaks contributed to an improved signal-to-noise ratio, thereby facilitating increased detection sensitivity. We also evaluated various mobile phase combinations, including acetonitrile, methanol and 0.1% formic acid. The results showed that using acetonitrile and 0.1% formic acid achieved excellent separation and peak

shape for all components within a short time. In terms of sample processing, the protein precipitation method selected for this study not only reduced the consumption of organic solvents but also minimized matrix interference. In summary, these parameters demonstrated that the developed method exhibited advantages in terms of speed, sensitivity and selectivity, making it suitable for pharmacokinetic studies.

Then, this validation method was successfully applied to the pharmacokinetic study of fexinidazole following a single oral dose in Sprague-Dawley rats. Observations revealed that fexinidazole exhibited rapid absorption but underwent first-pass metabolism. In stark contrast, the systemic exposure of its metabolites was substantially higher. This finding was consistent with the established prodrug mechanism of fexinidazole, where its pharmacological activity was primarily driven by M1 and M2.[8] Notably, a sequential delay in T_{max} was observed, where M2 appeared significantly later than M1.[21] This phenomenon confirmed that the metabolic pathway of fexinidazole involved its oxidation to M1, which was subsequently converted to M2.[22] Overall, this metabolic pattern was consistent with human pharmacokinetic data. Although the qualitative metabolic pattern was comparable between species, notable quantitative differences were observed when comparing the present rat data with previously reported human pharmacokinetic results.[21] In rats, the terminal $t_{1/2}$ of fexinidazole, M1 and M2 were approximately 2–3 h, which were markedly shorter than those reported in humans (approximately 10–21 h). In addition, the metabolite-to-parent exposure ratios were substantially higher in rats, with M1 and M2 AUC values exceeding the parent drug by approximately 45-fold and 61-fold, respectively, whereas in humans the corresponding ratios were considerably lower. Furthermore, the T_{max} of M2 occurred earlier in rats (approximately 8 h) compared with humans (approximately 24 h). These findings suggested faster metabolic turnover and more rapid systemic elimination in rats. Such quantitative differences may reflect species-dependent variations in metabolic capacity, enzyme activity, formation–elimination balance of sequential metabolites, and oral disposition characteristics. Therefore, although the rat model reproduced the overall metabolic trend observed in humans, quantitative extrapolation across species should be interpreted with caution.

It should also be noted that this study has certain limitations. First, the pharmacokinetic data were derived from healthy Sprague-Dawley rats. Given the inherent physiological and metabolic differences between rats and humans, caution is warranted when directly extrapolating these findings to humans. Second, this study examined pharmacokinetic characteristics following a single dose only. The accumulation potential and metabolic stability of fexinidazole and its metabolites under multiple-dose regimens remain to be elucidated. Despite these limitations, the bioanalytical method established and the preliminary pharmacokinetic insights obtained in this study lay a solid foundation for future pharmacodynamic research and deeper exploration of species differences.

Conclusion

In summary, this study established a rapid and efficient UPLC-MS/MS method for the simultaneous determination of fexinidazole, M1 and M2 in rat plasma for the first time. The method offered significant advantages including short run time, wide dynamic range and negligible matrix effects. Furthermore, the method was successfully applied to pharmacokinetic studies, providing detailed insights into the pharmacokinetic characteristics of fexinidazole in Sprague-Dawley rats. This study delivered crucial reference data for subsequent clinical toxicology evaluations and optimization of dosing regimens.

Ethics Approval

The animal study has been supervised and approved by Institutional Animal Care and Use Committee of The First Affiliated Hospital of Wenzhou Medical University. (WYYY-IACUC-AEC-2025-083).

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Disclosure

The authors report no conflicts of interest in this work.

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