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

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La variabilité des taux plasmatiques de voriconazole mesurés par de nouvelles méthodes de chromatographie liquide à haute performance et de bioassay

Le voriconazole (VRC) est un antifongique à large spectre de la classe des triazoles avec une pharmacocinétique non linéaire. L'utilité de la mesure de ses taux sanguins pour optimaliser le traitement est débattue. Les méthodes de dosage actuellement disponibles par chromatographie liquide à haute performance (HPLC) et par bioassay sont techniquement complexes. Elles prennent du temps ou ont un intervalle analytique étroit. Les objectifs de cette étude étaient de développer de nouvelles méthodes d'analyse simple et d'évaluer la variabilité des taux sanguins de voriconazole chez des patients avec une infection mycotique invasive. Pour le dosage par HPLC, nous avons utilisé une précipitation par acétonitrile, une séparation par "reverse-phase" et une détection par rayons UV . Une souche mutée de *Candida albicans* hypersensible pour le voriconazole a été utilisée pour le bioassay. Cette souche a été construite par délétion des gènes pour les transporteurs multidrogues *(cdr1Δ/cdr1Δ, cdr2Δ/cdr2Δ, flu1Δ/flu1Δ et mdr1Δ/mdr1Δ)* et la sous-unité A de la calcineurin *(cnaΔ/cnaΔ)*.
L'exactitude des principales valeurs de l'intra- /interrun sur des concentrations de voriconazole allant de 0.25 à 16 mg/l était de 93.7 % ± 5.0% / 96.5 % ± 2.4 % pour HPLC, et de 94.9 % ± 6.1% / 94.7% ± 3.3 % pour le bioassay.
Les coefficients de variation des principales valeurs intra- / interrun étaient de 5.2 % ± 1.5 % / 5.4 % ± 0.9 % pour HPLC, et de 6.5 % ± 2.5 % / 4.0 % ± 1.6 % pour bioassay .
Le coefficient de concordance entre l'HPLC et le bioassay était de 0.96.
Des mesures séquentielles sur 10 patients avec une infection mycotique invasive ont été réalisées. Elles ont montré, pour une courbe temps / concentration, que l'aire sous la courbe (AUC) était sujette à d'importantes variations inter- et intraindividuelles. Par exemple, pour le premier jour de traitement la valeur moyenne était de 43.9 mg·h/l (sur un intervalle de 12.9 à 71.1) et pour le dernier jour, de 27.4 mg·h/l (sur un intervalle de 2.9 à 93.1). Durant le traitement, l'AUC a diminué pour 5 patients, augmenté pour 3 et elle est restée stable pour 2. Une encéphalopathie toxique, probablement en relation avec l'augmentation de l'aire sous la courbe du voriconazole (de 71.1 à 93.1 mg·h/l), a été observée. L'AUC du VRC a diminué de 12.9 à 2.9 mg·h/l chez un patient présentant des signes persistants d'aspergillose. Ces observations préliminaires suggèrent qu'un sur ou sous dosage de voriconazole entraînant une variation des taux sanguins pourrait avoir un impact clinique. Ces méthodes simples d'HPLC et de bioassay offrent de nouvelles perspectives pour le suivi des patients traités par voriconazole.

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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

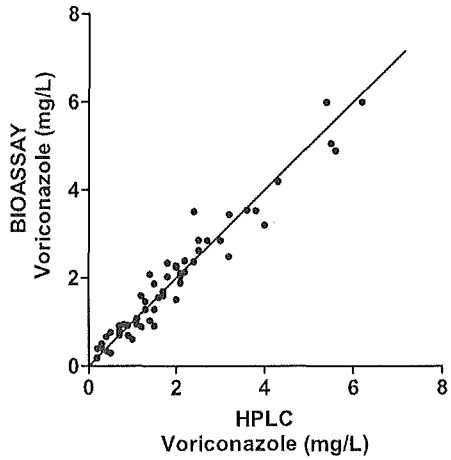


FIG. 1. Scatter plot of plasma VRC levels measured by HPLC and bioassay using the *C. albicans* mutant (*cdr1*Δ/*cdr1*Δ, *cdr2*Δ/*cdr2*Δ, *flu1*Δ/*flu1*Δ, *mdr1*Δ/*mdr1*Δ, and *cna*Δ/*cna*Δ). The line of congruence does not deviate from 1. The coefficient of concordance is 0.96.

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.

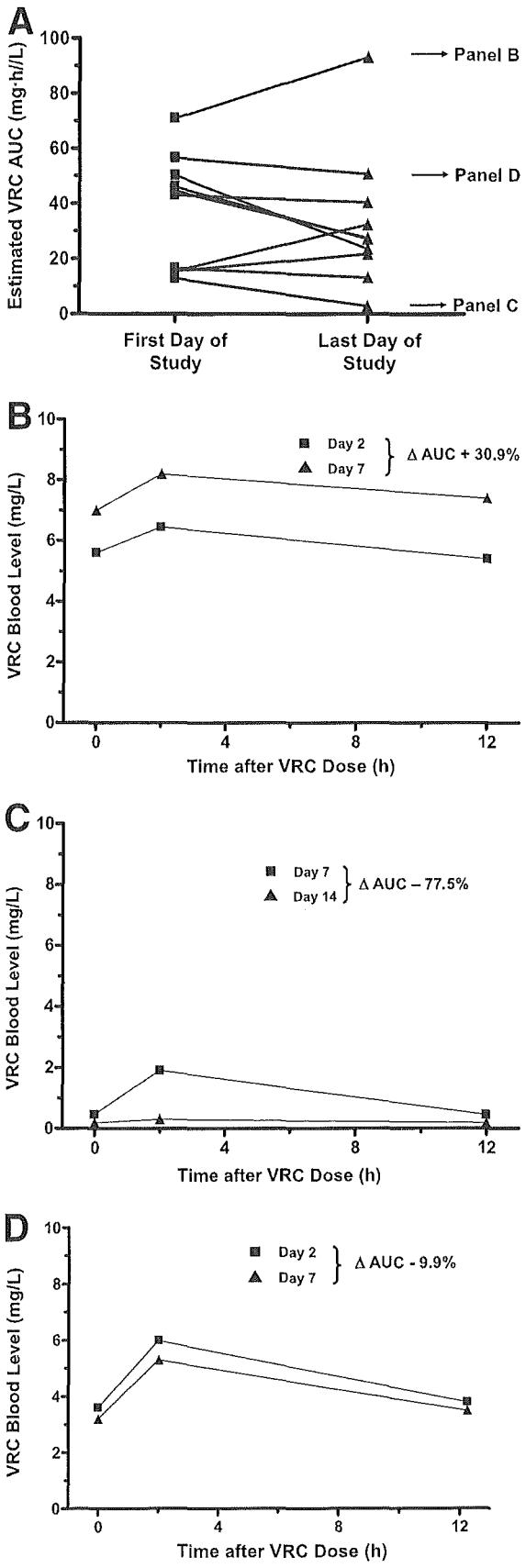
Figure 2 shows the course of the estimated VRC AUC values during the study period in the 10 patients (Fig. 2A) and three individual VRC concentration-time curves (Fig. 2B to D). A leukemic patient with proven pulmonary aspergillosis and increasing VRC exposure (at day 2, C_{max} was 6.4 mg/liter, trough 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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