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New analytical LC–MS/MS method for fluconazole and ivermectin estimation in combined pharmaceutical dosage form: development and validation

Popat Mohite[1*], Satish Balasaheb Nimse[2*], Jomon George Joy[2], Rohini Kulkarni[3], Ramdas Pandhare[3] and Anil Pawar[3]

Abstract

Background Fluconazole, an antifungal drug, prevents fungi growth by inhibiting the formation of a protective covering. Ivermectin has several biological activities, such as antibacterial, antiviral, and anti-cancer characteristics, and offers various therapeutic outcomes. There are several commercial products containing these two drugs. Therefore, developing a method that can allow the simultaneous estimation of Fluconazole and ivermectin is inevitable to monitor them in commercial dosage forms. The hyphenated methodology that combines spectroscopic and chromatographic techniques is gaining high interest in the pharmaceutical industry. Consequently, the objective of present research work was to investigate robust and sensitive LC–MS/MS avenue for simultaneous determination of Fluconazole and ivermectin in pure material and combined dosage form.

Results The simultaneous quantification of Fluconazole and ivermectin in tablet dosage form has been developed and validated using a straightforward, sensitive, practical, and repeatable LC–MS/MS approach. The separation was performed using a C_{18} (150 × 4.6 mm) column, injection volume of 10 µL, and elution with acetonitrile: formic acid at a ratio of 70:30, with the column temperature at 30 °C, and a flow rate of 4.0 mL/min. The retention times of Ivermectin and Fluconazole were 1.10 min and 1.05 min, respectively. The calibration curves for Fluconazole and ivermectin demonstrated significant linearities indicated by the correlation coefficients ($r^2 = 0.999$ and $r^2 = 0.997$) and precision (% R.S.D. of 1.58 and 1.13). The linear correlation between peak area and concentration allowed high percentage recoveries of 98.5%–99.4% and 97.8%–99.3% for Fluconazole and Ivermectin, respectively. The L.O.D.s for Fluconazole and ivermectin were found to be 0.0034 and 0.074 g/mL, respectively. The L.O.Q.s for Fluconazole and ivermectin were 0.010 and 0.225 g/mL, respectively.

Conclusion All the analytical parameters were identified and found to be within the acceptable range set forth by the ICH guidelines, demonstrating the devised method's acceptability in the simultaneous detection and estimation of Fluconazole and ivermectin in the commercial dosage forms.

Keywords Fluconazole, Ivermectin, LC–MS/MS, ICH, Validation

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Background

Fluconazole, an antifungal drug, prevents fungi growth by inhibiting the formation of a protective covering [1]. Ivermectin has several biological activities, such as antibacterial, antiviral, and anti-cancer characteristics, and offers various therapeutic outcomes [2]. There are several commercial products containing these two drugs. Therefore, developing a method that can allow the simultaneous estimation of Fluconazole and ivermectin is inevitable to monitor them in commercial dosage forms. The hyphenated methodology that combines spectroscopic and chromatographic techniques is gaining high interest in the pharmaceutical industry [3, 4]. In the hyphenated method, the chemical components are isolated from mixtures using chromatography, and the separated compounds are identified using the spectroscopic method [5]. Nowadays, hyphenated methods are frequently employed to address challenging analytical issues [6]. Analytical methods such as UV spectroscopy [7], HPLC (High-Performance Liquid Chromatography), HPTLC (High-Performance Thin Layer Chromatography) and LC/MS (Liquid Chromatography/Mass spectroscopy) are used for the estimation of drugs regularly. More people have utilized LC–MS/MS (Liquid chromatography-Mass spectroscopy/ Mass spectroscopy) than LC/NMR. The hyphenated method need not always be between two procedures; it can also incorporate more than one method of separation or detection, such as LC–MS/MS, LC-NMR/MS (Liquid chromatography-Nuclear Magnetic Resonance spectroscopy/ Mass spectroscopy), or LCPDA-NMR/MS [8]. The reliable analytical method known as liquid chromatography-mass spectroscopy (LC–MS) has extremely high sensitivity and specificity. LC–MS–MS is a combination of liquid chromatography (L.C.), which allows for the separation of components, and mass spectrometry (M.S.), which allows the detection, identification, and measurement of component masses even in the presence of other components [9]. The LC–MS/MS is a hyphenated method in which LC and Mass spectroscopy with two mass analyzers, whereas LC–MS instruments are essentially HPLC units and Mass spectroscopy with a single mass analyzer. The sample components are separated using liquid chromatography (LC), and the divided sample species are sprayed into an ion source at atmospheric pressure, changing them into ions in the gas phase. Ions are sorted using the mass analyzer based on their mass-to-charge ratio. The detector may additionally magnify the signal produced by each ion as it emerges from the mass analyzer. As a result, a mass spectrum is produced, which can be used to identify the elemental or isotopic composition of a sample, as well as the masses of particles and molecules, as well as to clarify the chemical structure of molecules [10]. HPLC-Q-TOF–MS/MS can be also used for metabolomics and the separation of compounds [11]. Sottani et al. has reported bioanalytical method UHPLC-MS/MS method for estimation of drugs [12].

According to Fig. 1, Fluconazole is chemically known as 2-(2,4-difluorophenyl)-1,3-bis(1H-1,2,4-triazol-yl) propan-2-ol (Fig. 1a) [13]. Antifungal drugs such as synthetic triazole derivatives have been demonstrated effective against various systemic and superficial fungal infections. It possesses advantageous pharmacological characteristics, such as an extended half-life and the flexibility to be taken parenterally or orally. Like other antifungals of the imidazole and triazole classes, Fluconazole inhibits the cytochrome P450 enzyme 14-demethylase. Because it can pass the blood–brain barrier, Fluconazole has a significant advantage over other antifungals [14].

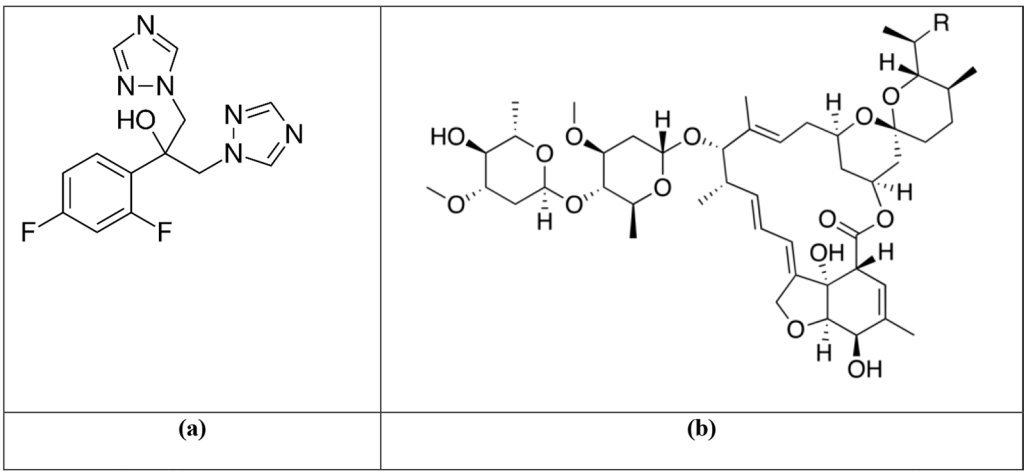


Fig. 1 Chemical structures of **a** Fluconazole, **b** ivermectin

Ivermectin is a broad-spectrum antiparasitic drug [15]. Ivermectin is chemically a (1R,4S,6R,10E,14E,16E,21R)-6'-Butan-2-yl-21,24-dihydroxy-12-[(2R,4S,6S)-5-[(2S,4S,6S)-5-hydroxy-4-methoxy-6-methyloxan-2-yl] oxy-4-methoxy-6-methyloxan-2-yl]oxy 5',11,13,22tetra methylspiro[3,7,19trioxatetracyclo[15.6.1.14,8.020,24] pentacosa-10,14,16,22-tetraene-6,2'-oxane]-2-one (Fig. 1b). Ivermectin binds specifically with high affinity to the glutamate-gated chloride ion channels in the microfilaria's invertebrate muscle and nerve cells. As a result of this binding interaction, the cell membrane becomes more permeable to chloride ions and becomes hyperpolarized, which paralyzes and eventually kills the parasite. Ivermectin has long been used to treat worm infections. It is also used in veterinary treatment [16].

Analytical method validation guarantees that LC–MS/MS analytical techniques produce reliable and repeatable results. It is an essential step in developing new dosage forms because it provides details about the accuracy, precision, linearity, detection and quantitation limits, and robustness [17]. ICH guideline states that the objective of validating an analytical procedure is to show that it is suitable for its intended purpose. Presently, providing authorities with the validation data is a requirement during the medication development process. ICH and U.S.P. guidelines are among the rules for validating analysis methods [18].

A review of the literature revealed that the UV–Vis spectrophotometric method [19, 20], HPLC [21], RP-HPLC [22–24], UPLC [25], Gas Chromatography [26], and other analytical methods [26] are a few techniques used for determining the dosage of fluconazole and ivermectin either alone or in combination with other medications. However, to our knowledge, the LC–MS/MS technique has never been used to detect Fluconazole and ivermectin in the tablet dosage form. To simultaneously estimate these two medications in tablet dosage forms, the LC–MS/MS method is developed and validated in this work.

Methods

Chemicals

The Fluconazole and Ivermectin used in the study were obtained from The Drug Product of India (Mumbai, India) and Shree Chem Pharmaceuticals Limited (Mumbai, India). The Nuforce-Plus tablet (Mankind Pharma Pvt Ltd., Delhi, India) that contains 6 mg of ivermectin and 150 mg of Fluconazole was also procured as a research sample. Methanol, formic acid, and acetonitrile of HPLC grade solvents were procured from Merck (Mumbai, India).

Instrumentation

ACQUITY Ultra Performance L.C. (Waters Corporation, Wilmslow, United Kingdom) and Quattro Premier XE (Waters Corporation, Wilmslow, United Kingdom) were used for chromatography and mass analysis, respectively. The simultaneous use of ACQUITY Ultra Performance L.C. and Quattro Premier XE mass spectrometer allowed us to develop and validate a method for determining fluconazole and ivermectin doses in tablet dosage form by LC–MS/MS technique. The device has an isocratic elution mode and uses a Synergi C18 (150 × 4.6 mm × 4.0 µm) column with a flow rate of 0.4 mL/min and a mobile phase of acetonitrile: formic acid (70:30).

Standard stock solution preparation

In a 10.0 mL volumetric flask, 10 mg of Fluconazole and 1 mg of Ivermectin are mixed and diluted with methanol to a concentration of 1 mg/mL. To create a mixed 10 ppm solution with diluents methanol: water, 0.100 mL of Fluconazole standard solution and 0.100 mL of ivermectin standard solution were added to a 10 mL volumetric flask (50:50). A 100% solution (1 ppm/1.001 ppb) was prepared by taking 0.1 mL of the stock as mentioned above solution and diluting it to 1.0 mL. To prepare a 150% (1.5 ppm/1500 ppb) solution, 0.150 mL of the stock solution was diluted to 1.0 mL.

Preparation of calibration curve

The various concentrations (100, 250, 500, 750, 1000, and 1250 ppb) of Fluconazole and Ivermectin were prepared in the mobile phase and subjected to LC–MS/MS. Peak areas at a particular retention time observed in LC–MS/MS spectra were used to obtain the respective calibration curves.

Preparation of the sample solution

Five tablets were firstly weighed and then finely powdered, an amount of powder equivalent to 150 mg of Fluconazole and 12 mg of Ivermectin was weighed precisely and transferred to a 10 mL volumetric flask. The required amount of methanol was added to make up the volume, the solution was sonicated for 15 min and filtered using a 0.45 µm nylon syringe filter. From the filtrate, the measured volume was taken and diluted with the diluent to achieve the final concentrations of 120 ppb of Fluconazole and 1500 ppb of Ivermectin.

Optimized chromatographic condition

The analysis was performed with the help of ACQUITY Ultra Performance L.C. with an ionization detector.

The chromatographic conditions were achieved using Synergi C18 (150×4.6 mm×4.0 µm) column with a flow rate of 0.400 mL/min. The isocratic mobile phase of acetonitrile: formic acid (70:30) was utilized to carry out the separation. The column temperature was maintained at 30 °C. The injection volume was kept at 10 µL. Detection was carried out using the multiple reaction monitoring (MRM) modes to measure the transition pair (precursor to product ion) of m/z 307–.70 for The molecular ion peak ($[M+H]^+$) corresponding to Fluconazole was observed at 307 g/mol which has highest mass to charge ratio. Similarly, the molecular ion peak ($[M]^+$) corresponding to Ivermectin was observed at 897 g/mol.

Results

Method development

Various physical and chemical characteristics of ivermectin and Fluconazole are listed in the literature. The LC–MS/MS method was chosen to determine the initial chromatographic parameters such as the M.S. spectra, mobile phase, stationary phase, and sample preparation process. The ratio and the total number of trials were changed across a series of tests. Synergi polar C18 (150×4.6 mm×4.0 µm) column was used for the highest quality chromatographic results. Acetonitrile and formic acid, in a 70:30 ratio, make up the mobile phase. The mobile phase was supplied at a rate of 0.400 mL/min. 10 µL of the prepared sample was used as the injection volume for the chromatography. The column was maintained at 30 °C, while the autosampler was maintained at 15 °C. The results obtained under these conditions are presented in Fig. 2.

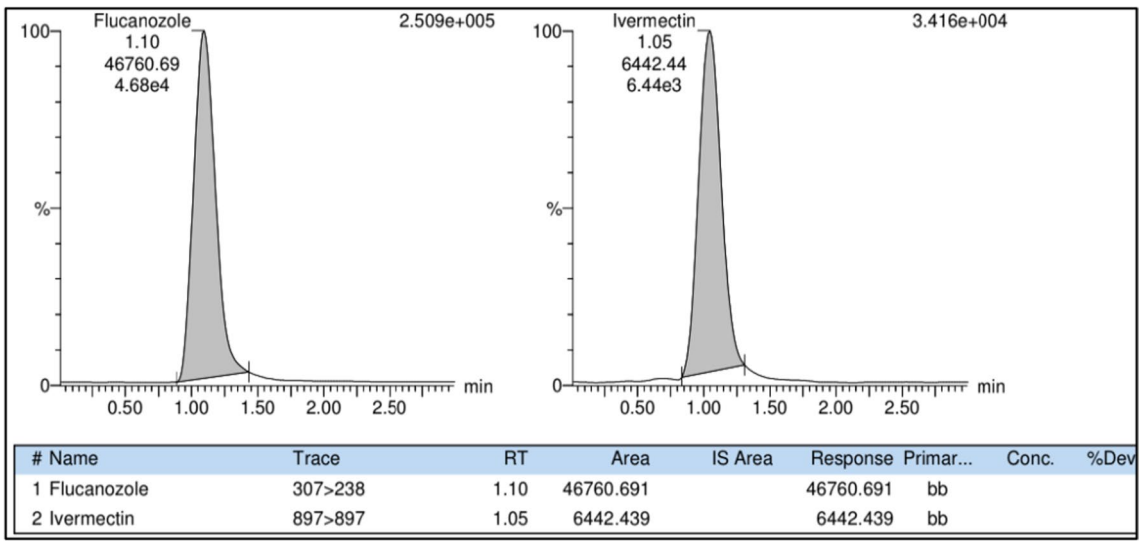
As shown in Fig. 2, retention times for Fluconazole and ivermectin were 1.10 and 1.05, respectively. The mass spectra of isolated Fluconazole and Ivermectin relevant to their specific retention times are displayed in Fig. 3a, b, respectively. The molecular ion peak ($[M+H]^+$) corresponding to Fluconazole was observed at 169 g/mol which has the highest mass-to-charge ratio. Similarly, the molecular ion peak ($[M+H_2O+4H]^+$) corresponding to Ivermectin was observed at 897 g/mol. Thus, we used the LC–MS/MS data to determine the area under the curve corresponding to the increasing concentrations (100, 250, 500, 750, 1000, and 1250 ppb) of Fluconazole and Ivermectin.

Method validation

The method was validated by following ICH guidelines, and the metrics for validation included robustness, specificity (L.O.Q. and L.O.D.), linearity, range, accuracy, and precision [27].

Linearity

The peak area obtained from the LC–MS/MS analysis for Fluconazole and ivermectin with the concentrations of 100, 250, 500, 750, 1000, and 1250 ppb were used to construct a calibration curve as presented in Fig. 4a, b (Additional file 1: Tables S1, S2, Figures S1, S2). Peak areas were plotted against corresponding concentrations, and the resulting curve was subjected to a linear regression analysis. The determination coefficients for linear relationship were found to be (r^2) of 0.999 and 0.998 for Fluconazole and ivermectin, respectively. Obtained results demonstrate high linearity in the peak area and increasing concentrations of Fluconazole and ivermectin.



#	Name	Trace	RT	Area	IS Area	Response	Primar...	Conc.	%Dev
1	Flucanozole	307>238	1.10	46760.691		46760.691	bb		
2	Ivermectin	897>897	1.05	6442.439		6442.439	bb		

Fig. 2 MRM chromatograms of fluconazole and ivermectin

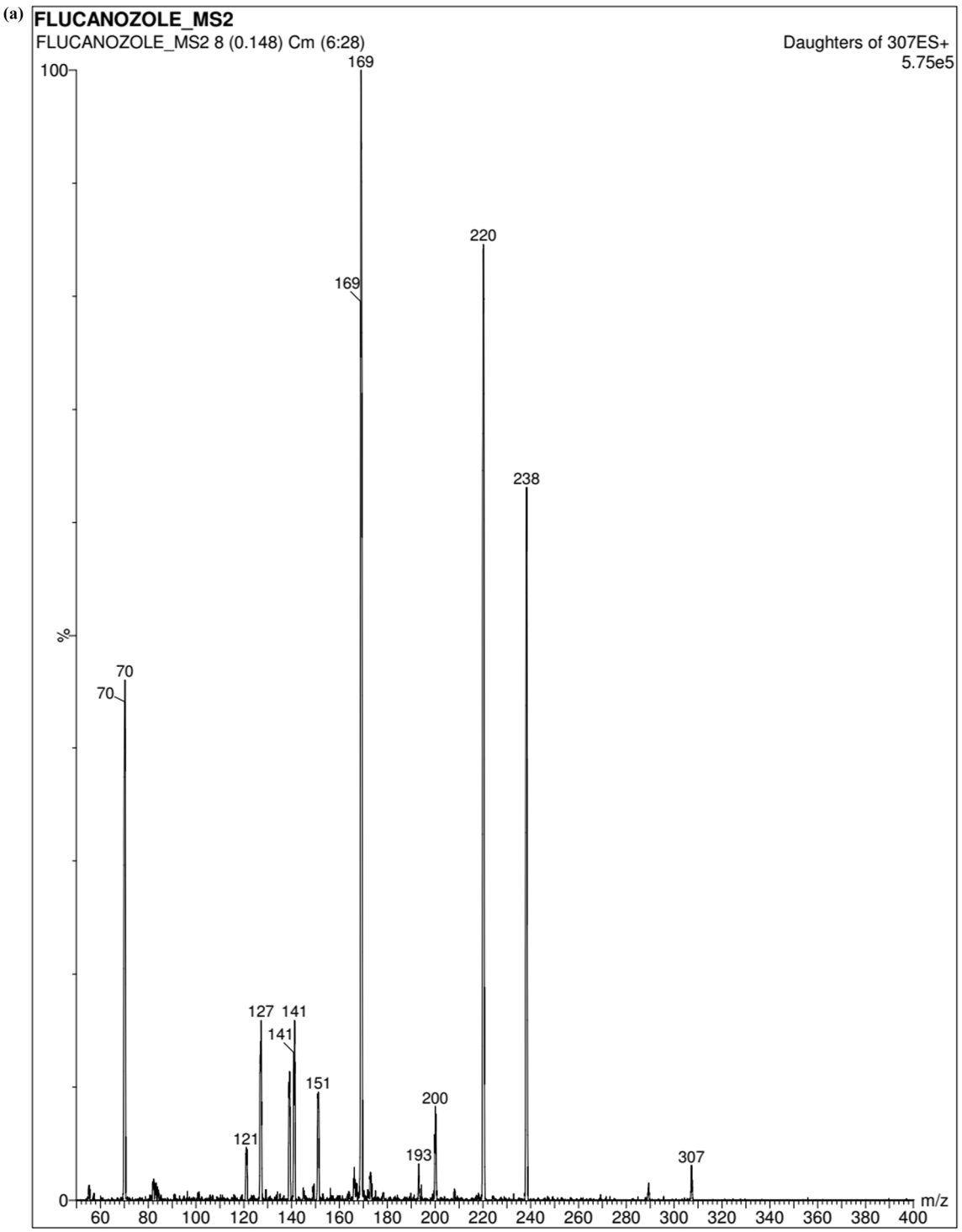


Fig. 3 Full scan mass spectra **a** fluconazole, **b** ivermectin

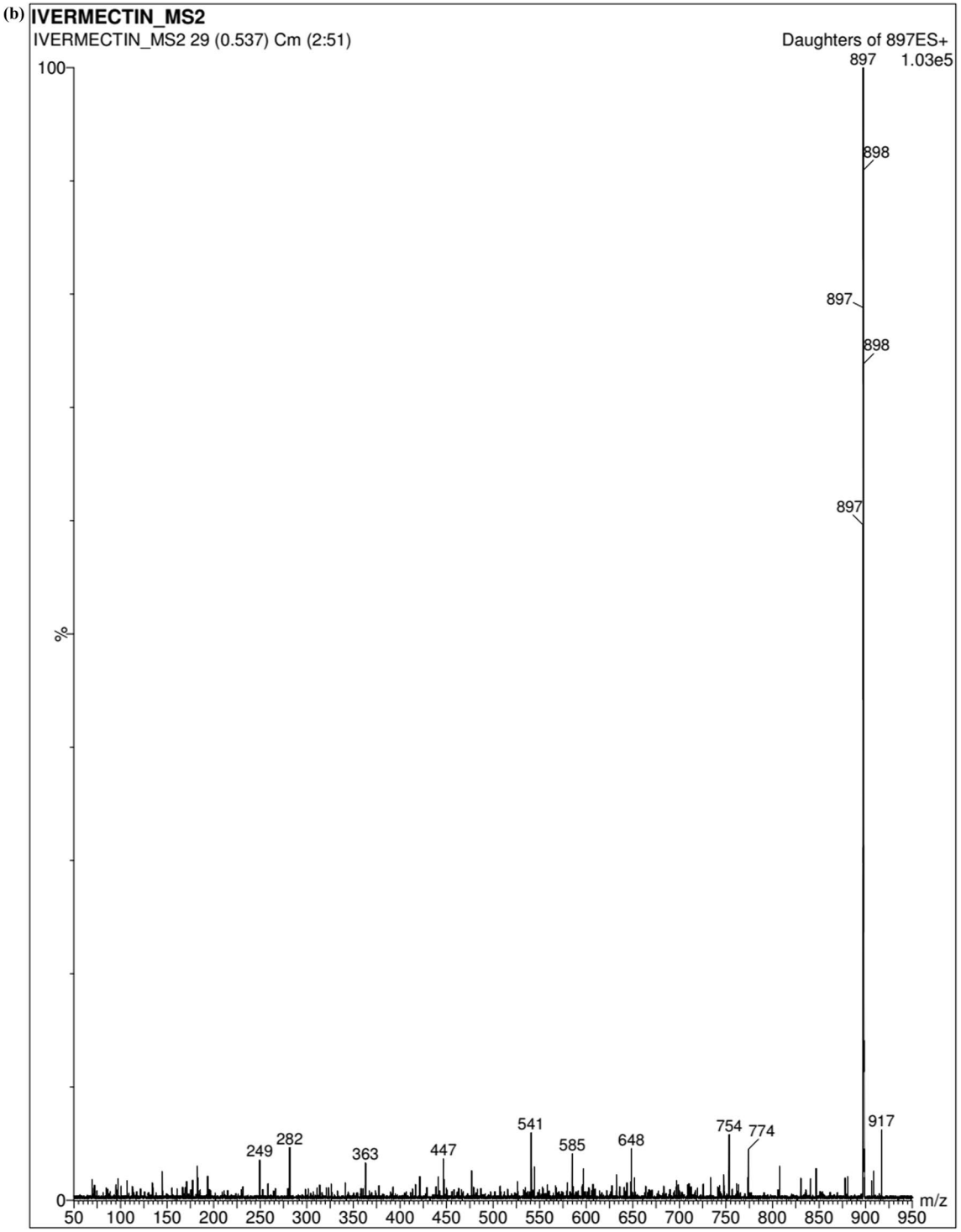


Fig. 3 continued

Precision

The method's precision was ascertained by examining the repeatability and intermediate precision of the data. Inter-assay precision is also called repeatability. Therefore, the method's precision was determined by analyzing five samples of Fluconazole (100 ppb) and ivermectin (100 ppb). The calibration curves presented in Fig. 4a, b were used to determine the concentrations. The

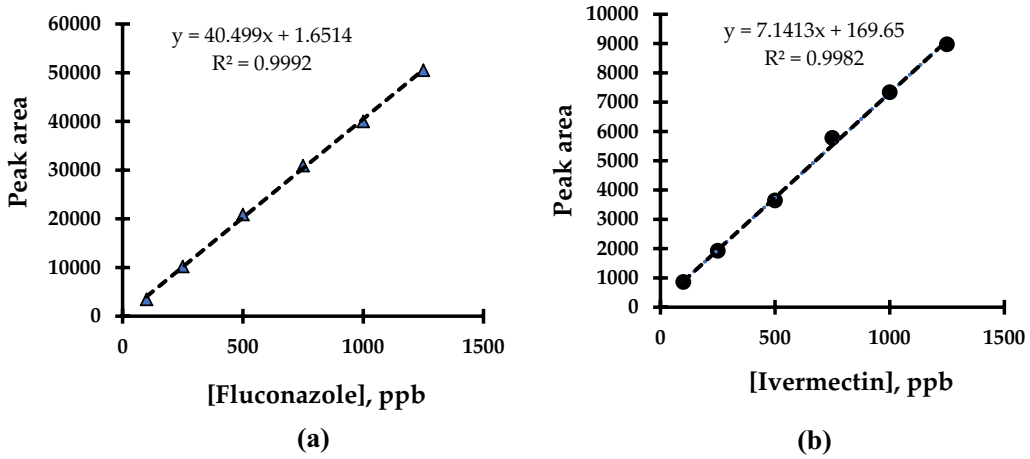


Fig. 4 Standard curve of **a** fluconazole and **b** ivermectin

Table 1 Precision results of fluconazole

Sample	Retention time (min)	Area	Concentration (ppb)
Fluconazole 100 ppb	1.10	3962.502	98
	1.10	4015.604	99.8
	1.10	3846.246	95.7
	1.10	3952.932	97.2
	1.10	3908.369	96.7
		Mean	97.5
		SD	1.54
		%RSD	1.58

Table 2 Precision results of ivermectin

Sample	Retention time (min)	Area	Concentration (ppb)
Ivermectin 100 ppb	1.05	790.37	95.4
	1.05	786.37	94.5
	1.05	798.769	95.8
	1.05	810.425	96.8
	1.05	812.044	97.2
		Mean	95.9
		SD	1.08
		%RSD	1.13

obtained results for the precision study are presented in Tables 1 and 2.

The mean, standard deviation, and % R.S.D. for Fluconazole were 97.5 ppb, 1.54, and 1.58, respectively. Similarly, the mean, standard deviation, and % R.S.D. for Ivermectin were 95.9 ppb, 1.08, and 1.13, respectively. The % R.S.D. of 1.58 and 1.13 for fluconazole ad ivermectin indicate the high precision of the developed method.

Accuracy

Accuracy was shown as the percentage of nominal concentration. The developed method's accuracy or recovery was assessed by doing recovery experiments at 50% and 150% of the anticipated assay value in the tablet dosage form. At each step, the drug recovery % was calculated. Each experiment was replicated thrice. The extraction recovery was calculated as a percentage by comparing the peak area in spiked concentration fortified with the known concentration samples. As shown in Table 3 and Table 4, the percentage recoveries for Fluconazole and ivermectin were determined to be 98.47–99.42% and 97.78–99.30%, respectively. The % R.S.D. in the range of 0.018–0.023 and 0.016–0.038 for fluconazole ad ivermectin indicate the high accuracy of the developed method.

L.O.D. and L.O.Q.

The L.O.D. and L.O.Q. for quantification of Fluconazole and ivermectin using the proposed method were determined by following IUPAC recommended Eqs. 1 and 2, respectively.

$$\mathrm{LOD} = \frac{3\sigma}{m} \tag{1}$$

$$\mathrm{LOD} = \frac{10\sigma}{m} \tag{2}$$

where σ is the standard deviation of blank samples (n = 10) and m is the slope of a calibration curve.

The L.O.D. and L.O.Q. for the detection and quantification of Fluconazole were found to be 0.0034 g/mL and 0.010 g/mL, respectively. The L.O.D. and L.O.Q. for detecting and quantifying ivermectin were found to be 0.074 g/mL and 0.225 g/mL, respectively.

Table 3 Accuracy results of fluconazole at various levels

Accuracy level	Area	Standard concentration (ppb)	Spike concentration (ppb)	% Recovery	Average %	SD	%RSD
50%	18,489.131	500	493.65	98.73	98.47	0.017	0.018
	19,015.820	500	490.6	98.12			
	19,154.805	500	492.85	98.57			
100%	38,003.746	1000	991.54	99.15	99.10	0.021	0.023
	36,333.129	1000	988.88	98.89			
	36,735.425	1000	992.55	99.26			
150%	56,544.449	1500	1488.85	99.26	99.42	0.017	0.018
	58,623.719	1500	1488.3	99.22			
	57,874.391	1500	1496.9	99.79			

Table 4 Accuracy results of ivermectin at various levels

Accuracy level	Area	Standard concentration (ppb)	Spike concentration (ppb)	% Recovery	Average %	SD	% RSD
50%	2061.290	500	487.95	97.59	97.78	0.015	0.016
	2435.510	500	488.5	97.70			
	2538.449	500	490.25	98.05			
100%	6382.333	1000	992.24	99.22	99.20	0.030	0.031
	5860.981	1000	990.6	99.06			
	6588.533	1000	993.2	99.32			
150%	9307.624	1500	1490.8	99.39	99.30	0.036	0.038
	10,646.158	1500	1485.3	99.02			
	10,829.113	1500	1492.5	99.50			

Table 5 Assay of Fluconazole and ivermectin in a tablet dosage form

Formulation	Drug	Claimed conc (%)	Recovery (% ± S.D.)
Nuforce plus	Fluconazole	100	99.18 ± 0.33
	Ivermectin	100	99.02 ± 0.36

Table 6 System suitability parameters

Parameters	Fluconazole	Ivermectin
Flow rate (mL/min)	0.400	0.400
Retention time (min)	1.10	1.05
Peak area	46,760.69	6442.63
Trace	307 > 238	897 > 897
Linearity (ppb)	100–1250	100–1250
MRM (M+)	169	897
LOD (g/mL)	0.0034	0.010
LOQ (g/mL)	0.074	0.225

Application to the pharmaceutical dosage form

We evaluated the applicability of the proposed method to simultaneously quantify the Fluconazole and Ivermectin in the pharmaceutical formulation using commercially available Nuforce Plus tablets. As shown in Table 5, the percent recoveries of Fluconazole and ivermectin were found to be 99.2% and 99.0%, respectively. It is important to note that the permissible range is 100 to 102%. Thus, these results indicate that the method proposed here can simultaneously quantify Fluconazole and ivermectin in pharmaceutical dosage forms.

System suitability

The results of optimized system suitability parameters are shown in Table 6. The system suitability parameters agree with the ICH guidelines.

Discussion

LC–MS/MS method is an effective hyphenated technique that can detect and quantify active pharmaceutical ingredients in pharmaceutical formulations. The combination of LC–MS with various spectrophotometric methods, such as UV–Vis, RP-HPLC, etc., increases assay sensitivity [18–23, 25, 26, 28]. The high sensitivity and specificity of LC–MS/MS can provide adequate component separation and better chemical structure clarity LC–MS/MS methodology has been widely used in the last decades in determining environmental pollutants [29, 30]. Several authors have reported the determination of the drugs, small molecules, pesticides, and even biomarkers using LC–MS/MS.

Our research group innovated the incorporation of the LC–MS/MS technique to quantify Fluconazole and ivermectin in pharmaceutical formulations simultaneously. The proposed LC–MS/MS method allowed us to quantify Fluconazole and ivermectin in the commercially available tablet dosage form. The standard stock solutions of Fluconazole and ivermectin were prepared in a methanol solution. Samples were injected in LC–MS/MS instrument with the mobile phase of acetonitrile and formic acid in a ratio of 70:30. Fluconazole and ivermectin showed a retention time of 1.10 and 1.05 min, respectively, at various concentrations. As the concentration of both drugs increased, the area under the curve also increased linearly. At a particular concentration, Fluconazole and ivermectin (100 ppb) showed retention of 1.10 and 1.05 repeatedly with an almost similar area under the curves. The % R.S.D. of 0.033 and 0.051 for Fluconazole ad ivermectin indicate the high precision of the developed method. The accuracy of the proposed LC–MS/MS method was found to be satisfactory and was in the acceptable range, with the % R.S.D. in the range of 0.018–0.023 and 0.016–0.038 for Fluconazole and ivermectin, respectively, indicating that the proposed method shows very high accuracy. Fluconazole's recovery percentage was 98.5–99.4%, and Ivermectin was 97.8–99.3%. The developed method shows all the values of validation within the acceptable range as per the ICH guidelines. Hence, we believe that the proposed LC–MS/MS technique is very effective in simultaneously detecting Fluconazole and ivermectin in pharmaceutical formulations.

Conclusion

To the best of our knowledge, LC–MS/MS method presented here is the first-ever report on the simultaneous detection and quantification of Fluconazole and ivermectin in pharmaceutical formulations. Results of the study indicate that the presented method shows high sensitivity, accuracy, and precision in the simultaneous detection of Fluconazole and ivermectin. The commercially available pharmaceutical dosage form was successfully subjected to the validated LC–MS/MS technique. Quality control laboratories can easily employ the devised method for the recently approved Nuforce Plus tablets. Further, the proposed method can be of interest to analysts in the field of drug control. The presented method has a high potential for further development to simultaneously detect more than two drugs in pharmaceutical formulations. We are also looking for its applications in pharmacokinetic research.

Abbreviations

HPLC	High Performance Liquid Chromatography
HPTLC	High Performance Thin lLayer Chromatography
LC/MS	Liquid chromatography/mass spectroscopy
LC–MS/MS	Liquid chromatography–mass spectroscopy/mass spectroscopy
ICH	International conference on harmonization
R.S.D.	Relative standard deviation
U.S.P.	United state Pharmacopoeia

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43094-023-00497-x.

Additional file 1. **Table 1:** Linearity of Fluconazole. **Table 2:** Linearity of Ivermectin. **Figure S1.** Retention time and peak area of fluconazole at a) 100 ppb, b) 250 ppb, c) 500 ppb, d) 750 ppb, e) 1000 ppb, and f) 1250 ppb concentrations. **Figure S2.** Retention time and peak area of ivermectin at a) 100 ppb, b) 250 ppb, c) 500 ppb, d) 750 ppb, e) 1000 ppb, and f) 1250 ppb concentrations.

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Author contributions

Conceptualization, PM, SBN; methodology, RK, JGJ, RP, AP; software, PM; validation, PM, RK, JGJ, and RP; formal analysis, RK, PM, JGJ, RP, AP; investigation, RK, JGJ, RP, AP; resources, PM, SBN, RP; data curation, RK, AP; writing—original draft preparation, AP, RK, PM, JGJ; writing—review and editing, PM, SBN; supervision, PM, SBN; project administration, SBN; funding acquisition, SBN. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are available upon request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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