Journal of Science and Mathematics Letters, Vol 10, Issue 1, 2022 (1-9)
ISSN 2462-2052, eISSN 2600-8718

RESEARCH PAPER

Development and Validation of Quantification Method for Fluconazole in Human Serum Using Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry

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Received: 9 December 2020; **Accepted:** 29 March 2021; **Published:** 27 May 2022

To cite this article (APA): Rofiee, M. S., Jamari, M. H., Kek, T. L., & Salleh, M. Z. (2022). Development and Validation of Quantification Method for Fluconazole in Human Serum Using Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry. *Journal of Science and Mathematics Letters*, *10*(1), 1-9. https://doi.org/10.37134/jsml.vol10.1.1.2022

To link to this article: https://doi.org/10.37134/jsml.vol10.1.1.2022

Abstract

Fluconazole is one of the commonly prescribed antifungal with recommendation for therapeutic level monitoring to ensure its levels are within therapeutic range to reduce the risk of adverse effects, toxicity of this otherwise safe drug and to prevent occurence of resistant fungi. A sensitive and robust method was developed to quantitate the level of fluconazole in human serum using ultra-high performance liquid chromatography-tandem mass spectrometry. Using Agilent SB-C18 column (50 x 2.1mm, 1.8 µm particle size), fluconazole was eluted separately from the internal standard using mobile phase (A) 0.1% formic acid in water and (B) methanol. The flow rate was set at 0.4 mL/min in positive ionization mode. The method was linear from 156.25 ng/mL to 5,000 ng/mL ($r^2 = 0.999$) with the recovery of 80-90%, and the accuracy and precision of 89-101% and 0-10%, respectively. Fluconazole was also proven stable under different test conditions with the accuracy of 87-114%. The developed method met the guidelines drawn by the European Medicines Agency, Food and Drug Administration and International Council of Harmonisation. The method used for purification of samples prior to injection was user-friendly and robust.

Keywords: fluconazole, quantitation, serum, validation, LC-MS/MS

INTRODUCTION

Fluconazole was first introduced in 1981 (Yamreudeewong et. al., 1993) and now considered as one of the most prescribed medications to treat fungal infection with more than 3 million in the USA (Kyle et al., 2010). This is because it is one of the most effective, safe as well cost-effective drugs according to the World Health Organisation (WHO, 2015). Fluconazole works by affecting

the cellular membrane of fungi (Kyle et al., 2010). It is found to be safer and have a predictable absorption when taken orally thus making it a better option as compared to the other azole antifungal (Rençber et al., 2019). There is still variability of the drug concentration in the blood due to the inconsistency in the pharmacokinetics of individuals (Andes et al., 2008). Therapeutic drug monitoring (TDM) is therefore recommended to ensure the levels are within therapeutic ranges (Ashbee et al., 2013). In addition, TDM is used to reduce the risk of adverse effects, toxicities and drug resistance so that drug therapy can be personalized i.e. therapy given to patients will not bring them more harm than it does good (Hope et al., 2008; Ashbee et al., 2013).

The oral dosage up to 400 mg or higher based on the patients' responses (Adedoyin et al., 2020), fluconazole can have adverse effects which include life-threatening liver failure (Clissold, 1990; Franklin et al., 1990; Muńoz et al., 1991), skin rashes (Stern et al., 1988; Sugar and Saunders, 1988; Mau et al., 1989), birth defects in pregnant women (Lopez-Rangel and Van Allen, 2005; Norgaard et al., 2008) and torsades de pointes (McMahon & Grayson, 2008) where the patient experience heart palpitations that can be fatal. These complications can be enhanced with pre-existing health conditions of patients. The medication is also reported to interact with other drugs (Nair and Morris, 1999), leading to an increase of the risk of side effects, thus consultation with healthcare professionals is a must before the medication is prescribed.

Various methods have been described for quantitation of fluconazole as summarised in Table 1. HPLC-UV has been proven to be insensitive whereas GC and GC-MS have problems with excessive injection carryover making LC-MS the preferred method (Hillis et al., 2004). Methods developed in other studies using the instrument LC-MS exhibited unsatisfactory peak resolution (Hillis et al., 2004) and the unpredictability of the method used (Bapiro et al., 2016).

Table 1. Comparison of Methods for the Quantitation of Fluconazole

Matrix	Sample Volume	Extraction Method	Instrument	Linear range	Lower limit of Quantitation	Reference
Plasma	100 uL	Protein Precipitation with deuteromethanol.	Liquid Chromatography Mass Spectrometry (LCMS)	50 ng/mL to 4000 ng/mL	50 ng/mL	Hillis et. al., 2004.
Serum	100 uL	Protein Precipitation with 0.1% Formic Acid in Acetanotrile	Liquid Chromatography Mass Spectrometry (LCMS)	0.2 µg/mL to 200 µg/mL	0.2 ug/mL	Xiao et. al., 2017
Serum and Plasma	50 uL	Protein Precipitation with Acetanotrile	Liquid Chromatography Mass Spectrometry (LCMS)	0.5 mg/L to 40 mg /L	0.5 mg/L	Müller et al., 2017
Plasma	1 mL	Liquid-liquid exraction using dichloromethane	High Performance Liquid Chromatogrphy	125 ng/mL - 10.0 µg/mL	125 ng/mL	Liew et al., 2012

In this study, Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS) has been used. It is a chemistry technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry. This technique is a powerful technique that has been used for many applications which have extremely high sensitivity and selectivity. Generally, it is accurate for the identification and quantification of the amount of chemicals or analytes in the complex mixture such as biological fluids. This study has successfully developed a method for the quantitative analysis of fluconazole

in serum where low volume of sample was used with a short run time ensuring high throughput of the instrument, relatively cheap extraction method as well as avoiding the need for the use of deuterated internal standard for analysis.

Ketoconazole was used as the internal standard as ketoconazole belongs to the same structural group as fluconazole in the azole group (Fig. 1). By selecting the internal standard from the same group as the target compound, this will ensure that the ion suppression and ion enhancement effects be experienced similarly across the run for both compounds and the analysis to be done smoothly as the ratio of the target analyte (fluconazole) and internal standard will be used throughout the analysis.

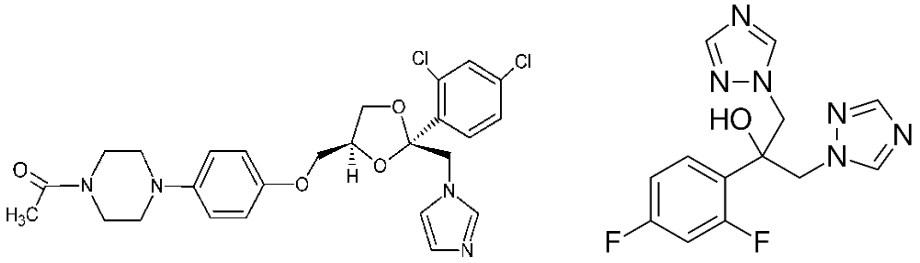

Figure 1. Comparison of the structure of ketoconazole (A) and fluconazole (B)

The developed quantification method satisfied the guidelines by European Medical Agency (EMA), 2011; US Department of Health and Human Services, Food and Drug Administration (FDA), 2018 and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, 2005 that includes selectivity, recovery and matrix effect, sampling, calibration curve, stability, sensitivity, accuracy and precision, dilution integrity as well as carryover criteria.

MATERIALS AND METHODS

Materials and Reagents

Reference standards for fluconazole and ketoconazole were obtained from Sigma Aldrich (St. Louis, USA). Formic acid was obtained from Sigma Aldrich (St. Louis, USA) whereas HPLC grade methanol and acetonitrile were obtained from Merck (Darmstadt, Germany) and purified water was obtained from an ELGA PURELAB ultra purification system (Wycombe, UK).

Chromatographic Condition

Chromatographic analysis was performed using Agilent 1260 Infinity II instrument control by Agilent Mass Hunter Workstation Acquisition (B.02.01). The separation was performed using Agilent Technologies' SB-C18 RRHT column (50 x 2.1 mm, 1.8 µm particle size) together with SB-C18 2.1 mm, 1.8 µm guard column. 0.1% formic acid in purified water was used as mobile phase A and 100% methanol was used as mobile phase B with the flow rate of 0.4 mL/min. The column temperature was kept at 40°C over the run whereas the autosampler was kept at 10°C to prevent degradation of the sample. The injection volume was set at 2 µL.

Mass spectrometry Conditions

Detection was carried out by Agilent 6460 triple quadrupole MS/MS fitted with Agilent Jet Stream Electrospray ionization (AJS-ESI) probe and operated in the positive ion mode. Detection was carried out in multiple reactions monitoring (MRM) mode. Nitrogen 99.999% was used as the collision gas. The optimized conditions were as follows: Nebulizer Pressure, 35 psi; Drying Gas Temperature, 250^{o} C; Sheath Gas Temperature, 350^{o} C; Drying Gas Flow, 9 (l/min); Sheath Gas Flow, 12 (l/min); Capillary, 4500 V and Nozzle Voltage, 500 V. The MRM transitions and the related optimized Fragmentor Voltage, collision energy for analyte and IS are shown in Table 2.

Table 2. Qualifier and quantifier transitions

Compound	Transition (m/z)	Type	Fragmentor	Collision Energy
Fluconazole	307.1>238.0	Qualifier	110	14
Fluconazole	307.1>220.0	Quantifier	110	14
Ketoconazole	531.1>489.1	Quantifier	200	34
Ketoconazole	531.1>244.0	Qualifier	200	38

Preparation of Standard Solutions

Standard stock solutions of fluconazole 1 mg/mL (w/v) and ketoconazole (IS) 1 mg/mL (w/v) were separately prepared in 10 mL volumetric flasks with methanol. Working solutions for calibration (20,000, 10,000, 5,000, 2,500, 1,250 and 625 ng/mL) and quality controls (16000, 8000, 1600 ng/mL) were prepared from the stock solution by adequate dilution using Methanol. The Internal Standard (IS) working solution (2000 ng/mL) was prepared by diluting the stock solution with methanol. All the stock and working solution were prepared in microcentrifuge tube and store in -80^{o}C until use.

Sample Preparation

100 µL of serum was spiked with 25 µL of working solution of internal standard and 25 µL of the working solution for calibration. The serum was then vortexed for 30 seconds. Six hundred (600) µL of acetonitrile was added to the serum and the mixture was then mixed again by vortexing for another 30 seconds. The mixture was subsequently centrifuged at 17,982x*g* and 4°C for 15 minutes. 500 µL of the supernatant was then transferred into a new tube containing 500 µL of purified water and mixed by vortexing for 30 seconds before transferring 100 µL of the mixture into an insert for analysis.

Validation of the Method

The proposed method was validated in accordance to the guidelines of Bioanalytical Method Validation from EMA, Bioanalytical Method Validation Guideline from Industry by FDA, Validation of Chromatographic Methods by Centre for Drug Evaluation and Research (CDER)-FDA as well as Validation of Analytical Procedure by ICH. The validation criteria of selectivity, recovery and matrix effect, sampling, stability, calibration curve, sensitivity, accuracy and precision, carryover and dilution integrity were all evaluated. The selectivity was done by comparing the response of individually extracted serum (blank serum) against the response of the lower limit of quantitation (LLOQ) for the target analyte (TA). Carryover was also analysed by the response of blank serum against the response of LLOQ for the TA but by using pooled serum instead of individual serum and by injecting the extracted serum after the upper limit of quantitation (ULOQ). Sensitivity on the other hand was evaluated by comparing the response for the TA of the blank serum spiked with IS against the response for TA of LLOQ.

The linearity of the calibration curve was evaluated by analyzing the slope, linear regression, weight, and correlation coefficients. For the analysis of accuracy and precision, duplicates of the 3 levels of QC were prepared and run in 3 different time. The QC levels were prepared for analysis of recovery and matrix effect by comparing the ratio of TA to IS of the extracted serum sample to the TA:IS of the standard prepared in deionized water and TA:IS of the serum spiked after the extraction, respectively. The stability was evaluated under various conditions including storage at room temperature, storage in autosampler of 10°C, 3 freeze-thaw cycles after storge at -20°C, and long term storage at -20°C. Two levels of QC were prepared for all the conditions.

RESULTS AND DISCUSSION

Optimisation of Quantitation Method using Liquid Chromatography-Mass Spectrometry (LCMS)

A method was developed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to quantitate the levels of fluconazole in human serum with a simple yet robust method that can be used to support a wide range of application including therapeutic drug monitoring. The analytes were separated using gradient elution of the mobile phase starting from 20% B at 0.0 min increased to 40% at 0.7 min and again linearly increased to 90% at 1.5 min. The ratio was kept at 10:90 for 2.5 min and decreased back to 20% in 0.1 min. The stop time was at minute 5 and an additional of a 2-minute post time was determined to stabilize the column before the next injection. The separation of the analytes was good with sharp and symmetrical peak shapes (Figure 2).

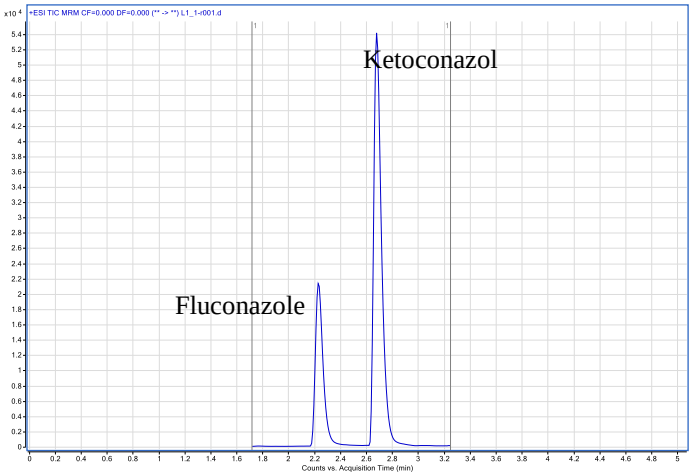

Figure 2. Chromatogram of the analytes

Determination of LOD

The limit of detection (LOD) was determined by analysis of the lowest concentration detected but could not give a consistent reading for the analysis. The LOD of this experiment was found to be at 79 ng/mL which was 8 times the noise or background.

Method validation

Linearity, accuracy and precision

Linearity of fluconazole was expressed over a six-point calibration curve ranging from the LLOQ of 156.25 ng/mL to the ULOQ of 5000 ng/mL as presented in Table 3 and Figure 3. The least-

square linear regression analysis used to study the relationship between the relative concentration (x) to the relative response (y) between the internal standard to the target analyte and the graph was expressed by using the equation y = mx+c and was found to be 0.797378x – 0.091525.

Table 3. The regression equation, correlation coefficients (r^2), linear range and lower limit of quantitation

Compound	Regression Equation	Correlation Coefficients (r^2)	Linear Range (ng/mL)	Lower limit of quantitation (ng/mL)
Fluconazole	0.797378x – 0.091525	0.999	156.25 - 5000	156.25

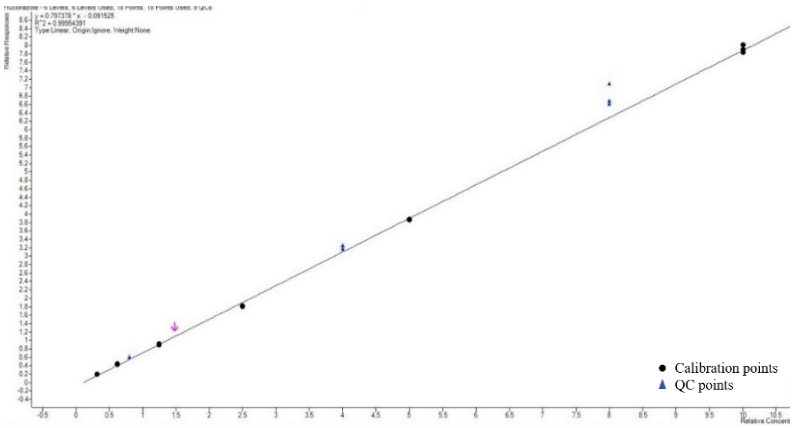

Figure 3. The linearity of the calibration levels with 3 QC levels

The overall accuracy and precision were assessed by calculating the difference between the ratio of the response between the samples and are presented in the form of percentage accuracy, standard deviation and percentage of relative standard deviation (RSD) as tabulated in Table 4. The method showed good accuracy and precision for all levels of QC (±12%) and LLOQ (±17%). This abide the limit of tolerance set by EMA and FDA which are (i) not more or less than 15% for nominal concentration; and (ii) 20% for the lowest level of quantitation (LLOQ) .

Table 4. Accuracy, SD and RSD (%) of accuracy and precision

Concentration (ng/mL)		Day 1		Day 2		Day 3		Inter-day	
		Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)	Mean ± SD (%)	RSD (%)
LLOQ	156.25	117.78 ± 1.94	1.65	114.74 ± 0.22	0.19	100.29 ± 0.30	0.30	100.94 ± 9.35	8.42
LQC	400.00	105.47 ± 8.23	7.81	106.18 ± 0.60	0.56	89.93 ± 1.19	1.32	100.53 ± 9.18	9.14
MQC	2000.00	106.94 ± 3.53	3.30	98.11 ± 2.92	2.98	88.00 ± 0.25	0.29	97.68 ± 9.48	9.70
HQC	4000.00	109.21 ± 0.97	0.89	99.22 ± 2.99	3.01	90.42 ± 2.10	2.32	99.62 ± 9.40	9.43

LLOQ – lowest limit of quantitation; LQC – Low level quality control; MQC – Medium level quality control; HQC– High level quality control

Selectivity and carryover

The mass chromatography of six blank sera from different individuals were analysed and found that no co-eluting peaks more than 20% of the area of the target analyte at the LLOQ level and 5%

for the internal standard (Table 5) were detected. This agrees with the reference guidelines from EMA, FDA and ICH.

Table 5. The selectivity result of the blank serum

Matrix	TA AUC (unit^2)	Response (%)	IS AUC (unit^2)	Response (%)
Spiked, pooled LLOQ serum	22921		111698	
Blank, individual serum	1612	7.03	496	0.44
	1103	4.81	445	0.40
	2901	12.66	438	0.39
	2310	10.08	360	0.32
	950	4.14	435	0.39
	4146	18.09	375	0.34

The carryover test met the acceptance criteria outlined by EMA and FDA by assessment of the peaks of injected blank samples after the injection of the highest concentration of the calibration standard (ULOQ). The guidelines stated that the peak of the blank sample should not be more than 20% of the LLOQ value. The developed method showed good elution as all the injections done to assess the carryover were found to be less than 5% of the LLOQ target analyte (TA) value (Table 6).

Table 6. The carryover result of blank serum after injection of ULOQ

Matrix	TA AUC (unit^2)	Response (%)	IS AUC (unit^2)	Response (%)
LLOQ serum	22921		111698	
Blank serum	594	2.59	546	0.49
	347	1.51	582	0.52
	744	3.25	532	0.48

The recovery of the TA proved to be consistent using the extraction method. All three levels of QC were able to recover more than 80% of the TA spiked with the RSD value not more than 15% which fulfilled the guidelines. The data of the mean recovery of the response ratio of the analytes, standard deviation and precision are recorded and tabulated in Table 7.

Table 7. Mean, SD and RSD of LQC, MQC and HQC recovery

Level	LQC	MQC	HQC
Mean	82.02	81.19	88.34
Standard deviation	0.17	1.13	1.40
RSD	8.63	11.77	7.89

By evaluating the effects of ion suppression or enhancement of the analytes in the presence of endogenous samples in the biological sample, we can measure and decide on the effect of the matrix (Jessome et al., 2006) which is serum in this case on our target analyte. The result suggested that there is little matrix effect on our target analyte.This matrix effect could be due to differences in the efficiency of ionization of the target analytes and other molecules in the sample. This may result in ion suppression or enhancement. In this study, the the matrix effect was less than 15% (Table 8) and is within the prescribed ranged recommended by the guidelines.

Table 8. Mean, SD and RSD of LQC and HQC matrix effect

Level	**LQC**	**HQC**
Mean	86.40	92.39
Standard deviation	0.10	1.24
RSD	4.28	6.42

Stability

Target analyte was proven to be stable and in agreement with the guidelines by EMA (the mean concentration at each level should be ±15% of the nominal value), FDA (the accuracy at each level should be ±15%) and ICH (stability should be robust). The stability of our TA in the serum was assessed in four different conditions by preparing four sets of low and high QC. The sera were subjected to different conditions after the TA and IS were spiked in. Four sets of low and high QC were prepared and the first condition was to extract the serum immediately after the TA and IS were spiked in whereas one group was left for four hours on the bench top at room temperature before extraction. A group of spiked sera was kept in the -20˚C freezer for three cycles of freeze-thaw (FT) before they were extracted whereas the final group was kept in the -20˚C freezer for one month before the stability was tested. The first set was left in the autosampler overnight (at 10˚C) for 24 hours before they were re-injected. The target analyte was stable in all four conditions by comparing the accuracy of the tested samples to the freshly prepared samples (Table 9).

Table 9. Mean, SD and RSD for LQC and HQC stability in three different conditions

	4 hours at 25˚C		**24 hours at 10˚C**		**FT**		**1 month**	
	LQC	**HQC**	**LQC**	**HQC**	**LQC**	**HQC**	**LQC**	**HQC**
Mean	99.82	105.56	109.95	103.47	113.18	114.09	94.64	87.33
Standard deviation	0.09	<0.00	0.21	<0.00	<0.00	<0.00	0.07	<0.00
RSD (%)	5.56	0.23	11.01	0.43	0.43	0.43	4.32	6.44

CONCLUSION

The quantitative method developed using LC-MS/MS exhibited excellent selectivity for the quantitation of fluconazole. The short run time also suggested that there is an opportunity for high sample throughput. The simple preparation method used allow quantitation of target analytes using a less laborious step. The method is reliable and reproducible and had fulfilled the validation criteria of EMA, FDA and ICH

Acknowledgement

This work was financially supported by the Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA (241810/2019/TLK/17). All the experiment was conducted at the Bioanalytical Unit, Integrative Pharmacogenomics Institute (iPROMISE), Universiti Teknologi MARA (UiTM), Malaysia.

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