A Sensitive Amphotericin B Immunoassay for Pharmacokinetic and Distribution Studies

SOPHIE MACHARD, FREDERIC THEODORO, HENRI BENECH, JEAN-MARC GROGNET, and ERIC EZAN

CEA, Service de Pharmacologie et d’Immunologie, F-91191 Gif-sur-Yvette, France

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Since currently used assays of amphotericin B (AMB) lack sensitivity or are not easily adaptable in all laboratories, we have developed an enzyme immunoassay for AMB in biological fluids and tissues. Antibodies to AMB were raised in rabbits after administration of an AMB-bovine serum albumin conjugate. The enzymatic tracer was obtained by coupling AMB via its amino group to acetylcholinesterase (EC 3.1.1.7). These reagents were used for the development of a competitive immunoassay performed on microtiter plates. The limit of quantification was 100 pg/ml in plasma and 1 ng/g in tissues. The plasma assay was performed directly without extraction on a minimal volume of 0.1 ml. The intra- and inter assay coefficients of variation were in the range of 5 to 17%, and the recoveries were 92 to 111% for AMB added to human plasma. The assay was applied to a pharmacokinetic study with mice given AMB intraperitoneally at the dose of 1 mg/kg. The drug distribution in selected compartments (plasma, liver, spleen, lung, and brain) was monitored until 72 h after administration. In conclusion, our assay is at least 100-fold more sensitive than previously described bioassays or chromatographic determinations of AMB and may be useful in studying the tissue pharmacokinetics of new AMB formulations and in drug monitoring in clinical situations.

MATERIALS AND METHODS

Reagents. Common salts or solvents were of analytical grade and were from Sigma (St. Louis, Mo.) or Merck (Darmstadt, Germany). AMB from Sigma or the sodium deoxycholate form (Fungizone; Bristol-Myers-Squibb, Princeton, N.J.) was used. The substances used for cross-reaction testing were fluconazole, griseofulvin, nystatin, miconazole, and ketoconazole (Sigma) and penicillin and streptomycin (Blockron, Berlin, Germany). Acetylcholinesterase (AChE) (EC 3.1.1.7) extracted from the electric organ of the Electrophorus electricus cell was used as an enzymatic tracer. The purified enzyme was obtained from Spi-Bio (Massy, France). Enzyme activities were measured using Ellman’s reagent, an AChE substrate comprising 2.2 g of acetylthiocholine and 1 g of dithionitrobenzene (Sigma) in 200 ml of 0.05 M phosphate buffer, pH 7.4. One Ellman unit is defined as the concentration of enzyme producing an absorbance increase of 1 during 1 min in 1 ml of substrate medium for an optical path length of 1 cm at 414 nm.

Immunoassay preparation and incubation. AMB was coupled to bovine serum albumin and administered to rabbits in order to induce the synthesis of antibodies as follows. AMB was dissolved in water at a concentration of 14 mg/0.8 ml and incubated for 1 h at room temperature with bovine serum albumin (fraction V; Sigma) (20 mg in 0.3 ml of 0.1 M phosphate buffer, pH 7.4) and 12 μl of 25% glutaraldehyde (Merck) as a coupling agent. The control of coupling was performed by elution of 0.1 ml of the mixture in molecular sieve chromatography (G25; Pharmacia, St-Quentin-en-Yvelines, France). Absorbance measurement at 406 nm in fractions containing bovine serum albumin and free AMB indicated that 1 mol of AMB was covalently coupled to 1 mol of carrier protein. The immunogen was emulsified in an equal volume of complete Freund’s adjuvant (Sigma) and injected intradermally at multiple sites on the backs of three adult male rabbits, each weighing 2.5 kg (Blanc du Bouscat, Evic, France). Each animal received 40 μg of coupled AMB at the first administration. Booster injections (20 μg of coupled AMB in complete Freund’s adjuvant) were repeated every 2 months for 8 months. Rabbits were bled from the central ear artery 10 and 20 days after booster injections. Blood was centrifuged, and sera were stored in 0.1% sodium azide at 4°C. Antibody induction was tested by incubation of various dilutions (1/50 to 1/1,000) of the antisera with the enzymatic tracer AMB-AChE. The incubation was performed according to the procedure described in “Enzyme immunoassay” below. The presence of antibody to AMB was revealed when the signal obtained for bound tracer was twice that obtained in the absence of antisera. The titer was the antibody dilution giving a bound tracer absorbance of 0.1 in 1 h.

Enzymatic tracer preparation. Tracer was obtained by conjugation of AMB to AChE. Thiol groups were first introduced into AMB (0.46 mg) by reaction with succinimidyl-S-acetyl-thioacetate (SATA) (Sigma; 1.1 mg in 40 μl of dimethylformamide). After incubation for 30 min at 30°C, unreacted SATA was eliminated on a SepPak column (Waters, Milford, Mass.) previously activated by methanol and water. The column was washed with 30 ml of water, and AMB was eluted with methanol-water (80:20, vol/vol). SATA-activated AMB was subsequently purified by high-performance liquid chromatography (HPLC) using a
C14 150- by 4.6-mm Kromasil column (Touzet et Matignon, Vitry-sur-Seine, France) and a mobile phase comprising triethylamine (pH 5.2)-acetone–tetrahydrofuran (56:34:10, vol/vol/vol). Activated AMB (16 µg) was then diluted with 2 µl of the buffer (pH 9) and, thioester groups were hydrolyzed by treatment with 20 µl of 1 M hydroxylamine for 15 min. Thiol-containing AMB was mixed with AChE (0.1 nmol) previously activated with N-succinimidyl-4-(N-maleimidylcyclohexane-1-carboxylate (8) and incubated overnight at 4°C. The enzymatic tracer was purified by molecular sieve chromatography using a 90- by 1.5-cm Bio-Gel A 1.5-m column (Bio-Rad, Paris, France) eluted with 0.1 M phosphate buffer (pH 7.4) containing 0.4 M NaCl, 5 mM EDTA, 0.1% bovine serum albumin, and 0.01% sodium azide. Two-milliliter fractions were collected, and the peak exhibiting AChE activity was collected and stored at −20°C.

Enzyme immunoassay. Plasma samples were assayed without extraction. For tissues, AMB was extracted as follows. Approximately 100 to 200 mg of tissues were taken from the animals and immediately frozen in liquid nitrogen. Two milliliters of ice-cold methanol-water (70:30; vol/vol) was added to 100 µg of tissue, and the mixture was immediately homogenized mechanically for 30 s. One milliliter of the homogenized tissue was then centrifuged for 30 min at 10,000 × g and 4°C. The supernatant was evaporated with a rotary evaporator (Speed Vac; Jouan, Saint-Herblain, France) and the dry residue was dissolved in human plasma (0.5 ml for 100 mg tissue initially extracted) before assay. The assays were performed in 96-well microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) coated with mouse monoclonal antibodies specific for rabbit immunoglobulins at a concentration of 5 µg/ml in 0.05 M phosphate buffer, pH 7.4 (Spio-Bio, Massy, France). The coating was performed for 18 h at room temperature, and the plates were then washed for 24 h at 4°C with 0.1 M phosphate buffer (pH 7.4) containing 0.15 M NaCl, 5 mM EDTA, 0.1% bovine serum albumin, and 0.01% sodium azide (enzyme immunoassay buffer). Before use, the coated plates were washed with 0.1 M phosphate buffer (pH 7.4) containing 0.05% Tween 20 (washing buffer) (300 µl/well and five wash cycles) (Auto washer; 96; Labystems, Eragny, France). Tracer (dilution of 1:100 from the stock preparation), and antiserum (bleeding 148784, diluted 1/300) were diluted in enzyme immunoassay buffer. Standards (2, 1, 0.5, 0.25, 0.125, 0.062, and 0.031 ng/ml) and quality control samples were diluted in drug-free human plasma (Etablissements de Transfusion de Sanguine, Les Ulis, France). The assay was performed in a total volume of 150 µl. Reagents were dispensed as follows: 50 µl of sample, quality control, or standard and 50 µl of antiserum. After incubation at room temperature for 24 h, the plates were washed as described above, and Ellman's reagent containing 0.15 M Na2HPO4, 0.045 M NaCl, 0.02 M sodium azide (enzyme immunoassay buffer) and 0.003 M EDTA was added. Plates were incubated overnight at 4°C. The best conditions were those optimized enzymatic incubations at 4°C. The best conditions were those used for 2 to 5 h of enzymatic reaction, the plate was read at 414 nm (Multiskan RC; Labsystems). AChE activity was standardized by the standard curve modeled with a cubic spline function. The standard curve relating the percentage of bound tracer (ordinate) to the log of the concentration (abscissa) (ImmunoAssist; Beckman, Gagny, France). All measurements for standards and samples were made in duplicate.

Validation studies. The specificity of the anti-AMB antibody was determined by evaluating its ability to bind to various compounds likely to be present with AMB in treated human subjects. The percent cross-reactivity of each compound was calculated as the ratio between the 50% inhibitory concentration of AMB and that of the tested compound. The 50% inhibitory concentration was the concentration able to displace by 50% the tracer-antibody binding in the absence of competitor. Assay precision was estimated in terms of repeatability (intraassay precision) and reproducibility (intersay precision). Repeatability was estimated by the coefficient of variation (CV) (standard deviation divided by the mean and multiplied by 100). Quality control samples (drug-free human plasma and those with AMB) assayed eight times in the same run. Reproducibility was estimated by the CV for the quality control samples assayed in five independent assays. The relative accuracy of the assay was determined by measuring overlap for each of the quality control concentrations. Accuracy was calculated as the ratio between measured and theoretical concentrations multiplied by 100. The limit of quantification of the assay was defined as the concentration allowing good assay precision (CVs of repeatability and reproducibility of less than 20%) and accuracy (accuracy ratio in the range of 85 to 115%). In order to establish assay specificity, we evaluated the recognition of AMB metabolites or endogenous compounds by the antibodies. Thus, plasma or tissue extracts from animals given AMB were fractionated by HPLC to establish if the immunoreactivity corresponded to a single immunoreactive compound. The HPLC consisted of a Kromazil C18 5-μm column (Touzart et Matignon, Vitry-sur-Seine, France) and a fraction collector (Roucaire, Velizy-Villacoublay, France). Plasma or tissue extracts (50 µl) were injected, and 1-min fractions were collected and evaporated (Speed Vac). The dry extracts were dissolved in human plasma before enzyme immunoassay.

Pharmacokinetics of AMB after administration of single doses to mice. Swiss mice from Ifa Credo (St-Germain sur l’Arbresle, France) were maintained on a 12-h light-dark cycle, with light from 7:00 a.m. to 7:00 p.m., in a temperature (21 to 22°C) and humidity (50% ± 10%)-controlled room. The mice were treated after the 7-day acclimation period, at which time their body weights were 25 g. Studies on animals complied with the Décret de l’Expérimentation Animale (French law on rules for animal experimentation; decree 87-848, 19 October 1987). AMB (Fungizone) diluted in sterile water was administered by the intraperitoneal route at a dose of 1 mg/kg to 36 mice. At selected times (5 min, 30 min, and 1, 2, 4, 8, 24, 48, and 72 h), four animals were sacrificed with pentobarbital and their spleens, livers, lungs, and brains were collected and immediately frozen until extraction. Blood was collected from the aorta using a heparinized syringe and immediately centrifuged to obtain plasma, which was then stored at −20°C before assay. AMB in plasma and tissue samples was measured by enzyme immunoassay, and the area under the concentration-time curve (AUC) was calculated for each tissue and compared to that for plasma. Pharmacokinetic analysis (AUC and elimination half-life) was performed on the mean values, since the animals were sacrificed at each sampling time. The trapezoidal AUC was calculated between the first time and the last time (72 h). The half-life was calculated between 24 and 72 h. The time to maximum concentration (Tmax) corresponded to the time at which the mean concentration reached its maximum (Cmax). Pharmacokinetic analysis was performed using Siphar software (Simed, Creteil, France).

RESULTS

Since AMB is not immunogenic per se, it was coupled to a carrier protein through its amino group in order to raise antibodies in rabbits. The immunizations allowed the preparation of antiserum containing antibodies specific to AMB, and bleedings were selected according to their affinities by binding inhibition studies. The specificity of the selected antiserum (148784) was tested against various antifungal agents and structurally related compounds (flucytosine, griseofulvin, nystatin, miconazole, ketoconazole, and penicillium). No interference (cross-reactivity of less than 0.01%) was seen with any tested compound except nystatin (cross-reactivity of 250%), which is structurally related to AMB. The concentrations of reagents (antiserum and tracer) were optimized after immunological incubations at 4°C. The best conditions were those detailed in Materials and Methods. Assay precision and accuracy were evaluated by measuring intra- and interday variabilities and the recovery of AMB added to plasma and tissue. As shown in Table 1, mean variabilities were 5% (intraday) and 15% (interday) for concentrations of above 100 pg/ml. The mean recovery was 103 ± 8% in the interday experiments. Intra- and interday variabilities were between 9 and 26% for mouse lung and brain tissue spiked with AMB at the concentration of 20 ng/g. These data allowed us to fix the limit of quantification of AMB in plasma at 0.1 ng/ml. This indicates an LOD of 1 ng/g in tissue, since in our extraction protocol 50 µg of tissue was processed (centrifuged, evaporated, and reconstituted in 0.5 ml of plasma). Because of the tissue extraction protocol, the limit of quantification in tissues was set at 1 ng/g when 100 mg of tissue was initially extracted.

The assay was used in pharmacokinetic studies with mice given AMB at the dose of 1 mg/kg. AMB was measured in plasma and selected tissues. The pharmacokinetic profiles are shown Fig. 1. AMB was measured in all compartments until the last sampling time. The estimated pharmacokinetic

<table>
<thead>
<tr>
<th>Parameter (%)</th>
<th>Value:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma2 with AMB at (pg/ml):</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1,000</td>
</tr>
<tr>
<td>Intraday CVa</td>
<td>3</td>
</tr>
<tr>
<td>Interday CVa</td>
<td>16</td>
</tr>
<tr>
<td>Accuracya</td>
<td>102</td>
</tr>
</tbody>
</table>

a Human plasma spiked with AMB at the concentrations indicated.

b Mouse tissues spiked with AMB at the concentration of 20 ng/g.

1 n = 8 for plasma; n = 4 for tissues.

2 n = 5 for plasma; n = 4 for tissues.

3 Recovery of added AMB in the interday experiment.

TABLE 1. Validation parameters for the enzyme immunoassay of AMB in plasma and selected tissues
AMB at the dose of 1 mg/kg by the intraperitoneal route. Each point is the mean for four animals.

In order to estimate assay specificity and the potential interference of AMB metabolites or endogenous compounds with the antibodies, a pool of plasma or tissue obtained 48 h after AMB administration in mice was fractionated by HPLC, and the antibodies could detect one or several AMB metabolites. Cross-reacting compounds interfered little with assay accuracy, since, and at least with our samples, they represented less than 15% of the total immunoreactivity recovered. Renal excretion of AMB is minor, indicating that AMB probably undergoes metabolization, although no metabolites have been identified so far (14). Our antibodies may therefore be applicable to the selective extraction of cross-reacting metabolites, thus enabling further identification.

Despite numerous articles addressing the pharmacokinetics of AMB, studies of its distribution in tissue are limited by the poor sensitivity of the analytical techniques currently used. There are, however, data indicating that AMB is distributed mainly in liver and spleen, with concentrations 5 to 10 times those encountered in other tissues, such as lung and kidney (10, 24). In postmortem studies with humans, 6% and 14 to 20% of the total dose were recovered in spleen and liver, respectively (10). The present pharmacokinetic study examined the usefulness of our assay in specifying the drug distribution in tissue. We defined the AMB kinetic profile over 72 h after administration. AMB pharmacokinetics were characterized by rapid uptake from tissues. As expected, we found that administration of AMB resulted in high concentrations in liver and spleen and lower concentrations in lung. AMB was cleared revealed only a single immunoreactive peak, the immunoreactivity may represent an AMB metabolite recognized by the antibodies.

**DISCUSSION**

Pharmacokinetic evaluations of AMB in preclinical and clinical studies support the development of new and less toxic AMB formulations (1, 13). AMB assays may be valuable in at least two situations: distribution studies with animals and monitoring of patients in order to correlate drug concentration and clinical outcome.

The challenge of designing highly sensitive AMB assays that are easy to perform has led to the development of AMB immunoassays (6, 18). Those authors adopted a format of enzyme-linked immunoabsorbent assay in which AMB to be assayed competes with AMB absorbed onto the wells of a microtiter plate. To each well is added a fixed amount of the enzymatic tracer and either known standards or samples. The amount of tracer bound to the antibodies is inversely proportional to the quantity of AMB present in the standard or sample. Quantification of the enzyme label allows measurement of the sample concentration from the standard curve. The use of high-affinity antibodies and AChE, which has a high turnover, led to an assay with a limit of quantification of 100 pg/ml, i.e., 1,500-fold better than those previously achieved (6, 18). Our assay may be performed in less than 24 h, does not require an extraction step for plasma, and may be easily applied by any laboratory familiar with immunoassays. The accuracy and precision after repeated intra- or interday AMB measurement indicate performance suitable for pharmacokinetic studies. Among drugs and endogenous compounds tested for cross-reactivity, only nystatin was recognized by the antibodies. This would be a problem only if nystatin was previously administered by the parenteral route, since this compound administered by the oral or topical route is not absorbed and not present in the circulation (6).

Investigation of assay specificity by HPLC fractionation of tissue samples revealed that the antibodies could detect one or several AMB metabolites. Cross-reacting compounds interfered little with assay accuracy, since, and at least with our samples, they represented less than 15% of the total immunoreactivity recovered. Renal excretion of AMB is minor, indicating that AMB probably undergoes metabolization, although no metabolites have been identified so far (14). Our antibodies may therefore be applicable to the selective extraction of cross-reacting metabolites, thus enabling further identification.

**TABLE 2. Pharmacokinetic parameters of AMB administered intraperitoneally to mice at the dose of 1 mg/kg**

<table>
<thead>
<tr>
<th>Sample</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/ml or ng/g)</th>
<th>AUC (ng/ml·h)</th>
<th>AUC ratio&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Half-life&lt;sup&gt;b&lt;/sup&gt; (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td>1</td>
<td>340</td>
<td>6200</td>
<td>1</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver</td>
<td>0.5</td>
<td>1,480</td>
<td>46,400</td>
<td>7.5</td>
<td>50.9</td>
</tr>
<tr>
<td>Lung</td>
<td>8</td>
<td>1,660</td>
<td>42,800</td>
<td>6.9</td>
<td>50.9</td>
</tr>
<tr>
<td>Brain</td>
<td>24</td>
<td>90</td>
<td>4,400</td>
<td>0.7</td>
<td>72.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Tissue/plasma ratio.

<sup>b</sup> Determined from the sampling points between 24 and 72 h.

<sup>c</sup> ND, not determined.
more rapidly from plasma and lung than from other tissues, indicating that after distribution to the tissues, AMB may be released at different and delayed rates.

Although brain penetration has been reported (5, 9, 17), the concentrations observed were close to the limit of the quantification of the chromatographic techniques used to monitor the tissue concentrations. Compared to that in other tissues, AMB penetration in brain was slower and reduced, since the maximum concentration was observed later and levels were 10- to 20-fold lower than those in spleen and liver. Contamination of brain tissue by AMB present in blood capillaries could not be excluded, but its importance should be minimized since there is less than 3% blood in rodent brain (16), a value much lower than the brain/blood ratio (70%) observed in our study. A second, indirect argument is that kinetics in plasma and brain are not parallel, at least for the first sampling times.

The possibility of monitoring low brain AMB concentrations, in order to compare the different formulations pharmacokinetically, is of particular interest in view of a recent study in which the relative efficiencies of three AMB lipid formulations were compared in the treatment of systemic murine cryptococcosis, a model whose primary target is infection of the central nervous system (7). These formulations displayed organ-specific differences, particularly in the brain. Monitoring of AMB brain penetration is also potentially interesting since this drug prolongs the course of experimental prion diseases and modifies the kinetics of abnormal prion protein accumulation in the central nervous system (2). The mechanism of action is unclear, and hypotheses involving the disruption of membrane structures or the inhibition of the conversion of the prion protein into an abnormal form in the central nervous system may require quantification of the drug at these putative sites of action.

Besides the potential advantages of AMB enzyme immunoassay in studies of tissue pharmacokinetics, as demonstrated here, the technique should also be useful in the clinical laboratory for monitoring patients on antifungal therapy. The pharmacokinetic rationale for adapting dosage in order to influence clinical outcome has been reviewed elsewhere (21). So far this is partly limited by the fact that currently used AMB bioassays and chromatographic methods require specific equipment not available in all clinical centers, disadvantages that may be circumvented by the assay presented here.

FIG. 2. Immunochromatographic profiles of mouse samples taken 48 h after administration of AMB at the dose of 1 mg/kg by the intraperitoneal route. (A) Plasma; (B) liver; (C) spleen. The elution time of synthetic AMB spiked in drug-free plasma was 14 to 18 min.
liposomal formulation of amphotericin B (AmBisome) in rabbits. Antimi-