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Development of an HPLC-QAMS method for simultaneous quantification of two morinidazole impurities, three ornidazole impurities and one levornidazole impurity in morinidazole sodium chloride injection

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Abstract: This study developed and validated a High-Performance Liquid Chromatographic (HPLC) method for the determination of related substances in Morinidazole Sodium Chloride Injection. **Background**: As a novel nitroimidazole antimicrobial, the quality control of Morinidazole is crucial for ensuring clinical drug safety, particularly in monitoring specific impurities that may arise during production or storage. **Objectives**: To establish a method capable of simultaneously quantifying two Morinidazole impurities, three Ornidazole impurities, and one Levornidazole impurity (Levornidazole impurity II), and to evaluate its environmental friendliness. **Methods**: Separation was performed using a Waters Symmetry C18 column with a mobile phase consisting of 0.05 mol/L potassium dihydrogen phosphate buffer (pH 7.0) and acetonitrile in a gradient elution program. The flow rate was 1.0 mL/min, the column temperature was maintained at 30°C, the detection wavelength was set at 319 nm, and the injection volume was 20 µL. Impurity quantification was performed using the main component self-compare method (Quantitative Analysis of Multi-components by a Single Marker, QAMS), and the results were compared with those obtained by the external standard method. Method validation was conducted in accordance with ICH guidelines. **Results**: The method demonstrated good specificity. The limit of quantification (LOQ) and limit of detection (LOD) were 0.2 µg/mL and 0.06 µg/mL, respectively. Excellent linearity ($r \geqslant 0.995$) was achieved within the range of 0.2–4.0 µg/mL. The average recoveries ranged from 95% to 105% (RSD < 2.0%). Both the system suitability solution and the test sample solution remained stable for 30 hours. The Analytical Greenness (AGREE) and Blue Applicability Grade Index (BAGI) scores for the QAMS method were 0.66 and 80.0, respectively. No significant difference was found between the results obtained by the QAMS method and the external standard method. **Conclusion**: The established HPLC-QAMS method is accurate, sensitive, and environmentally friendly. It is suitable for the quality control of related impurities in Morinidazole Sodium Chloride Injection and provides technical support for the clinical safety evaluation of this preparation.

Keywords: HPLC; Morinidazole; Stability-indicating; Validation

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INTRODUCTION

Morinidazole exhibits significant anti-infective and anti-inflammatory effects. In recent years, with the global rise in antibiotic resistance, the research and development of novel antimicrobial agents have become particularly critical (Cao *et al*., 2017; Zhong *et al*., 2014; Zhou *et al*., 2022; Wang *et al*., 2014). Compared to earlier generations of nitroimidazole antimicrobials, morinidazole demonstrates stronger anaerobic antibacterial activity, faster onset, better tolerability and higher safety. Its antimicrobial efficacy against Bacteroides fragilis, Veillonella spp. and Clostridium perfringens is comparable to ornidazole and superior to metronidazole and tinidazole. For Bacteroides distasonis and Bacteroides ovatus, its activity matches ornidazole and exceeds that of metronidazole and tinidazole. As a novel antimicrobial agent, morinidazole not only addresses current antibiotic resistance challenges but also holds significant implications for advancing pharmaceutical development (Wu *et al*., 2020; Tan *et al*., 2013; Wang *et al*., 2012; Morais *et al*., 2010). In this context, morinidazole sodium chloride injection, developed by Jiangsu Hansoh Pharmaceutical Co., Ltd., was launched in China in 2014 as an innovative drug. However, QAMS (Xu *et al*., 2010; Wang *et al*., 2021) and cross-verifying results with an external standard method to ensure the accuracy of the developed approach. This method not only enhances drug safety (Amer *et al*., 2019; Du *et al*., 2014; Wang *et al*., 2024; Sahoo *et al*., 2016) but also contributes to combating antimicrobial resistance. It offers technical support for clinical drug quality assessment, pharmacokinetic studies, therapeutic drug monitoring, stability testing and residue detection, thereby fostering innovation in the pharmaceutical industry. This study has important guiding significance for the quality research of morinidazole sodium chloride injections and has a positive impact on the formulation of commercial standards (Zhang *et al*., 2023).

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The findings of this study are expected to provide valuable insights for researchers and industry decision-makers in related fields.

MATERIALS AND METHODS

Experimental

Chemicals and reagents

The following standards were used: Morinidazole sodium chloride injection (HANSOH PHARMA) (100 mL: 0.5 g sodium chloride: 0.9 g Morinidazole); Morinidazole impurity 1 reference standard (96.4% purity CONTROL CHEMICALS INC.); Ornidazole impurity 1 reference standard (91.5% purity) (China National Standard Pharmaceutical Corporation Limited); Ornidazole impurity 2 reference standard (98.5% purity); Morinidazole impurity 2 reference standard (100.0% purity) (Taizhou Pharmaceutical City Guoke Huawu Biopharmaceutical Technology Co., Ltd.); Levornidazole impurity II reference standard (100.0% purity) (National Institute for Food and Drug Control); Ornidazole reference standard (99.9% purity) (National Institute for Food and Drug Control); Sodium Chloride (JiangSu Province Qinfen Pharmaceutical Co., Ltd.) (Injection grade); Potassium dihydrogen phosphate (China National Pharmaceutical Group Chemical Reagent Co., Ltd.) (AR grade); acetonitrile (TEDIA) (HPLC grade); sodium hydroxide (China National Pharmaceutical Group Chemical Reagent Co., Ltd.) (AR grade).

Instrumentation

For the development and validation studies, the following instruments were used: An Agilent 1260 HPLC system (Agilent, USA), XS205DU electronic analytical balance (Mettler Toledo, Shanghai, China) and Five Easy Plus FE28 pH-meter (Mettler Toledo, Shanghai, China).

Chromatographic conditions

The optimal chromatographic conditions were determined by optimizing the methods for the substances related to morinidazole. The columns used were as follows: Waters symmetry C18, 4.6×250 mm, 5 μm; the column temperature was 30°C; the mobile phase A consisted of 6.8 g of KH_2PO_4, 3 mL of triethylamine and 1000 mL of water were added to dissolve the mixture and the pH was adjusted to 7.0 with phosphoric acid solution; and the mobile phase B consisted of acetonitrile. Mobile phase A was filtered through a 0.22 μm nylon membrane filter and degassed before use. The flow rate was 1.0 mL/min and the injection volume was 20 μL. The analyte was monitored at a wavelength of 319 nm. The gradient elution program was as follows: 0min: 90%A; 5mins, 90%A; 20mins, 80% A; 40mins: 40%A; 45mins: 40%A; 46mins: 90%; 50mins: 90%A. The morinidazole peak was eluted at approximately 20 minutes.

Method validation

After determining the optimal chromatographic conditions and morinidazole impurity calculation method, a comprehensive methodological validation of the method was required to ensure its applicability to this product (Marson *et al*., 2020; Al-Hakkani *et al*., 2021; Wang *et al*., 2012; Kako *et al*., 2024; Subramanian *et al*., 2020. The verification of the system suitability, specificity, LOD, LOQ, linearity, range, accuracy, repeatability, robustness and solution stability of the proposed technique was conducted in accordance with the principles set out by the ICH guidelines (Mahr *et al*., 2024; Elder *et al*., 2024).

System suitability

Six impurity reference standard stock solutions were prepared: Appropriate amounts of impurities A, B, C, D, E and F reference standards were weighed and diluted with water to prepare stock solutions with a concentration of 0.2 mg/mL.

Preparation of the system suitability solution: An appropriate amount of morinidazole was weighed into a 100 mL volumetric flask, an appropriate volume of each impurity reserve solution was transferred into the volumetric flask and the resulting solution was diluted with water to prepare a system suitability solution containing 1 mg of morinidazole per 1 mL and 2 μg of impurities A, B, C, D, E and F.

Preparation of the impurity positioning solution: An appropriate amount of impurity reference stock solution was used to prepare a separate impurity positioning solution. The concentration of the impurity positioning solution was 2 μg/mL.

Then, the solution was injected into the liquid chromatograph, at least 2 injections of blank solvent were injected, 5 injections of system suitability solution were continuously injected and one injection of Morinidazole impurity 1, Ornidazole impurity 1, Ornidazole impurity 2, Morinidazole impurity 2, Levornidazole impurity II and Ornidazole positioning solution was injected. The resolution between the main peak and adjacent impurity peaks in the system suitability solution should meet the requirements. The resolution between known impurities and adjacent impurity peaks should not be less than 1.2. The RSD of peak area of five replicate injections should be ≤ 5.0% and the RSD of the 5-needle retention time should be ≤ 1.0%.

Forced degradation studies

Forced degradation (Bhaskar *et al*., 2020; Sonawane *et al*., 2011; Yulianita *et al*., 2018) studies on morinidazole sodium chloride formulations can identify possible degradants, which can help to validate whether the HPLC method is stable.

Non-destructive test sample preparation

5 mL of morinidazole and sodium chloride solution was accurately pipetted into a 25 mL volumetric flask. The flask was then filled to the mark with water, shaken thoroughly to ensure homogeneity and the resulting solution was collected as the undamaged test sample.

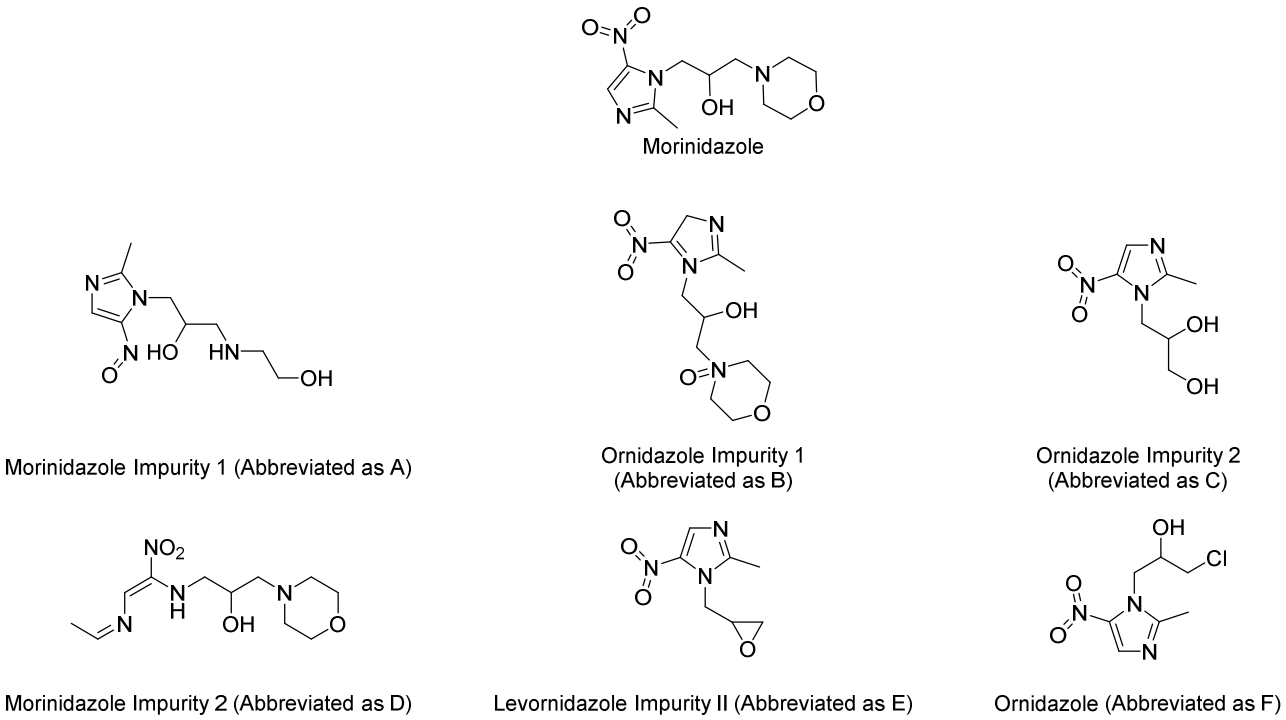

Fig. 1: Structures of Morinidazole, Morinidazole Impurity1, Morinidazole Impurity2, Ornidazole, Ornidazole Impurity 1, Ornidazole Impurity 2 and Levornidazole Impurity II

Table 1: Robustness parameter changes

Chromatographic parameters	Standard parameters	Scope of changes
Flow rate	1 ml/min	0.9 ml/min和1.1 ml/min
Initial column temperature	30℃	27℃ and 33℃
pH value of mobile phase A	7.0	6.9 and 7.1
Column	Waters symmetry C18, 4.6x250 mm, 5μm	Waters symmetry C18, 4.6x250 mm, 5μm

Acid degradation procedure

5 mL of morinidazole and sodium chloride solution were added to a 25 mL volumetric flask. Subsequently, 5 mL of 1 mol/L HCl was introduced and the mixture was allowed to react for 2 hours. After that, 5 mL of 1 mol/L NaOH was added to neutralize the solution. The flask was then filled to the mark with water and shaken well to obtain the acid - degraded solution.

Alkali degradation procedure

5 mL of morinidazole and sodium chloride solution was transferred to a 25 mL volumetric flask. Next, 5 mL of 0.5 mol/L NaOH was added and the reaction was allowed to proceed for 2 hours. Following this, 5 mL of 0.5 mol/L HCl was added for neutralization. The flask was filled to the mark with water and shaken vigorously to prepare the alkali - degraded solution.

Oxidation degradation procedure

5 mL of morinidazole and sodium chloride solution was injected into a 25 mL volumetric flask. Then, 1 mL of 30% H_2O_2 was added to the mixture and the reaction was carried out for 10 minutes. After the reaction, the flask was filled to the mark with water and shaken well to obtain the oxidation - degraded solution.

Thermal degradation procedure

5 mL of morinidazole and sodium chloride solution was accurately pipetted and incubated at 80°C for 48 hours. Subsequently, the solution was transferred to a 25 - mL volumetric flask, filled to the mark with water and shaken well to obtain the thermally degraded solution.

Photolytic degradation procedure

5 mL of morinidazole and sodium chloride injection solution was accurately measured and exposed to illumination (2500 ± 500 lux) for 48 hours. After the exposure, the solution was placed in a 25 - mL volumetric flask, filled to the mark with water and shaken well to obtain the photolytically degraded solution.

Degradation of blank excipient solution

Approximately 900 mg of blank excipients was precisely

weighed and placed in a 100 mL volumetric flask. The excipients were dissolved in water and the flask was filled to the mark and shaken well to prepare the blank excipient stock solution. 5 mL of the stock solution was transferred to a 25 mL volumetric flask, filled to the mark with water and shaken well to obtain the blank excipient solution. Multiple blank excipient solutions were prepared and subjected to degradation under different harsh conditions using the same procedures as those for the sample solutions.

1% self - controlled solution preparation

To assess precision, 2 mL of the undamaged sample was placed in a 200 - mL volumetric flask, diluted with water to the appropriate volume for measurement and shaken well.

There was at least 1 injection of blank solvent, 1 injection of system suitability solution and 1% self-controlled solution, 1 injection of blank excipient solution before and after destruction and 1 injection of destruction solution. The requirement for forced degradation was that the blank solvent and excipients have no interference. Under various conditions of the strong degradation and destruction test, the resolution between the main peak and adjacent impurity peaks should be ≥ 1.5 and the resolution between known impurities and adjacent impurity peaks should be ≥ 1.2. The material balance was within the range of 90% to 110%.

LOQs and LODs

For precision measurements, specific volumes (either 2 mL or 1 mL) of various impurities and morinidazole stock solutions were transferred into a single 100 mL volumetric flask. Subsequently, the flask was filled to the mark with water, resulting in a solution where each mL contained approximately 2 μg of morinidazole and each impurity. The concentration of this precisely prepared contrast mixing solution was regarded as the reference concentration for subsequent calculations. The solution was then gradually diluted with water. The dilution process continued until the S/N reached a value of at least 10. At this point, the concentration of the solution was defined as the LOQ, which was found to be 50% of the total initial concentration. Six parallel samples were prepared at this LOQ concentration to ensure the reproducibility of the results. The solution at the LOQ concentration was further diluted appropriately. The dilution was carried out until the S/N ratio was at least 3. The concentration at this stage was defined as the LOD. After determining the LOD and LOQ, the detection limit and the maximum percentage content of each impurity in the sample were calculated. For the six parallel samples at the limit concentration, the relative standard deviation (RSD) of the peak areas should not exceed 10.0%. Additionally, the limit concentration of the solution should not be less than 30% of the maximum concentration.

Linearity and range

Linearity stock solution: Examples of impurities A, B, C, D, E and F were mixed with morinidazole stock solution in the same volumetric flask and diluted with water to prepare solution A containing impurities A, B, C, D, E and F and 20 μg of morinidazole per mL.

Linear solution: The corresponding volume of linear reserve solution was precisely measured and the solution was placed in the corresponding volumetric flask and diluted to produce linear solutions at concentrations of 10% (LOQ), 25%, 50%, 100% (2 μg/mL), 150% and 200%.

There was at least 1 injection of blank solvent, 1 injection of system suitability solution and 2 injections of linear solution each. The linear correlation coefficient r was not less than 0.995; the y-axis intercept should not exceed 10% of the peak area at 100% concentration. The correction factor for impurities was calculated through a linear curve.

Accuracy

Four concentrations with limits of 20% (0.4 μg/mL), 50% (1 μg/mL), 100% (2 μg/mL) and 150% (3 μg/mL) were used to investigate the recovery rate of impurities and three test solutions were prepared for each concentration. The background solution was prepared by injecting 5 mL of morinidazole sodium chloride in a precise volume, placing it in a 25 mL volumetric flask, diluting it to scale with water and shaking it well. Self-controlled 1% solution: A 2 mL aliquot of undamaged sample was weighed, placed in a 200 mL volume bottle, diluted with water for measurement and shaken. The recovery rate of each impurity was required to be between 80% and 120% and the recovery rate RSD% was ≤ 10%. The results of the self-control method should be compared with those of the external standard method for Levornidazole impurity II.

Precision

The precision of the method was evaluated through repeated experiments and intermediate precision experiments. Using a 100% recovery solution for repetitive solutions, 6 consecutive injections were performed and the RSD of each content in the 6 repeated solutions was calculated. Subsequently, on different dates and with different experimenters, intermediate precision tests were conducted on different liquid chromatography methods, while the RSDs of various contents in 12 intermediate precision solutions were calculated.

Robustness

The system applicability and its effect on the results were investigated by adjusting the flow rate, column temperature, chromatographic column and pH of the mobile phase A. The changes in the chromatographic conditions are shown in table 1. The resolution between the main peak and the adjacent impurity peak in the system applicability solution should be ≥ 1.5 and the resolution between the known Morinidazole impurity and the adjacent impurity peak should be ≥ 1.2.

Sample and standard solution stability

The stability of the system suitability solution, impurity

reference solution and relevant test solution were assessed. The system suitability solution, test sample solution and impurity reference solution were all injected once at 0 hours and after being left for different times. The peak area RSD of the reference solution should be ≤ 5.0% and the peak area of each impurity in the system suitability solution should not be less than 50% compared to that at 0 h. The resolution between the main peak and adjacent impurity peaks in the system suitability solution should be ≤ 1.5 and the resolution between known impurities and adjacent impurity peaks should be ≤ 1.2. The difference between the content (X) and the 0 h content in the test solution met the standard.

RESULTS

Full wavelength scanning

5 mL of morinidazole sodium chloride was injected into a 25 mL volumetric flask, the solution was diluted with the mobile phase to the mark, the mixture was shaken and a concentration of 1 mg/mL was achieved. As shown in Fig. S1, the sample was scanned at full wavelength using a built-in DAD detector for high-performance liquid chromatography and the maximum absorption wavelength of morinidazole was determined to be 319 nm. At the same time, the elution time of morinidazole was approximately 20 minutes, the peak purity of the morinidazole chromatographic peak was greater than 990 and the system determined that the peak purity was qualified. This result laid the foundation for the development of the morinidazole method.

System suitability results

Fig. 2 shows the chromatogram of the system suitability solution. No interference was detected between the blank solvent and the excipients. The resolution between the main peak and adjacent impurity peak was ≥ 1.5 and the resolution between the known Morinidazole Impurity 1nd adjacent impurity peak was ≥ 1.2. The theoretical plate number of impurities was greater than 2000, the RSD of the peak area of the 5 needles was ≤ 5.0% and the RSD of the retention time of the 5 needles was ≤ 1.0%. At the same time, the localization times of morpholine nitrazole and impurities A, B, C, D, E and F were approximately 20 mins, 4mins, 5mins, 8mins, 9mins, 19mins and 25mins, respectively.

Specificity (forced degradation behavior)

As shown in Fig. S2, the blank excipient did not produce any new impurities under various destructive conditions, which proved that the excipient solution containing morinidazole sodium chloride was extremely stable. Figure 6 shows the spectra of the substance before and after damage by acid, alkali, oxygen, high temperature and light. The resolution between the main peak and adjacent peaks was greater than 1.5 and the resolution between known impurities and adjacent peaks was greater than 1.2, all of which met the standards. The blank excipients had no interference and the material balance of the disrupted sample was between 95% and 105%. The purity factor of the main peak was greater than 990, which met the requirements. The results showed that the substance related to morinidazole was stable under acidic conditions and did not produce new impurities. Under other conditions, new unknown impurities were produced. By using the 1% self-controlled method to calculate the size of impurities and calculate the material balance, the following results were obtained (Table 2). After alkaline conditions and oxygen damage, the decrease in the main component content of the sample was obvious. From the perspective of clinical use, relevant personnel should be reminded to avoid opening the injection and use it all at once as much as possible. At the same time, for enterprises, to ensure product quality, it was possible to consider simultaneously filling with nitrogen gas during filling to protect the product.

LOQs and LODs

Table 3 shows that the LODs and LOQs of morinidazole, Morinidazole impurity 1, Ornidazole impurity 1, Ornidazole impurity 2, Morinidazole impurity2, Levornidazole impurity II and Ornidazole were all much lower than the concentration limits for each impurity. The quantification limit of the solution was measured 6 times and the relative standard deviation (RSD) of each peak area was less than 10.0%. The reproducibility was good at low concentrations. This method has strong detection ability and high sensitivity for relevant substances. The LOQ was 0.2 μg/mL and the LOD was 0.06 μg/mL.

Linearity and correction factor

As shown in fig. 3, Morinidazole, Impurity A, Impurity B, Impurity C, Impurity D, Impurity E and Impurity F exhibited a good linear relationship with the peak area in the LOQ (10%-200% concentration range. Fig. S3 showed 100% concentration linear solution spectrum. The linear correlation coefficient (r) was not less than 0.995. The y-axis intercept did not exceed 10% of the peak area at 100% concentration, which met the standard. Based on the average values of the correction factors obtained from the two measurements, a correction factor of 1.0 was proposed for Impurity A, 1.2 for Impurity B, 0.8 for Impurity C, 1.2 for Impurity D, 0.8 for Impurity E and 0.8 for Impurity F.

Accuracy

As shown in table 4, by calculating the spiked recovery rate, it was confirmed that the recovery rates of impurities were all between 95% and 105% and the RSD of the recovery rates of individual impurities was less than 10%. This indicated that the method was accurate in detecting content and had a relatively high precision.

Repeatability

As shown in table 5, the 1% self-control method used in this article had a difference of no more than 0.05% compared to the external standard method and the RSD of the six parallel results was less than 5%. This indicated that the repeatability of the method was good and the proposed Ornidazole Impurity 2alculation method was accurate.

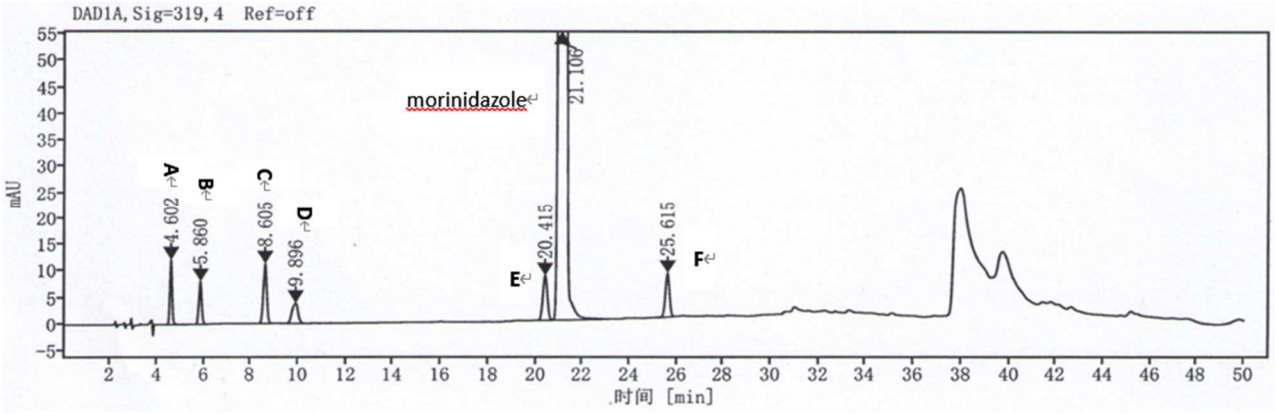

Fig. 2: System suitability solution

Table 2: Forced degradation results

Name	Undamaged test sample	Acid degradation	Alkali damage	Oxidation degradation	Thermal degradation	Photolytic degradation
Morinidazole impurity 1%	0.006	0.008	0.007	0.007	0.074	0.031
Ornidazole impurity 1%	0.014	0.014	0.013	8.931	0.116	0.077
Ornidazole impurity 2%	0.021	0.022	0.013	0.024	0.042	0.029
Morinidazole impurity2%	0.011	0.012	0.010	0.012	0.120	0.123
Levornidazole impurity II%	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Ornidazole%	Not detected	Not detected	Not detected	Not detected	Not detected	Not detected
Total amount of unknown impurities%	0.009	0.006	Not detected	0.045	0.156	0.143
Total impurities%	0.060	0.062	0.277	9.019	0.507	0.402
Principal component content %	98.7	97.2	93.7%	89.2	96.2	97.9
Material balance %	/	98.5	95.2	99.5	97.9	99.5
Main peak purity	998.9	999.5	999.4	999.6	999.3	998.9
Minimum resolution between the main peak and adjacent peaks	25.5	21.9	7.0	16.5	5.0	4.4

Table 3: LOQ and LOD verification results

Sample name	Morinidazole		Morinidazole Impurity 1		Ornidazole Impurity 1		Ornidazole Impurity 2		Morinidazole Impurity2		Levornidazole Impurity II		Ornidazole	
Concentration μg/ml	0.1983		0.1930		0.1907		0.2048		0.2000		0.1996		0.1952	
Approximately% of the limit concentration	9.9		9.7		9.5		10.2		10.0		10.0		9.8	
S/N	Area	S/N	Area	S/N	Area	S/N	Area	S/N	Area	S/N	Area	S/N	Area	S/N
LOQ-1	7.692	14.5	7.939	27.1	6.322	17.5	11.401	24.7	8.037	9.6	10.854	17.6	11.276	16.6
LOQ -2	7.089	16.3	7.851	31.1	6.915	20.8	11.676	28.2	7.929	10.9	10.81	20.1	11.702	19.5
LOQ -3	7.419	15.4	7.942	30.0	6.668	19.7	11.435	27	7.271	10.1	11.045	19.4	12.255	18.4
LOQ -4	7.769	15.2	8.102	29.4	6.661	19.1	11.667	25.7	8.794	10.4	11.139	18.6	10.003	17.4
LOQ -5	7.687	16.0	7.913	30.3	6.570	19.8	11.334	26.5	7.706	10.7	11.025	19.5	10.498	18.1
LOQ -6	7.722	15.7	8.015	29.9	6.597	19.4	11.206	25.7	7.404	10.1	10.987	19.1	10.726	17.4
Mean value	7.563	15.5	7.960	27.1	6.622	17.5	11.453	24.7	7.857	9.6	10.977	17.6	11.077	16.6
RSD%	3.47	/	1.10	/	2.89	/	1.63	/	6.94	/	1.13	/	7.48	/
LOD														
Concentration μg/ml	0.0595		0.0579		0.0572		0.0614		0.0600		0.0599		0.0586	
S/N	5.5		10.1		6.7		9.5		3.5		6.5		5.9	

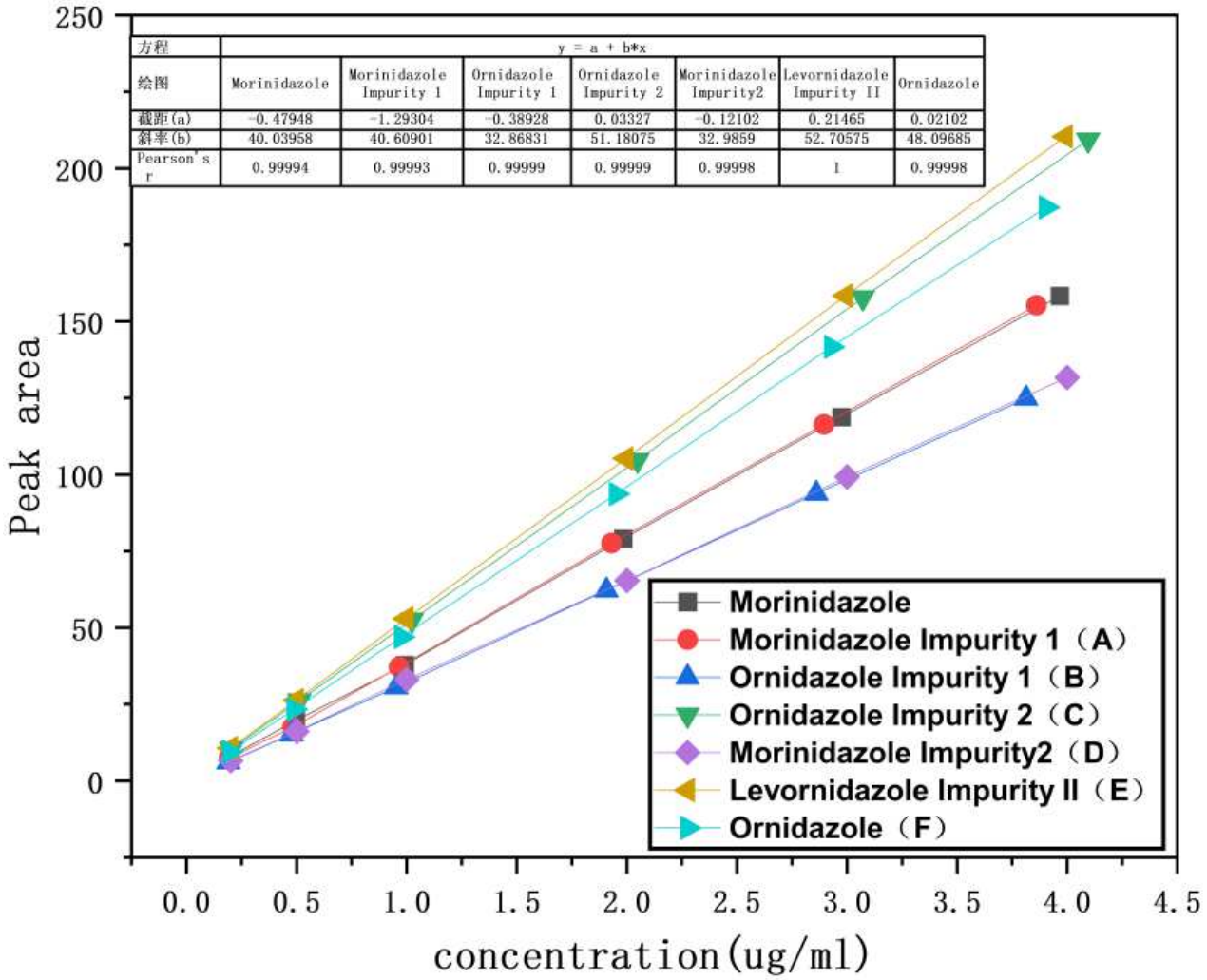

Fig. 3: Linear results for impurities A, B, C, D, E and F and morinidazole

Table 4: Recovery rates of impurities A, B, C, D, E, and F

Impurity name	A	B	C	D	E	F
Recovery rate	Recovery	Recovery	Recovery	Recovery	Recovery	Recovery
Concentration level	%	%	%	%	%	%
	105.1	102.5	105.1	102.1	106.0	105.3
20%	103.4	103.4	103.1	102.9	105.0	106.2
	103.2	101.7	103.1	102.7	106.7	105.9
	102.5	102.6	102.5	104.2	103.3	102.9
50%	103.3	102.2	104.0	104.5	103.0	103.1
	102.0	102.8	104.6	104.7	103.4	102.0
	103.6	102.6	104.1	104.3	102.2	101.1
100%	103.8	103.6	103.5	105.4	102.5	101.7
	103.5	102.5	104.1	105.0	102.2	101.7
	104.3	103.9	104.5	105.8	102.6	102.5
150%	103.9	103.4	104.4	105.4	102.1	103.3
	103.9	103.3	104.0	105.3	101.9	103.7
Average%	104	103	104	104	103	103
RSD%	0.8	0.6	0.7	1.2	1.6	1.7

Table 5: Comparison of the repetitive results between the two calculation methods

Calculate content using 1% self-control method						
Name	A%	B%	C%	D%	E%	F%
Repeatability -1	0.221	0.233	0.246	0.240	0.221	0.217
Repeatability -2	0.231	0.243	0.261	0.251	0.232	0.229
Repeatability -3	0.233	0.244	0.260	0.252	0.229	0.229
Repeatability -4	0.228	0.239	0.255	0.248	0.228	0.225
Repeatability -5	0.225	0.235	0.250	0.243	0.222	0.220
Repeatability -6	0.221	0.232	0.247	0.240	0.220	0.217
Average	0.226	0.238	0.253	0.246	0.225	0.223
RSD%	2.3	2.2	2.5	2.3	2.2	2.5
Calculating content using external standard method						
Name	A%	B%	C%	D%	E%	F%
Repeatability -1	0.204	0.214	0.228	0.221	0.203	0.199
Repeatability -2	0.204	0.213	0.230	0.221	0.202	0.200
Repeatability -3	0.205	0.214	0.228	0.221	0.199	0.200
Repeatability -4	0.203	0.213	0.228	0.221	0.201	0.200
Repeatability -5	0.205	0.214	0.228	0.221	0.200	0.199
Repeatability -6	0.204	0.213	0.228	0.221	0.201	0.199
Average	0.204	0.214	0.228	0.221	0.201	0.199
RSD%	0.3	0.2	0.4	0.1	0.6	0.2
The absolute value of the difference between the two calculation methods	0.022	0.024	0.02	0.025	0.024	0.024

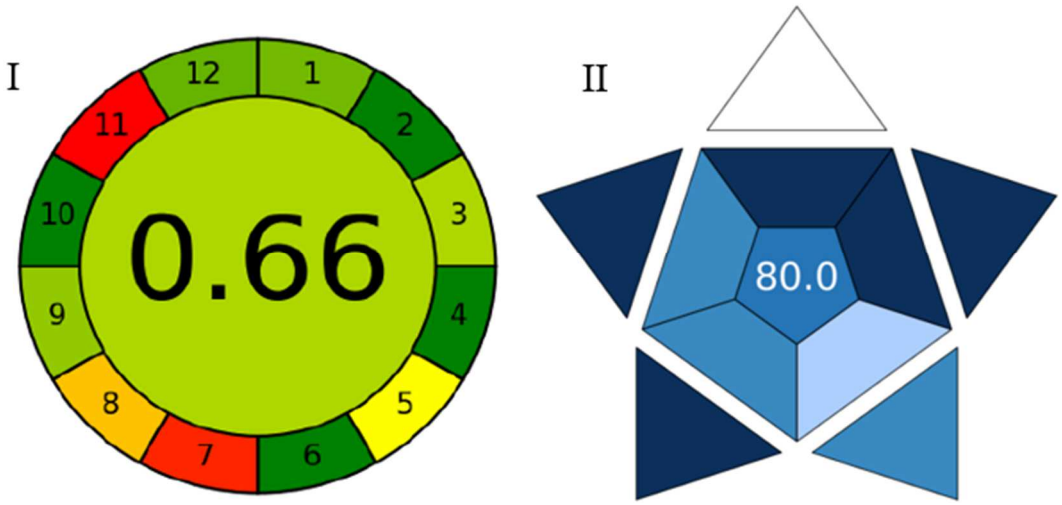

Fig. 4: Score of greenness and blueness. Note I: Greenness of QAMS, II: Blueness of QAMS

Intermediate precision

As shown in table S1, the RSD% of impurities between 0.10%-0.50% in 6 medium-precision solutions and 12 precision solutions (6 repetitive and 6 medium precision) met the standard; the difference in average content measured by different experimenters on different dates met the standard; the method had good repeatability; and the self-control method with correction factors could accurately calculate the content of this product.

Robustness

As shown in table S2, in the system applicability solution, the resolution of the main peak and the adjacent impurity peak was ≥ 1.5 and the resolution of the known impurities and the adjacent impurity peak was ≥ 1.2; the difference between the measured content (X) and the value measured under normal conditions was in line with the standard. The method demonstrated good durability.

Solution stability

The peak area RSD of the impurity standard solution at 0 hr, 20 hrs and 30 hrs was ≤5.0%. The peak area of each impurity in the system suitability solution at 20 hrs and 30 hrs was not less than 50% compared to that at 0 hr. The resolution between the main peak and adjacent impurity peaks in the system suitability solution was ≥1.5 and the

Table 6: Chromatographic column and gradient optimization scheme

Protocol	1			2			3		
Column	Inersil ODS-3 V 4.6×250 mm, 5μm			Welch AQ C18 4.6×250 mm, 5μm			Waters symmetry C18 4.6×250 mm, 5μm		
Gradient	Time/min	Mobile phase A/%	Mobile phase B/%	Time/min	Mobile phase A/%	Mobile phase B/%	Time/min	Mobile phase A/%	Mobile phase B/%
	0	90	10	0	90	10	0	90	10
	5	90	10	5	90	10	5	90	10
	20	80	20	25	80	20	20	80	20
	40	40	60	45	40	60	40	40	60
	45	40	60	50	40	60	45	40	60
	46	90	10	51	90	10	46	90	10
	50	90	10	60	90	10	50	90	10
Protocol	4			5			6		
Column	Inersil ODS-3 V 4.6×250 mm, 5μm			Welch AQ C18 4.6×250 mm, 5μm			Waters symmetry C18 4.6×250 mm, 5μm		
Gradient	Time/min	Mobile phase A/%	Mobile phase B/%	Time/min	Mobile phase A/%	Mobile phase B/%	Time/min	Mobile phase A/%	Mobile phase B/%
	0	90	10	0	90	10	0	90	10
	5	90	10	5	90	10	5	90	10
	25	80	20	20	80	20	25	80	20
	45	40	60	40	40	60	45	40	60
	50	40	60	45	40	60	50	40	60
	51	90	10	46	90	10	51	90	10
	60	90	10	50	90	10	60	90	10
Protocol	7			8			9		
Column	Inersil ODS-3 V 4.6×250 mm,5μm			Welch AQ C18 4.6×250 mm, 5μm			Welch AQ C18 4.6×250 mm, 5μm		
Gradient	Time/min	Mobile phase A/%	Mobile phase B/%	Time/min	Mobile phase A/%	Mobile phase B/%	Time/min	Mobile phase A/%	Mobile phase B/%
	0	90	10	0	95	5	0	90	10
	5	90	10	5	95	5	5	90	10
	20	85	15	20	80	20	20	85	15
	40	40	60	40	40	60	40	40	60
	45	40	60	45	40	60	45	40	60
	46	90	10	46	95	5	46	90	10
	60	90	10	60	95	5	55	90	10

resolution between known impurities and adjacent impurity peaks was ≥1.2. The difference between the Content and the 0 h content of the test solution at 20 hrs and 30 hrs met the standard.

Practical and environmental evaluation of methods

The AGREE scores of QAMS were 0.66 and BAGI scores were 80.0 (Fig. 4), indicating that the method is environmentally friendly and practical.

DISSCUSION

Mobile phase composition and pH optimization

In general, the concentration of buffering salt in the mobile phase should be in the range of 0.01-0.05 mol/L and 0.05 mol/L potassium dihydrogen phosphate solution was chosen as the buffering salt in this method. Considering that the pH of the Morinidazole sodium chloride injection was 7.0, the pH of the mobile phase was adjusted to 7.0. However, the potassium dihydrogen phosphate buffering solution was acidic, so some alkaline substances were added to the mobile phase to neutralize the acidity and alkalinity. After consulting relevant information (Wu *et al*., 1986), it was determined that triethylamine eliminated peak tailing and was alkaline. Therefore, a certain amount of triethylamine was added (Potassium dihydrogen phosphate (6.8 g) → 1000 mL, add triethylamine (3mL) to the mobile phase to increase the pH of the mobile phase to 7.0. A C18 4.6×250 mm, 5 μm chromatographic column was used for the investigation and the sample used was a mixed solution of system suitability solution and a high-temperature 30-day sample. The reason for using this

solution was that the product will degrade and generate new impurities under high-temperature conditions, which are far more abundant than the impurities in normal samples and the method's ability to separate impurities can be better tested. The figure shows the blank solution and system suitability chromatogram under this system. It can be seen from the chromatogram that there is a tailing phenomenon in the chromatographic peaks of impurities and the main components detected by this method. There two impurities did not achieve baseline separation and the main component morinidazole was subjected to chromatographic overload. Therefore, this method requires further optimization. At the same time, the calculation of impurities should adopt a 1% self-control method or an external standard method. The calculation of area normalization was obviously inaccurate. Considering that there were more degradation impurities in this product, the external standard method imposed a notable economic cost burden, primarily due to the high demand for high-purity standard substances and the need for frequent calibration to maintain accuracy. Therefore, a 1% self-control method was used to calculate the impurities. In addition, for the separation and tailing phenomena existing in the method, a further optimization strategy was to replace the chromatographic columns of different manufacturers and models and optimize the elution gradient.

Optimization of chromatographic columns and gradient elution

In this study, several combinations of elution gradients and chromatography columns were used in an attempt to completely separate impurities. The basis of gradient design was to change the proportion of the organic phase and the elution time to change the peak time of impurities and achieve baseline separation. The design scheme is shown in table 6. By implementing 9 schemes, it was confirmed that the chromatographic column and gradient of scheme 3 were the optimal solution for separating impurities, that is, using a Waters Symmetry C18, 4.6×250 mm, 5 μm chromatography column and gradient in scheme 3. This method achieved good separation of all impurities, with a minimum resolution of 2.5, a minimum theoretical plate count exceeding 6,000 and tailing factors ranging from 0.9 to 1.1. And it took the shortest amount of time.

Comparison with previous literature

LOD refers to the lowest concentration of an analyte that an analytical system can reliably identify with a high probability under specific experimental conditions. It is a key indicator for evaluating the sensitivity and accuracy of an analytical method. The LOD of the proposed method is 0.06 μg/mL, which is significantly lower than that of other reported method (Fang *et al*., 2025) (0.3 μg/mL). A lower LOD indicates a stronger ability of the analytical method to detect low-concentration samples, i.e., higher sensitivity. We also compared our AGREE score (0.66) with the scores reported in other relevant literature (Fang *et al*., 2025), where the scores of the methods proposed in those literatures were lower (0.59). This comparison highlights the superiority of our method in terms of "greenness".

The limitations of the study

In the process of method verification, we tried to destroy the sample and the results showed that the sample was stable only under acidic conditions, which meant that the product should be strictly sealed during clinical use and storage to prevent a decrease in sample quality. Moreover, we investigated the stability of the solution. Due to time limitations, we investigated only 30 hrs and the system solution, sample solution and standard solution were stable after 30 hrs. Obviously, the stability time was insufficient because impurities are usually very expensive. We hope that through the study of prolonging stability, the enterprise can reuse the reference solution reserve liquid, thus reducing costs. Therefore, we will further study the stability time of the solution. In addition, an obvious disadvantage of this method is that the detection time is 50 mins, which increases the time cost in practical applications and is not conducive to large-scale detection. We hope to shorten the detection time of this method through follow-up research. We hope to provide some inspiration for researchers and researchers at pharmaceutical companies.

This paper independently developed a method for the detection of substances related to Morinidazole sodium chloride injection and conducted a methodological verification of the method. Overall, the verification results were satisfactory. The method had good applicability and the LOQ and LOD were 0.2 μg/mL and 0.06 μg/mL, respectively. The method had good precision and accuracy, the recovery rate was between 95-105% and the RSD met the requirements.

CONCLUSION

The reversed high-performance liquid chromatographic method developed in this article can be accurately applied to the detection of substances related to morinidazole. Methodological validation was carried out according to the ICH guidance principles and the validation results were reliable. In the future, we will optimize the existing methods, including shortening the detection time and optimizing the baseline. At the same time, we will study the relevant substances of different manufacturers of Morinidazole sodium chloride injection, providing a basis for further improving the quality of the product.

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Authors' contributions

Jie Lin: Conceptualization, methodology, validation,

investigation and writing – original draft; Penghe Xu: Resources, formal analysis and data curation; Huina Zhang: Investigation, validation and visualization; Menggan Wang: Methodology, validation and data curation; Jianjun Liu: Supervision and project administration; Chunrui Zhang: Investigation and resources; Hangyu Zhao: Supervision, writing – review and editing and funding acquisition; Yonghua Yu: Supervision, writing – review, editing and funding acquisition.

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Ethical approval
This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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