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Disposition kinetics of Amphotericin-B in rats

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DISPOSITION KINETICS OF AMPHOTERICIN-B IN RATS

by

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APPROVAL BY THESIS DIRECTOR

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Michael Mayersohn

Michael Mayersohn, Ph.D.
Professor of Pharmaceutical Sciences

April 1, 1991
Date

TO MY PARENTS

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DISPOSITION KINETICS OF AMPHOTERICIN-B IN RATS

Yuli Cai, M.S.

The Univerisity of Arizona, 1991

Director: Michael Mayersohn

The disposition kinetics of Amphotericin-B (Am-B) were investigated in rats in three different dose groups. Each group contained four rats. The serum and urine Am-B concentrations were analyzed by reversed-phase HPLC.

There were no significant differences for total body clearance and half-lives as a function of dose. Those observations suggested systemic dose-independent behavior of Am-B. However, renal clearance of Am-B decreased significantly as Am-B dose increased; whereas, no renal damage was observed during the experiment.

The present studies suggested that renal clearance was dose-dependent and that there may be a saturable tubular secretion process for Am-B renal excretion.

Chapter 1

INTRODUCTION

Physical and Chemical Characteristics

Amphotericin B (Am-B) is the drug of choice for the treatment of most disseminated mycoses affecting humans and it has remained the least toxic and most reliable effective systemic antifungal agent for the past 30 years (Graybill et al., 1983). Am-B was discovered in 1953 and is elaborated by selected strains of Streptomyces nodosus growing under defined and regulated conditions. The drug accumulates as a sludge on the mycelia and in the culture medium. One important pharmaceutical property of this drug is that it is insoluble in water when the pH is between 2 and 11. However, aqueous solutions at the extremes of pH are unstable. The dry-powder form of Am-B, if stored at room temperature in the dark and under anaerobic conditions, will remain stable for long periods of time (Gold et al., 1956; Vandeputte et al., 1956). Am-B was found to be stable (<5% loss) in human serum at -20°C

for up to 14 days with only a 10% loss of drug after 6 months. In dimethylsulfoxide (DMSO), more than 60% was lost in 6 months (Edmonds et al., 1989).

The chemical structure of Am-B was deciphered in 1970. The drug, which has a molecular weight of 924, is a deep yellow-colored macrolide and contains a chromophore made up of a rigid, haptenic, planar, hydrophobic portion which is linked through an oxygen bridge to a flexible, polyhydroxylated, hydrophilic residue (Mechlinski et al., 1970). The structure is depicted in Figure 1.

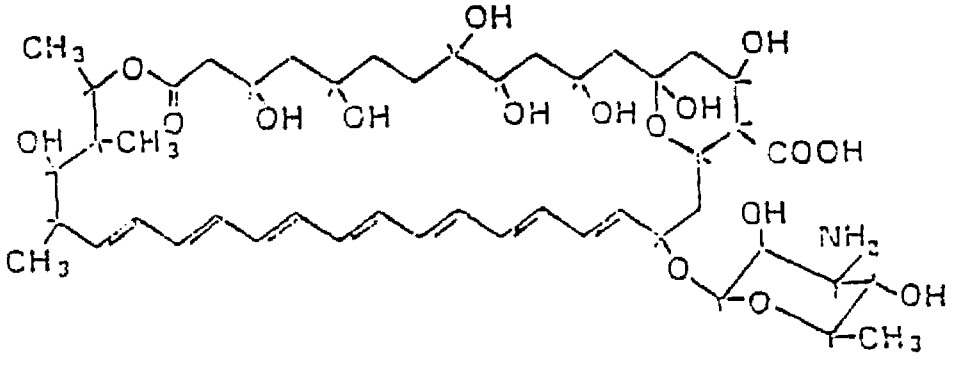


Figure 1. Chemical structure of Amphotericin-B

Pharmacology

Am-B exerts its effect on fungal and mammalian cells by forming complexes with the fungal membrane sterol, ergosterol, and with the mammalian membrane sterol, cholesterol. After forming a complex, membrane permeability is increased, which causes the leakage of small intracellular molecules such as potassium. At higher concentrations, Am-B increases membrane permeability to larger molecules such as hemoglobin and enzymes (Kobayashi et al., 1977). Am-B has no effect on bacteria since there is no sterol in the bacterial cell membrane (Kobayashi et al., 1977). The effects of Am-B in low concentrations are reversible, while at higher concentrations, irreversible cell damage occurs (Graybill et al., 1983).

The relative specificity of Am-B for fungi compared to mammalian cells is based on a more avid binding to ergosterol than to cholesterol, a phenomenon first reported by Gruda et al. (1980). Under experimental conditions, the spectral change of Am-B in the ultraviolet-visible region is due to the formation of antifungus-sterol complexes, which are more pronounced in the presence of ergosterol than with

cholesterol. This greater binding to ergosterol allows Am-B to be given parenterally to treat patients with invasive fungal infection. By contrast, filipin, a polyene with a small molecular weight, is too toxic for clinical use, because it has been shown to bind stronger to cholesterol than to ergosterol (Brajtburg et al., 1990).

Figure 2 presents two types of binding of Am-B to ergosterol or cholesterol. Figure 2A shows how Am-B and sterols, with the participation of H_2O, may form a "cage" resulting from hydrogen bonds. These bonds are regulated by proton donor-acceptor forces. The functional group involved in the hydrogen bonds are the hydroxyl groups of the sterols and the carboxyl group at the end of the C-18 of the Am-B molecule. This binding is strengthened by participation of the amino group of the amino sugar side chain (Brajtburg et al., 1990). Since both ergosterol and cholesterol are 3-ß-hydroxy sterols, it can be assumed that their reactions with Am-B involving hydrogen bonds are equivalent. Figure 2B shows the second type of interaction which involves the rigid chain of the seven conjugated double bonds of Am-B and the whole sterol molecule. This interaction is governed by van der Waals forces. The alkyl side chain of ergosterol at the C-22 double bond is responsible for the greater sensitivity of ergosterol-containing membranes to Am-B than to that of cholesterol-containing membranes (Brajtburg et al., 1990).

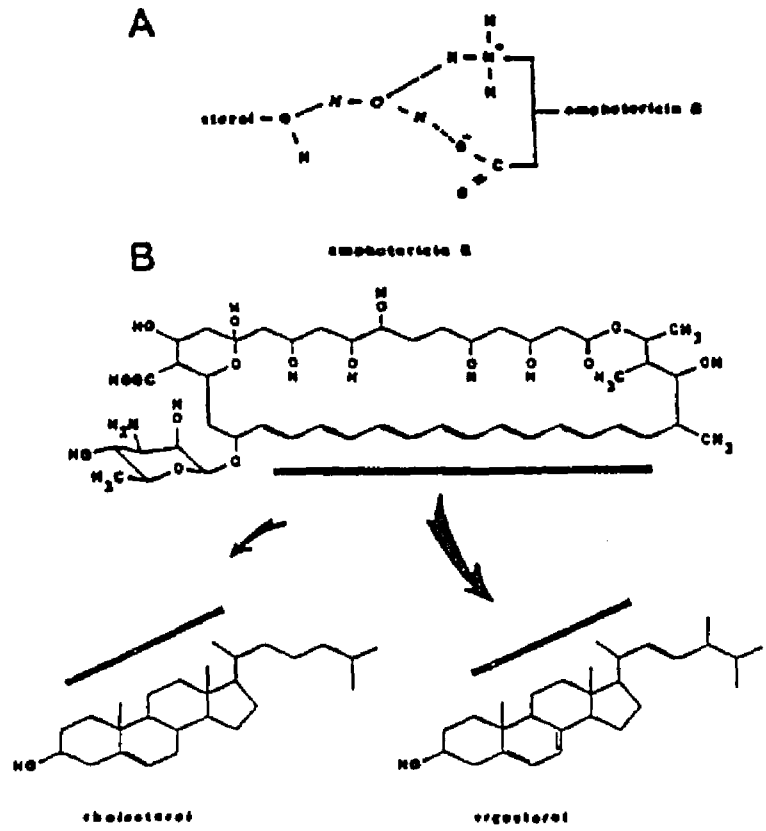


Figure 2. Schematic representation of hydrogen bond formation (2A) and nonspecific interactions between Am-B and sterols (2B). (Brajtburg et al., 1990)

Clinical Use and Toxicity

Am-B is used primarily for the treatment of Valley Fever, which is caused by Coccidioides immitis. This disease is prevalent in the southwest region of the United State. It is also an important drug widely used to treat serious systemic fungal infections. Such fungal infections can develop in the presence of conditions such as AIDS, cancer and organ transplant surgery, as a result of a defective immune response. Thirty percent of AIDS patients in the southwest part of the country died of systemic Coccidioides infections (Bronnimann et al., 1987); Am-B plays an important role in adjunct therapy associated with that condition. Because of its hydrophobicity, Am-B has been administered intravenously as a colloidal suspension. Fungizone, the commercial form of Am-B, is a mixture of 50% Am-B, 39% sodium deoxycholate and 19% sodium phosphates. The side effects of this compound include chills, fever, nephrotoxicity, hypokalemia, weight loss and febrile reaction (Fisher et al., 1989).

The recommendations for Am-B dosage are complex because of the acute development of nephrotoxicity and because the drug is generally used for long term treatment. Current clinical use of Am-B is based on limited pharmacokinetic

information available for this agent, therefore, dosage regimens are often dictated by concern for and signs of toxicity rather than by the findings of controlled clinical studies. Decisions on selecting daily dosage, total dosage, duration of therapy and whether or not to use Am-B are often determined by the clinical experience of the physician in treating fungal infections. Am-B is usually given intravenously over a 2 to 4 hour interval once a day or in double doses every other day. The maximum tolerated dose is about 2 mg/kg daily. The duration of therapy is not well defined for most deep fungal infections, but usually ranges from 6 to 12 weeks. AIDS patients who usually cannot tolerate flucytosine, should receive Am-B daily for 8 to 12 weeks (Benson et al., 1989).

Although Am-B remains the primary drug for the treatment of life-threatening fungal infections, the use of Am-B is often limited by its acute and chronic toxic effects. In humans, long term administration of Am-B may produce renal impairment, hypokalemia and anemia. A major limitation is the high incidence of nephrotoxicity. Soon after the initiation of therapy, the glomerular filtration rate (GFR) decreases by nearly 40% in most patients (Medoff et al., 1980). The GFR often stabilizes at 20% to 60% of normal throughout the course of therapy (Fisher et al., 1989).

Human and animal studies have shown that Am-B causes

renal insufficiency in at least two ways. First, intense intrarenal arteriolar constriction occurs shortly after the initiation of intravenous Am-B therapy. Renal plasma flow and GFR then decrease but may be reversed by saline loading (Butler et al., 1964; Gerkens et al., 1980). Second, after more prolonged treatment, afferent arteriolar smooth muscle cells become damaged, causing renal insufficiency (Bhathena et al., 1978). In addition, calcium deposition in the tubules and renal tubular acidosis occur and these may contribute to the impairment in renal function (Weldon et al., 1974).

Am-B and other polyene type drugs cause damaging effects to different types of cells, including erythrocytes. When Am-B is administered intravenously, rapid damage to erythrocytes can be produced at the site of infusion of solutions containing Am-B at a concentration of 75 μg/ml. Similar Am-B concentration also produced hemolysis in dogs. The common feature of this effect associated with infusion is loss of intracellular constituents. This is due to the effect on cell membrane permeability caused by Am-B binding with the cholesterol in the blood cell membrane (Butler et al., 1971).

Recently, encapsulation of Am-B in liposomes has been shown to greatly reduce the toxicity of this drug, including nephrotoxicity, while still maintaining its therapeutic effects. Gabriel et al. (1983) used mice as their experimental model and noticed that the LD_{50} increased by a factor of six as

a result of encapsulation. They also did preliminary studies in systemic fungal infection. The results indicated that there was almost no toxic effect associated with liposomal Am-B compared with the administration of free (i.e; non-liposomal) Am-B. Liposomal Am-B has the additional advantages of requiring only a short period of infusion (10-15 min vs 3-4 hr) and a low fluid volume (40 ml vs 500-1000 ml) (Gabriel et al., 1983). It seems likely that a major aspect of the behavior of liposomal Am-B is due to an altered partitioning of the drug between binding sites (sterols) in fungal and mammalian cell membranes. Kirsh et al. (1988) also showed that the mixing of Am-B with a lipid-emulsion carrier significantly reduced its hemolytic potency in vitro, as well as reduced its acute lethality and nephrotoxicity in vivo without altering its antifungal activity. These features allowed higher doses of the Am-B emulsion to be administered as compared to those of the conventional commercial formulation, resulting in a more effective maintenance of therapeutic concentrations in vivo.

The targeting of Am-B with antibody-directed liposomes has been reported by Hospenthal et al. (1989). Liposomal Am-B, bearing specific immunoglobulin (LAMB-Ab) was compared with the unencapsulated drug and with other liposomal Am-B formulations in the short-term survival of mice infected with disseminated candidiasis. LAMB-ab increased survival rates in

the murine model of candidiasis more than did other liposomal preparations containing Am-B. Liposomal forms of Am-B, in general, prolong survival in comparison to non-liposomal Am-B.

Pharmacokinetic Characteristics

Animal studies

Orally administered Am-B is poorly tolerated and less than 5% is absorbed systemically (Craven et al., 1979). Those investigators used dogs with bile duct cannulation as an experimental model and found that Am-B excretion in the urine accounted for 21% of the total dose; whereas, bile excretion accounted for only 3% of the dose and the stool contained no Am-B. However, bile salt depletion may have depressed biliary excretion of Am-B because in a dog with an intact biliary system, 19% of the dose was excreted in the stool. The half-life of Am-B in dogs is 4 days, the volume of distribution is 6.7 liter/kg and systemic clearance is about 44 ml/hr/kg (see Table 1). A reason for the failure to find most of the administered dose might be attributed to prolonged tissue storage of Am-B . The large volume of distribution and prolonged excretion in the urine (approximately 20 days after serum and bile concentrations were no longer detectable) are

compatible with tissue storage. Another reason for the failure to find most of the administered dose might be metabolic conversion of Am-B. The presence of a biologically active metabolite would probably have been detected in the above study by noting a discrepancy between bioassay and HPLC assay results.

Vadiei et al. (1990) examined the pharmacokinetics and toxicity of Am-B in the hyperlipidemic obese rat model compared with lean litter-mates. They reported that there was a 2-fold increase in the area under the serum concentration-time curve of Am-B in obese rats compared to lean rats; no differences in elimination rate constants were found between groups. Weight-corrected volume of distribution and total body clearance were significantly lower in obese compared with lean rats whereas no differences were found in absolute clearance or volume. The pharmacokinetic parameters of Am-B in lean rats is summarized in Table 1.

Human studies

Christiansen et al.(1985) reported that tissue distribution in patients who died of infection after systemic Am-B administration is initially widespread and includes liver, spleen, lungs, kidney, muscle, skin and adrenal glands. The liver is the major site of storage among those tissues and as much as 41% of the total dose could be recovered there. The

Table 1. Pharmacokinetics of Am-B in Humans and Animals[a]

Reference	Assay method	Subject (n)	Half-life, days	Systemic clearance, ml/hr/kg	V_{area}, L/kg
Chabot et al.	HPLC	Human (14)	11.0	10.0	3.8
Atkinson et al.	Bioassay	Human (2)	15.0	25.7	13.4
Benson et al.	HPLC	Children (12)	0.8	27.6	0.8
Jagdis et al.	Bioassay	Monkey (2)	11.5	1.0	0.4
Craven et al.	HPLC	Dog (4)	3.8	44.0	6.7
Vadiei et al.	HPLC	Rat (6)	0.7	177.0	4.4

a: Am-B was given as an intravenous infusion in all experiments

drug was also found in the kidney and lungs. The high percentage recovery of unchanged drug in tissue and the failure to detect metabolites in vivo and in vitro suggest that the drug is not metabolized.

Based on a study in two human subjects conducted by Atkinson et al. (1978), the elimination half-life of Am-B was found to be 15 days, largely because of the prolonged return from the slowly equilibrating peripheral compartment. The volume of this peripheral compartment accounted for 80% of the total distribution volume of 13.4 liters/kg and the total systemic clearance was 25.7 ml/hr/kg (see Table 1). Renal excretion appeared to be a relatively minor pathway for elimination since it only accounted for 3% of total clearance and the renal clearance of Am-B averaged only 3% of creatinine clearance in those patients.

The efficacy of hemodialysis in removing Am-B from blood was studied by Block et al. (1974) in eight adult patients with renal failure who required chronic hemodialysis. The results were correlated with a protein-binding study. Since Am-B was highly (>90%) protein-bound and has a large volume of distribution and also because of its hydrophobicity, Am-B was found to be poorly dialyzable, and plasma concentrations did not change appreciably after 4 to 6 hours of hemodialysis. These data suggest that there is no need to alter the dosage schedule in patients with renal

insufficiency, if or when they require hemodialysis.

Brajtburg et al (1984) investigated the interaction of Am-B with lipoprotein and other plasma proteins in vitro by using co-migration in density gradient ultracentrifugation and circular dichroic spectrum methods. Cholesterol is the major component in lipoprotein, and the cholesterol-phospholipid molar ratio of high density lipoprotein (HDL), low density lipoprotein (LDL) and very low density lipoprotein (VLDL) is 0.29, 0.72 and 0.78 respectively. Am-B interacts more extensively with LDL and VLDL than with HDL, and about 4 to 10 cholesterol molecules bind to one molecule of Am-B. Am-B also binds to other proteins in blood, but much higher concentrations (about 100 times) of these protein are needed. The interaction of Am-B with lipoprotein stabilizes its antifungal activity. Interaction with lipoprotein and with much higher concentrations of other proteins in blood can also inhibit the effects of Am-B on red blood cells, which contain cholesterol in their cell membranes.

Analytical Methods

Quantitation of antifungal drugs in biological fluids such as serum or urine is very important, especially for drugs that are toxic when present at too high a

concentration. Before the HPLC assay was developed, most studies used the bioassay method. Bioassay for Am-B, which had been available for sometime, was usually used for routine clinical monitoring and in earlier research investigations. However, incubation times range from 24 to 36 hours, and interference from other antifungal agents or from natural antifungal activity is a common problem (Granich et al., 1986). Mayhew et al. (1983) reported an assay method for Am-B blood samples by using reversed-phase HPLC with gradient elution which contained EDTA and p-nitrophenol as the internal standard. The reproducible limit of sensitivity of the assay was 40 ng/ml. Kobayashi et al. (1987) reported another assay method for Am-B urine samples which used a C_{18} column. The mobile phase contained 10 mM acetate buffer (pH=7.0)-acetonitrile-methanol-tetrahydrofuran (50:25:20:5, v/v). A solid phase extraction procedure was used to prepare the samples, and no internal standard was used. The reproducible limit of sensitivity of the assay was 10 ng/ml at 0.0025 auf.

Statement of the problem

Because antifungal drugs, such as Am-B, have a large toxic potential, predicting toxicity as a result of dosing is

particularly important. Despite nearly three decades of basic research and clinical use, an understanding of the pharmacokinetics and pharmacodynamics of Am-B remains limited. As a result, the selection of an optimal dose and schedule of administration remains largely empirical.

While some pharmacokinetic studies have been done, detailed studies examining the disposition kinetics of Am-B have not been reported. It is difficult to use humans as experimental subjects in order to examine the possible dose-dependence of Am-B since the compound is a very toxic agent.

The purpose of this investigation is as follows. It is necessary to examine the linearity in systemic and renal elimination and distribution processes. Therefore, kinetic studies were performed in groups of rats given three different doses. These studies will provide evidence for and an understanding of possible dose-dependent pharmacokinetics of Am-B. Furthermore, this information may potentially help in the rational design of treatment regimens which minimize drug toxicity and maximize therapeutic effect.

CHAPTER 2

EXPERIMENTAL

Materials and Methods

Assay Methodology

Chemicals and reagents

Fungizone (Lot# C0217A/43730, E.R. Squibb & sons, Princeton, NJ, U.S.A.) was supplied by the pharmacy in the Medical Center at the University of Arizona (Tucson, AZ, U.S.A.). Pure Am-B powder was supplied by E.R. Squibb & sons (Batch# 20-914-59718). 1-Amino-4-nitronaphthalene (ANN) was purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, U.S.A.) and was used as the internal standard (I.S.). Stock solutions (1 mg/ml Am-B in 50% methanol and 50% DMSO, 1.6 μg/ml I.S. in methanol) were prepared in light-protected glassware and stored at -20°C. Other reagents included: methanol (HPLC grade, Fisher Scientific Co. Fair Lawn, NJ, U.S.A.), acetonitrile (Optima, Fisher Scientific Co.),dimethyl sulfoxide (DMSO) and KH_2PO_4 (Aldrich Chemical Co), Na_2HPO_4 (Fisher Scientific Co,) and creatinine colormetric kits

(lot# 50H-6042, Sigma, St. Louis, MO, U.S.A.).

HPLC Instrumentation

The chromatographic system consisted of a single piston pump (Model 110 B Beckman Associater, San Ramon, CA, U.S.A.), a syringe loading sample injector (Model 210 A, Beckman) and a reversed-phase column (12.5 cm x 5.0 mm, ODS-3, particle size 5 micron, Whatman Inc. Clifton, NJ, U.S.A.) with a guard cartridge containing the same solid phase (Whatman Inc.). All chromatography was performed at ambient temperatures. The column effluent was monitored at 405 nm using a fixed wavelength UV-visible absorbance detector (Model 160, Beckman) and set at a sensitivity of 0.01 auf. The output from the detector was connected to both a 10-mv potentiometric integrator (Model 3380 A Hewlett Packard, Avondale, PA, U.S.A.) set at a chart speed of 0.4 cm/min and a recorder (Model 585, Linear Instruments Corporation, Irvine, CA, U.S.A.)at a chart speed of 0.5 cm/min.

The mobile phase, which contained 63% 1.6 mM Na_2HPO_4-KH_2PO_4 buffer (pH=7.0) and 37% acetonitrile (ACN), was filtered through a 0.45 μm Nylon 66 filter (Alltech Associater Inc, Deerfield, IL, U.S.A.). The mobile phase was pumped at a flow rate of 1.5 ml/min (2,5000 psi) and was not recycled.

Extraction procedures

Serum sample: According to Granish et al. (1986), a 200 μl aliquot of the cold methanolic internal standard solution (0.16 μg/ml) was added to 100 μl standard serum samples and authentic samples. The samples were vortexed for 10 second and centrifuged at 3000 rpm for 15 minutes. The supernatant was injected directly onto the column.

Urine sample: urine samples were diluted 4 times with water. A 200 μl volume of cold I.S. solution was added to the diluted urine samples, and the mixture vortexed and centrifuged. The supernatant was injected onto the column.

Calibration curves and data analysis

Standard calibration curves of the peak height ratio of Am-B to internal standard versus serum or urine concentrations were constructed by adding varying quantities of Am-B stock solution and a fixed amount of I.S. to blank rat serum or blank diluted rat urine. Concentrations of blood and urine samples were obtained from the peak height ratios and the regression equation of the appropriate calibration curve. On each day of analysis, a standard curve was constructed and the Am-B concentrations of the authentic samples were determined by comparing their peak height ratios

with those of the standard curve. In addition, the control samples (0.5 μg/ml) were injected after every ten samples, which were used as the standard samples to test the stability of the HPLC system.

Accuracy and precision

The intra-day and inter-day reproducibilities of the method were determined from repeated injection experiments. Serum containing 1 μg/ml, 0.5 μg/ml and 0.1 μg/ml of Am-B were prepared in triplicate and injected each day for three different days. The precision was estimated from the coefficients of variation (CV) around the measured concentrations.

Creatinine measurement

Jaffe described the color reaction between creatinine and alkaline picrate in 1886 (Slot 1965). Since then this reaction, named after Jaffe, has become the most commonly used method for creatinine analysis. However, this reaction suffers from lack of specificity. A number of other substances such as glucose and acetoacetate which called "non-creatinine chromogens" present in serum are known to interfere, thereby increasing the color yield. In 1965, Slot modified Jaff's

reaction by noticing that under acidic conditions, the creatinine-picrate color faded faster than the interfering chromogens (Slot 1965).

The creatinine diagnostics kit is based on the modified Jaffe's reaction. A 1.0 ml alkaline picrate solution (5 volumes of 0.6% picric acid and 1 volume 1.0 N sodium hydroxide) was added to different cuvettes which contained 0.1 ml H_2O, 0.1 ml creatinine standard (0.03 mg/ml creatinine in 0.02 N hydrochloric acid) and 0.1 ml of 10 times diluted urine or serum samples. The absorbance at 500 nm was determined with a DU-8 spectrophotometer (Beckman Associater) after those mixtures stood at room temperature for 8-12 minutes (initial absorbance). A 0.1 ml acid reagent, a mixture of sulfuric acid and acetic acid, was added to all cuvettes after recording the initial absorbance. The final absorbance was determined after 5 minutes at room temperature, and was used as the blank.

Animal Surgery

A male Sprague-Dawley rat, which was under ether anesthesia, was shaved on the dorsal neck-shoulder-blade area and right ventral neck. Two small incisions were made in the skin between the shoulder blades and the place on the ventral neck where the jugular can be seen to pulsate below the skin

surface. A cut was made at the point of pulsation, the tissue around the jugular was separated immediately and the point of bifurcation was ligated. A probe was pushed subcutaneously from the ventral side incision through the dorsal neck incision and the silastic (Cat# 602-135, Dow Corning Corporation, Midland, MI, U.S.A.) end of the cannula was pulled through the probe to the ventral side. A small incision was made in the vein about half way between the chest muscle and the ligation. A small probe was then inserted and along it the cannula was positioned into the hole. Some saline was injected and blood was withdrawn and reinjected in order to test the cannula viability. The vein was tied off around the cannula and the cannula was sutured to the neck muscle. Both incisions were finally closed and the cannula was plugged with an appropriate pin.

In vitro testing

Since Am-B was found to adsorb to the ultrafiltration plastic filtrate cup (Morgan et al., 1983), it was necessary to determine if the cannula adsorbed Am-B. An in vitro study was performed by preparing a Fungizone saline solution (0.5 μg/ml) and twice passing 2.0 ml of this solution through the cannula. Am-B concentrations were measured before and after each flush of Fungizone through the cannula.

Disposition Kinetics of Am-B in Sprague-Dawley rats

Animal study

Male Sprague-Dawley rats having a body weight of 200-300 g were supplied by Harlan Sprague Dawley Inc. (HSD, Indianapolis, U.S.A.). The rats were equilibrated in a standard environment in the animal care room one week before the study. A right external jugular vein cannula was implanted 24 hours prior to drug administration. The rats were housed separately in metabolic cages (Nalge Company, NY, U.S.A.) after the surgery and blank urine collected. The rats had access to water and food throughout the study. A 2.0 ml Fungizone suspension was infused at 0.13 ml/min for 15 minutes by an infusion pump (Sage Instrument, Model 355, MA., U.S.A.) and 1 ml of saline was used to flush the cannula immediately after the infusion. Based on the results of preliminary studies in two rats, the Am-B doses chosen were: 0.25 mg/kg, 0.5 mg/kg and 1.0 mg/kg. These doses were chosen with consideration given to the HPLC detection limits of our assay and the presumed toxic concentrations of the drug.

Blood and urine sampling

The blood samples were collected just prior to drug administration and at the following times after the end of

infusion: 0, 15, 30, 45, 105, 225, 345, 405, 705, 945, 1425, 1785 and 2865 minutes. Approximately 0.25 ml of blood was obtained at each time point after withdrawing 0.1 ml of fluid in the cannula in order to avoid an artifact due to sample contamination by saline or blood trapped in the cannula. (This volume was subsequently reinjected and followed by 1 ml of saline). The serum was separated and stored frozen at -20°C until analyzed.

The urine samples were collected at 465, 1425 and 2865 minutes after the end of infusion. The volume of each sample was measured and an aliquot of each sample was stored frozen at -20°C until analyzed.

Drug preparation

The Fungizone suspension was prepared immediately before drug administration by suspending the desired amount of Fungizone in sterile normal saline. After the infusion, an aliquot of the dosing solution was stored frozen at -20°C until analyzed in order to accurately determine Am-B concentration and the dose administered.

Data Analysis

Am-B serum concentrations were plotted as a function of time on semi-logarithmic graph paper. Data in the post-distribution phase were analyzed by linear regression assuming

log-linear decline according to the equation:

$$\text{Ln } C = \text{Ln } B - K*t \qquad (1)$$

where C is serum concentration at any time t; K is the apparent terminal first-order elimination rate constant; B is the Y-intercept.

The elimination half-life $t_{1/2}$ was calculated from the equation:

$$t_{1/2} = 0.693/K \qquad (2)$$

AUC is the total area under the serum concentration versus time curve from time zero to infinity. AUC was calculated using the linear trapezoidal rule, given by:

$$AUC_{0-\infty} = 1/2*C_1*t_1 + 1/2*\sum_{i=1}^{n}[(C_i+C_{i+1})*(t_{i+1}-t_i)] + C_n/K \qquad (3)$$

where the concentration used to determine the terminal area was not the observed concentration, but the theoretical value calculated by the linear regression equation.

A model-independent approach was used to determine the pharmacokinetic parameters. The systemic clearance CL_s was determined from:

$$CL_S = Dose/AUC \quad (4)$$

where dose was the total infused dose and AUC was obtained from equation (3).

The apparent volume of distribution V_{area} was determined from:

$$V_{area} = CL_S/K \quad (5)$$

The volume at steady state V_{ss} is equal to:

$$V_{ss} = AUMC/AUC \times CL_s \quad (6)$$

where AUMC was the total area (i.e., time zero to time infinity) under the first moment of the serum concentration-time curve, which is equal to:

$$AUMC_{(0-\infty)} = 1/2*C1*t1^2+1/2*\sum_{i=1}^{n}[(C_i*t_i+C_{i+1}*t_{i+1})*(t_{i+1}-t_i)] + t_n*C_n/K+C_n/K^2 \quad (7)$$

the concentration values (C_n) used to determine the terminal area was the value on the regression line.

The renal clearance CL_R was defined as:

$$CL_R = X_{u(t1-t2)}/AUC_{(t1-t2)} \quad (8)$$

where Xu was the amount of drug excreted into urine upon certain time intervals and AUC was determined over the corresponding time intervals.

The non-renal clearance, CL_{nr}, was calculated by the relationship:

$$CL_{nr} = CL_S - CL_R \quad (9)$$

The fraction excreted into urine was determined by:

$$F_u = CL_R/CL_S \quad (10)$$

Creatinine clearance, CL_{cr}, was obtained from the ratio of the urinary excretion rate and the serum creatinine concentration:

$$CL_{cr} = dX_u/dt/C_{cr,serum} \quad (11)$$

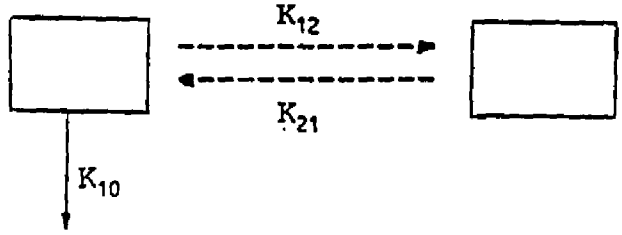
where

$$dXu/dt = C_{cr,urine} * Vol/t \quad (12)$$

The urinary excretion rate was obtained from the amount of creatinine excreted into the urine and from the collection time interval.

The data were also analyzed by a model-dependent approach, the curves were fit using the PCNONLIN program

(algorithm 1) and different weighting factors (1, 1/y, $1/y^2$) were chosen in order to obtain the best fit of the data. The model and equations can be described as follows:



$$C_p = R_1 * e^{-\alpha t'} + R_2 * e^{-\beta t'} \quad (13)$$

$$A_1 = T * R_1 * \alpha / (1 - e^{-\alpha T}) \quad (14)$$

$$A_2 = T * R_2 * \beta / (1 - e^{-\beta T}) \quad (15)$$

$$AUC = A_1/\alpha + A_2/\beta \quad (16)$$

$$V_c = Dose/(A_1 + A_2) \quad (17)$$

$$K_{21} = (A_1 * \beta + A_2 * \alpha)/(A_1 + A_2) \quad (18)$$

$$K_{10} = \alpha * \beta / K_{21} \quad (19)$$

$$K_{12} = \alpha + \beta - K_{21} - K_{10} \quad (20)$$

$$V_{ss} = dose * (A_1/\alpha^2 + A_2/\beta^2)/AUC^2 \quad (21)$$

where T is the infusion time, t' the post infusion time. V_c the volume of the central compartment, K_{21}, K_{10} and K_{12} the micro rate constants. R_i are the Y-intercepts from the post-infusion data.

The above disposition parameters for Am-B were statistically compared by using the Minitab program and a one-way analysis of variance and Tukey's test (Lyman Ott, 1988).

Chapter 3

RESULTS

Assay Methodology

Developing a specific and sensitive analytical procedure for quantitating concentrations of Am-B in serum and in urine is essential in order to characterize Am-B disposition kinetics. The procedure that we adapted involved a one-step extraction using methanol to precipitate proteins in serum and salts in urine (Granich et al., 1986). Subsequent analysis by reversed-phase HPLC was performed on a C_{18} column using an aqueous mobile phase with ultraviolet-visible detection (405 nm). 1-amino-4-nitronaphthalene (ANN) was chosen as an internal standard since it was readily obtained, absorbed UV light well at both 382 and 406 nm and was also readily soluble in methanol which was used for the extraction. ANN has been used successfully as an internal standard in an HPLC assay for polyene compounds (Granich et al., 1986). The Am-B concentrations were determined by comparing the peak height ratios (Am-B versus internal standard) measured from

the samples and regression equation of the standard curve obtained the same day.

The maximum UV absorbances are at 363 and 405 nm for Am-B, while the internal standard absorbs maximally at 406 nm. The chosen wavelength of 405 nm was near the maximum for both compounds.

Typical chromatograms of extracted blank rat serum and serum spiked with a known amount of Am-B are shown in Figure 3. Similarly, chromatograms of extracted Am-B from blank urine and urine spiked with a known amount of Am-B are shown in Figure 4. The compounds eluted from the C_{18} reversed-phase column as symmetrical peaks with retention times of 3.5 and 5.7 minutes for Am-B and internal standard, respectively. Direct injection of supernatant did not result in column deterioration.

The standard curves for Am-B in serum were linear over the concentration ranges from 0.022 μg/ml to 1.79 μg/ml, (R^2=0.999, see Figure 5). As a measure of reproducibility, the within-day and between-day coefficients of variation were determined at three different concentrations (see Table 2). Based on triplicate determinations, the within day coefficients of variation ranged from 5% to 15% for Am-B; the coefficients of variation increased as Am-B concentration decreased. The between-day variation was calculated by performing triplicate analyses of serum samples on three

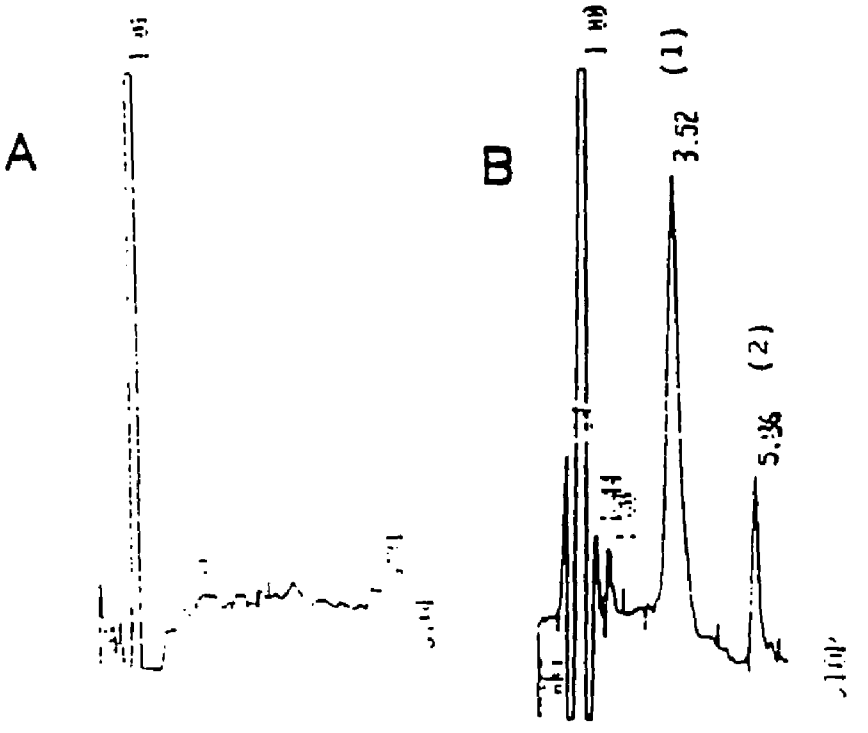


Figure 3. Representative chromatograms from a 100 µl serum extract showing retention times (min) for (1) Am-B; (2) ANN (I.S.). A: serum control after extraction, B: serum spiked with 0.89 µg/ml Am-B and 0.16 µg/ml I.S. The detector setting was 0.01 auf and injection volume was 20 µl.

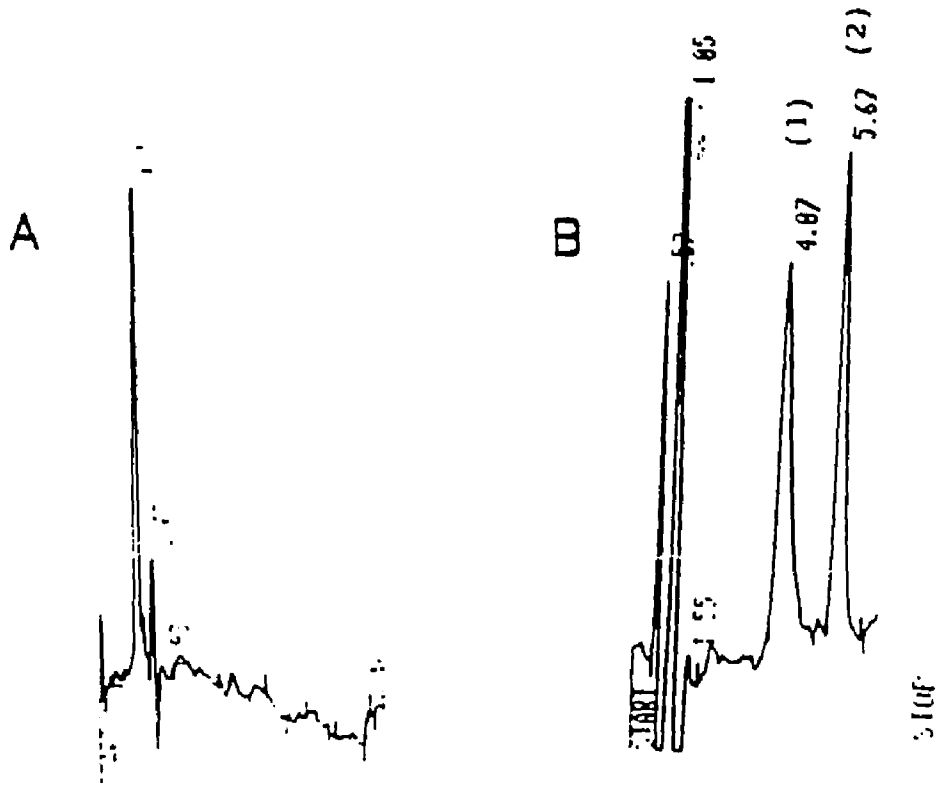


Figure 4. Representative chromatograms from a 100 µl diluted (4 fold) urine extract showing retention times (min) for (1) Am-B; (2) ANN (I.S.). A: diluted blank urine after extraction; B: diluted urine spiked with 0.45 µg/ml Am-B and 0.16 µg/ml I.S. The detector setting was 0.01 auf and injection volume was 50 µl.

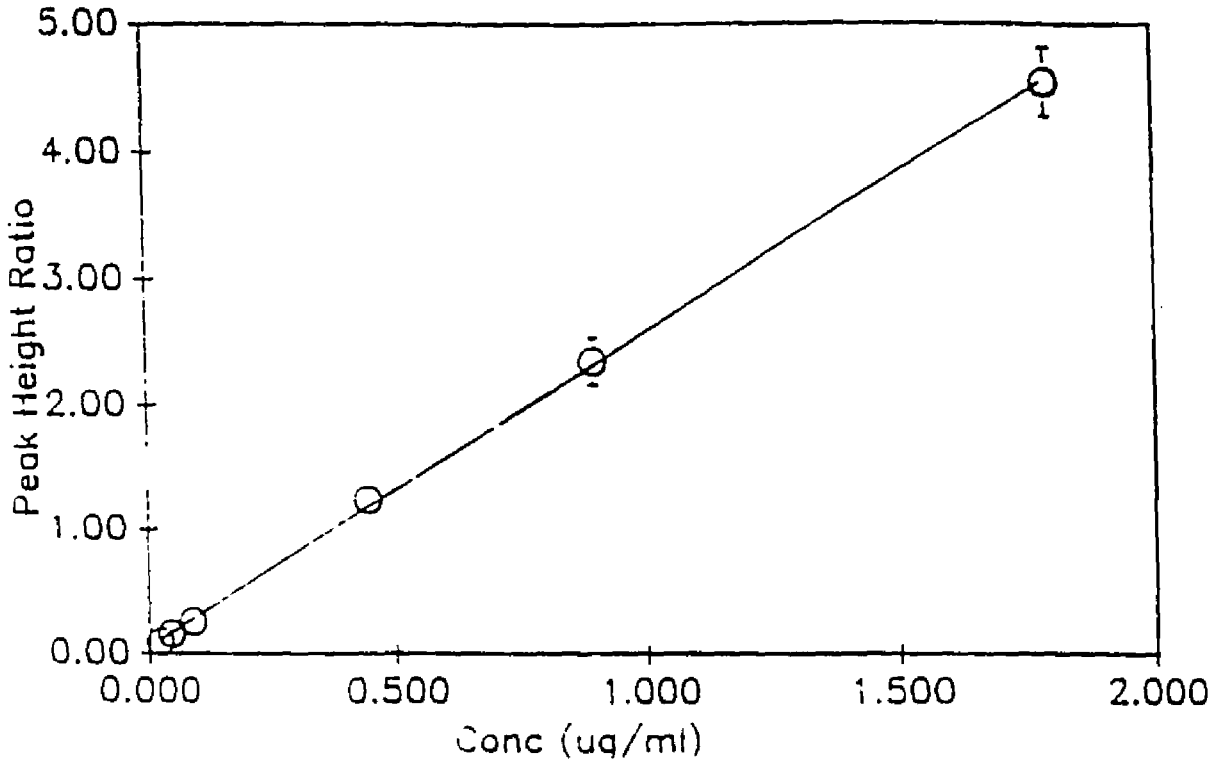


Figure 5. Standard curve of Am-B in serum. Peak height ratio versus concentration. The regression line is based upon three determination at each concentration, The equation of the line is, $y = 2.59 X + 0.05$ ($R^2 = 0.999$)

Table 2. Summary of Intra-day and Inter-day Variability of Am-B Serum Assay

Intra-day results

Set	Concentration (μg/ml)		SD	CV %	Difference[b] %
	Spiked Conc.	Measured[a]			
	1.00	1.18	0.065	5.54	-18
1	0.50	0.58	0.059	10.11	-16
	0.10	0.11	0.011	10.23	-10
	1.00	0.97	0.051	5.20	+ 3
2	0.50	0.46	0.039	8.32	+ 8
	0.10	0.13	0.007	4.91	-30
	1.00	1.20	0.043	3.60	-20
3	0.50	0.53	0.030	5.61	- 6
	0.10	0.11	0.018	16.76	-10

Inter-day results

Concentration (μg/ml)		SD	CV %	Difference[b] %
Spiked Conc.	Measured[c]			
1.00	1.12	0.119	10.59	-12
0.50	0.53	0.065	12.28	- 6
0.10	0.12	0.018	15.02	-20

a. mean of 3 determinations.
b. difference between spiked and measured concentrations x 100 divided by spiked concentrations.
c. mean of 9 determination.

different days; the coefficient of variation was 15% for 0.1 μg/ml.

The minimum detectable quantity was determined to be 22 ng/ml for Am-B by injecting 100 μl supernatant at a sensitivity setting of 0.01 auf.

Disposition Kinetics of Am-B in Sprague-Dawley Rats

The Sprague-Dawley rats used in the study were randomly assigned to three different dose groups. All animals tolerated the intravenous doses of Am-B during the course of the experiment.

In order to verify that the cannula did not adsorb Am-B, an in vitro study was performed. Am-B concentrations were measured before and after flushing Fungizone through the cannula. Identical Am-B concentrations (0.52 ± 0.015 μg/ml) suggested that the cannula itself did not adsorb Am-B.

Figures 6 to 8 demonstrate the serum concentration versus time profiles obtained after administration of three different Am-B doses. Serum concentrations of Am-B showed a biexponential decline with time, suggesting that Am-B disposition could be described by a multi-compartment model. For the high dose group, especially for the dose of 842 μg/kg, it seemed that the serum concentration versus time curves

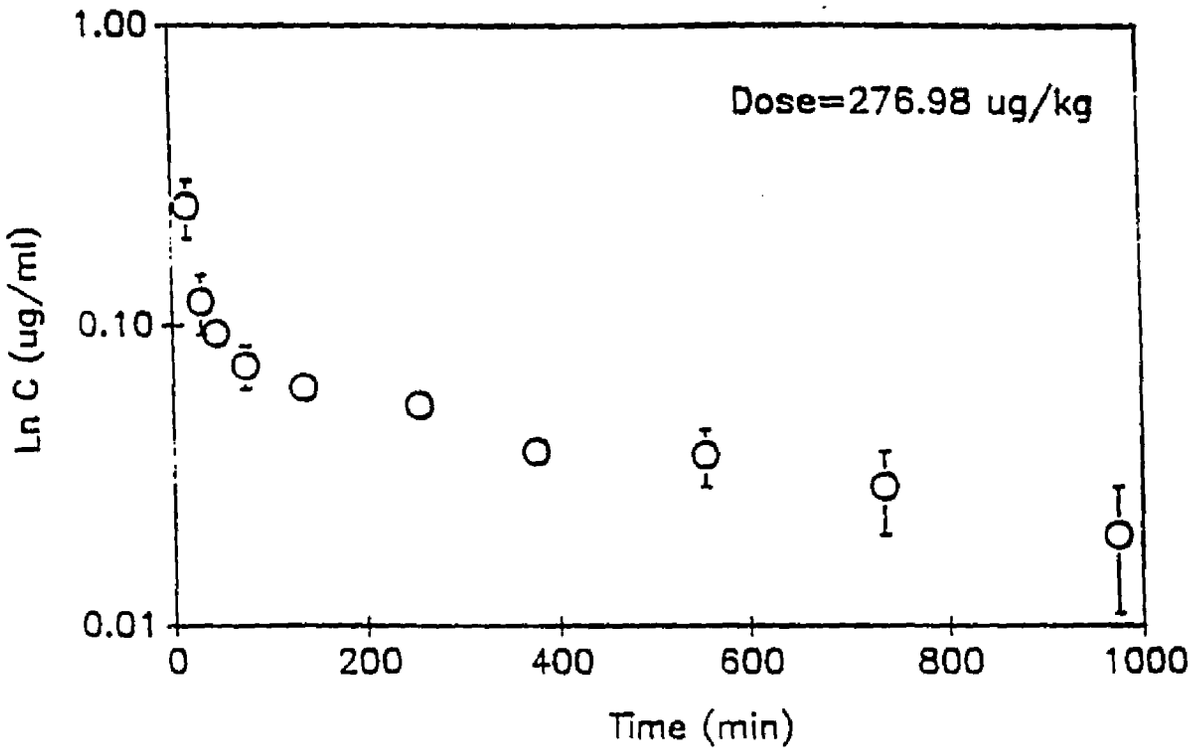


Figure 6. Plot of mean Am-B serum concentration versus time for the low dose group following an intravenous infusion of 276.98 µg/kg. Each point is the mean of four animals. The cross-hatched vertical bars represent the standard deviation of the mean.

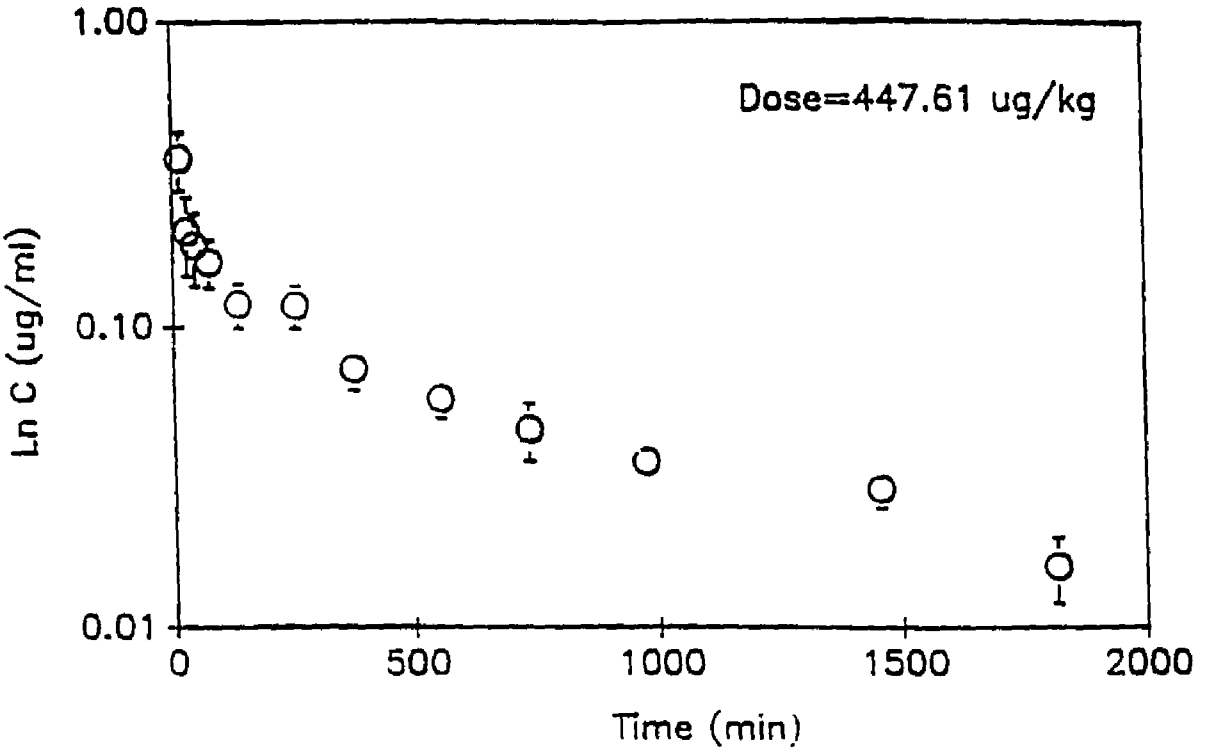


Figure 7. Plot of mean Am-B serum concentration versus time for the middle dose group following an intravenous infusion of 447.61 μg/kg. Each point is the mean of four animals. The cross-hatched vertical bars represent the standard deviation of the mean.

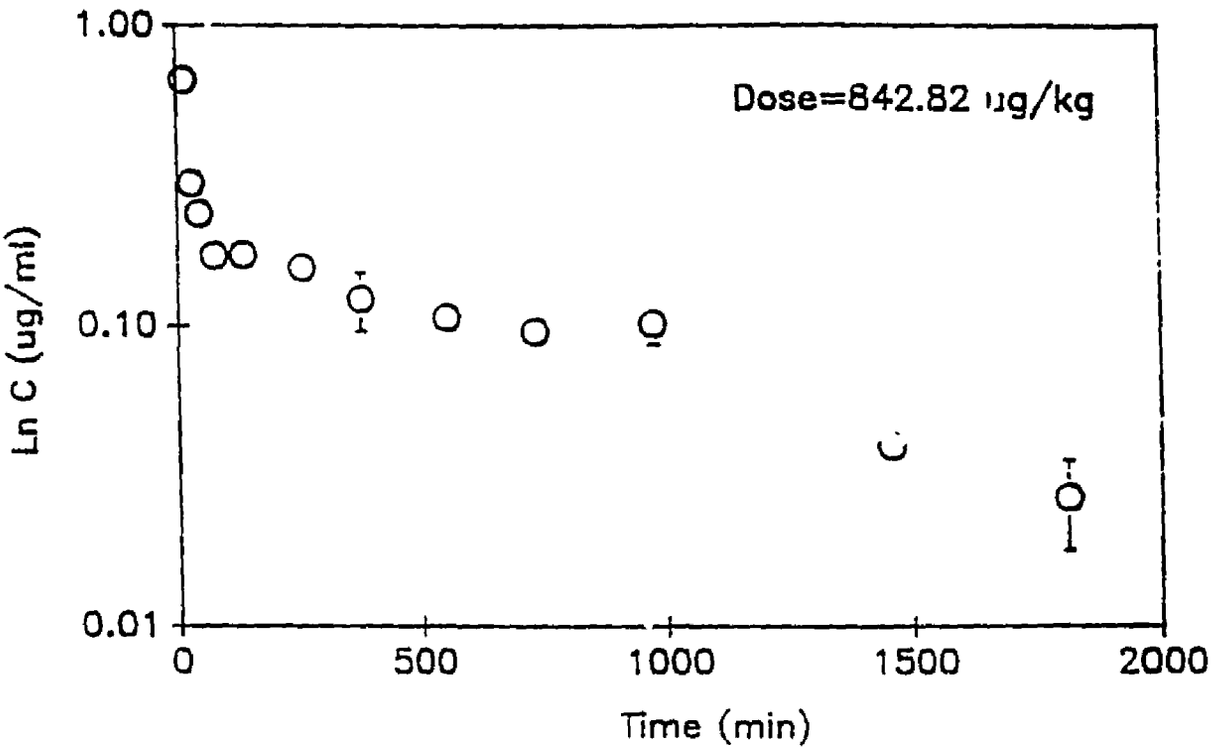


Figure 8. Plot of mean Am-B serum concentration versus time for the high dose group following an intravenous infusion of 842.82 µg/kg. Each point is the mean of four animals. The cross-hatched vertical bars represent the standard deviation of the mean.

declined in a pattern which is not consistent with a linear polyexponential profile. The serum concentrations remained essentially the same from 450 minutes to 1000 minutes.

The principle of linearity can be examined by normalizing the serum concentrations for dose. Figure 9 is the dose normalized concentration versus time profile for three different doses. All three curves were superimposable, consistent with the principle of linearty. Moreover, there were no statistically significant differences ($P>0.05$) among those data points based on a one-way analysis of variance, again, suggesting the absence of nonlinearity.

The pharmacokinetic parameter estimates obtained by the "model independent" approach are listed for each of the animals in each dose group (Tables 3 and 4). In addition to individual animal data, each table summarizes the mean value for each dose, as well as any statistical differences in those parameters. The serum and urine Am-B concentration versus time data, as well as the individual profiles for each animal, can be found in Appendices A and B, respectively.

For creatinine measurement, since the accuracy of this color reaction was dependent on time, it was necessary to prepare only 3 test samples along with a standard sample, as well as a blank sample. This allowed the reading of all absorbances in a certain time period. Table 5 is the summary of creatinine clearance data at different times before and

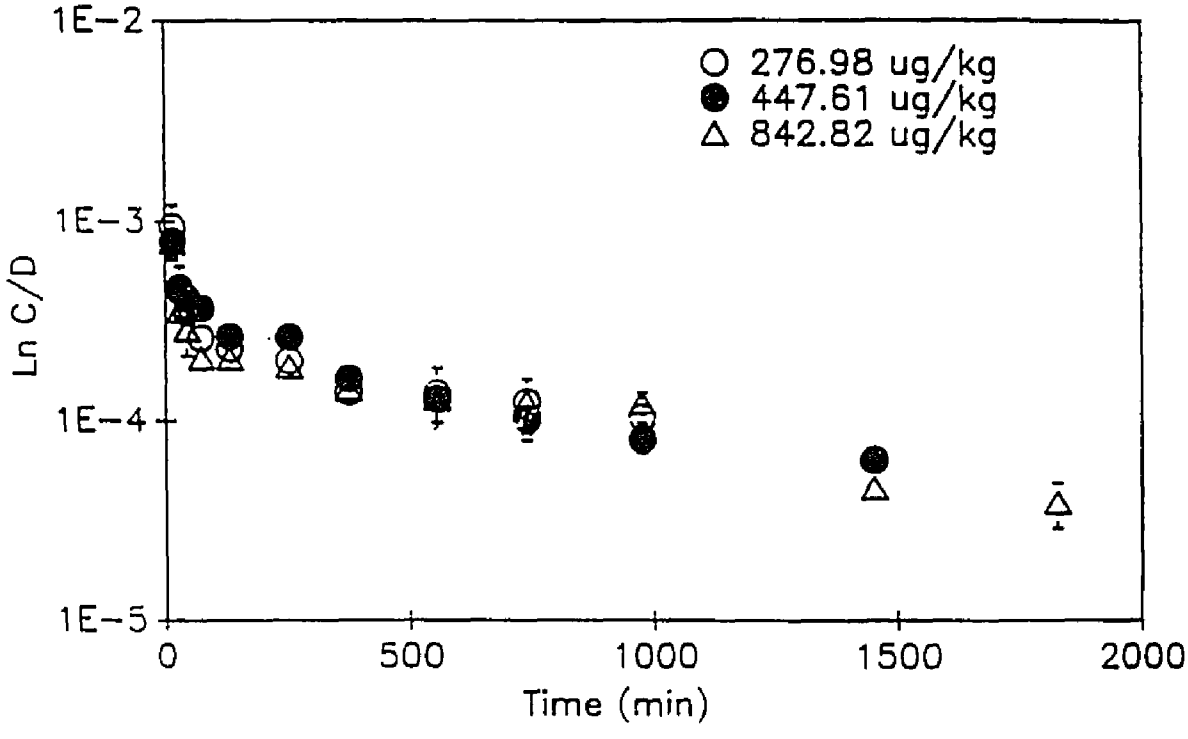


Figure 9. Plot of mean Am-B concentration normalized for dose versus time following the intravenous infusion (15 min) of three different doses: 276.98 μg/kg, 447.61 μg/kg and 842.82 μg/kg of Am-B.

after Am-B dosing. The urine and serum creatinine concentration data at different times can be found in Appendix C.

The harmonic mean half-life which was obtained from the model-independent approach for the low, middle and high doses were: 582.7 ± 205.7, 605.2 ± 108.7 and 692.1 ± 92.2 minutes, respectively. There were no significant differences ($P>0.05$) in half-life among those three doses.

Similarly, the average values of Varea, the apparent volume of distribution in the elimination phase, were 3.62 ± 0.13, 3.43 ± 0.71 and 3.89 ± 0.25 L/Kg for the low to high dose, respectively. No significant differences were observed among those values.

The renal clearance of Am-B decreased as dose increased and there was a significant difference among the three doses ($P<0.05$, see Table 4). However, the total body clearance was shown to be dose independent (see Table 3). Figures 10 and 11 graphically demonstrate the relationship between systemic clearance and renal clearance and dose. The percentage of the Am-B dose excreted into the urine as the unchanged form ranged between 20% to 40% depending on the dose (see Figure 12).

Figure 13 is a "population" plot of Am-B renal clearance at different time intervals versus Am-B serum concentration at the mid-time of the collection interval. The

Table 3. Summary of Am-B Pharmacokinetic Parameters (Model-independent Approach)

Dose (μg/kg)	$t_{1/2}$ (min)	K (min^{-1})	$AUC_{i.v.}$ (μg*min/ml)	CL_s (ml/min/kg)	V_{area} (L/kg)	V_{ss} (L/kg)
238.6	1005.5	$0.69*10^{-3}$	97.4	2.45	3.56	3.39
297.8	520.7	$1.33*10^{-3}$	60.1	4.95	3.72	3.54
294.5	415.5	$1.67*10^{-3}$	51.1	5.76	3.45	3.41
277.0	648.4	$1.07*10^{-3}$	69.3	3.99	3.73	3.50
416.6	642.4	$1.08*10^{-3}$	118.8	3.51	3.25	3.04
486.0	723.0	$0.96*10^{-3}$	118.0	4.12	4.29	4.08
466.5	487.3	$1.42*10^{-3}$	126.6	3.68	2.59	2.38
421.2	618.1	$1.12*10^{-3}$	104.6	4.03	3.60	3.46
711.2	846.6	$0.82*10^{-3}$	228.9	3.11	3.80	3.66
853.8	682.2	$1.02*10^{-3}$	204.8	4.17	4.09	3.89
967.8	605.0	$1.15*10^{-3}$	205.3	4.71	4.10	3.96
838.9	675.8	$1.03*10^{-3}$	227.4	3.69	3.58	3.50
Mean ± S.D.						
low dose	582.7 ±205.6	$1.19*10^{-3}$ $\pm 0.41*10^{-3}$	69.5 ±20.0	4.29 ±1.42	3.62 ±0.13	3.46 ±0.07
middle dose	605.2 ±108.7	$1.15*10^{-3}$ $\pm 0.20*10^{-3}$	117.0 ± 9.2	3.84 ±0.29	3.43 ±0.71	3.24 ±0.71
high dose	692.1 ± 92.2	$1.01*10^{-3}$ $\pm 0.14*10^{-3}$	216.6 ± 13.4	3.92 ±0.68	3.89 ±0.25	3.75 ±0.21
statistical significance	ns,	ns		ns	ns	ns

a: Harmonic mean ± pseudo standard deviation (Lam et al.1985).
ns: $P>0.05$.

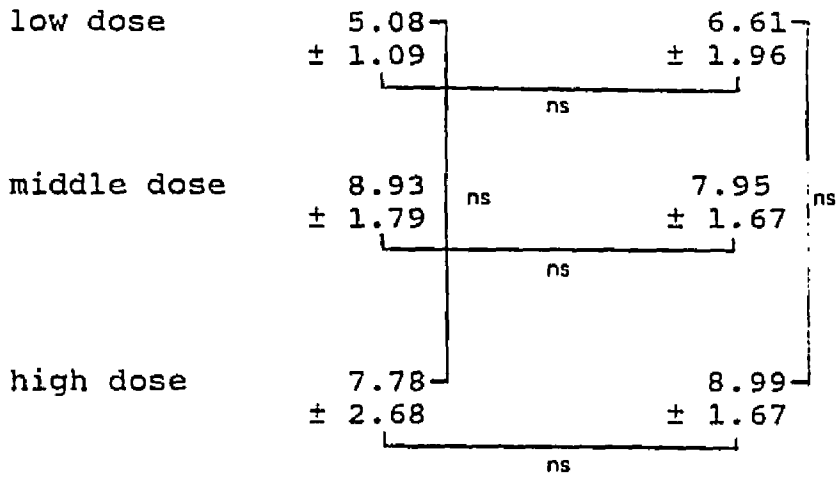
Table 4. Summary of Am-B Clearance Data

Dose (μg/kg)	Clearance (ml/min/kg)			F_u
	CL_s	CL_R	CL_{NR}	
238.64	2.45	1.09	4.57	0.44
297.75	4.95	1.78	3.17	0.36
294.45	5.76	2.20	3.56	0.38
277.04	3.99	1.67	2.32	0.42
416.61	3.51	1.50	2.01	0.43
486.00	4.12	1.21	2.91	0.29
466.53	3.68	1.14	2.54	0.31
421.24	4.03	1.21	2.82	0.30
711.19	3.11	0.75	2.36	0.24
853.57	4.17	0.76	3.41	0.18
967.75	4.71	1.14	3.57	0.24
838.94	3.69	0.72	2.97	0.20
Mean ± S.D.				
low dose	4.29 ±1.42	1.69 ±0.46	2.60 ±0.98	0.40 ±0.04
middle dose	3.84 ±0.29	1.27 ±0.16	2.57 ±0.41	0.33[a] ±0.07
high dose	3.92 ±0.68	0.84[b] ±0.20	3.08[b] ±0.54	0.22[b,c] ±0.03
statistical significance	P>0.05	P<0.05	P<0.05	P<0.01

a: low dose vs. middle dose
b: low dose vs. high dose
c: middle dose vs. high dose

Table 5. Summary of Creatinine Clearance Data

Dose (μg/kg)	creatinine clearance (ml/min/kg)	
	before Am-B	after Am-B
238.64	3.66	4.57
297.75	4.77	5.34
294.45	5.86	8.62
277.04	6.01	7.91
416.61	7.46	9.33
486.00	8.54	7.38
466.53	8.19	5.85
421.24	11.53	9.19
711.19	11.44	10.39
853.57	5.01	7.53
967.75	7.51	10.50
838.94	7.15	7.56
Mean ± S.D.		
low dose	5.08 ± 1.09	6.61 ± 1.96
middle dose	8.93 ± 1.79	7.95 ± 1.67
high dose	7.78 ± 2.68	8.99 ± 1.67



ns: P>0.05

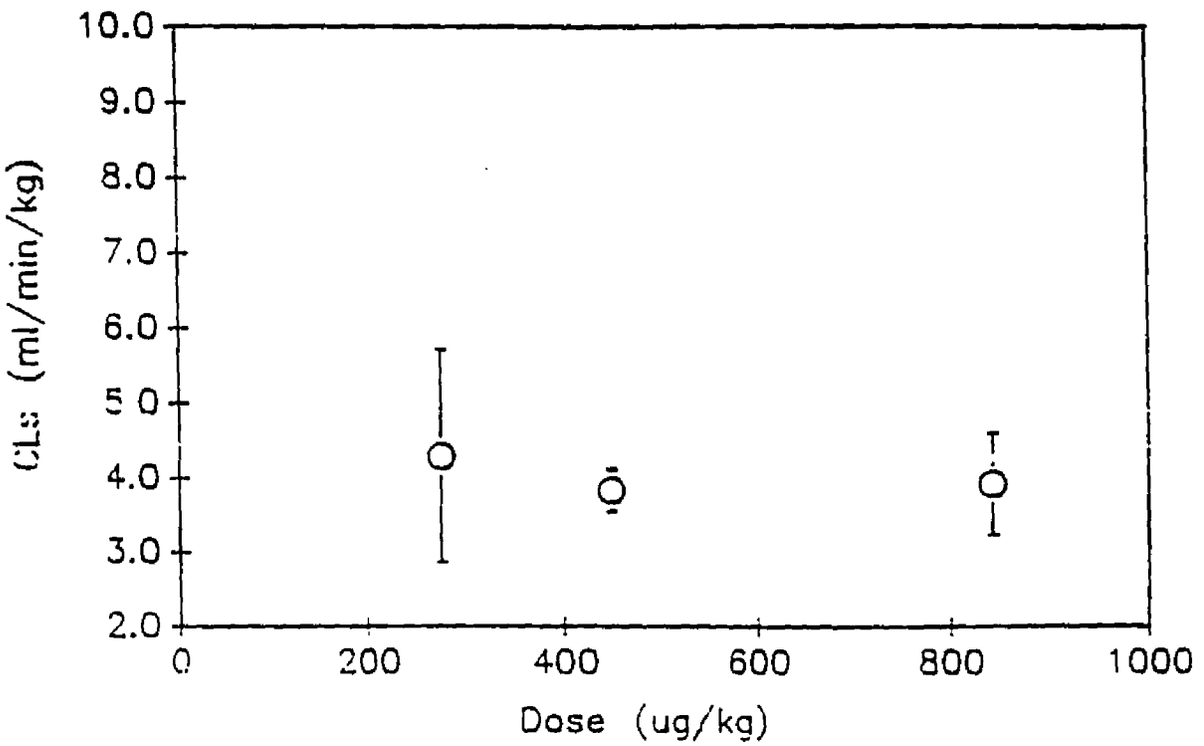


Figure 10. Plot of Am-B systemic clearance versus dose for three different doses after intravenous infusion (15 min.) of 276.98 µg/kg, 447.61 µg/kg and 842.82 µg/kg of Am-B. Each point is the mean of 4 determinations and the vertical cross-hatched bars represent the standard deviation of the mean.

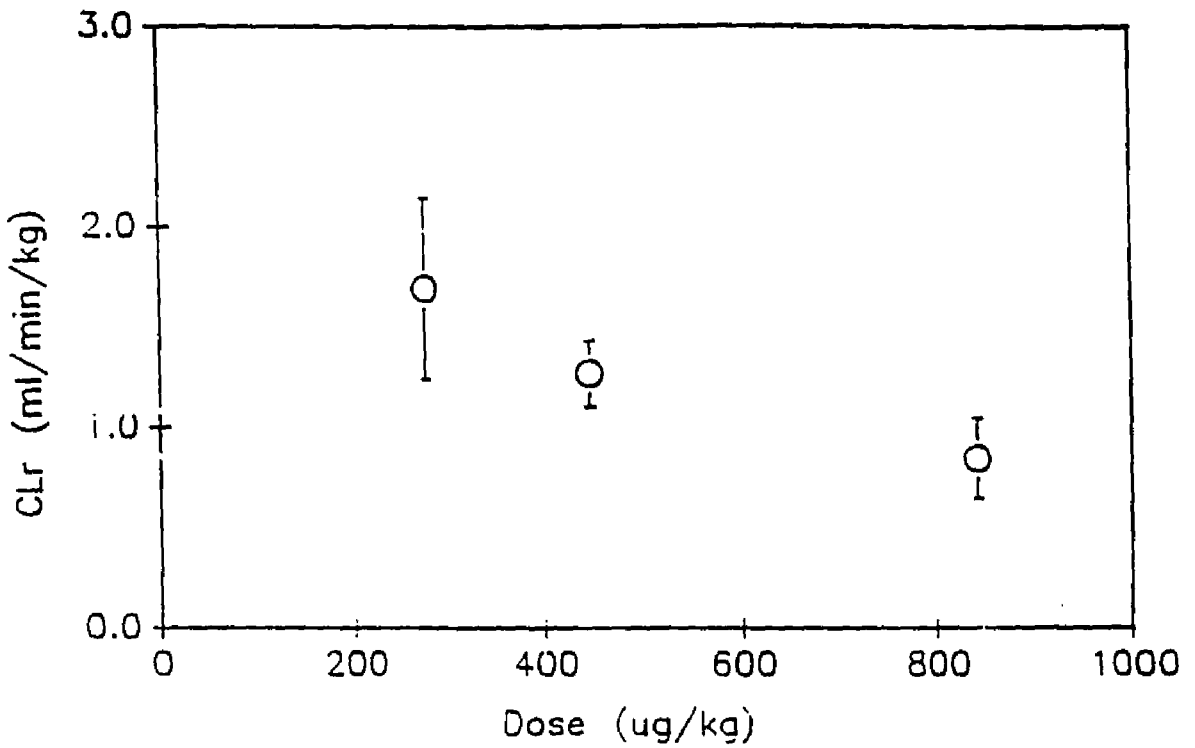


Figure 11. Plot of Am-B renal clearance versus dose for three different doses after intravenous infusion (15 min.) of 276.98 µg/kg, 447.61 µg/kg and 842.82 µg/kg of Am-B. Each point is the mean of 4 determinations and the vertical cross-hatched bars represent the standard deviation of the mean.

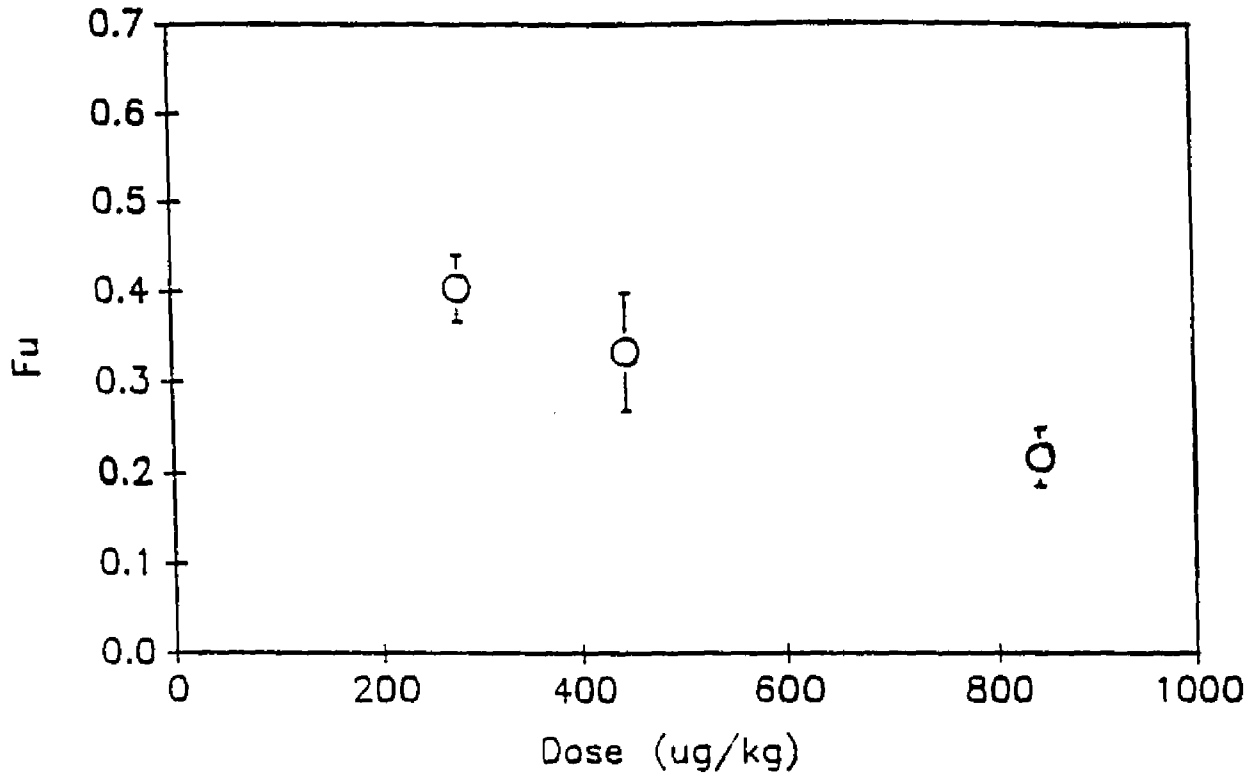


Figure 12. Plot of fraction of Am-B excreted in the urine in the unchanged form versus dose after intravenous infusion (15 min.) of 276.98 μg/kg, 447.61 μg/kg and 842.82 μg/kg of Am-B. Each point is the mean of 4 determinations and the vertical cross-hatched bars represent the standard deviation of the mean.

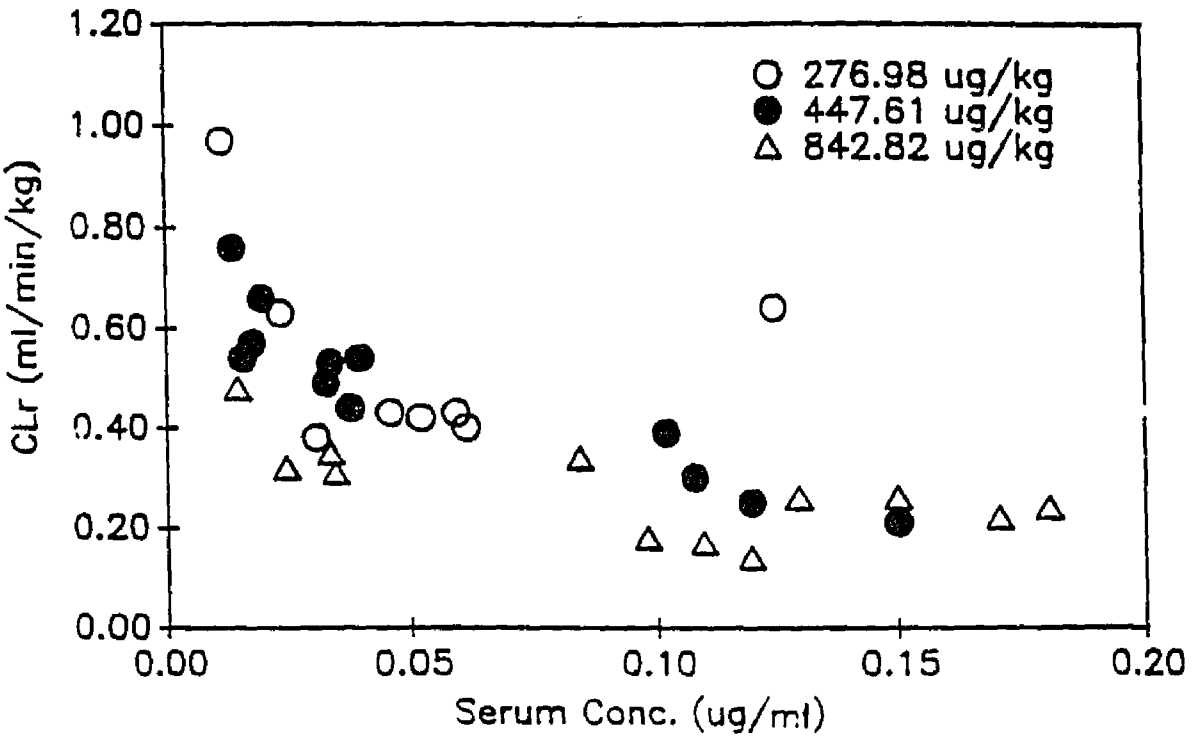


Figure 13. Plot of Am-B renal clearance versus Am-B serum concentration at mid-time points after intravenous infusion (15 min) of different doses of Am-B. Each point represents one value of renal clearance in an individual animal.

observed renal clearance appears to be inversely proportional to the Am-B serum concentration. Figure 14 is a plot of Am-B renal clearance determined at three different doses as a function of time. There were a statistically significant difference in renal clearance ($P<0.05$) as a function of time for the middle dose (2nd vs. 1st time period) and large dose (3rd vs. 1st time period). No difference with time was seen for the low dose. These data also demonstrate the inverse relationship between renal clearance and Am-B serum concentrations since high Am-B concentrations exist at early times. Figure 15 is a "population" plot of Am-B renal clearance determined at different time intervals as a function of urine flow, which suggests that there is no relationship between renal clearance and urine output.

Creatinine clearance values at different time intervals associated with three different Am-B dose groups fell within the observed normal range of creatinine clearance (determined prior to Am-B dosing, see Table 5).

Table 6 summarizes several pharmacokinetic parameters including micro-constants obtained by compartmental analysis. Figures 16 through 20 illustrate the relationship of these parameters obtained from model-independent and model-dependent methods of analysis.

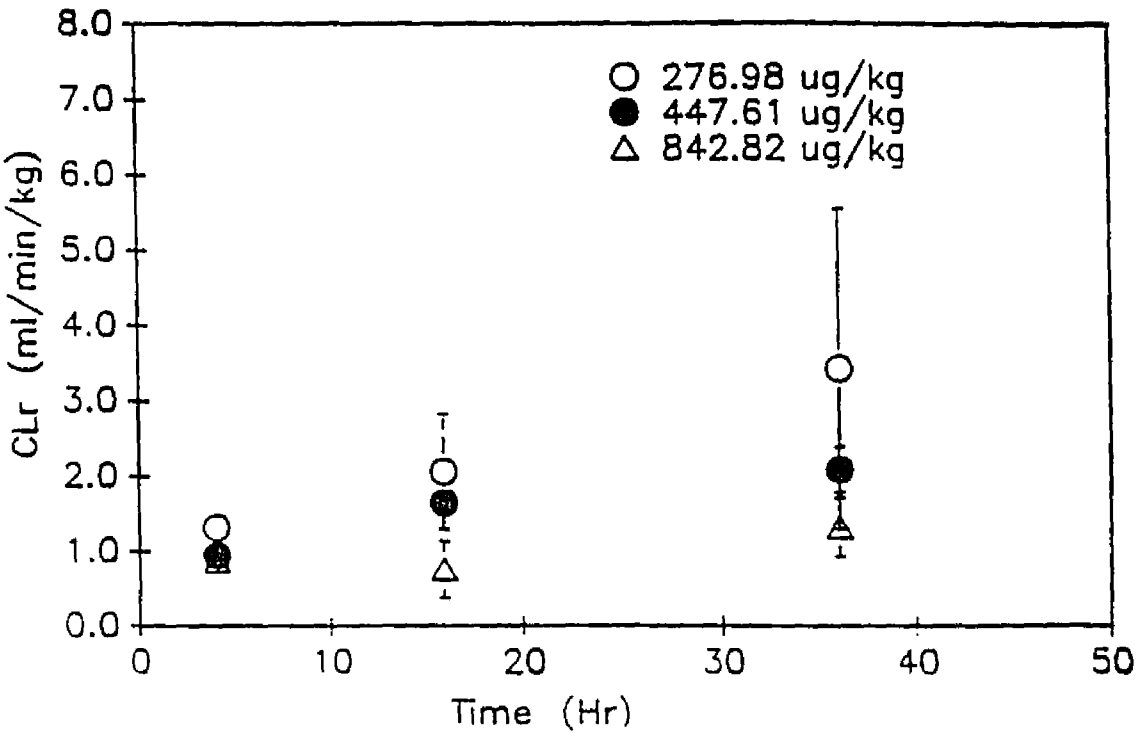


Figure 14. Plot of Am-B renal clearance versus time for three different doses after intravenous infusion (15 min) of 276.98 µg/kg, 447.61 µg/kg and 842.82 µg/kg of Am-B. Each point is the mean of 4 determinations and the vertical cross-hatched bars represent the standard deviation of the mean.

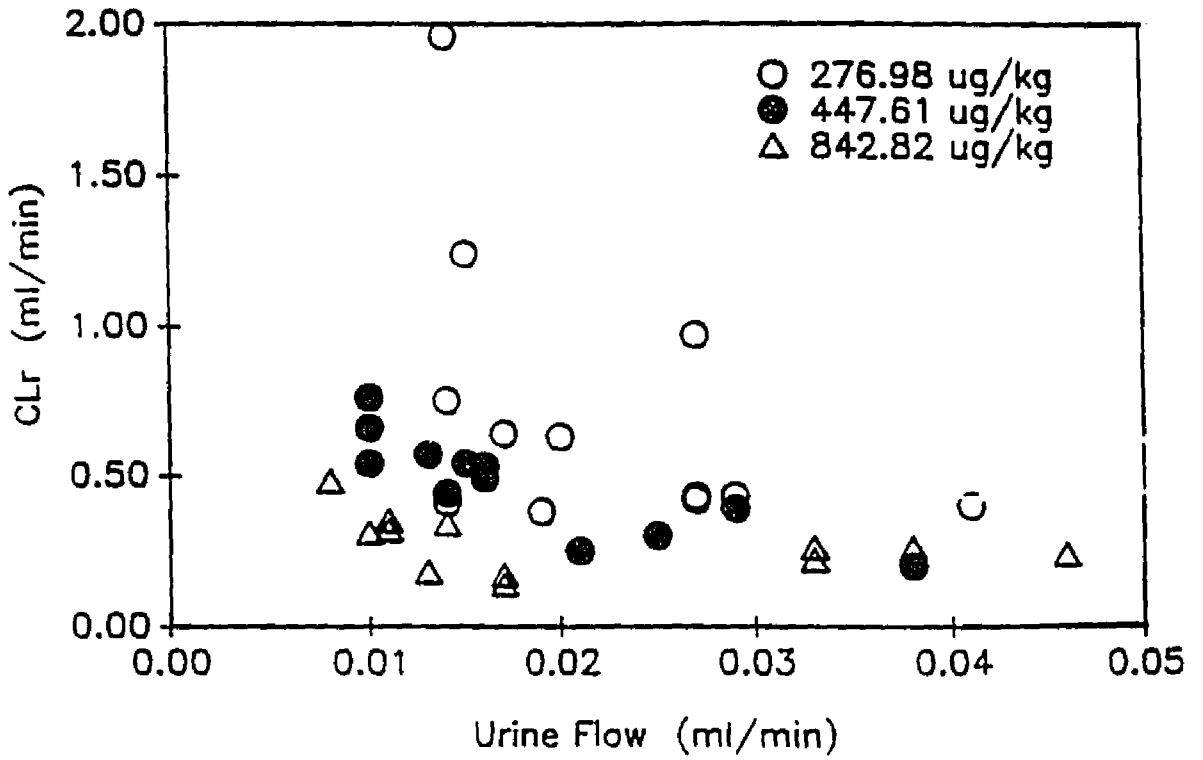


Figure 15. Plot of Am-B renal clearance versus urine flow at different times after intravenous infusion (15 min) of different doses of Am-B. Each point represents one value of renal clearance in an individual animal.

Table 6. Summary of Am-B Pharmacokinetic Parameters (Compartmental Analysis)[a]

Dose	R_1	R_2	α	ß	$T_{½}$	AUC	CL_s	V_c	V_{area}	V_{ss}	Wt.[b]
238.64	0.25	0.06	0.08	0.0007	1039.1	87.3	2.74	0.48	4.08	3.58	$1/y^2$
297.75	0.27	0.08	0.18	0.0019	361.4	48.5	6.14	0.34	3.23	2.91	$1/y^2$
294.45	0.18	0.08	0.04	0.0016	424.9	53.2	5.53	0.91	3.46	3.04	1
277.04	0.12	0.07	0.05	0.0010	628.8	66.5	4.17	1.19	4.17	3.61	1
416.61	0.19	0.13	0.03	0.0012	555.1	111.2	3.75	1.14	3.12	2.82	1
486.00	0.22	0.12	0.13	0.0012	562.4	105.7	4.60	0.78	3.83	3.73	$1/y^2$
466.53	0.27	0.16	0.03	0.0014	479.0	121.3	3.85	0.96	2.75	2.43	$1/y^2$
121.24	0.20	0.11	0.05	0.0012	563.1	92.0	4.58	1.09	3.82	3.50	$1/y^2$
711.19	0.50	0.17	0.06	0.0008	850.1	216.2	3.29	0.79	4.01	3.79	$1/y_2$
853.57	0.52	0.18	0.10	0.0010	676.4	191.2	4.46	0.72	4.46	4.15	$1/y^2$
967.75	0.57	0.21	0.12	0.0011	628.4	199.1	4.86	0.66	4.42	4.18	$1/y^2$
838.94	0.47	0.20	0.12	0.0009	732.6	220.7	3.80	0.69	4.00	3.87	1/y
Mean± S.D											
Low dose	0.21 ±0.07	0.07 ±0.01	0.09 ±0.06	0.0013 ±0.0005	613.6 ±305.8	63.9 ±17.4	4.64 ±1.52	0.73 ±0.39	3.73 ±0.46	3.29 ±0.36	
Middle dose	0.22 ±0.04	0.13 ±0.02	0.06 ±0.04	0.0013 ±0.0001	539.9 ± 40.8	107.6 ± 12.2	4.19 ±0.46	0.99 ±0.16	3.38 ±0.54	3.12 ±0.60	
High dose	0.52 ±0.04	0.19 ±0.02	0.10 ±0.03	0.0010 ±0.0001	721.9 ± 95.5	206.8 ± 13.9	4.10 ±0.70	0.71 ±0.05	4.22 ±0.25	3.99 ±0.20	
statistical significance		ns	ns	ns		ns	ns	ns	ns		

a, Units:
R_1: μg/ml; α,ß: 1/min; $T_{½}$: min.
AUC: μg*min/ml; CL_s: ml/min/kg.
V_c, V_{area}, V_{ss}: L/kg.
b, Weighting function.
ns: P>0.05

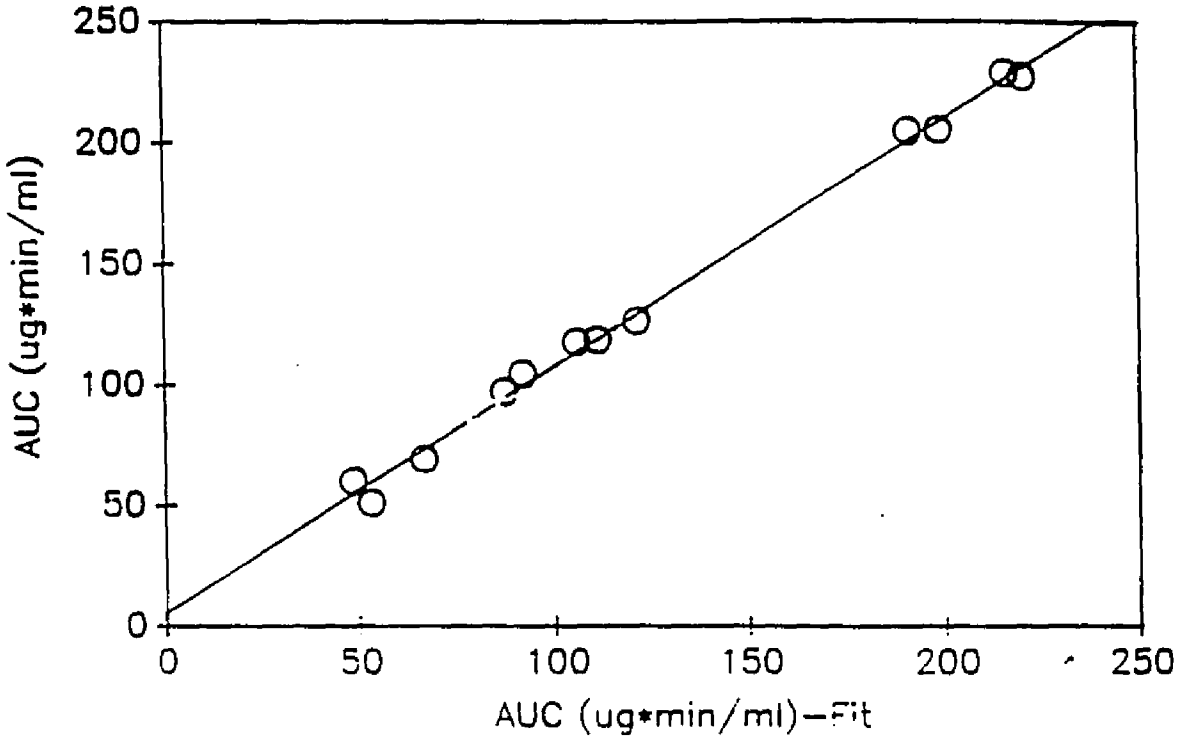


Figure 16. Plot of comparison of AUC values. Model-independent analysis (Y axis) versus curve-fitting of data (X axis). The equation of the line is, Y = 1.02 X + 5.46 (R^2 = 0.99).

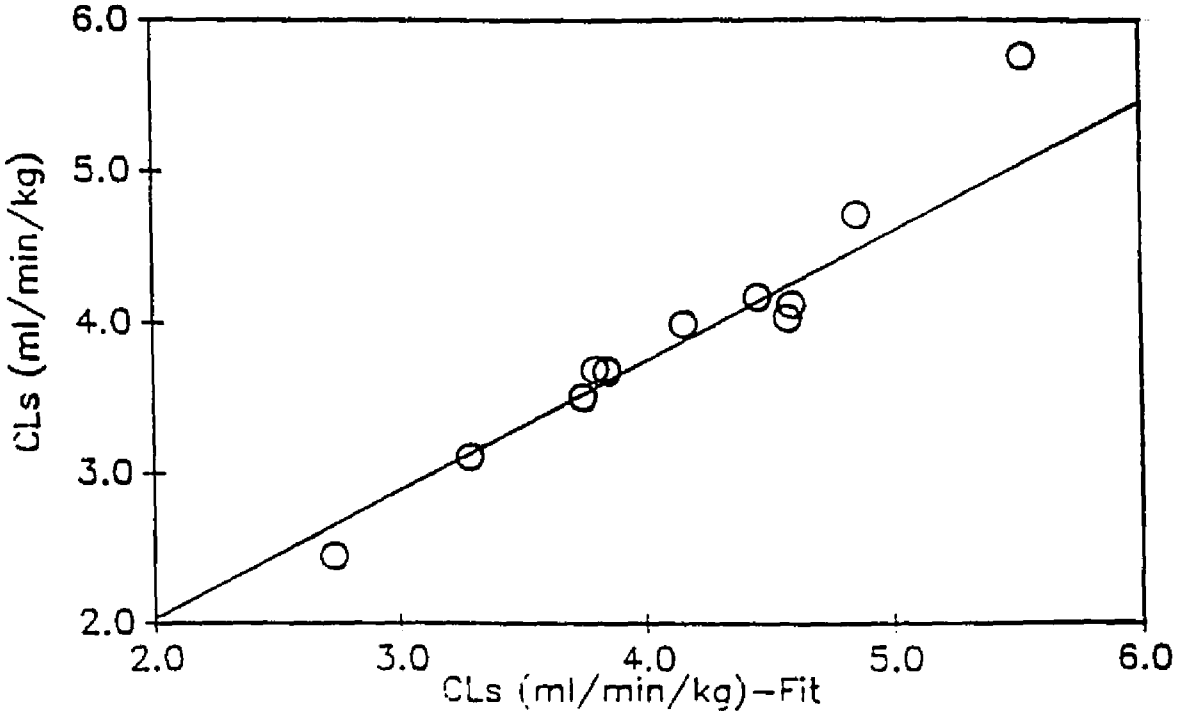


Figure 17. Plot of comparison of CL_s values. Model-independent analysis (Y axis) versus curve-fitting of data (X axis). The equation of the line is, Y = 0.86 X + 0.32 (R^2 = 0.87).

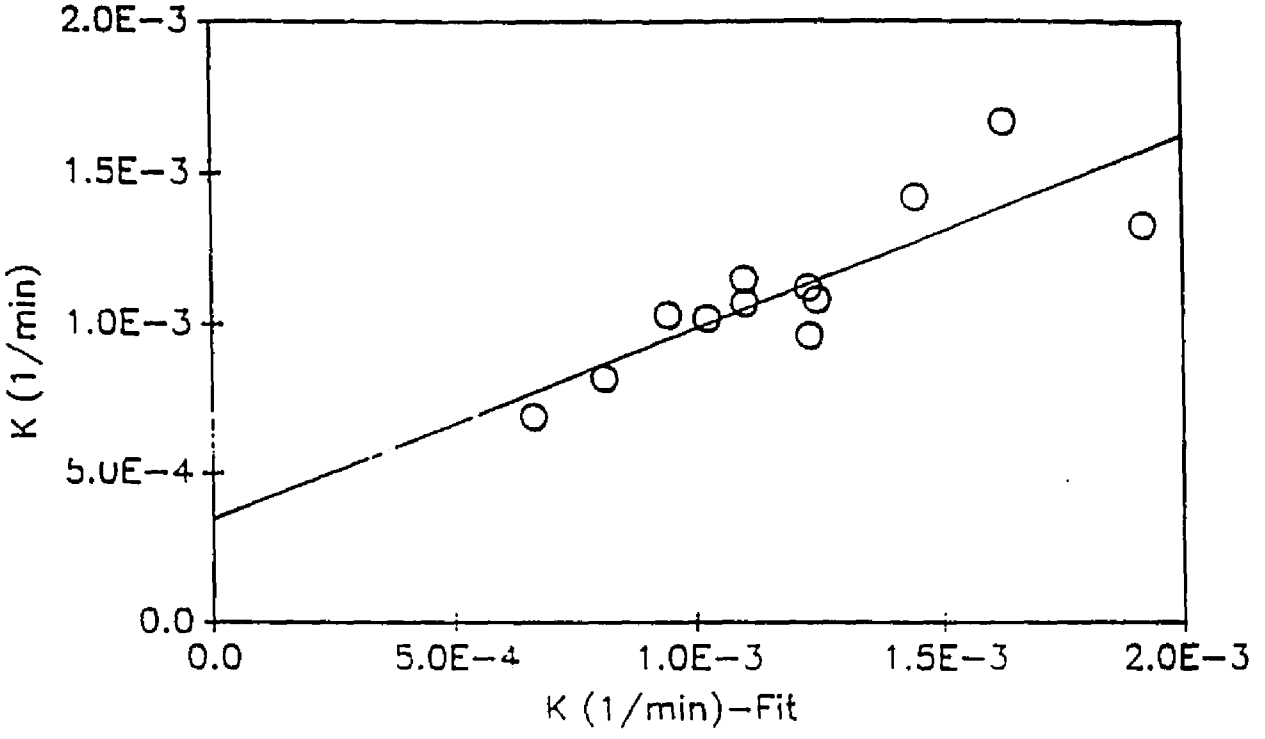


Figure 18. Plot of comparison of K values. Model-independent analysis (Y axis) versus curve-fitting of data (X axis). The equation of the line is, Y = 0.64 X + 0.0003 (R^2 = 0.71).

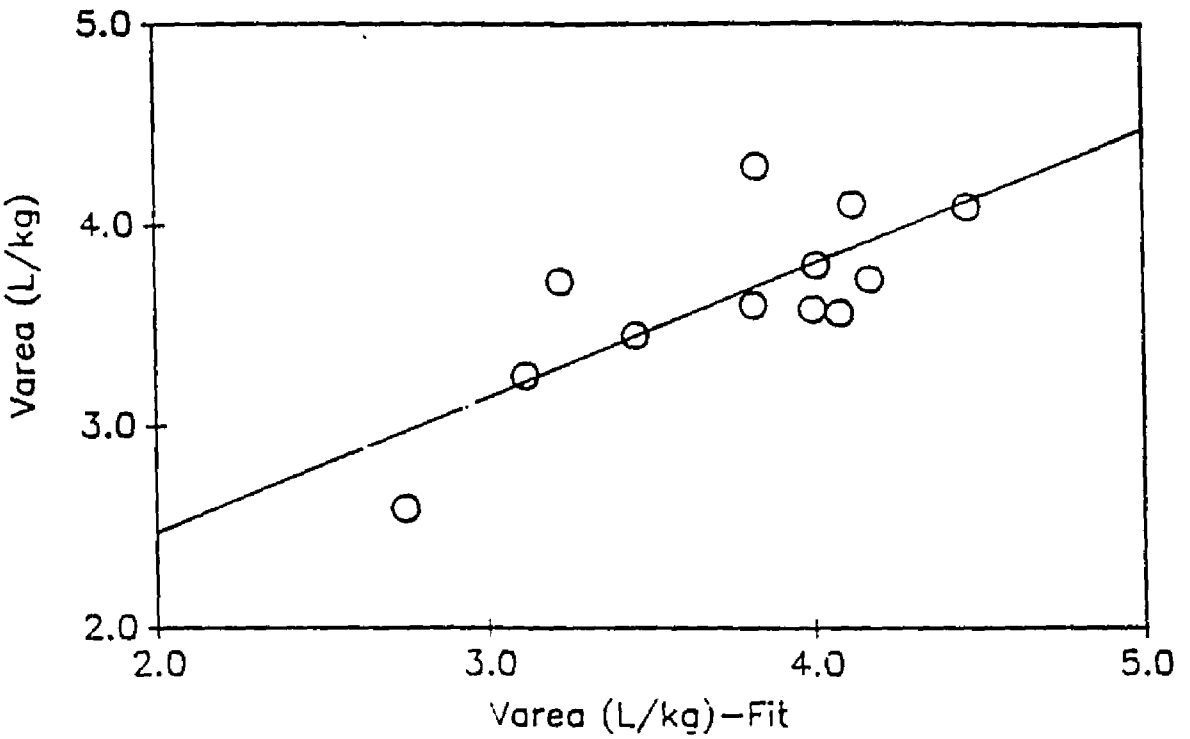


Figure 19. Plot of comparison of V_{area} values. Model-independent analysis (Y axis) versus curve-fitting of data (X axis). The equation of the line is, Y = 0.65 X + 1.20 (R^2 = 0.60).

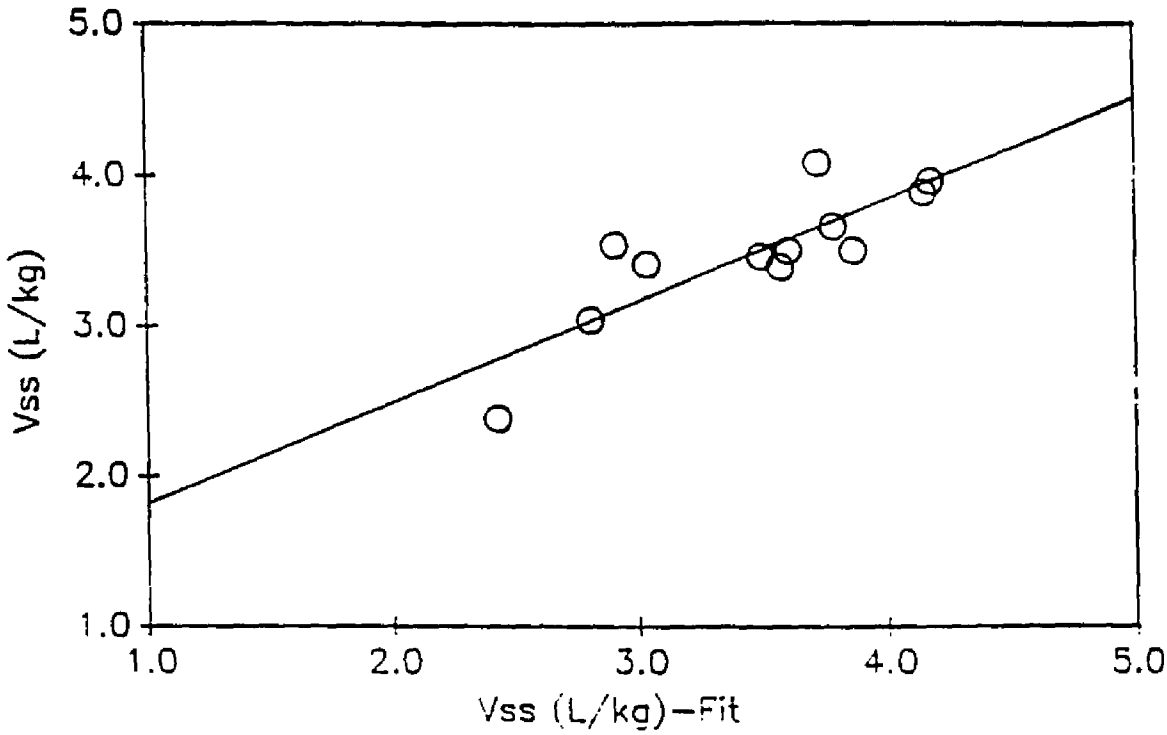


Figure 20. Plot of comparison of V_{ss} values. Model-independent analysis (Y axis) versus curve-fitting of data (X axis). The equation of the line is, $Y = 1.03\ X - 0.11$ ($R^2 = 0.69$).

Chapter 4

DISCUSSION

To be useful for both pharmacokinetic studies and routine clinical purposes, a drug assay must be simple and rapid as well as precise, accurate, sensitive, specific and reproducible. The previous studies for quantitating Am-B by using high-performance liquid chromatography with ultraviolet detection have demonstrated excellent specificity and sensitivity. Granich et al. (1986) reported a reversed-phase HPLC method which employed 0.01 M EDTA in the mobile phase, the detection limit of this assay was reported to be 40 ng/ml. However, there are a number of disadvantages when EDTA salt was used in the mobile phase. First, since EDTA is a metal chelating agent, the metal parts of the HPLC system may corrode after running the mobile phase through the system for a prolonged period of time. Second, this salt is not easily washed out of the column and it may cause the column to degrade.

The HPLC method described here permits the

quantitation of Am-B in both rat serum and urine samples without employing EDTA in the mobile phase. The structure of Am-B suggests the existence of two ionizable groups and therefore two pKa values. The carboxyl group is a hydrogen donating group with a potential pKa around 5 and the amino group is a hydrogen acceptor group with a potential pKa value of 10 to 11. However, the compound remains water-insoluble between pHs 2 to 11. One possible reason for the aqueous insolubility between pHs 2 to 11 is that the negative carboxyl charge is neutralized by the positively charged amino group in water. A variety of different mobile phase compositions have been tested in developing this assay. The current mobile phase condition which contains 1.6 mM KH_2PO_4-Na_2HPO_4 (pH=7) buffer demonstrates a reproducible and reliable chromatograph. This low concentration of buffer in the mobile phase, is likely to act as an ion-pair agent.

Although Am-B has been used clinically for more than 30 years, the available pharmacokinetic information remains limited (Benson et al., 1989). The results of the present study provide significant insight into the pharmacokinetic characteristics of Am-B in the animal model. These results may also provide some of the only insight regarding the renal handling and dose-dependency of this widely used antifungal agent.

In the first preliminary study, Am-B was given by

intravenous bolus, but no Am-B could be detected in serum, even in the first few samples. In contrast, reasonable results were obtained by using an intravenous infusion method. The reason for those differences may be due to precipitation of Am-B in the jugular vein around the cannula used for dosing when the intravenous bolus dose was given.

The systemic clearance, volume of distribution and elimination half-life are shown to be dose-independent in this study (see Table 2). This result tends to support the results in the recent study reported by Chabot et al. (1989). Those investigators have examined the pharmacokinetics of Am-B following continuous infusion in 14 patients with advanced carcinomas (CLs = 0.01 L/h/kg; Vss = 3.2 L/kg; $t_{\frac{1}{2}}$ = 11 days;). They concluded that total body clearance did not change significantly with dose, and the large volume of distribution may indicate extensive binding of Am-B to tissue membrane cholesterol or plasma proteins. However, they reported that the urinary excretion of Am-B was consistently low, and only accounted for 3.7% of the total dose (based on 5 patients data). These results are quite different from those in the present study. For example, the fraction of Am-B excreted into urine in the unchanged form ranged from 20% to 40% of the dose, depending upon the dose administrated. Craven et al. (1979) used dogs with biliary diversion and found that the excretion of Am-B in the urine accounted for 21% ± 5% of the

dose. This difference may be attributed to species differences.

The AUC values obtained from both methods of data analysis are almost identical (see Figure 16); similar results can be observed for systemic clearance (Figure 17). However, there is a poor regression relationship for K values (Figure 18), the reason for this discrepancy is due to the weighting factor chosen and the data points used by the computer for the regression. Since the V_{area} calculation is largely dependent upon the K value, a discrepancy is also observed for V_{area} values (Figure 19). K_{12} is about 10 fold higher than K_{10} and K_{21} (Table 6), suggesting that Am-B release from the tissues and clearance from the body are relatively slow processes.

Surprisingly, renal clearance decreased significantly ($P<0.05$) as dose increased. There are two possible explanations for this observation. First, renal function is somehow damaged, especially in the high dose groups. This nephrotoxicity may cause renal clearance to decrease. Second, renal excretion could be dose-dependent although the systemic clearance is dose-independent. It is possible that non-renal clearance increases as renal clearance decreases but we have no evidence to support that suggestion.

Since Am-B excreted as the unchanged form through the kidney only accounts for about 20 to 40% of the total dose (see Table 2), and since no metabolites have been identified

(Christiansen et al., 1985), the incomplete recovery of the drug may be due to extensive distribution (see Table 2), and very slow release of Am-B from tissues (Table 6).

Creatinine, an end product of muscle metabolism, which is mainly filtered by the glomerulus, is generally used as a measurement of renal function (Ritschel, 1976). Medoff et al.(1980) reported that the glomerular filtration rate (GFR) decreased nearly 40% in most patients who had developed nephrotoxicity caused by Am-B therapy. However, in this study, creatinine clearances were essentially the same among all three dose groups and all of the creatinine clearance values were within the normal range (5.08 ± 1.09 to 8.93 ± 1.79 ml/min/kg). Those normal values prior to Am-B dosing, correspond to the normal values reported by Morris et al. (1988), which range from 4.83 ± 2.11 to 7.0 ± 2.0 ml/min/kg. This suggests that there is no apparent renal toxicity produced by the doses of Am-B used in this study. It is not likely that the lack of alteration in creatinine clearance is due to a delayed nephrotoxic effect caused by Am-B. Wasan et al. (1990) reported that the renal damage caused by Am-B could be detected after only 100 minutes of Am-B dosing.

Renal excretion is generally defined as a combination of glomerular filtration, active tubular secretion and tubular reabsorption. Glomerular filtration is a passive process, only the unbound form of a compound can be filtered. In contrast,

tubular secretion is an active process. Reabsorption can either occur via an active or passive process. Non-ionic, lipid-soluble drugs are rapidly and extensively reabsorbed through a passive process whereas polar compounds and ions are unable to diffuse across the renal epithelium and are excreted in the urine. Active transport processes require a transport system and energy, and they are characterized as being a saturable process (Gibaldi, 1984; Ritschel, 1976).

Creatinine clearance, which represents GFR, is much larger than the renal clearance of Am-B as may be seen by comparing Tables 4 and 5. CL_R accounts for 9% to 25% of CL_{cr}. Similarly, Atkinson et al. (1978) reported that CL_R averaged only 3% of CL_{cr} in their study. However, Am-B is a highly protein bound drug whose unbound fraction is about 0.1 (Block et al., 1974). The glomerular filtration of Am-B is small since only the unbound drug can be filtered through the glomerulus.

High serum Am-B concentrations can be found at early times following dosing. Transport carriers are more easily saturated at high serum concentrations which would occur at early times following dosing. Figure 14 shows that CL_R increases as time increases, and there is a significant difference between the middle and high dose groups ($P<0.05$). Those data suggest that there is an active secretion process but not active reabsorption. The same suggestion is apparent

from the data in Figure 13, which is a plot of the "population" CL_R as a function of serum Am-B concentration. The nature of the relationship shown in that figure is characteristic of active secretion.

If passive reabsorption occurs, renal clearance generally would increase as urine flow increases. However, Figure 15 shows that there is no relation between renal clearance and urine flow, those data suggest that passive reabsorption does not exist or is only a minor component of the excretion mechanism.

From this study, we conclude that systemic clearance, volume of distribution and elimination half-life of Am-B are dose-independent in the Sprague-Dawley rat model. In contrast, renal clearance appears to be dose-dependent, and there may be an active secretion process. Passive and active renal reabsorption processes are relatively insignificant. Further studies should emphasize an examination of renal function and mechanisms of excretion.

APPENDIX A

Serum and urine Am-B concentration versus time data after infusion (15 min) of different doses of Am-B

Low dose group (serum conc.)

Dose = 238.64 µg/kg

Time (min)	Conc. (µg/ml)
16.02	0.3105
30.10	0.1166
45.30	0.0956
75.03	0.0643
135.20	0.0589
256.61	0.0462
375.20	0.0379
554.50	0.0479
734.80	0.0396
978.00	0.0307

Dose = 297.75 µg/kg

Time (min)	Conc. (µg/ml)
15.60	0.2840
29.91	0.0975
45.70	0.0892
75.80	0.0848
135.30	0.0626
255.30	0.0512
376.50	0.0395
554.90	0.0306
734.90	0.0168
974.40	0.0236

Dose = 294.45 µg/kg

Time (min)	Conc. (µg/ml)
16.10	0.2480
32.20	0.1611
45.52	---
74.70	0.0802
134.80	0.0634
256.80	0.0608
377.80	0.0315
555.30	0.0299
740.30	0.0265
975.30	0.0118

Dose = 277.04 µg/kg

Time (min)	Conc. (µg/ml)
16.30	0.1785
31.00	0.1231
46.20	0.0974
75.90	0.0612
136.40	0.0634
255.50	0.0594
375.50	0.0420
555.00	0.0394
735.50	0.0324
974.20	0.0125

Middle dose group (serum conc.)

Dose = 416.61 μg/kg

Time (min)	Conc. (μg/ml)
16.02	0.3322
29.70	0.2108
46.20	0.1936
74.80	0.1662
136.70	0.1189
256.60	0.1017
377.80	0.0744
557.00	0.0532
734.00	0.0518
977.00	0.0400
1457.00	0.0282
1817.00	0.0202

Dose = 486.00 μg/kg

Time (min)	Conc. (μg/ml)
16.50	0.3082
28.70	0.1481
43.89	0.1631
75.30	0.1492
133.80	0.1042
254.10	0.1077
374.00	0.0686
554.00	0.0532
733.80	0.0512
976.50	0.0329
1447.50	0.0344
1814.00	0.0184

Dose = 466.53 μg/kg

Time (min)	Conc. (μg/ml)
15.30	0.4722
30.80	0.2895
47.10	0.2451
75.80	0.2042
136.50	0.1521
254.30	0.1451
374.30	0.0879
554.80	0.0690
739.60	0.0492
974.30	0.0337
1450.30	0.0270
1814.80	0.0136

Dose = 421.24 μg/kg

Time (min)	Conc. (μg/ml)
16.30	0.3143
30.50	0.1807
45.30	0.1406
75.30	0.1361
136.20	0.1005
256.00	0.1177
375.30	0.0605
553.20	0.0550
735.30	0.0300
974.60	0.0376
1456.20	0.0256
1820.30	0.0108

High dose group (serum conc.)

Dose = 711.19 µg/kg

Time (min)	Conc. (µg/ml)
17.30	0.6224
30.10	0.3264
46.32	0.2790
73.20	0.1848
134.60	0.1592
259.00	0.1468
388.00	0.0845
554.00	0.1247
750.00	0.0876
970.00	0.0980
1460.00	---
1815.00	0.0347

Dose = 853.57 µg/kg

Time (min)	Conc. (µg/ml)
15.50	0.6792
30.30	0.2846
45.20	0.2133
75.40	0.1674
135.00	0.1647
259.00	0.1333
375.30	0.1240
560.00	0.1004
734.50	0.0922
975.00	0.1065
1461.00	0.0427
1889.60	0.0245

Dose = 967.75 µg/kg

Time (min)	Conc. (µg/ml)
15.90	0.7172
29.40	0.3023
45.20	0.2140
74.20	0.1753
134.50	0.1737
254.10	0.1699
374.40	0.1445
556.30	0.1091
735.40	0.1086
973.60	0.0837
1455.20	0.0377
1827.60	0.0154

Dose = 838.94 µg/kg

Time (min)	Conc. (µg/ml)
16.11	0.6127
29.10	0.2723
45.40	0.2352
79.61	0.1565
135.01	0.1892
255.40	0.1746
375.40	0.1375
555.30	0.0940
734.20	0.1284
971.40	0.1203
1455.01	0.0403
1809.90	0.0337

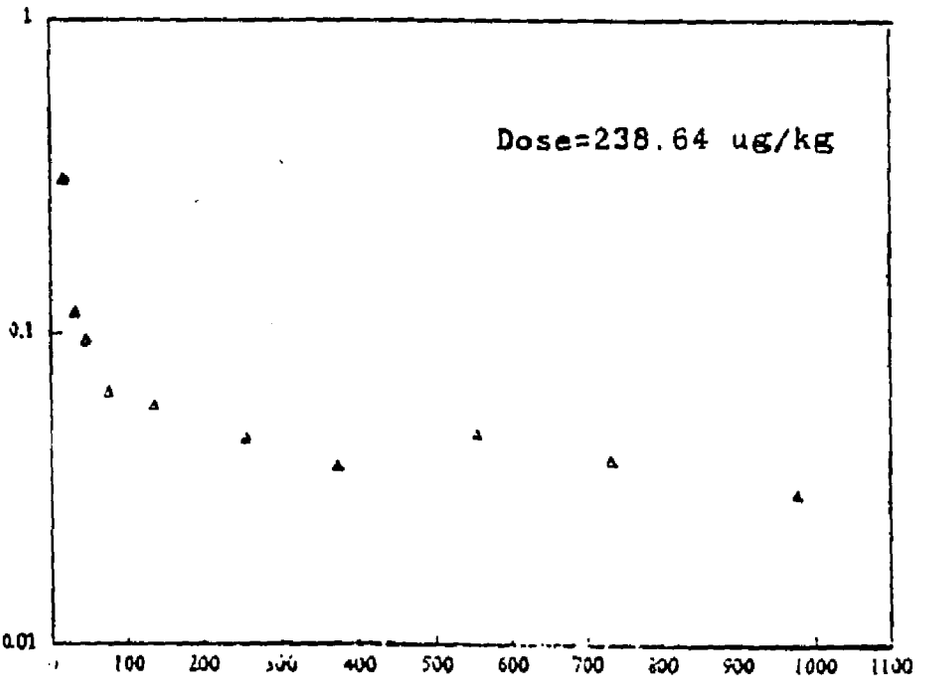
Urine Am-B concentration versus time data

Dose (µg/kg)	Time interval (hour)	Urine volume (ml)	Am-B Conc. (µg/ml)
238.64	8	14	1.0261
	16	18	0.6254
	24	20	0.4474
297.75	8	13	1.0581
	16	18	0.6612
	24	22	0.4144
294.45	8	20	0.6206
	16	26	0.5721
	24	20	0.4180
277.04	8	13	1.0975
	16	16	0.8716
	24	20	0.4195
416.61	8	14	1.6383
	16	14	1.4194
	24	15	0.8019
486.00	8	12	1.3639
	16	15	1.1575
	24	18	0.6626
466.53	8	18	0.8736
	16	15	1.2287
	24	14	0.7079
421.24	8	10	1.3478
	16	13	1.0471
	24	14	0.6030

Dose (μg/kg)	Time interval (hour)	Urine volume (ml)	Am-B Conc. (μg/ml)
711.19	8	18	1.2088
	16	12	1.2365
	24	12	1.1444
853.57	8	16	1.3821
	16	16	0.8282
	24	16	0.6458
967.75	8	16	1.2910
	16	14	1.7147
	24	12	1.2441
838.94	8	22	1.0250
	16	16	0.7630
	24	16	0.7748

APPENDIX B

Serum Am-B concentration versus time plots in each rat after infusion (15 min) of different doses of Am-B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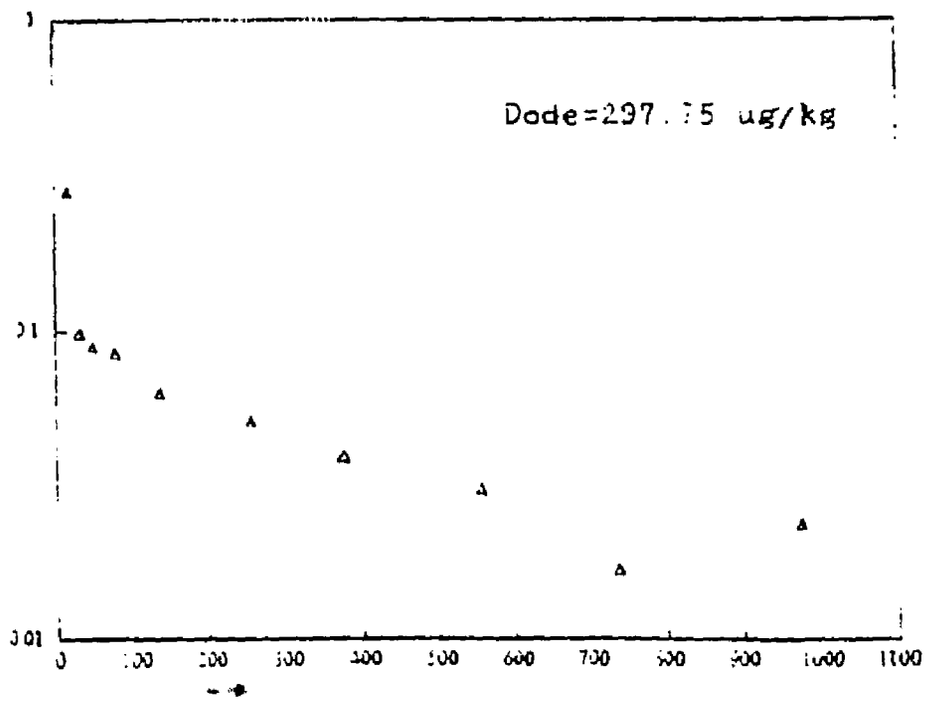
200
400
500
600
700
800
900
1000
1100


Dose=297.75 ug/kg
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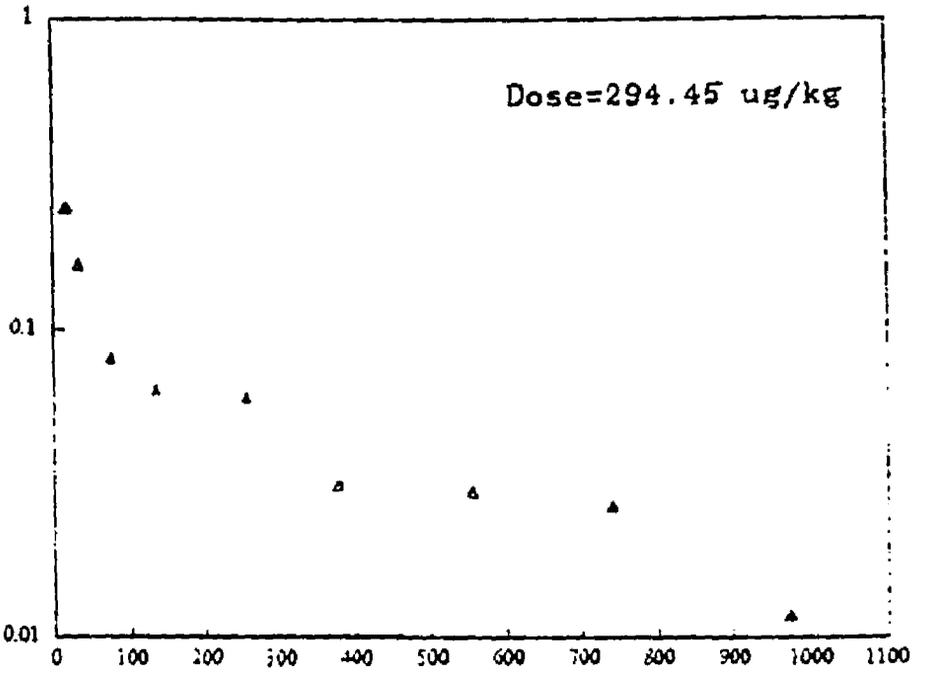
0.1
0.01
0
100
200
300
400
500
600
700
800
900
1000
1100


Dose=294.45 ug/kg
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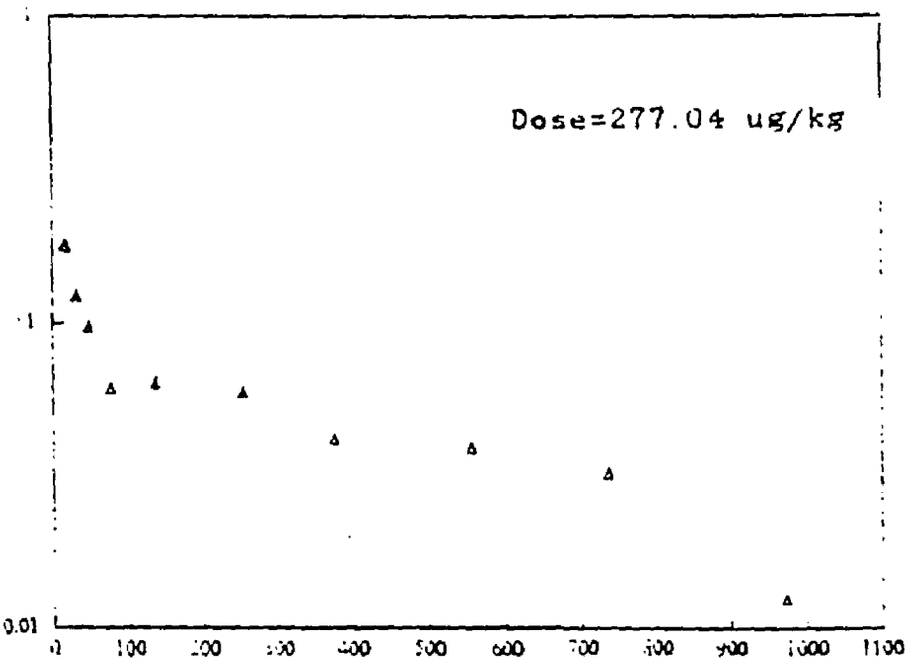
0.1
0.01
0
100
200
300
400
500
600
700
800
900
1000
1100


Dose=277.04 ug/kg
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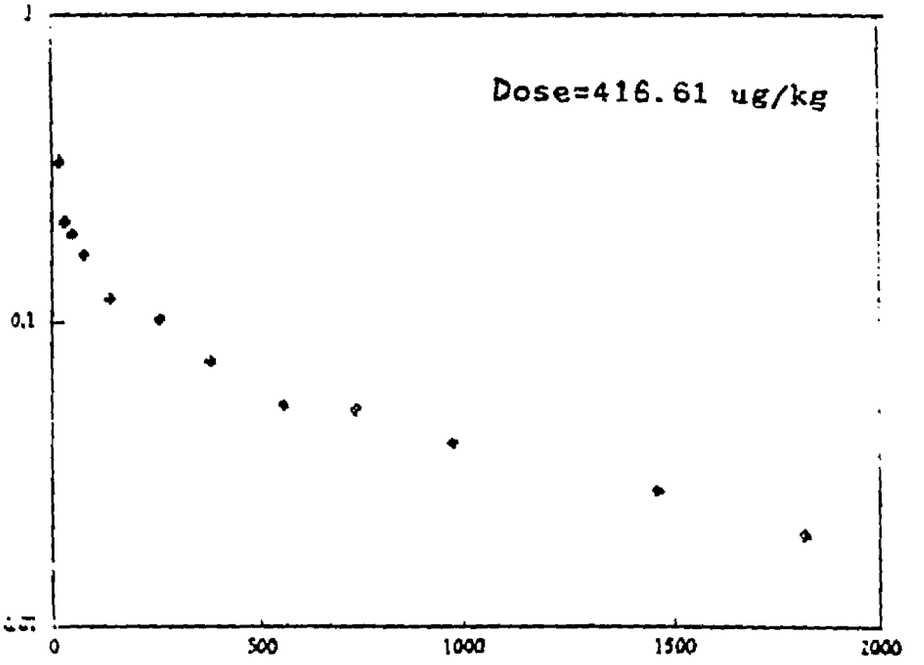
0.01
100
500
600
700
1100


Dose=416.61 ug/kg
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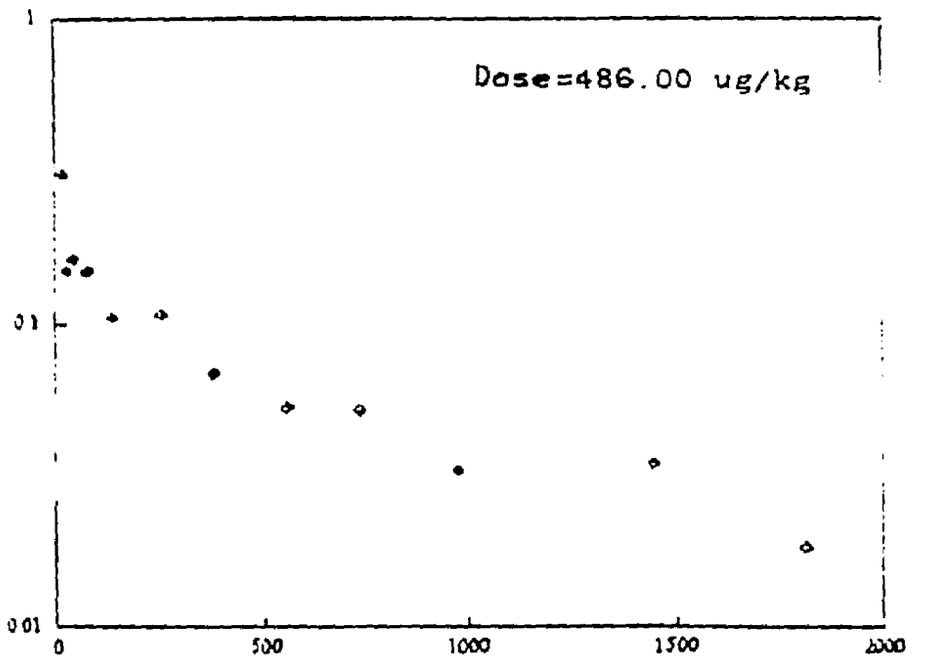
0.1
0
500
1000
1500
2000


Dose=486.00 ug/kg
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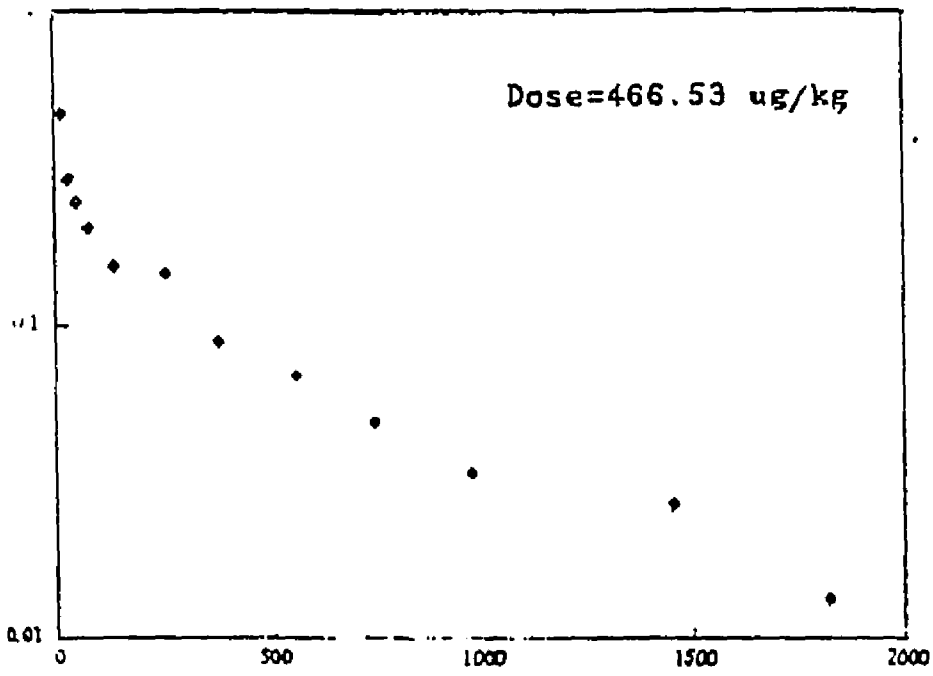
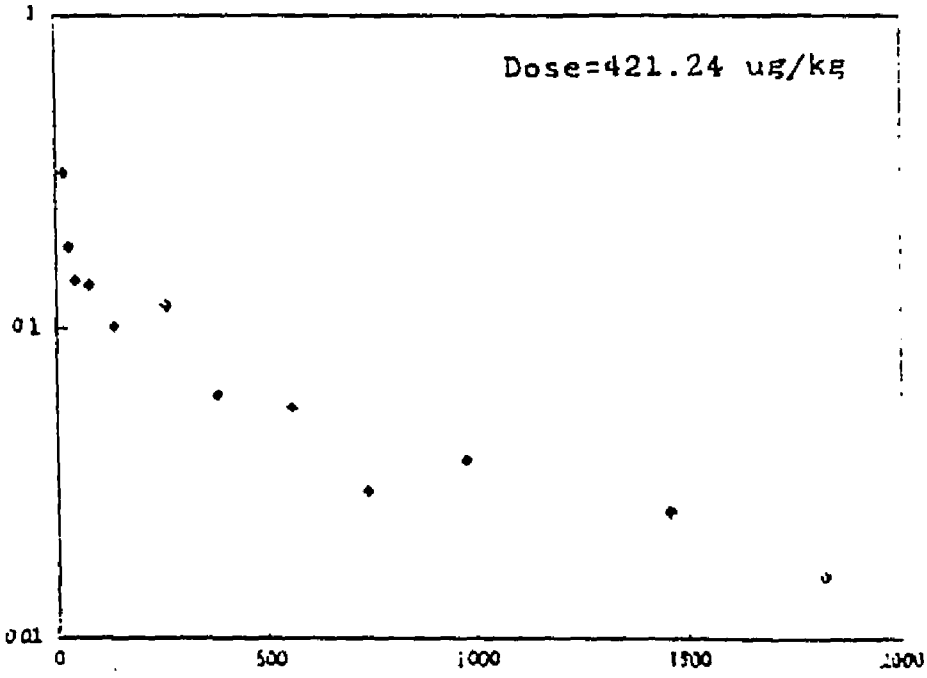
0.1
0.01
0
500
1000
1500


Dose=466.53 ug/kg
0.1
0.01
0
500
1000
1500
2000


Dose=421.24 ug/kg
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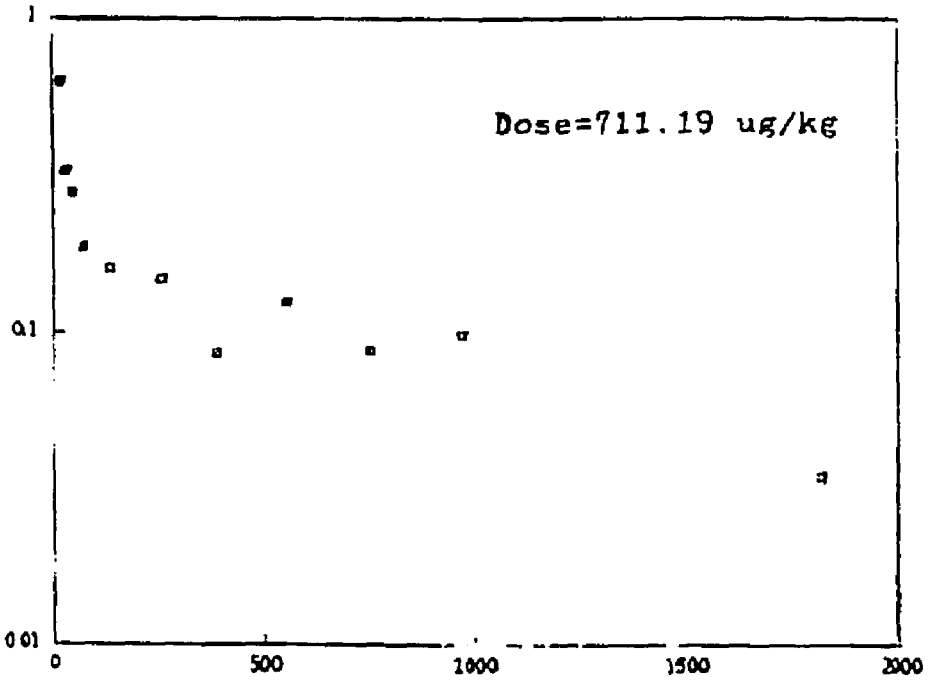
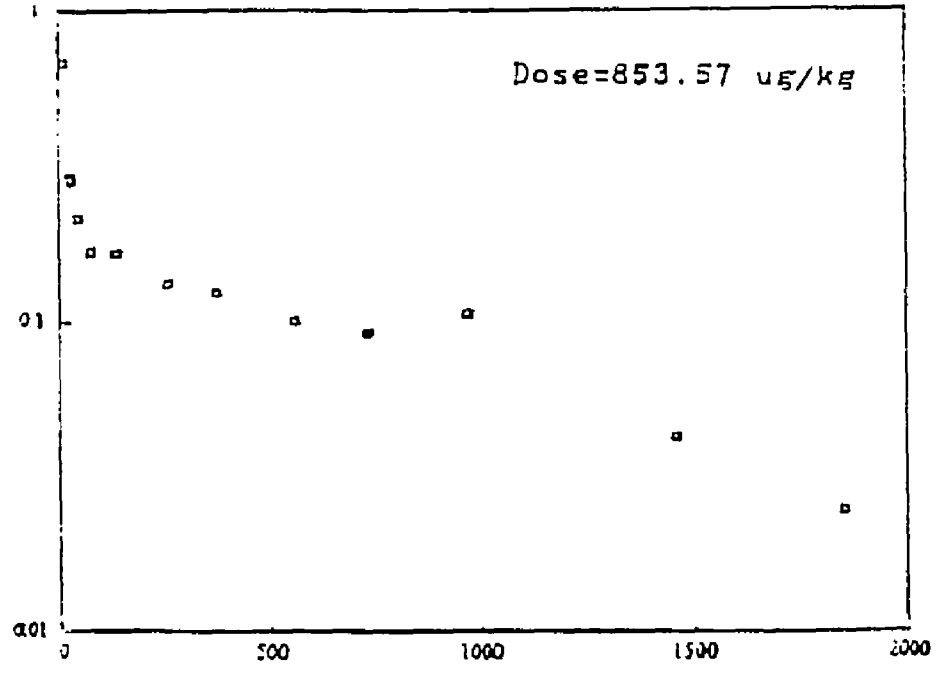
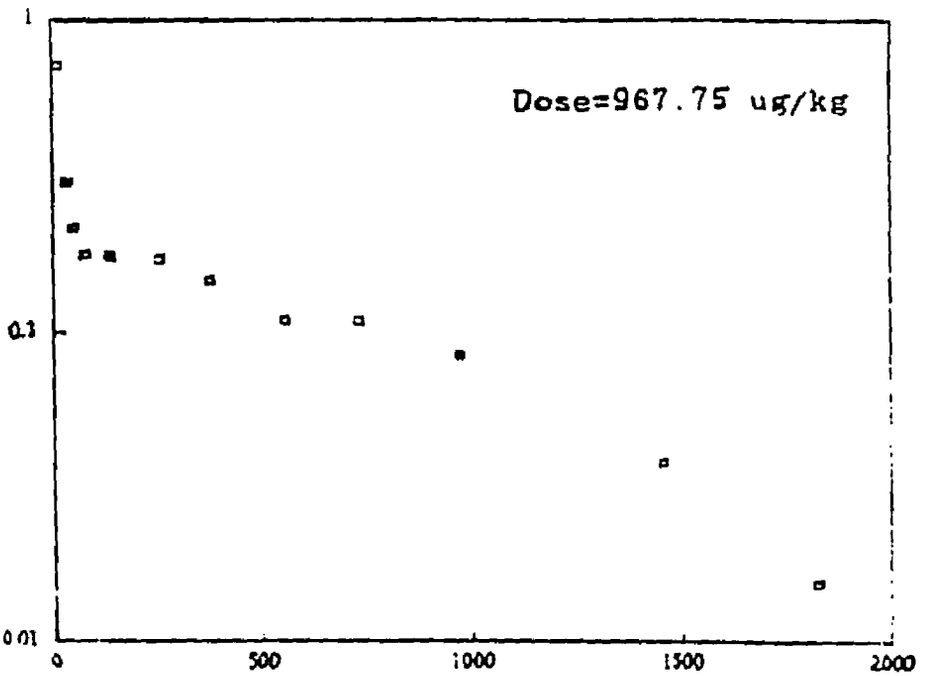
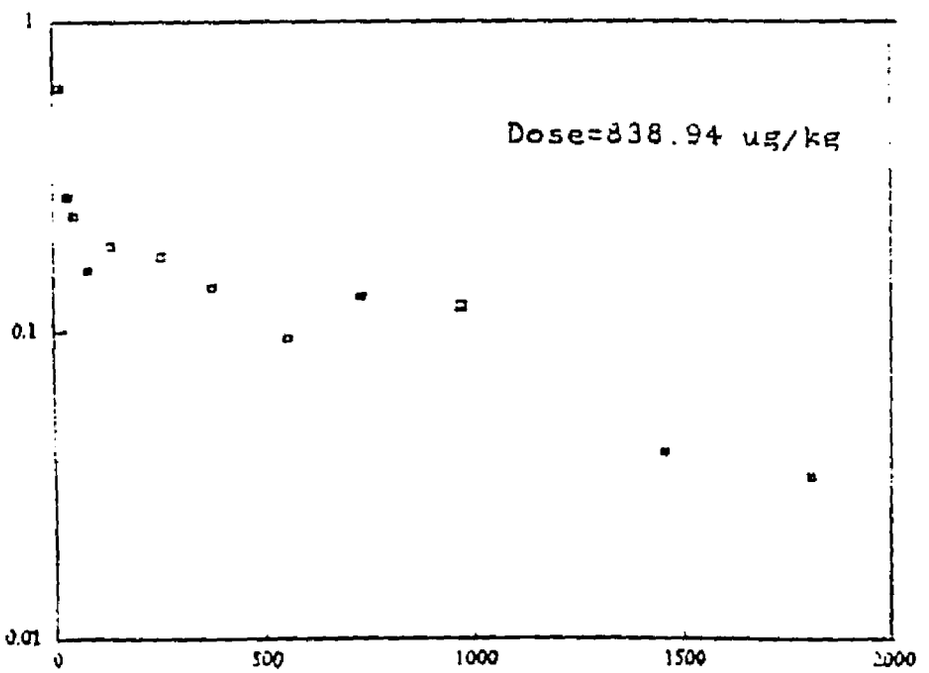
0.1
0.01
0
500
1000
1500
2000


Dose=711.19 ug/kg
1
0.1
0.01
0
500
1000
1500
2000


Dose=853.57 ug/kg
1
0.1
0.01
0
500
1000
1500
2000


Dose=967.75 ug/kg
1
0.1
0.01
0
500
1000
1500
2000


Dose=838.94 ug/kg
1
0.1
0.01
0
500
1000
1500
2000

APPENDIX C

Urine and serum creatinine concentration versus time data after infusion (15 min) of different doses of Am-B

Urine and serum creatinine concentration versus time data

Dose (μg/kg)	Time interval (hour)	Urine volume (ml)	Creatinine conc.(mg/100ml) urine	serum (mid.time)
238.64	17(blank)	16	67.8	0.899
	8	14	57.6	0.782
	16	18	48.6	0.740
	24	20	57.9	0.769
297.75	17(blank)	16	81.0	0.833
	8	13	59.4	0.706
	16	18	68.1	0.684
	24	22	45.6	0.709
294.45	17(blank)	22	48.9	0.566
	8	20	31.8	0.570
	16	26	83.4	0.444
	24	20	28.3	0.479
277.04	17(blank)	16	85.2	0.699
	8	13	62.7	0.598
	16	16	86.7	0.490
	24	20	72.9	0.577
416.61	14(blank)	12	88.8	0.556
	8	14	64.8	0.500
	16	14	74.7	0.514
	24	15	89.7	0.344
486.00	14(blank)	12	96.9	0.505
	8	12	79.8	0.710
	16	15	83.7	0.500
	24	18	67.5	0.501

Dose (μg/kg)	Time interval (hour)	Urine volume (ml)	Creatinine conc.(mg/100ml) urine	serum (mid.time)
466.53	14(blank)	10	121.2	0.576
	8	18	48.0	0.729
	16	15	30.9	0.455
	24	14	93.0	0.491
421.24	14(blank)	10	124.5	0.459
	8	10	77.4	0.559
	16	13	77.7	0.379
	24	14	81.6	0.385
711.19	17(blank)	12	79.8	0.281
	8	18	43.5	0.353
	16	12	69.9	0.403
	24	12	70.8	0.255
853.57	17(blank)	16	58.8	0.639
	8	16	44.7	0.531
	16	16	66.3	0.447
	24	16	49.8	0.449
967.75	17(blank)	18	59.1	0.547
	8	16	38.4	0.544
	16	14	68.7	0.368
	24	12	134.1	0.382
838.94	17(blank)	20	68.4	0.640
	8	22	42.0	0.575
	16	16	65.7	0.476
	24	16	53.4	0.594

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