AMERICAN JOURNAL OF PHARMTECH RESEARCH
Journal home page: http://www.ajptr.com/

Rapid Determination of Ketoconazole Level in Human Plasma by High Performance Liquid Chromatography

Rajaa F Hussein[1],Muhammad M Hammami[1]*
1.Clinical Studies and Empirical Ethics Department King Faisal Specialist Hospital & Research Center MBC-03, P.O. Box 3354, Riyadh 11211, Kingdom of Saudi Arabia

ABSTRACT

A simple, precise, and rapid high performance liquid chromatography (HPLC) method for the determination of ketoconazole level in human plasma using itraconazole as an internal standard (IS) was developed and validated. 0.25 ml plasma samples containing ketoconazole were mixed with 15 µg of the IS. After adding 0.25 ml acetonitrile, the mixture was vortexed for two minutes and then centrifuged for 10 minutes at 16000 rpm at room temperature. The clear supernatant was transferred into an auto-sampler vial, and 100 µl was injected into the HPLC system with a run time of 10 min. The compounds of interest were efficiently separated on 4.6 x 150 mm, Symmetry Shield $^{TM}RP_{18}$ 5-µm steel column, using a Guard Pak pre-column module with Radial-Pak C_{18} 5-µm insert, and detected using Waters 2475 multi λ fluorescence detector with an the excitation and emission wavelengths set at 260 and 375 nm, respectively. The mobile phase consisted of 0.02 M potassium dihydrogen phosphate (pH = 6.0, adjusted with 0.1 M sodium hydroxide) and acetonitrile (40:60, v:v), and was delivered at a flow rate of 1.0 ml/min. No interference from blank plasma or commonly used drugs was observed; and the detection limit of ketoconazole was 0.1 µg/ml. The relationship between ketoconazole concentration in plasma and peak area ratio of ketoconazole /IS was linear ($r^2 \geq 0.9979$) in the range of 0.1– 20 µg/ml. Intra- and inter-day coefficients of variation (CV) were ≤ 8.1% and ≤ 9.7%, respectively, with corresponding biases of ≤ -13% and ≤ 0.9%, respectively. Mean extraction recovery of ketoconazole and IS were ≥ 85% and 92%, respectively. Using the method, ketoconazole was found to be stable for 48 hrs at -20°C (≥ 95%) in processed samples and for 8 weeks at -20°C (100%) in unprocessed samples.

Key words: Ketoconazole, Itraconazole, Human plasma, HPL

*Corresponding Author Email: muhammad@kfshrc.edu.sa
Received 12 December 2016, Accepted 20 December 2016

Please cite this article as: Hammami MM *et al.*, Rapid Determination of Ketoconazole Level in Human Plasma by High Performance Liquid Chromatography. American Journal of PharmTech Research 2017.

INTRODUCTION

Ketoconazole sodium (CAS: 65277-42-1), cis-1-Acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl] methoxy]phenyl]piperazine[1], is an imidazole fungicidal agent with a broad spectrum activity that is used for treatment of superficial and systemic fungal infections[2,3]. It is a weak dibasic agent that requires high acidity for dissolution and absorption [4]. Its bioavailability is maximal when taken with meals[5]. Following oral administration of a single 200 mg dose with meals, a mean peak plasma concentration of 3.5 µg/ml is reached within 1 to 2 hours[6] with an elimination half-life of 2 hours[7].

Several high-performance liquid chromatography (HPLC) methods have been reported for the determination of ketoconazole in pharmaceutical preparations [8-12] and human plasma samples[13-15]. Some methods suffered from lack of sensitivity[13,14] and some required relatively large plasma volume (1 ml)[14]. Further, the reported information on stability of ketoconazole is limited[15].

We describe a simple, precise, and accurate HPLC method for ketoconazole level determination in human plasma. The method uses 0.25 ml plasma and a simple extraction method, was fully validated, and was successfully used to determine ketoconazole stability under several clinical laboratory conditions.

MATERIALS AND METHOD

Apparatus

Chromatography was performed on a Waters Alliance HPLC e2695D Separations Module, an 4.6 x 150 mm, Symmetry Shield ^{TM} RP_{18}, 5-µm (particle-size) steel column, a Guard Pak pre-column module with Radial-Pak C_{18}, 5-µm insert, and Waters 2475 multi λ fluorescence detector with an the excitation and emission wavelengths set at 260 and 375 nm, respectively (Waters Associates Inc., Milford, MA, USA). Data were collected with a Pentium D computer using Empower Chromatography Manager Software (Waters Associates Inc., Milford, MA, USA).

Chemicals and reagents

All reagents were of analytical grade unless stated otherwise. Ketoconazole and itraconazole standards were obtained from Sigma-Aldrich Co., St. Louis, MO, USA. Potassium dihydrogen phosphate and acetonitrile (HPLC grade) were purchased from Fisher Scientific, Fairlawn, NJ, USA. Sodium hydroxide pellets were purchased from BDH Chemicals Ltd, Poole, England. HPLC grade water was prepared by reverse osmosis and was further purified by passing through a Synergy UV (Millipore, Bedford, MA, USA). Drug-free left over human plasma was obtained

from the blood bank of King Faisal Specialist Hospital & Research Centre (KFSHRC) Riyadh, Saudi Arabia.

Chromatographic conditions

The mobile phase consisted of a mixture of 0.02 M potassium dihydrogen phosphate (pH = 6.0, adjust with 0.1 M sodium hydroxide) and acetonitrile (40:60, v:v) and was delivered at a flow rate of 1.0 ml/min. The analysis was carried out under isocratic condition maintaining column at room temperature.

Preparation of standard and quality control samples

Stock solutions (1 mg/ml) of ketoconazole and itraconazole (internal standard, IS) were prepared in methanol. They were diluted with blank human plasma or water, respectively, to produce working solutions of 20 µg/ml ketoconazole in plasma and 300 µg/ml IS in mobile phase. Calibration curve standards (nine concentrations) in the range of 0.1 – 20 µg/ml were prepared in human plasma. Four quality control (QC) samples (0.1, 0.3, 10, and 18 µg/ml) were also prepared in human plasma. 0.25 ml aliquots in 1.5 ml eppendorf micro centrifuge tubes (Fisher Scientific Co., Fairlawn, NJ, USA) were stored at –20ºC until used.

Sample preparation

Aliquots of 0.25 ml of calibration curve standards and QC samples in 1.5 ml eppendorf micro centrifuge tubes were allowed to equilibrate to room temperature. To each tube, 50 µl of the IS working solution containing 15 µg IS were added and the mixture was vortexed for 20 seconds. After the addition of 0.25 ml acetonitrile, the mixture was vortexed again for 2 min and then centrifuged for 10 min at 16000 rpm at room temperature. The clear supernatant organic layer was carefully transferred into an auto-sampler vial, and 100 µl were injected into the HPLC system. The run time was 10 min.

Stability studies

Three QC samples (concentration 0.1, 0.3, and 18 µg/ml) were used for stability studies: five aliquots of each QC sample were extracted and immediately analyzed (baseline), five aliquots were allowed to stand on the bench-top for 24 hours at room temperature before being processed and analyzed, five aliquots were stored at -20ºC for 8 weeks before being processed and analyzed, and five aliquots were processed and stored at room temperature for 24 hours or at -20 ºC for 48 hours before analysis. Fifteen aliquots of each QC sample were stored at -20ºC for 24 hours. They were then taken out of freezer and left to completely thaw unassisted at room temperature. Five aliquots of each sample were extracted and analyzed and the rest were returned to -20ºC for another 24 hours. The cycle was repeated three times.

Method validation

The method was validated according to standard procedures described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance[16]. The validation parameter included: specificity, linearity, accuracy, precision, recovery and stability.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Optimal experimental conditions consisted a mobile phase composed of 0.02 M of potassium dihydrogen phosphate (pH = 6.0, with 0.1 M sodium hydroxide) and acetonitrile (40:60, v:v) that was delivered at a flow rate of 1.0 ml/min. Under these conditions ketoconazole, IS, and components of plasma exhibited a well-defined separation within a 10 min run. The retention times of ketoconazole and itraconazole were around 3.5 and 8.6 min, respectively.

Specificity

Specificity is defined as the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. Potential interfering substances in plasma samples include endogenous components, metabolites, and decomposition products. We screened six batches of blank human plasma and eight frequently used medications (aspirin, acetaminophen, ranitidine, caffeine, nicotinic acid, ascorbic acid, omeprazole, and diclofenac sodium) for potential interference. No interference was found by plasma components and none of the drugs co-eluted with ketoconazole or the IS. Figure 1 depicts a representative chromatogram of drug free human plasma used in preparation of calibration standards and QC samples.

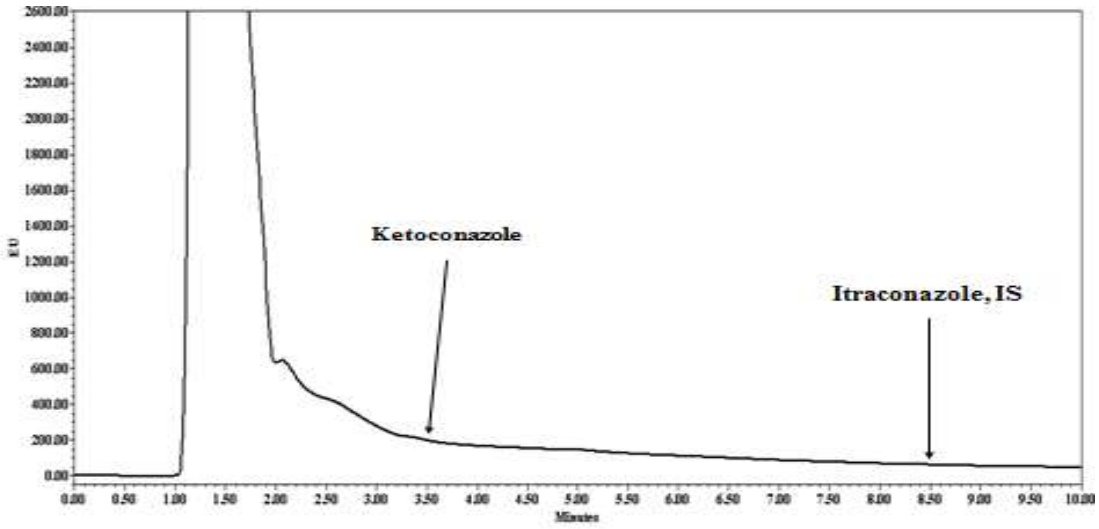

Figure 1: Representative chromatogram of drug-free human plasma. The arrows indicate retention times of ketoconazole and the internal standard (itraconazole, IS).

Limit of detection & quantification and linearity

The limit of quantification, defined as the lowest concentration on the calibration curve that can be determined with acceptable precision and accuracy (i.e., coefficient of variation and bias ≤15%), was 0.1 µg/ml. The limit of detection (signal to noise-ratio ≥3) was 0.05 µg/ml. Linearity of ketoconazole was evaluated by analyzing ten curves of nine standard concentrations (plus zero concentration) prepared in human plasma. Figure 2 depicts an overlay of chromatograms of a typical calibration curve. Mean (SD) of slope, intercept, and coefficient of determination (R^2) of the ten curves were 0.0971 (0.0088), 0.0049 (0.0093), and 0.9979 (0.009), respectively. The suitability of the calibration curves was confirmed by back-calculating the concentration of ketoconazole from the calibration curves (Table 1). All the calculated concentrations were well within the acceptable limits.

Table 1: Back calculated ketoconazole concentrations from ten calibration curves

Nominal level (µg/ml)	Measured level (µg/ml)	CV (%)	Bias (%)
	Mean (SD)		
0.1	0.09 (0.01)	11.9	-10
0.2	0.19 (0.02)	9.5	-5.0
0.4	0.39 (0.03)	6.6	-2.5
0.8	0.83 (0.07)	7.9	3.75
1.0	1.03 (0.07)	7.2	3.0
4.0	4.00 (0.25)	6.2	0
8.0	8.39 (0.52)	6.2	2.3
16.0	16.03 (0.64)	4.0	4.9
20.0	19.93 (0.49)	2.5	4.7

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100.

Bias = (mean measured concentration – nominal concentration) divided by nominal concentration × 100.

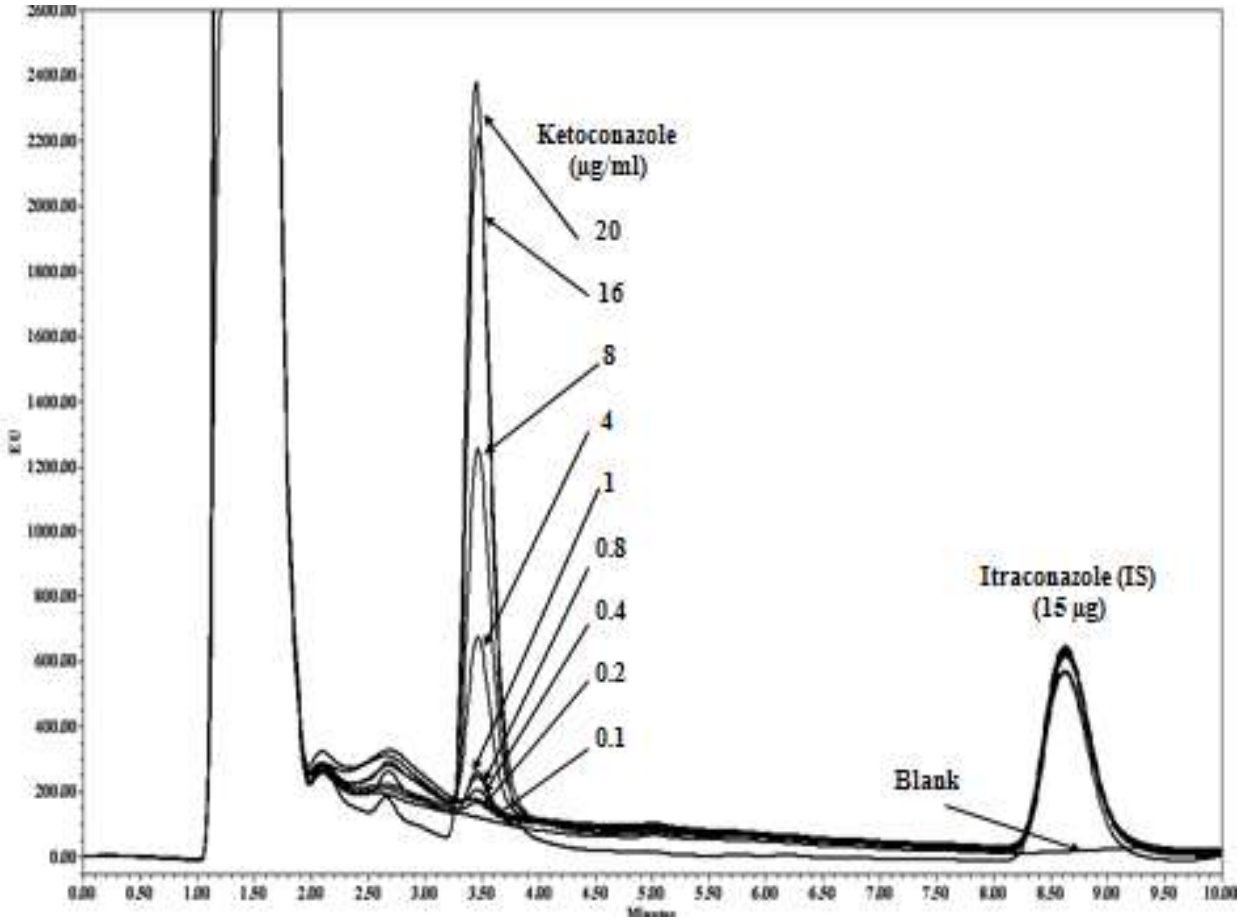

Figure 2: Overlay of chromatograms of extracts of 0.25 ml human plasma spiked with the internal standard (IS) or one of nine concentrations of ketoconazole.

Precision and bias

The intra-day and inter-day precision and bias of the method were evaluated by analyzing three QC concentrations (0.1, 0.3, and 18 µg/ml). Intra-day precision and bias (n = 10) ranged from 2.1% to 8.1% and from -13% to -2.4%, respectively. Inter-day precision and bias were determined over three different days (n = 20) and ranged from 4.1% to 9.7% and from -10% to 0.9%, respectively. The results are summarized in Table 2.

Table 2: Intra and inter-day precision and bias of ketoconazole assay

Nominal level (µg/ml)	Measured level (µg/ml) Mean (SD	CV (%)	Bias (%)
Intra-day (n= 10)			
0.1	0.09 (0.01)	8.1	-10
0.3	0.26 (0.02)	7.3	-13
10	9.17 (0.19)	2.1	- 8.3
18	17.56 (0.87)	4.9	-2.4
Inter-day (n= 20)			
0.1	0.09 (0.01)	7.5	-10
0.3	0.27 (0.02)	6.6	-10
10	10.09 (0.98)	9.7	0.9
18	17.90 (0.73)	4.1	-0.6

SD, standard deviation. CV, standard deviation divided by mean measured concentration x100.

Bias = (mean measured concentration – nominal concentration) divided by nominal concentration × 100.

Recovery

Extraction recovery of ketoconazole was assessed by direct comparison of peak areas from plasma and mobile phase samples, using five replicates for each of the four QC samples (0.1, 0.3, 10, and 18 µg/ml). Similarly, the recovery of the IS was determined by comparing peak areas of the IS in five aliquots of human plasma spiked with 50 µl of 3000 µg/ml IS solution with the peak areas of equivalent samples prepared in the mobile phase. The results are presented in Table 3.

Table 3: Recovery of ketoconazole and the internal standard from 0.25 ml human plasma

Concentration (µg/ml)	Human plasma Mean (SD)	Mobile phase Mean (SD)	Recovery (%)
Cefazolin			
0.1	1443707 (107479)	15539944 (76914)	93
0.3	4658398 (502005)	5303843 (156914)	88
10	164875551 (7839652)	193442190 (2315314)	85
18	325440139 (3760434)	351132610 (2622281)	93
Internal standard			
60	253058677 (96657)	274210375 (26203)	92

Data represent mean peak area (standard deviation), n = 5. Recovery is calculated as mean peak area in human plasma divided by mean peak area in mobile phase x 100.

Robustness and ruggedness

The robustness of a method is a measure of its capacity to remain unaffected by small variations in analysis conditions. The robustness of the current assay was evaluated by slightly altering proportions of acetonitrile (± 2.0%), and the pH of the mobile phase (± 0.2). No significant

changes were observed. Ruggedness was tested by conducting split sample test. Two split samples (concentrations 0.3 and 18 µg/ml) were analyzed by two blinded technologists on two different instruments. The accuracy of the reported concentrations was within the acceptable limits (bias ≤ 5.0%).

Stability

Stability of ketoconazole and the IS in biological matrices is an important pre-analytical variable. It is necessary to perform stability studies to determine the range of appropriate conditions and time of storage. Ketoconazole and IS stability in processed and unprocessed plasma samples were investigated using three QC samples (0.1, 0.3, and 18 µg/ml). Ketoconazole in processed samples was found to be stable for 24 hours at room temperature (100%) and 48 hours at -20°C (100%). Ketoconazole in unprocessed samples was stable for at least 24 hours at room temperature (≥ 96%), 8 weeks at −20°C (100%), and after three freeze-and thaw cycles (≥ 92%). Table 4 summarizes the results of stability studies.

Table 4: Stability for ketoconazole in human plasma

Stability (%)							
Nominal level(µg/ml)	**Unprocessed**		**Processed**		**Freeze-Thaw Cycle**		
	24 hrs RT	**8 wks -20 °C**	**24 hrs RT**	**48 wks -20 °C**	**1**	**2**	**3**
0.1	97	100	103	98	93	94	97
0.3	96	100	102	100	92	92	92
18	111	106	101	95	92	96	97

Stability (%) = mean measured concentration (n=5) at the indicated time divided by mean measured concentration (n=5) at baseline x 100. Spiked plasma samples were processed and analyzed immediately (baseline, data not shown), after 24 hours at room temperature (24 hrs RT), or after freezing at –20 ºC for 8 weeks (8 wks, –20 ºC), or processed and then analyzed after storing for 24 hours at room temperature (24 hrs, RT) or 48 hours at –20 ºC (48 hrs, -20 ºC). Freeze-thaw (FT), samples were frozen at -20 ºC and thaw at RT.

CONCLUSION

The described HPLC assay is precise, simple, accurate, and rapid. It requires 0.25 ml plasma and utilizes a simple protein precipitation procedure. The assay was successfully applied to determine stability of ketoconazole under various conditions encountered in the clinical laboratory.

REFERENCES

1. https://www.tocris.com/dispprod.php?ItemId=2333 **Ketoconazole** Supplier | **CAS 65277-42-1** | Tocris Bioscience. Downloaded from http://aac.asm.org/ on August 2, 2016.

2. Dumaine R, Roy ML, Brown AM. Blockade of HERG and Kv1.5 by ketoconazole. J.Pharmacol.Exp.Ther. 1998; 286(2):727-35.
3. Zimmerman HJ. Antifungal agents. In, Zimmerman HJ. Hepatotoxicity: the adverse effects of drugs and other chemicals on the liver. 2nd ed. Philadelphia: Lippincott, 1999, pp. 609-11.
4. Carlson JA, Mann HJ, and Canafax DM. Effect of pH on disintegration and dissolution of ketoconazole tablets. Am. J. Hosp. Pharm. 1983; 40 (8):1334-1336.
5. Mannisto, PT,. Maintyla R, R Nykiinen S, Lamminsivu U, and Ottoila P. Impairing effect of food on ketoconazole absorption. Antimicrob. Agents Chemother. 1986; 30 (2) 206210.
6. Daneshmend TK, Warnock DW, Ene MD , Johnson EM , Parker G, Richardson MD, and Roberts CJ. Multiple dose pharmacokinetics of ketoconazole and their effects on antipyrine kinetics in man. J. Antimicrob. Chemother. 1983; 12:185-188.
7. Heel RC, Brogden RN, Carmine A, Morley PA, Speight TM, and Avery GS. Ketoconazole: a review of its therapeutic efficacy in superficial and systemic fungal infections. Drugs. 1982; 23:1-36.
8. Kedor-Hackmann ERM, Nery MMF, Santoro MIRM. Determination of ketoconazole in pharmaceutical preparations by ultraviolet spectrophotometry and high-performance liquid chromatography. Anal Lett., 1994; 27(2): 363-376.
9. Kedor-Hackmann ERM, Santoro MIRM, Singh AK, Peraro AC. First-derivative ultraviolet spectrophotometric and high performance liquid chromatographic determination of ketoconazole in pharmaceutical emulsions. Braz J Pharm Sci., 2006; 42(1): 91-98.
10. Velikinac I, Cudina O, Jankovic I, Agbaba D, Vladimirov S. Comparison of capillary zone electrophoresis and high performance liquid chromatography methods for quantitative determination of ketoconazole in drug formulations. Farmaco. 2004; 59(5): 419-424.
11. Vander HY, Nguyen MNA, Detaevernier MR, Massart DL, Plaizier-Vercammen J. Simultaneous determination of ketoconazole and formaldehyde in a shampoo: J Chromatogr A. 2002; 7- 958(1-2):191-201
12. Amrutiya N, Madan M, Bajaj A. Development and validation of RP-HPLC method for simultaneous estimation of prednicarbate, mupirocin and ketoconazole in topical dosage forms. J of Anal Chem. 2010; 65(11): 1148–1154.
13. Fraihat SM, Bahgat KM. Spectrophotometric Methods for the Determination of Ketoconazole in Pharmaceutical Dosage Forms. Tropical Journal of Pharmaceutical Research September 2014; 13 (9): 1511-1514.

14. Kumar Jat R, S Sharma S,. Chhipa RC, Rambir Singh R, Alam. Development and validation of reverse-phase HPLC method for estimation of Ketoconazole in bulk drug. Pharmacophore. 2012; 3 (2):123-129.
15. Venishetty VK, Parikh N, Sistla R,. Ahmed FJ, Diwan PV. Application of Validated RP-HPLC Method for Simultaneous Determination of Docetaxel and Ketoconazole in Solid Lipid Nanoparticles. J Chromatogr Sci. 2011;49 (2):136-41.
16. Bioanalytical Method Validation, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), May 2001.