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Determination of Itraconazole and its Metabolite From Human Plasma by High Performance Liquid Chromatography-Tandem Mass (LC-MS/MS) Spectrometry

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Introduction

Itraconazole, (ITR, CAS: 84625-61-6) is a classical member of the triazole class and is an important drug in our arsenal to treat fungal infections because it exhibits broad-spectrum anti-fungal activity. The mechanism of action for its antifungal activity is believed to involve efficient inhibition of the fungal -demethylase by itraconazole[1]. Following oral absorption, it is extensively metabolized including side chain hydroxylation with CYP3A4 to form hydroxyitraconazole (HITR) which is the major metabolite. HITR, is biologically active and its plasma concentration is two fold higher than ITR at steady state. The pharmacokinetics of orally administered ITR in humans are characterized by considerable interindividual variation in drug absorption, extensive tissue distribution, with the concentrations in tissue being many times higher than those in plasma, and an elimination half-life of 12 h. ITR is extensively metabolized in humans, yielding over 30 metabolites, including the major antifungally active metabolite HITR. The structure of ITR and HITR are shown in Figure I.

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The plasma concentrations of ITR and its active metabolite were very low (ng/mL level). Several liquid chromatographic – tandem mass spectrometric methods were reported for quantification of ITR and metabolite from biological samples. D.V. Bharathi et al. were reported LC-MS/MS techniques for determination of ITR and metabolite from plasma and the range of calibration concentration 0.5-263 ng/mL for ITR and 0.49-256 ng/mL for HITR[2]. Solid phase extraction process was used to extract ITR and HITR in this method. Vogeser et. al. were developed HPLC-MS/MS method with 2 ng/mL for ITR and 1 ng/mL limit of quantitation (LOQ) for metabolite, They used solid phase extraction for separation of drug and metabolite from plasma[3]. Young Wook Choi et.al. were published a method for determination of ITR from plasma by LC-MS/MS. The detection limit was 0.2 ng/mL for ITR[4].

The method developed for the determination of ITR and metabolite from plasma by LC-MS/MS system, which is reliable and robust. The method uses simple and economical liquid-liquid extraction of the drugs from human plasma. Solid phase extraction process was time consuming and reproducibility problem during analysis. Therefore this easy

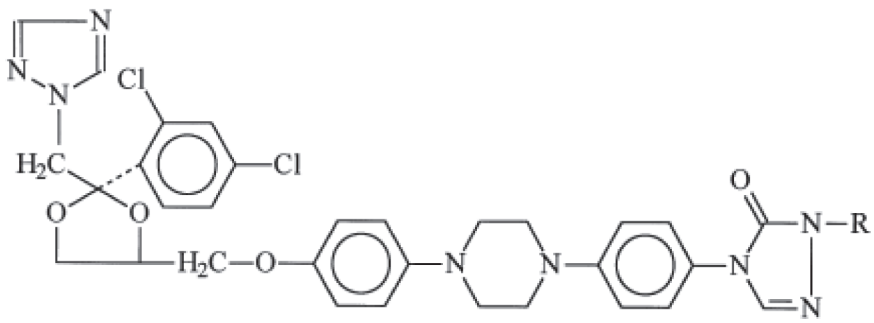

Figure 1
The chemical structures of ITR and HITR

applicable and cheap method can be used for analysing ITR and metabolite from plasma. The limit of detection of developed method is as low as LC-MS/MS system by liquid-liquid extraction procedure[3,4]. The validation results were included specificity, accuracy, extraction recovery, linearity and range. The assay can be applied easily successfully to the pharmacokinetic and bioequivalence studies.

Experimental

Apparatus and Analytical Conditions

The HPLC system of Agilent 1100 series with Micro Mass Quattro micro API detector was used for determination ITR and metabolite. The HPLC system was consisted of Agilent 1100 series G1311A pump, G1329A Auto sampler. MassLynx V4.1 was used as instrument software.

Chromatographic separations were carried out at 20°C temperature using a reversed phase Waters X-Terra RP18, 3.5 μm, 50 x 4.6 mm column. The mobile phase was a mixture of a mixture of [(acetonitrile: 0.066 % ammonia solution) (80:20) (v/v). Flow rate of mobile phase was 0.4 mL/min. The Mass Spectrometer was operated at multiple reaction monitoring (MRM) and positive mode and set to select to m/z 705.30>392.30 for ITR, m/z 721.25 >408.30 for HITR and m/z 531.15 >489.20 for ketoconazole (Internal standard, IS). The electron-spray capillary (kV) was 3.50 Mass selective detector parameters was as shown in Table I. The injection volume was 15 μL.

Chemicals and reagents

ITR and ketoconazole (IS) were obtained from Nosch Labs Private Ltd and Amphar b.v. respectively. HITR was purchased from SYNCOM Inc. HPLC grade methanol and isoamylalcohol were purchased from Merck. HPLC grade acetonitrile was obtained from Lab-Scan. Hexane and ammonium hydroxide were purchased from Merck. All other chemicals were analytical grade. Drug free plasma sample was obtained from Turkish Blood Centre. Millipore HPLC grade water were used through the study.

Preparation of standard solutions and quality control samples

A stock solution of ITR, HITR and IS (each of them, 0.5 ng/mL) was prepared using MeOH. Secondary standard solution of ITR, HITR and

TABLE I
Detector parameters

Detector Parameters		Value
Source (ES+)	Capillary (kV)	3.50
	Extractor (V)	3.00
	RF Lens (V)	0.1
	Source Temperature (°C)	130
	Desolvation Temperature (°C)	350
	Cone Gas Flow (L/Hr)	50
	Desolvation Gas Flow (L/Hr)	900
Analyser	LM 1 Resolution	14.0
	HM 1 Resolution	14.0
	Ion Energy 1	0.6
	Entrance	-1
	Exit	1
	LM 2 Resolution	13.5
	HM 2 Resolution	13.5
	Ion Energy 2	1.0
	Multiplier (V)	650

IS were prepared by diluting stock solution with MeOH and acetonitrile, respectively. All solutions were stored at 4°C until end of the study. The seven calibration standard were prepared by plasma from 1.00 to 600.0 ng/mL and from 2.00 to 600.0 ng/mL) independently for ITR and HITR, respectively. The quality control (QC) samples at a concentration 1.00; 3.00; 30.00; 480.0; 600.0 ng/mL for ITR and 2.00; 6.00; 30.00; 480.0; 600.0 ng/mL were made by diluting the secondary standard solution with human blank plasma. All calibration standard and QC sample were stored at -80°C until the end of study.

Plasma sample processing

ITR and HITR were extracted from plasma samples by using liquid-liquid extraction. 1 ml plasma samples in polypropylene tubes were spiked with 100 microliter 100 ng/mL Internal standard (IS) solution and vortexed for 20 seconds. 5 ml extraction solvent, (Hexan:Isoamylalcohol) (97:3; v/v) were added to each tube and vortexed 30 seconds to extract ITR and metabolite. The tubes were centrifuged at 5000 rpm, 5°C for 10

min. The supernatants were transferred into clean tubes and evaporated to dryness under nitrogen atmosphere at 40°C temperature. After evaporation samples were reconstituted by mobile phase and 15 µL injected to LC-MS/MS system.

Bioanalytical Method Validation

The method was validated according to FDA guidelines for validation of bioanalytical methods[5,8]. In order to show the acceptable nature of the analytical method, the following protocol was implemented during the method evaluation.

Selectivity

The selectivity of method was assessed by analyzing six different drug free human control plasma (four different source human plasma, one hemolyzed plasma and one liphophilic plasma). Chromatograms were compared for any interference from the matrix or any of the assay reagents.

Sensitivity

The lowest standard 1.0 ng/mL for ITR and 2.0 ng/mL for HITR on the calibration curve was identified as the lower limit of quantification (LOQ) with a precision of less than or equal to 20 %.

Linearity

The calibration curve was prepared from seven spiked plasma samples within the range of 1.0-600.00 ng/mL, including LOQ for ITR and 2.0-600.00 ng/mL, including LOQ for HITR . The acceptance criteria of back calculated standard concentration was 15 % deviation from nominal value except the LOQ (for LOQ 20 % deviation was applied). The calibration curve was obtained by plotting the area ratios of ITR, metabolite and IS as a function of the concentrations using least squares linear regression analysis. The LOQ was defined as a reproducible lowest concentration with signal to noise ratio greater than 10.

Recovery

Recovery of the method was performed comparing the three quality control (QC) samples at low, medium and high. The recoveries of ITR and

metabolite and IS were determined by comparing peak area obtained for QC samples that were subjected to the extraction procedure with those obtained from blank plasma extracts that were spiked post extraction to the same nominal concentrations.

Accuracy and precision

Intra batch accuracy and precision were determined by analysis of six replicates of 5 concentrations including low, medium and high concentration QC samples. Inter-batch accuracy and precision were determined by the analysis of these QC samples on three separate states. The overall precision of the method was expressed as percentage of coefficient of variation and the accuracy of the method was expressed in terms of relative errors.

Stability

The stability of the processed samples were tested in 5 terms. First, autosampler stability was tested by analysis after storage in the autosampler for 30h at room temperature. Second, freeze-thaw stability of samples was obtained over four freeze-thaw cycles at room temperature. Third, short-term stability was evaluated by keeping QC samples 6h and then reanalysing. Fourth, long term stability was tested by using QC samples stored at -80°C for 2 months and still continuing stability analysis every 3 months during one year. Fifth, stock solution stability was determined immediately after preparation of stock solution of ITR and metabolite and IS after 10 days at room temperature and +5°C.

Results and Discussion

Specificity and selectivity

Chromatographic separation of analyte and IS was optimized to provide acceptable resolution, good peak shape and intensity of the response. Mobile phase composition was changed systematically to establish chromatographic conditions giving an acceptable resolution. Mobile phase [(acetonitrile: 0,066 % ammonia solution) (80:20) (v/v)] provided good resolution for ITR and metabolite and IS. Retention time of ITR, metabolite and IS are given in Table II. Endogenous interference at the retention times of ITR, HITR and IS was not found in six different drug

TABLE II
Retention time of analyte, metabolite and IS

Analyte	**Retention Time (min.)**
ITR	2.35
HITR	1.86
Ketoconazole (IS)	1.80

free human control plasma extraction. Figure II and III shows representative chromatograms for blank and standards. The LOQ was defined as a reproducible lowest possible concentration within the calibration curve. The LOQ was found to be 1 ng/mL for ITR and 2 ng/mL for HITR. The intra and inter day CV% value are given Table III. The LOQ value of proposed analytical method was not low as found in MS/MS system reported before with lower LOQ values[2,3]. The proposed method showed sufficient selectivity and sensitivity for the determination of ITR and metabolite from plasma for pharmacokinetic and bioequivalence studies.

Linearity and sensitivity

The standard calibration curves was linear over the concentration range from 1.0-600 ng/mL with mean r^2=0.99509 for ITR and r^2=0.99252 for HITR respectively. The LOQ was 1.0 ng/mL for ITR and 2.0 ng/mL for HITR. The calibration curve had a regression equation of y=0.04118x -0.00102 for ITR, y=0.00534x-0.00129 and for HITR respectively where y is the peak area ratios of ITR and metabolite to IS and x is the plasma concentrations of ITR and metabolite.

Accuracy and precision

The precision was expressed as the percentage of coefficient of variation. Table IV and V gives a summary of the accuracy and precision at ITR and metabolite concentrations 1-600 ng/mL and 2-600 ng/mL.

Recovery

Acetonitrile:methyl tertiary butyl ether, hexan:dichloromethan, Hexan: dichloromethan:ethylacetate, hexan: methyl tertiary butyl ether, cyclohexan: methyl tertiary butyl ether, cyclohexan:isoamylalcohol mixtures were tested with different percentages as a solvent for the extraction

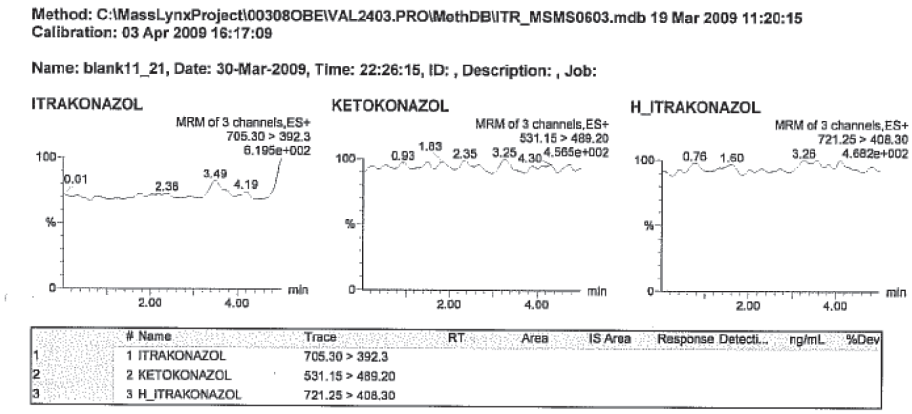

Figure 2
Chromatogram of blank plasma sample which was not contained ITR, HITR and IS

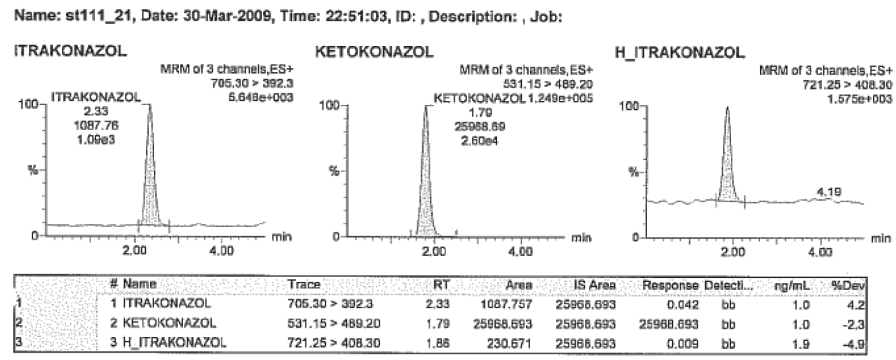

Figure 3
Chromatogram of spiked plasma sample which was contained 1ng/mL ITR and 2 ng/mL HITR

TABLE III
The intra and inter day CV% value

	Itraconazole (1-600 ng/mL)		Hydoxy-ITR (2-600 ng/mL)	
	Min.	Max.	Min.	Max.
Inter-day CV% Range(n=6)	2.43	5.53	1.48	9.79
Intra-day CV% Range(n=18)	3.55	5.47	4.84	8.30

TABLE IV

Intra-day coefficient of variation and relative error% for determination of HITR

Sample	Conc. of HITR (ng/mL)	Mean conc. of HITR (ng/mL)	Relative Error%	SD	CV%	n
QC1	2	1.98	98.14	0.13	6.73	18
QC2	6	5.62	100.55	0.47	8.30	18
QC3	30	26.73	89.73	1.29	4.84	18
QC4	480	489.02	102.25	31.73	6.49	18
QC5	600	577.57	101.79	45.02	7.80	18

TABLE V

Intra-day coefficient of variation and relative error% for determination of ITR

Sample	Conc. of ITR (ng/mL)	Mean conc. of ITR (ng/mL)	Relative Error%	SD	CV%	n
QC1	1	0.98	98.88	0.05	5.47	18
QC2	3	3.02	93.71	0.16	5.36	18
QC3	30	26.92	89.11	1.02	3.78	18
QC4	480	490.80	101.88	17.43	3.55	18
QC5	600	610.71	96.26	21.95	3.59	18

of ITR, metabolite and IS from plasma. Hexan:isoamylalcohol (97:3 / v:v) mixture showed sufficient yield and clear baseline. Thus, this solvent mixture was selected as a solvent for extraction.

The recovery of ITR and metabolite in the liquid-liquid extraction procedure from 1 mL of plasma was measured at low, medium and high concentrations (1.00-30.00-600.0 ng/mL and 2.00-30.00-600.0 ng/mL for ITR and HITR, respectively). The mean relative recoveries of ITR and metabolite from plasma ranged from 30 to 90 % with CV between 1.95 and 15.54 %. These results indicate sufficient recovery for ITR and HITR and allowed us to conclude that our method is able to quality ITR and metabolite from human plasma samples.

Stability

The stability was assessed under a variety of conditions and the data are shown in Table VI-X. All samples stored at -80°C. Short-term stability

TABLE VI

The freeze-thaw stability of ITR and HITR

Active	Conc. (ng/mL)	0. Cycle	3. Cycle			
		Mean of Conc. (ng/mL)	Mean of Conc. (ng/mL)	Relative Error %	SD	CV %
	1	1.04	0.99	95.18	0.035	3.54
ITR	40	40.4	36.34	89.94	2.871	7.18
	600	636.31	594.28	93.4	29.71	4.95
	2	1.9	2.03	106.72	0.092	4.60
HITR	50	45.77	46.11	100.73	0.240	0.48
	600	683.68	667.27	97.6	11.60	1.93

TABLE VII

The short-term stability of ITR and HITR

Active	Concentration (ng/mL)	Short-term Stability (Relative Error%)	SD	CV %
ITR	1	76.37	12.30	16.11
	30	106.64	14.60	13.69
	600	107.34	14.22	13.24
HITR	2	107.73	10.60	9.84
	30	102.55	15.10	14.73
	600	84.66	12.30	14.53

TABLE VIII

The auto sampler stability of ITR and HITR

Active	Concentration (ng/mL)	Auto sampler stability (30 h) (Relative Error%)	SD	CV %
ITR	1	103.97	9.35	8.99
	30	112.84	4.40	3.90
	600	96.24	5.64	5.86
HITR	2	122.79	9.09	7.40
	30	124.74	7.05	5.65
	600	113.12	11.89	10.51

TABLE IX
The stock solution stability of ITR and HITR

Active	**ITR Stock**	**Metabolite**	**HITR Stock**	**Internal Standart**	**Ketoconozole Stock**
	Stability of 10 days (Relative Error%)		**Stability of 10 days (Relative Error%)**		**Stability of 10 days (Relative Error%)**
Mean	112.28	**Mean**	80.23	**Mean**	72.16
SD	3.63	**SD**	5.05	**SD**	2.71
%CV	3.23	**%CV**	6.29	**%CV**	3.75

TABLE X
The long-term stability of ITR and HITR

Active	**Cons. (ng/mL)**	**0^{th} hour Mean of Conc. (ng/mL)**	**12^{th} month Mean of Conc. (ng/mL)**	**Relative Error%**	**SD**	**CV%**
ITR	1	0.97	0.82	85.28	7.26	8.51
	30	28.15	31.00	110.16	4.36	3.95
	600	622.57	585.63	94.10	4.05	4.31
HITR	2	1.99	2.05	103.83	21.72	20.92
	30	26.93	28.24	105.18	7.52	7.15
	600	606.02	622.98	102.85	3.02	2.94

of ITR and metabolite at room temperature for 6h had no effect on quantification. Auto sampler stability and stock solution stability were tested and found to be 30 h and three freeze-thaw cycles of the quality control samples were appropriate for the quantification. Long term stability was tested by using QC samples stored at -80 C for 12 months. Stock solutions stability in methanol were found 10 days at +4°C.

Pharmacokinetic application

This method was used to examine the pharmacokinetics of ITR and HITR. Healthy volunteer was administrated of a single dose of itraconazole under fed condition. Figure IV shows the plasma concentration-time profile of ITR and HITR.

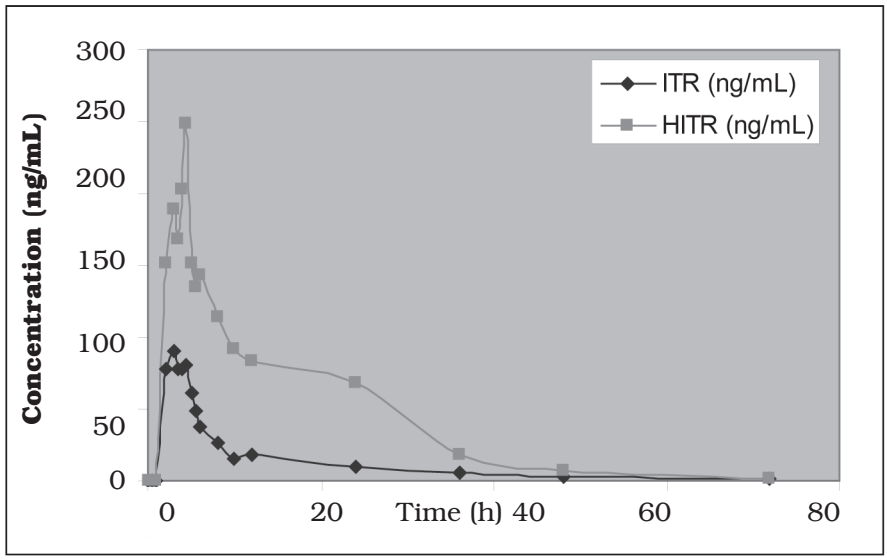

Figure 4
The plasma concentration- time profile of ITR and HITR

Conclusion

The developed method for the determination of ITR and metabolite from biological material has been found accurate, precise, selective, and suitable for the bioequivalence and pharmacokinetic studies.

Summary

Itraconazole is a triazole antifungal agent with a broad spectrum of activity. It acts primary by inhibiting the biosynthesis of ergosterol, an essential component of fungal cell membrans. It is used in the treatment of a variety of fungal infections. Following oral absorption, it is extensively metabolized by side chain hydroxylation (by CYP3A4) to form hydroxy-itraconazole.

A simple and sensitive high performance liquid chromatographic method with MS detection (HPLC-MS) for the determination of itraconazole and its metabolite from plasma was developed and validated. Liquid-liquid extraction was used for extracting itraconazole and its

metabolite from plasma. (hexan:isoamylalcohol) (97:3) was used as extraction solvent. The chromatographic separation of itraconazole, metabolite and ketoconazole (IS) was carried out using reverse phase X-Terra RP18, 3.5 μm, 50 x 4.6 mm with mobile phase of [(Acetonitrile: 0.066 % ammonia solution) (80:20) (v/v)]. The flow rate of mobile phase was 0.4 mL/min, injection volume was 15 μL. The mass spectrometric parameters were optimized to obtain maximum sensitivity at unit resolution. Atmospheric pressure ionization-electrospray mode (API-ES) was used at positive ionization. Data was collected by multiple reaction monitoring (MRM). The ions used to quantify were selected as m/z 705.30>392.30 for itraconazole, m/z 721.25 >408.30 for hydroxy-itraconazole, and m/z 531.15 >489.20 for IS. The calibration curve was linear within the concentration range 1-600 ng/mL for itraconazole and 2-600 ng/mL for hydroxy-itraconazole. The limit of quantification was 1 ng/mL and 2 ng/mL itraconazole, hydroxy-itraconazole, respectively, with good accuracy and precision. The stability was assessed under a variety of conditions and found that appropriate for the quantification. The method developed can be used for bioequivalence and pharmacokinetic studies.

Keywords: Itraconazole, Hydroxy-itraconazole, ketoconazole, human plasma, LC-MS/MS, bioanalytical method validation

Özet

İtrakonazol ve Metabolitinin İnsan Plasmasından HPLC-Tandem Mass (LC-MS/MS) Spektrometresiyle Tayini

İtrakonazol (ITR), triazol türevi bir antifungal ilaçtır. Öncelikli olarak mantar hücre membranlarının gerekli bileşeni olan ergosterol sentezini inhibe etmede rol oynar. Çeşitli sistemik mikozların tedavisinde önemli yer tutar. Oral absorbsiyonun arkasından, yan zincir hidroksillenme (CYP3A4 ile) ile yaygın bir şekilde metabolize olarak hidroksi-itrakonazole (HITR) dönüşür.

ITR ve metabolitinin insan plasmasından HPLC-MS sistemiyle tayininde basit ve hassas bir biyoanalitik metod geliştirildi ve valide edildi. İtrakonazol ve metaboliti, plazmadan sıvı-sıvı ekstraksiyonla ekstrakte edildi. Ekstraksiyon solventi olarak (hekzan: isoamilalkol) (97:3) karışımı kullanıldı. X-Terra RP18, 3.5 μm, 50 x 4.6 mm HPLC kolonu ve [(asetonitril: 0.066 % ammonia solution)(80:20)(v/v)] mobil fazı kullanılarak ITR,

metaboliti ve ketakonazolün (IS) kromatografik ayrımı sağlandı. Mobil faz akış hızı 0.4 mL/dak, enjeksiyon hacmi 15µL'dir. Kütle spektrometresine ait parametreler birim rezolüsyonda maksimum hassasiyet sağlayacak şekilde optimize edildi. API-ES pozitif modda çalışıldı. Datalar MRM (multiple reaction monitoring) olarak toplandı. ITR için m/z 705.30>392.30, HITR için m/z 721.25 >408.30, IS için m/z 531.15 >489.20 kütleleri üzerinden miktar tayini yapıldı. ITR için 1-600 ng/mL, HITR için 2-600 ng/mL derişim aralığında doğrusal kalibrasyon eğrisi oluşturuldu. LOQ değerleri ITR için 1 ng/mL, HITR için 2 ng/mL'dir. Çeşitli koşullarda stabilite sonuçları değerlendirildi ve analiz sonuçları uygun bulundu. Geliştirilen bu metod bioeşdeğerlik ve farmakokinetik çalışmalarda kullanılabilir.

Anahtar Kelimeler: İtrakonazol, Hidroksi-itrakonazol, Ketakonazol, insan plazması, LC-MS/MS, bioanalitik metod validasyonu

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