Comparison of High-Performance Liquid Chromatographic and Microbiological Methods for Determination of Voriconazole Levels in Plasma

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A new selective high-performance liquid chromatography (HPLC) method with UV detection for the determination of the investigational triazole voriconazole in human plasma by using acetonitrile precipitation followed by reverse-phase HPLC on a C18 column was compared with a simple agar well diffusion bioassay method with Candida kefyr ATCC 46764 as the assay organism. Pooled plasma was used to prepare standard and control samples for both methods. The results of analyses with spiked serum samples (run as unknowns) were concordant by the bioassay and HPLC methods, with expected values being obtained. HPLC demonstrated an improved precision (3.47 versus 12.12%) and accuracy (0.81 versus 1.28%) compared to those of the bioassay method. The range of linearity obtained by both methods (from 0.2 to 10 μg/ml for HPLC and from 0.25 to 20 μg/ml for the bioassay) includes the range of concentrations of voriconazole (from 1.2 to 4.7 μg/ml) which are considered clinically relevant. Although either methodology could be used for the monitoring of patient therapy, the smaller variability observed with HPLC compared to that observed with the bioassay favors the use of HPLC for pharmacokinetic studies.


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125, 1995). On the basis of its promising antifungal properties,
adequate tolerability, and optimal pharmacological profile, this
agent is undergoing evaluation in clinical trials with patients
with invasive mycoses.

The quantification of plasma voriconazole levels in clinical
trials is an important objective since it will permit a compre-
hsensive characterization of its pharmacological features and
monitoring of its levels in clinical plasma samples. For this
reason, there has been interest in the development of adequate
analytical methods, and a high-performance liquid chromato-
graphic (HPLC) method for the detection of voriconazole in
plasma has been described and validated (20). This method
involves direct injection of plasma onto a Sephadex column
and does not require protein precipitation. Although the re-
results regarding accuracy, precision, and lower limit of quanti-
fication are adequate, this method is laborious and technically
difficult.

This study describes and validates a new HPLC method that
uses precipitation of proteins with acetonitrile by use of a
reverse-phase column with UV spectrophotometric detection
as well as a biosay method for the detection of voricon-
azole in plasma. In addition, this report compares the accurac-
cies and precision of the two assays for the determination of
therapeutic concentrations of voriconazole in human plasma.

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Conference on Antimicrobial Agents and Chemotherapy, San
Diego, Calif., 24 to 27 September 1998.

MATERIALS AND METHODS

Standards and controls for both methods. Voriconazole (UK-109,496) was
kindly supplied by Pfizer Central Research (Sandwich, United Kingdom).
A stock standard of 1,000 μg/ml was prepared by transferring 10 mg of voriconazole into
a 10-ml volumetric flask and diluted in methanol to a total volume of 10 ml.
A working standard of 100 μg/ml was prepared by transferring appropriate
amounts of stock voriconazole in a 5-ml volumetric flask and diluted in ammox-
ium phosphate buffer to a total volume of 5 ml. Both stock and working
voriconazole solutions were stored at −10°C. Standards were prepared with serum
from human donors which had been screened to ensure the absence of
antifungal agents. Voriconazole standards with concentrations in the range of
0.2, 0.6, 1, 2, 3, 4, and 5 μg/ml and controls with concentrations of 0.8, 1.6, and
3.6 μg/ml were prepared by accurately transferring appropriate volumes of working
voriconazole solution to a 50-ml volumetric flask and QS with pooled plasma
(Table 1).

HPLC assay of voriconazole. The HPLC system consisted of a Beckman 126
solvent module binary pump, a Rheodyne model 7010 sample injection valve
with a 100-μl sample loop, a Waters symmetry C18 (5 μm, 4.6 by 250 mm) guard
column, a symmetry C18 (5 μm, 4.6 by 250 mm) analytical steel column, a
Beckman module 166 UV programmable detector. Integrations, calculations,
and plotting of chromatograms were performed with a Beckman computing
integrator (System Gold Software; Beckman Instruments Inc., Houston, Tex.).
The reagents and instruments used were as follows: methanol (HPLC grade),
aconitrile (HPLC grade), and ammonium hydroxide were purchased from EM
Science (Fair Lawn, N.J.), and ammonium dihydrogen phosphate (HPLC grade)
was purchased from Fisher Scientific (Gibbstown, N.J.). The mobile phase was
prepared by mixing acetonitrile with filtered 0.04 M ammonium dihydrogen
phosphate buffer, adjusted to pH 6.0 with ammonia, at 50/50 (vol/vol) propor-
tions. The mobile phase was filtered under vacuum through a 47-mm-diameter,
0.45-μm pore-size nylon membrane filter. The flow rate was 0.8 ml/min. The UV
detector was set at a wavelength of 255 nm. The HPLC apparatus was operated
at room temperature. No internal standard was used. All the samples were
swelled in a 37°C water bath. A 0.5-ml aliquot of each sample was pipetted into
a 2-ml Eme Mate microcentrifuge tube (ISC Bioexpress, Kaysville, Utah), and
the tube was vortexed briefly. The samples were then centrifuged at 3,000 rpm for 5
min in a Micro 14 Fisher Scientific microcentrifuge. After centrifugation, the
samples were transferred to a 5-ml disposable screw-cap tube (Pyrex, Corning,
N.Y.) and 0.8 ml of acetonitrile was added, and then the tube was vortexed and
the contents were mixed briefly. After 10 minutes, the samples were centrifuged
at 2,500 rpm with a Beckman model TJ-6 centrifuge for 5 min, and 50 μl of the
supernatant was injected into the liquid chromatography by using a 100-μl loop.
A standard curve was constructed by preparing standards in plasma and using
linear regression to correlate the peak height of the voriconazole spike versus
voriconazole concentrations.

Bioassay of voriconazole. Candida kefyr ATCC 46764 was maintained by
weekly passage on Sabouraud dextrose agar plates. Prior to use, the organism
was inoculated in a solution of yeast nitrogen base (YNB) broth (Difco) with 1%
glucose. The broth culture was incubated for 6 h at 35°C, and the turbidity
was then adjusted with sterile water to that of a no. 2 McFarland standard (63 to 70%
transmission at 590 nm). Agar for the bioassay was prepared by mixing 15
BL agar, 7 g of Sigma Dextrose glucose, and 7 g of BBL Trypticase peptone, and
water was added to give a total volume of 1,000 ml. Previously prepared 45-ml aliquots of the test agar were melted, allowed to cool
to 48°C, inoculated with 0.5 ml of adjusted C. kefyr suspension, and then gently
mixed by inversion and poured into round plastic disposable petri dishes (150 by
15 mm). The agar was allowed to gel at room temperature for 10 to 15 min. After
the solidification of the YNB, 5-mm-diameter wells were bored and with a sterile
cork borer and a 16-well template and aspirated. Ten microliters of each stan-
dard or control was pipetted into individual wells around the periphery of the
plate. The plates were incubated overnight at 37°C. Assay plates were prepared
in triplicate, resulting in nine and three measures for each control and standard,
respectively. The assay was repeated over 5 days. Zones of inhibition were
measured to the nearest 0.1 mm by using a metric caliper micrometer. A com-
puter program was used to calculate the best-fit curve for the standards and the
controls. Regression plotting of drug concentration versus bioassay zones
and cumulative zone was done with statistical analysis software (version 3.1; SAS
JMP computer software (version 3.1; SAS Institute, Cary, N.C.). The equation used was
Y = b0 + b1 + X, where Y is the log10 voriconazole concentration (in micrograms per milliliter), X is the zone of
inhibition (in millimeters), and b0 and b1 are a constant and the slope of the
curve, respectively.

Statistics. Least-squares linear regression was performed by using standard
techniques. Both procedures were repeated over 5 days, and each control was
run five (HPLC) and nine (bioassay) times. The within- and between-run vari-
abilities of the assays was estimated by computing the coefficient of variation
(CV). The agreement between both analytical methods has been evaluated by
computing the mean square error and bias (19). For these calculations, HPLC
was considered the reference method.

RESULTS

HPLC. Under the chromatographic conditions described
above, voriconazole was found to have a retention time of 7.5
min. Voriconazole was well separated from endogenous back-
ground peaks in serum. A chromatogram for a serum sample
spiked with 5 μg of voriconazole per ml is shown in Fig. 2.
Standard curves were constructed by plotting the peak heights
against the concentrations of the standards. Peak height values
were used instead of peak area values, as it has been demon-
strated that this gives improved precision and accuracy for low
concentrations of voriconazole (12). In practice, a linear rela-

![FIG. 1. Chemical structure of voriconazole.](http://aac.asm.org/Downloadedfromhttp://aac.asm.org)
A linear relationship was found over a range of drug concentrations from 0.2 to 10 μg/ml. A voriconazole concentration of 0.2 μg/ml was the lowest that could be reproducibly determined. HPLC results demonstrated a linear relationship between peak height ratios and voriconazole concentrations, giving standard curves with $r^2$ values of >0.99. To determine the accuracy of the HPLC assay, the same spiked controls used with the bioassay were assayed on five different occasions, and the values obtained were averaged and correlated with the expected values. There was an excellent agreement between the measured concentrations and the spiked concentrations (average CV, <2.5%). The interday variability of the HPLC, estimated as the average CV for the assays with the spiked controls on 5 different days, was <4%.

**Bioassay.** The zone margins of inhibition were sharp and clearly delineated, as shown in Fig. 3. To determine the accuracy of the bioassay, spiked controls containing 0.8, 1.6, and 3.6 μg/ml were assayed five times on five separate occasions. The values obtained were averaged and correlated with the expected values. The concentrations used in the bioassay standard curve were 0.6, 1, 2, 3, 4, and 5 μg/ml. Standard curves were linear, with $r^2$ always being >0.99. The intraday variability of the bioassay was estimated by computing the CV for the measurements of each of the spiked unknown samples containing 0.8, 1.6, and 3.6 μg/ml that averaged <3%. The interday variability of the bioassay, estimated as the average CV for the assays of the spiked controls run on 5 different days, was <13%. The bioassay was sensitive down to 0.25 μg/ml. Below this value the zones were poorly defined and too close to the well for accurate measurement.

**Correlation of HPLC and bioassay results.** The scatterplots in Fig. 4 depict the agreement between the HPLC and bioassay when used to assay the spiked controls at concentrations of 0.8, 1.6, and 3.6 μg/ml. The correlation between both methods has been assessed by calculating the mean square error and bias (Table 2), and the results demonstrate an excellent correlation between the two techniques ($r^2$ > 0.99), although the bioanalytical method has a greater degree of variability.

**DISCUSSION**

This study compares and validates an HPLC method and a bioassay method for the determination of voriconazole levels in human plasma. The HPLC method with UV detection involves a single-step protein precipitation with acetonitrile followed by reverse-phase HPLC on a C18 column and does not require the use of an internal standard. The bioanalytical method consists of a simple agar well-based microbiological method that determines voriconazole concentrations by measuring graded responses of *C. kefyr* and comparing unknown concentrations of voriconazole in serum against a standard curve. The results obtained in this study demonstrate an excellent correlation between the two techniques, which had similar lower limits of quantitation: 0.2 μg/ml for HPLC and 0.25 μg/ml for the bioassay. The HPLC method has improved precision (3.47 versus 12.12%) and accuracy (0.81 versus 1.28%) compared to those for the bioanalytical method.

Voriconazole represents a novel, broad-spectrum antifungal agent with interesting and promising features with potential clinical activity. In order to use this agent properly as treatment for patients with fungal disease, it is required that we have adequate knowledge of its pharmacological properties. The implication of pharmacokinetic studies in the clinical devel-
development of a new antifungal agent is patently illustrated by considering the data available for other relatively new triazoles including fluconazole and itraconazole. Pharmacokinetic studies have been pivotal in defining such critical issues as the necessity to monitor plasma itraconazole levels, given the erratic oral absorption and dose-dependent pharmacokinetics of itraconazole, and the requirement to modify fluconazole doses on the basis of renal function (1).

Another HPLC analytical method for the determination of voriconazole concentrations in plasma has been described previously (20). In contrast to the method described in this report, that method uses as the initial procedure injection of the plasma sample directly onto a Sephadex size-exclusion column without protein precipitation and requires the use of an internal standard. Although that method has an extremely low limit of quantitation, in the nanogram range, technical difficulties impair its widespread use for pharmacokinetic studies and monitoring of patients. The major disadvantage involves the requirement of a precolumn with a maximum pressure limit of about 180 lb/in\(^2\). This results in frequent blockage of the system after 100 injections and, consequently, repeated replacement of the precolumn. This problem is avoided by precipitation of plasma proteins, as used for the method described here.

Although the lower limit of quantitation of these two methods (on the order of micrograms) is relatively high compared to that of the other reported method, the linearity range obtained by both methods effectively covers what is currently believed to be the clinically relevant range for voriconazole concentrations in plasma. In humans, the range of concentration of voriconazole in plasma following the administration of multiple oral and intravenous doses ranging from 4 to 6 mg/kg of body weight varies from 1.2 to 4.7 \(\mu\)g/ml, which are within the limits in which both analytical methods were linear (0.2 and 10 \(\mu\)g/ml for HPLC and 0.25 and 20 \(\mu\)g/ml for bioassay; \(r^2 > 0.99\)).

The major advantage of the bioassay over the HPLC method is its relative simplicity. The HPLC method is moderately laborious and requires expensive equipment. For instance, the processing of 15 samples requires about 5 h for extraction and analysis by HPLC, which is twofold the time required to process the same number of samples by the bioassay (plus an overnight incubation to read the results). In addition, the bioassay is easy to perform and does not require special equipment. The major advantages of the HPLC method compared to the bioassay are its enhanced accuracy (0.81 versus 1.28%) and precision (3.47 versus 12.12%). A second difference between both methods pertains to their ability to measure active metabolites. This potential problem has been elegantly demonstrated with itraconazole. Itraconazole is metabolized through the cytochrome P-450 detoxification system to yield several metabolites, with one of them, an hydroxylate metabolite, being a very active antifungal agent. While the HPLC method detects only the parent compound with a predefined chemical structure and not the different chemical species that result from metabolic reactions, the bioanalytical method will detect any active substances, irrespective of their chemical characteristics. This factor explains the discrepancies observed between the two methods when analyzing clinical plasma samples for their itraconazole concentrations (8). These considerations are not applicable in the case of voriconazole, since the metabolism of this agent results in substances which do not possess antifungal activity.

Many questions about the measurement of voriconazole levels and the clinical relevance of those levels remain to be addressed. As additional data from ongoing and future studies are collected and analyzed, clearer concepts as to the utility of both analytic and bioassays may emerge. As with the azoles developed prior to the development of voriconazole, there will undoubtedly arise issues involving perceived and/or genuine resistance, drug-drug interactions, absorption difficulties, and metabolism nuances in specific patients or patient groups and the need for knowledge about the pharmacokinetics and pharmacodynamics of the drug. It is hoped that assays such as those described here will be of value and assistance in gathering such data and providing information for the optimal clinical use of a new and potentially significant weapon in the treatment of invasive mycotic infections.

In conclusion, the results of this study indicate that both analytical methods are adequate for analysis of voriconazole and are suitable for use in clinical laboratories. The HPLC

![FIG. 4. Scatterplot depicting the agreement between the HPLC method (x axis) and the microbiological method (y axis) for determination of voriconazole concentrations in spiked plasma samples.](http://aacl.asm.org/)

<table>
<thead>
<tr>
<th>Amt ((\mu)g/ml) of voriconazole added to sample</th>
<th>Mean squared prediction error (95% CI)</th>
<th>Bias (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8</td>
<td>0.010 (0.007–0.014)</td>
<td>0.018 (−0.025–0.060)</td>
</tr>
<tr>
<td>1.6</td>
<td>0.063 (−0.022–0.147)</td>
<td>−0.065 (−0.0166–0.037)</td>
</tr>
<tr>
<td>3.6</td>
<td>0.139 (0.064–0.212)</td>
<td>0.074 (0.078–0.229)</td>
</tr>
</tbody>
</table>

TABLE 2. Correlation between HPLC and microbiological method for assay of voriconazole in spiked plasma samples.
method, although more laborious, is more accurate and precise, being more appropriate for pharmacokinetic studies. The microbiological method, however, is relatively simple and has sufficient precision and accuracy to be used to monitor drug levels in patients for routine clinical work-ups.

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