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A novel chiral HPLC and LC-MS/MS method development for the triazole antifungal compound

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ABSTRACT

The objective of the present study was to separate and develop a chiral high performance liquid chromatography (HPLC) and sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) technique to estimate the (+) and (−) enantiomers of Albaconazole and validate the individual enantiomer of the drug. Albaconazole is used to treat for anti-fungal disease. The stationary phase was reverse phase Chiralpak IG-3 (250 × 4.6 mm, 5 µm) and (100 × 4.6 mm, 3 µm), whereas the isocratic mobile phase was ethanol and diethyl amine (100:0.1% *v*/*v* ratio HPLC) Acetonitrile and 10 mM ammonium bicarbonate (90:10 *v*/*v* ratio LC-MS/MS) and the flow rate was 1.0 and 0.5 ml/minute. The resolution of the (+) and (−) enantiomers were monitored using HPLC diode array detector (DAD) 240 signal and LC-electrospray ionization-MS/MS in positive transition at 432.0 *m*/*z* (M + H) for Albaconazole. The retention time of the (+) and (−) enantiomers of the drug was 6.952 and 9.955 minutes and 2.905 and 3.780 minutes by HPLC and LC-MS/MS. The major benefits of the LC-MS/MS are related to its improved selectivity, precision and accuracy and the lower variability in comparison to the HPLC-DAD. This study provided a rapid, sensitive and novel selective method to evaluate the (+) and (−) enantiomers in active pharmaceutical ingredients by HPLC and LC-MS/MS.

INTRODUCTION

Albaconazole is a triazole antifungal belongs to the class of 7-chloro-3-[(2R, 3R)-3-(2,4-difluorophenyl)-3-hydroxy-4-(1,2,4-triazol-1-yl) butan-2-yl]quinazolin-4-one (Amjad *et al*., 2016). Generally, azole compounds inhibit the steroid demethylation and the biosynthesis of a critical component of fungal membrane called ergosterol by blocking a cytochrome P_{450} dependent enzyme: lanosterol 14-α-demethylase which is crucial for the conversion of lanosterol to ergosterol. Lack of ergosterol and accumulation of lanosterol-14-α-demethylase will increase the membrane permeability and lead to disruption of several enzymes in the membrane, such as chitin synthase (Maertens, 2004). This does not only inhibit its DNA replication, but also distracts cell growth that causes the death of yeast and fungi. Azoles also decrease the adhesion potential of pathogen cells to host tissues and impede the transformation of yeasts to mycelial form (Ghannoum and Rice, 1999; Sumrra *et al*., 2022). Therefore, they are widely applied as veterinary drugs (Bhanderi *et al*., 2009), as fungicides in agriculture (Brauer *et al*., 2019) and as antifungal agents for both humans and animals (Scorzoni *et al*., 2017; Zafa *et al*., 2021). Chirality plays a significant role in determining the pharmacological actions of chiral compounds and vital importance at the drug discovery stage (Ates *et al*., 2013; Zhang *et al*., 2005). One-third of all marketed drugs are now sold in a single isomeric form and chirality is now a significant factor in the development of new pharmaceuticals, with regulatory and therapeutic considerations driving the process (Mukherjee and Bera, 2012). The enantiomers of a chiral drug molecule may behave differently after administration, so the pharmaceutical industry places a high value on chiral resolution. To have a therapeutic effect, a molecule must engage a target receptor when it is administered. Drug molecules that are chiral will only fit into this receptor in one of their enantiomers (the eutomer), producing the desired therapeutic effect. A lesser effect could result from the other enantiomer (distomer), interacting or not with the receptor. The distomer can

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occasionally interact with different receptors, leading to side effects or even toxicity. In order to distinguish the eutomer from the distomer during drug substance identification and impurity determinations, additional research is needed on the enantiomers of active compounds during the development process. Racemates resolution is still difficult because of their similar characteristics in chiral environments, and work on highly specialized separation techniques is ongoing to resolve individual enantiomers (Liu *et al*., 2015). Based on literatures survey (Azhari *et al*., 2020; Bhowmick *et al*., 2021; Gazzinelli *et al*., 2022; Shekar *et al*., 2014), revealed that few analytical methods were reported for chiral separation on triazole antifungal drugs by high performance liquid chromatography (HPLC). Furthermore, the reported methods on chiral separation were more retention time and less sensitivity. As a result, our objective of this research is to separate and develop a novel, fast, selective and sensitive method with less retention time for chiral separation and estimation of (+) and (−) enantiomers in active pharmaceutical ingredients using HPLC and liquid chromatography tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Reagents

YMC India private limited gifted pure Albaconazole (+/−) as a working standard. SD fine chemicals and Merck, Mumbai, India supplied the chemicals ammonium bicarbonate and solvents methanol and acetonitrile (HPLC and LC-MS grade). The Milli Q RO system was used to purify the water (Millipore, Bedford, UK).

Instrumentation (HPLC and LC-MS/MS)

HPLC-photo diode array (PDA) chromatographic fingerprints were obtained with an Agilent 1260 Infinity II HPLC instrument (Agilent Technologies, Waldbronn, Germany) equipped with a 1260 Infinity II quaternary pump, a 1260 Infinity II degasser, a 1260 Infinity II vial sampler, a 1260 Infinity II column thermostat, a 1260 Infinity II diode array detector (DAD) HS. PC with the Agilent open lab CDS software for data acquisition.

Ultra fast liquid chromatography coupled with tandem triple quadrupole mass spectrometer (Shimadzu LC-MS/MS, Tokyo, Japan) equipped with interfaced by electrospray ionization (ESI) and solvent delivery system LC-20AD pump, SPD M20 PDA detector, SIL-20AC auto sampler, CTO 20AC column oven, CMB-20 alite controller. Using LC lab solution software, the data acquisition was performed. For the study, optimized factors include heat block temperature, desolvation line, Nebulizer gas, collision energy, etc. The mass spectrometer was run in positive ionization detection mode (M + H) with an ESI source. The nebulizer pressure was set to 345 kPa, ionization temp was set to 300°C, the capillary voltage was 5,000 V and gas flow rate was 11 l/minute. The collision cell gas was ultrapure nitrogen and the ionization source gas was nitrogen.

Chromatographic conditions (HPLC and LC-MS/MS)

The HPLC enantio-selective separation was achieved using a chiral stationary phase as reverse phase (RP) Chiral ART cellulose—SZ (250 × 4.6 mm, 5 µm) and the isocratic mobile phase composition of ethanol: ethanol and diethyl amine (DEA):(100%: 0.1% *v*/*v* ratio) at the flow rate of the detection of analyte was 1.0 ml/minute. Injection volume of 20 µl of each sample injects into the system and employed at an ambient column temperature. The total run time for the chiral separation was 20 minutes. The resolution target was detected using Agilent HPLC 1260 infinity II with a DAD detector.

The LC-MS/MS enantio-selective separation was achieved using a chiral stationary phase as RP Chiralpak IG-3 (100 × 4.6 mm, 3 µm) and the isocratic mobile phase composition of acetonitrile and 10 Mm ammonium bicarbonate (90:10 *v*/*v* ratio) at the flow rate of the detection of analyte was 0.5 ml/minute. 10 µl of injection volume of each sample injects into the system and employed at an ambient column temperature. The total run time for the chiral separation was 5 minutes. The resolution targets was detected using a Shimadzu—8030 triple quadrupole mass

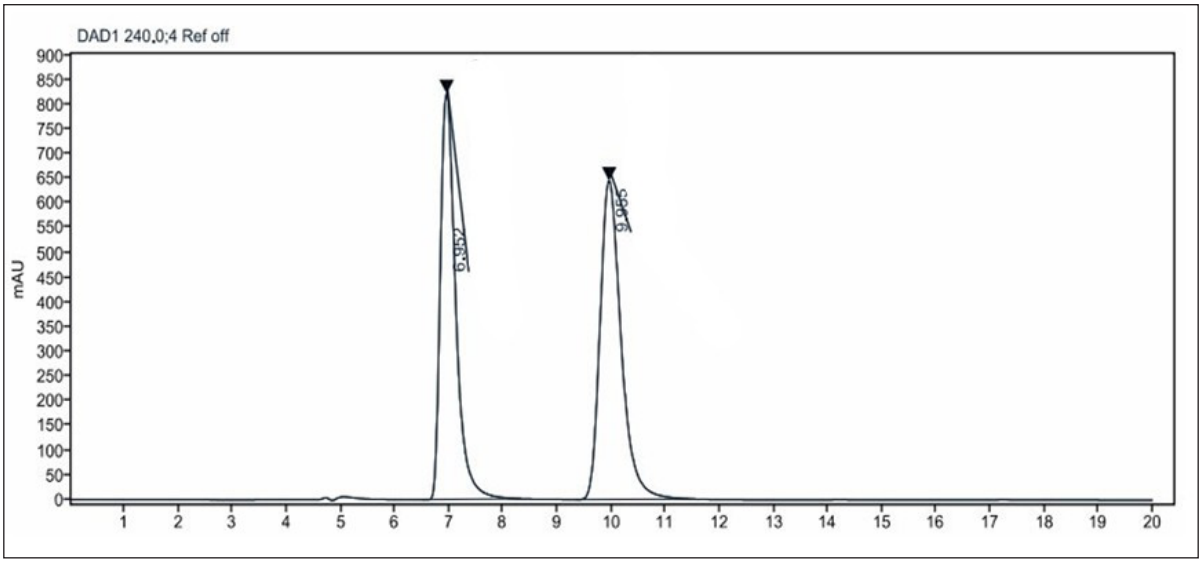

Figure 1. Chromatogram of albaconazole enantiomers by HPLC.

spectrometer with ESI interfaced with the mass analyzer (Hassan *et al*., 2022). The molecular ion spectra for albaconazole were found to be at *m/z*: 432.0 and the most prominent fragmentation peaks were observed at 45.0, 391.0, and 415.0. The most stable fragment of maximum intensity at 391.0 (daughter ion) (Sumrra *et al*., 2021). The mass spectrometer was run in positive ionization detection mode (M + H) with an multiple reaction monitoring (MRM) mode with the following transitions: *m/z* 432.0 (parent ion) → *m/z* 391.0 (daughter ion) for (+/−) Albaconazole, respectively (Figs. 2 and 3).

Standard solution preparation for (+/−) Albaconazole (HPLC and LC-MS/MS)

Working standard of (+/−) Albaconazole 1,000 µg/ml concentration was prepared by dissolving 10 mg of the enantiomeric drug in a 10 ml volumetric flask with methanol and make up the volume with methanol. A working concentration of 1,000 ng/ml was prepared from the above solution. The calibration curve for (+) and (−) Albaconazole of 10–100 ng/ml enantiomeric drug was prepared using the working standard.

Method validation for Albaconazole

The optimized HPLC and LC-MS/MS method was validated in accordance with the ICH guidelines in the aspects of specificity and carry-over, limit of quantification (LOQ), limit of detection (LOD), linearity, accuracy and precision and robustness, etc. (ICH, 1996).

Accuracy and precision

The recovery of the method was used to define the accuracy of the method. According to ICH guideline, the accuracy of the proposed HPLC and LC-MS/MS method was evaluated from the three levels of quality control samples by analyzing the six replicates. The recovery of the precision was carried out and the percent RSD was recorded.

Specificity and carry over

The ability to clearly assess the analyte in the presence of components that might be anticipated to be present is known as specificity. Typically, these could be degradants, impurities, etc.

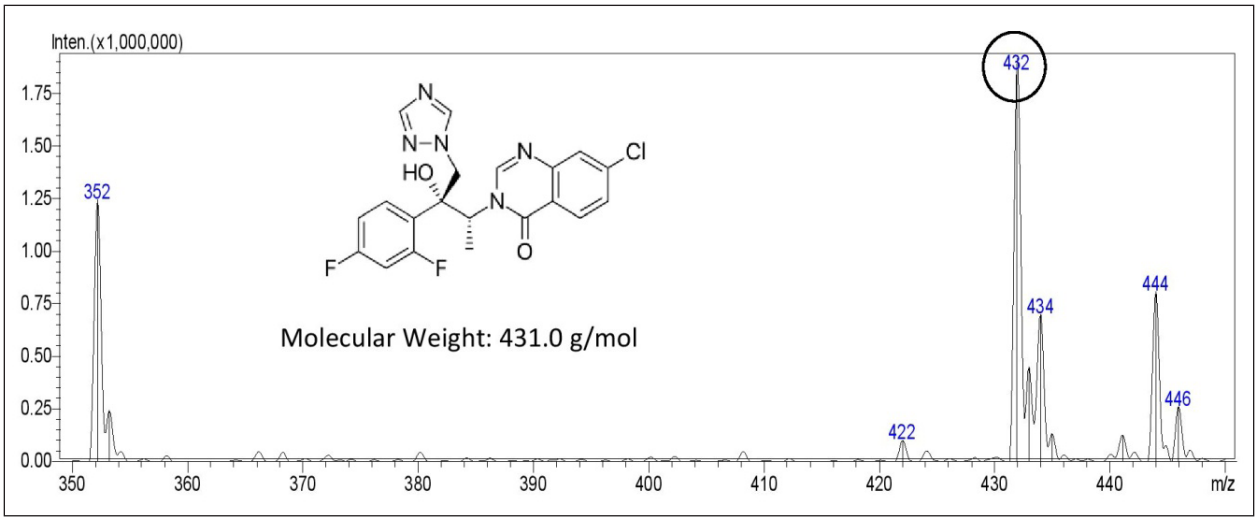

Figure 2. Molecular spectra for albaconazole enantiomers by LC-MS/MS.

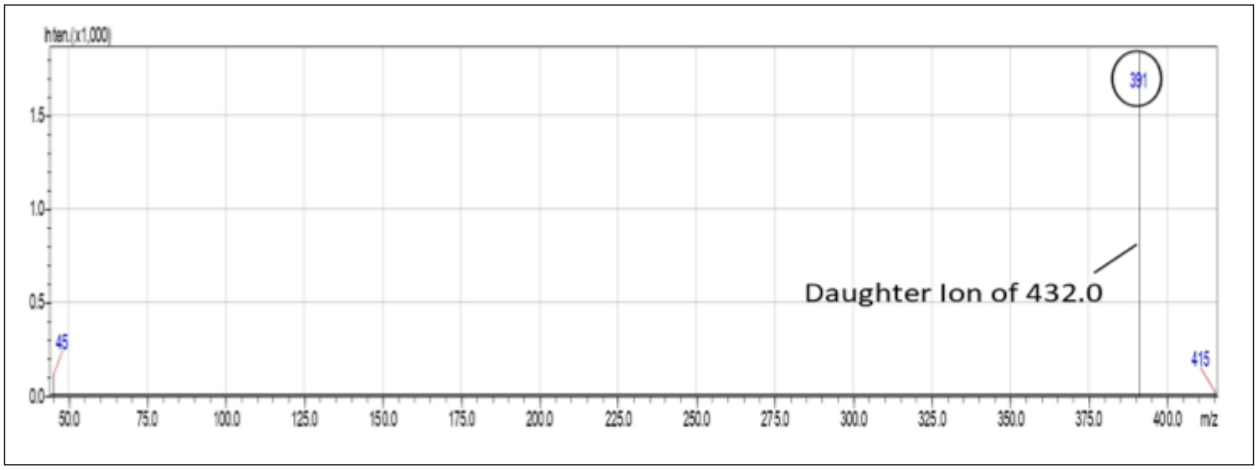

Figure 3. Daughter ion spectra for (+) and (−) enantiomers by LC-MS/MS.

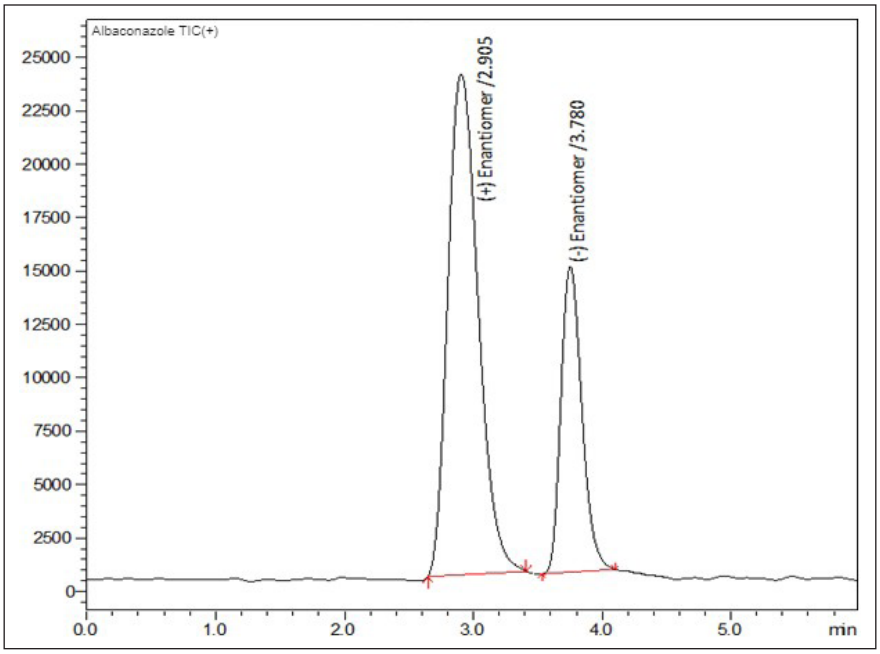

Figure 4. MRM chromatogram for (+) and (−) enantiomers by LC-MS/MS.

Table 1. Linearity range for (+) and (−) enantiomer by HPLC.

(+/−) Albaconazole concentration (µg/ml)	Peak area for (+) enantiomer	Peak area for (−) enantiomer
10	225,846	150,848
35	379,200	450,454
50	490,598	752,420
70	713,747	955,639
90	834,414	1,255,326
110	985,585	1,559,238

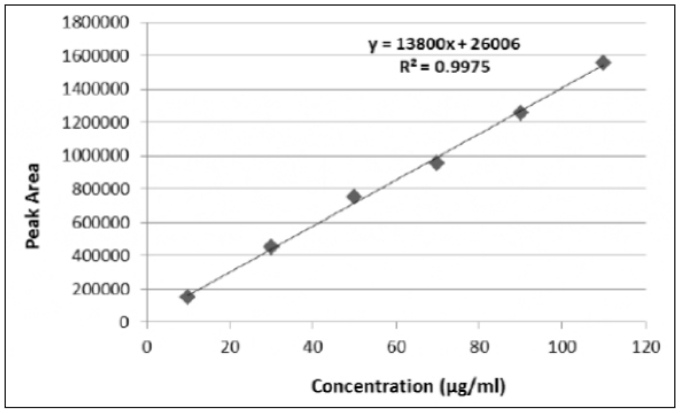

Figure 5. Calibration curve for (+) enantiomers by HPLC.

Linearity

The linearity concentrations were used to measure the slope, intercept values and R^2 value using regression equation ($Y = mx + C$). Six different concentrations of individual enantiomers of Albaconazole in the range of 10–100 ng/ml, respectively.

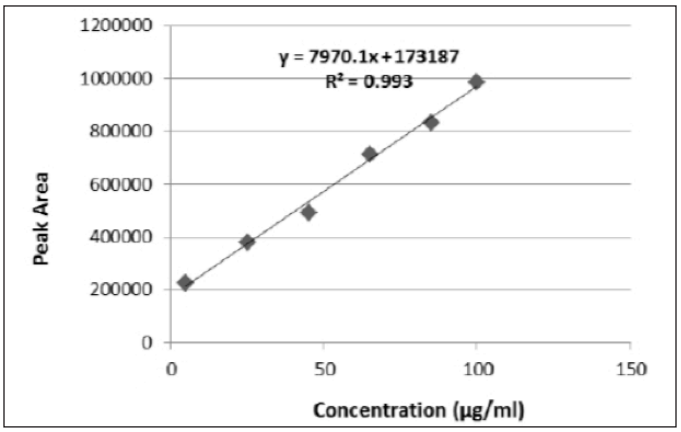

Figure 6. Calibration curve for (−) enantiomer by HPLC.

LOD and LOQ

LOD and LOQ were determined by signal to noise ratios (S/N) of 3:1 (LOD) and 10:1 (LOQ), respectively, in accordance with ICH guidelines a method is considered sensitive if it can detect incredibly low concentrations.

Robustness

The robustness of the developed method was determined by altering experimental conditions are mobile phase, flow rate and injection volume, etc. was studied.

RESULTS AND DISCUSSION

In this study, RP direct chiral HPLC and LC-MS/MS technique has been developed and validated for the chiral resolution of (+/−) Albaconazole. In order to separate the enantiomers, develop a simple, sensitive and effective HPLC

and LC-MS/MS method for (+/−) Albaconazole were carried out by selected optimal conditions such as, the resolution factor, theoretical plates, tailing factor, peak area, and peak asymmetry factor. For HPLC, RP Chiral Art Cellulose—SZ (250 × 4.6 mm, 5 µm) as the chiral stationary phase and ethanol: DEA: (100%: 0.1% *v*/*v* ratio) as the isocratic mobile phase with a flow rate of 1.0 ml/minute constitute the optimal chromatographic conditions. The total chromatographic resolution run time was 20 minutes. The (+) enantiomeric retention time was found to be 6.952 minutes and the (−) enantiomeric retention time was found to be 9.955 minutes, respectively (Fig. 1). For LC-MS/MS RP-Chiralpak IG-3 (100 × 4.6 mm, 3 µm) as the chiral stationary phase and acetonitrile and 10 Mm ammonium bicarbonate (90:10 *v*/*v* ratio) as the isocratic mobile phase with a flow rate of 0.5 ml/minute constitute the optimal chromatographic conditions. The total chromatographic resolution run time was 5 minutes. The (+) enantiomeric retention time was found to be 2.905 minutes and the (−) enantiomeric retention time was found to be 3.780 minutes, respectively (Fig. 4).

Specificity

During the elution time of the individual enantiomers for both the methods, no potential interference peaks were noticed. As a result, the method was found to be specific and highly sensitive.

Linearity

The HPLC calibration curve for (+) and (−) Albaconazole was defined at six various concentration levels, and the R^2 value was 0.9975 and 0.993. Individual enantiomeric concentration of working linearity ranging from 10–110 µg/ml (Table 1). The results of (+) and (−) regression equation $Y = 13{,}800X - 26{,}006$ and $Y = 7{,}970.1X + 173{,}187$ were found to be linear (Figs. 5 and 6).

The LC-MS/MS calibration curve for (+) and (−) Albaconazole was defined at six various concentration levels, and the R^2 value was 0.9955 and 0.9938. Individual enantiomeric concentration of working linearity ranging from 10–100 ng/ml (Table 5). The results of (+) and (−) regression equation

Table 2. Ecovery studies for (+) and (−) enantiomer by HPLC.

Sample (µg/ml)	Area found ± SD	% Recovery (+) and (−) Albaconazole	% RSD	% Recovery (+)	% RSD	% Recovery (−)	% RSD
10	9.6 ± 0.08	96.0	0.83	4.8 ± 0.08	1.87	4.8 ± 0.05	1.04
50	49.3 ± 0.54	98.6	1.09	24.9 ± 0.35	1.40	24.4 ± 0.20	0.81
110	109.3 ± 0.31	99.3	1.15	55.9 ± 0.40	0.71	54.4 ± 0.25	0.45

Table 3. Precision studies for (+) and (−) enantiomer by HPLC.

Sample (µg/ml)	Area found ± SD (+) enantiomer	% RSD	Area found ± SD (−) enantiomer	% RSD
10	151,564.4 ± 2,071	1.36	228,922.8 ± 2,476.1	1.08
50	900,113 ± 7,898.42	0.87	544,952.2 ± 3,323.7	0.60
110	1,395,202 ± 8,793.54	0.63	885,664.7 ± 4,070.9	0.45

Table 4. System suitability studies for (+) and (−) enantiomer by HPLC.

Parameters	Values obtained for (+) enantiomer	Values obtained for (−) enantiomer
Theoretical plate (N)	3,189.07	3,287.76
Tailing factor	1.54	1.36
Resolution factor (R)	5.05	
LOD (µg/ml)	4.0 (ng/ml)	
LOQ (µg/ml)	10.0 (ng/ml)	

Table 5. Linearity range for (+) and (−) enantiomer by LC-MS/MS.

(+/−) Albaconazole concentration (ng/ml)	Peak area for (+) enantiomer	Peak area for (−) enantiomer
10	148,175	105,248
25	354,714	255,863
40	648,590	421,987
65	944,106	604,982
80	1,195,071	721,694
100	1,564,841	952,357

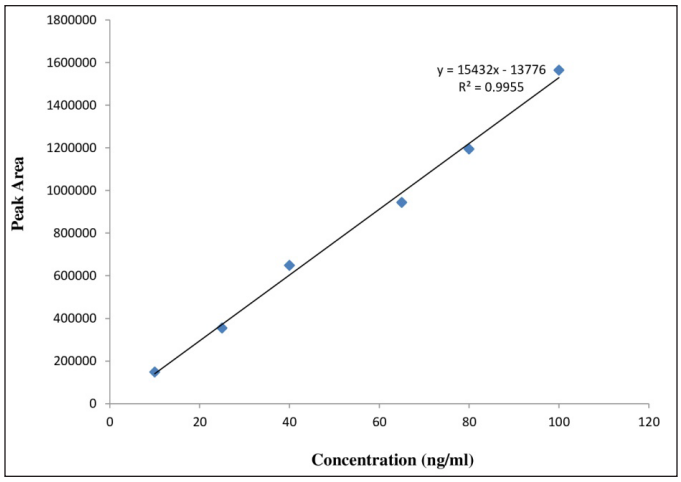

Figure 7. Calibration curve for (+) enantiomer by LC-MS/MS

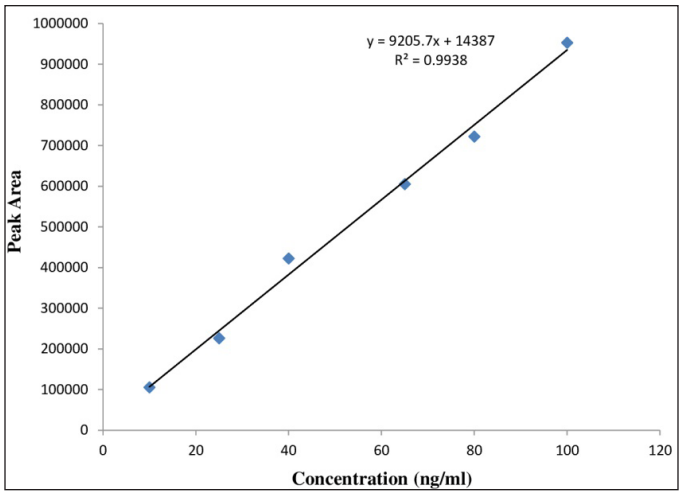

Figure 8. Calibration curve for (−) enantiomer by LC-MS/MS.

Table 6. Accuracy studies for (+) and (−) enantiomer by LC-MS/MS.

Sample (ng/ml)	Area found ± SD	% Recovery (+) and (−) Albaconazole	% RSD	% Recovery (+)	% RSD	% Recovery (−)	% RSD
5	4.9 ± 0.09	98.4	1.20	2.6 ± 0.05	1.92	2.3 ± 0.04	1.73
50	49.6 ± 0.52	99.2	1.81	26.6 ± 0.45	1.69	23.0 ± 0.36	1.56
100	99.7 ± 0.30	99.7	1.89	49.9 ± 0.40	0.80	49.8 ± 0.45	0.90

Table 7. Precision studies for (+) and (−) enantiomer by LC-MS/MS.

Sample (ng/ml)	Area found ± SD (+) enantiomer	% RSD	Area found ± SD (−) enantiomer	% RSD
10	152,011 ± 2,152	1.41	103,986 ± 1,943	1.86
100	756,232.2 ± 7,377	0.97	507,366 ± 5,234	1.03
200	1,507,440 ± 10,695	0.70	953,959 ± 6,775	0.71

Table 8. System suitability studies for (+) and (−) enantiomer by LC-MS/MS.

Parameters	Values obtained for (+) enantiomer	Values obtained for (−) enantiomer
Theoretical plate (*N*)	5,181	4,843
Tailing factor	0.93	1.0
Resolution factor (*R*)	3.10	
LOD (ng/ml)	3 (ng/ml)	
LOQ (ng/ml)	10 (ng/ml)	

$Y = 15{,}432X - 13{,}776$ and $Y = 9{,}205.7X + 14{,}387$ were found to be linear (Figs. 7 and 8).

Accuracy and precision

The HPLC and LC-MS/MS recovery studies were conducted to determine the method accuracy and the recovery percentage of enantiomers ranged between 96.0%–99.3% and 98.4%–99.7% (Tables 2 and 6). The percentage relative standard deviation (RSD) of the individual enantiomers were found to be (1.36%–0.63%) and (1.08%–0.45%) and (1.41%–0.70%) and (1.86%–0.71%) for the precision studies (Tables 3 and 7).

LOD and LOQ

Based on the S/N ratio and minimum level of peak area and the LOD (4 µg/ml) and (3 ng/ml) as well as LOQ (10 µg/ml) and (10 ng/ml) were determined for the (+) and (−) enantiomers for the developed method by HPLC and LC-MS/MS.

System suitability

As per ICH guidelines, a system suitability study were conducted to determine the system suitability parameters are resolution factor, retention time (R_T), tailing factor and theoretical plates (*N*). The results were found to be within the limits (Tables 4 and 8).

Table 9. Robustness studies for (+) and (−) enantiomer by LC-MS/MS.

Parameters	Conditions	Retention time ± % RSD for (+) enantiomer	Retention time ± % RSD for (−) enantiomer
Flow rate (ml/minute)	0.2	3.3 ± 1.64	4.9 ± 1.86
	0.5	2.8 ± 1.12	4.3 ± 1.21
	0.7	2.1 ± 0.51	3.8 ± 0.76
Methanol percentage (%)	90	3.1 ± 1.74	5.1 ± 1.74
	95	2.8 ± 1.08	4.3 ± 1.12
	98	2.4 ± 0.64	3.7 ± 0.81
pH	5.5	1.9 ± 1.89	4.0 ± 1.52
	6.0	2.8 ± 1.12	4.3 ± 1.02
	6.5	3.3 ± 0.74	4.8 ± 0.62

Table 10. Robustness studies for (+) and (−) Enantiomer by LC-MS/MS.

Parameters	Conditions	Retention time ± % RSD for (+) enantiomer	Retention time ± % RSD for (−) enantiomer
Flow rate (ml/minute)	0.8	8.3 ± 1.64	12.2 ± 1.86
	1.0	6.9 ± 1.12	9.9 ± 1.21
	1.2	4.8 ± 0.51	5.9 ± 0.76
Ethanol percentage (%)	90	4.2 ± 1.74	5.6 ± 1.74
	95	5.1 ± 1.08	6.4 ± 1.12
	100	6.9 ± 0.64	9.9 ± 0.81

Robustness

The conditions were followed for the robustness study are flow rate (0.2, 0.5, 0.7 ml/minute), pH (5.5, 6.0, 6.5) and percentage methanol (90%, 95%, 98%). The Individual percentage RSD for the selected conditions was calculated for pH (1.89%–0.74% and 1.52%–0.62%), flow rate (1.64%–0.51% and 1.86%–0.76%) and methanol percentage (1.74%–0.64% and 1.74%–0.81%) by HPLC and LC-MS/MS (Tables 9 and 10).

CONCLUSION

In conclusion although both methods here described are reliable, and fast to perform, the major benefits of the LC-MS/MS are related to its improved selectivity, precision and accuracy and the lower variability in comparison to the HPLC-DAD. As per ICH guidelines a sensitive direct chiral reverse HPLC and LC-MS/MS method for chiral separation for novel triazole antifungal compounds was developed and validated. As it provides good sensitivity and reproducibility. In spite of the elevated instrumentation cost, the LC-MS/MS method, here presented, is simple and rapid and therefore it could be applied to routine analysis of oxidative stress in clinical chemistry. This method will be useful for pharmaceutical, pharmacokinetics and bioequivalence study.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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