Performance Characteristics of Two Bioassays and High-Performance Liquid Chromatography for Determination of Flucytosine in Serum

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We compared the accuracy and precision of two microbiological methods and one high-pressure liquid chromatography (HPLC) procedure used to measure the concentrations of flucytosine in serum. On the basis of an analysis of six standards, all methods were judged reliable within acceptable limits for clinical use. With the biological methods, a slight loss of linearity was observed in the 75- to 100-µg/ml range. Compared with the bioassays, the HPLC method did not present linearity problems and was more precise and accurate in the critical zone of 100 µg/ml. On average, results obtained with patient sera containing 50 to 100 µg of flucytosine per ml were 10.6% higher with the HPLC method than with the bioassays. Standards for the biological assays may be prepared in serum or water.

Flucytosine (5-FC) is a synthetic oral antimycotic agent used in the treatment of several mycotic systemic infections, including cryptococcosis, candidiasis, and chromomycosis. High concentrations of this drug in serum have been associated with gastrointestinal and bone marrow toxicity (12). Presently, 5-FC appears to be the only antifungal agent for which there is a consensus regarding the importance of monitoring levels in blood during treatment (4, 11, 12; M. A. Saubolle, Clin. Microbiol. News1 14:113, 1987). Recently, Stamm et al. recommended that levels be measured 2 h after an oral dose once a week and that the dose be adjusted to maintain a level of 50 to 100 µg/ml. They also observed that toxicity was associated with peak levels greater than or equal to 100 µg/ml for 2 weeks or more (12).

Methods used to measure 5-FC levels in serum include bioassays (2, 6, 11), high-pressure liquid chromatography (HPLC) (1, 3, 7, 13), gas-liquid chromatography (14), fluorometry (9), and an enzymatic method (5, 8). However, the bioassay remains the simplest and therefore the most widely used method. In this study, the performance characteristics of a well diffusion method (2, 11), a cylinder diffusion method (6), and an HPLC method (7) are compared.

5-FC powder obtained from Hoffmann-La Roche, Inc., was used to prepare a stock solution containing 10,000 µg/ml in deionized water. A working solution of 1,000 µg/ml in water was used to prepare two sets of standards containing 6.25, 12.5, 25, 50, and 100 µg of 5-FC in pooled human serum and in water. The serum pool had showed no detectable bioactivity against the indicator organism. Portions (1 ml) of the standards were kept frozen at −70°C in polypropylene plastic tubes until tested. Fifty-seven serum samples were collected from 31 patients and stored at −20 or −70°C until tested. Most of these patients were being treated with amphotericin B and 5-FC for cryptococcosis.

The bioassays were performed on yeast morphology agar (YMA; Difco Laboratories, Detroit, Mich.) in NUNC 240835 bioassay dishes (25 by 25 cm). Saccharomyces cerevisiae ATCC 9763 was used as an indicator organism with both microbiological methods. The well diffusion method has been described in detail by a working group of the British Society for Mycopathology (2) and by Shadomy et al. (11). Standards and patient sera were tested in triplicate by distributing 10 µl of serum into each well. Incubation was performed at 37°C for 18 to 24 h. With the cylinder technique (6), 11 stainless steel bioassay cylinders were set on the surface of the agar, and each cylinder received 100 µl of serum. Samples were tested in duplicate in two separate bioassay dishes incubated at 30°C for 36 to 48 h. Data for the standard curve and patient sera were analyzed by linear regression.

In the HPLC procedure, plasma samples and standards (0.5 ml) were deproteinized with an equal volume of 10% trichloroacetic acid (7). The supernatant was injected through a 20-µl loop (7125 Rheodyne). Analyses were performed on a µBondapak reverse-phase column with Guard Pak C-18 (10-µm octadecylsilane; 250 by 4.6 mm; Waters Associates, Inc.). The mobile phase was a sodium acetate buffer (pH 8.1) at a flow rate of 1.0 ml/min at ambient temperature. The detector was an HP 1040A (Hewlett Packard diode array; wavelength, 276 nm; sensitivity, 0.1 milliunits of absorbance; and threshold, 0.3 milliunits of absorbance).

Statistical calculations for the computation of the mean, coefficient of variation, and least-square linear regression analysis were performed with a TI Programmable 59 calculator (Texas Instruments, Dallas, Tex.) and Statgraphics 1986 version software (STSC, Inc., Rockville, Md.).

To determine precision, each of the six standards prepared in serum and containing 6.25, 12.5, 25, 50, 75, or 100 µg/ml of 5-FC was tested on 17 different days. The coefficients of variation obtained ranged from 2.2 to 4.8, 3.7 to 7.9, and 2.5 to 18.8% for the well, cylinder, and HPLC assays, respectively (Table 1).

Linear regression was used to determine accuracy by comparing the experimental values obtained for the six standards of 5-FC tested with their theoretical values. Magnitudes of proportional and constant errors were assessed by the slope and y intercept of the regression line. Mean results for the entire 5-FC concentration range examined were within 10% for all three methods, with the exception of the 6.25-µg/ml standard tested by HPLC (Table 1). For the well, cylinder, and HPLC methods, respectively, the correlation

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coefficients were 0.996, 0.998, and 1.00; the y intercepts were -2.4, -2.3, and -0.2; and the slopes were 1.1, 1.1, and 1.0, indicating good overall agreement. However, bioassay results showed a slight loss of linearity in the therapeutically important zone of 75 to 100 µg/ml. The HPLC method produced good linearity as well as lower constant and proportional errors.

On average, with 57 patient serum samples, the HPLC procedure produced lower levels than the microbiological methods at low 5-FC concentrations and higher levels at high 5-FC concentrations (Fig. 1). Results with sera containing 50 to 100 µg of 5-FC per ml were 10.6% higher by HPLC than by bioassays. These differences were more and more significant as the concentrations of 5-FC increased. Similar observations were reported by Diario et al. (3) and Warnock et al. (13). Regression data are summarized in Fig. 1. Negative intercepts may be explained by lack of specificity of the biological methods.

To compare standards prepared in serum and in water, both were tested simultaneously in the same dish by the two bioassay procedures. This was repeated on five different days. The zone diameters for each standard in serum and in water were compared by linear regression for the cylinder and well methods, respectively, and produced slopes of 1.002 and 1.001, y intercepts of -0.329 and -0.183, and coefficients of correlation of 0.997 and 0.994, indicating no significant difference between the use of water or serum as a solvent for 5-FC. This is not altogether surprising, since protein binding is not known to occur with 5-FC.

During the study, we noticed that for a given concentration of 5-FC, the zones of inhibition appeared slightly larger in the corners of the bioassay dishes. We therefore decided to measure zones of inhibition produced with a 100-µg/ml standard placed in all cylinders or all wells within a bioassay dish. This was repeated three times. Differences in zone diameters of 0.7 and 1.1 mm between corner and center locations were obtained with the well and cylinder method, respectively. These differences may seem trivial, but they can account for differences of up to 15% in the final results.

Within the conditions of our study, the bioassays and the HPLC assay produced results with an accuracy and a precision adequate for routine clinical application. With the bioassays, a slight loss of linearity observed between 75 and 100 µg/ml indicates that for optimal results, testing should be performed on patient sera containing less than 100 µg of drug per ml. In the HPLC procedure, difficulties encountered were caused by column degradation. Even though the column was protected by a filter, detection of low concentrations of 5-FC (6.25 and 12.5 µg/ml) became gradually more and more difficult. Woolard (15) has also encountered this difficulty, but unfortunately, no remedy has been found. However, accuracy and precision in this range are not critical from a therapeutic standpoint. Despite this problem, HPLC presented lower constant and proportional errors than the microbiological methods. However, although HPLC analysis does present many advantages, including specificity and rapid turnaround time, it is unfortunately not available in most hospital laboratories. In our laboratory, a batch of four patient serum samples required approximately 4 h for analysis by HPLC. The bioassays require approximately 1 h of technician time for the same number of specimens, but results must await overnight incubation at best with the well method and 36 h with the cylinder method. This still allows for day-to-day dose adjustment with the well method, although weekly 5-FC determinations combined with biweekly serum creatinine level checks are sufficient according to Stamm et al. (12).

Although most of our patient sera contained both 5-FC

<table>
<thead>
<tr>
<th>Method</th>
<th>Mean result* (µg/ml) at target value of (coefficient of variation):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6.25</td>
</tr>
<tr>
<td>Well bioassay</td>
<td>5.8 (3.4)</td>
</tr>
<tr>
<td>Cylinder bioassay</td>
<td>6.0 (5.1)</td>
</tr>
<tr>
<td>HPLC assay</td>
<td>7.0 (18.8)*</td>
</tr>
</tbody>
</table>

* Means of 17 determinations for each standard.

* See the text.

FIG. 1. Comparison of results of the microbiological methods with those of the HPLC procedure for determination of concentrations of 5-FC in serum samples from 57 patients.
and amphotericin B, no significant interference was noted with the bioassays on the basis of comparisons with HPLC results. Amphotericin B is known for its poor solubility in water and may have only slight effects on results with sera containing low concentrations of 5-FC. These effects are negligible and do not warrant any efforts to inactivate amphotericin B before testing (3, 4). However, lack of specificity remains one of the major problems with the biological methods. As more and more antifungal drugs are made available, interference problems are more likely to be encountered. Presently it is known that the presence of azoles in serum does interfere with 5-FC determinations (10).

The main advantage in using large plates for bioassays is that all standards and patient sera are on the same plate and are thus subjected to nearly identical conditions. It is of paramount importance that these plates be poured on a level surface. Even so, we have noticed that localization of serum on the plate can influence zone diameters and that uniform distribution of standards and patient sera is also important. For routine testing with the cylinder method, we recommend that the cylinders be distributed in a 3 x 3 pattern, thus allowing for the simultaneous testing of four patient sera with five standards. Since the volume of testing for 5-FC is usually low, a maximum of four patient sera per test should not be a major drawback in the use of this technique. The 6.25-µg/ml standard should be placed in the center of the plate, and the other samples should be distributed at random around the plate. Since this method requires two plates per test, the specimens should be rotated in the second plate so that those located in corners in one plate are located on the sides in the second plate. The 5 x 6 pattern is recommended for the well method since it allows five clinical specimens and five standards to be tested. Specimens are to be distributed at random. From a practical point of view, we believe this method to be slightly superior to the cylinder method. It requires only overnight incubation as opposed to 36 h of incubation with the cylinder method. The use of only one bioassay dish and the testing of samples in triplicate may also provide better precision. In any event, when either of these procedures is set up, the effects of localization, linearity, and recovery levels should be verified.

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LITERATURE CITED


