Standardization of a Fluconazole Bioassay and Correlation of Results with Those Obtained by High-Pressure Liquid Chromatography

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An improved bioassay for fluconazole was developed. This assay is sensitive in the clinically relevant range (2 to 40 μg/ml) and analyzes plasma, serum, and cerebrospinal fluid specimens; bioassay results correlate with results obtained by high-pressure liquid chromatography (HPLC). Bioassay and HPLC analyses of spiked plasma, serum, and cerebrospinal fluid samples (run as unknowns) gave good agreement with expected values. Analysis of specimens from patients gave equivalent results by both HPLC and bioassay. HPLC had a lower within-run coefficient of variation (<2.5% for HPLC versus <11% for bioassay) and a lower between-run coefficient of variation (<5% versus <12% for bioassay) and was more sensitive (lower limit of detection, 0.1 μg/ml [versus 2 μg/ml for bioassay]). The bioassay is, however, sufficiently accurate and sensitive for clinical specimens, and its relative simplicity, low sample volume requirement, and low equipment cost should make it the technique of choice for analysis of routine clinical specimens.

Fluconazole is a recently marketed triazole antifungal agent that has been found useful in the treatment of mucocutaneous candidiasis and cryptococcal meningitis (1). While it is generally not necessary to measure fluconazole levels, the dose of fluconazole must be adjusted in patients with renal insufficiency (3) and may require adjustment in patients taking drugs which interact with fluconazole (10). The most commonly described assays for fluconazole levels are high-pressure liquid chromatography (HPLC) (5) and gas-liquid chromatography (4, 8, 14), but these methods require expensive equipment and considerable technician time. As an alternative, we considered several bioassay methods. When an itraconazole bioassay using Difco nutrient agar, pH 6.0, was tested for fluconazole (6), small zones of diffusion, a lower limit of sensitivity of about 4 μg/ml, and fuzzy zone edges were observed. The system using Pfizer High Resolution agar (used according to the manufacturer’s instructions) produced sharp zones and good sensitivity, but its requirement for special agar (obtained only from Pfizer Pharmaceuticals), and a dedicated 28°C incubator made it otherwise unattractive. A thin-layer yeast nitrogen broth-peptone agar system (2, 7) was designed for use with round petri dishes (150 by 15 mm), allowing only 10 samples per plate. When larger plates (245 by 245 mm) were used, the zone diameters of duplicate wells had excessive variability. Finally, a SAAMF (synthetic amino acid medium for fungi) agar-based assay that we developed to detect very low concentrations of fluconazole (12) required a difficult, tedious technique that was not suitable for large-scale work. In response to these difficulties, we developed a modified bioassay that was suitable for measuring fluconazole concentrations in the clinically relevant range of 2 to 40 μg/ml. Further, as we were unable to find any information correlating bioassay and HPLC results for fluconazole and as a further validation of the new bioassay technique, we performed a blind comparison of these two methods for measuring fluconazole concentrations.

MATERIALS AND METHODS

Reagents. Fluconazole powder (lot R29) was a gift from Pfizer Pharmaceuticals. All standards and spiked unknowns were prepared from this one lot. Pooled lots of human serum and plasma were prepared from groups of four to five healthy donors, while a pooled lot of human cerebrospinal fluid (CSF) was collected from patients undergoing lumbar puncture. Patient CSF was added to the pooled lot only when it was known that the routine analysis of the CSF was unremarkable and that the patient was not taking any antifungal drugs. Sufficiently large lots of each of the three fluids were collected to allow all standards and unknowns used throughout this study to be prepared from these pooled lots.

The HPLC method used spectral grade ethyl acetate and methanol (American Scientific Products, American Hospital Supply Corporation, Muskegan, Mich.), analytical grade sodium phosphate (Fisher Scientific, Silver Spring, Md.), and double-distilled water. All solvents were passed through a 0.45-μm-pore-size filter (Millipore, Bedford, Mass.) and degassed prior to use.

Preparation of spiked unknowns. Fluconazole was dissolved in sterile water at 1,000 and 100 μg/ml and used to prepare both standards and spiked unknowns in each of these body fluids, with the percentage of added water (vol/vol) in the final material never being greater than 0.64. Spiked unknowns were prepared by one of the researchers,

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labeled with code letters, and distributed to the other researchers for blind analysis. These unknowns were prepared at a central location and split, thus ensuring that the assays were done on identical aliquots. Materials were shipped on dry ice, although it was subsequently found that fluconazole in water, plasma, serum, and CSF was stable for at least 1 month at 4°C (data not shown).

Collection of patient material. Over a period preceding this study, aliquots of CSF, serum, and plasma were submitted for fluconazole assay were collected. These patient specimens were divided, coded, and distributed for analysis in a fashion similar to that for the spiked unknowns.

**Fluconazole bioassay.** *Candida* *kefyr* (*pseudotropicalis*) was maintained on Sabouraud dextrose agar slants. Prior to use, the organism was inoculated into SAAMF broth prepared with 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (0.165 M), pH 7.4, without Tris buffer, cysteine, or cystine (9). The broth culture was incubated overnight on a gyratory shaker (140 rpm) at 35°C in ambient air. Agar for the bioassay was prepared by mixing equal volumes of sterile, molten 3% BBL agar (at 50°C) and 2x SAAMF broth, buffered as described above. To this was added sulfisoxazole to C. *kefyr* to give a final concentration of 2.2 x 10^5 yeast cells per ml. Agar (150 ml) was poured into sterile, level, square plastic Nunc bioassay plates (245 by 245 mm). The agar was allowed to gel at room temperature for 45 min, and 5-mm-diameter wells were cut, using a sterile cork borer and a 36-well template. Wells were loaded with 20 µl of fluids. Standards were placed on each plate at the following concentrations (in micrograms per milliliter): 60.15 (2 wells), 31 (3 wells), 15.74 (3 wells), 7.94 (3 wells), 3.85 (3 wells), 1.96 (2 wells), 0.99 (1 well), and 0 (1 well). Unknowns were loaded in duplicate into the remaining 18 wells. The plate was then incubated at 4°C for 1 h and then at 35°C for 12 to 14 h.

Zone diameters were measured to the nearest 0.1 mm, using a vernier caliper and a magnifying lens. Each well was measured twice, and the average of the two measurements was taken as the zone diameter for that well. Zone areas were computed as the area of the clear zone minus the area of the sample well. A standard curve was constructed by plotting zone area versus log_{10} fluconazole concentration. Unknowns were always read by interpolating from the standard curve constructed from the same plate.

**Fluconazole HPLC assay.** A modification of the method of Troke et al. (11) was used. The liquid chromatograph was equipped with a UV detector (model 1050; Hewlett-Packard, Palo Alto, Calif.) operated at 260 nm and an isocratic solvent delivery system (2150 HPLC pump; LKB, Piscataway, N.J.). The C18 reverse-phase analytical column (25 cm long, 4.0 mm inside diameter, 5-µm particles) (Ultrasphere; Beckman, San Ramon, Calif.) was preceded by a guard column (30 mm long, 5.6 mm inner diameter, 5-µm particles) (Spheri-5 ODS; Brownlee Laboratories, Santa Clara, Calif.). The mobile phase consisted of methanol-0.025 M sodium phosphate buffer (45:55 [vol/vol]) adjusted to pH 7.0 with 85% (vol/vol) phosphoric acid. The flow rate was 1.0 ml/min, with a back pressure of 14 MPa and an operating temperature of 25°C.

Samples for analysis were prepared by adding 1.0 ml of 1.0 M NaOH, 4.0 ml of ethyl acetate, and 100 µl of internal standard (200 µg of UK-48,134 [Pfizer] per ml in mobile phase) to 1.0 ml of sample in a 15-ml glass test tube. The tube was shaken for 5 min and then centrifuged at 1,000 x g for 5 min. The organic layer was transferred to a 15-ml glass test tube, 2.0 ml of 1 M HCl was added, and the tube was shaken for 5 min and then centrifuged at 1,000 x g for 5 min. The organic layer was removed and discarded. To the remaining aqueous layer was added 1.0 ml of 5M NaOH and 4.0 ml of ethyl acetate. The tube was shaken for 5 min and centrifuged at 1,000 x g for 5 min, and the organic layer was transferred to a 15-ml glass test tube. The organic layer was then evaporated to dryness under nitrogen at 37°C, and the residue was dissolved in 200 µl of HPLC mobile phase, mixed for 30 s, and centrifuged for 2 min at 1,000 X g. Samples (25 to 100 µl each) were injected by an automatic sampler (WISP model 710B; Waters, Milford, Mass.). The total run time per sample was 10 min. A standard curve was constructed by preparing standards in plasma, serum, and CSF, extracting and analyzing them as described above, and using unweighted linear regression to correlate peak height ratios (sample/ internal standard) of the fluconazole spike versus fluconazole concentration.

**Statistics.** Least-squares linear regression was performed by using standard techniques (15). Within- and between-run variability of the assays was estimated by computing the coefficient of variation (CV) as (standard deviation/mean) x 100.

**RESULTS**

**Bioassay.** The zones of inhibition were clearly delineated. Duplicate wells gave the same zone diameters within 1 mm. The volume of agar in the bioassay plate was critical to achieving these results. With less agar (120 ml per plate) the zone diameters of duplicate wells were not consistent, while with more agar (180 ml per plate), the zone edges were fuzzy. Zone diameters ranged from ca. 10 (1.96 µg of fluconazole per ml) to ca. 26 mm (60.15 µg of fluconazole per ml). Standard curves were linear with r^2 > 0.96. This relatively low r^2 was due to a tendency toward nonlinearity at the lowest points on the standard curve (0.99 and 1.96 µg/ml).

To determine the accuracy of the bioassay, spiked unknowns containing 4.97, 9.9, 14.78, 19.6, 24.4, 29.1, 33.8, and 38.5 µg of fluconazole per ml were assayed three to six times on separate occasions. The values obtained were averaged and correlated with the expected values. This correlation was linear, giving a line that did not differ from the line of identity in any of the three fluids, with r^2 always >0.98. Detailed results of the measurements at each concentration are given in Table 1.

Between-run variability of the bioassay was estimated by computing the CV for the measurements of each of the spiked unknowns described above (Table 1). The average of these CVs was <12% for all three fluids. Within-run variability of the bioassay was estimated by making six measurements (each in duplicate) of samples containing 9.9 or 29.1 µg/ml, each on a single bioassay plate. The average of the CVs at these concentrations was <11% in all three fluids (Table 1). Bioassay was sensitive down to 2 µg/ml. Below this value the zones were poorly defined and too close to the well for accurate measurement.

**HPLC assay.** HPLC results demonstrated a linear relationship between peak height ratios and fluconazole concentration, giving standard curves with r^2 > 0.99. To determine the accuracy of the HPLC assay, the same spiked unknowns used with the bioassay (with the addition of a 0.99-µg/ml specimen) were assayed on two separate occasions, and the values obtained were averaged and correlated with the expected values. This correlation was linear, giving a line
TABLE 1. Accuracy and variability of the bioassay*

<table>
<thead>
<tr>
<th>Actual concn (µg/ml)</th>
<th>Plasma CV (%) (no. of repetitions)</th>
<th>Serum CV (%) (no. of repetitions)</th>
<th>CSF CV (%) (no. of repetitions)</th>
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<tr>
<td></td>
<td>Between run</td>
<td>Within run</td>
<td>Between run</td>
</tr>
<tr>
<td>4.97</td>
<td>4.6</td>
<td>4.5 (5)</td>
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<td>9.9</td>
<td>9.4</td>
<td>5.2 (4)</td>
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<td>16.5</td>
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<td>19.6</td>
<td>17.9</td>
<td>7.4 (4)</td>
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<td>24.4</td>
<td>24.3</td>
<td>2.4 (3)</td>
<td>26.8</td>
</tr>
<tr>
<td>29.1</td>
<td>29.2</td>
<td>7.4 (5)</td>
<td>29.3</td>
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<td>33.8</td>
<td>36.3</td>
<td>6.9 (3)</td>
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<td>38.5</td>
<td>41.0</td>
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<tr>
<td>Avg</td>
<td>7.7</td>
<td>7.7</td>
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* Results of measurements of the spiked unknowns are shown.

that did not differ from the line of identity in any of the three fluids, with $r^2$ always $>0.99$. Within-run variability of the HPLC assay, computed as the average CV for triplicate measurements of samples spanning the range of the spiked specimens was $<2.5\%$ for all three fluids. Between-run variability, computed as the average CV for the assays of the spiked unknowns, was $<5\%$ for all three fluids. The HPLC assay could detect as little as 0.1 µg of fluconazole per ml.

**Correlation of HPLC and bioassay results.** Figure 1 compares the results obtained by HPLC and bioassay when used to assay the spiked unknowns at concentrations from 5 to 40 µg/ml. There is excellent agreement between the two techniques ($r^2 > 0.98$ for all three fluids), though it is clear that the bioassay does have a greater degree of variability. When used to assay a series of patient specimens in plasma, serum, and CSF, the two techniques gave comparable results (Fig. 2). The correlations produced lines identical to the line of identity, with $r^2$ consistently $>0.98$.

**DISCUSSION**

In response to difficulties we encountered when attempting to adapt existing bioassays to assay fluconazole, we developed an alternative technique that is simple and reliable. We have validated this technique for concentrations from 2 to 40 µg/ml, a range that spans what is currently thought to be the clinically relevant range for fluconazole concentrations in body fluids. Compared with the HPLC assay, the bioassay performs...
quite well (Table 2). While it is neither as sensitive nor as accurate as HPLC, it is sufficiently accurate and sensitive for routine clinical work. The comparability of concentrations obtained by bioassay and HPLC from patient specimens is noteworthy and suggests that, unlike the case with itraconazole (13), no active metabolites go unmeasured in the HPLC assay.

The major advantage of the bioassay over the HPLC is its relative simplicity. The HPLC assay is moderately labor-intensive and requires expensive equipment. For example, processing 40 samples requires about 4 h to extract the samples, an evaporation step, and about 7 h of processing time on an automated HPLC machine. The bioassay, while requiring about 6 h of technician time and an overnight incubation to process 40 samples, is easy to perform and requires no special equipment.

An additional advantage of the bioassay is that the analysis can be performed with a very small amount of sample (20 μl per measurement for bioassay versus 1 ml for HPLC). This feature would make bioassay the technique of choice for doing detailed pharmacokinetic studies of fluconazole in small animals or human neonates.

In summary, we have developed and validated a bioassay for fluconazole that, while not as accurate or as sensitive as the HPLC assay, is satisfactory over the clinically relevant range of 2 to 40 μg/ml. Because of its relative simplicity, low sample volume requirements, and lack of a requirement for special equipment and supplies, this assay could easily be used by any clinical laboratory that wishes to offer a fluconazole assay.

**REFERENCES**


**TABLE 2. Comparison of bioassay and HPLC**

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Lower limit of detection (μg/ml)</th>
<th>Avg CV (%)</th>
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<th>Between run</th>
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<td>&lt;12</td>
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<tr>
<td>HPLC</td>
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