Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2011, 3(2): 316-328
(http://scholarsresearchlibrary.com/archive.html)

ISSN 0975-5071
USA CODEN: DPLEB4

Development and Validation of High-Throughput Liquid Chromatography - Tandem Mass Spectrometric Method for Quantification of Itraconazole and its Metabolite in Human Plasma

I. Sarath chandiran[1], K. N. Jayaveera[2] and Raghunadha Reddy. S*[3]

[1]Gokula Krishna College of Pharmacy, Sullurpet, Andhra Pradesh, India
[2]Jawaharlal Nehru Technological University, Anantapoor, Andhra Pradesh, India
[3]Actimus Biosciences (I) Pvt. Ltd., Vizag, Andhra Pradesh, India

---

ABSTRACT

A simple, sensitive and selective method is described for the determination of itraconazole and its metabolite in human plasma, Miconazole as internal standard by using HTLC-MS/MS. The method consists of a online coupling of extraction with cyclone P (50mm x 0.5mm, 50µm) HTLC column by injecting 15µL sample and chromatographic separation is performed with C_{18} Reverse phase column using 90:10 Acetonitrile: 10mM Ammonium Formate Buffer (pH 6.8) as gradient mobile phase followed by quantification with Tandem Mass spectrometry (MS/MS) in selective reaction monitoring mode using Electro spray ionization mode (ESI) as an interface. The method was fully validated in terms of specificity, sensitivity, precision, accuracy and stability over a concentration range of 1 to 500g/ml for both Drug and its metabolite using 0.5ml of human plasma per assay. Stability assessment was also included. The total run time for sample analysis was 1.5 min and the lower limit of quantification was 1ng/mL for both drug and its metabolite. The validated method was applied in bioavailability and bioequivalence study.

Key words: LC-MS/MS, Human Plasma, Bioanalytical, Itraconazole, Validation.

INTRODUCTION

Itraconazole [1-5] is a synthetic triazole antifungal agent. Itraconazole is a 1:1:1:1 racemic mixture of four diastereomers (two enantiomeric pairs), each possessing three chiral centers. It may be represented by the following nomenclature: 4-[4-[4-[4-[[2-(2,4-dichlorophenyl)- 2-(1H-1,2,4-triazol-1-ylmethyl)- 1,3-dioxolan-4- yl]methoxy]phenyl] piperazin-1-yl]phenyl]-2-(1-methylpropyl)-2,4-dihydro-1, 2,4-triazol- 3-one. Itraconazole has a molecular formula of $C_{35}H_{38}Cl_2N_8O_4$ and a molecular weight of 705.64. It is a white to slightly yellowish powder. It is insoluble in water, very slightly soluble in alcohols, and freely soluble in dichloromethane. It has a pKa of 3.70 (based on extrapolation of values obtained from

methanolic solutions) and a log (n-octanol/water) partition coefficient of 5.66 at pH 8.1. The mechanism of action of Itraconazole is the same as the other azole antifungal: it inhibits the fungal cytochrome P450 oxidase-mediated synthesis of ergosterol. Itraconazole has a broader spectrum of activity. In particular, it is active against aspergillus. It is also licensed for use in blastomycosis, histoplasmosis and onychomycosis. Itraconazole is over 99% protein bound and has virtually no penetration into cerebrospinal fluid. Therefore, it should never be used to treat meningitis or other central nervous system infections.

Itraconazole is extensively metabolized by the liver into a large number of metabolites, including hydroxyitraconazole, the major metabolite. Fecal excretion of the parent drug varies between 3-18% of the dose. Renal excretion of the parent drug is less than 0.03% of the dose. About 40% of the dose is excreted as inactive metabolites in the urine. No single excreted metabolite represents more than 5% of a dose. The plasma protein binding of Itraconazole is 99.8% and that of hydroxyitraconazole is 99.5%. Itraconazole is not removed by hemodialysis.

Analytical methods so far reported for quantification of itraconazole and its hydroxyl metabolite, employing HPLC method in human serum [6-9] and in human plasma [10-15], employing flourimetric method in human plasma [16-17], employing LC-MS-MS method in human plasma [18-19]. No method was reported for quantification of itraconazole and its hydroxyl metabolite in human plasma employing HTLC-MS/MS.

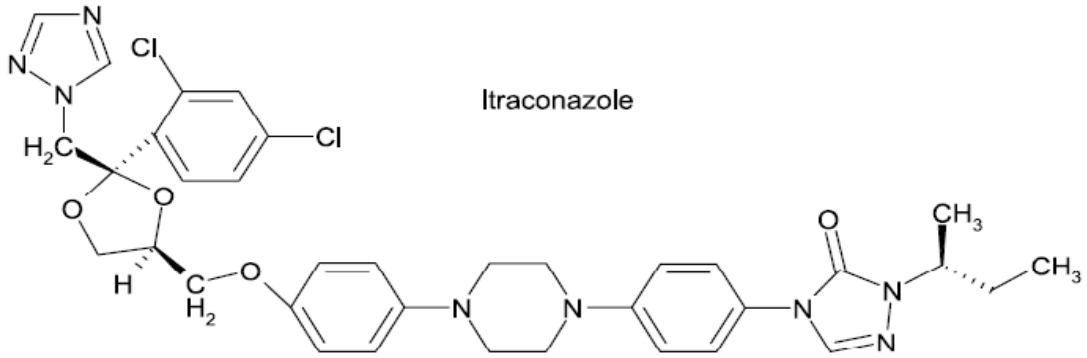
We now report a highly sensitive High throughput liquid chromatography/tandem mass spectrometry (HTLC/MS/MS) method developed and validated for the quantification of Itraconazole and its metabolite (hydroxy itraconazole) in human plasma using miconazole as an internal standard. The current method includes a simple and rapid sample preparation as well as significantly shorter analysis run time compared to previously published methods. At the same time, it was expected that this method would be efficient in analyzing large number of plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies after therapeutic doses of itraconazole.

MATERIALS AND METHODS

2.1 Chemicals and Reagents

Itraconazole ($C_{35}H_{38}Cl_2N_8O_4$, Mol.wt = 705.640) and its metabolite (Hydroxy itraconazole) ($C_{35}H_{39}Cl_2N_8O_5$, Mol.wt = 717.640) was obtained from IDDS, Hyderabad. Miconazole nitrate ($C_{18}H_{17}Cl_4N_3O_4$, Mol.wt = 481.160) was commercially procured from Shilpa Medicare limited. The chemical structures for Itraconazole, its metabolite and internal standard are shown in Fig.1A and Fig.1B. All the solvents used in this are of HPLC grade. Acetonitrile and methanol were of HPLC grade and obtained from J.T.Bakers. Formic acid, ammonium formate were obtained from Merck. Itraconazole free human plasma was obtained from Body care labs, Hyderabad. Double distilled water is obtained from Sartorius apparatus.

Fig.1A. the chemical structures for Itraconazole and its metabolite



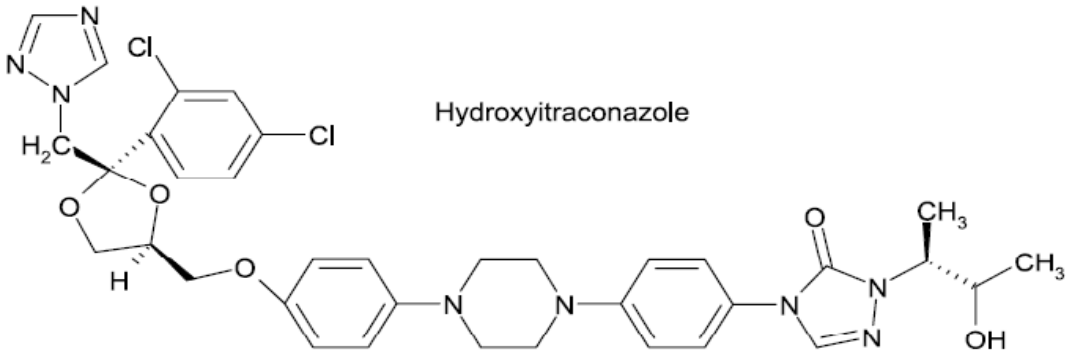
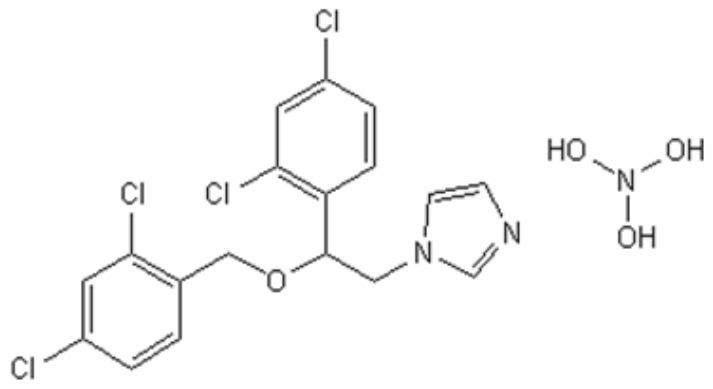


Fig.1B. the chemical structure for Miconazole nitrate



Miconazole nitrate

2.2 Instrumentation:

Chromatograms were acquired on a TSQ tandem mass spectrometry (Thermo Finnigan, Sanjose, CA, USA) equipped with Electrospray ionization (ESI) and connected to a PC runs with the standard software Xcalibur 2.0.7 and LC Quan 2.5.6. Mass spectroscopic detection was performed on a Triple quadrapole instrument (Thermo, TSQ Quantum Discovery Max). Robotic liquid handling system is operated using the software package supplied from the cohesive technologies Aria^{TM}.

2.3 Liquid chromatography – Tandem mass spectrometry:

The HTLC/MS/MS system consisted of four pumps for gradient solvent delivery, and a divert valve to direct LC effluent to the mass spectrometer in the analyte elution window.

The analytical column effluent is directed through the divert valve to a thermo electron TSQ quantum discovery mass spectrometer. The Mass spectrometer was operated in Positive ion mode; selective reaction monitoring using spray voltage of 5000, sheath gas of 25 and auxiliary gas of 15. The capillary temperature was maintained at 300°C. The duration of analytical run was 1.5 minutes.

2.4 Standard solutions preparation

2.4.1. Stock solution preparation

Approximately 5 mg of itraconazole(A) / 5 mg of hydroxy itraconazole(B)/ 2mg of miconazole (IS) working standard was weighed and transferred to 10.0 mL volumetric flask, to this 5.0 mL of Methanol was added and sonicated to aid dissolution and the final volume was made up with Methanol.

2.4.2. Preparation of internal standard dilution

The miconazole (ISTD) dilution of about 50ng/mL from the ISTD stock solution (IS stock) using (80:20 Acetonitrile: water) as the diluent was prepared.

2.4.3. Preparation of calibration curve (CC) standards and quality control (QC) samples

Appropriate dilutions of the stock solutions with diluent were made subsequently in order to prepare the working standard solution in the range of 0.050 to 25µg/ml for itraconazole and its metabolite. All the solutions were stored in a refrigerator between 2°C and 8°C. Calibration standards and quality control samples, in the range of 1.0 to 500 ng/ml for itraconazole and its metabolite were prepared for calibration. Accuracy and precision, quality control and stability assessment was done by spiking 0.5mL of drug free plasma with appropriate volume of working solution.

2.5. Solutions used for robotic on-line sample extraction system

10 mM ammonium formate buffer(pH 6.8) was used in pump A, Pure Acetonitrile is used in pump B, 0.2% formic acid is used in pump C and washing solution in the ratio of 60:25:15 (acetonitrile:IPA:acetone) was employed in pump D.

2.6 Sample Preparation:

Retrieved the frozen CC, QC samples and subject samples from the deep freezer and thaw in water bath maintained at room temperature, vortexed to mix. Removed the caps from the polypropylene tubes. Aliquoted 0.5 mL of CC, QC and subject samples into pre-labelled polypropylene tube. Added 50.0 µL of ISTD dilution (about 50ng/mL) followed by 0.150mL of 10mM ammonium formate buffer of pH (6.8) in to vials, cap it, vortex to mix and transfer vials to auto sampler.

2.7. Steps involved in on-line robotic method development

A typical two-column setup featuring two six-port switching valves as described by Herman [20] is employed for method development. The procedure consisted of four steps:

(a) The eluent loop was filled with 50% acetonitrile in 10 mM ammonium formate buffer.

(b) 15µL sample was added onto the Cyclone P (50 mm × 0.5 mm, 50 µm) HTLC column at a flow rate of 2 mL/min during 60 s.

(c) The eluent loop was discharged at 0.5 mL/min for 60 s to transfer the analytes from HTLC column onto the Cohesive Propel C18 (50 mm × 2.1 mm i.d., 5 µm) column and 0.5% aqueous formic acid at 0.2 mL/min in added post column.

(d) LC–MS/MS is performed using ballistic gradient at 2.0 mL/min (10–90% acetonitrile in 0.5% formic acid).

2.8. On-line sample extraction

The gradient program accomplished a Cyclone HTLC column for sample extraction, elution with four pumps as reported in Table 1. TLX turbo flow on-line technique is employed for separation of analyte from sample molecules. The mechanism involved in sample preparation may be affinity. The small drug molecules bind to the HTLC column, and molecules that have lower binding affinity quickly diffuse into the column particles and large sample molecules are flushed to waste, then the mobile phase elutes the analyte molecules that are binded at HTLC column to analytical column, from this analytical column analytes are entered to mass detector. To achieve required chromatograms with consistency we have performed different combinations of the solvents and gradient system. Finally we succeeded with the solution combinations as mentioned in Table 1 and analyzed more than 150 samples with out overloading of the chromatographic columns with improved real throughput efficiency.

Table 1: Steps involved in on-line robotic method

Step	Start	Sec.	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B
1	0.00	30	2.00	Step	0.0	100.0	0.0	0.0	–	Out	0.80	Step	10.0	90.0
2	0.31	90	0.40	Step	50.0	0.0	50.0	0.0	T	In	0.80	Step	10.0	90.0
3	2.01	60	2.00	Step	0.0	0.0	0.0	100.0	–	In	0.80	Ramp	10.0	90.0
4	3.01	60	2.00	Step	50.0	50.0	0.0	0.0	–	In	0.80	Step	10.0	90.0
5	4.01	60	2.00	Step	0.0	100.0	0.0	0.0	–	Out	0.80	Step	10.0	90.0

One important factor that can affect the quantitative performance of a mass detector is ion suppression. Sample matrix, co eluting compounds and cross-talk can contribute to this effect. Ionization suppression typically observed in sample extracts from biological samples is not likely to be caused ionization suppression is the result of high concentrations of nonvolatile materials present in the spray with the analyte. King et al. [21] suggested that the effect is more generally applicable to any nonvolatile solute, including analyte. The exact mechanism by which the nonvolatile materials inhibit release of analyte into the gas phase has not been clearly demonstrated, although a likely list of effects relating to the attractive force holding the drop together and keeping smaller droplets from forming should account for a large portion of the ionization suppression observed with ESI. Once nonvolatile materials has been removed from sample preparation, there is no guarantee that suppression of ionization will no longer be a problem, other mechanisms such as impairing agents (e.g. trifluoro acetic acid) may play a role in ionization suppression. Bonfiglio et al. [22] reported the effects of sample preparation methods on the variability of ESI response. According to their results precipitation method showed the greatest amount of ESI response suppression followed by solid-phase extraction while liquid–liquid extracts demonstrated the least. In our study robotic liquid handling system was employed for sample extraction from plasma and 0.2% formic acid was employed as mobile phase additive to minimize ion suppression.

2.9 Validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose" (International Conference on Harmonization Guideline Q2A). "Methods validation is the process of demonstrating that analytical procedures are suitable for their intended use" (US Food and Itraconazole Administration Draft Guidance for Industry, 2000).

2.9.1 Selectivity:

Six human plasma samples from six individual healthy donors receiving no medication were extracted and analyzed for the assessment of potential interferences with endogenous substances. The apparent response at the Retention time of itraconazole, its metabolite and internal standard were compared to the response at the lower limit of quantification (LLOQ) for itraconazole, its metabolite and to the response at the working concentration for internal standard.

2.9.2 Recovery:

Recovery experiments should be performed by comparing the analytical results for extracted samples at three concentrations with unextracted standards that represent 100% recovery. Recovery of the analyte need not be 100% but the extent of recovery of an analyte and an internal standard should be consistent, precise and reproducible.

2.9.3 Calibration and Sample Quantification:

Calibration standards at ten different levels including the replicates at higher and lower side were extracted and assayed as described above on three different days. Calibration curves (y=mx+c) represented by plots of the peak area ratio(y) of itraconazole/its metabolite to internal standard Vs concentration(x) of the calibration standards were generated using weighted ($1/x^2$) linear least square regression as the mathematical mode. Actual Quality control, stability samples were calculated from the resulting area ratio of itraconazole/its metabolite and the regression equation of the calibration curve.

2.9.4 Accuracy and Precision:

Intra-day accuracy and precision were evaluated by analysis of quality control samples at 4 different levels (n=6 at each level) on the same day. These levels were chosen to demonstrate the performance of the method and to determine the Lower limit of quantification of the method. The upper limit of quantification was given by the highest level of the calibration curve. Samples with concentration above this upper limit of quantification should be diluted prior to reanalysis. To assure the interday accuracy and precision, the intraday assays were repeated on 3 different days. The overall performance was calculated.

2.9.5 Stability:

The effect of 3 Freeze thaw cycles, the analyte stability at room temperature in human plasma and in working solutions were evaluated by repeated analysis (n=6) of stability control sample spiked with working solution at low and high concentration. The stability was expressed as a percentage of the theoretical value. The analyte stability was established for its stability in auto sampler and in dry extract form. For those samples to be diluted for analysis (i.e. whose values is above the upper limit of quantification) dilution integrity was also performed.

RESULTS

3.1 Mass Spectrum analysis

The mass spectrometric parameters were optimized to obtain the higher response for the pairs 704.656 → 392.077 m/z for Itraconazole, 721.654 → 408.136 m/z for hydroxy itraconazole and 416.373 → 158.824 m/z for internal standard. The method was fully validated using these selective reaction monitoring pairs. The fragmented ion spectrums for Itraconazole, its metabolite and miconazole were given in Fig.2A, 2B and 2C.

Fig.2A. Fragmented ion spectrum for Itraconazole

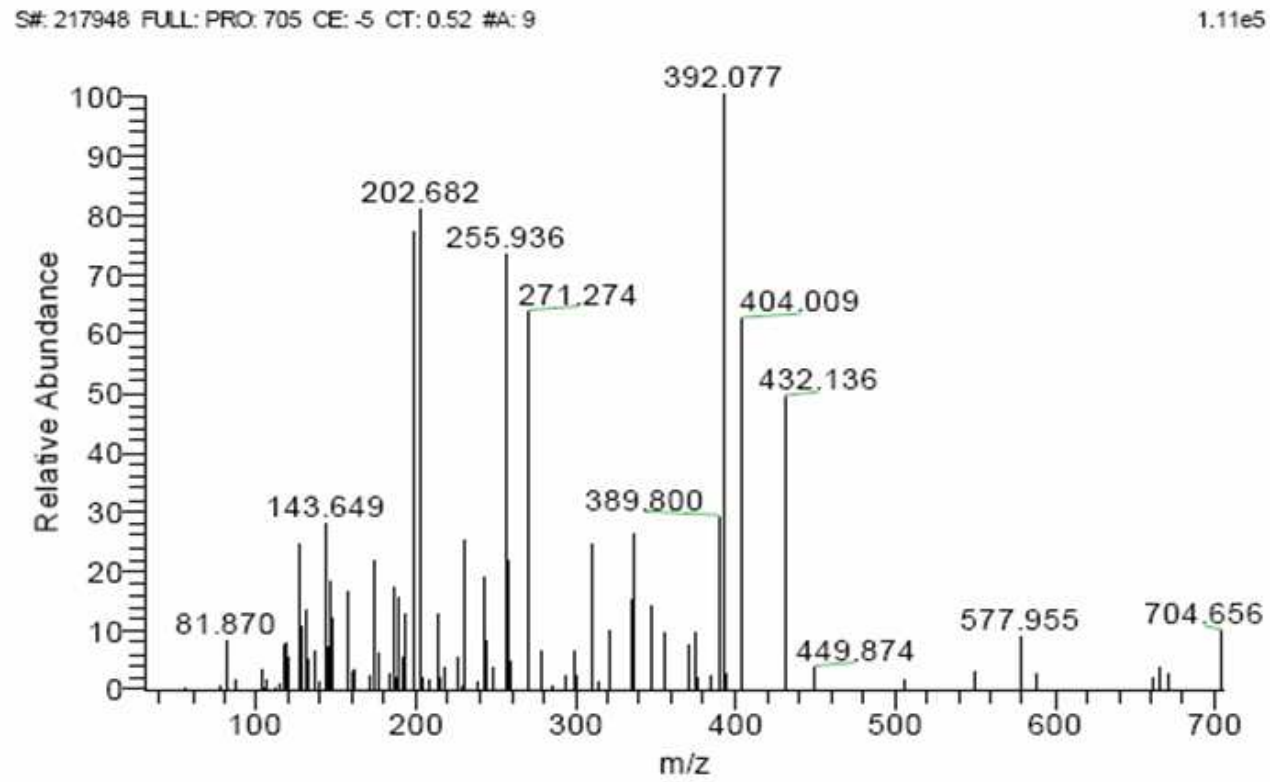


Fig.2B. Fragmented ion spectrum for Hydroxyitraconazole

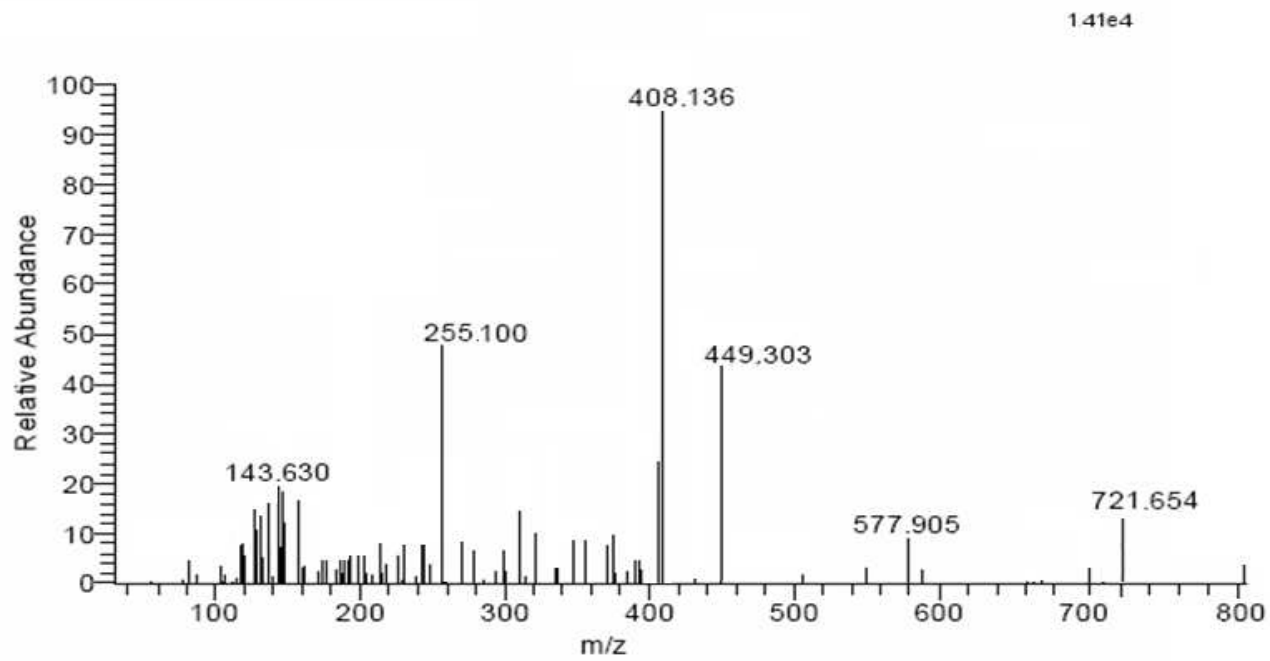
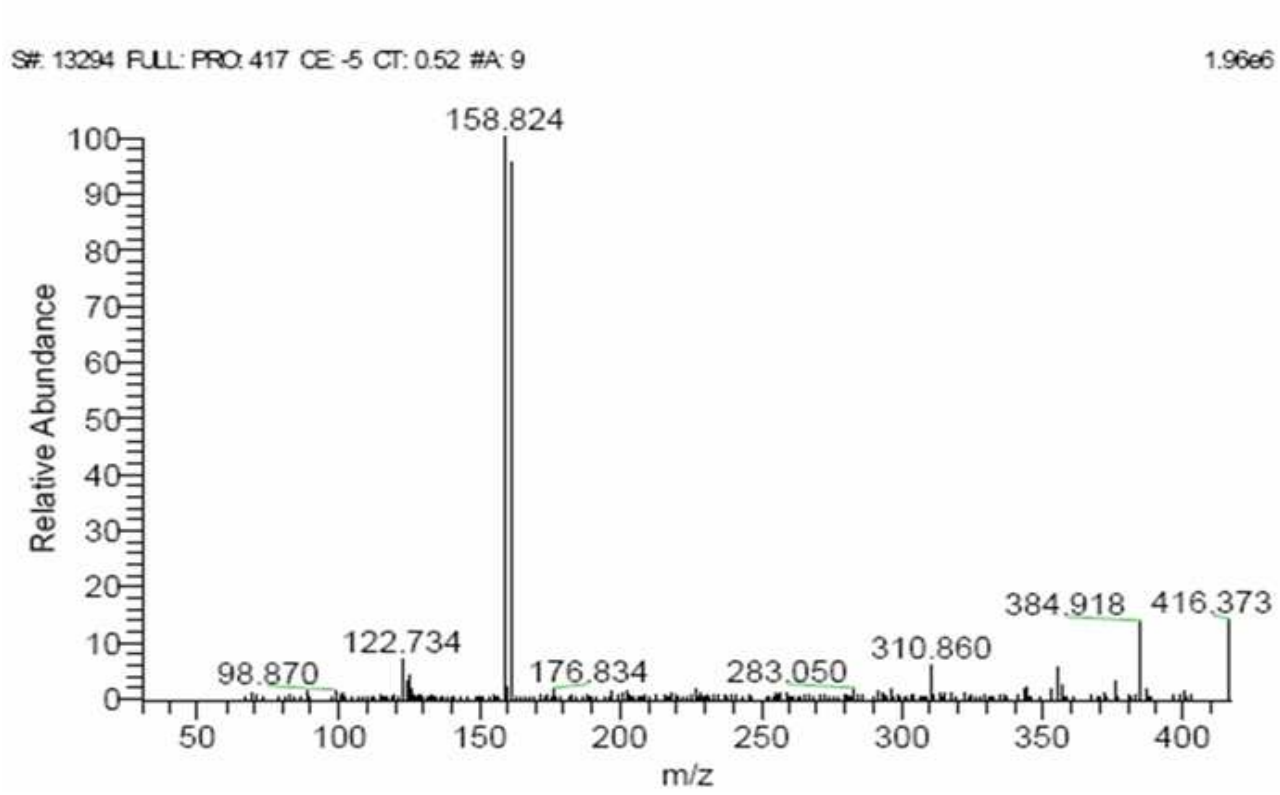


Fig.2c.Fragmented ion spectrum for Miconazole



3.2 Retention times and selectivity

Observed Retention times were about 0.61 min (itraconazole), 0.62 min (hydroxyl metabolite) and 0.57 min (Internal standard) respectively. No additional peak due to endogenous substances that could have interfered with the detection of the compounds of interest was observed. Representative chromatograms from an extract of human blank plasma spiked with miconazole as IS and from an extract from an extract human blank spiked with Itraconazole, its metabolite and miconazole (as IS) were given in Fig.3A and 3B.

Table 2.Back calculated concentrations from calibration curves

Nominal Conc. Itraconazole(ng/mL)	1.0	3.0	15.2	30.4	60.8	90.7	120.9	151.2	302.9	504.9
Mean Accuracy (%)	101.1	91.4	88.8	102.6	94.4	99.8	103.8	98.5	94.7	107.4
Precision (%)	1.9	2.4	5.9	9.0	10	6.6	10.5	9.6	3.9	5.6
Hydroxyitraconazole										
Mean Accuracy (%)	95.6	98.3	90.8	111.3	89.4	92.3	99.2	104.2	100.7	104.1
Precision (%)	2.5	8.2	1.5	10.2	5.3	2.8	1.8	2.2	0.9	1.9

Accuracy: 100%measured concentration/Nominal concentration.
Precision: Coefficient of variation (100%standard deviation/Mean)

Fig. 3. (A) Representative Chromatograms from an extract of Human blank plasma spiked with Miconazole as IS

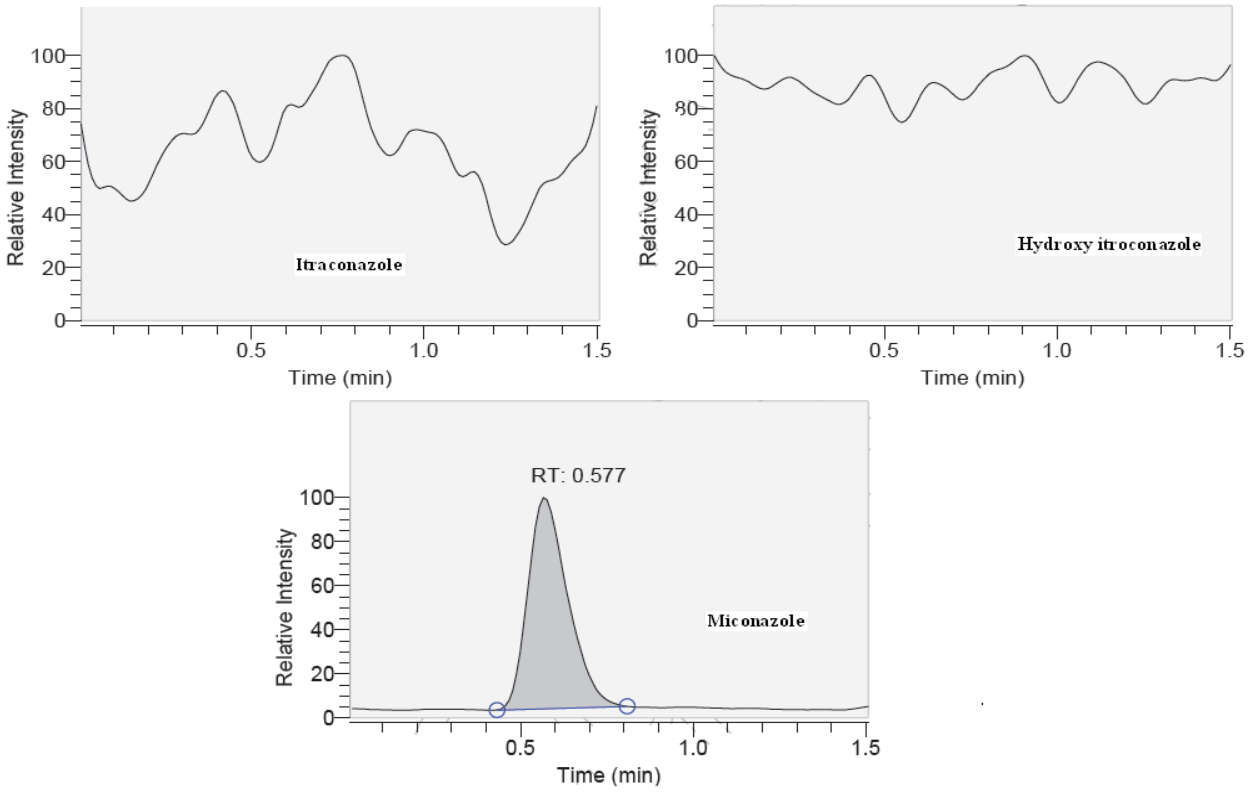
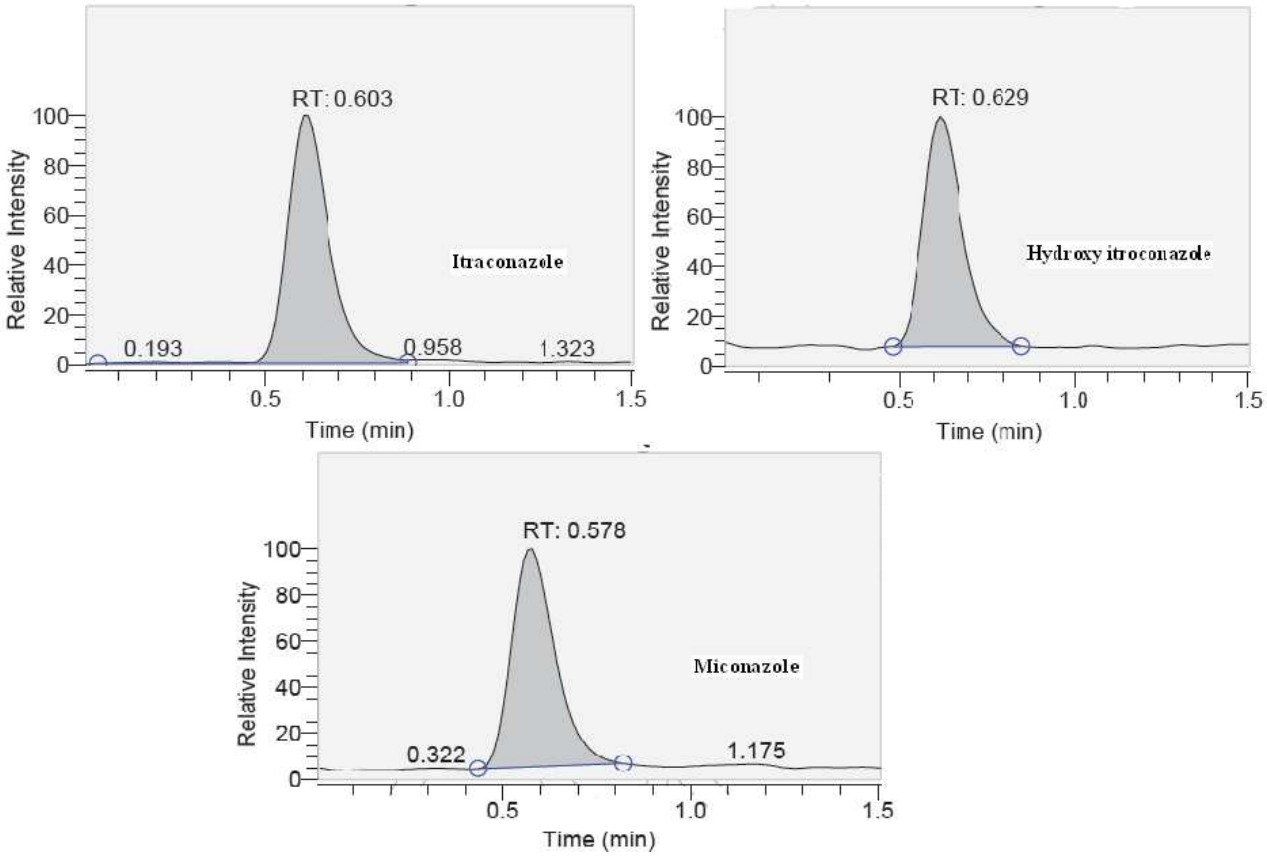


Fig.3B. Representative chromatograms from an extract from an extract human blank spiked with Itraconazole, its metabolite and miconazole (as IS)



3.3 Recovery: The recoveries of drug, its metabolite and internal standard were evaluated with 6 replicates at 3 different concentration levels**.** The mean recovery was 89 % for itraconazole, 75% for its metabolite respectively. These results suggested that there was no related difference in extraction recoveries at different concentration levels for itraconazole and its metabolite. . Internal standard recovery was also tested and was 63 % at the working concentration of 50ng/ml.

3.4 Linearity

Linear calibration curves were obtained with a coefficient of correlation (r^2) usually higher than 0.995. For each calibration standard level, the concentration was back calculated from the linear regression curve equation. The mean accuracy and precisions for back calculated concentrations of each standard calculated from calibration curves were tabulated as Table 2.

3.5 Precision and accuracy

The Accuracy and precision were established on three different days and calculated for individual days as well as overall. The results were found to be quite comfortable as per international guidelines. The accuracy and precision for inter day and intra day was tabulated in Table 3.

Table 3.Assessment of Accuracy and precision of the method

Nominal Conc. Itraconazole(ng/mL)	358.9	208.1	3.1	1.3
Intra-day accuracy(%)(day1)	96.1	99.4	103.7	105.6
Intra-day precision(%)(day1)	6.8	13.9	5.7	13.0
Intra-day accuracy(%)(day2)	92.0	92.5	94.9	92.1
Intra-day precision(%)(day2)	12.6	4.7	9.2	16.6
Intra-day accuracy(%)(day3)	95.6	100.9	101.4	106.4
Intra-day precision(%)(day3)	4.0	7.3	11.4	8.5
Overall accuracy (%)	94.6	97.6	101.5	101.4
Overall Precision (%)	1.9	3.7	2.0	6.5
Number of determinations	18	18	18	18
Hydroxy itraconazole (ng/mL)				
Intra-day accuracy(%)(day1)	95.2	98.2	107.2	113.2
Intra-day precision(%)(day1)	3.2	10.4	4.2	10.5
Intra-day accuracy(%)(day2)	98.6	95.2	99.4	111.0
Intra-day precision(%)(day2)	9.8	7.2	2.5	14.1
Intra-day accuracy(%)(day3)	89.2	105.4	110.7	104.3
Intra-day precision(%)(day3)	2.0	3.6	14.2	5.2
Overall accuracy (%)	96.8	98.4	110.7	107.0
Overall Precision (%)	1.2	7.3	1.4	5.2
Number of determinations	18	18	18	18

3.6 Stability

Stability experiments were performed to prove the stability of the compound in different conditions and results were found meeting the acceptance criteria. The accuracy and precisions of some of the stability studies were given in Table 4. Dilution integrity was performed and that the accuracy was quite good even after diluting the sample by ten times.

4.0 Application of the method

The present method was applied for a randomized cross-over bioequivalence study of two different formulations in 8 healthy male volunteers. After single oral administration of the drug blood samples were collected at a suitable time intervals up to 96 h. This method was

successfully used to measure the plasma concentrations of itraconazole. Various pharmacokinetic parameters established and compared for the both of the preparations were given in Table 5. Plasma concentration-time profiles were given as graph (Fig.4).

Table 4.Stability results

Experiment	Itraconazole				Hydroxy itraconazole			
	Accuracy		Precision		Accuracy		Precision	
	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC
Freeze-thaw stability (3 Cycles)	93.7	103	7.4	1.3	89.9	114.2	5.2	4.3
Bench top Stability (09 hours)	98	88.8	5.6	6.4	95.6	95.2	2.4	8.9
Auto sampler Stability (27 hours)	113.4	98.3	3.8	13.6	110.2	94.7	7.1	14.4
Dry extract stability (25 Days and 03 hrs)	93.8	107.2	3.1	4.3	98.4	88.2	1.9	2.8

Fig.4. Mean and Log Mean plasma concentration (ng/mL)-time (in hrs) profiles of itraconazole

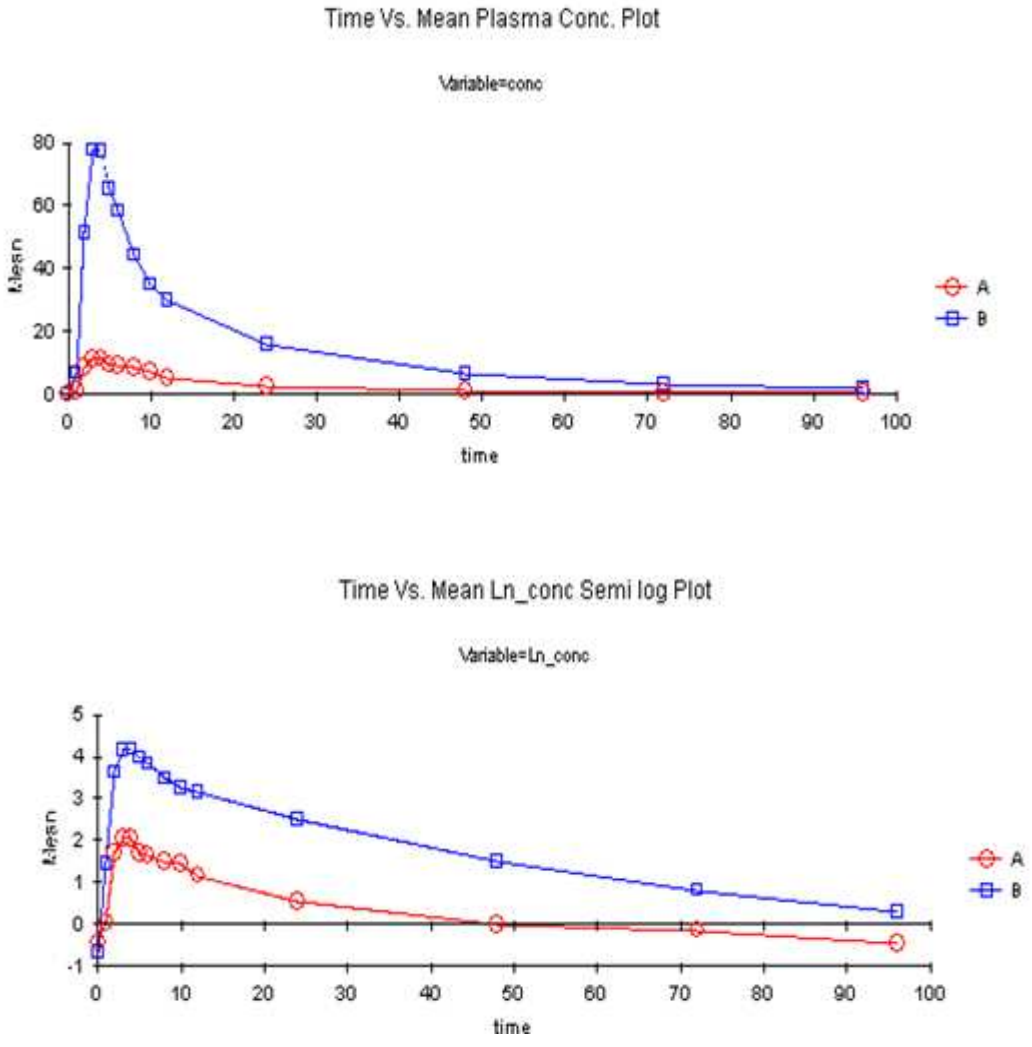


Table 5.Pharmacokinetic parameters of Itraconazole

PK Parameters	Formulation	
	Test	Reference
C_{max} (ng/ml)	15.04	80.70
AUC_{0-t} (ng.h/ml)	203.65	1274.26
AUC_{0-inf} (ng.h/ml)	249.12	1346.45
T_{max} (H)	4.83	3.69
K_{el} (H^{-1})	0.04	0.04
$T_{1/2}$ (H)	41.86	25.95

CONCLUSION

On-line coupling requires some modifications to the offline extraction techniques. The coupling is most commonly performed with the help of multiport valves and one or more pumps for the dynamic extraction or transfer of the extract to the chromatographic system, the extraction can be performed in either static or dynamic mode or as a combination of these so long as the extraction system allows the on-line transfer of the extract to the chromatographic system. In on-line systems, the whole extract is transferred to the chromatographic column, in contrast to traditional off-line techniques where only a small part is injected. This means that the sensitivity of the on-line method is much better. However, the high sensitivity easily leads to overloading of the analytical column. Miniaturisation of the extraction system is often required to avoid this. In our method Miniaturisation is achieved with small extraction in extraction vessels and the total analysis means sample extraction, chromatographic separation and mass spectrometric detection has been completed within 7.0 min for one sample quantitation.

REFERENCES

[1] De Beule K and Van Gestel J., Itraconazoles, Pharmacology of Itraconazole, **2001**; **61**(S1): 27–37.
[2] Florea NR, Capitano B, Nightingale CH, Hull D, Leitz GJ and Nicolau DP. , *Transplantation Proceedings* **2003**; 35: 2873–2877.
[3] Conway SP, Etherington C, Peckham DG, Brownlee KG, Whitehead A and Cunliffe H., *Journal of Antimicrobial Chemotherapy* **2004;** 53: 841–847.
[4] Janssen Pharmaceutica Products. Sporanox® (itraconazole) injection. US Food and Itraconazole Administration, **2002.** Available at: www.fda.gov/cder/foi/label/2002/20966s6lbl.pdf (access date: 3 May 2005).
[5] Kramer M, Marshall S, Denning D, Keogh A, Tucker R and Galgiani J. *Annals of Internal Medicine* **1990;** 113: 327–329.
[6] T.K.C. Ng, R.C.Y. Chan, F.A.B.Adeyemi-Doro, S.W. Cheung and A.F.B.Cheng, *Journal of Antimicribial Chemotherapy* **(1996)**37,465-472.
[7] Chan L, Potter JM, Miranda V and Dutton J., A simple high-performance liquid chromatography assay for quantitation of Itraconazole and hydroxy Itraconazole in serum. In 8th International Congress of Therapeutic Itraconazole Monitoring and Clinical Toxicology, Basel, **2003.**
[8] Compas D, Touw DJ and de Goede P., *Journal of Chromatography* B **1996**; 687: 453–456.
[9] Gubbins P, Gurley B and Bowman J, *Journal of Pharmaceutical Biomedicine* **1998**; 16: 1005–1012.
[10] Badock NR, *Journal of Chromatography* **1990**; 525: 478–483.

[11] Brandsteterova E, Kubalec P, Rady A and Krcmery V, *Pharmazie* **1995;** 50: 597–599.
[12] Koks C, Sparidans R, Lucassen G, Crommentuyn K and Beijnen J, *Journal of Chromatography B* **2002**; 767: 103–110.
[13] Poirier J-M and Cheymol G., *Therapeutic Itraconazole Monitoring* **1997**; 19: 247–248.
[14] Poirier J-M, Lebot M, Descamps P, Levy M and Cheymol G., Determination of Itraconazole and its active metabolite by column liquid chromatography Therapeutic Itraconazole Monitoring **1994;** 16: 596–601.
[15] Cox S, Orosz S, Burnette J and Frazier D, *Journal of Chromatography* B **1997;** 702: 175–180.
[16] Stefanie Redmann and Bruce G. Charles, *Biomedical Chromatography*, www.interscience.wiley.com.
[17] Al-Rawithi S, Hussein R, Al-Moshen I and Raines D, *Therapeutic Itraconazole Monitoring* **2001**; 23: 445–448.
[18] Vijaya Bharathi D, Kishore Kumar Hothvidya Sagar P. V, Sanagapati Sirish Kumar, Pandu Ranga Reddy, Naidu A., Mullangi Ramesh, Journal Of Chromatography. B **2008,** vol. 868, n°1-2, pp. 70-76.
[19] Constantinos Kousoulos, Georgia Tsatsou, Constantinos Apostolou, Yannis Dotsikas and Yannis l. Loukas; *Analytical And Bioanalytical Chemistry*; volume 384, number 1 / January, **2006;** 199-207.
[20] J.L. Herman, Rapid Commun. *Mass Spectrom.* 16 **(2002)** 421.
[21] R.C. King, R. Bonfiglio, M.C. Fernandez, S.C. Miller, T. Olah, *J. Am. Soc.Mass Spectrom*. 11 **(2000)** 942.
[22] R. Bonfiglio, R.C. King, T.V. Olah, K. Merkle, *Rapid Commun. Mass Spectrom*. 13 **(1999)** 1175.