THE INFLUENCE OF pH ON THE SOLUBILITY OF MICONAZOLE AND ITS EFFECT ON SURVIVAL OF *C. ALBICANS*

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Summary

The in-vitro activity of miconazole against *Candida albicans* (NCPF 3262) was investigated using Time-survival curves. An HPLC assay was also developed to produce a pH-solubility profile which showed that miconazole solubility varied from (2.5 ± 0.3) mg/L at pH 12 to (28.9 ± 0.6) mg/L at pH 5. Time survival curve determinations demonstrated a relationship between miconazole concentration and the area under the curve (AUC). In controlled conditions using buffered aqueous solutions of miconazole (8 mg/L), a change in pH from 8 to 5 resulted in an increase in the antifungal activity of miconazole. Experiments performed in buffered YNB using the same concentration of miconazole showed that a change in pH from 7 to 5 resulted in a decrease in miconazole antifungal activity. Such results indicate that apart from the well known inhibitory effects that the growth media have on azole activity, pH at which tests were performed may also influence growth of C. albicans in batch culture.

Keywords

Candida, miconazole, pH, solubility, viable counts, area-under-the-curve (AUC), high performance liquid chromatography (HPLC).

Introduction

Time-survival curves have recently been used to evaluate the effect of variation of the test conditions on antimicrobial susceptibility of microorganisms to antimicrobial agents. Interpretation of this kind of data entails the comparison of the pattern of survival, rates of kill or reduced growth and viable count at 24h[1,2].

Every organism has a range of pH over which growth is possible and an optimal pH at which growth is most favoured, different microorganisms are known to have different tolerances to environmental change including pH. Fungi are reported to be more tolerant to pH changes than bacteria[3]. Miconazole is an azole antifungal agent with a pK_a of 6.7[4],that is used in the treatment of candidosis[5]. Reports in literature indicate that the unprotonated form of the drug is the one that is responsible for antifungal activity[6].

A series of experiments to investigate the effect that pH has on *in vitro* activity against *Candida albicans* in a defined growing medium were designed. To complement this *in vitro* microbiological work a simple reversed phase High Performance Liquid Chromatography (HPLC) assay was developed. Reports in the literature suggest that the chromatographic techniques represent the favourite approach for quantitation of azoles such as miconazole[7]. The assay was a simple chromatographic separation involving a binary mixture on a reversed phase column. From the data obtained a pH solubility profile of miconazole in aqueous medium constructed. The data obtained was used to investigate the possibility of a correlation between miconazole activity and pH.

Method

Materials required for the HPLC assay:
The apparatus used throughout the HPLC assay for the determination of the solubility of miconazole in aqueous solution (at pH values that range from 5 to 8) consisted of: An Ultracentrifuge (MSE® Superspeed 50) fitted with a 30° angle set to reach an average RCF of 100,000 **g** and a rotor speed of 35,000 rpm, a Shimadzu® L.C. 6A chromatograph fitted with a rheodyne injector and a 20 ml injection loop, a Shimadzu® UV-visible variable wavelength detector, a 25 cm x 4.6 mm i.d., 5 µm particle size reverse phase column (Spherisorb® ODS 2. Phase Separations, UK), and a single channel strip chart recorder. (Gallencamp®, UK). Reference materials, chemicals and solvents used were, miconazole (Janssen®, Belgium), ketoconazole (Janssen®, Belgium), dimethyl formamide (DMF) (Sigma®, UK), ammonium phosphate (BDH®, UK) deionised water, methanol HPLC grade (Labscan®, UK). The mobile phase selected for this assay consisted of a 95% methanol and 5% 0.05M ammonium dihydrogen phosphate mixture. After mixing, the mobile phase was filtered through 0.45 µm PTFE filters to degas and remove any residual particulate matter.

Materials required for the microbiology experimentation:
C. albicans (NCPF 3262), maintained on Bacto Yeast Morphology Agar (BYMA, Difco, USA) slopes at 4°C., Yeast Nitrogen Base (Bacto® YNB, Difco, USA) prepared according to the Wickerham Formula[8], Sorensen's Buffer, filtration apparatus (Millipore®, USA), 0.22 µm and 0.45 µm filters and stock solutions of miconazole (1000mg/L) which were allowed to self sterilise[9].

Methods used in the HPLC assay:
Samples used in the HPLC assay were prepared from a stock solution of miconazole (1000 mg/L) in methanol (100% $^{v}/_{v}$, HPLC grade). A series of standard solutions that ranged from 0.5 to 10 mg/L were used to construct calibration curves of miconazole in Sorensen's buffer (pH 7). The solutions were vortexed for 1 min and a 2 ml aliquot taken and shaken, on a rotary mixer, for one hour with a volume (2 ml) of chloroform. The extraction process was repeated a second time with a further 2 ml volume of chloroform. The chloroform layers were removed, dried under a stream of nitrogen at a constant temperature of 35°C and reconstituted in a total volume of 1 ml of a 2.5 mg/L solution of ketoconazole (internal standard) in mobile phase, previously filtered through 0.45µm PTFE filter. Triplicate injections (20 µl) were made for each solution, peak heights for miconazole (m) and ketoconazole (k) were measured manually and the mean peak height ratio (m/k) and standard deviation (sd) were calculated for each concentration. Samples for analysis consisted of saturated solutions of miconazole in a 1% solution of methanol in Sorensen's buffer, prepared at pH 5, 5.5, 6.0, 7.0 and 8.0 respectively. The saturated solutions were left for 24h in a shaking water bath set to 25°C and 100 strokes/min. About 20 ml of each solution was placed in ultracentrifuge tubes that were balanced to the nearest 10 mg. The solutions were spun down for 90 min at an average RCF of 100,000 **g**. An aliquot (2 ml) of supernatant was taken from each tube and subjected to a similar extraction procedure and then finally reconstituted in 1 ml of mobile phase containing 2.5 mg/L ketoconazole internal standard. This process was carried out over forty eight hours, during which a total of three sets of test solutions for each pH value were prepared. On each test day, the calibration standards and the test solutions were run. Reversed phase chromatography using an octadecylsilane (ODS) column together with a mobile phase that consisted of 95% methanol and 5% 0.05M ammonium dihydrogen phosphate was used throughout the experiment. The mobile phase was pumped through the chromatograph at ambient temperature, at a rate of 2 ml/min and the peaks detected at a wavelength of 230 nm with 0.02 attenuation value. Traces obtained from the manually injected samples (Figure 1) were recorded on a strip chart recorder set at a chart speed of 5 mm/min. The miconazole concentration in each sample and therefore solubility was determined from the measurement of miconazole and ketoconazole peak heights.

Figure 1: HPLC Chromatogram of miconazole (m) and ketoconazole (k).

Methods used in the microbiology experimentation:

MIC and MFC determinations were carried out using a (macro) broth dilution technique, using a two-fold dilution series.

The experiments designed to investigate the survival of *Candida albicans* challenged with miconazole in YNB and aqueous medium required the same materials needed for the MIC and MBC determinations, the procedure adopted was as follows:

One loopful of *C. albicans* from a YMA slope was inoculated into buffered YNB (100 ml) and placed overnight in a shaking water bath (37°C, 150 strokes/min).The experimental challenge was prepared by addition of 10 ml overnight culture into warmed buffered YNB and grown until an $O.D._{.540}$ value of 0.5 was obtained. This culture (100 ml) was collected on a 0.45 µm filter and rinsed with warmed media. The culture was then resuspended in 10 ml of warmed media.

Sterile solutions of miconazole, 8 mg/L and 16 mg/L in buffered YNB and 1% DMF, were prepared by hundred-fold dilution of the appropriate sterile stock solutions (in 100% DMF) with sterile buffered YNB.

A volume (1 ml) of culture was used to inoculate the test media that consisted of miconazole in buffered YNB and 1% DMF. A control flask that did not contain miconazole was also inoculated with this culture The flasks were then incubated for a total of 24h during which samples were taken for viable count determinations. Throughout the exercise, the pour plate technique was used exclusively for viable count determinations.

In this set of experiments replicate cultures exposed to concentrations of miconazole were used for each pH and concentration.

Results

The results of the theoretical solubilities of miconazole in buffer, obtained with the use of the Henderson-Hasselbalch equation[10], and those determined by HPLC varied with changes of pH. Both sets of data show that the solubilities of miconazole at pH values above 6 are low (Table 1) and therefore media that contained more than 3.99 mg/L miconazole in solution could not be prepared at pH 6 and above. Due to these results, the process of double membrane filtration, as a means of sterilisation, could not be directly and safely applied to aqueous growing media (with 1% $^{v}/_{v}$ organic solvent) that contained more than 3.99 mg/L miconazole.

pH	Mean solubility* (mg/L)	Theoretical solubility† (mg/L)
5.0	28.90	129.84
5.5	10.28	42.79
6.0	3.99	15.27
7.0	2.79	3.81
8.0	2.37	2.67

*(HPLC determination)
†(Henderson-Hasselbalch equation calculation)

Table 1: Mean solubility profile determined by HPLC and the theoretical pH solubility profile for miconazole estimated by the Henderson-Hasselbalch equation as functions of pH.

Data from MIC and MFC determinations at pH 6 and 7 showed that not more than a two-fold difference was obtained with determinations carried out at pH 6 and 7. Time survival experiments were performed with the MFC values obtained at these two pH values (Table 2).

The data obtained from the time-survival experiments, carried out in YNB, was used to calculate the Area Under the Curve (AUC) with the use of the Trapezoidal rule (Table 3). In YNB, at pH 6 and 7, at both 8 and 16 mg/L, the algebraic sum of the AUC obtained is negative thus indicating that the deleterious effect exceeded that of growth. At pH 5 and 5.5 the algebraic sum of the AUC is positive. It should be noted that the AUCs obtained for both 8 and 16 mg/L have been estimated to have the same area.

The results obtained from experiments carried out in an aqueous medium (Tables 4), indicate a relationship between the extent of the killing process of the antifungal agent and pH of the medium.

pH	MIC (mg/L)	MFC (mg/L)
6.0	4.00	16.00
7.0	4.00	8.00

Table 2: MIC and MFC value for miconazole (mg/L) against *C. albicans* (NCPF 3262).

pH	AUC Conc. Miconazole (Mean, ± sd) 8mg/L	16mg/L
5.0	+ (11.90 ± 0.23)	+ (11.22 ± 0.61)
5.5	+ (3.36 ± 0.42)	—
6.0	- (2.25 ± 8.85)	- (43.92 ± 2.40)
7.0	- (18.62 ± 16.47)	- (32.61 ± 26.10)

Table 3: The relationship between AUC, miconazole concentration and pH of growing medium for cultures of *C. albicans* growing in buffered YNB.

pH	AUC (Mean, ± sd)
5.0	+ (17.53 ± 1.09)
5.5	+ (12.95 ± 0.91)
6.0	- (11.18 ± 3.69)
7.0	- (4.70 ± 1.86)
8.0	- (3.22 ± 1.81)

Table 4: The relationship between AUC and pH of cultures of *C. albicans* growing in aqueous solutions of miconazole (8mg/L) containing 1% DMF.

Discussion

The sensitivity of *C. albicans* (NCPF 3262) to miconazole was assessed by MIC and MFC determinations. This isolate was sensitive to miconazole and the values were within the expected sensitivity range according to previous in-vitro determinations[11]. Results at pH 6 and 7 were similar with no result differing by more than one dilution.

The inocula used throughout were obtained from cultures that were in log phase It has been suggested that the most effective killing occurs with organisms in mid-log phase. Less effective killing was reported with late log phase organisms with the least effective killing occurring with organisms in the stationary phase; which factors could influence the outcome of tests[12].

Time-survival determinations. give a better understanding of the changes in viability that can occur over the test period and measurement of the AUC permits an overall view of the time-survival curve and has recently been used to examine the activity of antibiotics against *Ps. aeruginosa* [5]. The results of the HPLC assay show that there is a direct relationship between pH and solubility of miconazole in a liquid medium. In other microbiological experimentation it has been shown that at pH 7, that there is a relationship between miconazole concentration and both rate of kill and AUC. A rapid increase in AUC was noted in the lower concentration range, but as concentration increased, the effect was considerably reduced[13].

These effects may be due to the fact that at pH 7 the solubility of miconazole in an aqueous medium is low. The actual solubility ranges between 2.8 and 3.8 mg/L, depending on whether the solubility is taken as that previously determined by HPLC or that determined by application of the Henderson-Hasselbalch equation. Above pH 6, solubility of miconazole in an aqueous medium is thus a problem when experimental procedures are designed to investigate the effect that specific concentrations of miconazole have on the behaviour of batch cultures of *Candida albicans*. Low solubility of miconazole in aqueous media, therefore did not permit the standard sterilisation by filtration of solutions, or rather suspensions, of miconazole in aqueous buffer solution or in aqueous buffered solutions of YNB. The two constituents had to be prepared separately and sterilised and then mixed together under aseptic conditions[9].

Apart from solubility, the deleterious effect exerted by miconazole may also be dependent on the degree of ionisation of the molecule. With a pK_a of 6.7 miconazole exists predominantly in its unionised form at pH 7 ($\alpha_{pH\ 7}$ = 33.4%), which moiety is reported to be required for antimicrobial activity; in this case, direct lethal action that causes damage to *C. albicans* cells. This has led to the opinion that pH regulation of the direct lethal action of miconazole against *C. albicans* reflects the influence of the H^+ ion on drug molecules, rather than on yeast cells. In fact non-protonated miconazole appears to be required for direct lethal action (DLA)[6].

The effect of pH on solutions of miconazole (8 and 16 mg/L in 1% DMF as solvent) in buffered aqueous solutions or in YNB (buffered to the required pH with Sorensen's buffer) were markedly different. In YNB, the efficacy of miconazole increased with increasing pH, in which conditions solubility decreased and a the degree of ionisation decreased. In contrast, the efficacy of miconazole in aqueous solution, at lower pH was greater.

In a growth medium such as YNB, components of the growth medium itself may exert some effect on the drug. As an example, the medium may adsorb the drug thus reducing the active concentration in solution. A further factor associated with assessment of time-survival in growth medium compared to the aqueous solution, is the behaviour of the organism. The growth medium may protect the organism and aid its survival.

The influence of media on the activity of azoles is well known[11]. However, it has not been previously demonstrated that this is complicated by the pH of the test conditions and this may account for some of the variability in the results reported.

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