

STUDIES ON THE ABSORPTION, DISTRIBUTION AND EXCRETION
OF RADIOACTIVITY AFTER INTRAVENOUS AND INTRAPERI-
TONEAL ADMINISTRATION OF ^{14}C -METHYL ESTER
OF AMPHOTERICIN B

NOBUO MONJI, DANIEL P. BONNER, YOSHINOBU HASHIMOTO
and CARL P. SCHAFFNER

Waksman Institute of Microbiology, Rutgers University
New Brunswick, New Jersey 08903, U.S.A.

(Received for publication February 3, 1975)

Distribution and balance studies with carbon-14-labeled amphotericin B methyl ester (AME) were carried out in mice. The radioactive AME was administered by either the intraperitoneal (i.p.) or intravenous (i.v.) route. In the organ distribution study, the percent radioactivity accumulating in the lung of i.v. treated mice at 1 hour after administration was about 150 times greater than that observed when the intraperitoneal route was used. No accumulation of radioactivity with time was detected in the kidneys of either the i.v. or i.p. treated mice. After 4 days, about 51% of the total radioactivity was excreted into the urine and feces of mice after i.v. administration, but only about 15% of the total radioactivity was excreted in the case of mice receiving radioactive AME by the i.p. route. In the identification of the substances excreted in the urine, thin-layer chromatography (TLC), radioactivity, and bioautographic evidence suggest that there was no detectable de-esterification of AME to the parent compound in mice treated either intraperitoneally or intravenously with AME.

The most effective antibiotic now available for the treatment of systemic fungal infections in man is intravenously administered amphotericin B. This drug is used for the treatment of a number of mycoses that are invariably fatal¹⁻³. The use of amphotericin B, however, is restricted to a significant degree by a variety of toxic side effects, the most serious of these being nephrotoxicity^{4,5}. It is suspected that these toxic side effects may be related to the inherent insolubility of this compound in water.

Amphotericin B methyl ester (AME), a water-soluble derivative of amphotericin B, was recently developed at the Waksman Institute of Microbiology, Rutgers University, New Brunswick, N.J.⁶. This compound not only retains the *in vitro* antifungal activity of the parent compound, but exhibits a substantially reduced toxicity from that observed with the parent compound^{7,8}. In light of this reduced toxicity without a loss in activity, the absorption distribution, and excretion of the radioactive derivative, amphotericin B- ^{14}C -methyl ester has been studied in mice and is reported here.

Materials and Methods

Preparation of Amphotericin B- ^{14}C -Methyl Ester (AME)

^{14}C -Methyl ester of amphotericin B (AME) was prepared by chemical conversion of purified amphotericin B with ^{14}C -diazomethane, following the method of MECHLINSKI *et al.*^{8,9} Fig. 1 shows the location of the labeled methyl group in the AME structure.

Distribution of Radioactivity in Organs and Tissues of Mice

In this study 10 mg of radioactive AME was dissolved in 1 ml 1% aqueous ascorbic acid and 5% dextrose (pH 2.8~3.0). The pH of this solution was adjusted to pH 5.0~5.5 with aqueous sodium hydroxide. The solution of AME so prepared was administered to male mice (Swiss strain, 30 ± 1 g body weight) either by intraperitoneal injection (0.36 μ Ci/20 mg/head) or by intravenous injection (0.036 μ Ci/2 mg/head) via the lateral tail vein.

After administration of the compound, each animal was kept in a metabolic cage with a supply of food and water. Feces and urine were collected separately. Where measurement of the radioactivity of expired carbon dioxide gas was required, the metabolic cage was housed in an apparatus furnished with the necessary equipment to trap the carbon-dioxide gas. The experimental procedures followed the method of KAWAGUCHI¹⁰.

At the end of each assigned time interval, as shown in Tables 1 and 2, animals (3 animals per group) were sacrificed by bleeding from the eye. A 0.1-ml aliquot of serum was used for the measurement of the serum radioactivity. Various organs, such as liver, spleen, kidney, lung, testis, intestine, stomach, thymus, brain, and heart were excised for the radioactivity measurements. All samples (20~40 mg) of excised tissues were repeatedly washed with saline solution, then completely digested with Soluene 350 (Packard Instrument Co.) and finally decolorized with 0.2 ml of 30~35% H_2O_2 . As an example, to each sample in a vial, 1 ml of Soluene 350 was added and incubated at 50°C for 2~3 hours. After cooling the homogenous solution, 0.2 ml isopropanol was added, followed by an addition of 0.2 ml H_2O_2 . The samples were shaken immediately and kept at 40°C for about 30 minutes. After cooling, 10 ml of Aquasol (New England Nuclear Co.) was added. The radioactivity of the samples was counted by the Packard liquid scintillation spectrometer model 3320. For the digestion and decolorization of serum, the same experimental procedure was followed.

As shown in Tables 1 and 2, the accumulation of radioactivity per g organ basis was expressed in terms of L-value¹¹. This expressed the relative ratio of distributed radioactivity per g organ to the total radioactivity administered per g organ basis. Therefore, L-value equals dpm/g organ/total dpm given per animal/total body weight of the animal. When the L-value is multiplied by the amount administered in mg/kg, the value for the amount in μ g of the drug localized per g organ is obtained.

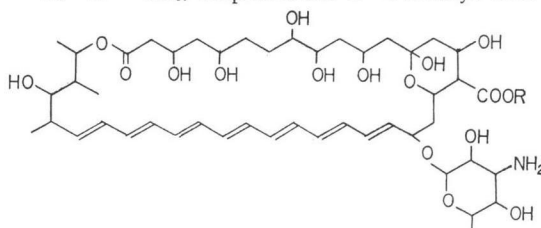
The bioassay and spectrophotometric measurement of tissue distribution in mice were done by the following procedures. The excised organs of mice sacrificed at each assigned time interval, as shown in Table 3, were lyophilized and ground thoroughly. The ground organs were carefully weighed out (50~100 mg). The possible lipids present in the tissues were extracted twice with 2-ml portions of petroleum ether and diethyl ether. To the defatted tissues 0.5 ml of dimethyl sulfoxide (DMSO) was added for the extraction of the drug. The residual drug in the tissues was further extracted twice with 1.5-ml volumes of methanol. Methanol and DMSO extracts were then combined and centrifuged to precipitate the proteins dissolved in DMSO. The supernatant solution so obtained was used for the spectrophotometric and bioassay measurement. For the spectrophotometric measurement, absorption of the drug was measured at 383 nm. The drug concentration was then calculated from the base that the absorption of 10 μ g/ml of amphotericin B methyl ester was 1.65 ($E_{1\%}^{1\text{cm}}$ 1,650).

For the bioassay measurement, a broth dilution method was employed, using *Saccharomyces cerevisiae* (ATCC 9763) as a test organism. The medium used for the incubation of the organism was SABOURAUD's broth (4% dextrose, 1% peptone). The incubation time was 36

Fig. 1. Structure of amphotericin B and amphotericin B-¹⁴C-methyl ester

I. R=H, amphotericin B

II. R=¹⁴CH₃, amphotericin B-¹⁴C-methyl ester



hours at 28°C.

Balance Study

Excreted urine, feces, and expired carbon dioxide gas from the mice described in the previous section were collected for the detection of radioactivity.

(a) Expired gas: Using the method of KAWAGUCHI¹⁰, expired CO₂ was trapped in monoethanolamine in the form of carbonate. At 3, 6, 12, 24, and 48 hours, 0.2 ml of the collection solution was taken out and added to 10 ml of Aquasol for radioactivity measurement.

(b) Urine: In all cases, 0.1 ml of each urine sample was taken and added to 10 ml of Aquasol.

(c) Feces: For the digestion and decolorization of feces, the following method was used. Approximately 7~10 mg of ground, lyophilized feces were put into a scintillation vial. To this 0.2 ml of distilled H₂O and 1 ml of Soluene 350 were added. After 2 hours of incubation at 50°C, 0.5 ml of isopropanol and 0.2 ml of H₂O₂ (30~35 %) were added. The samples were kept at room temperature for 10 minutes and then kept another 2 hours at 50°C. After the 2 hours, 10 ml Aquasol was added, and the radioactivity was measured.

Thin-Layer Chromatography (TLC) of the Urine Sample of Mice i.v. Treated with AME

The urine of the i.v. treated mice was collected up to 24 hours after drug administration. A group of 4 animals was used. The urine was extracted three times with an equal amount of butanol. The collected butanol was then evaporated under *vacuo* to near dryness. The concentrated extract was dissolved in 1 ml DMSO and further diluted with 1 ml methanol. The TLC of the urine extract was developed on Eastman 6060 chromatogram, silica gel with fluorescent indicator. The solvent systems used for the TLC were chloroform-methanol-borate buffer (0.025 M, pH 8.3) (2:2:1, lower phase) and chloroform-ethanol-20 % acetic acid (1:1: saturation). For the radioactivity measurement of the chromatogram, the developed TLC was scraped out at 5.0-mm intervals, and the scraped silica gel was collected in a test tube. The substance adsorbed in the silica gel was extracted with 0.5 ml DMSO. From it 0.2 ml was taken and put into a scintillation vial. There 10 ml of Aquasol was added and radioactivity measured.

For the bioautography, *S. cerevisiae* was used as a test organism. The organism was inoculated into 50 ml of SABOURAND's broth in a 250 ml Erlenmeyer flask which was then incubated at 28°C on a 245 rpm shaker for 24 hours. At the end of the incubation, 0.1 ml of the culture was taken and put into 50 ml of liquified SABOURAND's agar (40°C). To a Petri dish (150 mm × 15 mm), uninoculated medium was added to about a 5-mm thickness. After solidification, a thin-layer of the seeded medium was layered on top. When the top layer had solidified, the developed TLC was carefully layered with the silica gel side down. The plate was incubated for 24 hours at 28°C. Then, the TLC was carefully taken out and the plate was incubated for another 24 hours and examined for the bioactivity. Bioactive spots were compared with the spots detected by UV light.

Results

I. Distribution of Radioactivity in Organs and Tissues of Mice

Tables 1 and 2 show the tissue distribution of radioactivity at various time intervals in the i.p. and i.v. treated mice. The most striking difference observed in the distribution of radioactivity after i.p. and i.v. administration of labeled AME with the dosages indicated in the method, was the radioactivity appearing in lung. By i.v. route, the highest level of radioactivity was detected in 15 minutes after the administration. About half of the radioactivity disappeared in another 15 minutes. In percentage radioactivity per organ basis, the percentage in the lung of mice treated by i.v. route was about 150 times greater than the percentage in the lung of mice given AME by i.p. route at 1 hour after the administration. By both i.v.

Table 1. Comparative tissue distribution of ¹⁴C-methyl ester of amphotericin B in mice given single dose intravenously (66 mg/kg) or intraperitoneally (660 mg/kg). L-value*

| Organs | 1 hour | | 3 hours | | 6 hours | |
|-----------|--------------|-------------|--------------|-------------|--------------|-------------|
| | I.V. | I.P. | I.V. | I.P. | I.V. | I.P. |
| Liver | 4.408±0.646 | 0.178±0.013 | 3.851±0.230 | 0.312±0.040 | 5.407±0.146 | 0.207±0.036 |
| Spleen | 3.934±0.088 | 0.455±0.086 | 4.102±0.788 | 1.001±0.061 | 5.777±1.069 | 1.058±0.354 |
| Kidney | 1.181±0.300 | 0.503±0.018 | 0.987±0.128 | 0.232±0.039 | 1.274±0.272 | 0.571±0.090 |
| Lung | 20.899±4.532 | 0.128±0.025 | 11.306±1.255 | 0.087±0.008 | 20.058±3.663 | 0.094±0.010 |
| Testis | 0.161±0.043 | 0.307±0.070 | 0.149±0.013 | 0.301±0.056 | 0.100±0.010 | 0.309±0.056 |
| Intestine | 0.597±0.155 | 0.549±0.052 | 0.848±0.302 | 0.675±0.243 | 0.525±0.068 | 0.414±0.111 |
| Stomach | 0.462±0.050 | 0.613±0.143 | 0.521±0.127 | 0.235±0.315 | 0.459±0.305 | 1.262±0.621 |
| Thymus | 0.516±0.207 | 0.374±0.255 | 0.505±0.141 | 0.076±0.004 | 0.323±0.023 | 0.078±0.008 |
| Brain | 0.176±0.043 | 0.022±0.000 | 0.207±0.018 | 0.021±0.001 | 0.053±0.002 | 0.022±0.004 |
| Heart | 0.751±0.119 | 0.042±0.004 | 0.779±0.130 | 0.031±0.006 | 0.518±0.063 | 0.041±0.003 |

$$*L\text{-value} = \frac{\text{d.p.m./g. organ}}{\text{Total d.p.m./g. body weight}} \quad (\bar{X} \pm S.E., n=3, \text{ respectively})$$

Table 2. Comparative tissue distribution of ¹⁴C-methyl ester of amphotericin B in mice given single dose intravenously (66 mg/kg) or intraperitoneally (660 mg/kg). L-value*

| Organs | 24 hours | | 72 hours | | 96 hours | |
|-----------|--------------|-------------|--------------|-------------|--------------|-------------|
| | I.V. | I.P. | I.V. | I.P. | I.V. | I.P. |
| Liver | 4.741±0.300 | 3.908±0.547 | 5.982±0.238 | 1.816±0.122 | 4.324±0.361 | 2.340±0.410 |
| Spleen | 7.582±0.510 | 1.450±0.469 | 11.455±2.735 | 1.559±0.298 | 12.148±1.147 | 3.421±0.303 |
| Kidney | 1.273±0.056 | 0.325±0.143 | 0.944±0.036 | 0.432±0.030 | 0.638±0.079 | 0.262±0.047 |
| Lung | 13.175±0.539 | 0.045±0.002 | 4.366±1.235 | 0.092±0.005 | 3.522±0.474 | 0.146±0.034 |
| Testis | 0.099±0.008 | 0.822±0.213 | 0.224±0.006 | 0.291±0.023 | 0.553±0.013 | 0.340±0.058 |
| Intestine | 0.475±0.057 | 0.976±0.273 | 0.307±0.032 | 0.456±0.025 | 0.606±0.136 | 0.776±0.298 |
| Stomach | 0.600±0.377 | 0.562±0.077 | 0.457±0.009 | 0.587±0.083 | 0.946±0.391 | 1.640±0.352 |
| Thymus | 0.422±0.033 | 0.193±0.133 | 0.638±0.049 | 0.168±0.017 | 0.384±0.388 | 0.255±0.118 |
| Brain | 0.060±0.004 | 0.014±0.001 | 0.241±0.061 | 0.013±0.001 | 0.334±0.074 | 0.017±0.004 |
| Heart | 0.478±0.052 | 0.045±0.022 | 0.432±0.069 | 0.035±0.004 | 0.282±0.027 | 0.032±0.004 |

$$*L\text{-value} = \frac{\text{d.p.m./g. organ}}{\text{Total d.p.m./g. body weight}} \quad (\bar{X} \pm S.E., n=3, \text{ respectively})$$

and i.p. routes, slow accumulation of radioactivity was noticed in the spleen. No threshold accumulation of radioactivity in the kidney with time was detected.

Rather high radioactivity was still detected after 24 hours and 96 hours in the liver, spleen, testis, intestine, and stomach after i.p. administration. This seems to come partly from the direct absorption of AME onto the outer tissues of these organs since even after 96 hours a considerable amount of AME was still found in abdominal cavity of mice treated by the i.p. route.

In order to determine if the radioactivity accumulated in tissues is related to the biopotency of the drug, the tissue localization levels of the drug calculated from the radioactivity measurement were compared with the levels calculated from the UV and bioassay measurements. The results on four important organs, liver, lung, spleen and kidney are shown in

Table 3. Comparison of tissue localization level of AME (i.v., 66 mg/kg) with time, calculated from radioactivity, UV absorption, and bioassay measurement.
Average concentration, mg/g. organ. (n=3)

| Time | Liver | | | Kidney | | | Lung | | | Spleen | | |
|---------|-------|-------|-------|--------|-------|-------|-------|-------|-------|--------|-------|-------|
| | RA | UV | BIO | RA | UV | BIO | RA | UV | BIO | RA | UV | BIO |
| 15 min. | 0.229 | 0.223 | 0.116 | 0.090 | 0.088 | 0.017 | 2.909 | 2.648 | 1.796 | 0.210 | 0.269 | 0.191 |
| 30 min. | 0.247 | 0.244 | 0.163 | 0.060 | 0.082 | 0.019 | 1.287 | 1.813 | 1.248 | 0.270 | 0.317 | 0.136 |
| 60 min. | 0.290 | 0.286 | 0.203 | 0.080 | 0.103 | 0.055 | 1.379 | 1.717 | 1.223 | 0.360 | 0.386 | 0.141 |

Abbreviations: RA=The amount calculated from radioactivity measurement.
UV=The amount calculated from UV absorption measurement.
BIO=The amount calculated from bioassay measurement.

Table 3. The tissue localization levels calculated from the radioactivity measurements appear to be comparable to that of spectrophotometric measurement in all organs. Bioassay measurements in the organs showed about one half of the tissue localization levels as calculated from the spectrophotometric measurements. This difference may be due to either partial inactivation within the organ or co-extraction of substances from the organ that interfere with the bioassay. The resolution of this difference through the chemical characterization of the radioactive substances isolated from the various tissues is currently under study.

Our finding of high accumulation of radioactivity in the lung of mice treated by the i.v. route appears to be quite significant since the therapeutic potency of AME has been examined so far only by the i.p. route and found to be equally or somewhat less potent than the parent compound¹²⁾. Our results suggest that the administration of AME by the i.v. route may be a preferred therapeutic approach, especially in the treatment of pulmonary fungal infections.

II. Balance Study

(1) Serum: Table 4 shows the radioactivity profiles in the serum of mice receiving radioactive AME by i.p. or i.v. routes. For mice receiving AME by the i.p. route, the half life of the drug in the blood was calculated to be 30 minutes. In the case of the i.v. treated mice, the half life in blood was calculated to be 15 minutes.

(2) Urine: Table 5 and Fig. 2 show the radioactivity excretion pattern of mice given radioactive AME by i.v. or i.p. route. In both cases the maximum excretion of radioactivity occurred in the first 3~6 hours. The cumulative radioactivity in the urine of mice receiving AME by the i.v. route after 4 days was about 27%. On the other hand, the radioactivity recovered in the urine of mice after i.p. administration was only about 9% after this same period. With the dosages employed, these results suggest a poor absorption of AME into the

Table 4. Serum radioactivity level in mice given single dose of amphotericin B-¹⁴C-methyl ester intravenously or intraperitoneally. (n=3, respectively)

| Dose (mg/kg) | Route | Serum radioactivity level (expressed in L-value) | | | | | | |
|--------------|-------|--|---------|--------|--------|--------|---------|---------|
| | | 15 min. | 30 min. | 1 hrs. | 3 hrs. | 6 hrs. | 12 hrs. | 24 hrs. |
| 66 | i.v. | 0.526 | 0.225 | 0.246 | 0.215 | 0.148 | 0.132 | 0.072 |
| 660 | i.p. | 0.151 | 0.198 | 0.102 | 0.086 | 0.090 | 0.046 | 0.046 |

Table 5. Cumulative radioactivity recovery in urine and feces of mice given single dose of ^{14}C -methyl ester of amphotericin B intravenously (66 mg/kg) or intraperitoneally (660 mg/kg) ($n=3$, respectively) ($\bar{X}\pm\text{S.E.}$)

| | Urine (% Recovery) | | Feces (% Recovery) | |
|----------|--------------------|-------------------|--------------------|-------------------|
| | i.v. | i.p. | i.v. | i.p. |
| 6 hours | 10.678 \pm 0.635 | 5.549 \pm 0.344 | 0.947 \pm 0.424 | 0.016 \pm 0.000 |
| 12 hours | 14.522 \pm 1.555 | 6.206 \pm 0.481 | 1.679 \pm 0.371 | 0.307 \pm 0.247 |
| 24 hours | 19.617 \pm 3.074 | 6.689 \pm 0.537 | 3.744 \pm 1.326 | 1.336 \pm 0.399 |
| 48 hours | 22.727 \pm 1.057 | 7.763 \pm 0.602 | 16.727 \pm 5.590 | 3.717 \pm 0.674 |
| 72 hours | 25.050 \pm 1.163 | 8.340 \pm 0.586 | 20.268 \pm 5.682 | 4.442 \pm 0.732 |
| 96 hours | 27.461 \pm 1.304 | 9.121 \pm 0.632 | 23.368 \pm 4.162 | 5.398 \pm 0.595 |

Fig. 2. Cumulative radioactivity (%) in the excreted urine of mice after intravenous or intraperitoneal administration of AME (^{14}C)

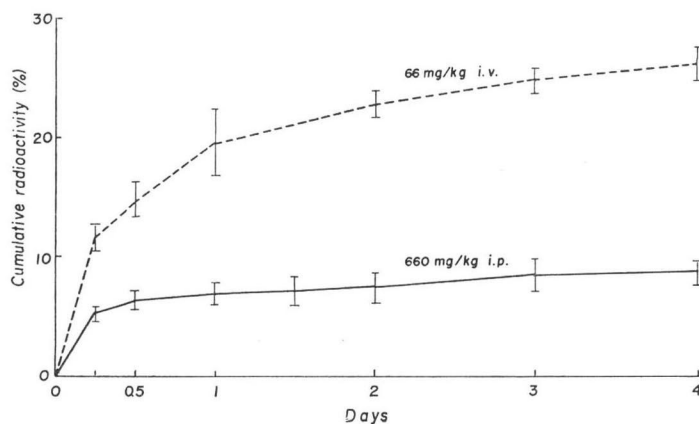
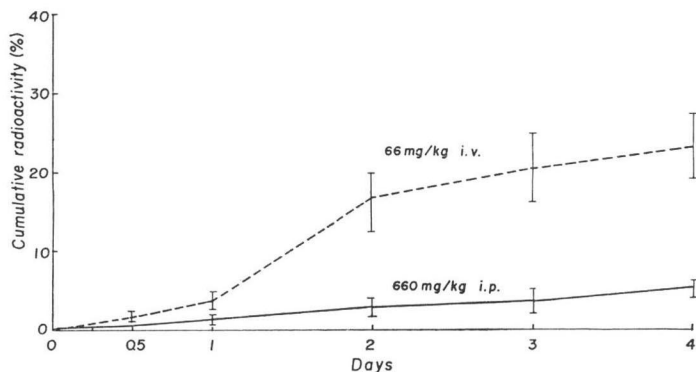


Fig. 3. Cumulative radioactivity (%) in the excreted feces of mice after intravenous or intraperitoneal administration of AME (^{14}C)



general circulation when the drug is administered by i.p. route.

(3) Feces: Table 5 and Fig. 3 show the radioactivity excretion pattern of mice receiving AME by i.v. and i.p. routes. For both the i.p. and the i.v. route, the maximum excretion of radioactivity occurred in the first 48 hours. The cumulative radioactivity after 4 days in the feces of mice after i.v. administration was about 23% of total radioactivity administered.

ed. In contrast, only 5% was recovered from the feces of mice receiving AME by the i.p. route.

(4) Expired gas. No significant radioactivity was detected in the expired gas of mice receiving radioactive AME by either i.p. or i.v. administration up to 48 hours after the administration.

III. Identification of Metabolites in Urine of Mice

Fig. 4 shows the TLC of the authentic AME and the urine samples of i.p. and i.v. treated mice developed by two solvent systems as described in materials and methods. Radioactivity measurements and bioautography indicate the presence of only AME. No spot comparable to amphotericin B was detected under UV light or by bioautography. The small amounts of radioactivity detected below the major spot appear to be caused by a tailing effect. Since no bioactivity was detected at the spot comparable to amphotericin B, the administered AME was evidently not de-esterified to the parent compound. The generally smaller zones of inhibition in the bioautogram developed by the solvent system 1 as opposed to solvent system 2 were possibly caused by the high acidity of solvent system 1 which may have inactivated some of the AME in the development of the chromatogram. Therefore, the radioactivity measurement and bioautography of TLC with both solvent systems strongly suggested that there appeared to be no apparent de-esterification of AME to the parent compound in mice receiving AME either by the intravenous or intraperitoneal route.

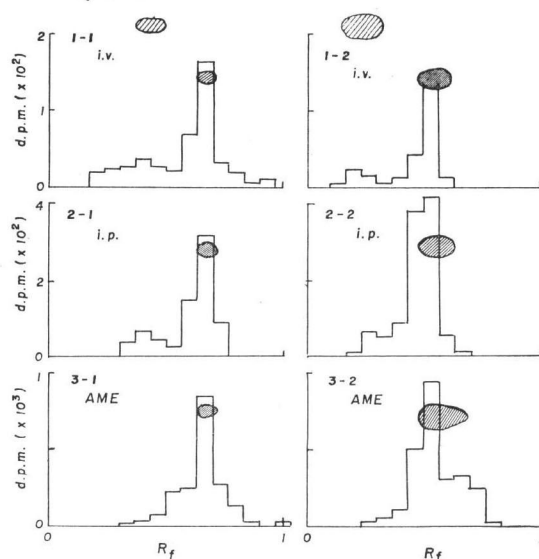
Discussion

Amphotericin B methyl ester hydrochloride (AME) was previously found to be active against various systemic fungal infections in mice but the efficacy of AME was somewhat lower than that observed for Fungizone¹²⁾. Our finding that AME showed poor absorption from the i.p. route partially explains this lower efficacy against systemic fungal infections. The high concentration of AME in the lung after i.v. administration is of considerable interest due to the involvement of this organ in many systemic mycoses¹³⁾. Measurement of the drug extracted from the lung showed that the AME retained much of its bioactivity (Table 3). Thin-layer chromatograms of the extracted urine showed a spot com-

Fig. 4. Thin-layer chromatograms of the urine samples of mice treated either by i.v. or i.p. with amphotericin B-¹⁴C-methyl ester: radioactivity measurements and bioautographs.

Two solvent systems were employed; (1) chloroform-ethanol-20% acetic acid (1:1: saturation). (2) chloroform-methanol-borate buffer (0.025M, pH 8.3) (2:2:1, lower phase). The shaded area shows the bioautograph of amphotericin B as a reference in each solvent system. The blackened area shows the bioautograph of the developed TLC in each solvent system.

- 1-1. Urine extract of mice i.v. treated with AME developed by solvent system 1
- 1-2. Urine extract of mice i.v. treated with AME developed by solvent system 2
- 2-1. Urine extract of mice i.p. treated with AME developed by solvent system 1
- 2-2. Urine extract of mice i.p. treated with AME developed by solvent system 2
- 3-1. Authentic AME developed by solvent system 1
- 3-2. Authentic AME developed by solvent system 2



parable to that of AME. These findings suggest that AME might be highly effective against systemic mycoses, particularly those involving the lung when administration is by the i.v. route. A possible explanation for the high accumulation of the drug in the lung may be that AME has a high affinity for the lung tissue due to the lipophilic nature of the drug. Alternatively, since a large amount of drug is administered, the AME may be simply retained in the lung through a filtration effect on the circulatory system in the alveoli. To clarify these points, the tissue localization pattern with a low dosage range using a high specific radioactive AME and autoradiographic examination of lung tissues are currently under investigation and will be reported elsewhere.

The de-esterification of AME to the highly toxic parent compound is undesirable and would possibly lead to potential danger. Our finding that no apparent de-esterification of AME was found in the urine sample of both i.p. and i.v. treated mice would seem to rule out this possibility. To confirm our argument, however, it would be essential to show that the methyl group of the ester is intact in animal tissues throughout the metabolic processes. One approach would be to synthesize ^3H -amphotericin- ^{14}C -methyl ester and monitor the radioactivity level of ^3H and ^{14}C independently. The synthesis of such a compound is currently in progress and the metabolic fate of the compound would be reported elsewhere.

Acknowledgments

This research was supported in part by contract NIHGMS 69-2161 from the National Institute of General Medical Sciences and by grant AI-02095 from the National Institute of Allergy and Infectious Diseases both under Public Health Service. The authors also wish to acknowledge the assistance of Dr. HARALD A.B. LINKE in the preparation of the radiocarbon-labeled amphotericin B methyl ester.

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