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Amphotericin B Enzyme-Linked Immunosorbent Assay

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Our purpose was to develop and characterize an enzyme-linked immunosorbent assay (ELISA) which could measure the concentration of amphotericin B in serum. Amphotericin B was assayed by competition ELISA. Multiwell ELISA plates coated with amphotericin B (1.0 μg/ml) conjugated to bovine serum albumin were used to test replicates of serum samples spiked with amphotericin B. Purified rabbit polyclonal antibody against amphotericin B (1.4 μg/ml) was added subsequent to the instillation of samples spiked with unknown amounts of amphotericin B. Experiments were performed to test the sensitivity, specificity, precision, and accuracy of the assay. The ability to measure lipid-associated amphotericin B was also evaluated in preliminary studies. Analysis of reference samples containing amphotericin B yielded a traditional sigmoidal curve. The limits of detection were 0.15 to 156 μg/ml. The sensitivity of the assay was affected by light and temperature exposure. Assay specificity was altered only by the presence of nystatin, a polyeone antifungal agent similar to amphotericin B. Intrarun (coefficient of variation = 3.0%) and interrun (coefficient of variation = 12.8%) coefficients of variation were calculated and were comparable to those in similar assays. The assay’s correlation coefficient (r = 0.907) demonstrated a statistically significant correlation between the optical density of the sample and the concentration of drug in the sample. The amphotericin B ELISA’s ease, precision, and overall accuracy suggest that this assay could be used for assessments of serum amphotericin B concentrations. Multiple research questions concerning the role of serum amphotericin B concentrations in toxicity and efficacy have gone unanswered because of the labor-intensive nature of the assays which have been available to date. The ability to easily and rapidly measure 40 duplicate samples containing amphotericin B should also prove to be a distinct advantage for clinical research or reference laboratories in addressing these questions.

Materials and Methods

Reagents. AB (Sigma Chemical Co., St. Louis, Mo.) solubilized with deoxycholate and conjugated with either keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) was purchased from Eastacres Biologicals (Southbridge, Mass.) for initial experiments. In subsequent experiments, AB was conjugated with BSA (AB-BSA) in our laboratory as described below.

AB-BSA conjugation. A range of AB-BSA conjugation ratios was evaluated in order to achieve optimal coating on polystrene plates. AB (5 mg/ml), BSA (1 mg/ml), and glutaraldehyde (25%; lot 4215003; Sigma Chemical Co.) at various concentrations were mixed and incubated for 3 h. One molar i-Lysine was added to block the binding of AB to BSA. The final solutions were dialyzed for 4 h in a cold room (4°C) against a phosphate-buffered saline (PBS) solution containing 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na 2HPO4, 1.4 mM KH2PO4, 5.0 mM NaHCO3, and 0.013 mM CaCl2 at pH 7.3.

Animal immunizations. AB-KLH was homogenized with complete Freund’s adjuvant (CFA) containing Mycobacterium butyricum (Difco Laboratories, Detroit, Mich.) for primary immunizations and incomplete Freund’s adjuvant (IFA) for secondary immunizations. Immunizations of 2- and 6-week-old New Zealand White rabbits began on day 0 with subcutaneous injections of 1.0 mg of AB-KLH (based on the AB concentration) with CFA. Secondary injections of 0.5 mg of AB-KLH with IFA occurred on days 21, 42, and 63. After validation of antibody production by direct ELISA, 30 ml of blood was obtained weekly from an ear vein. After clotting, the blood was centrifuged at 1,500 rpm (500 × g) for 15 min, and the separated serum was frozen at −70°C.

Antibody purification. An affinity column was prepared by diluting AB with sterile water to a concentration of 2.5 mg/ml, and the mixture was reacted with beaded agarose containing active aldehyde functional groups as directed by the manufacturer (Immunopure Ag/Ab; Pierce Chemical Co., Rockford, Ill.). AB was mixed with the activated beads for 2 h with gentle agitation and was then allowed to stand for 4 h at 27°C. AB-coated beads were transferred to a 5-ml column containing a base filter. Rabbit serum in aliquots of 30 ml was diluted 1:2 in NET buffer (0.14 M NaCl, 0.01 M Tris-HCl, 1.0 mM EDTA [pH 7.4]) and filtered through a 0.45-μm-pore-size filter. The antisera was then perfused over an AB-coupled affinity column at a rate of 15 ml/h. The column was then washed with NET buffer until the optical density at 280 nm was at the background level. Glycine (1.0 M; pH = 2.8) was used to disassociate antibody against AB from the column. The eluents were collected in 1-ml samples, and the pH was neutralized to between 7.4 and 7.8 by titration with Tris (pH 9.8; Sigma). Effluent aliquots which had an optical density at 280 nm or greater were pooled. Purified antisera was passed over a protein G column (Pierce Chemical Co.) in order to separate IgG from IgM. The purity of the IgG preparation was

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ELISA characterization. (i) antibody direct ELISA. One hundred microliters of AB-BSA at an AB concentration of 1.0 µg/ml in NET buffer was coated onto 96-well, flat-bottom polystyrene plates. One hundred microliters of serial twofold dilutions of ABIGG beginning from 180 µg/ml was added to each well, and the plate was incubated for 1 h at 37°C. The remaining b of the assay was performed as described below for the AB competition ELISA.

(ii) AB competition ELISA. Microtiter plates were coated with AB-BSA (1.0 µg/ml) as noted above; this was followed by triplicate washings with PBS containing Tween. Wash volumes of 300 µl of PBS were used. This was followed by incubation and tapping on a paper towel. The 96-well plates were then blocked with 1% BSA in PBS for 1 h at 37°C. The addition of the AB-spiked serum samples (100 µl) was followed by the addition of 100 µl of ABIGG at 1.4 µg/ml and then incubation for 1 h at 37°C. The plates were emptied and washed three times. Horseradish peroxidase-labeled anti-rabbit IgG diluted 1:1,000 in a buffer was added after blot drying. The plates were again incubated for an additional 1 h at 37°C and washed three times. An o-phenylenediamine dihydrochloride peroxidase substrate (Fast P-9187; Sigma Chemical) solution (200 µl) was pipetted into each well, and after 10 min the ELISA plates were evaluated spectrophotometrically in an ELISA plate reader (Dynatech multiscan; Flow Laboratories, McLean, Va.) with a 405-nm filter. Ten independent samples at each concentration were assayed in duplicate.

(iii) Sensitivity and reproducibility. The sensitivities of the assays for AB were determined by an evaluation of mean independence. If a given mean concentration varied beyond one standard error of the means immediately proximal (in descending mean), the mean was used for assay determination and utilization. This method allowed for estimation of the limits of detection.

The ability of light exposure to affect assay reproducibility was studied. Identified coated microtiter plates were evaluated after 72 h of storage under the following conditions: (i) 37°C covered by aluminum foil, 37°C unprotected from fluorescent light (approximate average luminance, 1.13 cd/cm²), and 37°C unprotected from natural light during daylight hours (approximate average luminance, 0.6 cd/cm²). These plates were then used for AB concentration determination, which was performed in triplicate as described above. The effect of storage temperature was also studied by using identically coated microtiter plates after 72 h of storage at either refrigerated (3°C) or room (27°C) temperature. These experiments were also performed in triplicate.

(iv) Specificity. Additional experiments were performed to explore the ability of a variety of compounds to interfere with the assay, including micronazole (2,000 to 9.8 µg/ml; lot 110H8473; Sigma), fluconazole (5,000 to 2.4 µg/ml; lot 24F0196; Roeing, Pfizer, New York, N.Y.), itraconazole (5,000 to 2.4 µg/ml; lot 930016; Janssen, Piscataway, N.J.), miconazole (2,000 to 0.96 µg/ml; lot 33H0762; Sigma), ketoconazole (5,000 to 2.4 µg/ml; lot 92C239; Janssen), and penicillin (10,000 to 4.8 U/ml; lot 1F4-0524; Sigma), streptomycin (5,000 to 2.4 µg/ml; lot 108F00915; Sigma), and cholesterol (2,000 to 0.0085 µg/ml; lot 110F48473; Sigma). These experiments were performed in triplicate and included a negative control consisting of serum alone.

Finally, samples of liposomal AB (LAB; Vestar Pharmaceutical, San Dimas, Calif.) and AB-lipid complex (ABL; Liposome Co., Princeton, N.J.) diluted in human serum were measured by ELISA in a similar fashion. Triplicate samples of twofold dilutions of LAB from concentrations of 4.000 to 0.009 µg of AB per ml and ABL from concentrations of 5,000 to 0.0012 µg of AB per ml were assayed as unknowns for the validation of standard curves. ABL was also diluted at these concentrations in saline and was assayed with saline controls in an attempt to discriminate bound versus unbound drug.

Statistical and mathematical analyses. (i) Mathematical analysis. A standard curve of AB concentrations versus optical density was generated and plotted (Table Curve 2D; Jandel Scientific, San Rafael, Calif.) with the following asymmetric sigmoidal equation:

\[
Y = a + b \left(1 - \left[1 + \exp \left(\frac{x - \text{d} \times \text{m}^{2/3} - 1 - c}{\text{d}}\right)\right]^{-1}\right)
\]

where a through e are constants established with each curve. This is a linear and nonlinear least-squares program which uses a Marquardt search algorithm. No weighting of the data was performed. ABL and LAB concentrations were also plotted by using the same equation. A composite standard curve was constructed from these data.

The relationship between the independent variable (standard AB concentration on the basis of the pharmaceutically labeled product; x) and the dependent variable (measured AB concentration; y) was determined by least-squares regression curve fitting in an attempt to approximate how well the ELISA predicted the AB concentration in spiked serum samples. Least-squares regression variance was analyzed by using an F distribution. A correlation coefficient (r) was generated to estimate the strength of an association between variables.

RESULTS

Antiserum production. All animals tolerated the immunization without recognizable reactions. High-titer antibody production was noted by day 60. The final secondary injection was administered on day 63, and production bleeds were initiated on day 90.

AB-BSA conjugate. The free AB amino group allowed for aggressive cross-linking of AB to BSA. However, a dark yellow-brown precipitate was routinely observed during conjugation and interfered with the performance of the ELISA. In order to reduce this precipitate, twofold dilutions of glutaraldehyde between 1.75 and 0.05% were studied to optimize conjugation. The AB-BSA conjugate obtained at each glutaraldehyde concentration was then plated, and the AB antibody competition ELISA was performed (Fig. 1). A maximal optical density was obtained at a concentration of 0.05%, which was selected for use in the AB competition ELISA. The optical densities of all other glutaraldehyde concentrations were significantly below 0.05%. A dark yellow precipitate occurred at glutaraldehyde concentrations of greater than 1.0%. Protein analysis of the AB-BSA conjugate revealed a final protein concentration of approximately 0.30 µg/ml. Samples with the following glutaraldehyde concentrations contained the indicated amount of protein per ml: 1.75% contained 1,022 µg/ml, 1.5% contained 868 µg/ml, 1.25% contained 72 µg/ml, 1.0% contained 66 µg/ml, 0.75% contained 0.6 µg/ml, 0.5% contained 0.6 µg/ml, 0.25% contained 0.48 µg/ml, and 0.1% contained 0.4 µg/ml.
AB specific antibody. Affinity-purified rabbit antisera contained two antibody populations, IgG and IgM, when analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). After passage of the affinity-purified rabbit antisera over a protein G affinity chromatography column, only IgG was noted. Only AB IgG was used to characterize the AB ELISA. Samples of AB IgG were diluted in NET buffer to a stock concentration of 180 µg/ml and were frozen at −70°C until further use. Evaluation of antibody requirements for the AB competition ELISA was performed through a 47-fold antibody dilution repeated 12 times by direct ELISA. Eighty percent of the maximal optical density at 405 nm (A405) was observed with an AB IgG concentration of 1.4 µg/ml (Fig. 2). This AB IgG concentration was used in all further ELISA experiments.

Competition ELISA. A composite standard curve was generated, as described for AB, from 10 replicates performed in duplicate. A traditional sigmoidal curve was observed (Fig. 3), with limits of detection initially selected to be 0.15 to 156 µg/ml. These concentrations were then further criticized (see below). Observation of the concentrations commonly observed during patient therapy (0.5 to 10 µg/ml) found that these concentrations were at the upper portion of the curve, yet they were very reliable. Sensitivity was characterized after generation of the standard curve and was affected by several factors. The most pronounced effect was observed with variable light and temperature exposures. Storage of AB-BSA-coated plates at either room temperature (27°C) or exposure to light decreased sensitivity by at least 1 logarithmic concentration. When ELISA plates were exposed to both light (UV or incandescent) and room temperature (27°C), assay sensitivity was decreased greater than 3 logarithmic concentrations. Therefore, all AB-BSA-coated plates were immediately protected from light by covering them with aluminum foil and were refrigerated. The results of assays performed with light-protected plates stored at 4°C for up to 60 days were equivalent to those performed with freshly coated plates. The test with a control serum sample resulted in a small change in optical density. Thus, wells containing control sera were used as absolute zero for a ratio scale of the assay.

Assay specificity was of tremendous concern, especially with clinical samples, which may contain other potentially interfering agents. Of the agents tested, only nystatin interfered with the assay. At all concentrations tested, nystatin affected our ability to measure the AB concentration in serum. ELISA optical density results showed an increase of 60% ± 12% for wells containing nystatin and AB compared with that for spiked wells containing only AB.

Precision and accuracy. Intrarun analysis determined by the 23 reference samples containing AB assayed 10 times within the same run revealed a coefficient of variation of 3% for all assays. Interrun precision resulted in coefficients of variation of between 8.5 and 17% (average, 12.8% ± 3.8%). Accuracy estimations based on linear regression analysis were performed for AB concentrations of between 0.15 and 156 µg/ml. A correlation coefficient of 0.907 was statistically significant (P = 0.3298; P < 0.01). The coefficient indicates good linearity and a strong association between the known concentrations of drug and the concentrations measured by ELISA over the concentration range tested. The equation of the regression line was y = 0.32 + 0.906x (Fig. 4). The proportional and constant errors within the ELISA are small, as indicated by a slope of 0.906 (90.6%) and an intercept of less than 0.32 µg/ml.

Lipid AB preparations. Additional experiments were performed to determine if incorporation of AB into a lipid formulation would alter the sensitivity of the assay for AB. Twenty-three dilutions of each LAB and ABLC were assayed, and standard curves were generated as described for AB (Fig. 5). Both agents fit linear components of sigmoidal curves for LAB (3.9 to 312 µg/ml) and ABLC (9.76 to 156 µg/ml). Standard curves were established for these concentrations. Regression analysis of these concentrations revealed a correlation coefficient of 0.95 for LAB and one of 0.91 for ABLC when compared with the AB concentrations on the label of the pharma-
ceutical container. The average coefficient of variations over this range were 19% for LAB and 26% for ABLC. Analysis of ABLC diluted in saline resulted in curves similar to those observed for ABLC in sera (data not shown). Evaluation of the optical density of ABLC or LAB obtained from ABLC and LAB ELISAs in the AB ELISA demonstrated approximately 30% concordance. Spectrophotometric analysis of lipid products not containing AB explained this discordance (data not shown).

**DISCUSSION**

AB is structurally too small (<2,000 Da) to be antigenic. However, AB contains an amino and a carboxyl side chain which allows for conjugation to antigenic carrier molecules. The amino group provided an excellent reactive site for conjugation to either KLH or BSA. The AB-KLH conjugate was highly immunoreactive, leading to significant polyclonal antibody production in rabbits after 2 months. This antibody response remained constant over a 1-year period, with no waning of the titers observed. The quality of the affinity-purified AB IgG on PAGE revealed a protein with an apparent homology comparable to that of a purchased rabbit polyclonal IgG control. Interestingly, previous attempts in our laboratory to raise antibodies against AB by a similar protocol in sheep and rats were unsuccessful.

Our results clearly demonstrate the precision, accuracy, specificity, and sensitivity of the AB ELISA. The precision of the assay was 97%, with only 3% variability for intra-assay analysis. Three percent variability is excellent, but it is overshadowed by the 12.8% interrun variation. The large variation about the mean (12.8%) can be partially attributed to sample biodegradation, variability in antigen coating on ELISA plates, pipetting errors, and variance in the ELISA optical density reader. However, our results were comparable to those of other assays for measuring AB concentrations. By using high-pressure liquid chromatography, interrun variation has been reported to vary between 2 and 9.1%, with interrun variations of between 4.9 and 25% (3). The accuracy of the AB ELISA was also excellent (90.7%). When one considers the variation in pharmaceutical preparations in addition to our errors in dilution, conjugation calculations, and coating errors, we could easily account for a proportion of the 9.3% dissociation observed with our correlation coefficient. No assay interference was noted with commonly prescribed pharmaceutical agents, especially the systemic azoles, although interference was noted with nystatin. This polyene is not absorbed by topical or oral administration and, thus, should not affect the assay results when it is administered by these routes.

Sensitivity to light and temperature exposure was of tremendous concern. Previously, AB in spiked sera stored at 27, 4, -20, and -70°C was found to have shelf half-lives of 21 h at 27°C and 20 days at 4°C, but no change was found in samples frozen at -70°C (1). It was not discussed previously (1) whether these samples were protected from light. Therefore, on the basis of previous data, the serum samples were immediately frozen and protected from light until use. We also felt that it was critical to evaluate the effects of UV or incandescent light-exposed AB ELISA plates. It was not surprising that storage of AB-BSA-coated plates at either room temperature (27°C) or exposure to light decreased the sensitivity of the assay by at least 1 logarithmic concentration. ELISA plates were not stored for more than 60 days (data not shown).

Our assay is also capable of measuring the concentrations of ABLC and LAB in serum. These results were very exciting in light of current ongoing clinical trials with these products. If these products are eventually approved for use by the U.S. Food and Drug Administration, there will be expanded use of AB formulations. Identification of therapeutic endpoints will be needed, and serum drug concentrations could play a role in defining one of these endpoints. The addition of ABLC in saline was used to evaluate free versus bound AB. The results suggested that there are not clear answers concerning free versus lipid- or cholesterol-bound AB. The measured ABLC concentration was the same whether ABLC was diluted in saline.
serum or saline. This would indicate that the immunoreactive epitope of AB is exposed, even when it is complexed with lipid. Furthermore, this might also indicate that the AB remains complexed with lipid, despite dilution with saline. The measured AB concentrations did not increase, as might occur if the antigenic epitope of AB was protected by the lipid complex and was dissociated in PBS. Further work will be necessary to evaluate these issues. In addition, the standard curves generated with the lipid preparations were shifted to the right when compared with the curves for AB-deoxycholate. We do not believe that there is increased antibody binding to the lipid formulations. Lipid alone, without AB, does increase the optical densities of the samples, approximating the difference in the optical density of AB and that of lipid-associated AB. Thus, the use of AB-deoxycholate standards to estimate lipid-AB concentrations would not be accurate. Lipid-associated AB formulations must be used to create standard curves for the measurement of the respective agents.

There are no data to guide a clinician in the interpretation of serum AB concentrations for therapeutic response or toxicity. The utility of an easy, inexpensive, and reproducible assay for AB would provide several distinct contributions. First, this would allow the clinician to correlate serum AB concentrations with clinical outcomes. Data from studies evaluating other antifungal agents lead us to believe that this goal is not unrealistic. Serum ketoconazole or itraconazole concentrations of less than 0.25 μg/ml have been associated with antifungal prophylaxis (2). Second, the relationship of AB toxicity and serum (tissue) AB concentrations could be studied in more depth. A better understanding of the pharmacokinetics of AB will allow a more careful appraisal of the antifungal susceptibilities of clinical isolates. Finally, the AB IgG used for the ELISA described here could also be used for other studies. For example, we are currently using gold-labeled AB IgG to study intracellular trafficking and localization of AB, LAB, and AB LC in cells and tissue.

Conclusion. The AB ELISA’s ease, precision, and overall accuracy suggest that this assay could be useful in assessing serum AB concentration. The serum AB concentrations routinely achieved by therapeutic AB doses appeared to be readily measurable. The ability to rapidly measure the concentration of AB in a large number of samples should also prove to be a distinct advantage for clinical research or reference laboratories. We are excited about the prospect of this rapid and easy assay for AB. Multiple questions concerning the role of serum (tissue) AB concentrations in toxicity and efficacy have gone unanswered because of the difficulty of performing and the labor-intensive nature of the assays which have been available to date.

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