Bioassay for Serum Itraconazole Concentrations Using Hydroxyitraconazole Standards

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Low concentrations of itraconazole in serum have been associated with therapeutic failure. Variable interpatient bioavailability and detrimental drug interactions with p450 enzyme-inducing agents are well documented. Thus, routine monitoring of serum itraconazole concentrations in patients with life-threatening mycoses is essential for patient care. Present high-performance liquid chromatography (HPLC) methods measure only concentrations of itraconazole and not its active metabolite hydroxyitraconazole. Bioassay methods using itraconazole standards overestimate concentrations in serum as measured by HPLC. We have developed a bioassay for total serum itraconazole and hydroxyitraconazole concentrations using hydroxyitraconazole standards. Itraconazole and hydroxyitraconazole concentrations in 40 clinical samples were assayed by HPLC. Total drug concentrations were measured in the same samples by bioassay with itraconazole or hydroxyitraconazole standards. The correlation of concentrations measured by the last bioassay method with HPLC determinations of both compounds was excellent (r = 0.98, slope = 0.5), with acceptable reproducibility. Small errors were seen at extremes of concentrations. The ratio of hydroxyitraconazole to itraconazole in serum varied from 0.76 to 3.2. The use of hydroxyitraconazole standards rather than itraconazole standards for determination of total itraconazole and hydroxyitraconazole concentrations in serum by bioassay gives accurate and reproducible results that correlate well with total itraconazole and hydroxyitraconazole concentrations as measured by HPLC. Our data show that although hydroxyitraconazole gives larger inhibition zones than itraconazole in bioassay standards, this is not true of patient samples, in which the two compounds make equivalent contributions.

Itraconazole (ITZ) is a triazole antifungal agent with therapeutic activity against a range of yeasts, moulds, and dimorphic pathogens (10). It is used in the treatment of invasive aspergillosis, the endemic mycoses, cryptococcal meningitis, and dermatophyte infections and as prophylaxis against fungal infections in leukemic patients (1–9, 11, 14). Although ITZ is generally free of side effects even at high concentrations in serum (11), therapeutic monitoring must be performed in patients with life-threatening mycoses to identify those with low concentrations in serum. This may be due to variable drug absorption when given by the oral route or to drug interactions caused by p450 enzyme-inducing agents (1, 2, 4–6, 10, 12).

Both biological and high-performance liquid chromatography (HPLC) assays have been described for ITZ (10, 13). HPLC has good sensitivity and does not suffer from interference with other drugs. Bioassay methods, although suitable for use in most microbiology laboratories, lack sensitivity, and the results obtained to date have correlated poorly with those obtained by HPLC (13); bioassay results for ITZ are always higher than HPLC results.

Recently, a biologically active metabolite of ITZ, hydroxyitraconazole (H-ITZ), was described (10). A possible explanation for discrepancies between results from bioassay and those from specific HPLC applied to clinical samples is a failure of HPLC to detect H-ITZ. This may be due to poor recovery of H-ITZ by the solvent used in HPLC sample extraction methods (10), leading to underestimation of biologically active drug concentrations by this method. However, bioassay results are still higher than combined H-ITZ and ITZ concentrations measured by HPLC (10). A more likely explanation of the discrepancy between bioassay and HPLC results is the marked insolubility of ITZ in aqueous solutions. This phenomenon may affect the preparation of bioassay standards in serum, because of the formation of ITZ precipitates when the compound is introduced into an aqueous environment. Such precipitates may adhere to glass or diffuse poorly through agar.

This would reduce the zone sizes of standards, leading to overestimation of drug concentrations in clinical samples in which ITZ behaves differently and may not form precipitates.

HPLC assay techniques are the optimum method of quantitation of ITZ and H-ITZ concentrations in serum. Such facilities are not available in most clinical microbiology laboratories, although bioassay facilities are. A simple bioassay providing an indication of total active drug concentration in serum to identify those patients with problematic absorption or metabolism is required in hospital laboratories. The current bioassay method does not do this; we have therefore examined alternative methods of determining concentrations in serum.

H-ITZ is reported to have biological activity similar to that of ITZ (10) but has greater aqueous solubility and diffusibility. We set out to examine whether total serum ITZ and H-ITZ concentrations can be accurately measured by bioassay with standards prepared with H-ITZ rather than ITZ. We compared combined serum H-ITZ and ITZ concentrations as determined by HPLC with concentrations obtained by this new bioassay method for 40 serum samples from patients receiving ITZ therapy.
MATERIALS AND METHODS

Reagents. ITZ, H-ITZ, and internal standard for HPLC (R51012) were obtained from the Janssen Research Foundation (Beerse, Belgium). All solvents were obtained from BDH (Dorset, United Kingdom) and were Analar grade, except methanol, which was HPLC grade. Stock solutions (200 mg/liter) of ITZ, H-ITZ, and R51012 for HPLC were prepared by dissolving 10 mg of compound in a small volume (0.5 ml) of dimethylformamide and making the solution up to 50 ml with methanol in volumetric glassware. Solutions were stored at −20°C. ITZ stock solution for bioassay (10 mg/ml) was prepared in aqueous 0.05 M HCl (50:50) with heating at 60°C for 2 h or dimethyl formamide-methanol as specified. The H-ITZ stock used for HPLC was also used for bioassay.

Serum samples. Pooled serum from normal human subjects was used to prepare drug standards for HPLC and bioassay. Forty serum samples were obtained from patients receiving ITZ as sole antifungal therapy. Samples were from patients with different medical problems including chronic respiratory disease, AIDS, prophylaxis of leukemia, and prophylaxis following bone marrow or solid-organ transplants.

All 40 samples were tested by HPLC and by bioassay with H-ITZ standards, and 36 samples were tested by bioassay with ITZ standards (there was insufficient serum for four samples).

HPLC. (i) Standard preparation. Standards for HPLC were prepared as follows. The stock solutions of ITZ and H-ITZ were diluted in methanol to produce a range of dilutions of each compound from 20 to 0.2 mg/liter. Samples (1 ml) of pooled serum were mixed with 1 ml of 0.05 M sodium borate, and then 100-μl volumes of the methanolic stock solutions of ITZ and H-ITZ were added to the pooled serum to give standards containing final serum ITZ and H-ITZ concentrations ranging from 2 to 0.02 mg/liter.

(ii) Preparation of samples from patients. Samples (1 ml) of each patient's serum or of pooled serum were mixed with 1 ml of 0.05 M sodium borate. Each specimen serum and the standards were then spiked with 100 μl of internal standard (final concentration, 0.5 mg/liter). Each patient sample was spiked with 200 μl of methanol so that samples and standards contained equivalent volumes (300 μl) of methanol.

(iii) Assay procedure. The method of extraction was that developed by Janssen Research Foundation. Samples and standards were extracted twice with heptane-isooamyl alcohol (4 ml, 95:5 [vol/vol]) on a rotary mixer. These proportions give better recovery of H-ITZ (10) than the 98.5:1.5 [vol/vol] mixture used by Warnock et al. (13). The combined organic phases were then back extracted with 1 M H2SO4 (2 ml). After removal of the organic phase, the aqueous phase was made alkaline by the addition of concentrated NH4OH (0.6 ml). The aqueous phase was then extracted twice with heptane-isooamyl alcohol (2.5 ml, 95:5 [vol/vol]). The organic phases were combined and dried under a stream of nitrogen at 60°C, and the residue was taken up into 100 μl of water-acetonitrile (40:60) and then assayed by HPLC.

(iv) Chromatography. The chromatograph consisted of a Waters model 600A pump connected to a C18 guard column and a steel cartridge (100 mm by 4.5 mm inside diameter) containing Hypersil octylcyclceline silane (particle size, 3 μm) (Alltech, Carchfort, United Kingdom). The mobile phase consisted of water-acetonitrile (40:60) containing 0.03% diethylylamine; it was adjusted to pH 7.8 with dilute orthophosphoric acid and then filtered and degased under reduced pressure before use. The flow rate of the mobile phase was 1 ml/min, and 20-μl aliquots of sample or standard were injected onto the column via a rheodeyne valve. A fixed-wavelength UV absorbance detector (254 nm) was used to continually monitor the absorbance of the eluent. Plotting and integration of the chromatogram were performed with a Waters M730 data module.

(v) Interpretation. For each serum standard, the peak areas of ITZ, H-ITZ, and the internal standard were calculated. The peak area ratios (PAR) of ITZ to internal standard and H-ITZ to internal standard were calculated. Calibration lines were prepared for ITZ and H-ITZ by plotting the PAR for each drug against the drug concentration on log-log paper to ensure linearity, and a linear regression equation was derived by computer with the program Minitab (Minitab Data Analysis Software, Philadelphia, Pa.). ITZ and H-ITZ concentrations in patient serum samples were determined by calculating the PAR for each drug and then substituting the value obtained into the appropriate regression equation.

Bioassay. (i) Method. ITZ serum standards were prepared by dilution of the 10-mg/ml stock solution initially in water and finally in pooled serum to give concentrations of 0.2 to 25.6 μg/liter. H-ITZ standards (range, 0.16 to 10 μg/liter) were prepared by dilution of the stock solution with serum.

The medium used for the bioassay was yeast nitrogen base (Difco, Surrey, United Kingdom) supplemented with 1% glucose and 0.5% trisodium citrate and solidified with 2% Oxoid agar no. 1 (Unipath Ltd., Basingstoke, United Kingdom). The medium was prepared and seeded with Candida pseudotropicalis (C. kefyr) San Antonio (a kind gift of D. Stevens), as described by Warnock et al. (13). Forty-microliter volumes of serum and standards for H-ITZ or ITZ standards were applied in triplicate in a randomized fashion to 8-mm wells cut into the plate. The plate was allowed to stand for 30 min at room temperature and then incubated at 37°C for 18 h. The inhibition zone diameters were measured with dial calipers, and a standard curve was constructed by plotting the mean zone diameter of standards against the log10 drug concentration. Drug concentrations of patient samples were then determined by reference to the standard curve. When the concentration in serum was higher than the value of the top standard, the assay was repeated following dilution of the patient's serum in normal human pooled serum.

To directly compare the activities of ITZ and H-ITZ, identically prepared standards of each drug were prepared directly in serum from methanolic stock solutions. Both sets of standards were tested at the same time on the same assay plate.

(ii) The influence of solvents and containers on bioassay standard preparation. To investigate whether preparation of ITZ bioassay standards was a serious source of discrepancies between HPLC and bioassay results, ITZ standards prepared in a number of different ways were compared. Stock solutions of ITZ (5 mg/ml) were made in acetone-HCl and dimethyl formamide-methanol as described above. Further dilutions of each stock were prepared in either water or methanol. Four serum standards (25.6, 6.4, 1.6, and 0.4 μg/liter) were then prepared from these solutions. All four standards were tested in duplicate on the same bioassay plate. After incubation overnight, the zone diameters were measured, and the mean zone size was plotted against the log10 drug concentration.

Similarly, four ITZ serum standards were prepared from the acetone-HCl stock solution in either glass, polystyrene, or polypropylene containers to assess the effect of container material on zone diameter.

(iii) Reproducibility of the bioassay method. The reproducibility of the bioassay method was assessed by assaying four standards on different days and calculating the percent error for each sample.

(iv) MIC determinations of bioassay indicator organism.

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The MICs for the C. pseudotropicalis strain used in the bioassay of H-ITZ and ITZ were determined by a broth macrodilution method with drug concentrations in the range of 0.016 to 8 mg/liter and an organism inoculum of ∼1 × 10^5. The medium used was yeast nitrogen base with 1% glucose and 0.59% trisodium citrate, and the inoculated medium was incubated in air for 48 h at 37°C.

RESULTS

As determined by the HPLC system described, capacity factors for H-ITZ, ITZ, and the internal standard were 2.7, 7.4, and 10.8, respectively. A linear relationship between log_{10} PAR and log_{10} drug concentration of both ITZ and H-ITZ was demonstrated for drug concentrations between 20 and 2,000 µg/liter. The lowest level of quantifiable drug was 20 µg of both ITZ and H-ITZ per liter. In all patient samples, both ITZ and H-ITZ peaks were detectable. The ratios of H-ITZ to ITZ in sera varied from 0.76 to 3.2. ITZ and H-ITZ concentrations in serum were combined to give the total drug concentration (TDC) for comparison with results obtained by bioassay.

Drug concentrations in the 40 patient samples measured by bioassay with ITZ standards were approximately sevenfold higher than the HPLC TDC. The correlation coefficient of the two methods was 0.85. By linear regression analysis, the equation HPLC TDC = 0.14 × bioassay concentration + 1.15 was obtained. High values in the bioassay were prone to considerable error in comparisons with HPLC results (Fig. 1). If the bioassay values below 10 mg/liter are compared with HPLC results, the correlation is 0.79 (n = 23); for bioassay samples with drug concentrations of >10 mg/liter, the correlation is 0.58 (n = 13).

In a comparison of the bioassay values of the 40 samples obtained with H-ITZ standards with HPLC TDC, the correlation coefficient was 0.98; by linear regression analysis, the equation HPLC TDC = 2.09 × bioassay concentration − 0.053 was derived (Fig. 2).

For each sample, the drug concentration obtained by bioassay with H-ITZ standards was corrected by use of the expression 2.09 × bioassay concentration − 0.053. The derived result was then compared with the HPLC TDC. The percent error between values derived from the equation and HPLC TDC values was determined; 22 of 40 (55%) samples had an error of <10%, and 33 of 40 (83%) samples had errors of <20%. Five of the remaining seven samples gave percent error values between 20 and 30%. Two samples gave errors of 30 and 31%, respectively. In both of these instances, HPLC and bioassay values were at the extremes of the concentration range. There was no correlation between the percent error and the ratio of H-ITZ to ITZ or between the percent error and the bioassay value obtained by use of H-ITZ standards.

Serum ITZ standards prepared from two different stock solutions each diluted in either water or methanol before final dilution in serum were examined by bioassay. Calibration lines for all four sets of standards were identical (data not shown). Similarly, calibration lines obtained for ITZ standards prepared in glass, polythene, or polypropylene containers were identical. A comparison of identically prepared H-ITZ and ITZ serum standards assayed together showed that H-ITZ standards gave larger zones of inhibition than did equivalent concentrations of ITZ (Fig. 3).

The MIC of both ITZ and H-ITZ for C. pseudotropicalis was 0.06 mg/liter in the broth dilution test, showing that both the parent compound and the metabolite have similar antifungal activities against this isolate.

Four serum samples were assayed by bioassay with H-ITZ standards on four separate occasions. The method is clearly reproducible, with a maximum mean interexperiment error of 7.4% (Table 1).

DISCUSSION

For determination of serum ITZ concentrations in the clinical setting, an assay which is sensitive, precise, and rapid and can be performed in most microbiology laboratories is needed. In some studies, bioassay results with ITZ standards have approximated HPLC results (ITZ and H-ITZ combined) (10), although the method was not described. The bioassay...
Bioassay results (mg/l) using hydroxy-itraconazole standards

HPLC results (mg/l)

FIG. 2. Comparison of TDCs as determined by HPLC and bioassay with H-ITZ standards: HPLC TDC = 2.09 × bioassay concentration − 0.05; r = 0.98.

Currently in use with standards prepared with ITZ gives results markedly higher than those obtained by HPLC (2, 13). In addition, we found the accuracy of the bioassay to be poor at high drug concentrations. We sought to develop a bioassay which gives results more closely approximating those obtained by HPLC and to examine possible reasons for the elevated bioassay results obtained with ITZ standards.

Forty patient serum samples were assayed by bioassay with H-ITZ standards; total serum drug concentrations were approximately half of the TDCs measured by HPLC. A correction factor derived by regression analysis is therefore required (in this study, the correction factor was ≈ 2). This factor may change under different test conditions, and may need validation with each different bioassay system (different test organism or culture medium). After application of the correction factor to bioassay results, there was excellent correlation with concentrations determined by HPLC. Concentrations in serum therefore could be predicted with much greater accuracy by use of this bioassay rather than the ITZ standard bioassay. The relationship between bioassay and HPLC results was valid over a wide range of serum concentrations and was unaffected by the ratio of H-ITZ to ITZ. This suggests that ITZ and H-ITZ in biological samples have equivalent activities in the bioassay. However, in spiked samples the two drugs behave differently from each other and also from drug in patient serum with respect to diffusion kinetics in an agar plate.

With the H-ITZ bioassay, 88% of sera gave values within 20% of those measured by HPLC. This level of error is most
likely due to the inherently subjective aspects of bioassay methodology such as zone diameter measurement errors; however, minor variation in sample extraction and/or HPLC measurements may have contributed some discrepancies.

Bioassay values with H-ITZ standards were approximately half those obtained by HPLC. The reason for this is unknown but may be due to a lower amount or different affinity of protein binding of H-ITZ in spiked samples than in serum samples, allowing a greater degree of diffusibility.

The reason why ITZ standards give poor results is unclear. We have shown that solvent systems and the type of container used did not affect standard potency. Differences in protein binding between H-ITZ (99.5%) and ITZ (99.8%) (9a) are unlikely to account for the marked difference in zone sizes between these compounds. We suggest that diffusion of spiked drug in ITZ standards through agar is poor because of precipitation of free drug in the standard or in the aqueous environment of the agar gel.

H-ITZ is the principal metabolite of ITZ and is formed by first-pass metabolism (10). In vitro studies show that it has antifungal activity similar to that of the parent compound (10), and we obtained identical MICs of both ITZ and H-ITZ for the C. pseudotropicalis assay organism used in this study. In vivo H-ITZ appears to be less active than ITZ, possibly because of differences in pharmacokinetics (10). H-ITZ has a shorter half-life than ITZ, although concentrations of the metabolite in serum are often twice those of the parent compound (10). We found the ratio of the metabolite to the parent compound in clinical samples to vary from 0.76 to 3.2, the lowest ratios tending to occur at high TDs. Little is known of the factors which affect the metabolism of ITZ to H-ITZ or the distribution of H-ITZ in tissues and body fluids or, indeed, of the significance of H-ITZ in serum.

There are some data suggesting a poorer outcome from fungal infections treated with ITZ if serum concentrations are low or undetectable (1, 5, 6, 12). It has proven difficult to establish a cutoff value which would reliably distinguish responders from nonresponders. Most data are derived from bioassay with ITZ standards or from HPLC values for ITZ alone.

HPLC concentrations of ITZ in serum in a steady-state system below 0.25 mg/liter for prophylaxis in neutropenia predicted failure (1). ITZ bioassay concentrations below 1 mg/liter predicted persistently positive cerebrospinal fluid cultures in cryptococcal meningitis in AIDS (5). Among six patients with allergic bronchopulmonary aspergillosis who received 400 mg of ITZ daily, the mean concentration in serum was 5.1 mg/liter. Five responded to ITZ and one failed; he had a serum drug concentration of only 1.8 mg/liter (6). Interactions with rifampin, phenytoin, phenobarbital, and carbamazepine lowered serum ITZ concentrations to almost zero with loss of antifungal efficacy in a number of patients (12). As H-ITZ is biologically active and circulating concentrations of this compound vary from 76 to 320% of ITZ concentrations, interpretation of existing data relating to concentrations in serum requires reevaluation. Likewise, the documented toxicity observed with high oral doses of ITZ which did not correlate with concentrations in serum determined by ITZ HPLC requires reevaluation (11).

We propose that H-ITZ is more appropriate for preparing standards for bioassay of sera because of its greater solubility and diffusion characteristics combined with its equal biological potency against the assay organism. Results with the H-ITZ bioassay correlate very well with results obtained by HPLC over a wide range of drug concentrations and are unaffected by the ratio of ITZ to H-ITZ in serum. A disadvantage of bioassay is that it does not separately quantify ITZ and H-ITZ but gives the TDC. This is of little concern to practitioners unless differential activity between ITZ and H-ITZ against certain organisms is observed or particular toxicities are associated with one or the other component. A further disadvantage is the need for a correction factor; however, as this factor approximates to 2 we feel that this will not affect the utility of the assay in the clinical setting. Bioassay is a technique which can be performed in the majority of microbiology laboratories, and by using H-ITZ rather than ITZ to prepare standards for assay, total active drug in serum can be easily and accurately measured.

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TABLE 1. Reproducibility data for serum samples each tested by bioassay with H-ITZ standards on four separate occasions

| Sample | Mean ITZ conc ± SD | Mean % error |
|--------|-------------------|--------------|
| 1      | 0.86 ± 0.08       | 7.4          |
| 2      | 1.42 ± 0.08       | 3.9          |
| 3      | 1.46 ± 0.12       | 6.1          |
| 4      | 3.8 ± 0.12        | 2.6          |
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