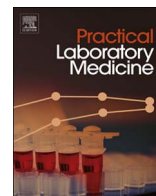




Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Performance evaluation of enzyme immunoassay for voriconazole therapeutic drug monitoring with automated clinical chemistry analyzers



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ARTICLE INFO

Keywords:

Voriconazole
Antifungal agents
Therapeutic drug monitoring

ABSTRACT

Objective: Voriconazole is a triazole antifungal developed for the treatment of fungal infectious disease, and the clinical utility of its therapeutic drug monitoring has been evaluated. Recently, a new assay for analyzing the serum voriconazole concentration with an automated clinical chemistry analyzer was developed. We evaluated the performance of the new assay based on standardized protocols.

Methods: The analytical performance of the assay was evaluated according to its precision, trueness by recovery, limit of quantitation, linearity, and correlation with results from liquid chromatography-tandem mass spectrometry (LC-MS/MS). The evaluation was performed with the same protocol on two different routine chemistry analyzers. All evaluations were performed according to CLSI Guidelines EP15, EP17, EP6, and EP9 [1–4].

Results: Coefficients of variation for within-run and between-day imprecision were 3.2–5.1% and 1.5–3.0%, respectively, on the two different analyzers for pooled serum samples. The recovery rates were in the range of 95.4–102.2%. The limit of blank was 0.0049 µg/mL, and the limit of detection of the samples was 0.0266–0.0376 µg/mL. The percent recovery at three LoQ levels were 67.9–74.6% for 0.50 µg/mL, 75.5–80.2% for 0.60 µg/mL, and 89.9–96.6% for 0.70 µg/mL. A linear relationship was demonstrated between 0.5 µg/mL and 16.0 µg/mL ($R^2 = 0.9995–0.9998$). The assay correlated well with LC-MS/MS results ($R^2 = 0.9739–0.9828$).

Conclusions: The assay showed acceptable precision, trueness, linearity, and limit of quantification, and correlated well with LC-MS/MS. Therefore, its analytical performance is satisfactory for monitoring the drug concentration of voriconazole.

1. Introduction

Voriconazole is a triazole antifungal developed for the treatment of fungal infections. It is currently the drug of choice for invasive

Abbreviations: CLSI, Clinical and Laboratory Standards Institute; CV, coefficient of variation; G6PDH, glucose-6-phosphate dehydrogenase; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation; MRM, multiple reaction monitoring; NAD, nicotinamide adenine dinucleotide; RMSSD, root mean square standard deviation; SD, standard deviation; TDM, therapeutic drug monitoring; UV, ultraviolet

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<http://dx.doi.org/10.1016/j.plabm.2017.05.002>

Received 31 October 2016; Received in revised form 22 April 2017; Accepted 5 May 2017

Available online 13 May 2017

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aspergillosis, and is available for both oral and intravenous administration [5,6]. Voriconazole shows potent activity against a broad spectrum of clinically significant pathogens, including *Aspergillus*, *Candida*, *Cryptococcus*, *Fusarium*, and *Scedosporium* [7]. In both adults and children, marked inter- and inpatient variability in voriconazole pharmacokinetics has been observed, and hence recommendations for therapeutic drug monitoring (TDM) were proposed [8–11]. Recent guidelines indicated that the pharmacokinetic profile of voriconazole is influenced by numerous factors, including sex, age, race, genotypic variation, liver dysfunction, and drug–drug interactions with CYP450 inhibitors and inducers [5,12,13].

The therapeutic range for voriconazole has not been well established, although the reference therapeutic range of 1.0–5.5 µg/mL was proposed [14]. Another study recommended that a minimum lower target concentration for TDM in the treatment of established disease is a trough concentration of > 1 µg/mL or a trough: minimum inhibitory concentration ratio of 2–5 [15,16]. Voriconazole toxicity may manifest as visual disturbances, liver dysfunction, skin reactions, and neurotoxicity [17,18]. Trough concentrations that are associated with a greater probability of toxicity vary among studies, but have been reported at ≥5 µg/mL and ≥6 µg/mL [16,18,19]. Voriconazole concentrations have been determined in a variety of biological fluids such as plasma and serum [9,20,21]. The methods employed included agar well diffusion bioassays and high-performance liquid chromatography (HPLC) with fluorescence, mass spectrometry (MS), or ultraviolet (UV) detection [9,22,23]. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods are attractive because they are highly sensitive and specific [12,24,25]. However, the equipment is expensive and requires a high degree of specialty and experience to operate effectively [12].

The ARK™ Voriconazole Assay (ARK Diagnostics, Inc., Fremont, CA, USA) is a homogeneous enzyme immunoassay intended for the quantitative determination of voriconazole in human serum or plasma on automated clinical chemistry analyzers. To date, there is one study which has been conducted to evaluate the analytical imprecision and bias in the quantitation of voriconazole with routine clinical chemistry analyzers [26]. Thus, we confirmed the results of the first study and assessed the previously unevaluated performance of voriconazole quantitation based on an enzyme immunoassay using automated clinical chemistry analyzers in a routine clinical laboratory.

2. Methods

2.1. ARK™ Voriconazole immunoassay

The ARK™ Voriconazole Assay (ARK Diagnostics, Inc., Fremont, CA, USA) is based on competition between the drug in the specimen and voriconazole labeled with the enzyme glucose-6-phosphate dehydrogenase (G6PDH) for binding to the antibody reagent. Upon binding to the antibody, G6PDH enzyme activity decreases. However, in the presence of the drug from the specimen, enzyme activity increases in a manner that is directly proportional to the drug concentration. Quantitative analysis of voriconazole was performed using the Roche Modular P800 automated clinical chemistry analyzer (Roche Diagnostics, Basel, Switzerland) and the Toshiba TBA-200FR analyzer (Toshiba Medical Systems Corporation, Tochigi, Japan).

2.2. LC-MS/MS assay

For method comparison, the results were evaluated with LC-MS/MS using the Agilent 1260 HPLC system (Agilent Technologies, Santa Clara, CA, USA) connected to Agilent 6490 Triple Quadrupole MS/MS (Agilent Technologies). Blood samples in serum-separation tubes were centrifuged for 10 min at 2200g. Fifty microliters of serum was immediately vortexed with a reagent containing 50 µL of internal standard working solution, 50 µL of methanol, and 400 µL of acetonitrile. After centrifugation (5 min at 13,200 rpm), 2 µL of supernatant was injected in the autosampler. Chromatographic separation was carried out on an Agilent 1260 HPLC separation module (Agilent Technologies) with a Poroshell 120, EC-C18 reversed-phase column (50 mm × 3 mm, 2.7 µm; Agilent Technologies). The mobile phase was a mixture of water (buffer A) and acetonitrile (buffer B) containing 0.1% formic acid. An isocratic mobile phase (30:70, v/v) was used. This composition was maintained for 3 min to allow for elution of all compounds, and the system was then re-equilibrated for the next injection. The flow rate was set at 0.3 mL/min. MS analysis was performed using Agilent 6490 MS/MS (Agilent Technologies) equipped with an electrospray ionization source operating in the electrospray-positive mode. The source and nebulizing temperature were each set to 200 °C. Nitrogen was used as the nebulizing gas and was set at a flow rate of 16 L/min. The capillary voltage was set to 3.5 kV, the cone voltage was 14 V, and the collision energy was 16 eV. Voriconazole was detected by multiple reaction monitoring (MRM) with a dwell time of 0.1 s. We used deuterium (d) labeled internal standards for improving the accuracy of quantitation in complex matrices. They work by normalizing differences in extraction, injection, chromatography, ionization and detection between samples. The following MRM transitions were monitored: m/z 350.1 → 281 for voriconazole and m/z 353.1 → 284 for d₃-voriconazole. This MS method was developed by our laboratory and its analytical performance was validated. Within-run and between-run imprecision according to the Clinical Laboratory Standards Institute (CLSI) Guideline EP15-A3 were evaluated using 3-level quality control materials and ranged 1.92–5.61% [1]. We also tested certified reference material samples (Cerilliant Corporation, Round Rock, Texas, USA) ranging from 0.075 to 8.0 mg/L. Median recovery within the measuring range was 99.5% (recovery range 97.9–102.7%).

2.3. Precision

Precision experiments were conducted according to the CLSI Guideline EP15-A3 [1]. Both tri-level controls of the assay and three pooled human serum samples were tested in each run. The pooled serum samples were used to demonstrate equivalent precision in

both the synthetic calibrator/control matrix and human serum. Mean determinations of voriconazole and standard deviations (SDs) were used to calculate within-run and between-day coefficients of variation (CVs). Precision was determined using Analyze-it® Standard Edition (Version 2.04) for Microsoft Excel (Microsoft Corporation).

2.4. Trueness by recovery

Trueness-by-recovery experiments were conducted to determine the trueness of the quantitative measurements of voriconazole across the assays. Evaluation of trueness (analytical recovery) was performed by adding concentrated voriconazole to a human serum sample that was negative for voriconazole. Serum samples were prepared by the gravimetric addition of voriconazole to methanol and volumetric addition of this stock solution to the human serum negative for voriconazole. Test sample concentrations were 1.5, 3, 6, 9, 12, and 15 µg/mL, representing the drug concentrations across the assay range. This yielded a total of six replicates for each test sample. The results of the six replicates were averaged and compared to the theoretical target concentration, and the recovery percentage was calculated as follows: % Recovery = $100 \times (\text{Mean recovered concentration} / \text{Theoretical concentration})$.

2.5. Detection capability estimates

The limit of blank (LoB) and limit of detection (LoD) were evaluated by testing 60 replicates of the blank (normal pooled human serum). LoB is the highest measurement result that is likely to be observed for a blank sample. It is required to calculate LoD as recommended by ISO (International Organization for Standardization) [2]. Sixty replicates of a positive level (approximately 0.3 µg/mL voriconazole in serum) that exceeded the LoB. Three calibrated analytical runs were performed. In each run, 20 replicates of the blank and 20 replicates of a low-concentration voriconazole sample were analyzed. The grand mean and root mean square standard deviation (RMSSD) were calculated. If calibration was verified, eight replicates of each test sample were assayed. This procedure was repeated five times, yielding a total of 40 replicates of each LoQ sample tested. The grand mean, RMSSD, and CV for each test sample were calculated. The LoQ was defined as the lowest concentration for which acceptable inter-assay precision and recovery is observed. Samples were prepared to test three LoQ levels that were below the lowest positive calibrator concentration (1 µg/mL). Samples were prepared by the gravimetric addition of voriconazole to methanol and volumetric addition of this stock solution to human serum negative for voriconazole. Pooled human serum representative of the patient specimen matrix was supplemented with voriconazole to obtain concentrations of 0.5, 0.6, and 0.7 µg/mL. The criteria of LoB, LoD, and LoQ were determined according to CLSI Guideline EP17-A2 [2].

2.6. Linearity

The proportional linearity of quantitative measurements of voriconazole was evaluated across the assay range according to the recommendations of CLSI Guideline EP6-A [3]. Voriconazole concentrations were prepared proportionally across the assay range (0–20 µg/mL). A 20 µg/mL serum sample was prepared by the gravimetric addition of voriconazole to methanol and volumetric addition of this stock solution to human serum negative for voriconazole. Dilutions were achieved with human serum negative for voriconazole. LC-MS/MS was used to confirm the high drug concentration. Linearity, together with the recovery and LoQ were used as determinants of the assay range. If calibration was verified, three replicates of each test sample were assayed. This procedure was repeated twice, yielding a total of six replicates of each sample tested. The grand mean, SD, and CV for each test sample were calculated. Regression analyses were performed between the measured mean voriconazole concentration and the nominal values for each dilution using first-order and second-order polynomial determinations. Linear bias was estimated based on the predicted results according to first-order and second-order mathematical regressions and by the percentage difference between the predicted values according to CLSI EP6-A.

2.7. Method comparison

Method comparison was performed to evaluate the agreement between the assay and LC-MS/MS results, according to CLSI Guideline EP9-A2 [4]. One hundred eighty-seven leftover serum specimens that were not individually identifiable were obtained with their associated LC-MS/MS values. This protocol was approved by the Institutional Review Board of Seoul National University Hospital for acceptable use of leftover specimens that were not individually identifiable without informed consent for the evaluation of *in vitro* diagnostic methods. Groups of 10–20 specimens were assayed per run with the assay, and specimens were tested individually from April 7th to 24th, 2014. Results were analyzed using Deming regression analysis. The statistical parameters such as slope, y-intercept, coefficient of determination (R^2), and number of samples were compared between methods. The samples showing results that differed by greater than 30% between the assay and LC-MS/MS method were run again with both methods.

3. Results

3.1. Precision

The mean concentration of the LOW QC level was 1.52 µg/mL on Roche analyzer and 1.57 µg/mL on Toshiba analyzer. The mean concentration of the MID QC level was 4.99 µg/mL on Roche and 5.03 µg/mL on Toshiba, and that of the HIGH QC level was 9.84 µg/mL

Table 1

Precision of the ARK™ Voriconazole Assay for ARK™ Voriconazole Control and human serum specimens on two different analyzers.

Sample	Chemistry analyzer	Level	N	Mean (µg/mL)	Within-run		Between-day	
					SD	CV (%)	SD	CV (%)
ARK™ Voriconazole Control	Roche ^a	LOW	40	1.52	0.092	6.0	0.049	3.2
		MID	40	4.99	0.261	5.2	0.119	2.4
		HIGH	40	9.84	0.624	6.3	0.202	2.1
	Toshiba ^b	LOW	40	1.57	0.056	3.6	0.033	2.1
		MID	40	5.03	0.191	3.8	0.097	1.9
		HIGH	40	9.89	0.302	3.0	0.151	1.5
Pooled serum specimens	Roche	LOW	40	1.46	0.063	4.3	0.029	2.0
		MID	40	4.93	0.242	4.9	0.076	1.5
		HIGH	40	9.93	0.504	5.1	0.163	1.6
	Toshiba	LOW	40	1.54	0.049	3.2	0.024	1.5
		MID	40	5.09	0.164	3.2	0.097	1.9
		HIGH	40	10.03	0.398	4.0	0.302	3.0

^a Roche Modular P800.^b Toshiba TBA-200FR.

mL on Roche and 9.89 µg/mL on Toshiba. The mean concentration of the LOW level of pooled human serum specimens was 1.46 µg/mL on Roche and 1.54 µg/mL on Toshiba, that of the MID level was 4.93 µg/mL on Roche and 5.09 µg/mL on Toshiba, and that of the HIGH level was 9.93 µg/mL on Roche and 10.03 µg/mL on Toshiba. The CVs for within-run and between-day imprecision were 3.0–6.3% and 1.5–3.2%, respectively, on the two different analyzers for the ARK™ Controls. CVs for within-run and between-day imprecision were 3.2–5.1% and 1.5–3.0% for the pooled serum samples (Table 1).

3.2. Trueness by recovery

The recovery rates ranged from 95.4% to 99.4% on the Roche analyzer, and from 97.8% to 102.2% on the Toshiba analyzer (Table 2).

3.3. Detection capability estimates

On the Roche analyzer, the LoB was 0.0049 µg/mL and the LoD was 0.0376 µg/mL. On the Toshiba analyzer, the LoB was 0.0049 µg/mL and the LoD was 0.0266 µg/mL. On the Roche analyzer, the percent recovery for 40 replicates of each test sample at three LoQ levels were 67.9% for 0.50 µg/mL, 75.5% for 0.60 µg/mL, and 89.9% for 0.70 µg/mL; the CVs were 7.2–8.1%. On the Toshiba analyzer, the percent recoveries were 74.6% for 0.50 µg/mL, 80.2% for 0.60 µg/mL, and 96.6% for 0.70 µg/mL; the CVs were 3.4–4.7% (Table 3). The acceptable criteria of percent recoveries for determining LoQ were further mentioned in the Discussion section.

3.4. Linearity

Ten levels of serum sample dilutions were prepared from 0 to 20 µg/mL. Linearity was considered acceptable if the percent difference was ± 10% between the predicted first- and second-order regressed values, or ≤ 0.2 µg/mL at concentrations ≤ 2.0 µg/mL.

Table 2

Analytical recovery of the ARK™ Voriconazole Assay.

Theoretical concentration (µg/mL)	Chemistry analyzer	Mean recovered concentration (µg/mL)	Percent recovery
1.5	Roche ^a	1.43	95.4
	Toshiba ^b	1.52	101.0
3.0	Roche	2.95	98.2
	Toshiba	2.98	99.3
6.0	Roche	5.86	97.6
	Toshiba	5.94	99.0
9.0	Roche	8.89	98.8
	Toshiba	8.80	97.8
12.0	Roche	11.80	98.3
	Toshiba	11.91	99.2
15.0	Roche	14.91	99.4
	Toshiba	15.32	102.2

^a Roche Modular P800.^b Toshiba TBA-200FR.

Table 3
Performance of the ARK™ Voriconazole Assay at three limit-of-quantitation levels.

Concentration (µg/mL)	Chemistry analyzer	Mean recovered concentration (µg/mL)	Percent recovery	RMSSD	CV (%)
0.5	Roche ^a	0.34	67.9	0.025	7.2
	Toshiba ^b	0.37	74.6	0.015	4.0
0.6	Roche	0.45	75.5	0.037	8.1
	Toshiba	0.48	80.2	0.023	4.7
0.7	Roche	0.63	89.9	0.050	8.0
	Toshiba	0.68	96.6	0.023	3.4

^a Roche Modular P800.

^b Toshiba TBA-200FR.

A linear relationship was demonstrated between 0.5 and 16.0 µg/mL with $R^2 = 0.9995$ on the Roche analyzer and $R^2 = 0.9998$ on the Toshiba analyzer (Table 4, Fig. 1).

3.5. Method comparison

Using LC-MS/MS as the comparison method, the voriconazole concentrations (n = 187) ranged from 0.05 to 12.61 µg/mL. Fifteen samples were excluded from the analysis because they had a concentration below the LoQ. The results on the Roche analyzer correlated well with those obtained from LC-MS/MS ($R^2 = 0.9739$). The slope and intercept of the regression equation were 0.9661 and 0.0989, respectively. The results on the Toshiba analyzer also correlated well with those from LC-MS/MS ($R^2 = 0.9818$), and showed a slope of 1.0377 and intercept of 0.0622 (Figs. 2 and 3).

4. Discussion

The importance of TDM of antifungal agents is becoming increasingly recognized, and accumulating evidence supports TDM for voriconazole. TDM is recommended for both immunocompromised patients receiving voriconazole for the prophylaxis of invasive fungal disease and patients receiving voriconazole for active invasive fungal diseases. Outcomes in patients undergoing TDM, who had plasma concentrations maintained between 1.0 and 5.5 µg/mL, were significantly better (81%) than those in the non-TDM group (57%) [14]. The ARK™ Voriconazole Assay showed good performance in the concentration range, well covering the therapeutic target range of 1.0–5.5 µg/mL.

Several methods have been used for measuring serum concentrations of antifungal agents, including bioassays [12]. Bioassays are known to be simple and inexpensive to perform, but they are also subject to potential interference from other drugs, including other antifungals, and may measure the combined activity of parent molecules and metabolites [27,28]. Some studies that used LC/MS for voriconazole quantification in aqueous humor rather in serum or plasma [21,29,30]. The majority of the available analytical methods for quantifying voriconazole in plasma by UV or LC/MS require large sample volumes or an additional step to improve the LoQ, such as solid-phase extraction, which raises the cost of analysis [24], except several methods using simple protein precipitation [31,32]. Antifungal TDM performed by LC/MS needs the expensive equipment and specialized technical personnel, and the fact that it is not random access makes this method less cost-effective for analyses of small numbers of samples, providing results within a few hours in a non-reference clinical laboratory. Moreover, each laboratory must prepare or purchase commercial multi-level calibration standards for MS analysis. The absence of a common calibrator thus significantly affects and compromises the trueness and basis for a proper metrological traceability statement for the certified values [33]. Differences in the calibrators used can also lead to differences in patient sample results between different laboratories. The ARK™ Voriconazole Assay is applicable onboard to any automated

Table 4
Difference between the predicted first- and second-order regressed values of the ARK™ Voriconazole Assay.

Concentration (µg/mL)	Roche ^a		Toshiba ^b	
	% Difference	Difference (µg/mL)	% Difference	Difference (µg/mL)
0.0	–58.7	0.11	–72.9	0.06
0.5	21.7	0.07	9.3	0.04
1.0	4.9	0.04	2.4	0.02
2.0	–0.8	–0.01	–0.4	–0.01
4.0	–2.5	–0.10	–1.3	–0.05
8.0	–1.9	–0.15	–1	–0.08
10.0	–1.2	–0.12	–0.6	–0.06
12.0	–0.4	–0.05	–0.2	–0.02
16.0	1.2	0.20	0.7	0.12

^a Roche Modular P800.

^b Toshiba TBA-200FR.

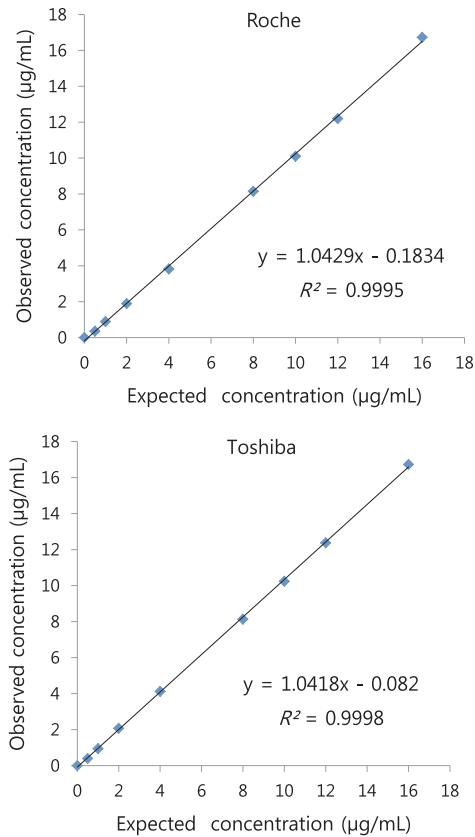


Fig. 1. Linearity of voriconazole values of the ARK™ Voriconazole Assay measured on the Roche Modular P800 and Toshiba TBA-200FR analyzer.

clinical chemistry analyzer, which consists of convenient, liquid-stable, ready-to-use reagents for homogeneous enzyme immunoassays.

Compared with the recently published study with ARK™ Voriconazole Assay, our results showed slightly better precision [26]. In the previous study with immunoassay, however, a method comparison was performed with ultra-high performance liquid chromatography method with photodiode array detection (UPLC-PDA), one of ultraviolet-detecting methods. To our knowledge, this is the second study showing results of the first voriconazole immunoassay, and is also the first showing those of trueness by recovery, linearity, validation of detection capability estimates, and method comparison of immunoassay and LC-MS/MS. In another previous study, LC-MS/MS analysis of voriconazole at six different concentrations showed within-run imprecision of 2.8–3.5% and between-run imprecision of 2.8–7.6% [32]. Similar imprecision was obtained for the ARK™ Voriconazole Assay compared to LC-MS/MS. The results of trueness by percent recovery showed no significant difference with LC-MS/MS (90.6–103.6%). The LoQ should be determined as part of the development of a method, which may be claimed by manufacturers. According to CLSI Guideline EP17, it may not be necessary to determine the LoQ for every method if the uncertainty (or total error) of measurement can be determined for low levels. For the present study, if the users set the acceptable recovery criterion at LoQ levels to 90%, then the LoQ is 0.7 µg/mL. If the criterion is set at 70%, the LoQ is 0.5 µg/mL. In these instances, it may be acceptable to simply report the estimated uncertainty of every low-level result, and allow the user to interpret whether it is suitable for use. For this study, we determined the LoQ to be 0.7 µg/mL for each analyzer. A good linear relationship ($CV < 5\%$) was demonstrated between 1.0 and 16.0 µg/mL on both analyzers. At 0.5 µg/mL, the CV was more than 10%, but the absolute difference was 0.04–0.07 µg/mL, which is tolerable for making an effective clinical decision. The ARK™ Voriconazole Assay correlated well with the LC-MS/MS result ($R^2 = 0.9739–0.9828$), which indicates that the assay can be an alternative method for voriconazole quantitation in clinical laboratories.

However, there are some limitations to this study that should be mentioned. Potential interference of endogenous materials should further be evaluated according to CLSI Guideline EP7-A2 [34]. For example, individual patient specimens containing high levels of cholesterol and triglycerides should be evaluated. Furthermore, the assay should be used in conjunction with information available from clinical evaluations and other diagnostic procedures. Clinicians should carefully monitor patients during therapy and dosage adjustments.

In conclusion, we introduced a simple, convenient, and low-cost analysis of voriconazole in human specimens for routine clinical practice. The ARK™ Voriconazole Assay will offer an alternative tool for the accurate and precise quantification of drug concentrations in patients receiving voriconazole therapy.

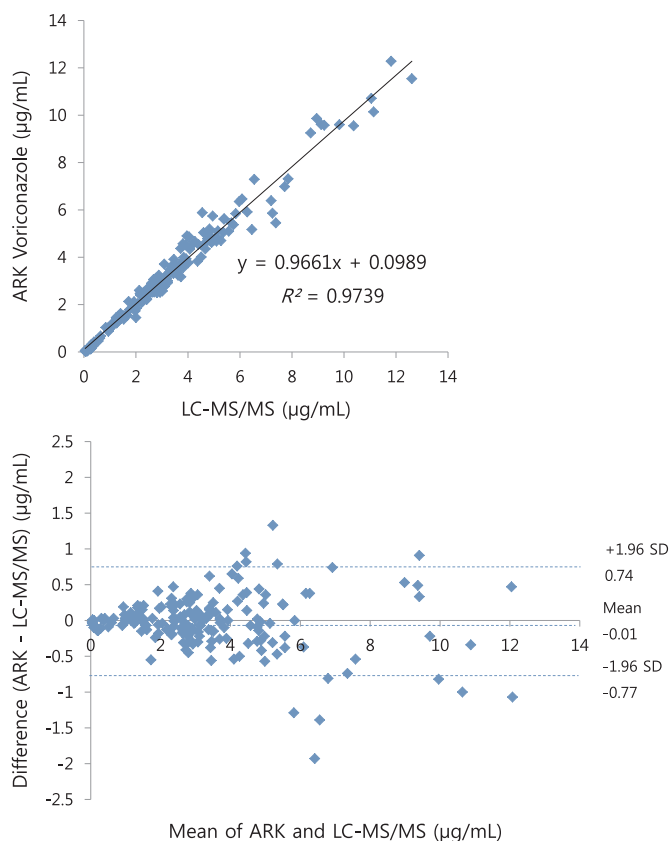


Fig. 2. Linear regression plot and Bland-Altman plot showing the comparison of voriconazole values of the ARK™ Voriconazole Assay measured by Roche Modular P800 and LC-MS/MS.

Author contributions

All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding

None declared.

Employment or leadership

None declared.

Honorarium

None declared.

Competing interests

None declared.

Acknowledgements

The authors appreciate the kind donation of materials for the voriconazole homogeneous enzyme immunoassay from ARK Diagnostics, Inc. (www.ark-tdm.com) and to Dr. Soon J. Oh for his kind advice in setting up the method. At the time of this manuscript, ARK has revised the assay calibrator scheme, and it is marketed as ARK Voriconazole II Assay. By adjusting the calibrator levels an improvement in analytical recovery below 1.0 $\mu\text{g/mL}$ was observed according to the manufacturer's information.

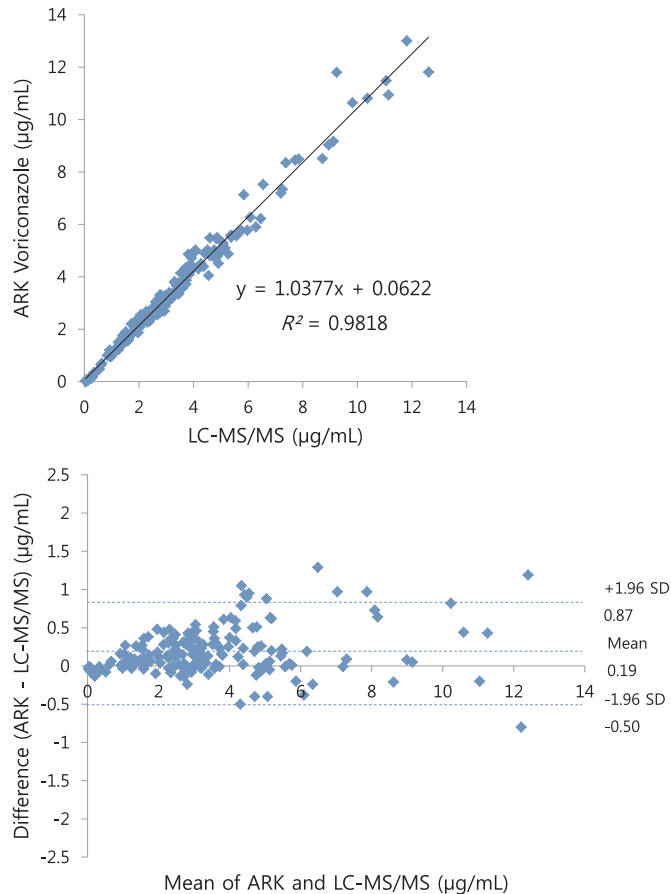


Fig. 3. Linear regression plot and Bland-Altman plot showing the comparison of voriconazole values of the ARK™ Voriconazole Assay measured by Toshiba TBA-200FR and LC-MS/MS.

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