Validation of an Assay for Voriconazole in Serum Samples Using Liquid Chromatography–Tandem Mass Spectrometry

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Abstract: A simple and rapid liquid chromatography tandem mass spectrometry (HPLC-MS/MS) method for the analysis of voriconazole has been developed. For comparison, serum voriconazole was measured using HPLC and bioassay. For the HPLC-MS/MS assay, samples were prepared in a deep-well block by adding 10 μL of serum to 40 μL of 0.1 M zinc sulfate solution. Proteins were precipitated by adding 100 μL acetonitrile containing ketoconazole as internal standard. After vigorous mixing and centrifugation, 3 μL of the supernatant was injected into the HPLC-MS/MS system. An HPLC system was used to elute a C_{18} cartridge (2 mm × 4 mm) at 0.6 mL/min with a step gradient of 50% to 100% methanol containing 2 mM ammonium acetate and 0.1% (vol/vol) formic acid. The column was maintained at 55°C, and the retention times were voriconazole 1.50 minutes and ketoconazole 1.47 minutes. Cycle time was 3 minutes, injection to injection. The analytes were monitored using a tandem mass spectrometer operated in multiple reaction monitoring mode using the following transitions: voriconazole *m/z* 350.0 > 224.1 and ketoconazole *m/z* 531.1>489.1. Within- and between-batch CVs were <5% and <8%, respectively, over the range 0.38 to 15.3 mg/L. The lower limit of quantification was 0.1 mg/L. Regression analysis showed HPLC-MS/MS = 1.06 ± 0.02 (HPLC-UV) − 0.07 ± 0.1, R^2 = 0.95, n = 99.

Key Words: voriconazole, tandem mass spectrometry, aspergillosis

(*Ther Drug Monit* 2004;26:650–657)

Voriconazole is a newly licensed azole antifungal drug with good clinical efficacy for invasive aspergillosis.[1,2] Voriconazole inhibits the cytochrome P450-dependent enzyme 14α-sterol demethylase, thereby disrupting the fungal membrane and stopping fungal growth. It is metabolized primarily by the cytochrome enzymes 2C19, 3A4, and 2C9, in order of priority.[3] It has a variable half-life in man, 6–24 hours, depending on many factors, the most important being age, concurrent therapy, hepatic dysfunction, and genetic polymorphism of CYP2C19.[4] Trough concentrations in adult patients varied from <0.25 mg/L to 9.7 mg/L.[1] Young children appear to metabolize the drug very rapidly, and much larger doses than in adults have been used to achieve adequate concentrations (D. W. Denning, unpublished data). Patients with severe liver dysfunction may metabolize the drug slowly. Concentrations exceeding 6.0 mg/L were associated with more frequent liver function test abnormalities in volunteers (P. Troke, personal communication), and some concerns have been expressed about toxicity occurring at much higher concentrations than this in patients.[5] Some drugs increase voriconazole exposure, and others reduce it, notably rifampicin. Because of this, there is an argument for therapeutic drug monitoring of voriconazole, as others have pointed out.[6]

Voriconazole has been measured using bioassay or HPLC techniques.[7] The bioassay is technically easier than the HPLC method but suffers from poor accuracy and precision and may be unsuitable for patients receiving combination therapy. The HPLC method is capable of measuring in the therapeutic range, but a relatively large sample volume is required to ensure sensitivity at lower concentrations. More recently liquid chromatography coupled with electrospray ionization mass spectrometry has been used to measure voriconazole concentration in the aqueous humor of rabbits in an attempt to improve the sensitivity of the method.[8] Sample cleanup was not required in this method because aqueous humor is a relatively clean matrix and could be injected directly. The chromatography time was the same as the HPLC-UV method because single quadrupole detection was used and interfering substances had to be resolved from the voriconazole peak. We therefore decided to use liquid chromatography tandem mass spectrometry (HPLC-MS/MS) in an attempt not only to increase the sensitivity of the method over the HPLC-UV method but also to decrease the chromatography time and hence increase the throughput and turnaround of the assay.

HPLC-MS/MS is a technique potentially capable of this because of the high specificity of the detector, which allows the measurement of compounds in complex biologic matrices

Received for publication September 8, 2003; accepted February 2, 2004.

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with minimal sample preparation. It is also possible to use smaller sample volumes and still maintain adequate sensitivity. We describe an HPLC-MS/MS assay for voriconazole in serum samples capable of a faster turnaround than previously reported assays. The aim of this study was to develop and validate an HPLC-MS/MS method that uses precipitation of proteins with zinc sulfate and acetonitrile before on-line chromatographic cleanup and then analysis with tandem mass spectrometric detection.

MATERIALS AND METHODS

Voriconazole (UK-109,496) was kindly supplied by Pfizer Central Research (Sandwich, UK), and ketoconazole (R41400) was kindly supplied by Janssen Pharmaceutical (Beerse, Belgium). A stock voriconazole solution was prepared by dissolving pure compound in methanol. Working serum standards were prepared by diluting the stock standard into a serum pool to give a range of calibrators 0, 0.1, 0.5, 1.0, 5.0, 10.0, and 20 mg/L. Internal standard was prepared by dissolving ketoconazole in methanol (1 mg/L).

HPLC-UV Procedure

We used a modification of the method of Perea et al.[7] Briefly, serum samples and standards (100 μL) were deproteinized with acetonitrile (200 μL) before injection (50 μL) onto a Sphereclone ODS2 3-μm column, 150 mm × 4.6 mm (Phenomenex, Macclesfield, UK). The mobile phase consisted of acetontrile/ammonium dihydrogen phosphate buffer (0.04 mol/L), pH 6.0, 40/60 (vol/vol). Analysis was performed isocratically at a flow rate of 0.8 mL/min with the UV detector operating at 255 nm. Under these conditions voriconazole eluted at 7 minutes with a total run time of 10 minutes. The determination was based on the external standard method.

Voriconazole Bioassay Method

Candida kefyr San Antonio strain (kindly provided by Dr D. A. Stevens) was suspended in 5 mL sterile distilled water and cell density adjusted to approximately 2×10^7 cfu/ml. Test agar was prepared by melting 100 mL of RPMI-1640 agar medium (Sigma, Dorset, UK) which was allowed to cool to 60°C before inoculating with the prepared yeast suspension and then poured into a levelled plastic bioassay dish (Nunc, Denmark). Once solidified, 36 (8 mm) wells were cut out in a 6 × 6 pattern with a sterile cork borer. The plate was dried at 37°C.

A stock solution of voriconazole (1000 mg/L) was diluted in pooled serum to provide a range of standard concentrations from 3.12 to 0.098 mg/L.

Standard, internal control, and patient specimens (neat and 1:2 and 1:5 dilutions) (40 μL) were pipetted in triplicate into randomly selected wells. The loaded samples were allowed to prediffuse for 30 minutes. The plate was incubated for approximately 18 hours at 37°C.

Zones of inhibition were measured using digital dial calipers (Mitutoyo 500-652, Hampshire, UK), and the mean zone diameters were entered into a computer program (Microsoft Excel) and plotted by regression curve. Drug concentrations in patient samples were calculated from the standard curve obtained.

HPLC-MS/MS

Serum samples or calibrators (10 μL) were added to zinc sulfate (100 mmol/L) solution (40 μL) in deep-well microtiter plates, and then acetonitrile containing ketoconazole as internal standard (1 mg/L) (100 μL) was added. The plate was sealed with thermosealing film and mixed vigorously for 30 seconds using a multivortex mixer to disperse the precipitated material. After centrifugation at 800 × *g* for 5 minutes, the sealed plate was transferred to the autosampler for analysis.

Chromatography was performed using a Waters 2795 Alliance HT HPLC system (Waters Ltd, Watford, UK). Supernatant (3 μL) was directly injected from the 96-well microtiter plate and was analyzed by on-line solid-phase extraction using a SecurityGuard C_{18} cartridge column (4.0 mm × 2.0 mm; Phenomonex, Macclesfield, UK). The extract was injected onto the guard column, and after a brief wash for 0.5 minutes with 20% aqueous methanol containing 2 mM ammonium acetate and 0.1% formic acid at a flow rate of 0.6 mL/min, the analytes were eluted from the column by stepping up the methanol concentration to 100% for 0.5 minutes. The column was maintained at 55°C, and the eluent was connected directly to electrospray probe of the mass spectrometer with no splitting or solvent diversion. The cycle time was approximately 3.0 minutes injection to injection.

A Quattro micro-HPLC tandem mass spectrometer fitted with a Z Spray ion source was used for all analyses (Micromass, Manchester, UK). The instrument was operated in electrospray positive ionization mode and was directly coupled to the HPLC system. System control and data acquisition were performed with MassLynx NT 4.0 software with automated data processing using the MassLynx Quantify and Neolynx programs provided with the mass spectrometer. Calibration curves were constructed using linear least-squares regression with 1/x weighting for the multiple reaction monitoring (MRM) quantification. The determination was based on the internal standard method. 1/x weighting was used because it gives more weighting to the lower calibrators, which should improve precision and accuracy at lower concentrations.

To tune the mass spectrometer, a solution of voriconazole or ketoconazole (1 mg/L in mobile phase) was infused into the ion source, and the cone voltage was optimized to maximize the intensity of the precursor ions for voriconazole and ketoconazole, *m/z* 350.0 and *m/z* 531.1, respectively. The collision energy was then adjusted to optimize the signal for the most abundant voriconazole and ketoconazole product ions, *m/z* 224.1 and *m/z* 489.1, respectively. Typical tuning

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conditions were as follows: electrospray capillary voltage 1.0 kV, sample cone voltage 25 V, and collision energy 20 eV at a collision gas pressure 1.8×10^{-3} mBar Argon. Sample analysis was performed in the MRM mode of the mass spectrometer with a dwell time of 0.1 second per channel using the following transitions: ketoconazole *m/z* 531.1 > 489.1 (Fig. 1) and voriconazole *m/z* 350.0 > 224.1 (Fig. 2).

Patient Samples

The patient samples were taken solely for the analysis of voriconazole concentrations. Patient treated prior to license gave informed consent and the protocols received ethical approval. The blank serum used for assay validation was subject to local ethical approval.

Validation

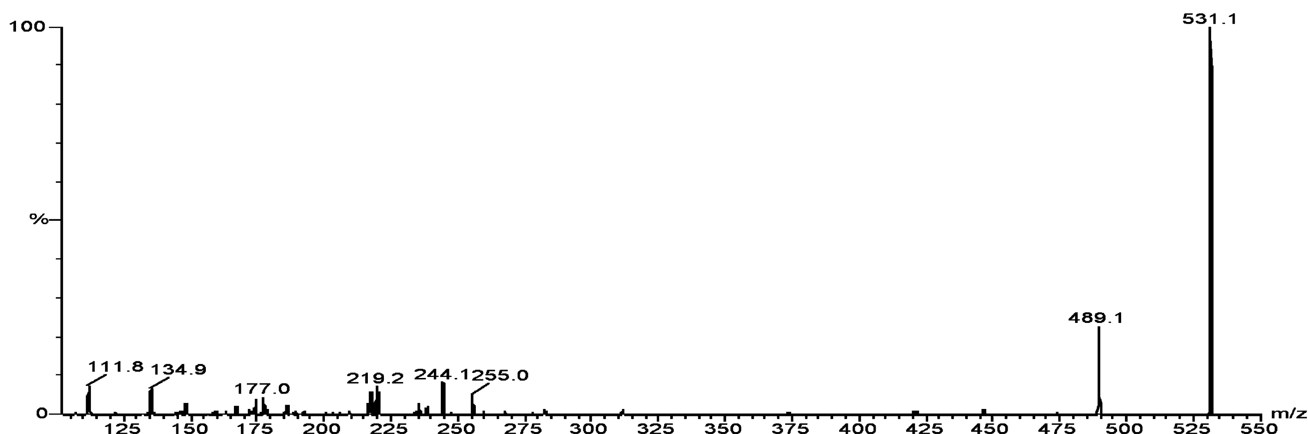
The assay was validated against published acceptance criteria for linearity, accuracy, precision, recovery, and sample stability.[11] In addition, ion suppression–caused matrix effects was investigated.

Linearity

To evaluate linearity of the calibration curves, 3 calibration curves were prepared and analyzed. The curves were judged linear if the correlation coefficient R^2 was better than 0.99 as calculated by weighted linear regression.

Accuracy and Precision

Accuracy and precision of the method was assessed against 4 samples: LLOQ, low, medium, and high. These samples were analyzed 3 times per day for 5 days to calculate between-assay accuracy and precision. To determine within-batch accuracy and precision, the same samples were analyzed 15 times within 1 batch. An overall coefficient of variation (CV) less than 20% was accepted for the LLOQ of this method. For the other samples a CV less than 15% was accepted. Ac-



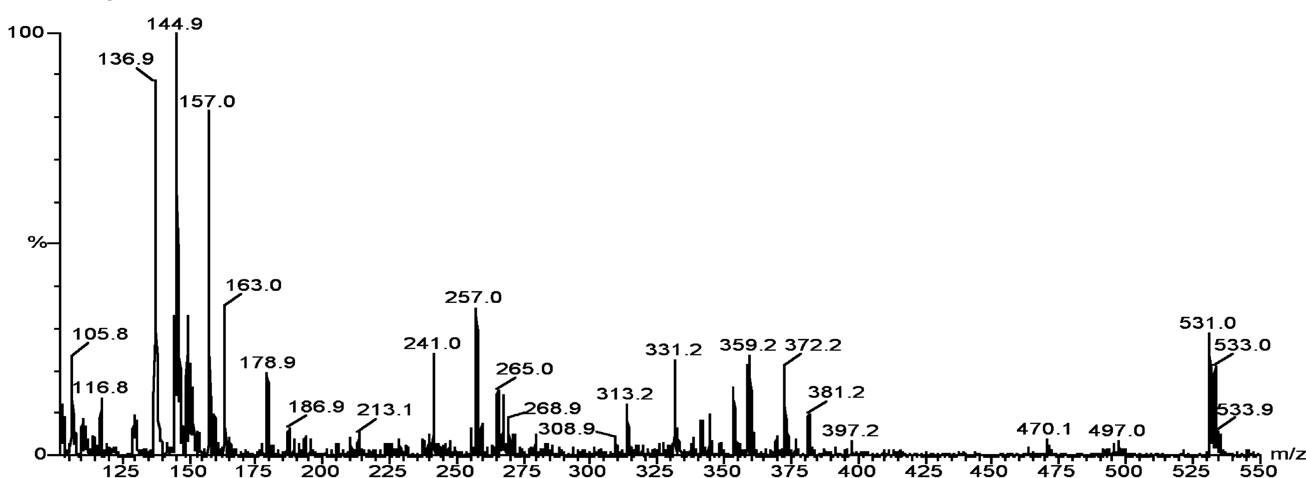


FIGURE 1. Positive ionization electrospray MS (upper) and MS/MS (lower) spectra for ketoconazole.

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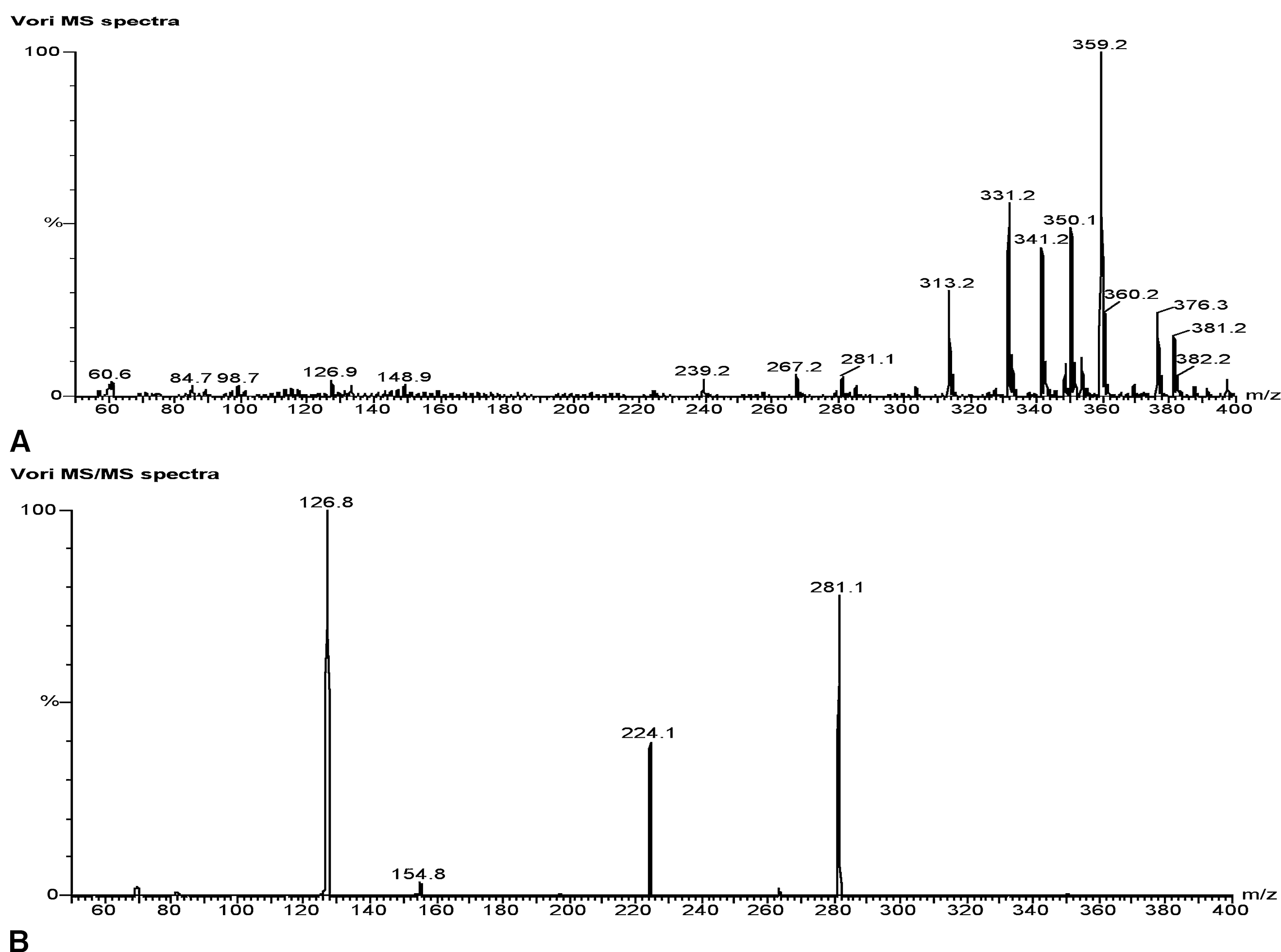

FIGURE 2. Positive ion electrospray MS (upper) and MS/MS (lower) spectra for voriconazole.

curacy was calculated from the difference between mean observed and nominal concentrations at a given level. The LLOQ was accepted if the mean result was within 20% of the theoretical concentration. For the other samples the mean was acceptable if it was within 15% of the theoretical concentration.[11]

Recovery

The recoveries of voriconazole and ketoconazole were determined by comparing the peak areas of samples before and after the addition of known amounts of voriconazole and ketoconazole.

Sample Stability

Two samples with voriconazole concentrations of 0.5 and 8.0 mg/L were subjected to 5 freeze–thaw cycles, after which the samples were analyzed. Samples were considered stable if bias was less than 10% after 5 freeze–thaw cycles.

Stability of extracted samples was assessed by analyzing the low, medium, and high control samples over a 24-hour period. The calculated response at t = 24 hours was compared with the calculated response at t = 0 hours. Samples were judged stable if the decrease in response was less than 10%. Stability of the extracted samples was also assessed by repeat analysis of the medium control sample every 7 minutes over a 14-hour period.

Dilution

Because some samples of clinical patients can have concentrations above the working range of the calibrators, these samples have to be diluted to obtain concentrations within the calibration range. To validate the dilution of samples, we diluted samples with a concentration of 40 mg/L, 100 mg/L, and 200 mg/L, 5 times or 20 times, to give 8 mg/L, 5 mg/L, and 10 mg/L, respectively. Dilution was considered possible if the measured bias was less than 10%.

Statistical Analysis

Validation results were analyzed using Analyze-It Software (Analyze-it Software Ltd, Leeds, UK).

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RESULTS

The dynamic range of the bioassay was found to be inadequate; consequently, patient specimens frequently required dilution (1:2 or 1:5) to achieve a value within the range 0.098 to 3.12 mg/L. Bioassay methodology was found to lack interassay reproducibility with the results of a QC sample (HPLC value of 3.026 mg/L) varying from 2.42 to >6.2 mg/L.

Under the chromatographic conditions described above for HPLC-MS/MS, voriconazole and ketoconazole were found to have retention times of 1.50 minutes and 1.47 minutes, respectively. The chromatograms in Figure 3 are typical for calibrator and patient samples and show no interfering peaks, indicating the specificity of the method. The assay time, injection to injection, was 2 minutes and 50 seconds, thus allowing the solvent gradient to return to baseline conditions before the next injection. The time taken to process a batch of 15 samples including controls and calibrators was less than 1.5 hours.

Quantification was performed by integrating the area under the extracted ion chromatograms for voriconazole and ketoconazole for a series of serum calibrators. A calibration curve was constructed by plotting the peak area ratio against voriconazole concentration. The calibration curve shown in Figure 4 is linear up to 20 mg/L and showed good agreement with the stated values $R^2 = 0.9995$ (n = 3).

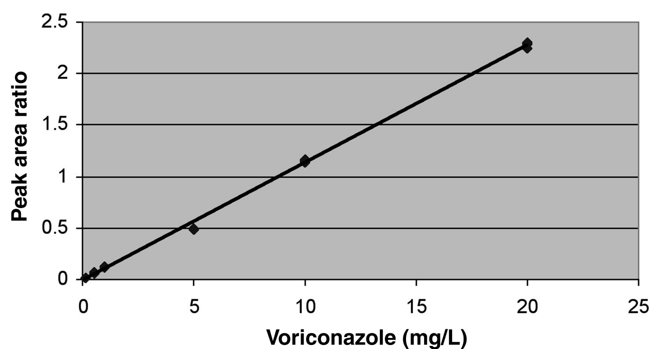

FIGURE 4. Linear concentration–response curve of voriconazole by HPLC-MS/MS (n = 3), $y = 0.101x - 0.008$, $R^2 = 0.9995$. Voriconazole/ketoconazole peak area ratio is shown plotted against voriconazole concentration.

Samples from patients receiving voriconazole therapy were analyzed by bioassay and then stored at −20°C for up to 104 months before analysis by HPLC-MS/MS and HPLC-UV methods. Comparison of LC-MS/MS against the other two methods using Passing and Bablock regression analysis showed HPLC-MS/MS = 1.25 (bioassay) − 0.73, $R^2 = 0.87$, n = 40, and HPLC-MS/MS = 1.06 ± 0.02 (HPLC-UV) − 0.07 ± 0.1, $R^2 = 0.95$, n = 99. Bland Altman analysis showed poor

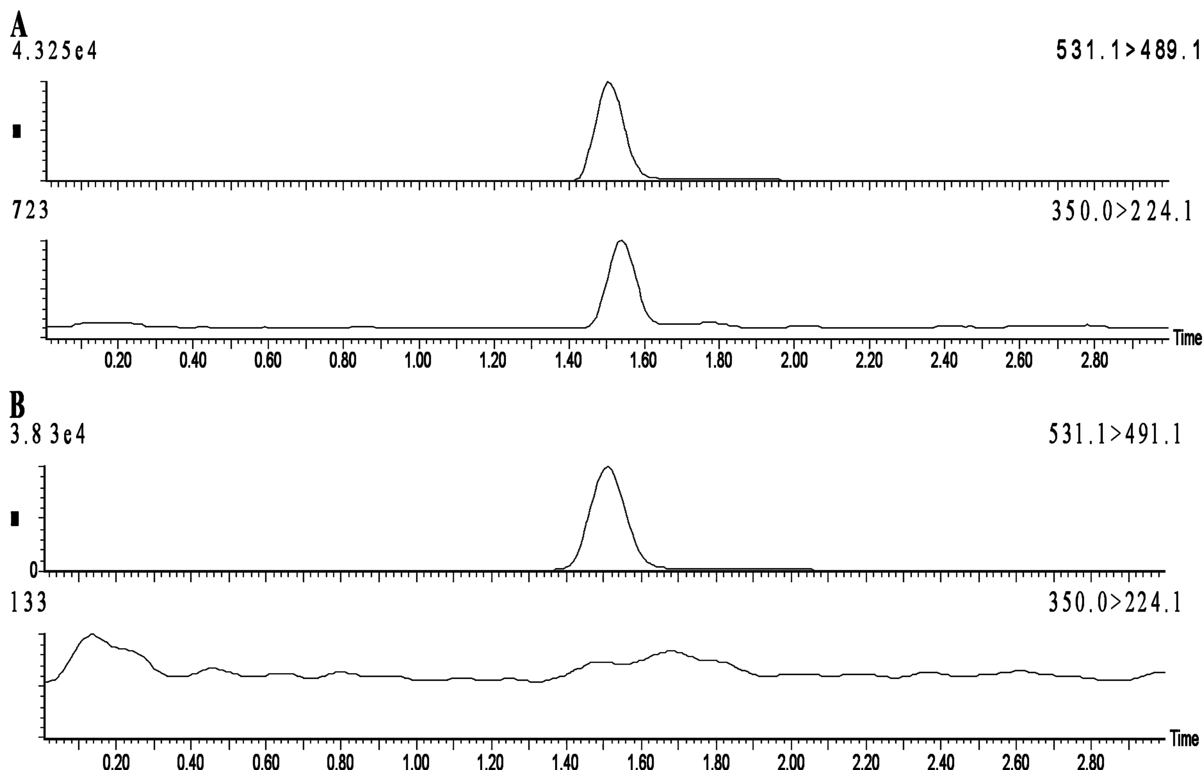

FIGURE 3. Liquid chromatography tandem mass spectrometry chromatograms for voriconazole (lower traces, m/z 350.0 > 224.1) and for ketoconazole (upper traces, m/z 531.1 > 489.1). Sample containing 0.1 mg/L voriconazole (A) and zero calibrator (B).

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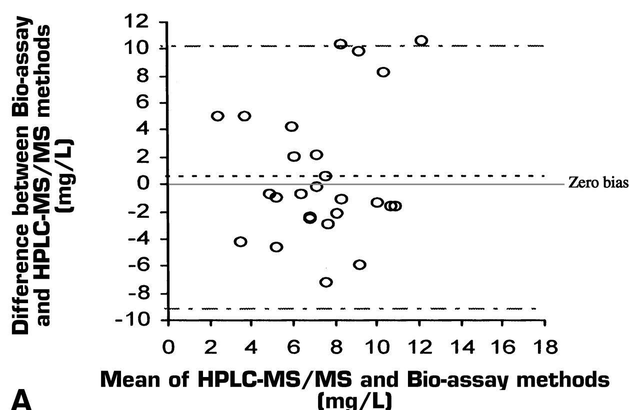
agreement between HPLC-MS/MS and bioassay with a small bias but reasonable agreement between HPLC-MS/MS and HPLC-UV assays (Fig. 5). The lower detection limit was calculated as the smallest detectable peak above baseline noise (signal-to-noise ratio > 3:1) and was found to be 0.01 mg/L. The lower limit of quantification (LLOQ) was found to be 0.1 mg/L. Accuracy and precision studies showed that coefficients of variation, within and between assays, were below 20% for LLOQ and below 15% for low, medium, and high concentrations. The bias was also less than 20% at LLOQ and less than 15% at the remaining levels (Table 1). To examine any suppression of ionization caused by matrix effects, a series of 6 serum samples were spiked with voriconazole at concentrations of 3.5 and 7.0 mg/L and prepared in the standard way. In addition, a series of aqueous standards were prepared in triplicate at identical concentrations. There was no difference in the area counts between serum samples and aqueous voriconazole solution, indicating that there was no evidence of ion suppression. Although we found that there was no interference in the assay from ion suppression, we did find that using ketoconazole as an internal standard improved the precision of the assay.



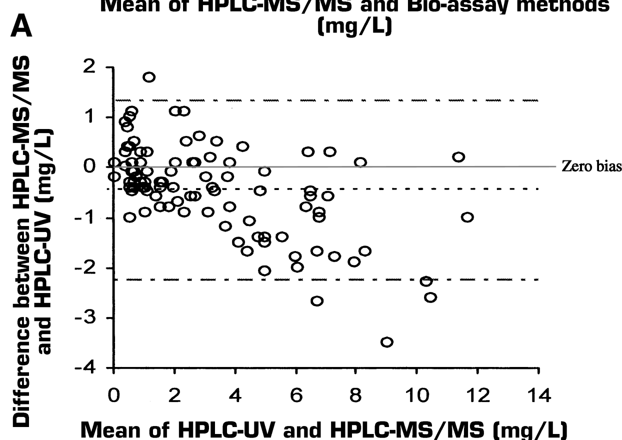


FIGURE 5. Bland Altman difference plots of voriconazole concentrations measured by HPLC-UV vs HPLC-MS/MS and HPLC-MS/MS vs bio-assay. Dot-dash lines indicate two standard deviations (2SD).

TABLE 1. Accuracy and Precision of Voriconazole in Serum Samples

Samples (n = 15)	Nominal conc. (μg/L)	Measured conc. (μg/L)	Within-day CV% (bias)	Between-day CV% (bias)
LLOQ	0.1	0.09	10.0 (−9.1)	17.0 (−7.0)
Low	0.38	0.40	4.4 (+2.6)	7.2 (+5.2)
Medium	3.8	3.84	3.9 (+1.0)	4.3 (+1.1)
High	15.3	15.20	3.6 (−0.6)	4.8 (−0.7)

The mean recovery of voriconazole across a range of concentrations between 3 mg/L and 10 mg/L was 93% (range 82–105%, n = 5). Recovery of ketoconazole was 95% (range 91–103%, n = 5). Freeze–thaw experiments showed acceptable bias (<2%). Repeat analysis of samples on different days showed that voriconazole is stable in serum at room temperature for at least 7 days. This should permit postage of samples for testing in central laboratories. Stability of a single extract injected over a 14 hour period is shown in Figure 6. No systematic loss in sensitivity was observed in the peak area ratio (analyte/internal standard), and the CV for this ratio was 4.0%. Stability of a batch of extracted samples measured over a 24-hour period was also found to be acceptable, with the low, medium, and high control samples having a bias of 2%, −1.5%, and 4.6%, respectively.

Dilution experiments showed acceptable bias between observed and theoretical concentrations. The bias for 40 mg/L, 100 mg/L, and 200 mg/L diluted samples was −4.6%, −7.2%, and −8.2%, respectively.

DISCUSSION

We have developed a rapid assay for voriconazole that is considerably faster than the currently available bioassay or

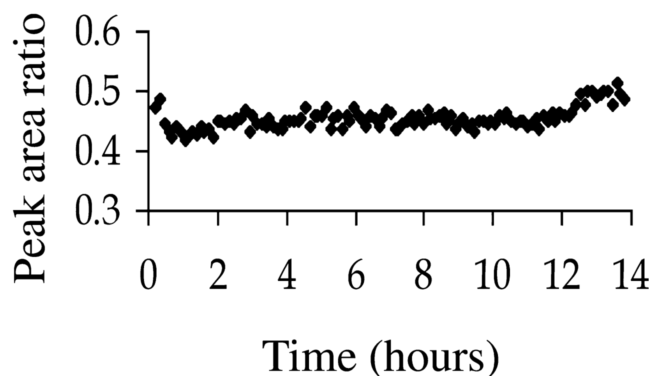


FIGURE 6. Replicate injections of an extracted serum sample containing voriconazole at a concentration of 4.0 mg/L, over a 14-hour period. Voriconazole/ketoconazole peak area ratio is shown plotted against time.

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HPLC-UV procedures. The HPLC-MS/MS assay is also highly sensitive compared with the other techniques and has the capacity for even further improvement in sensitivity because only 10 μL of serum was taken for extraction, and only 3 μL of this was used for injection. This represents a 10-fold decrease in sample requirement over the HPLC-UV method. The method of Zhou et al[8] used direct injection of untreated aqueous humor, did not incur the sample dilution associated with a protein precipitation step, and achieved a sensitivity of 5 μg/L. The difference in sensitivity between the 2 methods can be explained by the dilution of serum with acetonitrile in our method.

The ideal internal standard is a stable isotope of the compound: the isotope will ionize in the same way and will behave the same chromatographically. We believe that ketoconazole is a good internal standard from a theoretical perspective because it coelutes with voriconazole in our procedure and will therefore be subject to the same matrix effects. From a practical perspective, ketoconazole is unlikely to be coadministered with voriconazole, and it has the distinct advantage of being commercially available.

Although long-term stability data for voriconazole in serum does not exist, we would have expected a significant bias between the bioassay and HPLC-MS/MS results if the samples had denatured with time. It is therefore unlikely that the storage conditions of these samples have resulted in denaturation.

The bioassay method is inexpensive in terms of equipment and consumable costs; however, it suffered from poor assay precision in our hands. We cannot explain our poor performance with the bioassay because others have found acceptable precision and good agreement with HPLC-UV.[7] Nevertheless, the bioassay has several other deficiencies compared with the HPLC methods. The bioassay turnaround is poor compared with the chromatographic methods because of the long 18-hour incubation time, and in addition, combination therapy may also be a problem with the bioassay but not with the chromatographic techniques. The inability of the bioassay to accurately determine high concentrations of voriconazole (>6 mg/L) without sample dilution is a significant limitation (a minimum of 300 μL serum is required for bioassay analysis). And finally, the large dynamic range required for voriconazole measurement, from <0.25 to ~20 mg/L, cannot be achieved with any accuracy using bioassay. The clinical benefit of the more rapid and specific HPLC-MS/MS procedure is therefore clear.

Discrepancies have been previously reported with direct comparison of HPLC and bioassay results for itraconazole, primarily because of bioactive metabolites.[9] This is not the case for voriconazole, however, because the drug has no known metabolites with antifungal activity. Therefore, comparison between the assays will not be affected by this mechanism. HPLC is therefore a useful alternative method but requires more sample and more time to generate a result than HPLC-MS/MS. Both HPLC-UV and HPLC-MS/MS have the advantage over bioassay for patients on combination antifungal therapy, an increasingly common phenomenon. HPLC-UV and HPLC-MS/MS systems are costly in terms of equipment and are not available to all laboratories. However, there are an increasing number of laboratories using HPLC-MS/MS for therapeutic monitoring of other drugs, notably immunosuppressants. The consumable cost of 1 bioassay (which may be used for up to 5 patient samples) is less than £6, and the techniques required for the bioassay method are available to all routine laboratories. Nevertheless, we would still argue that the HPLC-MS/MS procedure is technically superior to the bioassay and should be the method of choice for analysis of voriconazole.

The optimal voriconazole concentrations for efficacy and minimization of adverse events have not been fully determined. The substantial interpatient variability in peak and time to peak concentration, as well as β half-life, make simple algorithms for therapeutic monitoring of voriconazole (as with the aminoglycosides, for example) difficult. Patients with undetectable concentrations of drug (usually because of noncompliance or drug interactions, or in young children) are unlikely to respond to therapy. Good clinical responses in invasive aspergillosis have been recorded in patients with random voriconazole concentrations above 0.25 mg/L,[1] and it may be that this is a useful threshold trough concentration. More data are required on this point, however, particularly in challenging situations such as cerebral infections. Patients with plasma concentrations higher than 6 mg/L may be at risk for concentration-related toxicity. These patients are typically elderly, have hepatic impairment (which may be subclinical), and probably are genetically determined slow metabolizers of the drug (2C19 polymorphism, ~3–5% of whites and 15–20% Asians).[10] High trough concentrations (ie, >6 mg/L early after initiation of therapy) have been associated with some serious clinical events (hypoglycaemia, electrolyte disturbance, confusion, pneumonitis),[5] and dose reduction is probably appropriate.

In conclusion, we have developed and validated a rapid and sensitive assay for voriconazole with the advantage of a small sample size that should prove useful for TDM and pharmacokinetic studies, especially in small children.

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