UNIVERSITE DE LAUSANNE - FACULTE DE BIOLOGIE ET DE MEDECINE

Département de Médecine
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1) Variability of Voriconazole Plasma Levels Measured by New High-Performance Liquid Chromatography and Bioassay Methods
2) Voriconazole Therapeutic Drug Monitoring in Patients with Invasive Mycoses Improves Efficacy and Safety Outcomes

THESE

préparée sous la direction du Docteur Oscar Marchetti, Privat-Docent et Maître d'Enseignement et de Recherche

et présentée à la Faculté de biologie et de médecine de l'Université de Lausanne pour l'obtention du grade de

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par

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1) Variability of voriconazole plasma levels measured by new high-performance liquid chromatography and bioassay methods

2) Voriconazole therapeutic drug monitoring in patients with invasive mycoses improves efficacy and safety outcome

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de la Faculté de Biologie et de Médecine

S. Clarke

Madame le Professeur Stephanie Clarke
Directrice de l'Ecole doctorale

Rapport de synthèse

1. Partie de laboratoire

Cette première étude décrit le développement et la validation, selon les standards internationaux, de deux techniques de mesure des concentrations sanguines de voriconazole, un nouvel agent antifongique à large spectre: 1) la chromatographie en phase liquide à haute pression et 2) le bio-essai utilisant une souche mutante de *Candida* hypersensible au voriconazole. Ce travail a aussi permis de mettre en évidence une importante et imprévisible variabilité inter- et intra-individuelle des concentrations sanguines de voriconazole malgré l'utilisation des doses recommandées par le fabriquant. Ce travail a été publié dans un journal avec "peer-review": *"Variability of voriconazole plasma levels measured by new high-performance liquid chromatography and bioassay methods"* by A. Pascual, V. Nieth, T. Calandra, J. Bille, S. Bolay, L.A. Decosterd, T. Buclin, P.A. Majcherczyk, D. Sanglard, O. Marchetti. *Antimicrobial Agents Chemotherapy, 2007; 51: 137-43*

2. Partie Clinique

Cette deuxième étude a évalué de façon prospective l'impact clinique des concentrations sanguines de voriconazole sur l'efficacité et sécurité thérapeutique chez des patients atteints d'infections fongiques. Des concentrations sanguines élevées étaient significativement associés à la survenue d'une toxicité neurologique (encéphalopathie avec confusion, hallucinations et myoclonies) et des concentrations sanguines basses à une réponse insuffisante au traitement antifongique (persistance ou progression des signes cliniques et radiologiques de l'infection). Dans la majorité des cas, un ajustement de la dose de voriconazole, sur la base des concentrations mesurées, a abouti à une récupération neurologique complète ou à une résolution de l'infection, respectivement. Ce travail a été publié dans un journal avec "peer-review": "*Voriconazole Therapeutic Drug Monitoring in Patients with Invasive Mycoses Improves Efficacy and Safety Outcomes*" by A. Pascual, T. Calandra, S. Bolay, T. Buclin, J. Bille, and O. Marchetti. *Clinical Infectious Diseases, 2008 January 15; 46(2): 201-11.*

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Variability of Voriconazole Plasma Levels Measured by New High-Performance Liquid Chromatography and Bioassay Methods▽

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Voriconazole (VRC) is a broad-spectrum antifungal triazole with nonlinear pharmacokinetics. The utility of measurement of voriconazole blood levels for optimizing therapy is a matter of debate. Available high-performance liquid chromatography (HPLC) and bioassay methods are technically complex, time-consuming, or have a narrow analytical range. Objectives of the present study were to develop new, simple analytical methods and to assess variability of voriconazole blood levels in patients with invasive mycoses. Acetonitrile precipitation, reverse-phase separation, and UV detection were used for HPLC. A voriconazole-hypersusceptible *Candida albicans* mutant lacking multidrug efflux transporters (*cdr1*Δ/*cdr1*Δ, *cdr2*Δ/*cdr2*Δ, *flu1*Δ/*flu1*Δ, and *mdr1*Δ/*mdr1*Δ) and calcineurin subunit A (*cna*Δ/*cna*Δ) was used for bioassay. Mean intra-/interrun accuracies over the VRC concentration range from 0.25 to 16 mg/liter were 93.7% ± 5.0%/96.5% ± 2.4% (HPLC) and 94.9% ± 6.1%/94.7% ± 3.3% (bioassay). Mean intra-/interrun coefficients of variation were 5.2% ± 1.5%/5.4% ± 0.9% and 6.5% ± 2.5%/4.0% ± 1.6% for HPLC and bioassay, respectively. The coefficient of concordance between HPLC and bioassay was 0.96. Sequential measurements in 10 patients with invasive mycoses showed important inter- and intraindividual variations of estimated voriconazole area under the concentration-time curve (AUC): median, 43.9 mg · h/liter (range, 12.9 to 71.1) on the first and 27.4 mg · h/liter (range, 2.9 to 93.1) on the last day of therapy. During therapy, AUC decreased in five patients, increased in three, and remained unchanged in two. A toxic encephalopathy probably related to the increase of the VRC AUC (from 71.1 to 93.1 mg · h/liter) was observed. The VRC AUC decreased (from 12.9 to 2.9 mg · h/liter) in a patient with persistent signs of invasive aspergillosis. These preliminary observations suggest that voriconazole over- or underexposure resulting from variability of blood levels might have clinical implications. Simple HPLC and bioassay methods offer new tools for monitoring voriconazole therapy.

Voriconazole (VRC) is a new treatment option for aspergillosis, candidiasis, other emerging or refractory mycoses, and persistent fever during neutropenia (1, 4, 6, 8, 12, 15, 22, 25, 36). Combinations of VRC with other antifungal agents are being investigated for use with severe infections (3, 21). A correlation between in vitro MIC of VRC and response to antifungal therapy has been described previously (26). The VRC area under the concentration-time curve (AUC)/MIC ratio is the pharmacokinetic/-dynamic parameter which best predicts success in experimental candidiasis (2). Nonlinear pharmacokinetics, genetic polymorphism of the cytochrome P450 enzyme CYP2C19, changes in volume of distribution, drug interactions, hepatic dysfunction, and age may modify VRC pharmacokinetics (10, 13, 14, 16, 28). Interindividual variability of VRC blood levels has been reported previously, but it is unclear whether intraindividual variations also occur during therapy (10). VRC blood levels of <0.5 mg/liter (MIC_{90} for *Candida* and *Aspergillus* spp.) have been observed in 20% of patients with invasive aspergillosis (10). VRC trough blood concentrations of <0.25 mg/liter have been reported for children (35). Denning et al. described treatment failures in patients with VRC plasma concentrations of <0.25 mg/liter (6). VRC blood levels as high as 15 mg/liter were observed for subjects with liver toxicity or a "poor metabolizer" CYP2C19 genotype (5–7, 10, 14, 27). Whether measurement of VRC blood levels may optimize therapy in individual patients is a matter of debate (7).

Reported high-performance liquid chromatography (HPLC) methods are characterized by complex extraction, long analytical time, or limited analytical range (11, 23, 24, 34). A bioassay method using *Candida kefyr* ATCC 46764 (VRC MIC, 0.015 mg/liter) has been validated over a range of 0.8 to 3.6 mg/liter (24). Clinical observations suggest that quantification of VRC blood levels between 0.25 and 15 mg/liter is needed. Azole-hypersusceptible *C. albicans* mutants constructed by targeted deletion of genes encoding multidrug efflux transporters and calcineurin offer interesting tools for development of bioassays (19, 20, 31). The objectives of this study were to develop simple HPLC and bioassay methods with a clinically relevant analytical range and to assess the variability of VRC blood levels by sequential measurements in patients with invasive mycoses.

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MATERIALS AND METHODS

HPLC. (i) Sample preparation. Five hundred microliters of plasma and 500 μl of acetonitrile (Merck, Darmstadt, Germany) were vortexed/mixed (10 s), stored at 4°C (10 min), and centrifuged at 17,900 × *g* (5 min). The supernatant (500 μl) was transferred into an Eppendorf tube and evaporated under nitrogen stream (1 h) (nitrogen dryer; bioMérieux, Marcy l'Etoile, France). The dry residue was reconstituted in 200 μl of ultrapure water (Direct-Q water system; Millipore, Bedford, MA).

(ii) System. The HPLC system (Hitachi Instruments, Ichige, Hitachinaka, Japan) consisted of an L-2200 Peltier autosampler, an L-2100 gradient pump (with low-pressure mixing), and an L-2450 diode array detector. The results were analyzed using EZChrom Elite software, version 3.1 (Hitachi Instruments, Tokyo, Japan).

(iii) Conditions. Samples were maintained at 4°C in the autosampler prior to injection, and the column temperature was maintained at 35°C. Fifty-microliter samples were injected and separated using a reverse-phase C_{18} column (Nucleosil 300-5, C_{18}, 15 cm by 4.6 mm; Macherey-Nagel, Oensingen, Switzerland) with a universal security guard cartridge (Macherey-Nagel, Oensingen, Switzerland), an isocratic gradient of 50% (vol/vol) methanol (Merck, Dietikon, Switzerland) in 0.01 M sodium acetate buffer, pH 5.0 (Merck, Dietikon, Switzerland), and a flow rate of the mobile phase of 1.0 ml/min. The eluent was monitored at a 255-nm wavelength (deuterium lamp; Hamamatsu Photonics, Toyooka, Japan) and absorbance measured in arbitrary units.

(iv) Analysis. Each analytical run included one blank, eight standards, and four quality control samples (see paragraph i in "Validation of HPLC and bioassay" section). All samples were analyzed in duplicate. The amount of VRC was calculated by an external standard method based on peak areas (see paragraph iii in "Validation of HPLC and bioassay" section).

Bioassay. (i) Strains. *C. albicans* mutant DSY2621 was constructed by targeted deletions of genes encoding membrane efflux transporters (*cdr1*Δ::*hisG*/*cdr1*Δ::*hisG*, *cdr2*Δ::*hisG*/*cdr2*Δ::*hisG*, *flu1*Δ::*hisG*/*flu1*Δ::*hisG*, and *mdr1*Δ::*hisG*/*mdr1*Δ::*hisG*) and calcineurin subunit A (*cna*Δ::*hisG*/*cna*Δ::*hisG-URA3-hisG*) (VRC MIC, 0.008 mg/liter) (31). *C. albicans* ATCC 95020 (VRC MIC, 0.015 mg/liter) and *C. kefyr* B11501, a clinical isolate from the collection of the microbiology laboratory of our hospital (VRC MIC, 0.015 mg/liter), were also used. Strains were maintained at 4°C on Sabouraud dextrose agar plates (Difco Laboratories, Basel, Switzerland).

(ii) Inoculum. A single CFU of the test strain was grown overnight under agitation (214 rpm) at a temperature of 35°C in 2 ml of yeast extract-peptone-dextrose medium (10 g/liter Bacto peptone [Difco Laboratories, Basel, Switzerland], 5 g/liter yeast extract [Difco Laboratories, Basel, Switzerland], and 20 g/liter glucose [Fluka, Steinheim, Switzerland]). An inoculum of 1.5×10^7 CFU/ml was prepared by diluting the overnight culture with 0.9% NaCl to an optical density of 0.4 arbitrary units of absorbance at a wavelength of 540 nm (Novaspec II; Pharmacia Biotech, Cambridge, England). The viable counts of the inoculum were determined by subcultures on Sabouraud dextrose agar plates.

(iii) Medium and agar plate. A broth medium (412.5 ml) containing 25 g/liter agar (Becton Dickinson, Sparks, MD) and 8 g/liter of yeast nitrogen base (Becton Dickinson, Sparks, MD) was autoclaved and then allowed to equilibrate at a temperature of 50°C. Fifty milliliters of a 20% glucose aqueous solution was added (Sigma-Aldrich Chemie, Steinheim, Germany). A buffer solution (12.8 ml) (7.15 g KH_2PO_4 [Sigma-Aldrich Chemie, Steinheim, Germany] and 50 g $Na_2HPO_4 \cdot 2H_2O$ [Merck, Darmstadt, Germany] diluted in 500 ml of bidistilled water with a final pH of 5.5), 115 ml of broth medium, and 2.6 ml of inoculum were mixed at a temperature of 50°C. The final volume of 130.4 ml was poured into a square glass plate (220 by 220 mm). The agar was allowed to solidify at room temperature. Thereafter, 16 round wells (diameter, 4 mm; capacity, 25 μl) were cut using a sterile cork borer over a standard template. Each well was filled with 25 μl of plasma, which was allowed to diffuse through the agar at 4°C for 2 h. The plate was then incubated at 35°C for 14 h.

(iv) Analysis. Each analytical run included the following plasma samples in duplicate: one blank, eight standards, and four quality controls. Growth inhibition around wells was quantified by measuring the horizontal, the vertical, and the two diagonal diameters to the nearest 0.1 mm with a Vernier caliper. The amount of VRC was calculated by an external standard method (see paragraph iii in "Validation of HPLC and bioassay" section).

Validation of HPLC and bioassay. HPLC and bioassay were internally validated over a concentration range of 0.25 to 16 mg/liter according to the international recommendations for analytical method validation (9, 32).

(i) Spiked plasma samples. Citrated plasma from healthy donors was kindly supplied by the blood transfusion service of our hospital. A batch of spiked human plasma samples was prepared from an aqueous stock solution containing 1 mg/liter of VRC. VRC pure substance was kindly supplied by Pfizer Central Research (Sandwich, United Kingdom). Eight standards (i.e., samples used to calculate a regression equation) were obtained by serial dilutions: 0.19, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, and 25 mg/liter. The four quality control samples (i.e., used for back calculation of VRC values by the regression equation) contained 0.25, 1, 4, and 16 mg/liter. Aliquots of each concentration were prepared for HPLC (500 μl) and bioassay (60 μl) and frozen at −80°C. Spiked plasma samples from a unique batch were used for the entire validation procedure.

(ii) Limits of detection and quantification. The limit of detection (LOD) was defined as the lowest VRC concentration that could be differentiated reliably from background noise (mean of 12 different background noise measurements ± 3 standard deviations for HPLC) or could be recognized as a delimited zone of growth inhibition (for bioassay). The limit of quantification (LOQ) was defined as the lowest amount of VRC that could be quantified in a plasma sample with ±20% accuracy and precision (9, 32).

(iii) Standard curves. Curves consisting of eight points (0.2 to 25 mg/liter) were calculated by linear regression (best fit obtained by exploring different weighting factors when plotting peak areas measured by HPLC against VRC concentrations) or by quadratic regression (best fit obtained by plotting diameters of growth inhibition measured by bioassay against VRC concentrations).

(iv) Recovery of VRC from plasma. The absolute recovery of VRC from plasma (biological matrix) was calculated for the four quality control samples as follows: measured concentration in plasma/measured concentration in water × 100. To assess the efficiency of VRC extraction from plasma, a 50-μl volume of water spiked with VRC was injected into the HPLC (equaling 100% recovery); percent recovery of VRC from extracted plasma was calculated according to the formula given above.

(v) Influence of the anticoagulant on VRC quantification. The percentages of measured/nominal VRC values in quality control samples were compared in plasma containing citrate, heparin, or EDTA and in serum. Citrated standard plasma samples were used for the calibration curve.

(vi) Quantification of VRC over time under different storage conditions. Quality controls were tested for stability of VRC over time (percent deviation of measured from initial values) (a) in plasma at 4 and 21°C (7 days), (b) in plasma at −80°C (12 months), and (c) in plasma during four freeze-thaw cycles. Stability was also measured in whole blood from a patient treated with VRC at 4 and 21°C (7 days).

(vii) Accuracy and precision. Intra- and interrun accuracy (measured value/nominal value × 100) and precision [(coefficient of variation) = (standard deviation/mean of measured values × 100)] were assessed for the four quality controls with six duplicate measurements within the same experiment or in six experiments (each with duplicate measurements) performed on different days, respectively.

(viii) Selectivity. To assess the selectivity of the HPLC method for VRC, the following antifungals, antibiotics, antivirals, and immunosuppressants were tested with VRC: (a) amphotericin B, caspofungin, 5-flucytosine, fluconazole, and itraconazole; (b) amoxicillin-clavulanic acid, piperacillin-tazobactam, cefepime, ceftazidime, ceftriaxone, imipenem, meropenem, teicoplanin, vancomycin, clindamycin, clarithromycin, ciprofloxacin, levofloxacin, metronidazole, trimethoprim, amikacin, gentamicin, and rifampin; (c) amprenavir, efavirenz, indinavir, lopinavir, nelfinavir, nevirapine, saquinavir, and ritonavir; and (d) cyclosporine A and tacrolimus.

(ix) Concordance between HPLC and bioassay. The agreement between VRC levels measured by HPLC and bioassay in plasma samples from patients treated with VRC was analyzed by a method proposed by Lin (17). Measurements by HPLC and bioassay were performed by two different investigators who were blinded of the results obtained with the other method. The coefficient of concordance was calculated using the following parameters: μ_1 and μ_2, means of values measured by HPLC and bioassay; σ_1 and σ_2, standard deviations of values measured by HPLC and bioassay; location shift (u), $(\mu_1 - \mu_2)/\sqrt{(\sigma_1 - \sigma_2)}$; scale shift ($v$), σ_1/σ_2; precision (r), coefficient of correlation; congruence (A), $[(v + 1/v + \mu^2)/2]^{-1}$; concordance, $r \times A$.

Measurements of VRC blood levels in patients with invasive mycoses. Inpatients receiving VRC therapy in the ward of the Infectious Diseases Service over a 9-month period were studied after providing written informed consent. The study protocol was approved by the Institutional Ethical Committee. VRC loading and maintenance doses followed manufacturer's recommendations. Clinical data on demographics, diagnosis of fungal infection (according to EORTC/MSG

TABLE 1. Intra- and interrun validation of the HPLC method and the bioassay[a,d]

Nominal VRC concn(s) (mg/liter)	HPLC				Bioassay			
	Intrarun (n = 6)		Interrun (n = 6)		Intrarun (n = 6)		Interrun (n = 6)	
	Accuracy[b] (%)	Coefficient of variation[c] (%)	Accuracy (%)	Coefficient of variation (%)	Accuracy (%)	Coefficient of variation (%)	Accuracy (%)	Coefficient of variation (%)
0.25	100.7 ± 6.4	6.4	98.9 ± 4.2	5.1	89.0 ± 4.1	5.0	90.9 ± 3.6	4.2
1	89.8 ± 3.5	3.9	93.2 ± 5.6	6.3	99.2 ± 10.2	10.3	96.3 ± 5.8	6.1
4	90.1 ± 6.0	6.6	96.2 ± 5.9	5.9	96.6 ± 5.0	5.2	97.8 ± 2.1	2.2
16	94.3 ± 3.6	3.9	97.5 ± 5.0	4.3	94.7 ± 5.2	5.5	93.9 ± 1.7	3.6
All	93.7 ± 5.0	5.2 ± 1.5	96.5 ± 2.4	5.4 ± 0.9	94.9 ± 6.1	6.5 ± 2.5	94.7 ± 3.3	4.0 ± 1.6

[a] The bioassay method used the *C. albicans* mutant lacking four multidrug efflux transporters (*cdr1*Δ/*cdr1*Δ, *cdr2*Δ/*cdr2*Δ, *flu1*Δ/*flu1*Δ, and *mdr1*Δ/*mdr1*Δ) and the calcineurin subunit A (*cna*Δ/*cna*Δ).

[b] The accuracy was calculated as follows: measured concentration/nominal concentration × 100.

[c] The coefficient of variation was calculated as follows: standard deviation of measured concentration/mean measured concentration × 100.

[d] Mean values ± standard deviations are shown.

criteria), response to therapy (complete or partial clinical/radiological resolution or persistent signs of infection), comedications, and adverse events (according to National Cancer Institute grading criteria) were prospectively collected. VRC blood levels were measured on days 2, 7, and 14 of antifungal therapy. Two milliliters of citrated blood was drawn just before and 2, 4, 6, and 12 h after VRC administration. VRC levels were measured by HPLC as described above. Average exposure to VRC was assessed by the estimated AUC over one dosing interval (0 to 12 h) calculated by the linear trapezoidal rule during increase and the log trapezoidal rule during decay (29). VRC peak concentrations in serum (C_{max}), trough concentrations, and estimated AUC values were analyzed by descriptive statistics.

RESULTS

HPLC. A plasma peak overlapping with that of VRC at a retention time of 8 min was observed using a recently reported HPLC method (24). The effect was independent from the type of organic matrix and occurred in plasma containing citrate, heparin, or EDTA and in serum. To eliminate this interference, we modified the experimental conditions by using a different mobile phase (50/50 [vol/vol] methanol and sodium acetate) and a shorter column (C_{18}, 150 by 4.6 mm, 5 μm). The run time was significantly shorter (12 min), and the chromatogram showed a VRC peak free of interferences after a retention time of 7.4 min regardless of the type of biological matrix (plasma with different anticoagulants or serum).

For the standard curve, a linear regression with a $1/x^2$ weighting factor was used (sum of the relative errors by back calculation of the VRC concentrations in standards, 2.9%; range of relative errors, 0.4 to 5.9%; constant variability of relative errors over the analytical range, 0.125 to 25 mg/liter). The mean curve parameters were as follows: a = 287,048 ± 27,801; b = −4,816 ± 7,372; r^2 = 0.0997 ± 0.002. The LOD and LOQ of VRC were 0.04 and 0.125 mg/liter, respectively. The absolute VRC recovery was 102% ± 6%. The influence of the biological matrix on VRC quantification was studied. The mean percent ratios of measured/expected VRC values in plasma containing citrate, heparin, or EDTA and in serum were 93.7% ± 5.0%, 87% ± 1.6%, 89.1% ± 5.7%, and 91.9% ± 1.9%, respectively. The quantifications over time of VRC in stored plasma (measured values/initial values × 100) were 95% ± 4% and 94% ± 3% at 4 and 21°C, respectively, over 7 days, 98% ± 9% at −80°C over 12 months, and 95% ± 7% after four freeze-thaw cycles. The ratios (measured values/initial values × 100) in whole blood drawn from patients treated with VRC and stored over 4 days were 92% ± 4% at 4°C and 95% ± 6% at 21°C.

Table 1 summarizes the results of intrarun and interrun validations over the 0.25- to 16-mg/liter concentration range. The mean intrarun accuracy was 93.7% ± 5.0%, and the mean coefficient of variation was 5.2% ± 1.5%. The mean interrun accuracy was 96.5% ± 2.4%, and the mean coefficient of variation was 5.4% ± 0.9%.

To assess the selectivity of the HPLC method, the retention times of the commonly used antifungals (n = 5), antibiotics (n = 18), antivirals (n = 8), and immunosuppressive drugs (n = 2) listed in Materials and Methods were studied. The antiretroviral agents indinavir and amprenavir had retention times overlapping with that of VRC, leading to potential interferences with calculation of the VRC concentrations. No interference was observed between VRC and the remaining drugs.

Bioassay. VRC bioassays using the azole-hypersusceptible *C. albicans* mutant (*cdr1*Δ/*cdr1*Δ, *cdr2*Δ/*cdr2*Δ, *flu1*Δ/*flu1*Δ, *mdr1*Δ/*mdr1*Δ, and *cna*Δ/*cna*Δ), the reference strain *C. albicans* ATCC 95020, and the clinical isolate *C. kefyr* B11501 were compared. The zones of growth inhibition of the standard curve obtained with the hypersusceptible *C. albicans* mutant were well delimited and ranged between 17 and 52 mm over the 0.2- to 25-mg/liter VRC concentration analytical range. Those obtained with *C. albicans* ATCC 95020 and *C. kefyr* B11501 ranged between 21 and 39 mm and between 23 and 39 mm, respectively. The LOD and LOQ of the methods using the azole-hypersusceptible *C. albicans* mutant were 0.1 mg/liter and 0.2 mg/liter, respectively, and those with *C. albicans* ATCC 95020 or *C. kefyr* B11501 were 0.39 mg/liter and 0.5 mg/liter, respectively. With the hypersusceptible mutant, excellent standard curve reproducibility was obtained (mean ± standard deviation): b_0 = −1.92 ± 0.42, b_1 = 0.04 ± 0.02, b_2 = 0.0007 ± 0.0003, and r^2 = 0.998 ± 0.035. The influence of the biological matrix on VRC quantification was studied. The absolute VRC recovery was 99% ± 0.5%. The mean percent ratios of measured/nominal VRC values in plasma containing citrate, heparin, or EDTA and in serum were 97.6% ± 3.6%, 95.4% ± 1.4%, 95.4% ± 1.4%, and 106.3% ± 3.7%, respectively. The quantifications over time of VRC in stored plasma (measured

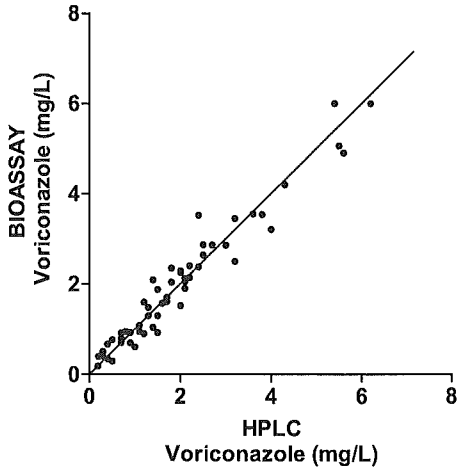


FIG. 1. Scatter plot of plasma VRC levels measured by HPLC and bioassay using the *C. albicans* mutant (*cdr1*Δ/*cdr1*Δ, *cdr2*Δ/*cdr2*Δ, *flu1*Δ/*flu1*Δ, *mdr1*Δ/*mdr1*Δ, and *cna*Δ/*cna*Δ). The line of congruence does not deviate from 1. The coefficient of concordance is 0.96.

values/initial values × 100) were 99% ± 3% and 99% ± 3% at 4 and 21°C, respectively, over 7 days, 99% ± 5% at −80°C over 12 months, and 104% ± 4% after four freeze-thaw cycles. The ratios (measured values/initial values × 100) in whole blood drawn from patients treated with VRC and stored over 4 days were 99% ± 1% at 4°C and 99% ± 0.5% at 21°C.

The intra-/interrun accuracy (mean, 94.9% ± 6.1%/94.7% ± 3.3%) and precision (mean coefficients of variation, 6.5% ± 2.5%/4.0% ± 1.6%) were well within the recommended validation limits (accuracy, 85% to 115%, and precision, ±15%) (Table 1) (9, 32).

Concordance between HPLC and bioassay. The comparison of the VRC levels measured in 58 clinical plasma samples by HPLC and bioassay methods handled by different laboratory technicians is shown in Fig. 1. The correlation coefficient was 0.97 ($P < 0.001$), and the coefficient of concordance calculated according to the method reported by Lin (17) was 0.96 (u [location shift], 0.007; v [scale shift], 1.283; A [congruence], 0.99).

Measurements of VRC blood levels in patients with invasive mycoses. Ten Caucasian adult patients were studied (Table 2). None had underlying hepatic or renal disease. During the study period, VRC dosing schedules remained unchanged.

VRC blood levels were measured over 7 days of therapy in five patients and over 14 days in the remaining five. In seven cases, the study ended at discharge from the hospital. In three cases, VRC therapy was discontinued after invasive mycosis was ruled out (one case), because of a serious adverse event (one case), or because of persistent signs of infection requiring a switch to a salvage regimen (one case).

The median C_{max} values were 6 mg/liter (range, 2.7 to 6.4) on the first day and 3.8 (range, 0.3 to 8.2) on the last day of study. The median VRC trough levels were 3 (range, 0.3 to 5.6) and 1.2 (range, 0.2 to 7.4), respectively. Average exposure was expressed by estimated AUC based on measurements of VRC blood levels at three to five time points. The median estimated VRC AUC value decreased over the study period from 43.9 mg · h/liter (range, 12.9 to 71.1) to 27.4 mg · h/liter (range, 2.9 to 93.1). In five patients, the AUC value decreased (19.9%, 38.3%, 52.9%, 40.9%, and 77.5%). One patient was treated with VRC intravenously (i.v.), two orally, and two had an intravenous-to-oral switch with identical VRC daily doses; two patients received concomitant medication with esomeprazole and one with imatinib. In three cases, the VRC AUC increased (30.6%, 30.9%, and 107.1%). Two patients were treated with VRC i.v., and one had an intravenous-to-oral switch with no change of the VRC daily dose; one patient received esomeprazole comedication. The VRC AUC remained unchanged in two cases (decreased 5.8% and 9.9%): one patient was treated with VRC orally, and one had an intravenous-to-oral switch with the same VRC daily dose.

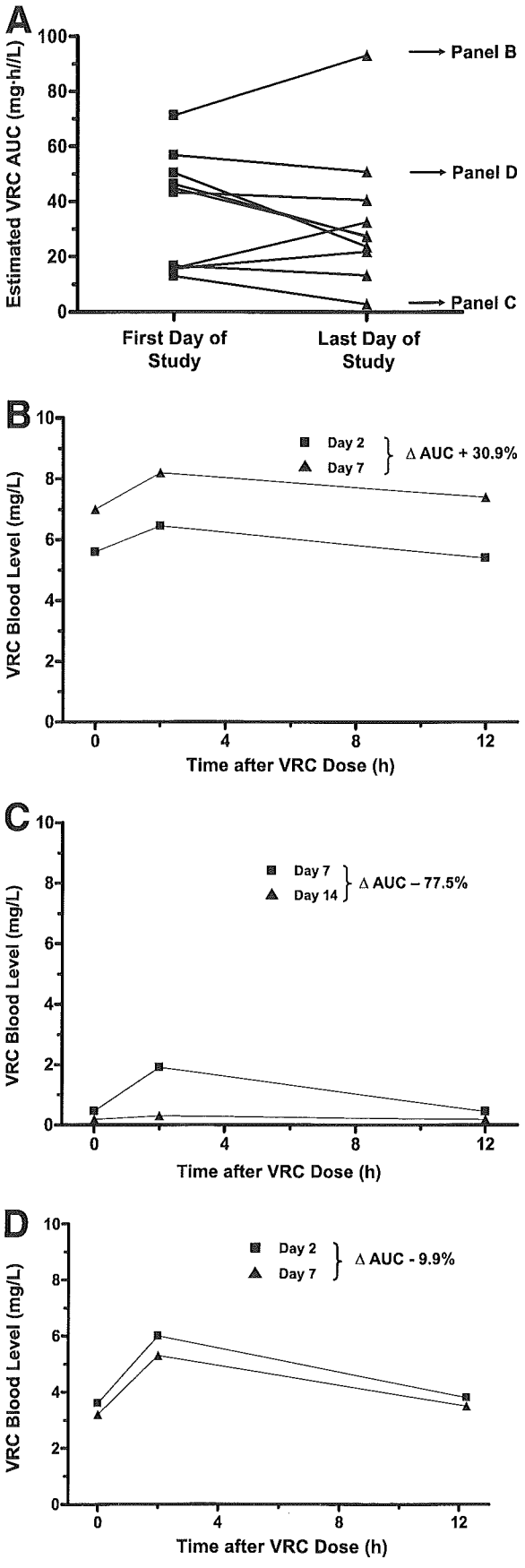
Figure 2 shows the course of the estimated VRC AUC values during the study period in the 10 patients (Fig. 2A) and three individual VRC concentration-time curves (Fig. 2B to D). A leukemic patient with proven pulmonary aspergillosis and increasing VRC exposure (at day 2, C_{max} was 6.4 mg/liter, trough was 5.6 mg/liter, and AUC was 71.1 mg · h/liter; at day 7, C_{max} was 8.2 mg/liter, trough was 7 mg/liter, and AUC was 93.1 mg · h/liter) under 200 mg orally twice daily developed a toxic encephalopathy (confusion, agitation, and hallucinations; grade 3 according to NCI classification) and a mild cholestasis (alkaline phosphatase three times the upper normal value) (Fig. 2B). This adverse event was probably related to high VRC blood levels and promptly resolved after interruption of therapy. The comedication with esomeprazole may have interacted with VRC metabolism and contributed to the increase of VRC exposure. Rechallenge with lower VRC doses (150 mg twice daily) was well tolerated. A persistence of signs of probable pulmonary aspergillosis (persistent fever and inflamma-

TABLE 2. Characteristics of 10 patients treated with VRC

Characteristic	No. of patients with characteristic
Gender (male/female)[a]	8/2
Underlying conditions	
Acute leukemia/neutropenia after myeloablative chemotherapy	8
None	2
VRC therapy	
Indications	
Invasive aspergillosis	7
Proven/probable	4/3
Pulmonary/rhinocerebral/disseminated	5/1/1
Possible pulmonary invasive mycosis	2
Persistent fever during neutropenia	1
Settings	
First-line therapy	4
Salvage therapy (failure of/intolerance to previous antifungal therapy)	4 (2/2)
Intravenous-to-oral switch after therapy with another antifungal agent	2
Initial route of administration (i.v./oral)[b]	8/2
Intravenous-to-oral switch during VRC therapy	4
Response of infection to antifungal therapy on day 14 (complete response/partial response/persistence of infection)	2/6/2
Adverse event leading to discontinuation of therapy	1

[a] The median patient age was 62 years (range, 28 to 64 years), and the median patient weight was 69 kg (range, 50 to 98 kg).

[b] The median dose, given twice daily, was 4 mg/kg of body weight (range, 2.5 to 4.6 mg/kg).



tory syndrome, radiological progression of the initial focus, and documentation of a new contralateral pulmonary lesion) was observed during the neutropenic period in a leukemic patient receiving 200 mg orally twice daily. A drop in VRC blood levels was documented (at day 7, C_{max} was 1.9 mg/liter, trough was 0.5 mg/liter, and AUC was 12.9 mg · h/liter; at day 14, C_{max} was 0.3 mg/liter, trough was 0.2 mg/liter, and AUC was 2.9 mg · h/liter) (Fig. 2C). No cause for the decrease of VRC exposure was identified: the dose had remained unchanged, no comedication interacting with VRC metabolism had been added, and drug compliance was appropriate. A clinical and radiological resolution of the infection was obtained after increase of the VRC dose to 300 mg orally twice daily. In another leukemic neutropenic patient, persistent signs of probable pulmonary aspergillosis (persistent fever, increasing inflammatory syndrome, and radiological progression of multiple pulmonary lesions) were observed. With a VRC dose of 300 mg orally twice daily, VRC C_{max} and trough levels at the time of assessment of persistence of infection were 4.3 and 2.6 mg/liter, respectively (AUC had remained unchanged, i.e., 43 mg · h/liter on the first day and 40.5 mg · h/liter on the last day of therapy). In this patient, therapy was switched to a salvage regimen with liposomal amphotericin B and a complete resolution of infection was obtained. Figure 2D shows stable concentration-time curves from a leukemic patient with an uneventful clinical course.

DISCUSSION

Reported HPLC methods for measurement of VRC blood levels are technically complex and/or time-consuming (e.g., extraction procedure of >4 h and/or run time of >30 min) (11, 23, 24, 34). With a recently described method using organic phase extraction, we have observed a peak interfering with the VRC quantification (24). The reported *C. kefyr* ATCC 46764 bioassay has a limited analytical range (0.8 to 3.6 mg/liter) (24). The present study reports the development of new, simple HPLC and bioassay methods with an analytical performance meeting clinical requirements.

Plasma VRC extraction by acetonitrile precipitation followed by evaporation under nitrogen stream and reverse-phase

FIG. 2. (A) Courses of estimated VRC AUC in 10 patients. (B to D) Individual VRC concentration-time curves. (B) Patient with proven pulmonary aspergillosis, toxic encephalopathy, and mild cholestasis. Increasing VRC blood levels were observed (ΔAUC + 30.9%) despite an unchanged VRC dose (200 mg orally twice daily; a switch from i.v. to oral treatment had occurred). During the entire study period, the patient received esomeprazole. This serious neurological adverse event was probably related to VRC overdosing and completely resolved after stop of VRC therapy. Rechallenge with lower VRC doses (150 mg orally twice daily) was well tolerated. (C) Patient with persistent signs of probable pulmonary aspergillosis. Decreasing VRC exposure was documented (ΔAUC − 77.5%) despite an unchanged VRC dose (200 mg orally twice daily) and absence of comedication interacting with VRC metabolism. The infection responded after increase of the VRC dose to 300 mg orally twice daily. (D) Patient with probable pulmonary aspergillosis and an uneventful clinical course. VRC concentrations were stable (ΔAUC − 9.9%). The VRC dose was 300 mg twice daily, the route was i.v. on days 2 and 7, and there was no comedication interacting with VRC metabolism.

separation followed by UV detection over 12 min were used for HPLC. The absolute recovery of VRC from plasma was close to 100%. The method has been validated according to international guidelines over the 0.25- to 16-mg/liter analytical range, which covers the clinically relevant concentration spectrum (5–7, 10, 14, 27, 35). Results are not influenced by type of organic matrix or anticoagulant and are reproducible under different storage conditions. The technique selectively detects VRC in the presence of a large number of frequently used antimicrobial and immunosuppressive drugs: analytical interferences have been observed only with the antiretroviral agents indinavir and amprenavir. Simple preanalytical processing and short analytical time (results available within 6 h) are advantages of this HPLC technique over those previously reported.

The new bioassay uses an azole-hypersusceptible *C. albicans* mutant constructed by targeted deletion of genes encoding multidrug efflux transporters and calcineurin subunit A (31). This simple test using very small volumes of plasma (50 μl, i.e., 10 times lower that those needed for HPLC) has an analytical range identical to that of HPLC (0.25 to 16 mg/liter). The concordance between VRC levels measured in clinical plasma samples by HPLC and bioassay was excellent. Despite a longer analytical time (16 h), bioassay may be a valid alternative to HPLC. Bioassay is particularly useful for analyses with small volumes of blood (e.g., from children), when analytical interferences alter detection of VRC by HPLC, or in laboratories not equipped for HPLC.

Multiple factors have been associated with interindividual variations of VRC blood levels (10, 13, 16, 28, 37). Decrease of VRC blood levels during therapy has been reported in experimental models with rats, mice, and dogs (30). Autoinduction of cytochrome P450 metabolism by VRC has been associated with this phenomenon. It is unclear whether intraindividual variations also occur in humans (10, 16, 28). The utility of measuring VRC blood levels to improve clinical management has been a matter of debate in recent reports (5–7, 10, 18, 27, 33, 35). We sequentially measured VRC blood levels in Caucasian adult patients receiving standard doses of VRC for invasive mycoses. Large inter- and intraindividual variations of VRC blood levels were observed. During the study period, VRC exposure decreased or increased in 8 out of 10 patients despite the absence of changes in VRC doses and of comedications interacting with VRC metabolism or liver function. Although oral bioavailability of VRC is excellent, an intravenous-to-oral switch might have influenced VRC blood levels in some patients (10, 16, 28). Three clinical cases were illustrated. The first patient received esomeprazole as comedication and developed a toxic encephalopathy probably related to increasing VRC exposure (C_{max} level, 8.2 mg/liter; trough level, 7 mg/liter; AUC, 93.1 mg · h/liter), which completely resolved after interruption of therapy. Rechallenge with lower doses of VRC was well tolerated. In a second neutropenic patient with persistent clinical and radiological signs of a probable invasive aspergillosis, a decrease in VRC blood levels and AUC (C_{max} level, 0.3 mg/liter; trough level, 0.2 mg/liter; AUC, 2.9 mg · h/liter) was observed. No cause of this drop in VRC exposure was identified. Infection responded after increase of the VRC dose. In a third leukemic patient, persistence of a possible pulmonary mycosis despite appropriate VRC blood levels required a switch to a salvage therapy. In the remaining seven patients, the clinical course was uneventful. These preliminary observations suggest that, in addition to interindividual differences, important intraindividual variations of VRC blood levels may occur during therapy. Small sample size and lack of data on genetic polymorphism of CYP2C19 are important limitations of this study. Nevertheless, in addition to those previously reported by other investigators, our clinical data suggest that VRC over- or underexposure might be associated with toxicity or persistence of infection, respectively. Simple, accurate, and precise HPLC and bioassay techniques offer new tools for monitoring VRC therapy.

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MAJOR ARTICLE

Voriconazole Therapeutic Drug Monitoring in Patients with Invasive Mycoses Improves Efficacy and Safety Outcomes

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(See the editorial commentary by Lewis on pages 212–4)

Background. Voriconazole is the therapy of choice for aspergillosis and a new treatment option for candidiasis. Liver disease, age, genetic polymorphism of the cytochrome CYP2C19, and comedications influence voriconazole metabolism. Large variations in voriconazole pharmacokinetics may be associated with decreased efficacy or with toxicity.

Methods. This study was conducted to assess the utility of measuring voriconazole blood levels with individualized dose adjustments.

Results. A total of 181 measurements with high-pressure liquid chromatography were performed during 2388 treatment days in 52 patients. A large variability in voriconazole trough blood levels was observed, ranging from ≤1 mg/L (the minimum inhibitory concentration at which, for most fungal pathogens, 90% of isolates are susceptible) in 25% of cases to >5.5 mg/L (a level possibly associated with toxicity) in 31% of cases. Lack of response to therapy was more frequent in patients with voriconazole levels ≤1 mg/L (6 [46%] of 13 patients, including 5 patients with aspergillosis, 4 of whom were treated orally with a median dosage of 6 mg/kg per day) than in those with voriconazole levels >1 mg/L (15 [12%] of 39 patients; $P = .02$). Blood levels >1 mg/L were reached after increasing the voriconazole dosage, with complete resolution of infection in all 6 cases. Among 16 patients with voriconazole trough blood levels >5.5 mg/L, 5 patients (31%) presented with an encephalopathy, including 4 patients who were treated intravenously with a median voriconazole dosage of 8 mg/kg per day, whereas none of the patients with levels ≤5.5 mg/L presented with neurological toxicity ($P = .002$). Comedication with omeprazole possibly contributed to voriconazole accumulation in 4 patients. In all cases, discontinuation of therapy resulted in prompt and complete neurological recovery.

Conclusions. Voriconazole therapeutic drug monitoring improves the efficacy and safety of therapy in severely ill patients with invasive mycoses.

Voriconazole is the first choice therapy for invasive aspergillosis and is a new treatment option for candidiasis, other emerging invasive fungal infections (IFI), such as fusariosis, and refractory IFI [1–5]. Status of the host immunity, localization and extension of infection, early start of antifungal therapy, lack of response to previous therapy, surgical management, and in vitro antifungal susceptibility of the pathogen are key determinants for a successful response [6–9]. The role of antifungal drug blood concentrations with respect to efficacy and safety is unclear. Liver metabolism plays a key role in the disposition of voriconazole, which acts simultaneously as a substrate and inhibitor of multiple enzymes of the cytochrome P450 system [7]. Multiple factors have been found to be associated with a large variability in voriconazole exposure following standard dose administration, such as nonlinear saturable pharmacokinetics, drug-drug interactions, liver disease, patient age (in particular, with respect to children), and genetic polymorphism of the cyto-

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chrome CYP2C19 [3, 7, 10, 11]. In experimental candidiasis, the ratio of voriconazole peak concentration and the 24-h area under the curve over the MIC have been identified as the best pharmacodynamic predictors of treatment success [12]. In contrast, no experimental data are available regarding the pharmacodynamics of voriconazole for the treatment of aspergillosis or other mycoses. Some recent clinical observations have suggested that under- and over-dosing of voriconazole may influence the efficacy and safety of therapy, respectively [1, 13–16]. Recently, experts have suggested that voriconazole trough blood levels should be measured after 1 week of therapy for dose adjustment to target values of 2–6 mg/L [7]. However, the utility of such measurements remains debatable, given that multiple confounding factors may influence the outcome of infection and the assessment of tolerance in severely ill patients [17–19]. This uncertainty highlights the need for additional drug exposure–related efficacy and safety data in patients treated with voriconazole. The objective of the present observational study was to assess the utility of voriconazole therapeutic drug monitoring (TDM).

PATIENTS AND METHODS

Patients. All consecutive adult patients treated with voriconazole at a single university hospital during the period 2004–2006 were identified from the pharmacy records. Patients who received voriconazole with TDM were studied prospectively, and those who received voriconazole without TDM were analyzed retrospectively. Treating physicians based the choice of voriconazole route and dose on the recommendations of the package insert, published guidelines, and the literature [1, 2, 4, 20–22]. IFIs were classified as either proven, probable, or possible according to definitions of the Invasive Fungal Infection Group of the European Organization for Research and Treatment of Cancer and Mycoses Study Group of the National Institute of Allergy and Infectious Diseases [23]. Empirical antifungal therapy was given to patients with persistent febrile neutropenia despite >96 h of broad-spectrum antibacterial therapy. Demographic data, clinical characteristics, diagnosis of IFI, and response to or experience of adverse events associated with voriconazole therapy were recorded.

Response of infection to antifungal therapy. The assessment of response to voriconazole therapy as partial or complete was based on clinical (fever, signs and symptoms of infection, and inflammatory markers) and radiological (CT or MRI findings) improvement or resolution and on proven or presumed eradication of the fungal pathogen [6]. Lack of response to voriconazole therapy was defined by persistent IFI after >14 days of treatment or by progressing IFI (clinical and radiological progression, persistently positive culture results, or death due to IFI) after >7 days of treatment.

Safety of antifungal therapy. The type and severity of severe adverse events and their causal relationship with voriconazole therapy were defined according to the criteria of the National Cancer Institute [24].

Measurement of voriconazole blood levels. Voriconazole trough blood levels were studied in citrated plasma by validated high-pressure liquid chromatography (analytical range, 0.125–25 mg/L) [25, 26]. Voriconazole trough levels were measured because of the lack of clinical data on other pharmacodynamic parameters that might better predict the efficacy and safety of therapy and because routine blood sampling just preceding the next voriconazole dose is practical and reliable for intrapatient and interpatient comparisons. The therapeutic interval for voriconazole troughs was 1–5.5 mg/L [1, 8, 13, 15, 27]. The choice of the 1-mg/L cutoff value had been based on in vitro susceptibility data (MIC_{90}, 0.5–1 mg/L for the majority of fungal pathogens), considering that only the free-circulating fraction of voriconazole (i.e., 40%–50%) is microbiologically active and available for penetration in infected organs [3, 8, 28, 29]. The upper cutoff value was based on data reported for patients who experienced adverse events [1, 13, 14, 30]. The following voriconazole therapy modifications were proposed: (1) a 50% increase in daily dose in patients with trough levels ≤1 mg/L and lack of response to therapy, as recommended in the package insert, and (2) discontinuation of therapy in patients with trough levels >5.5 mg/L plus/minus adverse events probably related to overdosing.

Statistical analysis. Proportions were compared by the χ^2 test or Fisher's exact test, as appropriate. Continuous variables were compared by the nonparametric Mann-Whitney *U* test or Kruskal-Wallis test, as appropriate. Statistical significance was defined by a 2-sided *P* value <.05. The Spearman method was used to study the correlation of 2 variables. A logistic regression analysis (Stata software, version 8.2; Stata) was performed to assess whether the log-transformed voriconazole trough concentration is a significant predictor of response to therapy (coded as success or lack of response) or safety (coded as absence or presence of severe toxicity).

RESULTS

Patients. Among 96 adult patients treated with voriconazole during the study period, 52 had TDM. All patients with TDM were white; 51 (98%) of 52 were inpatients. The most frequent underlying condition was acute myeloid leukemia with neutropenia following myeloablative chemotherapy. Patients demographic data and clinical characteristics are summarized in table 1.

Data were analyzed retrospectively for 39 (89%) of 44 patients without voriconazole TDM; of these patients, 13 (33%) were neutropenic, 21 (54%) had another type of immunosuppression, and 5 (13%) had no underlying condition.

Voriconazole therapy. A total of 2388 days of voriconazole

Table 1. Demographic and clinical characteristics, invasive fungal infections, and voriconazole therapy in 52 patients with therapeutic drug monitoring.

Variable	Patients with measured voriconazole blood levels (n = 52)
Demographic data	
Age, median years (range)	58.5 (23–78)
Sex, male:female	38 (73):14 (27)
Underlying condition	
Hematological malignancy, neutropenia <0.5 g/L	32 (61)
Solid-organ transplantation	3 (6)
Abdominal surgery	3 (6)
Chronic liver disease	3 (6)
Other condition[a]	7 (13)
None	4 (8)
Fungal infection	
Proven or probable invasive aspergillosis[b]	26 (50)
Lung	19 (36)
Sinus[c]	3 (6)
Disseminated[d]	3 (6)
Intra-abdominal	1 (2)
Proven or probable invasive candidiasis[e]	8 (15)
Bloodstream	3 (6)
Hepatosplenic	4 (7)
Bone	1 (2)
Other proven invasive fungal infection (*Pseudallescheria boydii* and *Paecilomyces* species): bone	2 (4)
Possible invasive fungal infection: lung	11 (21)
Persistent fever during neutropenia	5 (10)
Voriconazole therapy	
First line	33 (64)
Second line	19 (36)
Failure of first-line therapy	1 (2)
Intolerance to first-line therapy	9 (17)
Switch from intravenous to oral therapy	9 (17)
Route of administration	
Intravenous	40 (77)
Switch from intravenous to oral therapy	17 (33)
Oral	12 (23)
Voriconazole daily dose, median mg/kg/day (range)	
Loading dose (oral or intravenous)	12
Maintenance dose	
Intravenous	8 (6–11)
Oral	6.5 (2–11)[f]
Duration of therapy, median days (range)	
Overall	50 (4–1130)
Intravenous	6.5 (4–30)
Oral	55 (5–1130)

NOTE. Data are no. (%) of patients, unless otherwise indicated.

[a] Chronic lung disease (2 patients), diabetes mellitus (2 patients), HIV infection (2 patients), and open knee fracture (1 patient).

[b] Proven aspergillosis (19 patients) and probable aspergillosis (7 patients).

[c] With intracerebral extension (1 patient).

[d] Involvement of lung (3 patients), sinus (2 patients), liver (2 patients), and skin (1 patient).

[e] Proven candidiasis (4 patients) and probable candidiasis (4 patients).

[f] Intravenous vs. oral maintenance daily dose (P = .07).

therapy in 52 patients with TDM were studied. Types and sites of IFI and data on therapy are summarized in table 1.

Among 39 patients without voriconazole TDM, 31 (79%) were treated for aspergillosis (6 proven cases, 16 probable cases, and 9 possible cases), 2 (5%) were treated for proven candidiasis, and 6 (16%) were treated for persistent febrile neutropenia. Patients received voriconazole intravenously in 9 (23%) of the cases, intravenously followed by orally in 12 (31%), and orally in 18 (46%). The voriconazole loading dosage was 12 mg/kg per day, and the median maintenance dosage was 6.5 mg/kg per day (range, 5–8 mg/kg per day). The median duration of therapy was 30 days (range, 7–365 days).

Measurements of voriconazole trough blood levels. A total of 181 voriconazole trough levels were measured (median number per patient, 4.5; range, 1–9). The median time interval between dose administration and measurement of trough levels was 12 h (range, 11–13 h). The first trough levels were measured at a median of 5 days (range, 3–46 days) after starting therapy. The median time elapsed between consecutive blood level measurements was 7 days (range, 1–62 days). Figure 1 shows the trough levels in patients receiving different voriconazole daily doses. A large intradose variability in voriconazole levels was observed. No significant difference was found among trough levels at different daily doses, nor was any correlation between daily doses and trough levels observed ($r^2 = 0.07$). During therapy with identical daily doses in 13 patients, the intraindividual variability of trough levels was substantial. In 5 patients, levels increased (median increase, 61%; range, 22%–125%; 1 patient had an increase >100%); in 8 patients, levels decreased (median decrease, 52%; range, 42%–84%; 4 patients had a decrease >50%).

Response of infection to voriconazole therapy. At the time of clinical assessment, voriconazole trough levels were ≤1 mg/L in 13 cases (25%) and >1 mg/L in 39 cases (75%). A significantly higher proportion of patients with levels ≤1 mg/L received oral voriconazole. Lack of response to therapy was more frequently observed in patients with levels ≤1 mg/L (46%) than in those with levels >1 mg/L (12%; $P = .02$) (table 2). Among patients with levels ≤1 mg/L, the characteristics of those with lack of response (6 patients) and those responding to therapy (7 patients) were compared. Immunosuppression was present in 5 (83%) of 6 and 5 (71%) of 8 patients, respectively. Aspergillosis was diagnosed in 5 patients (83%; 3 patients received a diagnosis of extrapulmonary aspergillosis) and 4 patients (57%; no patients received a diagnosis of extrapulmonary aspergillosis), respectively. The clinical course of 6 patients with persistence or progression of IFI and voriconazole levels ≤1 mg/L is shown in table 3. In all 6 patients, IFI responded after an increase in voriconazole doses (no antifungal agent was added to the treatment regimen). Neutropenia resolved in 2 patients (after clinical response of IFI in 1 patient); immunosuppression persisted in 3 patients (in 1 patient receiving azathioprine and/or corticosteroids, 1 patient with AIDS, and 1 patient with liver cirrhosis), and 1 patient was not immunocompromised.

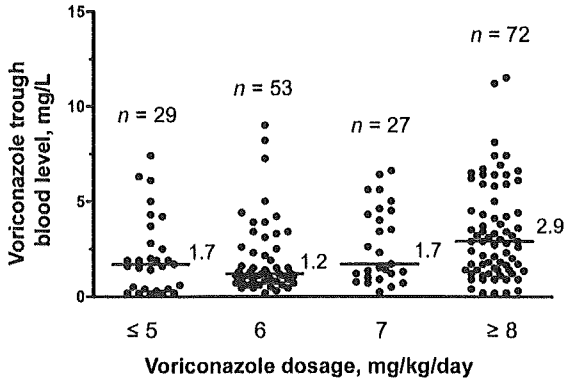

Figure 1. Relationship between voriconazole dosage and voriconazole trough blood level. Each point represents a single blood level measurement. Numbers of measurements for each daily dose are reported. Horizontal bars represent median values (the numerical values are reported on the right of the horizontal bar for each group). Voriconazole dosages have been rounded to the nearest unit.

Among the 5 patients with voriconazole levels >1 mg/L (median voriconazole level, 4.1 mg/L; range, 2.4–9 mg/L) and lack of response to therapy, 1 had probable invasive candidiasis, 1 had probable invasive aspergillosis, 2 had possible IFI, and 1 had breakthrough PCR-proven zygomycosis. In 4 cases, treatment was switched to a salvage regimen (1 patient received caspofungin, 2 received liposomal amphotericin B, and 1 received a combination of both agents) after a median of 9 days of voriconazole therapy (range, 4–21 days). Three patients had complete resolution of IFI (median duration of follow-up, 60 days; range, 45–60 days), and 2 patients died due to IFI (after 5 and 7 days, respectively).

The logistic regression analysis indicated that the log-transformed voriconazole trough level is a significant predictor of response to therapy: a 2-fold increase in blood level is associated with an OR for success of 1.8 (95% CI, 1.1–3.1; $P = .03$). The logistic regression model indicates a 70% probability of response at a voriconazole trough concentration of 1 mg/L (figure 2*A*).

Among the 39 patients without voriconazole TDM, 9 patients (1 with proven, 5 with probable, and 3 with possible IFI) did not respond to therapy (no MIC data were available). For 3 patients, voriconazole doses were increased (2 patients responded, and there was no follow-up in 1 patient); in 2 patients, therapy was switched to a salvage regimen (1 patient responded,

Table 2. Voriconazole trough blood levels and clinical response to antifungal therapy.

Variable	Voriconazole trough blood level		P
	≤1 mg/L (*n* = 13)	>1 mg/L (*n* = 39)	
Route of voriconazole administration			.05
Intravenous	4 (31)	24 (61)	
Oral	9 (69)	15 (39)	
Voriconazole dosage, median mg/kg/day (range)			
Overall	7 (2.5–9)	8 (2–11)	NS
Intravenous	7.5 (7–8)	8 (6–11)	NS
Oral	6 (2.5–9)	7 (2–11)	NS
Response to antifungal therapy			
Interval between start of voriconazle therapy and assessment, median days (range)	21 (10–120)	17.5 (10–180)	NS
Treatment success			
Overall	7 (54)[a]	34 (88)	.02
Complete response	5	27	
Partial response	2	7	
Lack of response	6 (46)	5 (12)	
Persistence	3 (23)	0 (0)	
Progression	3 (23)	4 (10)	
Breakthrough IFI	0 (0)	1 (2)	

NOTE. Data are no. (%) of patients, unless otherwise indicated. NS, not significant.

[a] In 1 patient, comedication with rifampin resulted in low voriconazole blood levels.

and 1 patient died of refractory IFI); and in 4 patients, therapy remained unchanged (all 4 patients died of refractory IFI).

Safety of voriconazole therapy. Voriconazole trough levels were ≤5.5 mg/L in 36 patients (69%) and >5.5 mg/L in 16 patients (31%) (table 4). The majority of patients with voriconazole levels >5.5 mg/L received voriconazole intravenously, and a significant proportion of these patients received omeprazole comedication. Five serious neurological adverse events (National Cancer Institute grade 3) were observed in patients with voriconazole levels >5.5 mg/L (31%), compared with none among patients with levels ≤5.5 mg/L (0%; $P = .002$). The clinical characteristics and the course of adverse events in these patients are summarized in table 5. When patients with trough levels >5.5 mg/L with and without toxicity were compared, voriconazole daily doses, route of administration, and blood levels were similar (data not shown). Absence of other causes and prompt and complete neurological recovery after therapy discontinuation suggest the probable relationship between these encephalopathies and voriconazole overdosing. The logistic regression analysis confirmed a significant association between voriconazole trough concentrations and neurotoxicity: the OR for severe toxicity after a 2-fold increase of voriconazole levels in blood was 284 (95% CI, 0.96–84,407; $P = .05$). The estimated probability of neurotoxicity at voriconazole trough levels of 5.5 mg/L and 8 mg/L was 15% and 90%, respectively (figure 2*B*).

A severe cholestasic hepatopathy (defined as 10 times the baseline or 3 times the baseline, if the baseline was >3 times the upper limit of normal) occurred in 6 patients (2 with pre-existing hepatopathy). These events were observed in 8% of patients with voriconazole levels ≤5.5 mg/L and in 19% of those with levels >5.5 mg/L (the difference was not statistically significant). Although this toxicity was possibly or probably related to voriconazole, no correlation was found between voriconazole trough levels and alkaline phosphatase ($r^2 = 0.01$) or γ-glutamyl transpeptidase ($r^2 = 0.01$). Voriconazole dose was reduced by 50% in 4 cases: cholestatic parameters remained >10 times the upper limit of normal in 2 patients and decreased to 5 times the upper limit of normal in 2 patients. After discontinuation of voriconazole, cholestasis decreased to 3–5 times the upper limit of normal in all patients within a median of 20 days (range, 10–30 days). In 2 patients who had long-term follow-up (10 and 12 months), the liver parameters remained elevated despite the absence of a pre-existing hepatopathy. The logistic regression analysis failed to demonstrate a significant association between voriconazole levels and hepatotoxicity; the OR for hepatotoxicity of a 2-fold increase in voriconazole levels was 1.4 (95% CI, 0.7–3).

Among 39 patients without voriconazole TDM, 7 (18%) experienced serious adverse events possibly related to therapy (2 of which were neurological and 5 of which were hepatic).

Table 3. Characteristics and clinical course of patients with voriconazole (Vor) trough blood levels ≤1 mg/L and lack of response to antifungal therapy.

Patient	Sex	Age, years	Weight, kg	Underlying disease/ immunosuppression	Invasive fungal infection	Vor daily dose, route	Duration of therapy at time of assessment of response, days	Vor trough blood level, mg/L	Assessment of response to Vor therapy	Modification Vor daily dose, route	Time to first control Vor blood level, days	Vor trough blood levels in follow-up, mg/L	Final assessment of response, days of follow-up
1	M	58	77	Acute leukemia/ neutropenia	Probable pulmonary aspergillosis	5 mg/kg/day (2 × 200 mg), oral	24	<0.2	POI: persistent fever and inflammatory syndrome, RP of initial focus, and new contralateral lesion (on CT)	Increase to 8 mg/kg/ day (2 × 300 mg), oral	10	1.1, 2.1, 1.7	Complete response, 75
2	F	65	56	Crohn disease, surgery for toxic megacolon/corticosteroids and azathioprine	Proven peritoneal aspergillosis:*Aspergillus fumigatus;* MIC of Vor, 0.25 mg/L	7 mg/kg/day (2 × 200 mg), oral salvage therapy after failure of fluconazole therapy	20	0.7	PI after surgery: persistent fever and inflammatory syndrome, persistence of *Aspergillus* species in peritoneal fluid	Increase to 11 mg/kg/ day (2 × 300 mg), oral	5	2, 2.4	Complete response, 60
3	M	68	45.5	HIV infection with AIDS	Proven pulmonary aspergillosis: *A. fumigatus;* MIC of Vor, 0.5 mg/L	9 mg/kg/day (2 × 200 mg), oral (concomitant lopinavir and ritonavir therapy)	14	0.9	POI: persistent fever, cough and inflammatory syndrome, RP of initial focus (on CT)	Increase to 13 mg/kg/ day (2 × 300 mg), oral	7	2.9, 2.9, 3.5	Complete response, 90
4	M	64	62	Hodgkin lymphoma/ neutropenia	Probable hepatosplenic candidiasis	6.5 mg/kg/day (2 × 200 mg), intravenous	34	0.7	PI: persistent fever and inflammatory syndrome, no radiological change (on CT)	Increase to 10 mg/kg/ day (2 × 300 mg), intravenous	7	2.3, 2.0, 2.1	Complete response, 65
5	M	70	81	Alcoholic liver cirrhosis	Proven sinus aspergillosis with cerebral extension: *Aspergillus flavus;* MIC of Vor, 0.25 mg/L	2.5 mg/kg/day (2 × 100 mg), oral	13	1	PI after surgery: persistent fever and symptoms (nasal congestion, facial pain), no radiological change (on MRI)	Increase to 3.5 mg/ kg/day (2 × 150 mg), oral	5	2.8, 1.6, 2.3	Complete response, 50
6	F	61	63	None	Proven sinus aspergillosis: *A. fumigatus;* MIC of Vor, 0.25 mg/L	6.5 mg/kg/day (2 × 200 mg), intravenous	22	<0.2	POI after surgery: RP, ethmoidal focus with extension to skull basis (on MRI)	Increase to 9 mg/kg/ day (2 × 300 mg), intravenous	7	2.7, 3.1, 2.8, 3	Complete response, 180

NOTE. PI, persistent infection; POI, progression of infection; RP, radiological progression.

Table 4. Voriconazole (Vor) trough blood levels and safety of antifungal therapy.

Variable	Vor trough blood level		P
	≤5.5 mg/L (*n* = 36)	>5.5 mg/L (*n* = 16)	
Vor route			.07
Intravenous	15 (42)	13 (81)	
Oral	21 (58)	3 (19)	
Vor dosage, median mg/kg/day (range)			
Overall	7 (2–11)	8 (6–11)	.13
Intravenous	7.5 (6–10)	8 (6–11)	NS
Oral	6 (2–11)	7 (6–8)	NS
Serious adverse event			
Encephalopathy			
Incidence	0	5 (31)	.002
Interval after start of Vor, days (range)	NA	9 (5–30)[a]	
Cholestatic hepatopathy			
Incidence	3 (8)	3 (19)	NS
Interval after start of Vor, days (range)	50 (5–150)	13 (6–20)	NS
Concomitant therapy			
Omeprazole	6 (17)	7 (44)	.04
Tacrolimus	0	1 (6)	NS

NOTE. Data are no. (%) of patients, unless otherwise indicated.

[a] The time interval elapsed between start of Vor therapy and documentation of Vor blood levels >5.5 mg/L in patients without encephalopathy was a median of 5 days (range, 2–7 days); $P = .04$, vs. time interval in patients with encephalopathy.

The 2 patients with neurological symptoms completely recovered after discontinuation of voriconazole.

DISCUSSION

We report the experience with voriconazole TDM during 2388 treatment-days in 52 white adult patients. A large variability in voriconazole trough levels was observed: voriconazole trough levels were ≤1 mg/L in 13 (25%) of the patients (the majority of whom were receiving oral therapy) and >5.5 mg/L in 16 (31%) (the majority of whom were receiving intravenous therapy). This observation is consistent with previous reports [16, 27, 31]. Intravenous daily doses were higher than oral daily doses. Confounding factors—such as drug-drug interactions, liver disease, and polymorphism of cytochrome CYP2C19—may have influenced voriconazole metabolism in some individuals. Among white individuals, 26% of heterozygous extensive metabolizers (who experienced 2-fold voriconazole exposure, compared with homozygous extensive metabolizers) and 2% of homozygous poor metabolizers (who experienced 4-fold exposure) have been described [10, 27]. CYP2C19 genotype was not determined in the present study.

Persistence or progression of IFI was observed in a significantly higher proportion of patients with voriconazole trough concentrations ≤1 mg/L, compared with patients with trough concentrations >1 mg/L. The association between trough levels and response to antifungal therapy was confirmed in a logistic regression model. No fungal in vitro resistance was documented as a cause of treatment failure. No difference in immunosuppressive conditions was found. In all cases involving a lack of response at low voriconazole levels, an increase in voriconazole doses resulting in levels >1 mg/L was successful. The choice of the 1-mg/L cutoff value for voriconazole trough concentrations had been based on (1) in vitro susceptibility data (MIC_{90}, 0.5–1 mg/L for the majority of pathogenic fungi), (2) the fact that only the free-circulating fraction of voriconazole (40%–50%) is microbiologically active and penetrates infected tissues, (3) the fact that blood sampling just preceding the next voriconazole dose is practical and reliable for intrapatient and interpatient comparisons, and (4) the lack of clinical data on other pharmacodynamic parameters that might better predict efficacy and safety [3, 8, 28, 29]. Patients with voriconazole levels ≤1 mg/L received oral treatment more frequently than did those with levels >1 mg/L. Variability in oral bioavailability associated with meals and hepatic first-pass effect might have contributed to lower exposure [1, 13, 27]. Degradation of oral voriconazole by intestinal cytochrome enzymes may contribute to impaired drug absorption: the reversing effect on this phenomenon of grapefruit juice has been associated with improved bioavailability in mice [32]. No drug-drug interaction could be identified in patients with voriconazole underexposure. In 2 patients, the recommended oral dosage not adjusted for body weight (200 mg twice per day) was lower than the intravenous dosage (4

Table 5. Characteristics of and clinical course in patients with voriconazole (Vor) trough blood levels >5.5 mg/L and serious neurological adverse events.

Patient	Sex	Age, years	Weight, kg	Underlying disease/invasive fungal infection	Vor daily dose, route	Concomitant therapy	Duration of therapy at time of occurrence of adverse event, days	Vor trough blood level, mg/L	Assessment of serious adverse events; causality of Vor; National Cancer Institute grade	Modification to Vor therapy	Time to follow-up Vor blood level after stopping Vor, days	Vor trough blood level during follow-up, mg/L	Final clinical assessment (days of follow-up)
1	F	35	54	Lung transplantation/ probable pulmonary aspergillosis	7.5 mg/kg/day (2 × 200 mg), oral	Tacrolimus	16	6.9	Confusion, agitation, myoclonies, EEG pattern of toxic encephalopathy (normal CT findings); probable; grade 3 (concomitant severe cholestasis)	Discontinuation	NA	NA	Complete resolution (5 days after discontinuation of Vor), prolongation of hospital stay
2	M	61	100	Acute leukemia/probable pulmonary aspergillosis	8 mg/kg/day (2 × 400 mg), intravenous	Omeprazole	30	11.2	Confusion, extrapyramidal signs, myoclonies, EEG pattern of toxic encephalopathy (normal CT findings); probable; grade 3	Decrease dosage to 6 mg/kg/day (2 × 300 mg), then 3 (2 × 150 mg), then discontinuation	5	4.3	Complete resolution (5 days after discontinuation of Vor), prolongation of hospital stay
3	M	59	80	Acute leukemia/ proven pulmonary aspergillosis	8 mg/kg/day (2 × 320 mg), intravenous	Omeprazole	9	5.9	Confusion, worsening hallucinations; probable; grade 3	Discontinuation	5	1.6	Complete resolution (3 days after discontinuation of Vor); rechallenge with a lower dose of Vor was well tolerated
4	M	66	57	Acute leukemia/ proven pulmonary aspergillosis	7 mg/kg/day (2 × 200 mg), intravenous	Omeprazole	6	5.6	Persistent visual and auditive hallucinations; probable; grade 3 (concomitant hepatic serious adverse event)	Decrease dosage to 5 mg/kg/day (2 × 150 mg), then discontinuation	3	1	Complete resolution (4 days after discontinuation of Vor)
5	M	31	74	Acute leukemia/possible pulmonary invasive fungal infection	8 mg/kg/day (2 × 300 mg), intravenous	None	3	9	Confusion, persistent visual hallucinations; probable; grade 3	Discontinuation	NA	NA	Complete resolution (2 days after discontinuation of Vor)

NOTE. NA, not available.

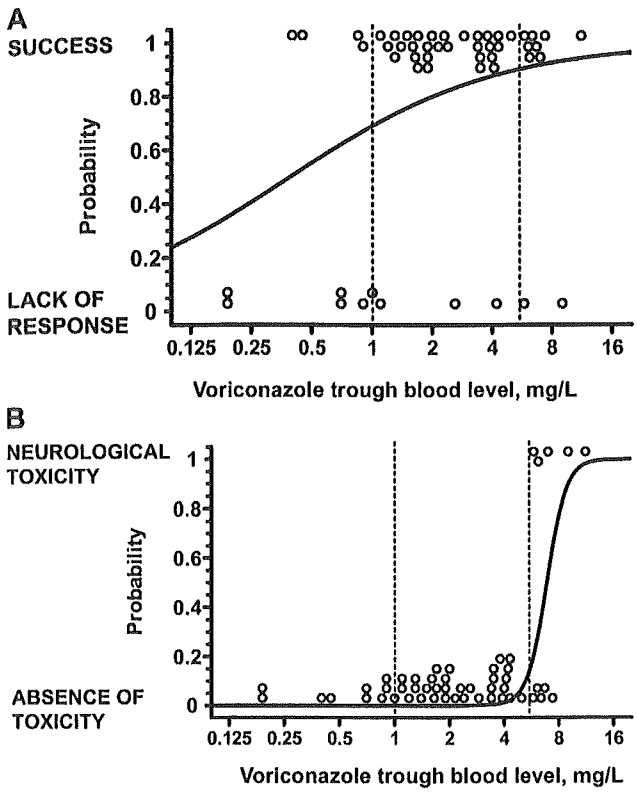

Figure 2. Voriconazole trough blood levels and logistic regression model for response to antifungal therapy *(A)* and for neurotoxicity *(B)*. The symbols represent the voriconazole levels observed in each individual patient with treatment success *(top)* or lack of response to therapy *(bottom)* and with *(top)* or without *(bottom)* signs of neurological toxicity. The continuous line represents the logistic regression model predicting the probability of response to antifungal therapy *(A)* or neurotoxicity *(B)* as a function of the voriconazole trough blood concentration. The vertical dotted lines indicate the proposed 1–5.5-mg/L therapeutic interval of voriconazole.

mg/kg twice per day). Our data confirm and extend previous observations that low voriconazole levels may impair the response of IFI to therapy. The US Food and Drug Administration's voriconazole briefing document reports a pharmacokinetic/pharmacodynamic analysis involving 280 cases of proven or probable IFI that suggests a trend to higher success rates in patients with mean voriconazole levels >0.5 mg/L (136 [56%] of 245 patients), compared with patients with levels <0.5 mg/L (16 [46%] of 35 patients; OR, 1.5; 95% CI, 0.6–3.4) [27]. In an open-label, multicenter trial of voriconazole for first-line or salvage therapy in 116 patients with aspergillosis, 6 treatment failures among 11 patients with mean voriconazole levels <0.5 mg/L were observed [1]. A relationship between voriconazole levels randomly measured during the dosing interval and treatment success was described in 28 patients with IFI: there was a 100% treatment response rate in patients with voriconazole levels >2 mg/L and a 56% response rate in patients with voriconazole levels <2 mg/L [15]. Among a group of 8 critically ill patients who received voriconazole via a nasogastric tube, 2 treatment failures that were possibly associated with low blood levels of voriconazole were observed [33]. Six breakthrough IFIs occurred during voriconazole prophylaxis in a group of 43 allogeneic hematopoietic stem cell transplant recipients with voriconazole trough concentrations <2 mg/L, and none occurred in a group of 24 such patients with voriconazole trough concentrations >2 mg/L [34]. These data are consistent with the exposure-response relationship recently described for posaconazole, another new-generation triazole [35].

Safety issues were also found to be associated with the variability of voriconazole blood levels. We observed a toxic encephalopathy in one-third of patients with trough levels >5.5 mg/L after >1 week of therapy. The association between overdosing and neurotoxicity was confirmed in a logistic regression model. These persistent or worsening neurological manifestations, which differed from transient and spontaneously resolving disturbances of light perception reported in 20%–25% of patients, promptly resolved after discontinuation of therapy. Moreover, in patients with high levels of voriconazole and no neurological symptoms or signs, prompt dose reduction or discontinuation of voriconazole might have prevented toxicity by limiting the duration of overexposure. The majority of patients with blood levels >5.5 mg/L were treated intravenously, with a trend towards higher daily doses. Drug-drug interactions, in particular with omeprazole, may have played a role in voriconazole overdosing [27, 36, 37]. The severe cholestasis that developed during therapy might also have contributed to voriconazole accumulation. Two studies have described visual hallucinations in 4% of cases and encephalopathy in 1% of cases during voriconazole therapy. However, no data on voriconazole levels were reported [2, 22]. Another trial reported voriconazole trough levels >10 mg/L in 6 (5%) of 116 patients, all of whom required discontinuation of therapy: in 3 (50%) of 6 patients, toxicity possibly related to overdosing was described [1, 13, 17]. Other investigators reported a significant association between visual adverse events and voriconazole levels; the rate of such events was 18% among patients with trough concentrations <1 mg/L and 31% among those with trough concentrations >9 mg/L [38]. Six neurological adverse events were described among a group of 28 patients; the hazard ratio was 2.3 (95% CI, 1.4–3.6) per 0.1 mg/L increase in voriconazole trough level [14]. A patient with liver cirrhosis treated with voriconazole (4 mg/kg per day) and pantoprazole developed coma while the voriconazole trough level was 13.9 mg/L and then rapidly recovered when the voriconazole level decreased to <10 mg/L after therapy discontinuation [30].

Abnormal liver function test results have been reported in 1%–10% of patients receiving voriconazole [22, 27, 39]. In our study, severe hepatic toxicity occurred independently of vori-

conazole levels. This finding confirms other observations, which reported only a weak correlation, if any, between voriconazole exposure and severity of hepatotoxicity [14, 16, 18, 27, 31, 38].

The lack of a prospective study design with systematic voriconazole TDM at fixed time points is a limitation of the present study. However, similar clinical observations in patients without TDM suggest that the reported experiences are representative of individuals receiving voriconazole. No IFI with a voriconazole MIC of 1–4 mg/L was documented. In patients with such infections, individualized dose adjustments targeting voriconazole trough levels exceeding these values may be required [3].

In conclusion, the present study shows the importance of voriconazole TDM in severely ill patients with invasive mycoses [7, 17]. Multiple intrinsic and extrinsic factors may unpredictably influence voriconazole pharmacokinetics. Because it is difficult to clinically identify individuals with inappropriate exposure, dose adjustment after documentation of low or high voriconazole levels is critical if infection is not responding to treatment or if toxicity is suspected. Moreover, detection of voriconazole trough levels outside the therapeutic interval of 1–5.5 mg/L during the first week of therapy may prevent treatment failures and neurological toxicity. Prospective trials including voriconazole TDM are needed.

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