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Measurements of antifungal levels in corneal tissue: a simplified bioassay for amphotericin B

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Abstract. Measuring precise antifungal levels in the cornea with broth-dilution bioassays is difficult, as standard techniques involving visual determination of endpoints are hindered by corneal debris. To increase the precision of the measurement, we modified the sample preparation for bioassay of rabbit corneas treated with subconjunctival amphotericin B. Endpoint determination and variance were compared for a freshly thawed corneal suspension and the supernatant after 24 h equilibration; bioassay of the corneal suspension after 24 h equilibration served as an additional control. All endpoints were read visually in a masked fashion and were verified by culture. The three methods gave comparable endpoint values with equivalent degrees of variance. Amphotericin B levels were consistent by both visual and culture determination; however, endpoints were clearly visible and easier to read for the supernatant. Visual determination of the endpoints for the supernatant following 24 h equilibration simplified and ensured the precision of the bioassay technique.

Introduction

Broth-dilution bioassay techniques for measuring levels of antifungal agents in the cornea are valuable but difficult to perform. The presence of corneal debris in the broth renders visual determination of endpoints imprecise. This problem is particularly evident when the levels of biologically active drug being measured are low, as is often the case with antifungal drugs in the cornea [1]. In an effort to increase the accuracy of the bioassay technique, we compared a standard method of sample preparation for bioassay with one modified to enhance visual endpoint determination. We evaluated the variance and difficulty of the two techniques using visual inspection and culture confirmation of the endpoints.

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Materials and methods

Six Dutch-belted rabbits (1.5–2.5 kg) were anesthetized with intramuscular ketamine hydrochloride (Bristol Laboratories, Syracuse, N.Y.) and xylazine hydrochloride (Fermenta Animal Health Co., Kansas City, Mo.). Topical anesthesia was achieved with 0.5% Opthaine (Squibb, Princeton, N.J.). A Kimura spatula was used for complete removal of the epithelium from both corneas of each rabbit. A 300-µl dose of Fungizone (1,500 µg amphotericin B in sodium deoxycholate; Squibb) was injected subconjunctivally via a 30-gauge needle adjacent to the superior limbus and anterior to the superior rectus muscle of each eye.

Rabbits were randomly divided into two groups of three animals each. One group was killed with T-61 euthanasia solution (Taylor Pharmacal Co., Decatur, Ill.) 72 h following subconjunctival injection of amphotericin B; the corneas from these animals comprised sample set 1. The second group was killed using T-61 96 h following drug administration; the corneas from these rabbits made up sample set 2. Each cornea was excised at the limbus, cut into small pieces, and suspended in 19 times its weight of distilled water. All samples were frozen at −70° C until the time of assay. The corneal tissue was thawed to room temperature and ground for three 10-s intervals with a Tissumizer (Tekmar, Cincinnati, Ohio), and bioassays for amphotericin B were performed sequentially.

Standard bioassay

Aliquots of 90 µl from each sample were removed immediately after grinding and added to 90 µl of double-strength M-3 medium (Difco, Detroit, Mich.) in a microtiter plate. Ten serial 2-fold dilutions of each sample were made in single strength M-3 medium. Duplicate rows of each sample, as well as duplicate rows of standards containing from 10 to 0.01 µg/ml amphotericin B, were used in each plate. The last wells in both rows of standards contained only M-3 medium and served as drug-free controls. All wells were inoculated with 10 µl of a *Candida albicans* suspension (1/10 dilution of a 95% T-suspension at 550 nm) made from overnight cultures. The plates were sealed and incubated at 35° C for 24 h at 100% relative humidity.

Bioassay of corneal suspension after 24 h equilibration

After removal of aliquots for the standard bioassay, the remaining corneal suspensions were allowed to equilibrate at room tempera-

ture in a dark environment for 24 h. Aliquots of 90 µl from each sample were then removed, and broth-dilution bioassays were performed as described above.

Bioassay of supernatant after 24 h equilibration

The remaining equilibrated samples of corneal suspension were centrifuged for 5 min in an International Clinical centrifuge. Aliquots of supernatant (90 µl) were removed for bioassay as described above.

Endpoint determination

For each of the three methods, endpoints were determined visually and by culture confirmation. After 24 h incubation the plates were examined from below with a reflective viewer, and the highest dilution of sample that inhibited visible growth of *C. albicans* was designated as the endpoint of the bioassay. The minimal inhibitory concentration for the indicator organism was simultaneously determined from inspection of the two rows of standards on each plate. The concentration of amphotericin B in each corneal sample was calculated as the product of this minimal inhibitory concentration and the reciprocal of the endpoint dilution for that sample [3].

Visually determined endpoints for each corneal specimen were verified by plating the entire contents of each microtiter well onto individual Sabouraud's dextrose agar plates (BBL, Cockeysville, Md.). After incubation at 25° C for 48 h, the plates were read. The culture endpoint was defined as the dilution on the plate directly preceding the first plate that exhibited confluent growth.

Results

As determined by visual endpoints, amphotericin B levels in sample set 1, were equivalent for all three bioassay techniques (Table 1). Overall drug levels in sample set 2 showed some variability between the three methods (Table 2). For individual corneas, however, this variability was the result of a difference of only one well between visually determined endpoints. This was seen with both high and low levels of amphotericin B. Similarly, for both sample sets, endpoints established by plate culture were either equivalent or differed by no more than one well for the three methods of bioassay.

For all three methods, culture determination of endpoints was consistent with the results obtained by visual inspection (P, not statistically significant). Variability in amphotericin B levels, calculated from the two endpoint determination techniques, again represented a maximal difference of one well between endpoints.

Discussion

Amphotericin B levels determined by bioassay of 24-h equilibrated supernatant were equivalent to those obtained by bioassay of both freshly thawed cornea and corneal samples that had been equilibrated for 24 h, de-

Table 1. Corneal amphotericin B levels determined from visual and culture endpoints 72 h following subconjunctival injection (1,500 µg/300 µl)

Sample set 1 Corneas	Methods									
	Freshly thawed (µg/g tissue)		24 h equilibration (µg/g tissue)		Supernatant (24 h) (µg/ml)		Visual		Culture	
	Visual	Culture	Visual	Culture	Visual	Culture	Mean	SD	Mean	SD
1 OD	56.7	56.7	56.7	56.7	56.7	56.7	56.7	0	56.7	0
1 OS	28.5	28.5	28.5	28.5	28.5	28.5	28.5	0	28.5	0
2 OD	1.8	1.8	1.8	1.8	<1.8	<1.8	1.5	0.5	1.5	0.5
2 OS	56.7	56.7	56.7	113.5	56.7	56.7	56.7	0	75.6	32.8
3 OD	14.2	14.2	14.2	28.5	14.2	10.7	14.2	0	17.8	9.4
3 OS	228.6	228.6	228.6	457.1	228.6	228.6	228.6	0	304.8	131.9

Table 2. Corneal amphotericin B levels determined from visual and culture endpoints 96 h following subconjunctival injection (1,500 µg/300 µl)

Sample set 2 Corneas	Methods									
	Freshly thawed (µg/g tissue)		24 h equilibration (µg/g tissue)		Supernatant (24 h) (µg/ml)		Visual		Culture	
	Visual	Culture	Visual	Culture	Visual	Culture	Mean	SD	Mean	SD
4 OD	1.8	<0.9	1.8	<0.9	3.6	<0.9	2.4	1.0	<0.9	0
4 OS	3.6	3.6	7.1	3.6	7.1	3.6	5.9	2.0	3.6	0
5 OD	0.9	<0.9	<1.8	<0.9	1.8	<0.9	1.2	0.5	<0.9	0
5 OS	3.6	5.3	7.1	3.6	7.1	5.3	5.9	2.0	4.7	1.0
6 OD	7.1	7.1	14.2	7.1	14.2	7.1	11.9	4.1	7.1	0
6 OS	14.2	21.3	28.5	28.4	28.5	28.4	23.7	8.3	26.0	4.1

spite the variability in corneal concentrations determined in different eyes. Leakage from the puncture wound following subconjunctival injection may account for this variability. The variance between endpoint values obtained with supernatant and those achieved with the corneal samples was minimal over the wide range of amphotericin B levels measured (<1.78–228.57 μg/g tissue). This difference of no more than one well is within the range of experimental error.

There appeared to be no loss of biologically active drug from corneas and supernatant in which bioassay was delayed for 24 h, since bioassays of freshly thawed corneal samples and those of corneal samples that had undergone 24 h equilibration gave comparable values for amphotericin B levels over the entire range of drug levels observed. Furthermore, amphotericin B in supernatant appeared to be in equilibrium with that in corneal tissue after 24 h incubation at room temperature. Although the minimal time required for equilibration was not established by these experiments, amphotericin B levels obtained by assaying the corneal samples 24 h after thawing and those obtained by assaying supernatant from the same samples were equivalent, indicating that complete equilibration had been achieved. These results suggest that any of the three bioassays performed are likely to give comparable amphotericin B levels, with minimal variance.

Amphotericin B levels calculated from culturing wells containing the corneal suspension and those derived from wells containing only the supernatant were equivalent to values determined by visual inspection of endpoints. This consistency of the two techniques supports the validity of using visual inspection as a method of determining endpoints. In addition, bioassays using visually determined endpoints appear to have an advantage over those using cultures in terms of expense, simplicity, and time. Furthermore, it seems unlikely that sophisticated optical techniques, such as spectrophotometry, would add precision and reliability to these results. Therefore, visual determination of endpoints appears to be sufficient for calculation of amphotericin B levels in the cornea.

The use of supernatant in our modified bioassay eliminated much of the difficulty inherent in the determination of endpoints by standard bioassay due to the presence of corneal debris in the broth. This particularly presents a problem in the first three wells of the microtiter plate. In this study, these wells often corresponded to the endpoints of the assay, as in sample set 2, in which amphotericin B levels were low, and it was difficult at times to distinguish corneal tissue from growth of the indicator organism. The use of supernatant enabled the endpoints to be clearly visible, thereby enhancing the ease and ensuring the precision with which the plates were read.

The pharmacokinetics of antifungal agents in the cornea are poorly understood, and with broth-dilution bioassays the measurement of drug levels is often complicated by the low levels of biologically active drug present [2]. Bioassay of equilibrated supernatant provides a measure of amphotericin B levels that appears to be as reliable and valid as the standard technique. In addition, use of the supernatant appears to enhance the ease of precise visual endpoint determination. This modification of sample preparation may be applicable to bioassays performed for other antifungal agents that are present at low biologically active levels in the cornea.

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References

1. O'Day DM (1987) Selection of appropriate antifungal therapy. Cornea 6:238–245
2. O'Day DM, Head WS, Robinson RD, Clanton JA (1986) Bioavailability and penetration of topical amphotericin B in the anterior segment of the rabbit eye. J Ocular Pharmacol 2:371–378
3. Sabath LD, Anhalt JP (1980) Assay of antimicrobics. In: Lennette EH, Balows A, Hausler WJ Jr, Truant JP (eds) Manual of clinical microbiology, 3rd edn. American Society for Microbiology, Washington, D.C., pp 485–490