ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Bio-Analytical Method Development and Validation of Voriconazole Using LC-MS/MS.

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ABSTRACT

A rapid, simple and sensitive LC–MS/MS analytical method was developed and validated for the determination of voriconazole in human plasma, using Fluconazole as an internal standard. A water Quattro Micro LC-MS/MS was used. Chromatographic separation was achieved using a Vertisep BDS C18 (4.6x 100mm), 5µm, maintained at 35 °C. The samples were eluted using a non-evaporating buffer system; (Phosphate buffer). Desired response was observed for mobile phase, 0.2% formic acid buffer solution: methanol in the ratio of (20:80), at a flow rate of 1 ml/min with a total run time of 2.2 min. The LC system was coupled with an atmospheric pressure ionization source (API-3200) triple quadruple mass spectrometer equipped with an electro spray ionization source, operating in positive mode. Analysis was performed in multiple reaction-monitoring (MRM) mode by monitoring the ion transitions from m/z 350.10→281.10.100 (Voriconazole) and m/z 307.20.→220.20 (IS). Calibration curves in spiked plasma were linear over the concentration range of 25–5000 ng/mL with determination coefficient >0.9989. The lower limit of quantification was 25ng /mL Intra batch and inter batch precision %CV ranged from 0.93% to 5.66% and 3.03% to 5.16%.While % of accuracy was within 92.44-107.61% and 93.69-99.23% respectively. This method has significant advantage over the other reported methods in terms of cost and gave reproducible data with a chromatographic runtime of 2.20 minutes. The reported method provided the necessary sensitivity, linearity, precision, accuracy, and specificity to allow the determination of Voriconazole in pre-clinical pharmacokinetic studies.

Key words: Bio-analytical, Voriconazole, LC-MS/MS, Human plasma

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ISSN: 0975-8585

INTRODUCTION

Voriconazole chemically, [(2*R*, 3*S*)-2-(2,4-difluorophenyl)-3-(5-fluoro-4-pyrimidinyl)-1-(1*H*-1,2,4-triazol-1-yl)-2-butanol] is a new antifungal agent that is a derivative of fluconazole, having one triazole moiety replaced by a fluropyrimidine ring and a methyl group added to the propanol backbone. This results in potent, wide-spectrum activity and a fungicidal action against Candida and Aspergillus species. Its primary mode of action is inhibition of fungal cytochrome P450-dependent 14α-sterol demethylase, an essential enzyme in ergosterol biosynthesis.[1,2] Voriconazole is metabolized by the Cytochrome P450 system, with < 2% of the dose excreted unchanged [3]. Compared to fluconazole, voriconazole has an enhanced antifungal spectrum that includes filamentous fungi. Voriconazole was designed to enhance the potency and spectrum of activity of fluconazole [4] used against a broad spectrum of significant clinical isolates like Aspergillus, Candida, Scedosporium and Fusarium[5,8].

Several methods are reported to determine the levels of voriconazole in biological fluids by using LC-MS-MS [9, 11], LC-EI-MS [12,13], HPLC [14,17], UV[18] and by microbiological methods[19,20].

These methods involve cumbersome and laborious extraction procedure with large sample volumes or have a narrow analytical range. Special emphasis was made to develop a time efficient, analyst friendly and cost efficient sample processing procedure. The present work aims to develop and validate an analytical method for estimation of Voriconazole in human K_2 EDTA plasma using LC-MS/MS technique and was validated in accordance with the ICH guidelines. [21, 23]

MATERIALS AND METHODS

Apparatus and Chromatographic Conditions

A water Quattro Micro LC-MS/MS was used. Chromatographic separation was achieved using a Vertisep BDS C18 (4.6x 100mm), 5µm, maintained at 35 °C. The samples were eluted using a non-evaporating buffer system, (Phosphate buffer) in the LC-MS/MS system, and to get good response with sharp peak shape ,mobile phase containing 0.2% formic acid buffer solution: methanol in different volume ratios were tried. Desired response was observed for mobile phase 0.2% formic acid buffer solution: methanol in the ratio of (20:80), at a flow rate of 1 ml/min with a total run time of 2.2 min. The LC system was coupled with an atmospheric pressure ionization source (API-3200) triple quadruple mass spectrometer (AB Sciex Instruments, Foster, CA; Model: 029345-K) equipped with an electro spray ionization source, operating in positive mode. Analysis was performed in multiple reaction-monitoring (MRM) modes by monitoring the ion transitions from m/z 350.10→281.10.100 (Voriconazole) and m/z 307.20.→220.20 (IS). The MS/MS conditions were as follows: spray needle voltage, 4.5 kV; source temperature, 400 ◦C; auxiliary gas flow, 71 l/min; equalizer gas pressure, 80 psi. The instrument was controlled and the data integration was performed with analyst 1.4.1 software version (AB Sciex Instruments, Foster, CA).

Selection of mobile phase

0.2% formic acid was prepared and tried with various ratios of methanol. Desired response was observed from mobile phase with 0.2% formic acid buffer solution and methanol, mixed in the ratio of (20:80).It was thoroughly mixed and degassed.

Selection of internal standard

With Itraconazole the peak response was not good. With Ketokonazole the peak shape was not up to the mark, finally Fluconazole gave a good peak shape with retention time less than the other compounds, and it was thus selected.

Tuning of Voriconazole

Tuning was carried out both in positive and negative modes by direct infusion of standard solution of Voriconazole and ISTD (100 ng/ml solution for an analytes and ISTD were prepared by using methanol solution) The quantification was carried with, selected ion monitoring mode (SIM) set to the protonated parent molecular ion at m/z=350.10amu for Voriconazole and m/z of 307.20amu for ISTD.

Preparation of Voriconazole standard stock solution

5.037mg of Voriconazole was weighed and transferred into a 5ml volumetric flask and 5.103 mg into another 5ml volumetric flask (for preparing quality control samples) dissolved in methanol and the volume was adjusted up to the mark. It was labelled and stored at 2-8ºC.

Preparation of internal standard stock solutions

5.052 mg of Fluconazole was weighed and transferred into a 5ml volumetric flask, dissolved in methanol and the volume adjusted. The concentration of the resulting solution was calculated by considering the purity of Fluconazole (99.87). It was labelled and stored at 2-8 ºC. The stock solution was further diluted with 60% methanol in water to get a concentration of 3.022µg/ml.

Biological Matrix

Plasma K_2EDTA was collected from commercially procured human whole blood. Plasma blank from six donors was chromatographically determined for interfering substances prior to use. Same human plasma batches, free from interferences were used for the preparations of calibration curve standards and quality control samples.

Spiking of plasma for the calibration curve standards

Stock dilutions of Voriconazole were prepared with 60% methanol in water, with concentration ranging of 25.00 to 5000.00ng/ml, using dilutions of main stock solution. For

calibration curve standards, Voriconazole stock solution was spiked with K_2EDTA plasma and was labelled accordingly.

Preparations of Quality control samples

Stock solution of Voriconazole was prepared in the concentration range of 1.500 to 80.000µg/ml with 60% methanol for the quality control samples. These were further diluted and spiked appropriate amount of with K_2EDTA plasma to get concentrations of 75, 390, 2000, 4000ng/ml and were labelled as, low concentration, Geometric mean concentrations, median concentrations and high concentrations quality control samples respectively.

Selection of extraction solvent

Extraction of Voriconazole from human plasma was carried out using different organic solvents, ethyl acetate, methanol and acetonitrile. The extraction efficiency (recovery) was evaluated by the determination of Voriconazole at different spiked concentrations levels. The recoveries for the three concentrations were calculated by comparing peak area obtained for plasma samples with those obtained by direct injection of standard solutions at the same concentrations and conditions. Results revealed that most efficient extraction of Voriconazole was achieved by acetonitrile. Mean %recovery of analyte from matrix samples by acetonitrile solvent was 72.38. The results of recovery data of Voriconazole is shown in Table No.1.

Table 1: Recovery data of Voriconazole with Different Solvents

Concentration of Analyte (ng/ml)	Ethyl acetate		Methanol		Acetonitrile	
	% recovery	CV%	% Recovery	CV%	% Recovery	CV%
75.000	70.22	7.14	73.44	5.45	76.55	3.14
2000.000	68.95	4.07	70.12	3.85	71.99	0.89
4000.000	69.64	2.13	72.78	4.68	70.63	1.07

Sample preparation procedure

Different extraction procedure were tried, both liquid-liquid extraction and protein precipitation methods gave reproducible results but protein precipitation method was selected, as it was the cheapest and less time consuming method. In order to avoid the orifice and detector saturation of mass spectrometer, evaporation and reconstitution steps were adopted.

Blank, calibration curve standards, quality control samples were withdrawn from the deep freezer and were allowed to thaw. It was vortexed to ensure complete mixing of the contents. 0.25ml of plasma sample was taken in a RIA vial; to it 25µl of internal standard Fluconazole (3µg/ml) was added followed by 25µl of 60% methanol solution. The sample was vortexed to ensure complete mixing of the contents.

Then approximately 1.75 ml of acetonitrile was added to the above samples. It was placed on a shaker for 10 min and centrifuged for 10 min at approximately 40000rpm at 20

ºC. 0.5 ml of supernant was transferred into another RIA vial and it was evaporated under a stream of nitrogen at 45ºC. The residue was reconstituted with 0.5 ml of mobile phase and was again vortexed. 15µl of that sample was injected into the LC-MS/MS system.

REASULTS AND DISCUSSION

Method Validation

A thorough and complete validation of the developed method for Voriconazole in human plasma was carried following the ICH guidelines. [21,23] The developed method was validated in terms of system suitability, selectivity, specificity linearity, sensitivity accuracy, intra-day and inter-day precision, stability, recovery studies and ruggedness.

System suitability

It was performed by injecting 6 sets of known concentrations of aqueous mixture for analyte and ISTD. The %CV for the retention time Retention time and the area ratio (Analyte area/ISTD area) was calculated. The results of system suitability were within the acceptance criteria, with a SD of 0.0052 and 0.0051. The %CV was 0.36 and 0.48 for the analyte and internal standard respectively. The mean Peak Response Area was 1.06 with a CV of 1.85%.

Selectivity

The selectivity of the present method was established by checking the four regular plasmas, one hyper lipemic and one haemolysed blank plasma (without spiking with Voriconazole and ISTD) obtained from 6 different volunteers. All the plasma samples were analysed using the proposed extraction procedure and compared the chromatographic conditions with lower limit of quantisation for analyte and ISTD. There was no interfering peak at the retention time for Voriconazole in blank plasma, when compared to LLOQ chromatogram of Voriconazole. In case of Fluconazole a small interference is seen, at the retention time in blank plasma, but the interference was within the acceptance criteria.

Specificity

It was evaluated, for any interference at the analytes retention time caused due to ISTD by injecting six replicates of matrix blank with ISTD, and also 6 replicates of matrix blank and analyte (MQC) at 2000ng/ml for detection of any interferences. There were no peaks obtained at the analyte retention time due to blank+ISTD, when compared with analytes LLOQ concentration. MQC chromatogram at concentration 2000ng/ml for Voriconazole was detected. Fig 1

Linearity

The analytical curves were constructed using eight none zero standards ranging from 25 to 5000ng/ml of Voriconazole. A blank sample (Matrix sample processed without internal standard) and a zero sample matrix processed with internal standard were used to exclude contamination. The linear regression analysis of Voriconazole was performed by plotting the

peak area ratio (y) against the concentration ratio (x). The coefficient of variation (%CV) and the accuracies were calculated for all the non-zero standards and were compared to the nominal value. The method was found to be linear between the ranges of 25 to 5004.250 ng/ml for Voriconazole. The correlation coefficient was found to be 0.9989, Intercept and slope was found to be 0.0138 and 0.00363. The data for linear concentration range of Voriconazole is given in Table 2.[Fig 2]

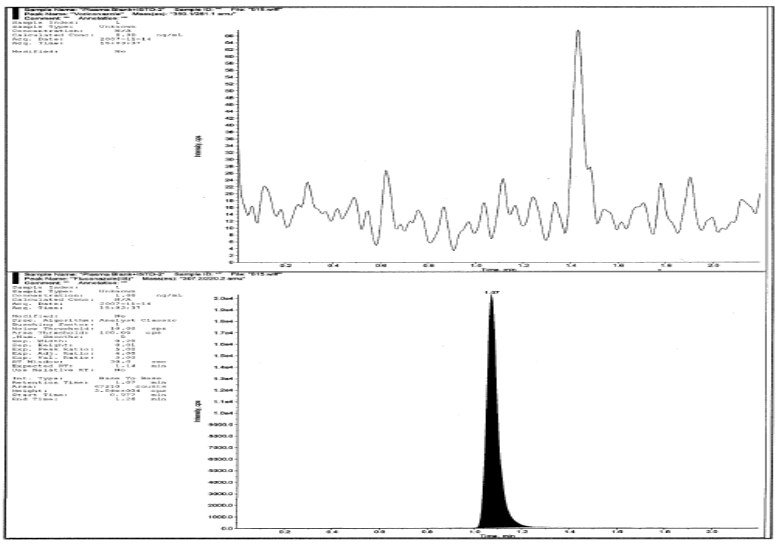

Figure 1: Chromatogram obtained by injecting (a) Deprotinated blank plasma (b) Blank plasma spiked with internal standard

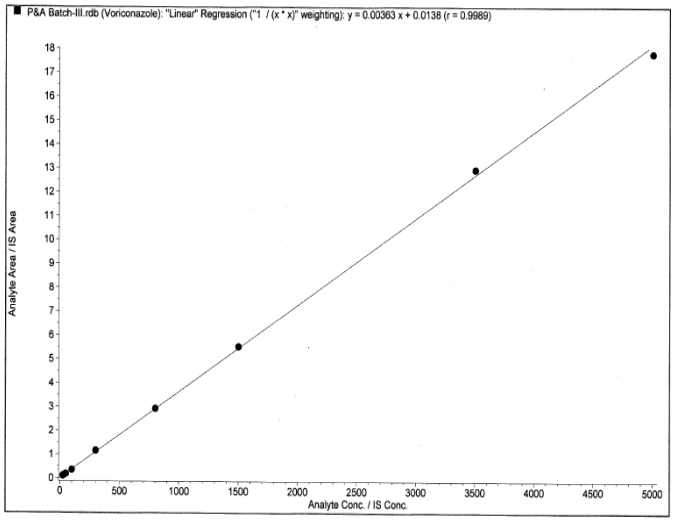

Figure 2: Calibration Curve for Voriconazole

ISSN: 0975-8585

Table 2: Linear Concentration Range of Voriconazole

Spiked Concentration (ng/ml)	Observed concentration (ng/ml)	% Accuracy
25.000	25.923	103.69
50.050	47.082	94.07
100.100	95.056	94.96
300.250	317.950	105.90
800.700	804.556	100.48
1501.300	1519.770	101.23
3503.000	3564.263	101.75
5004.250	4900.245	97.92

Sensitivity

The sensitivity of the method was performed at the lower limit of quantization (25 ng/ml) for Voriconazole. LLOQ-QC which is the lower limit of quantisation quality control sample that can be measured with acceptable accuracy and precision. The LLOQ was found to be 25.350 ng/ml for Voriconazole. The accuracy for Voriconazole at 25.350ng/ml was 87.03%, SD was 0.8439 and precession denoted by CV was 3.83 %.[Fig 3]

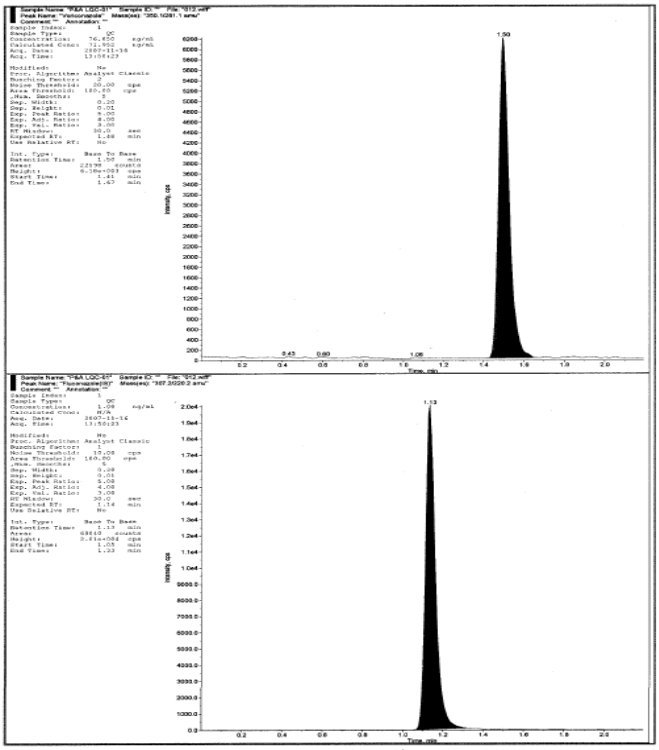

Figure 3 :(a) LQC chromatogram of voriconazole (b) LQC chromatogram of ISTD.

Precision and Accuracy

The precision and accuracy of the above method were evaluated by six samples at three concentrations levels. The calibration standards and quality controls were analysed on three different batches in order to determine intra-batch and inter-batch precession and accuracy To determine the accuracy and precision three runs for intra batch and a single run for inter batch were considered. Intra batch and inter batch precision CV% ranged from

0.93% to 5.66% and 3.03% to 5.16%.While % of accuracy was within 92.44%-107.61% and 93.69%-99.23% respectively. The precision and accuracy study indicated that the developed method was reproducible and accurate Table 3.

Table 3: Intra & Inter Batch Precision and Accuracy

Parameter	% Accuracy	GMQC± SD ng/ml	% Accuracy	MQC±SD ng/ml	% Accuracy	HQC±SD ng/ml	% Accuracy
Intra batch precision 1	92.435	375.716± 19.683	95.01	1999.258±60.236	98.58	3951.497±169.203	97.42
2	105.97	397.697± 17.90	100.57	2182.189±20.36	107.61	4171.182±236.21	102.84
3	96.79	370.498± 11.21	93.69	2012.392±62.98	99.23	3931.649±202.98	96.94
Inter batch precision	96.28	370.896± 11.21	93.69	2012.392±62.98	99.23	3931.649±202.988	96.94

Stability studies

Freeze and thaw stability

The frozen samples were thawed and were analysed using a fresh calibration curve. The concentrations obtained were compared with the actual concentrations of quality control samples. Stability after third cycle was 95.85% for LQC and 100.9% for HQC. The samples were found to be stable during above mentioned stability study Table 4.

Table 4: Stability Studies of Voriconazole in different conditions by using LC MS/MS.

Parameter	LQC±SD ng/ml	% Accuracy	HQC±SD ng/ml	% Accuracy
Freeze and thaw stability	72.891±1.402	95.84	4063.492±77.7439	100.186
Injector stability	69.729±2.563	91.68	3993.266±39.0633	98.458
Post processing stability	70.688±2.142	92.95	4034.635±37.2269	99.476
Short term plasma stability (15 hrs)	71.528±1.95	93.39	4034.705±83.19	99.47
Long term plasma stability (day 0)	75.489±2.62	99.26	4133.257±238.51	101.90
Long term plasma stability (day 46)	74.741±3.65	98.28	3578.148±116.53	90.68

Injector stability

It is also known as auto sample stability and was performed at two levels, LQC &MQC concentrations. The processed quality control samples at low concentration and high concentrations were kept in auto sampler at 20 ºC, and was analysed after 21 hours under a fresh calibration curve and the concentrations obtained were compared with the actual concentrations of quality control samples. The stability after 21 hours was 91.69% and 98.46% for LQC and HQC respectively as indicated in Table 4.

ISSN: 0975-8585

Post processing stability

The samples were processed and kept on the bench at ambient temperature and analysed after 18 hours under a fresh calibration curve. Post processing stability of processed quality control samples at low concentration and high concentration, in sets of six were determined at ambient temperature and the % of Voriconazole after 18 hours were compared to the actual concentration of quality control samples. The stability after 18 hours was 92.955% and 99.48% for LQC and HQC respectively. The results are given in Table 4.

Short term plasma stability: (Bench top stability)

The thawed quality control plasma samples in six sets, at low and high concentrations were processed after 15 hrs on bench top, and Voriconazole concentration was measured and compared with actual concentration of quality control samples. The stability after 15 hours was 93.40% and 99.48% for LQC& HQC respectively. Table 4.

Long term plasma stability

The plasma stability of Voriconazole was assessed at two levels of concentrations. The stability of Voriconazole in human plasma during storage under frozen conditions was determined after 46 days. The quality control samples were prepared and stored at -20 ºC in deep freezer. After 46 days these controls were analysed under a standard calibration curve prepared from a fresh stock solution.

$$\% \text{ Stability} = \frac{\text{Mean concentration in 46 days}}{\text{Mean concentration of Day 0 Samples}} \times 100$$

The stability of Voriconazole after 46 days when compared to mean of day zero concentrations, were found to be 99.01% and 88.99% at LQC and HQC respectively. The concentration measured on day 46 was also expressed at percentage of their spiked values, Table 4.

Recovery

Recovery experiments were performed in six replicates for analyte along with internal standard by comparing the analytical results for the extracted samples at three concentrations, (equivalent to LQC, MQC and HQC) with unextracted samples that represent 100% recovery.

$$\% \text{ Recovery} = \frac{\text{Mean response of Extracted samples}}{\text{Mean responce of un extracted samples}} \times 100$$

The % recovery of Voriconazole was 72.38% and that of Fluconazole was found to be 96.83 %. Table 5.

Table 5: Recovery study data of Voriconazole and Fluconazole from sample matrix

Analyte Voriconazole	Unextracted standard Peak area ±SD	Extracted matrix standard peak area±SD	% of Recovery
LOQ	29717±479.472	22823±450.03	76.80
MQC	791961±9901.310	559671±36222±380	70.67
HQC	1534229±19661.107	1068750±41015.661	69.66
ISTD Fluconazole	85228±3284.39	82528±3964.61	96.83

Ruggedness

The method was validated for ruggedness by changing the column from different batch and using the same matrix-spiked concentration, equivalent to MQC concentration and the CV% was with the acceptable limit.

CONCLUSION

The developed LC-MS/MS assay for Voriconazole was found to be sensitive, selective, rapid, precise and accurate when compared to those of other HPLC-UV methods. This method has significant advantage over the other reported methods in terms of cost and gave reproducible data with a chromatographic run time of 2.20 minutes. Most of the methods reported earlier, use the ESI source for plasma and serum analysis of Voriconazole. Voriconazole was found to be a stable compound and taking this data into consideration, a novel LC-MS/MS method using APCI source for plasma sample analysis was developed and results obtained were found to be satisfactory. Majority of the reported analytical methods quantifying Voriconazole in plasma by UV or LCMS require big sample volumes around 1-2 ml or introduce a step to improve the LOQ, for example in solid phase extraction sample preparation, when used raises the cost of analysis. Combination of LC with APCI-MS/MS enabled to detect Voriconazole in low concentration i.e. of about 25 to 5000ng/ml with maximum sensitivity.

This well developed and validated LC-MS/MS can further be used for routine measurement of Voriconazole in human plasma in support of clinical findings.

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