BIODISTRIBUTION OF 4-[14C]CHOLESTEROL-AMBISOME FOLLOWING A SINGLE INTRAVENOUS ADMINISTRATION TO RATS

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ABSTRACT:

A biodistribution study of 4-[14C]cholesterol-AmBisome, a unilamellar liposomal preparation of amphotericin B was conducted to support a radiolabeled human study. The radioactive plasma concentration profile (as measured in μg-Eq/ml of cholesterol) was best fit to a sum of three exponentials that yielded α, β, and γ half-life estimates of 3.0 ± 0.3, 11.8 ± 3.7, and 113.4 ± 32.4 h, respectively. Clearance and the steady state volume of distribution were 4.9 ± 0.2 ml/h/kg and 341 ml/kg. Recovery data collected through 96 h demonstrated mass balance and indicated that although the elimination profile in both urine and feces were incomplete, the dominant route of elimination (<2% in urine versus 33% in feces) was feces, presumably via biliary excretion of intact liposome and/or cholesterol. The liver, spleen, and lungs, organs of the reticuloendothelial system known for their rapid uptake of liposomes, presented with the highest levels of radioactivity. Levels in the kidney were 15% of that found in the liver and lungs.

Amphotericin B has remained the standard for antifungal therapy even though its use is limited by toxicity rather than by therapeutic response. The advent of lipid formulations (Bekersky et al., 1999b) has reduced the toxicity associated with amphotericin B in both animals (Proffitt et al., 1991; Fielding et al., 1992; Clemens and Stevens, 1993; Wasan et al., 1998) and man (Lopez-Berestein et al., 1985; Rapp et al., 1997; Sculier et al., 1988; Walsh et al., 1998, 1999; Bekersky et al., 1999a), while maintaining antifungal activity. AmBisome, a true liposome formulation that consists of small unilamellar vesicles made from rigid neutral and charged phospholipids, cholesterol, and amphotericin B, is designed to prolong drug residence time in the body (Bekersky et al., 1999b). AmBisome’s (amphotericin B) pharmacokinetic profile differs markedly from other amphotericin B formulations, exhibiting higher plasma exposures at a given dose (Boswell et al., 1998b). In an effort to address specific disposition issues of AmBisome (amphotericin B) by administering radiolabeled AmBisome to human volunteers, a radiolabeled drug study in rats was required. For these reasons, a biodistribution study using 4-[14C]cholesterol-AmBisome was conducted in Sprague-Dawley rats.

Materials and Methods

Test Animals. Young adult male and female Sprague-Dawley Rats (175–275 g) were obtained from Charles River Laboratories (Portage, MI). After dosing, animals were individually housed in Nalgene metabolism cages. General procedures of animal care and housing met current AALAC standards, current requirements published in the “Guide for the Care and Use of Laboratory Animals” (National Research Council, 1996) and the requirements of the United States Department of Agriculture through the Animal Welfare Act (Public Act 99-198). Certified Rodent Lab Diet (PMI Feeds, Inc., Richmond, IN) and municipal drinking water were available ad libitum.

Test Chemical. 4-[14C]Cholesterol-AmBisome, a liposomal amphotericin B preparation containing 5.1 mg of amphotericin B/ml, was synthesized with 4-[14C]-labeled cholesterol (PerkinElmer Life Science Products, Boston, MA; 46 mCi/mmol with a purity 97% by high-performance liquid chromatography and thin-layer chromatography) at 4.7 mg/ml in the liposomal lipid by NeXstar Pharmaceuticals (now Gilead Sciences, Inc., San Dimas, CA). The specific activity of liposomal 4-[14C]cholesterol-AmBisome preparation was 0.1 mCi/ml.

Dose Solution Preparation and Administration. Stock 4-[14C]cholesterol-AmBisome was diluted with 5% dextrose for injection to yield a dosing solution that contained 0.75 mg of amphotericin B/ml and 0.69 mg of 4-[14C]cholesterol/ml with a specific activity of 27.54 μCi/mg. The dosing solution, administered via a peripheral tail vein at 4 ml/kg, resulted in an AmBisome (amphotericin B) dose of 3.0 mg/kg (or 2.8 mg/kg 4-[14C]cholesterol; radioactive dose of 82.6 μCi/kg).

Experimental Design. Male and female rats were assigned to the study by body weight using a computerized procedure to provide homogeneous group mean body weights. There were eight groups of animals. Groups 1 through 6, and 8, composed of three rats/sex/group, were designed for distribution; group 7, comprising five rats/sex/group, was set up for elimination.

Sample Collection. Distribution. Preterminal blood samples (two per animal) were collected in heparinized tubes following puncture of the retro-orbital plexus before dosing and at 0.08, 0.25, 0.50, 0.75, 1, 2, 6, 10, 18, 30, 36, 60, and 84 h post dose from three rats/sex/time point under CO2/O2 anesthesia. Terminal blood samples obtained by cardiac puncture during exsanguination procedures concurrent with euthanasia (CO2/Asphyxiation) were obtained at 3, 8, 12, 24, 48, 72, and 168 h. Blood samples were stored on wet ice until harvested for plasma. Plasma was stored at 5°C until processed for radioactive measurement. At termination, adipose, eyes, heart, small intestines, kidneys, liver, lungs, skeletal muscle, skin, spleen, and residual carcass tissues were excised, weighed, and processed for radioactive measurements as described below.

Elimination. Rats were placed in Nalgene metabolism cages immediately after dosing for collection of urine and feces. Excreta were collected at 6, 12, 24, 48, 72, and 96 h post dose. Cage rinses with deionized water were performed after each collection interval. At 96 h, rats were euthanized by CO2 asphyxiation concurrent with exsanguination procedures via cardiac puncture. Whole blood and tissues were collected and processed as noted above.
Sample Processing and Analysis. Weighed aliquots of plasma and urine were mixed directly with liquid scintillation fluid without additional processing. Feces and residual carcasses were homogenized in deionized water while remaining tissues were minced before delivering aliquots into tared combustion cones with pads. Samples were allowed to dry before combusting in a Packard Tri-Carb Sample Oxidizer model 307 for collection of $^{14}$CO$_2$. Duplicate samples were analyzed by liquid scintillation for 5 min using a Packard 2300TR liquid scintillation analyzer. Raw counts were adjusted for quench and background to yield disintegrations per minute (Packard Instrument Co., Meriden, CT). The level of detection for the scintillation counter was set at 100 dpm. Standards were used between runs to verify counter accuracy. The concentration of $^{14}$C-derived radioactivity in tissues (or plasma), expressed in disintegrations per minute (dpm), was obtained by dividing the sample concentration (dpm/g) by the specific activity of the dosing solution (dpm/µg).

Pharmacokinetic Calculations. Plasma/tissues. 4-$^{14}$C]Cholesterol-AmBisome-derived plasma/tissue concentration-time data were analyzed by noncompartmental methods in WinNonlin (Pharsight Corporation, Mountain View, CA, version 3.1, models 200 and 201 for tissues and plasma, respectively). Plasma concentration-time data were also analyzed with compartmental methods using WinNonlin (model 18).

Excretion/mass balance. The cumulative percentage of the radioactive dose excreted in the urine (U) and feces (F) up through 96 h, the last sample collection time point, was calculated as $\sum U_d$ dose and $\sum F_d$ dose × 100, respectively. A mass balance estimate was calculated from the summation of the percentage of radioactivity recovered in excreta, tissues, and residual carcass 96 h post dose.

Tissues. The tissue distribution of 4-$^{14}$C]cholesterol-AmBisome and the percentage of dose recovered from the tissues were calculated and averaged. Total tissue weights of adipose, plasma, skeletal muscle, and skin were determined by extrapolating the fractional weights (as a percentage of the whole body weight) obtained from the literature (Brown et al., 1997) to the average of the pretreatment and necropsy body weights. The extrapolation procedure could not distinguish whether drug bound to receptors within these tissues. Thus, the values for total tissue weight obtained from the extrapolation procedure were gross estimates at best.

Results

Pharmacokinetics. Plasma. There were no apparent gender differences in the pharmacokinetic disposition of 4-$^{14}$C]cholesterol-AmBisome-derived radioactivity (Table 1). Therefore, male and female concentration-time data for 4-$^{14}$C]cholesterol-AmBisome-derived radioactivity was combined. The mean ($n=6$ rats/time point) concentration-time profile in microgram-equivalents per milliliter of cholesterol is presented in Fig. 1. A noncompartmental analysis of the data indicated that the $C_{max}$ of 66.2 $\mu$g-Eq/ml coincided with the first sample collection time point (0.08 h). The $AUC_{ss}$ was 576 $\mu$g-Eq·h/ml and $t_{1/2}$, $\lambda$ was 104.9 h. CL and $V_{ss}$ for 4-$^{14}$C]cholesterol-AmBisome-derived radioactivity were 4.9 ml/h/kg and 310 ml/kg.

A compartmental analysis of the plasma concentration data depicted in Fig. 1 was best fit to the following sum of three exponentials equation:

$$C_p = 49.9e^{-0.2303t} + 7.7e^{-0.0588t} + 1.4e^{-0.0061t},$$

where $C_p$ was the plasma concentration of cholesterol in $\mu$g-Eq/ml at any time, $t$. Pharmacokinetic parameters generated by this method were consistent with noncompartmental methods as evidenced by $AUC_{ss}$, CL, mean residence time, and $V_{ss}$ values presented in Table 1.

Excretion/mass balance. The mean (±S.E.) total recovery of radioactivity within 96 h of dosing was 109.69 ± 0.89% (Table 2). From this total, 1.37 ± 0.33% was recovered in the urine, 33.32 ± 1.36% in the feces, 0.16 ± 0.04% in the cage rinse, and 74.46 ± 1.94% in the body (selective tissues plus residual carcass), respectively. Recovery data indicated that radioactivity was still being excreted in both urine and feces 96 h post dosing and that the primary route of elimination was via feces.

Tissues. The mean radioactive tissue concentration-time data is provided in Table 3. Tissue concentration-time profiles for target organs are also depicted in Fig. 1. At 3 h post dose, the first sample collection time point, the highest concentrations of 4-$^{14}$C]cholesterol-AmBisome-derived radioactivity were found in the spleen, liver, and lungs at 21.0, 20.7, and 6.9 $\mu$g-Eq/g, respectively. In the case of the liver and spleen, radioactive concentrations decreased slowly and were still 65 and 88% of $C_{max}$ at 13.5 and 18.4 $\mu$g-Eq/g at 12 h, respectively. Over this same period, lung concentration increased by 44% to 10.0 $\mu$g-Eq/g, reaching $C_{max}$. From 12 h on, radioactivity in liver and spleen decreased at rates that paralleled the terminal phase of plasma, whereas radioactivity in the lung decreased at a slower rate than plasma. As such, by the last sample collection time point, the lung at 4.1 $\mu$g-Eq/g or 41% of $C_{max}$ had the highest reported level of radioactivity. The spleen and liver followed with 3.0 $\mu$g-Eq/g or 14% and 2.0 $\mu$g-Eq/g or 10% of $C_{max}$, respectively.

The kidney bears mention because of amphotericin B-related nephrotoxicity. As shown in Fig. 1, the radioactive concentrations in the kidney remain relatively constant throughout the entire collection period. A $C_{max}$ of 3.5 $\mu$g-Eq/g was observed at 3 h. By 168 h, the level was still 69% of $C_{max}$, or 2.4 $\mu$g-Eq/g.

Pharmacokinetic parameters were calculated for target tissues (Table 1). The estimated half-lives for $^{14}$C]AmBisome-derived

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**Table 1.** Pharmacokinetic parameters (±S.E.) of plasma 4-$^{14}$C]cholesterol-AmBisome-derived radioactivity in Sprague-Dawley rats 96 h after the intravenous administration of 3 mg/kg $^{14}$C]AmBisome (2.8 mg/kg cholesterol)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Matrix</th>
<th>$C_{max}$ (Obs)</th>
<th>$AUC_{0-168}$</th>
<th>$AUC_{ss}$</th>
<th>$t_{1/2}$ $\alpha$</th>
<th>$t_{1/2}$ $\beta$</th>
<th>$t_{1/2}$ ($\lambda$ or $\gamma$)</th>
<th>CL</th>
<th>MRT</th>
<th>$V_{ss}$</th>
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<tbody>
<tr>
<td>Male</td>
<td>Plasma$^a$</td>
<td>63.2</td>
<td>472</td>
<td>553</td>
<td>101.3</td>
<td>5.1</td>
<td>55.8</td>
<td>282</td>
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</tr>
<tr>
<td>Female</td>
<td>Plasma$^a$</td>
<td>69.2</td>
<td>521</td>
<td>600</td>
<td>101.1</td>
<td>4.7</td>
<td>64.9</td>
<td>303</td>
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<tr>
<td>Combined Plasma$^a$</td>
<td>66.2 ± 3.4</td>
<td>496</td>
<td>576</td>
<td></td>
<td>104.9</td>
<td>4.9</td>
<td>63.8</td>
<td>310</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined Plasma$^a$</td>
<td>59.9 ± 2.0</td>
<td>575 ± 28</td>
<td>3.0 ± 0.3</td>
<td>11.8 ± 3.7</td>
<td>113.4 ± 32.4</td>
<td>4.9 ± 0.2</td>
<td>70.1 ± 18.9</td>
<td>341 ± 75</td>
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<tr>
<td>Combined Liver$^a$</td>
<td>18.7</td>
<td>918</td>
<td>1149</td>
<td></td>
<td>80.7</td>
<td></td>
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<tr>
<td>Combined Lung$^a$</td>
<td>10.0</td>
<td>1098</td>
<td>2014</td>
<td></td>
<td>160.0</td>
<td></td>
<td></td>
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<tr>
<td>Combined Spleen$^a$</td>
<td>21.0</td>
<td>1294</td>
<td>1748</td>
<td></td>
<td>105</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Combined Kidney$^a$</td>
<td>3.1</td>
<td>478</td>
<td>473</td>
<td></td>
<td>N.C.</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

N.C., not calculated.

$^a$ Noncompartmental analysis.

$^b$ Compartmental analysis.

$^c$ Predicted value.

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1 Abbreviations used are: AUC, area under the concentration-time curve; $V_{ss}$, distribution volume at steady state; CL, clearance; RES, reticuloendothelial system.
radioactivity ranged from 80.7 in the liver to 160 h in the lungs. Half-life values were not estimated for the kidney because the terminal data points did not lend themselves to regression. AUC 0–168 values were greatest in the spleen, lungs, liver, and kidney, in that order.

**Discussion**

The convention for studies of this nature is to use radiolabeled drug substance. However, when it became apparent that 14C-labeled amphotericin B is not commercially available, we decided that 4-14C-labeled cholesterol, a major liposomal component of AmBisome, was the best possible choice by which to support the corollary radiolabeled AmBisome study in humans. The rationale was based upon the fact that cholesterol-rich liposomes are more stable in blood and plasma than conventional liposomes (Betageri et al., 1993). Furthermore, evidence indicates that AmBisome, a cholesterol-rich liposome containing amphotericin B, remains intact for extended periods upon intravenous administration (Fujisawa Healthcare, Inc., 1999).

The plasma profile of AmBisome collected through 168 h in the rat after a single dose of 4-[14C]cholesterol-AmBisome revealed the presence of a deep compartment. Best fit to a sum of three-exponentials with $t_{1/2}$ values of 3.0, 11.8, and 113.4 h for the $\alpha$, $\beta$, and $\gamma$-phases, respectively, the triexponential profile differed from the biexponential profile previously reported in the rat (Boswell et al., 1998a). The reason for the observed kinetic difference in the rat probably stemmed from the limited sample collection interval (24 h) used by Boswell. However, perhaps a more interesting finding was the observation that the $\beta$-phase $t_{1/2}$ of 11.8 h derived from radiolabeled cholesterol (AmBisome) was consistent with terminal $t_{1/2}$ values (7.3–9.7 h) of AmBisome derived from amphotericin B using high-performance liquid chromatography-UV. This was deemed to be of interest because similar findings were observed in the radiolabeled...
human study. This was a more complex study that used scintigraphic methods and liquid chromatography/tandem mass spectrometry to quantify both total radioactivity and amphotericin B concentrations in plasma, urine, and feces for 7 days (Bekersky et al., 2000b,c). In this study, the presence of a deep compartment was also established. Mean β- and γ-phase t1/2 values for [14C]cholesterol and amphotericin B of 8.1 and 6.0 h and 147 and 152 h, respectively, appear to be consistent with findings in the rat. It did not imply that the disposition of cholesterol-derived radioactivity and amphotericin B as obtained from AmBisome was the same; they are not. For example, in the consistent with findings in the rat. It did not imply that the disposition of cholesterol-derived radioactivity and amphotericin B obtained from AmBisome in the rat, where amphotericin B was the pharmacokinetic marker (Boswell et al., 1998a; Bekersky et al., 2000a). This provided additional support for using 4-[14C]cholesterol as a pharmacokinetic marker for amphotericin B within AmBisome. Moreover, mass balance was demonstrated at 96 h. The primary route of elimination was via feces, presumably after excretion of intact liposome or 4-[14C]cholesterol via bile. This was consistent with findings from the human study.

In conclusion, 4-[14C]cholesterol, an integral component of the liposome delivery device, was used as a marker through which the distribution/dispersion of AmBisome was evaluated. Uptake of AmBisome-derived radioactivity by the RES was rapid with the highest concentrations being found in liver and spleen. The elimination of AmBisome-derived radioactivity occurring predominantly via bile was slow. One possible reason for this is that AmBisome (a cholesterol-rich liposome) is relatively stable within plasma and tissues. Recall that AmBisome has a unique ability to sequester amphotericin B in stable liposomes for extended periods of time and that this appears to modulate the toxicity associated with drug substance (Boswell et al., 1998b). The other possible reason for slow elimination via bile comes from the human study. Recall that amphotericin B was cleared more rapidly than cholesterol. Furthermore, the fact that the primary route of radioactive elimination was via feces in both rat and human studies was consistent with cholesterol catabolism. One additional note stems from the interesting findings of half-life for total radioactivity and amphotericin B in both the rat and human studies. One can speculate that the pharmacokinetic profile of 4-[14C]cholesterol-AmBisome-derived radioactivity in Sprague-Dawley rats after intravenous administration of 3 mg/kg AmBisome (2.8 mg/kg cholesterol) was consistent with plasma AUC values and agrees with findings from the human study. This was a more complex study that used scintigraphic methods and liquid chromatography/tandem mass spectrometry to quantify both total radioactivity and amphotericin B concentrations in plasma, urine, and feces for 7 days (Bekersky et al., 2000b,c). In this study, the presence of a deep compartment was also established. Mean β- and γ-phase t1/2 values for [14C]cholesterol and amphotericin B of 8.1 and 6.0 h and 147 and 152 h, respectively, appear to be consistent with findings in the rat. It did not imply that the disposition of cholesterol-derived radioactivity and amphotericin B as obtained from AmBisome in the rat, where amphotericin B was the pharmacokinetic marker (Boswell et al., 1998a; Bekersky et al., 2000a). This provided additional support for using 4-[14C]cholesterol as a pharmacokinetic marker for amphotericin B within AmBisome. Moreover, mass balance was demonstrated at 96 h. The primary route of elimination was via feces, presumably after excretion of intact liposome or 4-[14C]cholesterol via bile. This was consistent with findings from the human study.

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### References


Boswell DW, Bekersky I, Buell D, Hiles R and Walsh TJ (1998a) Toxicological profile and


