Abstract □ The chronobiological effect on the pharmacokinetics of liposomal encapsulated ampicillin (LEA) was compared at noon (1200) and midnight (2400) after intravenous injection of 50 mg/kg of LEA in rats. The effects of fasting on the circadian rhythms of LEA were also investigated. Serial blood samples were collected for 2 h. Bile and urine were collected during the entire study. Plasma data was analyzed by noncompartmental methods. Dosing of LEA at 2400, when the animals were active, resulted in a 50% decrease in mean residence time (MRT) and a 20% increase in systemic clearance (Cl_total). The increase in Cl_total was reflected by increases in biliary (Cl_bile) and renal (Cl_renal) clearance at 2400. In addition, the steady-state volume of distribution (Vss) at 2400 was also decreased by about 50% as compared to dosing at 1200. Interestingly, no difference in the pharmacokinetic parameters were observed in fasting animals at 1200 and 2400. Since rats consume very little food during their sleep cycle, restriction of food intake did not have any effect on the pharmacokinetics of LEA at 1200. However, fasting rats had an approximately 36% decrease in Cl_total as compared to nonfasting rats at 2400. This decrease in systemic clearance was paralleled by a 60% and 24% decrease in Cl_bile and Cl_renal respectively. These variations could be attributed to changes in bile composition and/or lipoprotein concentrations in the plasma as a result of “forced” fasting at 2400 when the animals are generally more active and food intake is high.

Introduction

Research studies on animals and humans have indicated that a variety of drugs are influenced by circadian rhythms after administration into a biological system.1-3 A circadian rhythm is an inborn, genetically programmed, self-sustained rhythm in behavior, physiology, or metabolism that enables living organisms to cope with the 24-h daily cycle. These rhythms can also be influenced by external factors such as light, food intake, etc. The rhythm generally reaches the peak and nadir of its sinusoidal wave once daily during the active and resting phase, respectively.1-3 Some examples of drugs which exhibit circadian rhythms include calcium channel blockers, theophylline, and indomethacin.1-3 These studies found that experimental subjects were more susceptible to the toxic effects of drugs during the rest period as opposed to the active period.

Nakano et al.4 found that the percent mortality of mice was significantly higher when gentamicin was injected in the middle of the rest period (0700–1900) when compared to the same dose injected at midnight when the animals were active. In fact, Nakano et al.5 observed a significantly lower clearance, longer half-life and larger area under the concentration–time curve when gentamicin was administered during the rest period. Similar temporal changes in pharmacokinetics and nephrotoxicity of tobramycin in rats were also documented by Lin et al.6,7 The renal and biliary clearance (active secretion) of ampicillin in rats also exhibits a circadian rhythm which results in the plasma concentration–time profiles having a 2-fold longer MRT during the resting phase (1200) as compared to the active phase (2400).8

The observed chronobiological variations may be due to the changes in the rates of biliary and renal excretion. Renal clearance is generally lower during the rest cycle and reaches a maximum in the middle of the active cycle.9,10 Urinary excretion varies significantly with the time of day. Urinary excretion is the sum of glomerular filtration, active tubular secretion, and reabsorption. The circadian rhythms associated with glomerular filtration (mimic the renal blood flow) and reabsorption (influenced by rhythm of urine pH) are well documented.9,10 However, the rhythm associated with active tubular secretion has not been evaluated extensively and conflicting reports appear in the literature. Limited data is available regarding the circadian rhythm associated with biliary excretion. However, one study indicates that biliary excretion is higher during the active cycle than during the sleep cycle.11 The enhanced biliary excretion may be due to an increase in bile flow and/or modifications in the composition of bile due to food intake which is increased during the active phase.

Liposomes are microscopic lipid vesicles consisting of one or more concentric phospholipid bilayers enclosing discrete aqueous spaces. The major therapeutic advantage of liposomes lies in controlling the availability of the entrapped agents either by altering tissue distribution or prolonging release of entrapped drug. Since liposomal encapsulated drug is not readily available for elimination, administration of an entrapped drug can result in a significant decrease in the apparent systemic drug clearance and prolong the MRT.12-14 Several β-lactam antibiotics, such as cefazolin and ampicillin, have been formulated in liposomes. Studies done in our laboratory have found that LEA has a lower systemic clearance, greater uptake, and retention by the organs of the reticuloendothelial system (RES) and prolonged circulating blood levels as compared to free ampicillin.9 These results correlate well with other studies found in the literature.12-14 These characteristics of liposomal formulations may reduce the effect of diurnal biological rhythms on the apparent clearance of encapsulated drug. However, no information concerning the temporal changes in the pharmacokinetics of LEA or other liposomal encapsulated drugs has been reported in the literature. In addition, several factors, such as high-density lipoprotein (HDL)/low-density lipoprotein (LDL) plasma levels, which influence liposomal disposition, are known to follow a circadian rhythm which is strongly influenced by food intake.15 Food intake causes an increase in plasma HDL which acts to destabilize the phospholipid bilayers of liposomes resulting in release of entrapped drug; however, no reports on the effect of food on the pharmacokinetics of liposomal encapsulated drug after iv administration are available.

The purpose of these studies was to investigate if the incorporation of ampicillin into liposomes could reduce the effect of the circadian rhythms associated with the clearance of ampicillin. These studies were conducted in fasting and nonfasting rats.
nonfasting animals to determine the influence of food intake on the disposition of LEA.

Materials and Method

Chemicals—Cholesterol (CHOL), dioctyl phosphatidate (DCP), sphingomyelin (SPM), and ampicillin sodium salt were purchased from Sigma Chemicals (St. Louis, MO) and used as supplied. Spectra-Tor molecular porous membrane dialysis tubing (MW cutoff 12000–14000) was purchased from Fisher Scientific. Bacillus subtilis spore suspension and Antibiotic Medium No. 1 were purchased from Difco laboratories. HPLC-grade mobile-phase solvents and other analytical grade chemicals were obtained from J. T. Chemical Co. (Phillipsburg, N.J.).

Liposomal Formulation—Liposomes used in this study were freshly prepared as follows:16 lipids (SPM CHOL DCP, molar ratio 2:1:0.5, lipid concentration (mg/mL) 20.5:3.3:7) were completely dissolved in chloroform and the organic solvent was evaporated under vacuum at 50 °C, forming a thin lipid film. The lipid film is hydrated with ampicillin solution (50 mg/mL in normal saline) by a gentle hand-shaking method. The preparation was then sonicated (Model G.112 SPIG, Lab. Supplies, Hicksville, NY) for two 10 min periods separated by a 10 min interval followed by annealing at 50 °C in a shaking water bath for 1 h. Liposomes containing ampicillin were separated from unentrapped drug by dialysis against normal saline at 37 °C (1:50 ratio). The external medium was changed every 10 min for three intervals. The concentration of the ampicillin in the liposomes was determined by high-performance liquid chromatography after rupturing the liposomes with 1% Triton-X 100. The mobile phase consisted of a mixture of methanol/0.1 M sodium phosphate buffer solution (pH adjusted to 4.6 with phosphoric acid) (15:85). The flow rate of the mobile phase was set at 1 mL/min. The wavelength of detection was set at 254 nm and the detection was set at 0.05 absorbance unit full scale. Linear calibration curves were constructed with standard ampicillin solutions ranging from 0.5 to 200 μg/mL with a lower detection limit of 0.05 μg/mL. The inter- and intraday coefficient of variation were less than 5% and 10%, respectively. The liposomes were used within 2 h of preparation. A Nicomp Submicron Particle Sizer Autodilute model 370 was used to determine the liposome size distribution. The entrapment efficiency (EE) of ampicillin in liposomes was determined by ultrafiltration using an Amicon MPS-1 system at 2000 g for 20 min.

Animals—Adult male Sprague-Dawley rats (Charles River) with an average weight of 281.3 ± 20.3 g were used in this study. Animals were housed in a 12 h light/12 h dark cycle with constant temperature environment (22 °C). Animals were allowed to acclimatize for at least 7 days. Each animal underwent cannulation of the right external jugular vein with polyethylene 50 silastic catheter under ketamine/acepromazine/xylazine (50:3.3:3.4 mg/kg) anesthesia. The cannula was routed subcutaneously, externalized at the neck, and secured to the musculature. Animals were allowed to recover from the surgery for at least 24 h before the actual studies. Food but not water was withheld for 24 h in fasted rats. Food and water were allowed ad libitum in nonfasted rats. On the day of the experiment, each animal was anesthetized with pentobarbital solution (50 mg/kg) while the abdominal cavity was exposed for bile duct cannulation. Additional pentobarbital was administered as needed to maintain anesthesia throughout the study. The body temperature of each animal was maintained using heated surgical pads. Complete collection of urine was ensured by flushing the bladder at the end of the experiment.

Treatment—Animals were randomly divided into four groups. Each animal received via the cannula a single intravenous bolus injection of freshly prepared liposomal encapsulated ampicillin (LEA) (50 mg/kg) under fasting and nonfasting conditions at 1200 and 2400. The concentration of lipid in the final formulation ranges from 16 to 27 mg/mL, yielding an average lipid dose of 24–27 mg per animal. Blood samples (0.3 mL) were collected at 0.08, 0.25, 0.41, 0.58, 0.75, 0.91, 1.2, 1.5, and 1.8 h after dosing via the cannula into heparinized tubes. Normal saline was used to replace the body fluid lost through sample collection. Blood samples were immediately centrifuged and the plasma was collected. Urine was collected in 10 min intervals for the first hour and in 20 min intervals during the second hour. Urine was collected during the 2 h study and the bladder was drained and flushed with 6 mL of normal saline at the end of the study. All samples were kept frozen at -80 °C until analysis.

Sample Analysis

All samples collected were analyzed by a microbiological assay, employing an agar diffusion technique using B. subtilis as the test microorganism.17 All samples were frozen and thawed before the assay. The procedure of freezing–thawing disrupts the liposomal bilayers and enables an efficient measurement of the total drug (both free and encapsulated) in the plasma. Ampicillin standard solutions ranging from 1 to 100 μg/mL were assayed with each set of samples to obtain the standard curves. Samples were diluted so concentrations fell within the linear range of the standard curve. All samples were analyzed in triplicate. A pair of metric calipers was used to measure the zones of inhibition of growth after an 18 h incubation at 37 °C. Total ampicillin concentrations were determined from these measurements against the standard curves. Least-squares regression analysis was used to generate the slopes and intercepts. A weighting factor of 1/X² was employed in the analysis. The intra- and interday coefficients of variation for the assay were less than 10%. The concentrations of ampicillin as determined by the microbiological assay were confirmed by HPLC. There was less than a 5% variability between the two methods.

Pharmacokinetic Analysis

The pharmacokinetic parameters of total ampicillin concentrations were calculated using a noncompartmental analysis method. Area under the plasma concentrations vs time curve (AUC) and area under the first-moment curve (AUMC) from time zero to the last sample time were determined by La Grange computer analysis program18 with extrapolation to time infinity using the least square terminal slope (K) obtained by Rstripc computer program.19 Apparent total systemic clearance (Cloral), renal clearance (Clrenal), biliary clearance (Clbile), mean residence time (MRT), steady-state volume of distribution (VSS) and half-life (t1/2) were calculated using the following equations20 (fb denotes fraction excreted in bile):
animals were more active, resulting in a 50% decrease in the concentration of LEA after iv administration. Plasma concentrations of LEA declined in a biphasic manner with results obtained in the literature. 

Pharmacokinetic parameters are listed in Table 1. Consistent with results obtained in the literature, plasma concentrations of LEA after iv administration declined in a biphasic manner which is characteristic of MLV liposomes. The rapid initial decline in concentrations results from uptake of the aqueous compartment. Naccucio et al. has also reported a high % EE of 35–60% for piperacillin in neutral liposomes and concluded that piperacillin absorbs to the lipid bilayer.

The interaction between the antibiotic and the bilayer which results in the high entrapment of β-lactam antibiotics in liposomes is most likely of a hydrophobic nature since electrostatic interactions can be ruled out. Ampicillin and piperacillin bear a net negative charge in normal saline (pH = 5.3) and are unlikely to associate with a negatively charged or neutral lipid bilayer through electrostatic interactions.

Liposomal encapsulation significantly altered the disposition of ampicillin resulting in an increase in MRT at 1200 (22.7 ± 4.0 to 46.8 ± 6.9 minutes) and 2400 (12.3 ± 1.0 to 22.3 ± 3.0 minutes). These results suggest that release of encapsulated drug was the rate-limiting step in drug elimination at both 1200 and 2400. This hypothesis is supported by the observation that biliary clearance of encapsulated drug steadily decreases from a peak clearance of 0.93 ± 0.1 mL/min to a nadir of 0.25 ± 0.05 mL/min over a 2 h period, indicating that encapsulated drug in the blood is unavailable for excretion.

However, the timing of drug administration had a significant impact on the disposition of LEA in non-fasted animals. LEA had a significantly lower clearance at 1200 as compared to 2400 which is consistent with the circadian rhythm observed for the clearance of ampicillin and many other drugs. The decrease in clearance coupled with the increase in VSS resulted in a 2-fold increase in MRT. The release of ampicillin from the liposomes may occur at a faster rate at 2400 which would result in the observed increase in clearance of released drug and decrease in VSS. However, in the fasted animals no significant differences were observed.

**Discussion**

Biological rhythms have been detected in the disposition of many drugs. These time-dependent variations could be due to the parallel changes in physiological functions and variables involved in the absorption, distribution, metabolism, and excretion of drugs. The clearance of ampicillin is known to exhibit a circadian rhythm which peaks at 2400 and reaches a nadir at 1200. Liposomal encapsulation is known to significantly alter the disposition of drugs. The release of encapsulated drug is often the rate-limiting step in elimination. This factor may be able to override the influence of the circadian rhythm associated with renal and biliary excretion of ampicillin.

Previous experimental data has shown that the aqueous volume of multilamellar vesicles consists of only 7–10% of the total liposomal volume. The high percentage of entrapment observed in this study indicates that ampicillin interacts with the lipid bilayers in addition to entrapment within the aqueous compartment. Naccucio et al. has also reported a high % EE of 35–60% for piperacillin in neutral liposomes and concluded that piperacillin adsorbs to the lipid bilayer. The interaction between the antibiotic and the bilayer which results in the high entrapment of β-lactam antibiotics in liposomes is most likely of a hydrophobic nature since electrostatic interactions can be ruled out. Ampicillin and piperacillin bear a net negative charge in normal saline (pH = 5.3) and are unlikely to associate with a negatively charged or neutral lipid bilayer through electrostatic interactions.

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Ampicillin is actively secreted into the urine and renal clearance accounts for approximately 80% of the dose in rats. Active renal secretion has been shown to exhibit a circadian rhythm where renal excretion is increased during the active phase and reduced during the sleep cycle. A similar pattern for ampicillin was observed in the nonfasted animals receiving LEA. However, no rhythm was detected in the renal clearance of LEA in the fasted animals dosed at 1200 and 2400. In fact, the renal clearance of LEA was similar for the three groups with restricted food intake (fasting groups and nonfasting group dosed at 1200). Since entrapped drug cannot be eliminated via renal excretion, the release of ampicillin from the liposomes can be the rate-limiting step for renal clearance. Therefore, if ampicillin is released from the liposomes at a faster rate, renal clearance will increase as observed with the nonfasted rats dosed at 2400.

In addition to salts and electrolytes, many organic substances, such as cholesterol, fatty acids, and liposomal components, are excreted into the bile and are influenced by changes in bile flow and composition. When comparing LEA at 1200 and 2400 in nonfasted rats, the 50% higher C10 could be attributed to an increase in bile flow and/or changes in bile composition. Since no differences in bile flow were noted among the four treatment groups, the composition of the bile is most likely responsible for the differences in biliary clearance. Being nocturnal animals, rats are more active and have increased food consumption at night. Rodent chow contains phospholipids and cholesterol which will affect bile composition thereby altering ampicillin elimination into the bile. In addition to the reduced biliary clearance of ampicillin during the resting phase at 1200 (when food intake is low), additional decreases in biliary clearance were noted in the fasted animals dosed at 1200 and 2400. Thus, food intake appears to have a significant influence on the biliary excretion of ampicillin administered as the liposomal formulation.

The results clearly indicate that food intake has a significant impact on the disposition of LEA. There were no significant differences in the pharmacokinetics of LEA after administration to fasting animals at 1200 and 2400 and nonfasting animals at 1200 when food intake is normally low, suggesting that the rhythm observed in nonfasted animals is a food effect. Food appears to increase the release of entrapped drug during the terminal phase. A possible mechanism involves destabilization of the liposomes in the plasma. In addition, an increased turnover rate of the liposomes in the tissues could also explain, in part, the differences observed in the disposition of LEA in this study.

In a recent report in which continuous parenteral nutrition was the method of feeding, Saito et al. showed that there is no circadian rhythm for many endogenous substances, such as HDL, under conditions of continuous enteral feeding, whereas intermittent meal feeding did invoke rhythms in the plasma levels of these compounds. Intermittent food intake is a potent time cue and external stimulus for a variety of endogenous substances. Therefore, during periods of restricted food intake, the levels of HDL are lower which should decrease their interaction with liposomes and increase the plasma stability of the formulations. Lower food intake, as observed during the resting phase, lowers the HDL plasma levels and can, in part, explain the differences in the pharmacokinetics of LEA at 1200 and 2400. However, lower HDL levels should not influence the initial uptake of MLV liposomes by the RES. The similarities in the initial decline in plasma concentrations among the four treatment groups supports the argument that the initial uptake of MLV liposomes by organs of the RES was not significantly affected by food intake or time of drug administration. In addition, rat liver stearol carrier protein, a regulator for lipid metabolism and transport, also exhibits a circadian rhythm. Thus, the increase in activity during the active phase could result in a greater uptake and increased turnover rate of liposomes by the liver resulting in release of encapsulated drug as a function of time of drug administration.

In this study, we have established that food intake plays a significant role in the disposition of encapsulated drug. It appears that food increases the release of entrapped drug which can then be eliminated from the body and significantly decreases the MRT. Thus, parenteral liposomal encapsulated drugs should be administered under fasting conditions to optimize their usage and targeting ability.

References and Notes

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