Bioassay for Determining Voriconazole Serum Levels in Patients Receiving Combination Therapy with Echinocandins

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Voriconazole levels were determined with high-performance liquid chromatography (HPLC) and a microbiological agar diffusion assay using a *Candida parapsilosis* isolate in 103 serum samples from an HPLC-tested external quality control program ($n = 39$), 21 patients receiving voriconazole monotherapy ($n = 39$), and 7 patients receiving combination therapy ($n = 25$). The results of the bioassay were correlated with the results obtained from the external quality control program samples and with the HPLC results in sera from patients on voriconazole monotherapy and on combination therapy with an echinocandin (Spearman's rank correlation coefficient [r_s], > 0.93 ; mean $\pm$ standard error of the mean [SEM] % difference, $< 12\% \pm 3.8\%$).

Voriconazole is characterized by nonlinear pharmacokinetics due to saturation of its metabolism, resulting in unpredictable exposure with standard dosing regimens. Furthermore, it exhibits substantial inter- and inpatient variability (88 to 100%), as many physiological, pathological, and pharmacological variables affect its serum concentrations (1). A correlation between serum concentrations and toxicity or response has been reported (2), whereas the benefit of therapeutic-drug monitoring (TDM) of voriconazole in the clinical setting has been demonstrated in many clinical studies, including a randomized clinical trial (3–7). Therefore, TDM of voriconazole is an important tool in individualized therapy, leading to dosage optimization in order to maximize the therapeutic effect and minimize toxicity.

The clinical use and value of TDM are related to accurate, rapid, and cost-effective assays. Specifically, voriconazole levels in body fluids are often determined by using chromatographic or microbiological methods. Although high-performance liquid chromatography (HPLC) is still considered the gold standard, bioassays are frequently adopted and routinely performed because of their relative technical simplicity and low consumable and equipment costs, while there are several data indicating the concordance of results between the two methods (8–13). Nevertheless, current microbiological assays are lacking specificity in cases of antifungal combination therapy, as they do not allow the separation and simultaneous quantification of each individual compound. In light of the recent encouraging data from a large prospective randomized clinical trial on antifungal combination therapy (14), voriconazole may be combined with echinocandins in order to increase efficacy and overcome the limitations of voriconazole monotherapy, such as the long time to reach steady state, subtherapeutic levels, and difficult-to-treat infections (e.g., central nervous system [CNS] infections and those caused by azole-resistant pathogens) (15, 16). We therefore developed and validated an agar diffusion bioassay to determine voriconazole concentrations in the serum samples from patients on combination therapy with echinocandins.

Isolate. A *Candida parapsilosis* clinical isolate from the collection of the microbiology laboratory of our hospital (internal identifier no. 221) served as the test organism. The *in vitro* susceptibilities of the strain to voriconazole (0.015 mg/liter) and the three echinocandins (anidulafungin, caspofungin, micafungin, 0.25, 0.25, and 0.5 mg/liter, respectively) were tested using two broth microdilution techniques, the reference method of the Clinical and Laboratory Standards Institute (CLSI) (17), and the colorimetric Sensititre YeastOne antifungal panel (Trek Diagnostic Systems, Cleveland, OH, USA). The isolate was stored in normal sterile saline with 10% glycerol at -70°C until the study was performed; prior to testing, it was revived by subculture twice onto Sabouraud dextrose agar plates with gentamicin and chloramphenicol (SGC2) (bioMérieux) at 30°C for 24 h to ensure purity and viability. Distinctive CFU of the subcultured yeast were tipped and suspended in normal sterile saline. After counting viable cells in a Neubauer chamber, the *Candida* suspension was adjusted to give a final inoculum concentration of 3×10^5 CFU/ml. The CFU counts were affirmed each time by spread plate counts on SGC2 plates.

Antifungal drugs and medium. Laboratory-grade standard powders of voriconazole and anidulafungin (Pfizer, Inc., Groton, CT, USA), caspofungin (Merck & Co., Inc., Whitehouse, NJ, USA), and micafungin (Astellas Pharma, Inc., Osaka, Japan) were dissolved in sterile dimethyl sulfoxide (DMSO) (Carlo Erba Reac-

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tifs-SDS, Val de Reuil, France), and stock solutions of 10 mg/ml were stored in small portions at -70°C until use. The medium used throughout was RPMI 1640 medium (with L-glutamine, without bicarbonate) (AppliChem, Darmstadt, Germany) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (AppliChem).

Bioassay. The agar diffusion assay is a slight modification of a previously described assay (18). Briefly, the yeast suspension was inoculated in standard medium with 15 g/liter agar (60 ml at 50°C) (Oxoid Ltd., Basingstoke, England), which was then dispensed into square sterile plastic plates (10 by 10 cm) and left to solidify at room temperature. Thereafter, round wells were cut aseptically using a sterile cork borer in a well-spaced pattern. Sixty microliters of each standard, control, or clinical sample was pipetted into individual wells of the plate. After 24 h incubation at 37°C , growth inhibition was quantified by measuring the diameters of the zones of inhibited growth. Each run included one blank (drug-free serum control to exclude the possibility that the inhibitory activity was due to serum components), calibration standards, and two external quality controls (HPLC tested with known concentrations of voriconazole provided by the external quality control program UK NEQAS). Calibration standard samples containing 8 to 0.125 mg/liter voriconazole were prepared by serial 2-fold dilutions of the stock solutions in different pooled serum samples from healthy human donors.

HPLC assay. A previously validated HPLC method was used for cross-validation (19). Briefly, 40 μl of internal standard (12 $\mu\text{g/ml}$ naproxen) was added to 200 μl of serum standard, quality control, or serum sample. Voriconazole extraction was performed with 30 μl of 0.05 M phosphate buffer (pH 3.1) and 400 μl of methanol. After vortexing (30 s) and centrifugation ($1,000 \times g$ for 5 min), the supernatant was evaporated under nitrogen stream, reconstituted in 100 μl of methanol, and injected (30 μl) into the HPLC system. The chromatographic separation was performed using a LiChrosorb column (250 by 4.6 mm, 5 μm inside diameter) with a compatible LiChrosorb reverse-phase (RP)- C_{18} guard column. The temperature was maintained at 30°C throughout the measurement. The mobile phase consisted of a filtered and degassed mixture of acetonitrile-sodium dihydrogen phosphate (0.05 M, pH 3.1) (55:45 [vol/vol]) and was delivered at a flow rate of 1.2 ml/min in the isocratic mode. Detection was achieved by monitoring the absorbance at 255 nm. Measurements by each methodology (microbiological and chromatographic) were performed blindly by two different investigators in triplicate.

Serum samples. A total of 103 serum samples from patients who received voriconazole for different indications were analyzed: 39 samples from 21 patients receiving voriconazole monotherapy, 39 external quality control program HPLC-tested samples received from an interlaboratory proficiency testing program (NEQAS, North Bristol, United Kingdom) for an assessment of voriconazole levels, and 25 samples originating from 7 patients receiving concurrent therapy with echinocandins (5 with caspofungin and 2 with anidulafungin). Blood samples were collected in evacuated blood collection tubes containing potassium-EDTA (lavender top) just before and 0.5 h after drug administration in order to obtain trough and peak concentrations, respectively. The samples were then centrifuged at $4,000 \times g$ for 10 min and stored at -70°C .

Evaluation of bioassay. The diffusion assay was tested for linearity, analytical sensitivity, reproducibility, and specificity. The

diameters of the inhibition zones versus standard drug concentrations were analyzed with linear regression analysis. Intra- and interday reproducibility were assessed by running 16 external quality control program samples, with voriconazole concentrations ranging from 0.3 to 7.5 mg/liter in triplicate on nonconsecutive days, and by estimating the percent coefficient of variation (%CV). The effect of the presence of echinocandins in determining voriconazole levels was evaluated by measuring voriconazole levels with the bioassay in serum spiked with 0.5, 2, and 6 mg/liter voriconazole alone and together with concentrations of 1, 6, and 12 mg/liter of each echinocandin. The echinocandin concentrations were chosen based on the clinically achievable concentrations in patient serum (20–23). The data were analyzed by conducting repeated-measures analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test.

Correlation between bioassay and HPLC. The two methods were compared quantitatively and qualitatively, using HPLC as the reference method. For the quantitative analysis, the results of the two methods were analyzed with Spearman's rank correlation coefficient (r_s) and linear regression analysis in order to test whether the slope was significantly different than 1. For the qualitative analysis, the categorical agreement between the two methods was estimated as the percentage of serum samples lying lower than, within, or higher than the therapeutic window of 2 to 5 mg/liter with the two methods (8, 24).

All analyses were performed with the statistics software package GraphPad Prism, version 5.0, for Windows (GraphPad Software, San Diego, CA).

The standard curve of the diameter of the inhibition zone versus voriconazole concentration is depicted in Fig. 1. The lower limit of quantification (LLOQ) was determined to be 0.25 mg/liter, and the bioassay was internally validated over the range of 0.25 to 8 mg/liter, which includes the therapeutic window, as previously found (25, 26). Drug concentrations correlated linearly with the inhibition zone diameter ($r^2 = 0.98$, $P < 0.0001$) with mean (range) intra- and interexperimental variation of 6% (0 to 12%) among all drug concentrations tested, which is within the limits of acceptability of data established by international guidelines (27, 28). None of the echinocandins at the tested concentrations produced a discernible inhibition zone in the bioassay when tested alone, except for caspofungin at 12 mg/liter. Regarding the effect of echinocandins on voriconazole inhibition zones when the three echinocandins (at 1, 6, and 12 mg/liter) were combined with voriconazole (at 0.5, 2, and 6 mg/liter) in spiked human serum, there was no difference between the inhibition zones of voriconazole alone and in the presence of each echinocandin (ANOVA $P > 0.18$). No interaction between voriconazole and echinocandins against *C. parapsilosis* isolates has been reported (29–31).

Voriconazole levels measured by the bioassay were significantly correlated with the external quality control samples ($n = 39$; $r_s = 0.97$ [95% confidence interval {CI}, 0.94 to 0.99; $P < 0.0001$]; slope of the regression line, 1.000 ± 0.044 ; $P = 0.995$), with a mean $\pm$ SEM % difference of $9\% \pm 3.5\%$, which corresponded to mean $\pm$ SEM difference at concentrations of 0.17 $\pm$ 0.10 mg/liter (Fig. 2). Drug bioassay levels were also correlated with the HPLC results in serum samples from patients treated with voriconazole monotherapy ($n = 39$; $r_s = 0.93$ [95% CI, 0.87 to 0.96; $P < 0.0001$]; slope of the regression line, 1.013 ± 0.037 ; $P = 0.728$), with a mean $\pm$ SEM % difference of $9\% \pm 3.1\%$

FIG 1 The new bioassay and the standard curve of the diameter of inhibition zone (y axis) versus voriconazole concentration (x axis). Left panel, the numbers above the holes represent voriconazole concentrations, whereas the numbers below the holes are the mean diameters of the inhibition zones ($n = 35$). The standard deviations were <1 mm. Right panel, the bioassay showed linearity in the range of 0.25 to 8 mg/liter, with a correlation coefficient of $r^2 = 0.98$ in all runs. The error bars represent the standard deviations.

(0.34 ± 0.12 mg/liter). A high correlation was found between the bioassay and the HPLC results in clinical samples from patients treated with voriconazole-echinocandin combination therapy ($n = 25$; $r_s = 0.98$ [95% CI, 0.95 to 0.99; $P < 0.0001$]; slope of the regression line, 0.912 ± 0.047 ; $P = 0.074$), with a mean $\pm$ SEM % difference of $12\% \pm 3.8\%$ (0.50 ± 0.19 mg/liter) (Fig. 2). The aforementioned deviations from the HPLC values fulfill the criteria established by international recommendations for accepting the accuracy of a method (27, 28). The overall categorical agreement between the bioassay and the HPLC was 91%. For the remaining 9% (9/103) of the samples, the bioassay resulted in a mean $\pm$ SEM of $13\% \pm 10\%$ (0.54 ± 0.48 mg/liter) of HPLC concentrations (3 samples were lower and 6 were higher than the bioassay levels) at the upper (6) and the lower (3) limit of the therapeutic concentration range in 8 patients receiving voriconazole monotherapy and in 1 patient receiving combination therapy.

The present study reports, for the first time, the validation of a simple microbiological bioassay that can be used for TDM of voriconazole in patients on combination therapy with echinocandins.

The in-house-developed technique exhibits good sensitivity (LLOQ, 0.25 mg/liter) and reproducibility (%CV, 6%) across the entire concentration range tested, while its accuracy and reliability were ensured by validation with the reference method. The bioassay correlated well quantitatively and qualitatively with the HPLC assay in sera from patients treated with voriconazole alone and in combination with echinocandins ($r_s = >0.93$; 9 to 12% difference; slope, 0.912 to 1.013). The overall categorical agreement between the two methods was 91%, with the remaining 9% of the samples representing borderline deviations (13%) from the upper and lower limit of the therapeutic concentration range.

The ideal method for performing TDM is highly accurate (both sensitive and specific), reproducible, rapid, and inexpensive, and it should require a relatively small volume of sample for analysis. Several assays have been developed for the quantification of voriconazole in human blood. HPLC is still considered the gold standard, but the protocols used are characterized by moderately

FIG 2 Scatter plots of voriconazole serum concentrations measured by HPLC and bioassay in serum samples obtained from external quality control (QC) program (A), patients on voriconazole monotherapy (B), and patients on combination therapy with an echinocandin (C). r_s , Spearman's rank correlation coefficient with the P value, slope of the regression line $\pm 95\%$ confidence interval. The dotted lines represent the therapeutic window of 2 to 5 mg/liter. $C_{\text{voriconazole}}$, concentration of voriconazole.

laborious preanalytical processes and are costly, as specialized equipment and trained personnel are needed, hindering their implementation in daily clinical laboratory practice. In recent years, there has been a growing trend in the development of protocols of liquid chromatography in conjunction with mass spectrometry, which lead to rapid high-resolution separation analysis, but these are extremely expensive and not widely available for routine clinical workups (32). As a result, microbiological assays represent an attractive alternative testing methodology characterized by greater technical simplicity and lower cost.

When voriconazole is coadministered with other antifungal compounds, microbiological assays are not suitable to determine blood concentrations, since they lack specificity, as inhibition zones may be influenced by any metabolite or drug that possesses antifungal activity. However, for the first time in the literature, we developed and validated an agar diffusion bioassay to quantify voriconazole levels in serum from patients on combination therapy with echinocandins. For this purpose, a *C. parapsilosis* clinical isolate with high levels of susceptibility to voriconazole (0.015 mg/liter) and relatively low susceptibility to all three echinocandins (0.5 to 0.25 mg/liter) was used. Afterwards, the full range of serum drug concentrations that can be achieved in patients receiving the standard dosages, in accordance with previous pharmacokinetic studies (20–23), was tested *in vitro* in order to exclude potential interactions between them. Finally, the results were compared to reference method target values. The small number of serum specimens and patients on combination therapy (particularly with subtherapeutic levels) may be considered a limitation of this study. Nevertheless, the collection of such samples is difficult, because antifungal combination therapy has not yet been established, and fungal infections are rarely treated empirically in accordance with this strategy.

In the reported agar diffusion methods for measuring voriconazole blood levels from patients receiving monotherapy, several strains have been used as test organisms, i.e., a *Candida kefyr* isolate (8, 12, 13), a *Saccharomyces cerevisiae* isolate (11), an azole-hypersusceptible *Candida albicans* mutant (10), and recently, a *C. parapsilosis* isolate (33). A standard microorganism for the performance of this methodology has not yet been defined. Apparently, any voriconazole-susceptible isolate providing well-defined and symmetrical zones of growth inhibition not affected by the presence of echinocandins, as is the case with our own clinical *C. parapsilosis* strain, may be suitable after performing validation studies. In the present study, a simple and widely available medium was used, similar to the one recommended by the CLSI and EUCAST for antifungal susceptibility testing. A small volume of serum was utilized, which may be particularly important for neonates. The LLOQ and the linearity range obtained by our in-house technique are slightly better than those of previously reported methods (9, 11, 12), covering what is currently believed to be the therapeutic range for voriconazole concentrations in human blood (25, 26), while the concordance with the reference method is improved (9, 11, 12) or comparable (8, 10, 13) to that from other studies. Like in a previously published assay (9), voriconazole concentrations measured by HPLC were marginally higher than bioassay levels, although nonsignificantly higher drug concentrations were also found with bioassays than those with HPLC (8).

Since in all previous studies, the developed bioassays were suitable for use for TDM only in patients who received monotherapy with voriconazole, our study is unique, because the present bio-

assay has been extensively validated for patients both on voriconazole monotherapy and on combination therapy with an echinocandin. Studies with bioassays for patients on antifungal combination therapy are limited and restricted to patients treated with 5-fluorocytosine plus amphotericin B without extensive validation (34, 35).

In conclusion, since voriconazole is often coadministered with echinocandins for difficult-to-treat infections, and its blood concentrations are characterized by considerable variability and have been associated with adverse effects and unfavorable clinical response, periodic monitoring of voriconazole levels has been recommended in order to avoid subtherapeutic or toxic levels. In the present study, an easy and reliable microbiological assay was developed to determine voriconazole levels in serum from patients on combination therapy with echinocandins, with good reproducibility and sensitivity. This method may be a valid alternative tool to HPLC in clinical laboratories without specialized facilities.

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