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Simultaneous determination of five nitroimidazole antimicrobials in pharmaceuticals using HPLC-ESM and QAMS methods

Baoxia Fang[1†], Dan Jiang[1†], Sicen Wang[1,2*] and Fuchao Chen[1*]

Abstract

A green, rapid, and simple HPLC-External standard method (ESM) and a quantitative analysis of multi- components with a single-marker (QAMS) method were established for the simultaneous determination of five nitroimidazole antimicrobials (Metronidazole sodium chloride injection, Tinidazole injection, Ornidazole sodium chloride injection, Morinidazole sodium chloride injection, Secnidazole tablets) in pharmaceutical preparations. The five specified drugs were chromatographed via HPLC on a ZORBAX SB-C_{18} (150 mm×4.6 mm, 5 µm particle size) analytical column using a mobile phase consisting of methanol-0.1% v/v triethylamine (26:74 v/v, pH adjusted to 3.0 with phosphoric acid) with isocratic elution and monitored by photodiode array detector at 316 nm. The chromatographic separation was accomplished within a short run time (less than 20 min) for the studied analyte. Using metronidazole as internal reference, the relative correction factors of each constituent were calculated were established, and the contents of each component of 5 nitroimidazole were calculated to achieve QAMS. The measured results were verified by the ESM. The methods were validated in terms of linearity, intra- and inter-batch precision, accuracy, stability, and recovery. The proposed ESM and QAMS methods could simultaneously determination of the studied analyte, and they were successfully applied to the analysis of the above cited drugs in pharmaceutical preparations with excellent accuracy and precision. In addition, the analytical greenness (AGREE) and blue applicability grade index (BAGI) metric tools were used to evaluate the greenness and environmental friendliness of the developed methods. AGREE scores of QAMS and EMS were 0.66 and 0.59, and BAGI scored 82.5 and 77.5, respectively.

Keywords Nitroimidazole antimicrobials, HPLC, Simultaneous determination, Quantitative analysis of multi-components with a single-marker, External standard method

†Baoxia Fang and Dan Jiang contributed equally to this work and should be considered as equal first authors.

*Correspondence:
Sicen Wang
wangsc@mail.xjtu.edu.cn

Fuchao Chen
dfyycfc@163.com
[1]Sinopharm Dongfeng General Hospital, School of Pharmaceutical Sciences, Hubei University of Medicine, 16# Daling Road, Shiyan, Hubei 442008, PR China
[2]School of Pharmacy, Xi'an Jiaotong University, 76# Yanta West Road, Xi'an, Shanxi 710061, PR China

Introduction

Nitroimidazole represents a class of heterocyclic compounds characterized by the nitroimidazole ring structure. Nitroimidazole drugs are distinguished by their broad spectrum of activity against anaerobic bacteria, potent bactericidal properties, cost-effectiveness, and significant therapeutic efficacy, making them widely utilized in the treatment of infectious diseases in humans and poultry, either as monotherapy or in combination with other agents [1, 2]. Furthermore, these drugs have demonstrated notable success in areas such as anti- tuberculosis therapy, tumor sensitization, antihypertensive applications, radionuclide hypoxia imaging techniques, antiviral treatments, artificial probes development, promotion of animal growth and enhancement of feed utilization-drawing considerable attention from both domestic and international medical professionals [3–8]. Since the synthesis and clinical application of metronidazole commenced in 1959, there has been rapid advancement in the development and utilization of this drug class. Currently marketed products include metronidazole, tinidazole, ornidazole, secnidazole and morinidazole [9].

Metronidazole (Fig. 1-a), with a chemical structure of 2-methyl-5-nitroimidazole-1- ethanol, 1-(2-hydroxyethyl) -2-methyl-5-nitroimidazole, is mainly used clinically for the treatment of Helicobacter pylori infection, amebiasis, Giardia, trichomoniasis, bacterial vaginosis, Crohn's disease, etc., and can also be used as an antibiotic to prevent infection during surgery [10]. Tinidazole (Fig. 1-b), with a chemical structure of 2-methyl-1 -[2-(ethyl sulfonyl) ethyl] -5-nitro-1 H-imidazole, is a second-generation member that can be used effectively against metronidazole resistant strains of Trichomonas vaginalis and recurrent periodontitis [11]. Ornidazole (Fig. 1-c), with chemical structure of 1-(3-chloro-2- hydroxy propyl) -2-methyl-5-nitroimidazole, is the third generation of nitroimidazole derivatives, mainly used for the prevention of postoperative anoxic infection and the treatment of various infectious diseases caused by sensitive anoxic bacteria. Among the metronidazole, tinidazole and ornidazole, ornidazole has higher antibacterial activity and tolerance [12]. Secnidazole (Fig. 1-d), with the chemical structure of 1- (2-hydroxypropyl) -2-methyl- 5-nitroimidazole, is a novel nitroimidazole drug. Compared with the three nitroimidazole drugs mentioned above, Secnidazole has the advantages of good oral absorption, fewer adverse reactions and long half-life [13]. Morinidazole (Fig. 1-e), with chemical structure of 1-[3-(4-morphinyl) -2- hydroxypropyl] -2-methyl-5-nitro-1 H-imidazole, is a new nitroimidazole drug developed by Jiangsu Hausen Pharmaceutical Co. in 2014, which has strong antibacterial activity against both anaerobic gram-negative bacteria and positive bacteria isolated in clinic [14].

Analyses of different mixtures containing these antimicrobials were described in several articles. Metronidazole could be identified and detected by ratio spectra derivative spectrophotometry, titration analysis, ionization tandem mass spectrometry, ultraviolet spectrophotometry, etc [15–19]. On the other hand, the combination of Metronidazole together with tinidazole and secnidazole was analyzed using HPLC-UV, fast liquid chromatography-tandem mass spectrometry, extraction spectrophotometric analysis, capillary electrophoresis [20–25]. Notably, a substantial body of literature exists regarding the quantitative analysis of five nitroimidazole antimicrobials using HPLC; however, these published analytical methods primarily focus on the qualitative and quantitative

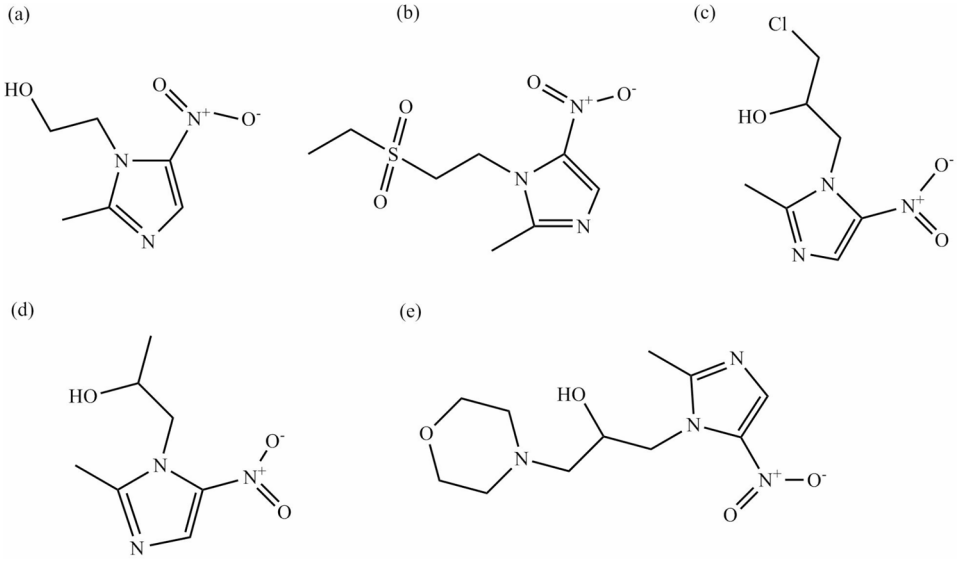

Fig. 1 Chemical structures of the five nitroimidazole **a**. antimicrobials metronidazole; **b**. tinidazole; **c**. ornidazole; **d**. secnidazole; **e**. morinidazole

detection of one or two to four nitroimidazole drugs in pharmaceuticals, biological samples, and environmental matrices [15–22]. To date, no reports have documented the simultaneous determination of all five nitroimidazole antimicrobials via HPLC.

The multi-component quantitative with a single-marker (QAMS) method is based on the structure and physicochemical properties of the components to be analyzed. It first selects an appropriate marker, which is easily quantifiable and has a known reaction factor to determine the content of representative components in the sample to be tested, and then calculates the relative correction factor (RCF) of each analyte relative to the marker compounds, and calculates the content of other components in the sample to be tested through RCF [26]. Several studies have shown that using this method for separation and simultaneous determination is economic and time-effective [27, 28]. This method helps solve problems such as the difficulty in obtaining some reference substances and the high cost of using multiple standard reference materials simultaneously. It is economical, convenient, accurate, environmentally friendly, and has good development and application prospects.

Consequently, this study aims to establish an accurate and rapid HPLC-ESM and quantitative analysis of the QAMS method for the simultaneous quantification of five nitroimidazole antimicrobials. This approach seeks to provide technical references for clinical drug quality assessment, pharmacokinetics studies, therapeutic drug monitoring, stability investigations, as well as drug residue detection and illegal addition assessments.

Materials and methods

Drugs and reagents

Metronidazole (100.0% purity), tinidazole (100.0% purity), ornidazole (100.0% purity), secnidazole (100.0% purity), morinidazole (99.8% purity), and urine pyrimidine (99.6% purity) reference standards were supplied by the National Institutes for Food and Drug Control (Beijing, China). Metronidazole sodium chloride injection (Hangzhou Minsheng Pharmaceutical Co., LTD., 0.5 g/100 mL, lot 22010403), Tinidazole injection (Sichuan Kelun Pharmaceutical Co., LTD., 0.4 g/100 mL, lot G22012004), Ornidazole sodium chloride injection (Sichuan Kelun Pharmaceutical Co., LTD., 0.5 g/100 mL, lot S22021611), Morinidazole sodium chloride injection (Jiangsu Hausen Pharmaceutical Co., LTD., 0.5 g/100 mL, lot 220304), Secnidazole tablets (Hunan Jiudian Pharmaceutical Co., LTD., 0.25 g/ tablet, lot 221204), and 0.9% sodium chloride injection (Hunan Kelun Pharmaceutical Co., LTD., 4.5 g/500 mL, lot E23072006) were purchased from Sinopharm Holding Company Limited (Hubei, China). HPLC grade Methanol was purchased from Tianjin Kemiou Chemical Reagent Co., LTD. (Tianjing, China). Triethylamine and ortho-phosphoric acid were analytical pure and delivered from Shanghai Pudong Chemical Reagent Factory (Shanghai, China).

Chromatographic system

The chromatographic system was Dionex Ultimate 3000 series (USA) equipped with a four-element low pressure gradient pump, a temperature controlled auto injector, and configuration of chameleon 7 data processing software. The Ultimate 3000 photodiode array detector was set at 316 nm for all drugs. Chromatography was performed on an Agilent ZORBAX SB-C_{18} column (150 mm×4.6 mm, 5 μm; Agilent Technology Co. LTD. Beijing, China). The mobile phase consisted of 0.1% triethylamine solution (pH adjusted to 3.0 with phosphoric acid) and methanol (74: 26, v/v). The detection wavelength was 316 nm. The flow-rate was set at 1.0 mL·min^{-1} and the column was kept at a temperature of 30 ℃.

Standard solutions

The appropriate amounts of metronidazole, tinidazole, ornidazole, secnidazole, and morinidazole reference standards were accurately weighed and placed in 25 mL bottle, dissolved with mobile phase, and diluted to the scale. The standard stock solutions of metronidazole 1.02 mg·mL^{-1}, tinidazole 1.02 mg·mL^{-1}, ornidazole 1.0 mg·mL^{-1}, secnidazole 1.04 mg·mL^{-1}, and morinidazole 0.998 mg·mL^{-1} were obtained, respectively. Then, 1 mL of each standard stock solution was taken and evenly mixed into a 10 mL bottle to prepare the mixed reference solution with concentrations of 102.0, 102.0, 100.0, 104.0, and 99.8 μg·mL^{-1}, respectively. The standard stock and the mixed reference solution were frozen in the refrigerator at -20℃ for use.

Preparation of pharmaceutical dosage sample

Ten tablets of secnidazole were accurately weighed and finely powdered [29]. Subsequently, precisely measured approximately 500 mg of the powdered drug and transferred it to a 100 mL volumetric flask. Dissolved the powder with the mobile phase and diluted to the mark, then shaken and filtered the solution. Finally, withdrew 1.0 mL of the filtered solution and put it in a 100 mL volumetric flask, added the mobile phase, dissolved it ultrasonically, and filtered it with a microporous filter membrane to obtain the sample solution of secnidazole. Metronidazole, tinidazole, ornidazole, and morinidazole injections were accurately measured in 1mL volume and transferred to 100 mL volumetric flask. It was diluted with mobile phase and diluted to the mark. The solution was filtered through a 0.45 μm microporous filter membrane, and the resulting filtrate was used as the sample solution of other four nitroimidazoles.

Method validation for HPLC-ESM

The linear range, limit of quantification (LOQ), limit of detection (LOD), precision, stability, and recovery of the five nitroimidazole drugs were analyzed and validated according to the Chinese Pharmacopoeia and International council for harmonization of technical requirements for pharmaceuticals for human use (ICH) method validation guidelines [30, 31]. To assess linearity, precise volumes of 0.25, 0.5, 1.0, 0.75, 1.5, and 2.0 mL for each of the five nitroimidazoles were placed into a 10 mL volumetric flask and dissolved with mobile phase to prepare mixed reference solutions at varying concentrations. Following the chromatographic conditions described above, each sample was injected 20 μL for analysis. The determination was repeated for three times, and the chromatogram was recorded. Linear regression was performed with the drug concentration (X, $\mu g{\cdot}mL^{-1}$) as the horizontal coordinate and the peak area (Y) of each drug chromatographic peak as the longitudinal coordinate, and the linear equation and correlation coefficient (r) were obtained. The LOD and LOQ were determined by diluting the standard solutions of the five nitroimidazole antimicrobials in the blank control solutions at a signal-to-noise ratio (S/N) of three-fold and ten-fold respectively. Intra-day and inter-day precisions for these methods were evaluated by measuring responses from mixed reference solutions at three distinct concentrations under standard curve conditions six times within one day as well as over three consecutive days respectively. For the stability studies, the sample solution of the above five nitroimidazoles was taken and placed at room temperature for analysis at 0, 2, 4, 6, 8 and 12 h, respectively. For determining accuracy, the five nitroimidazole antimicrobials reference standard were accurately weighed and spiked to the pharmaceutical dosage sample at three different concentration levels to gain 50%, 100%, and 150% [32]. At each level, samples were prepared in triplicate, and the percent recovery was determined.

Method validation for QAMS

In this study, UV spectral similarity, relative retention time (RRT), and RCF were used as qualitative and quantitative indicators of QAMS [33–38]. In order to calculate the UV spectral similarity, RRT, and RCF, a mixture of the five nitroimidazole antimicrobials reference standard (about 50 $\mu g{\cdot}mL^{-1}$) under the above standard curve was taken and 20 μL was injected in HPLC system according to the above chromatographic conditions. The chromatogram was recorded, each drug's ultraviolet spectrum diagram was collected, and a UV spectrum database was established. The original, first derivative, and second derivative spectra of studied analytes were extracted by ChemStation software C.01.10 for similarity analysis. The RRT was calculated using the following formula [1].

$$RRT = (t_i - t_0)/(t_s - t_0) \quad (1)$$

RRT = $(t_i\text{-}t_0)$ / $(t_s\text{-}t_0)$ [1]

Where t_i, t_0 and t_s are the retention time of analyte, urine pyrimidine and internal standard metronidazole, respectively. The RCF of the QAMS method were then calculated based on the following formulas:

$$f = \frac{f_s}{f_i} = \frac{A_s/C_s}{A_i/C_i} \quad (2)$$

$$Ci = f \times C_s \times \frac{A_i}{A_s} \quad (3)$$

Where A_s is the peak area of metronidazole, C_s is the concentration of metronidazole, A_i is the peak area of component i, and C_i is the concentration of component i in the sample solution.

From Eq. [2] and Eq. [3], we can calculate the concentration of the studied analytes in the sample solution. To evaluate the robustness of the QAMS method, changing levels of some important factors as three HPLC instruments (Agilent 1260, Shimadzu LC-20AT, and UltiMate 3000), different brands of columns (Agilent ZORBAX SB-C_{18}, Shimadzu ShimNex UP-C_{18}, and Kromasil 100-5- C_{18}), column temperatures (± 1 °C), pH values (± 0.2), and flow rates (± 0.02) of the mobile phase was assessed.

Determination of five nitroimidazoles in pharmaceutical preparations

Appropriate amounts of metronidazole, tinidazole, ornidazole, morinidazole sodium chloride injection and secnidazole tablets were taken from the inpatient pharmacy of the hospital. The sample solution was processed according to the preparation method in item 2.4 and filtered by a 0.45 μm microporous filter. The further filtrate of 20 μL was injected and determined according to the above chromatographic conditions. HPLC-ESM and QAMS methods were used to calculate the contents and relative standard deviation (RSD) values of five nitroimidazole antimicrobials, respectively. The tablets solution and injection solutions were injected to the column in separate solutions.

Assessment of the proposed method's greenness

Analytical GREEnness metric (AGREE) conducted a green evaluation of the established method. AGREE is calculated based on the 12 basic principles of the Green Analytical Chemistry (GAC). The center of the circular hieroglyphics is the final score, which is a score of one unit, from 0 to 1. The tool is accessed via the link mentioned in the AGREE publication [39]. Additionally, the blue applicability grade index (BAGI) approach was

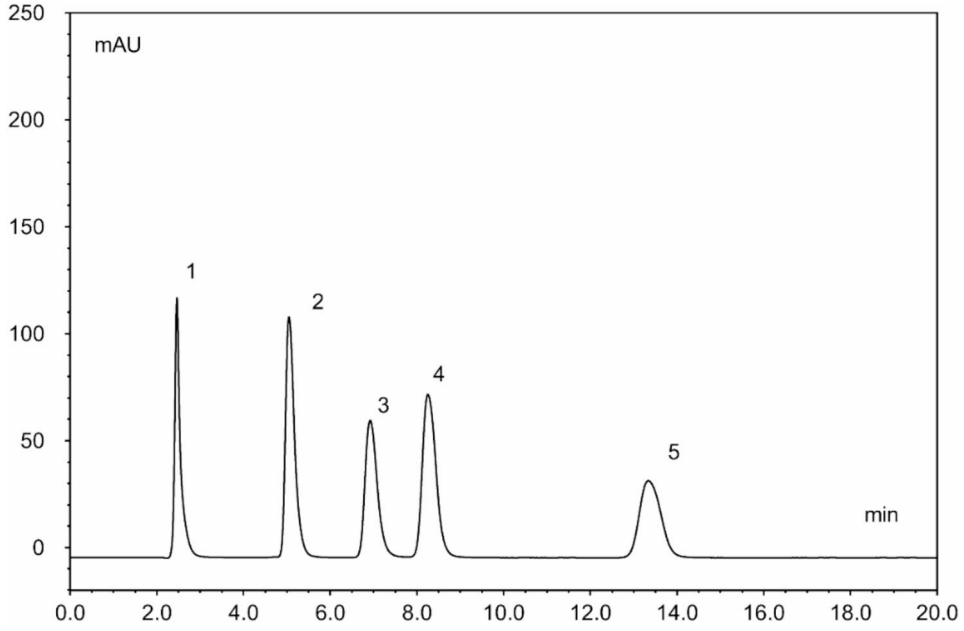

Fig. 2 Chromatogram of the standard drug mixture solution. Note (1) 99.8 μg·mL^{-1} of morinidazole; (2) 102.0 μg·mL^{-1} metronidazole; (3) 102.0 μg·mL^{-1} of tinidazole; (4) 104.0 μg·mL^{-1} of secnidazole; (5) 100.0 μg·mL^{-1} of ornidazole

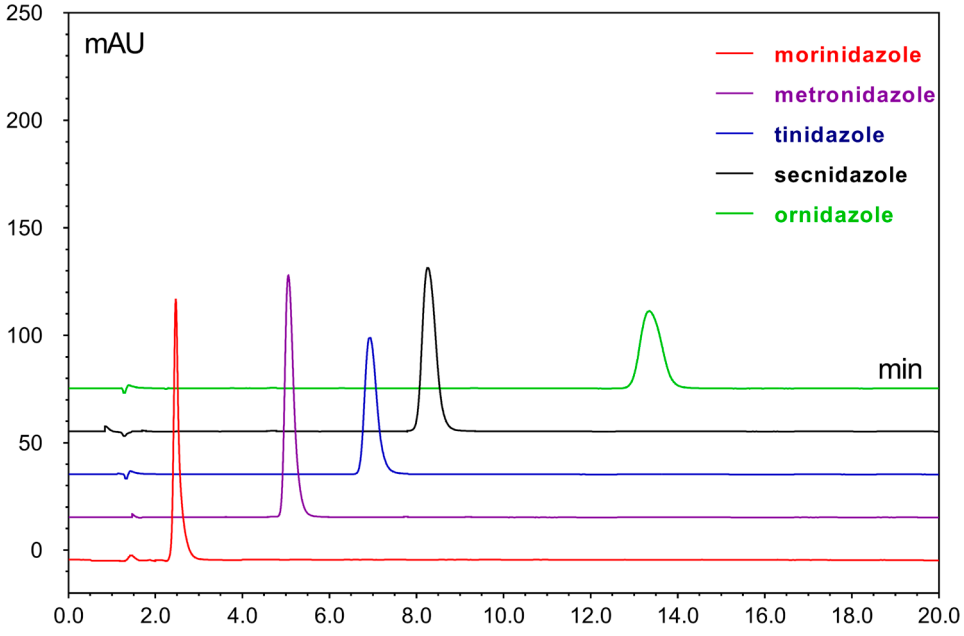

Fig. 3 HPLC Chromatograms of five individual samples. Note (1) 100.6 μg·mL^{-1} of morinidazole; (2) 100.2 μg·mL^{-1} metronidazole; (3) 100.4 μg·mL^{-1} of tinidazole; (4) 104.2 μg·mL^{-1} of secnidazole; (5) 100.4 μg·mL^{-1} of ornidazole

employed to assess the method's functionality and practicality [40, 41].

Results

Methodological verification results of HPLC-ESM method

Under the above chromatographic conditions, the linear range, LOQ, LOD, precision, stability, and recovery of the five nitroimidazole drugs were analyzed and validated. The separation of five nitroimidazole antimicrobials was shown in Figs. 2 and 3. The retention times of morinidazole, metronidazole, tinidazole, secnidazole, and ornidazole were 2.5, 5.1, 7.0, 8.3, and 13.4 min, respectively. The theoretical plate number of each chromatographic peak is greater than 3000, and the separation degree is greater than 1.5. The results showed that the rapid separation of studied analytes could be achieved within 20 min. The

Table 1 System suitability parameters and assay validation parameters for the HPLC-DAD method

Analytical parameter	ornidazole	tinidazole	metronidazole	secnidazole	morinidazole
Retention time(min)	13.4	7.0	5.1	8.3	2.5
Resolution factor (Rs)	6.78	4.29	9.27	2.40	1.96
Asymmetry factor (A)	1.12	1.27	1.30	1.19	1.56
HETP (mm)	0.0389	0.0441	0.0433	0.0401	0.0467
N (EP)	3859	3396	3467	3737	3209
Linear range (μg·mL^{-1})	12.5~200.0	12.75~204.0	12.75~204.0	13.0~208.0	12.48~199.6
Linear equation	Y = 0.8850X + 0.8883	Y = 0.9317X + 0.4143	Y = 1.2085X-5.1059	Y = 1.1587X + 0.4257	Y = 0.7526X + 0.0917
Coefficient of correlation (r^2)	0.9994	0.9963	0.9982	0.9997	1.0000
Quantification limit (μg·mL^{-1})	2.0	1.2	0.8	1.0	0.8
Detection limit (μg·mL^{-1})	0.75	0.4	0.25	0.35	0.3

Reference values: 1: A = 1 for a symmetric peak; 2: Rs > 1.5; 3: N (Theoretical plate number) > 3000; 4: HETP: The smaller the value, the higher the column efficiency

Table 2 Determination results of recovery tests for the five nitroimidazole antimicrobials

Drug name	Concentration tested (μg·mL^{-1})	Recovery %	Precision RSD (%)	
			Intra-day	Inter-day
ornidazole	50.0	99.7		
	100.0	100.3	0.8	1.8
	150.0	101.0		
tinidazole	51.0	98.3		
	102.0	97.9	1.0	1.7
	153.0	99.5		
metronidazole	51.0	102.6		
	102.0	100.4	0.9	1.5
	153.0	101.7		
secnidazole	52.0	98.9		
	104.0	97.8	1.4	1.8
	156.0	99.2		
morinidazole	49.9	97.6		
	99.8	99.8	1.1	1.5
	149.7	97.8		

Reference values: RSD of recovery < 2%, indicating that the methodology had good accuracy

results of the linear range, LOQ, LOD, precision, and recovery tests are shown in Tables 1 and 2. It can be seen from the table that the five nitroimidazole drugs have a good linear relationship within a certain range ($r > 0.999$), and the LOD and LOQ are 0.25–0.75 μg·mL^{-1} and 0.8-2.0 μg·mL^{-1}, respectively. The relative standard deviations (RSDS) of inter-day and intra-day precision and stability tests were all lower than 2%, and the recoveries of studied analytes were between 96.8 and 102.6%, with RSDS lower than 2.0%, indicating that the methodology had good accuracy.

Methodological verification results of QAMS method

Qualitative and quantitative analysis of QAMS method generally adopts spectral similarity, RRT and RCF as indicators. The ultraviolet spectra of the five nitroimidazoles are shown in Fig. 4. The similarity results of the original spectra, first derivative spectra and second derivative spectra of the studied analytes are shown in Tables 3, 4 and 5. The similarity comparison between the first derivative spectrum and the second derivative spectrum magnifies the difference effect of UV spectrum, but the qualitative identification effect is not obvious. As can be seen from Fig. 4, the UV spectral absorption curves of metronidazole, tinidazole, ornidazole, morinidazole and secnidazole are very similar, and it is difficult to distinguish different nitroimidazole drugs by spectral similarity and maximum absorption wavelengths. In terms of RRT calculation, metronidazole was used as the internal standard reference substance, and the RRT of four nitroimidazole drugs, tinidazole, ornidazole, morinidazole and secnidazole were 1.33, 3.66, 2.68 and 2.17, respectively. The effects of RRT on different instruments, columns, mobile phase ratio, column temperature, flow rate and mobile phase pH are shown in Table 6. The RSDs of the above durability tests on RRT are all less than 3%, indicating that the RRT can be used to distinguished of the studied analytes. In the calculation of RCFs, metronidazole was used as the internal standard reference, and the RCF of tinidazole, ornidazole, secnidazole, and morinidazole were 1.184, 1.237, 0.987, and 1.473, respectively.

Determination results of 5 nitroimidazole in pharmaceutical preparations

The contents of the five nitroimidazoles in pharmaceutical preparations were determined by the ESM and QAMS, as shown in Table 7. ESM refers to adding a certain amount of reference substance in the blank solvent according to the gradient to make a control sample, which is processed in parallel with the unknown sample and detected by HPLC. It can be seen that the relative percentage of the above five drugs is between 90 and 110%. ANOVA analysis showed that there was no significant difference between QAMS and ESM at 95% confidence level ($P > 0.05$), indicating that the established method was accurate and reliable.

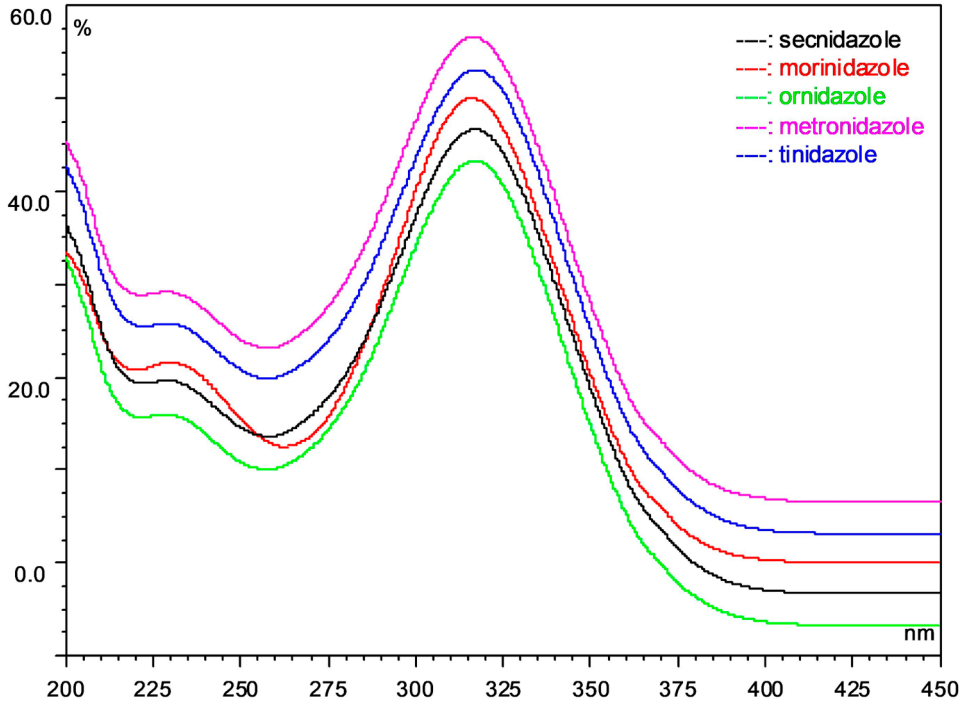

Fig. 4 UV spectra of the five nitroimidazole antimicrobials. Note (1) 99.8 μg·mL^{-1} of morinidazole; (2) 102.0 μg·mL^{-1} metronidazole; (3) 102.0 μg·mL^{-1} of tinidazole; (4) 104.0 μg·mL^{-1} of secnidazole; (5) 100.0 μg·mL^{-1} of ornidazole

Table 3 Original spectra similarity between the five nitroimidazole antimicrobials

Drug name	ornidazole	tinidazole	metronidazole	secnidazole	morinidazole
ornidazole	1.0000				
tinidazole	0.9982	1.0000			
metronidazole	0.9991	0.9956	1.0000		
secnidazole	0.9989	0.9948	0.9999	1.00000	
morinidazole	0.9987	0.9993	0.9958	0.9954	1.0000

Table 4 1st spectra similarity between the five nitroimidazole antimicrobials

Drug name	ornidazole	tinidazole	metronidazole	secnidazole	morinidazole
ornidazole	1.0000				
tinidazole	0.9903	1.0000			
metronidazole	0.9937	0.9736	1.0000		
secnidazole	0.9933	0.9708	0.9997	1.00000	
morinidazole	0.9936	0.9967	0.9761	0.9749	1.0000

Assessment of the proposed method's greenness and blueness

According to the AGREE evaluation tool, the score of QAMS is 0.66, and the score of ESM is 0.59 (Fig. 5A). The proposed QAMS and ESM methods are more environmentally friendly, and the results show that the proposed technology has less impact on the environment. Additionally, the BAGI score of 82.5 assigned to the QAMS and 77.5 assigned to the ESM demonstrated that the methods had good applicability (Fig. 5B).

Discussion

In recent years, nitroimidazole antimicrobials have gained widespread application in the treatment and prevention of diseases affecting humans, poultry, and other species, as well as in animal and plant breeding due to their versatile applications, proven efficacy, and

Table 5 2st spectra similarity between the five nitroimidazole antimicrobials

Drug name	ornidazole	tinidazole	metronidazole	secnidazole	morinidazole
ornidazole	1.0000				
tinidazole	0.9851	1.0000			
metronidazole	0.9955	0.9694	1.0000		
secnidazole	0.9949	0.9653	0.9994	1.00000	
morinidazole	0.9911	0.9966	0.9783	0.9756	1.0000

Table 7 Results of the comparison of the five nitroimidazole antimicrobials samples

Drug name	the QAMS methods		the ESM methods	
	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Metronidazole injection	101.3	0.8	99.8	1.2
Ornidazole injection	98.5	0.9	98.2	0.7
Tinidazole injection	95.7	0.6	96.3	0.8
Morinidazole injection	98.8	1.4	99.4	0.5
Secnidazole tablets	99.4	1.2	101.1	1.5

cost-effectiveness [42]. However, drug residues found in animal and plant tissues pose significant risks to food safety. Meanwhile, the illegal addition of nitroimidazole in proprietary Chinese medicines, health products and cosmetics is harmful to human health. At present, the development and application of rapid drug testing technology is progressing rapidly. With the advantages of fast analysis speed, good selectivity, good reproducibility and wide application, QAMS method combined with HPLC rapid detection technology has been widely used in the qualitative and quantitative analysis of chemical drugs, Chinese medicinal materials and decoction pieces, and various active ingredients or related substances in traditional Chinese medicine preparations [33–38]. Consequently, the development of analysis methods for nitroimidazole plays an important role in the qualitative and quantitative studies of pharmaceutical preparations, drug interactions, pharmacokinetics, therapeutic drug monitoring, stability, drug residues and illegal additions.

As the chemical structures and physicochemical properties of the five nitroimidazole drugs are very similar, in order to effectively separate these compounds, methanol water was first used as the mobile phase in our preliminary experimental study after reviewing literature and the Chinese Pharmacopoeia [15–17, 20–22, 43]. The results showed that metronidazole and tinidazole were not completely separated and the peak shape of morinidazole was poor. Moreover, continuous optimization of mobile phase ratio has not solved the problem. Then the aqueous solution containing triethylamine as the mobile phase can effectively improve the separation effect. In this study, the effects of different concentrations of triethylamine aqueous solution (0.05-0.2%) on the separation were investigated, and the results showed that the aqueous solution containing 0.1% triethylamine obtained better results. In order to further optimize the chromatographic conditions, the ratio of triethylamine aqueous solution and mobile phase at different pH (3.0–6.0) was investigated. The results showed that the chromatographic separation of the studied analyte was best when methanol – 0.1% triethylamine solution (pH adjusted to 3.0 by phosphoric acid) was used (26:74).

The traditional HPLC qualitative identification method is usually compared with the retention time of reference standard, but it is greatly affected by the instrument, column and flow equality, and the reproducibility is poor. Especially for compounds with similar physical properties or structures, it is difficult to achieve accurate characterization. In order to improve the accuracy of qualitative identification, UV spectral similarity was first adopted as one of the indicators for identification. However, the structures of the studied analytes were too similar, and there was little difference in their original UV spectral similarity. Generally speaking, the spectra of compounds will show more characteristic details than the original spectra after derivation, so the microscopic differences between the spectra will be more obvious and easier to distinguish. The UV spectra of 5 nitroimidazoles were treated with first and second derivatives, and

Table 6 Effects of different instruments, columns, column temperatures, flow rates, volume and pH on RRTs ($n = 3$)

	Effects factor	morinidazole	tinidazole	metronidazole	ornidazole	secnidazole
RSD %	Chromatographic Columns	2.54	1.56	-	1.73	2.05
	HPLC Instruments	1.39	1.20	-	0.87	1.29
	Column Temperatures	1.26	0.34	-	0.72	0.32
	Flow Volume of Mobile Phase	2.15	1.60	-	0.97	0.58
	Flow Rate of Mobile Phase	1.62	0.48	-	1.14	0.80
	pH Value of Mobile Phase	2.78	1.71	-	0.95	1.32

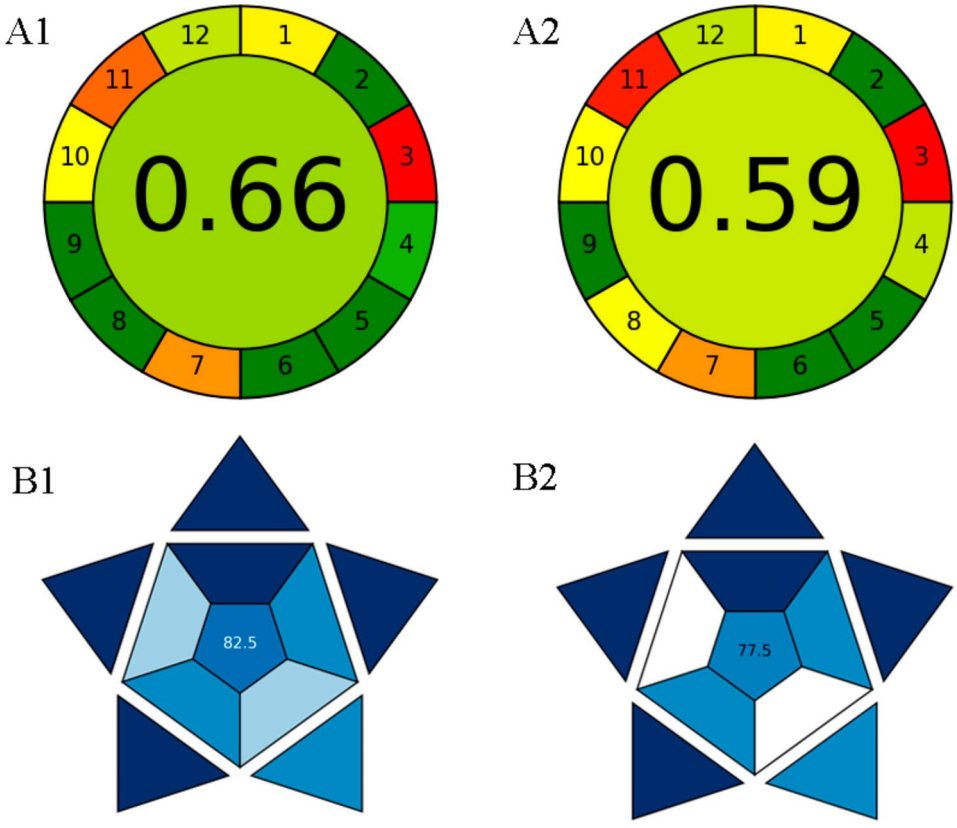

Fig. 5 Score of greenness and blueness. Note A1: Greenness of QAMS, A2: Greenness of ESM, B1: Blueness of QAMS, B2: Blueness of ESM

different spectra and spectra were obtained. In order to overcome the above disadvantages, the RRT was adopted as the qualitative method in this experiment. By correcting the dead volume of the index, the reproducibility of the data is improved, so as to achieve the purpose of accurate qualitative.

According to the literature, numerous studies reported the determination of the five nitroimidazole antimicrobials by HPLC alone. Three HPLC methods were developed to determine two components of nitroimidazole drugs simultaneously in pharmaceutical formulations and bioequivalence studies [15–17]. In addition, only one method was identified for quantifying four nitroimidazole drugs in human saliva [21]. Compared with the above quantitative analysis methods, the QAMS method adopted in this study uses metronidazole, a commonly used, inexpensive and stable reference substance, as the reference material, and can rapidly and simultaneously detect the contents of other four nitroimidazole drugs through the RCF. There was no significant difference between QAMS method and HPLC external standard method. The analysis method established in this research is accurate, can greatly simplify the operation process, shorten the detection time, and improve the detection efficiency.

However, the proposed methods possess certain limitations. The qualitative and quantitative parameters of QAMS, including RRT and RCF, are influenced by the column and mobile phase composition. Therefore, in the qualitative and quantitative analysis of nitroimidazole drugs using the QAMS method, it is imperative to optimize and define the chromatographic conditions. Additionally, the proposed method may be susceptible to factors such as changes in manufacturer, production process, and the composition of pharmaceutical excipients. Consequently, in subsequent stages, the developed method will be employed to determine the content, and further exploration will be conducted to assess the universality and practicability of the method. Moreover, efforts will be made to reduce the amount of mobile phase used and employ bio-based organic solutions to minimize environmental impact.

Conclusions

In summary, HPLC-ESM and HPLC-QAMS methods have been developed for the simultaneous determination of five nitroimidazole in pharmaceutical preparations. Notably, the QAMS method requires only one standard reference to achieve qualitative and quantitative analysis of multiple drugs. In the HPLC-QAMS process, metronidazole was used as an internal reference to calculate

the relative correction factor of the other four studied analytes, and the influencing factors such as different chromatographic columns, instruments, column temperatures, mobile phases, and pH value of mobile phases were investigated. Five compounds of different chemical structures and medicinal uses were effectively separated and accurately quantified in a run time less than 20 min, and through the results of greenness and blueness, the described methods can be considered cost- and time-effective. The method can satisfy the rapid detection and stability research of pharmaceutical preparations industrial settings, while also serving as a valuable technical reference for pharmacokinetic research, therapeutic drug monitoring, and illegal drug addition.

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Author contributions
Baoxia Fang and Dan Jiang: Formal analysis, Investigation, Software, Data curation, and Writing the original draft. Fuchao Chen and Sicen Wang: Methodology development, Validation, Investigation, and Validation and Reviewing the original draft. All authors read and approved the final manuscript.

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Data availability
The data used and/or analyzed during this study are available from the corresponding author on a reasonable request.

Declarations

Ethical approval
Not applicable.

Consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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