AN ABSTRACT OF THE THESIS OF

Tharin Limsakun for the degree of Doctor of Philosophy in Pharmacy presented on May 19, 1994.

Title: The Disposition of $C_{27}H_{22}N_4O_3S$ in Rats and Three Antiulcer Agents in Llamas

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John Mark Christensen, Ph.D.

The solubility and disposition of a new anticancer agent $C_{27}H_{22}N_4O_3S$ was investigated. The cosolvent system of 70% PEG, 25% N, N-dimethylacetamide, and 5% water, having solubility parameter of 11.4 $(cal/dec)^{1/2}$, yielded the highest solubility (72 mg/ml). This solvent but not $C_{27}H_{22}N_4O_3S$ was toxic to mice, however $C_{27}H_{22}N_4O_3S$ was toxic to rats but the cosolvent was not. The compound is rapidly distributed and eliminated from rats. The average half-lives and mean residence times for $C_{27}H_{22}N_4O_3S$ in rats given 15 mg/kg, 30 mg/kg, and 60 mg/kg doses were 6.8, 20.9, and 24.2 minutes for half-lives and 8.0, 11.1 and 19.2 minutes for mean residence times, respectively.

The pharmacokinetic study of a gastric acid suppression agents in llama showed omeprazole the most effective agent. Ranitidine (1.5 mg/kg dose) showed little gastric acid suppression (less than two hours) compared to omeprazole. Ranitidine's half life, volume of distribution, and clearance were 1.57 hrs, 1.91 l/kg, and 0.8456 l/kg/hr, respectively. IV administration of omeprazole reduced gastric

acid production to a greater extent than ranitidine and misoprotol. Oral omeprazole was ineffective in reducing gastric acid. The half-life of omeprazole increased with increasing dose from 0.13 hrs, 1.38 hrs, and 6.2 hrs to for 0.2, 0.4, and 0.8 mg/kg doses respectively. The pharmacokinetics of misoprostol could not be determined as plasma concentrations were below detectable limits.

The Disposition of $C_{27}H_{22}N_4O_3S$ in Rats and Three Antiulcer Agents in Llamas

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF TABLES

CHAPTER I
SOLUBILITY, BLOOD COMPATIBILITY, AND PHARMACOKINETICS OF $C_{27}H_{22}N_4O_3S$, A NEW CYTOTOXIC AGENT

ABSTRACT

A co-solvent vehicle was developed for $C_{27}H_{22}N_4O_3S$ for intravenous administration. $C_{27}H_{22}N_4O_3S$ is a new cytotoxic agent with poor aqueous solubility. Studies were performed to develop an intravenous dosage form compatible with blood. Solubility parameters of the drug and selected solvents were estimated using the group contribution method. A variety of co-solvent systems of known solubility parameters were prepared to dissolve $C_{27}H_{22}N_4O_3S$ in to determine the optional solubility parameter of the drug, which was determined to be is around 11.4 $(Cal/cc)^{1/2}$. HPLC with UV detection was employed to determine the solubility of the compound in the various cosolvent systems studied. The cosolvent systems of 90% PEG + 5% Ethanol + 5 H_2O and 10% PEG + 25% N,N-dimethylacetamide + 5 H_2O have identical solubility parameters 11.4 $(Cal/cc)^{1/2}$ but the second yielded a two-fold increase in solubilizing power for C_{27} H_{22} N_4 O_3 S. In vitro hemolytic red blood cell studies were conducted to determine compatibility of the cosolvent systems and the drug with blood. Three doses (15, 30, and 60 mg/kg) in 70% PEG 400 + 25% N,N-dimethyl- acetamide + 5% H_2O were slowly infused to avoid solvent toxicity to determine the pharmacokinetics. The compound is rapidly distributed and eliminated, and its average half-life, and mean residence times increase as the dose increased ($t_{1/2}$

20.9, and 24.2 mins, MRT 8.0, 11.1, and 19.4 mins for 15, 30, and 60 mg/kg doses, respectively). Upon autopsy, the physical appearance of the liver, intestines and kidneys of drug treated rats clearly indicated damage.

INTRODUCTION

$C_{27}H_{22}N_4O_3S$ (Figure I.1) is a new cytotoxic agent which has shown promise against carcinomas in laboratory tests.[1]

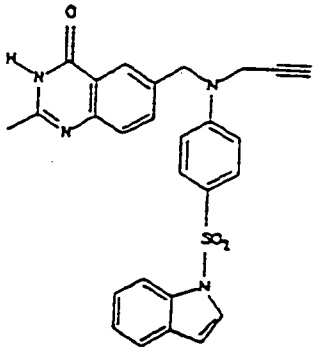

Figure 1 Structure of $C_{27}H_{22}N_4O_3S$ having the molecular weight of 481.54

The purpose of this research was to develop an intravenous dosage form for systemic administration of $C_{27}H_{22}N_4O_3S$ ($C_{27}H_{22}N_4O_3S$). Other parenteral dosage forms, a sterile suspension, had been considered but difficulty in terms of drug absorption from the site of injection, and tissue damage on i.m. injection outweigh the

benefits. An i.v. solution can be manufactured simply and be administered directly to the systemic circulation.

In developing an i.v. solution, determination of drug solubility is a necessary step. The initial objective was to quantitatively determine $C_{27}H_{22}N_4O_3S$ solubility in mixed ternary solvents that may be used for parenteral drug formulation based on prior evidence of physiological compatibility with biological fluids.[2]

The maximum solubility of $C_{27}H_{22}N_4O_3S$ in 1% DMSO/water is approximately 5 μg/ml.[1] The solubility of $C_{27}H_{22}N_4O_3S$ could not be accurately determined in water. The extended Hildebrand Solubility Approach[3] can be applied to predict drug solubility in ternary cosolvent systems using a range of solubility parameters. Potential cosolvent systems useful in parenteral drug formulation can be determined with this approach.

Differential Scanning Calorimetry (DSC) and Capillary Melting Point Apparatus (CMPA) were used in an attempt to characterize the enthalpy for $C_{27}H_{22}N_4O_3S$. The enthalpy data generated from DSC and CMPA are valuable in guiding solubility studies in various solvents.[4]

The possibility of hemolysis occurring upon i.v. injection of $C_{27}H_{22}N_4O_3S$ led to the in-vitro evaluation of the formulation in conditions that simulate the drug concentration at the site of injection immediately after administration and following dilution of the drug concentration by its circulation throughout the body. The degree of hemolysis is greater when the ratio of test solution to blood is higher than when the ratio of test solution to blood is low. Thus, injected solution is more physiologically

compatible if the solution is infused slowly into the circulation as the dilution by circulation is rapid.

Three different doses of $C_{27}H_{22}N_4O_3S$ dissolved the solvent system of 5% water + 25% N,N- dimethylacetamide (DMA) + 70% PEG 400 or the cosolvent system alone were slowly infused into one femoral artery of Male Sprague-Dawley rats via cannulation, while the other femoral artery was cannulated for sample collection. This study was performed to determine the pharmacokinetics of $C_{27}H_{22}N_4O_3S$ in the rats at various doses.

THEORETICAL

Solids in liquids are the most frequently encountered, and probably the most important type of pharmaceutical solutions. Prediction of solubility of such systems is difficult to attain due to many factors, including non-ideality.[3] For an ideal solution, solubility of a solid depends on temperature, melting point of the solid, and molar heat of fusion. A non-ideal solution requires additional terms to account for deviation from ideality.

Scatchard and Hildebrand introduced "regular solution theory" to describe systems of non-ideal solutions which exhibit complete freedom of motion and randomness of distribution in the solution.[3] If the system truly behaves like an ideal solution, then specific interactions and chemical effects of the Scatchard and Hildebrand theory can be excluded from consideration, and heat of mixing can be

related simply to cohesive energy densities (i.e., solubility parameters) of individual components.[5]

The Hildebrand-Scatchard equation for solubility of solid X_2 (represented by subscript 2) in a regular solution can be expressed mathematically as follows:

$$- \log X_2 = \frac{\Delta H_f}{2.303RT}\frac{T_m - T}{T_m} + \frac{V_2\Phi_1}{2.303RT}(\delta_1 - \delta_2)^2 \qquad \text{Eq. 1}$$

where X_2 is the mole fraction solubility of the drug, ΔH_f is the molar heat of fusion of the crystalline drug, T_m is the absolute temperature of the melting point, T is the absolute temperature of the solution, R is the molar gas constant in Cal/K.mole, V_2 is the molar volume of the drug as a hypothetical supercooled liquid, Φ_1 is the volume fraction of the solvent (as subscript 1), and δ_1 and δ_2 are solubility parameters of the solvent, and the drug or solute respectively.

The first group of terms on the right side of equation (1) is for an ideal solution and indicates that solubility of drug depends only on molar heat of fusion of crystalline drug, melting point of drug, and solution temperature.[3] A DSC can determine the heat of fusion and melting point of drug, i.e., ΔH_f and T_m.

The second term on the right side of the equation (1), which represents deviation from ideal solution, consists of the difference between the solubility parameters of the drug and the solvent mixture (δ_1 and δ_2) or, theoretically, the difference in cohesive energy densities.[3] There have been many approaches proposed to estimate solubility parameters (δ_1 and δ_2). One way is to use the group contribution method proposed by Fedors.[5]

From regular solution equation (1), solubility is described mathematically as the negative index (power), i.e., -log X_2. The less negative number of -log X_2 of the solution system, the more soluble the drug is. Thus, the way to increase drug solubility is to find a solvent system that has a solubility parameter equal, or close to, that of the drug. The effects contributing to nonideal solution conditions in the solvent system would diminish or cease existing to affect the solubility of the solute.

Solubility parameters of the drug $C_{27}H_{22}N_4O_3S$ and solvents desired for use in the solvent mixture were estimated by using the group contributing method, which is expressed as :

$$\delta_2 = \frac{\Sigma\Delta E_i}{\Sigma\Delta V_i}^{1/2} \qquad \text{Eq. 2}$$

where $\Sigma\Delta E_i$ = the summation of the cohesive energy densities of atom or chemical groups,

$\Sigma\Delta V_i$ = the summation of the molar volume of atoms or chemical groups.

With data compiled for group contributions to the molar vaporization energy and molar volume by Hansen[6], the solubility parameter of a compound is estimated from its structure. For $C_{27}H_{22}N_4O_3S$ the molar vaporization energies and molar volumes are listed below.

Group	Frequency	$\Sigma\Delta E_i$ (KJ/mole)	$\Sigma\Delta V_i$(cc/mole)
$-CH_3$	1	4.71	33.5
-HN-C-	1	33.5	9.5
-C=	8	24.48	-44
=N-	1	11.7	5.0
≥5membered ring	5	5.25	80
conjugated double bond in ring			
	13	21.71	-28.6
-HC=	13	56.03	175.5
$-CH_2-$	2	9.88	32.2
-N<	2	8.4	-18
-C≡	1	17.07	6.5
HC≡	1	3.85	27.4
0=S=0	1	n/a	n/a

Since there is no such group in Hansen's table, what was assumed in the estimation was each of the four possibilities, as these are similar chemically.

		ΔE_i	ΔV_i
L_1	>SO	39.1	20*
L_2	SO_3	18.8	27.6
L_3	SO_4	28.5	31.6
L_4	$-SO_2Cl$	37.1	43.5

*(not reported, but approximated to be 20)

So the solubility parameter $\delta_2 = [(\Sigma\Delta E_i)/(\Sigma\Delta V_i)]^{1/2} =$ 13.73, 12.96, 13.16, or 13.16 $(Cal/cc)^{1/2}$ for the keto form (I) of $C_{27}H_{22}N_4O_3S$ according to the sulfur dioxide group used for estimation. The enol form (II) has δ_2 estimated to be 13.88, 13.12, 13.32, and 13.31.

Solubility of mixed solvents: The solubility parameter, δ_2, for a mixture of three solvents a, b, c can be calculated by the following equation (5):

$$\delta_1 = \frac{\delta_a\Phi_a + \delta_b\Phi_b + \delta_c\Phi_c}{\Phi_a + \Phi_b + \Phi_c} \qquad \text{Eq. 3}$$

where Φ_1 is the total volume fraction for three solvents ($\Phi_1 = \Phi_a + \Phi_b + \Phi_c$) and δ_a, δ_b, and δ_c are the solubility parameters of solvents a,b, and c, respectively.

For our calculation, it was assumed that Φ_1 was unity. For instance, for 80% ethyl lactate + 10% ethanol +10% water, δ_1 can be calculated as followed:

$$\delta_1 = \frac{(80)(10.56) + (10)(12.96) + (10)(23.39)}{(80 + 10 + 10)} = 12.08 \quad (Cal/cc)^{1/2}$$

where δ is 10.56 for ethyl lactate, 12.96 for ethanol, and 23.39 $(Cal/cc)^{1/2}$ for water. In this fashion δ_1 can be estimated for various mixtures of solvents.

Volume fraction and Mean Molar Volume of mixed solvents: The total volume fraction, Φ_1, of mixed solvents is calculated as follows (5):

$$\Phi_1 = \frac{X_1V_1}{X_1V_1+X_2V_2} = \frac{(1-X_2)V_1}{(1-X_2)V_1 + X_2V_2} \qquad \text{Eq. 4}$$

When the mole fraction solubility of $C_{27}H_{22}N_4O_3S$ is determined experimentally, the actual value for Φ_1 can be expressed and substituted back to equation (3); hence an adjustment could be obtained ($\Phi_1 = \Phi_a + \Phi_b + \Phi_c$). V_2 is the molar volume of the drug $C_{27}H_{22}N_4O_3S$,and V_1 is the mean molar volume of the mixed solvents (a, b, and c) in various proportions and can be calculated as

$$V_1 = \frac{(X_aM_a + X_bM_b + X_cM_c)}{\rho_1} \qquad \text{Eq. 5}$$

where X_i and M_i are the mole fraction and molecular weight of individual solvent in the mixture, and ρ_1 is the density of the mixed solvent at the experimental temperature which can be measured by pycnometer.

MATERIALS AND METHODS

$C_{27}H_{22}N_4O_3S$ was used as received from Agouron Pharmaceuticals, Inc. All other chemicals and reagents were of analytical grade or better and used as obtained. Distilled water was deionized prior to use. Acrodisc disposable filter assemblies were obtained from Gelman Sci.

Perkin Elmer DSC system with TADS (Thermal Analysis Data Station) model 3500 with system 4 microprocessor controller and DSC-4 Analyzer and Capillary Melting Apparatus (Arthur H. Thomas Company: Unimelt) were used to characterize thermodynamic properties of $C_{27}H_{22}N_4O_3S$.

UV scanning was performed with Beckman DB-GT and recorder. Spectronics 20 (Bausch & Lomb) was used to measure heme concentration during hemolysis study.

Solubility Determination

Sample Preparation Solvent mixtures were prepared according to percentages shown in the tables I.1-I.3. These solvent combinations were selected as they provide calculated solubility parameters (δ_2) which covered a range (11.3-15.9) encompassing the calculated solubility parameter (δ_1 between 12.9 - 13.9) for $C_{27}H_{22}N_4O_3S$. Further, the solvent systems investigated may be clinically acceptable. 2 ml. of the cosolvent was placed in each test tube and $C_{27}H_{22}N_4O_3S$ was added to the mixtures. Test tubes were capped with stoppers and wrapped with parafilm to prevent solvent leakage. Test tubes were then secured to a rotator rack which was immersed in a water tank with the temperature controlled at 25 °C. Each day, an additional 5 mg $C_{27}H_{22}N_4O_3S$ was added into each test tube containing a cosolvent mixture. After visual observation for drug in solution determined saturation conditions were reached in the cosolvent mixture the solution was assayed.

Spectrophotometry 5.1 mg of $C_{27}H_{22}N_4O_3S$ was dissolved in 25 ml of DMSO to ensure of complete dissolution. One ml. of the solution was further diluted to 100

ml. with deionized water to make the solution become 1% DMSO in water, yielding the concentration of $C_{27}H_{22}N_4O_3S$ of 2.04 μg/ml. With the concentration below half of its maximum solubility in 1% DMSO/H_20 to ensure complete dissolution, UV scannings compared to a solvent blank of 1% DMSO/H_2O were performed over the range of 200-360 nm.

Chromatograph and Data System

$C_{27}H_{22}N_4O_3S$ was determined by an HPLC assay procedure. The chromatographic system consisted of an HPLC Pump (model 6000A; Water Associate, Inc.), a 25-cm Bondapak C-18 column (5 μm ODS; Water Associate, Inc.), a WISP autosampler (model 710B; Water Associate, Inc.), a detector (Series 440-UV Absorbance; Water Associate, Inc.) with Mercury lamp with filter at wavelength 313 nm. and sensitivity set at 0.1, and an integrator (C-R3A Chromatopac; Shimadzu) set attenuation at 4. The mobile phase was 50% acetonitrile in 5 mM ammonium dihydrogen phosphate buffer (pH 3.5) at a flow rate of 1 ml/min.

Preparation of Standards for Solubility Assay

Stock solution of $C_{27}H_{22}N_4O_3S$ (15.0 mg/ml in PEG 400) was prepared. Serial dilutions with acetonitrile of this stock solution were made in duplicate to obtain the following concentrations: 1.5, 3.0, 6.0, 12.0, 24.0 and 48.0 μg/ml. Internal standard solution of butylparaben (50.4 mg/50 ml in methanol) was used for all assays. 30 μl

of internal standard solution was added to 200 μl of each $C_{27}H_{22}N_4O_3S$ dilution in microcentrifuge tubes (polyethylene, 250 μl) and vortexed before analysis.

Sample Preparation for Solubility Assay

All samples were filtered (0.2 μm Acrodisc Filter assembly) before analysis. Sample nos. 1, 2, 3, 4, 5, 6 of table I.2 were diluted with acetonitrile by dilution factors of 1250, 1250, 1562.5 2500, 3333.3, and 4000, respectively in order that the concentrations were within the concentration range of the standard solutions for solubility assay. Two hundred μl of each diluted sample was pipetted into a microcentrifuge tube, followed by 30 μl of internal standard solution.

Sample Preparation for a New Cosolvent System

Theoretically when the solubility parameter value of the drug matches the solubility parameter value of the cosolvent system the maximum solubility of $C_{27}H_{22}N_4O_3S$ is obtained. The results from the previous solubility study in the PEG 400 ethanol water system led to the use of another cosolvent system. The determination of the solubility parameter of $C_{27}H_{22}N_4O_3S$ in the PEG 400 + ethanol + water system allowed another cosolvent system of 25% DMA + 5% H_2O + 70% PEG 400 to be attempted to increase solubility. Based on the solubility parameter. DMA was used to replace ethanol in the new cosolvent system due to past experience that the drug solubility is enhanced with the presence of DMA in the solution. In

addition, if the solubility is enhanced, the volume of solvent containing drug needed to be administered would be decreased.

25 μl of deionized water, 125 μl of DMA, and 350 μl of PEG 400 were measured and mixed in a small glass vial. The solvent mixture was calculated by percentage volume of 0.5 ml of total volume. The drug, $C_{27}H_{22}N_4O_3S$, was added (approximately 40 mg) to one-half ml of the solvent system, and the solution did not visually turn cloudy. To a second one-half ml volume of solvent in a vial of the same solvent combination approximately 50 mg $C_{27}H_{22}N_4O_3S$ added, but the contents remained cloudy. Only the first vial was analyzed for this report.

This drug sample was diluted 1000 times with acetonitrile in duplicate. 200 μl of diluted sample was pipetted into a microcentrifuge tube, followed by 30 μl of internal standard solution and vortexed prior to drug assay.

Thermodynamic Parameter Evaluation

Calibration of the DSC was made using an encapsulated indium sample (6.805 mg) at a heating rate of 10°C/min, following the recommendations of the Perkin-Elmer Co.

3.0 to 6.0 mg of $C_{27}H_{22}N_4O_3S$ was weighed into an aluminum DSC pan (for each run the exact sample weight was noted) and sealed with the lid. Weighings were made with a Cahn 28 balance. The sample was loaded into the cell and heated at 10°C per minute. The total area under each peak was integrated and determined.

The temperature (T_{max}) where the curves peak and the change of enthalpy (ΔH) of each peak was recorded.

Capillary Melting Point Studies

A small amount of $C_{27}H_{22}N_4O_3S$ was sealed into a glass capillary tube. The tube was immersed in an oil bath and heated from 45 to 290°C. The melting point was visually observed through the installed magnifying lens of the Melting Point Apparatus.

Hemolysis of Red Blood Cells

To study the hemolysis effect of the solution with drug and the solvents themselves, reference standards are needed to compare the degree of hemolysis (i.e. complete hemolysis and a control of no hemolysis). Saponin, known for its hemolytic properties, in a 100 mg/ml saponin solution was used to produce 100% hemolysis. Normal saline solution was used as a control for no hemolysis due to its isotonicity.

There are two conditions to be simulated when studying the hemolysis of RBC's after i.v. drug administration. The first is to simulate the condition at the site of i.v. administration when the drug solution is injected causing its proportions to be higher than the blood present in the vessel. The other condition is to simulate the dilution of the i.v. drug solution that occurs in the systemic circulation as it mixes with the blood and distributes throughout the body.

Hammarlund's method[7], a direct method, was employed to test the ability of the cosolvents and $C_{27}H_{22}N_4O_3S$ in the cosolvents to hemolyze red blood cells. In the case of cosolvent alone, 10 ml of cosolvent (test solution) : 0.1 ml of blood (100:1) and 0.1 ml of cosolvent : 2 ml of blood (1:20) were evaluated to simulate the conditions occurring in the blood at the site of injection and after distribution by circulation of the blood after the test solution is diluted, respectively. Due to the limited supply of $C_{27}H_{22}N_4O_3S$, the hemolysis study for the compound was modified to have 0.05 ml of test solutions : 0.025 ml of blood (2:1) to simulate the conditions at the injection site and 0.1 ml test solution : 10 ml of blood (1 : 100) to simulate the condition when the test solution becomes diluted in the blood stream. After the test solution had been gently mixed with blood, the supernatant, which contained ruptured red blood cells and heme, was decanted and measured spectrophometrically at 600 nm. The control for this direct method was normal saline solution, which produced minimal hemolysis of red blood cells (RBC). Saponin solution caused complete hemolysis and resulted in 100% hemolysis of RBC. This method works well unless there is a large ratio of test solution which may alter measured absorbance: by shifting the visible spectrum of hemoglobin, a color change, or precipitation of hemoglobin (e.g. zinc ion).

Pharmacokinetic Studies

Ten milliliter Vacutainer tubes (Becton Dickson VACUTAINER Systems), containing 143 U.S.P. units of sodium heparin, with 2 ml. normal saline solution

(0.9% sodium chloride injection U.S.P., American McGaw) were added and mixed well to make sure that the heparin sodium was completely soluble. 50 μl of heparin sodium solution was pipetted into each centrifuge tube containing a 200 μl blood sample in order to have an equivalent concentration of heparin contained in a 10 ml Vacutainer tube.

Twelve Male Sprague-Dawley rats obtained from OSU Lab Animal Resources, weighing 300 - 400 g., were used in this study. The animals were initially anesthetized with 40 mg/kg pentobarbital sodium intraperitoneally before they were cannulated with PG - 50 tubing in both femoral arteries, one for drug administration and the other for blood sampling. When needed, the animals were given an anesthetic maintenance dose of 10 mg/kg.

A 70 mg/ml solution of $C_{27}H_{22}N_4O_3S$ was prepared in a cosolvent system of 5% water + 25% DMA + 70% PEG 400. The drug was slowly infused over 1- 3 minutes intraarterially via the cannula, followed by a 1 ml normal saline solution flush. Doses of 15 mg/kg, 30 mg/kg, or 60mg/kg $C_{27}H_{22}N_4O_3S$ each were administered to three rats to study the pharmacokinetics of $C_{27}H_{22}N_4O_3S$ and determine if the drug follows dose-dependent pharmacokinetics. Blood samples (200 μl) were taken at 1, 2, 3, 5, 10, 15, 30, 60, 90, 120, 150, and 180 min. postinfusion. Blood samples were mixed well with 50 μl solution of heparin sodium and kept refrigerated at all the times. The cosolvent only was infused into three rats with the volume equivalent to that used when the maximum dose was administered.

Sample Preparation & Analytical Procedures for Pharmacokinetic Studies Each sample was deproteinated with 200 ml of acetonitrile, and 30 μl internal standard was added. The sample was centrifuged for 30 - 40 minutes in a microcentrifuge (Eppendorf Centrifuge 5415C) at 10,736 xg. The supernatant solution was removed for HPLC analysis. HPLC system and integrator were the same as previously reported. Standards were prepared in the same manner as samples.

Pharmacokinetic Analysis

$C_{27}H_{22}N_4O_3S$ plasma concentrations versus time data were analyzed using RSTRIP.[9] The pharmacokinetic parameters were used to evaluate the total area under the curve (AUC) by converting the postinfusion-fit parameters to i.v. bolus-fit parameters for calculation of pharmacokinetic parameters Vd, AUC etc.[10] Both compartmental and noncompartmental pharmacokinetic parameters were estimated.

RESULTS AND DISCUSSION

Differential Scanning Calorimetry (DSC)

DSC thermograms of $C_{27}H_{22}N_4O_3S$ showed three endotherms between 40 and 300°C. (Fig I.2) The first endotherm occurring between 80 and 90°C is the vaporization of water contained in the $C_{27}H_{22}N_4O_3S$ sample either as a part of the crystal structure or associated with the drug powder. This interpretation was supported by heating a fresh sample of $C_{27}H_{22}N_4O_3S$ from 40 to 110°C (passing through the temperature range of the first endotherm, but not heating it high enough to

reach the second endotherm), cooling the sample back to 40.0°C, and then reheating the sample above 110°C to 300°C. The first endotherm vanished during the second heating (figure I.3) indicating possible water evaporation during the first heating. The change of enthalpy value for the first endotherm, ΔH_1 = 3.66 cal/gram (Fig I.2, first heating from 40 to 200°C) does not represent the heat of vaporization of water because the ΔH calculated in this case was based on the whole sample and not on the water alone.

The second endotherm occurring between 120 and 140°C represented the transition of $C_{27}H_{22}N_4O_3S$ from one state to another. For the second endotherm, ΔH_2 = 3.80 cal/gram (Fig I.2, first heating from 40 to 200°C) and ΔH_2 = 5.66 cal/gram (Fig I.3, second heating from 40 to 300°C). The difference in two heats of transition values was due to the effect of water loss from $C_{27}H_{22}N_4O_3S$. Once water left the lattice, the endothermic process of the second transition for the two endotherms should not be identical. The interpretation of $C_{27}H_{22}N_4O_3S$ undergoing a transition was supported by visually observing physical changes in a fresh drug sample during heating from 40 to 290°C using the Capillary Melting Point Apparatus. It was observed that between 130 and 135°C, $C_{27}H_{22}N_4O_3S$ began to transform from a yellowish powder to an orangy-red substance without melting. This suggests that the second endotherm involved a heat of transition.

The third endotherm occurring between 175 and 195°C was related to a second heat of transition in which the drug changed from the orangy-red substance to a darker reddish color as the sample was heated higher.

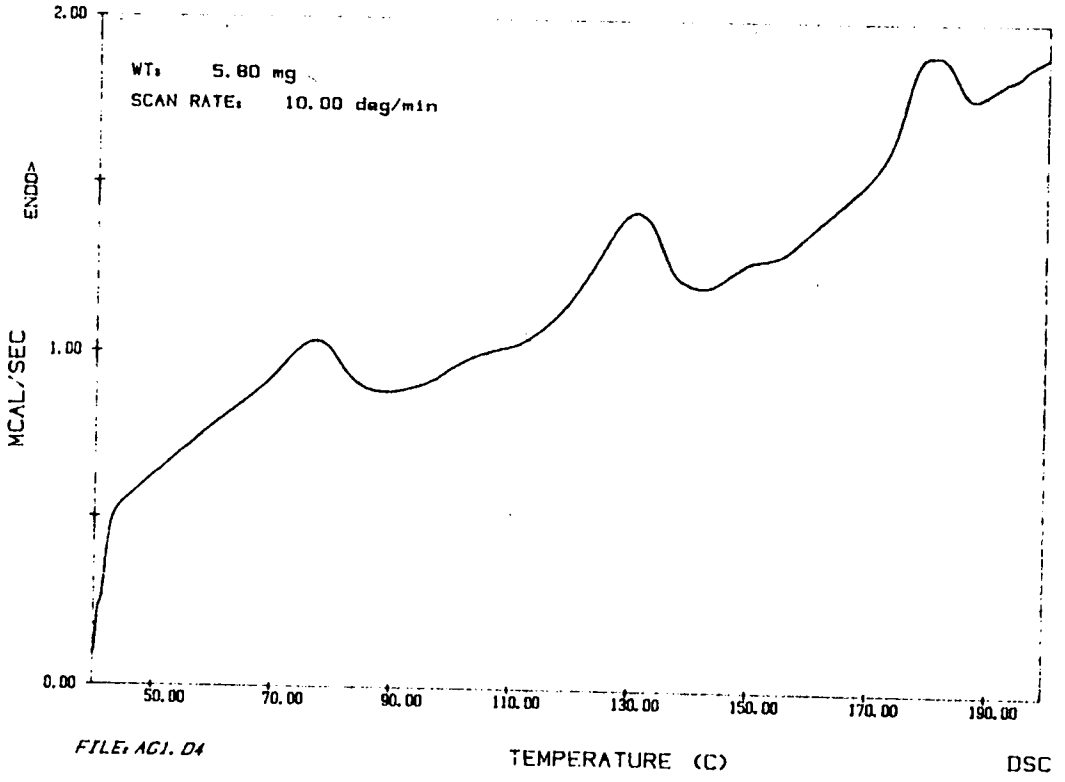

Figure I.2: DSC thermogram of $C_{27}H_{22}N_4O_3S$ from 40 to 300°C with the heating rate of 10°C/min.

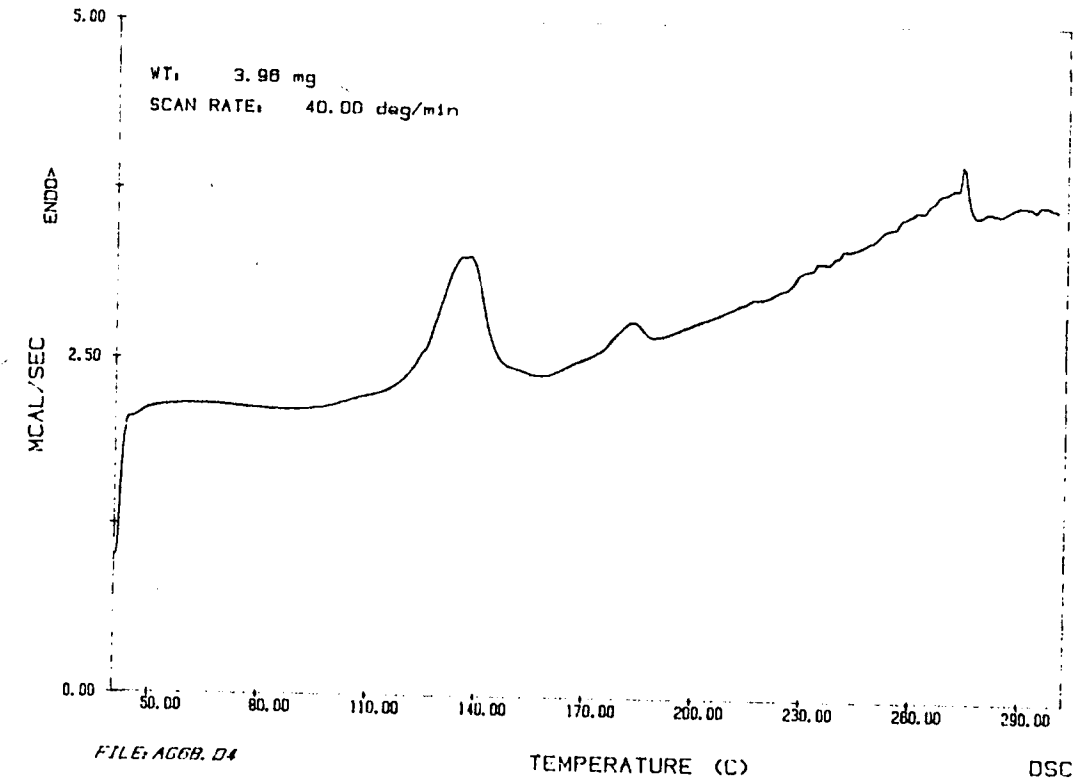

Figure I.3: DSC thermogram of reheating $C_{27}H_{22}N_4O_3S$ after it was heated up to 110°C and was cooled down to room temperature with the heating rate of 40°C/min.

Capillary Melting Point Studies

Between 130 -135°C, the compound began to transform from a yellowish powder to an orangy-red substance without melting. The orangy-red substance became darker in color and turned into crystalline-like particles between 185-190°C. At 210°C it began to melt. The melting process was complete at 215°C. Finally at 290°C, it became charred.

Based on the DSC and Melting Point Studies, it appears that $C_{27}H_{22}N_4O_3S$ may have a polymorphic structure with at least two transition states. The first transition occurred around 130 to 135°C and the second at about 175 to 195°C.

Solubility Studies

The expected thermodynamic parameters were not obtained from DSC studies, i.e., the molar heat of fusion and melting point of $C_{27}H_{22}N_4O_3S$ were not clearly obtained. Thus, the solubility of the drug cannot be predicted using equation (1) since the first term on the right side could not be determined for the regular solution theory approach.

However, the combination solvent mixture approach (Tables I.1 and I.2) where the drug's maximum solubility in a cosolvent mixture should reflect the solubility parameter of the drug. This is more time consuming as it requires experimentally determining solubility in a variety of solvents, but the calculations presented earlier allows initial choice of solvent combinations in a useful range of solubility parameters. One of the combinations of solvents would theoretically produce an ideal solution for

the drug. It can be visualized as allowing the second term on the right side of equation 1 to be dropped, if δ_1 and δ_2 are equal. From Tables I.1-I.3 the solubility parameter of $C_{27}H_{22}N_4O_3S$ is between 11.34 and 12.0 $(Cal/deg)^{1/2}$.

Dosage Consideration & Use of PEG 400 as a Vehicle in Parenteral Preparations

The highest desired dose of $C_{27}H_{22}N_4O_3S$ is 60 mg/kg, or 4200 mg/70 kg. A drug solution of 15 mg/ml would require an injection volume of approximately 280 ml to attain this dose. The solvents used to deliver the drug must be safe physiologically, and not accumulate in the body when long-term treatment is used. The volume of 280 ml can be administered as a slow i.v. infusion. Ethyl lactate + ethanol + water cosolvent mixture was initially investigated because it is an excellent cosolvent mixture for some low dose compounds. Solutions needing 50% or more of ethyl lactate to solubilize the drug into solution require at least 140 ml to be given. Ethyl lactate is hydrolyzed into lactic acid and ethanol in the blood. The LD_{50} for ethyl lactate and ethanol are 618 mg/kg and 2 gram/kg respectively.[2] The toxicities of ethyl lactate and ethanol are relatively low but are still far too toxic to be used in the volumes needed to administer the drug. The drug solution would deliver 140 g of ethyl lactate which is well above the LD_{50} of 43.3 g for a 70 kg person. The primary value of the ethyl lactate system in this study was for investigating and identifying the drugs solubility parameter (δ_2).

Since PEGs have been approved in i.v. preparations PEG 400 + ethanol + water was studied. PEG 400 has a solubility parameter of 10.6 $(cal/cc)^{1/2}$ and is

similar to ethyl lactate's solubility parameter of 9.5 $(cal/cc)^{1/2}$ making it a desirable alternative to ethyl lactate. PEG 400 is a colorless, moderately viscous, somewhat hygroscopic liquid, and does not hydrolyze or deteriorate. It dissolves in water in all proportions to form clear solutions.[11] PEG 400 does not elicit a foreign body reaction in animals.[11] In dogs, the removal of PEG from the site of injection is rapid, since the material diffuses freely into surrounding tissue. When PEG 400 was injected intravenously into humans, 77% was recovered in the first 12 hours.[11] For intrathecal injection into mouse, the median lethal dose (LD_{50}) of PEG 400 is 4200 mg/kg and (i.p.) 49.0 gm/kg[12].

Tables I.1 and I.2 summarize the preliminary solubility results of the two solvent systems:

Table I.1: Composition of Ethyl Lactate, Ethanol and Water Cosolvent System: Solubility Parameters (δ_1) and Amount of $C_{27}H_{22}N_4O_3S$ Added

Test tube	% Ethyl Lactate	% Ethanol	% water	δ_1	$C_{27}H_{22}N_4O_3S$ added (mg)
1	80	10	10	12.08	35.2
2	75	10	15	12.72	36.3
3	70	10	20	13.36	35.9
4	65	10	25	14.0	35.9
5	60	10	30	14.64	36.0
6	55	10	35	15.28	15.5
7	50	10	40	15.92	15.2

5 to 10 mg of $C_{27}H_{22}N_4O_3S$ was added daily to the test tubes to achieve the reported amount in table I.1. Test tubes 6 and 7 turned cloudy after the addition of approximately 15 mg of drug on day 3. No more drug was added to either tube. On day 7, test tube 5 turned cloudy after approximately 25.8 mg of drug had been added; the rest of the tubes (1 to 4) remained clear. Saturation had not yet been attained in tubes 1,2,3 or 4. Unfortunately, no more drug was available for further addition. No solutions reached saturation, and more drug should be added. This experiment was discontinued due to a shortage of drug. Later the saturation study was continued as shown in Table I.2.

Table I.2. Composition of PEG 400, Ethanol, Water Cosolvent System: Solubility Parameter (δ_1) Systems

% PEG 400	% Ethanol	% water	$C_{27}H_{22}N_4O_3S$ added(mg/2ml)	δ_1^*(Cal/cc)
90	5	5	83.800	11.36
85	5	10	79.200	12.02
80	5	15	63.500	12.66
75	5	20	41.600	13.30
70	5	25	30.800	13.93
65	5	30	31.500	14.57

δ_1^* is the calculated solubility parameter of mixture with the assumption that Φ_1 or the total volume fraction for the three solvents to be unity.

UV Scanning

UV scan of $C_{27}H_{22}N_4O_3S$ in 1% DMSO/H_20 with 1% DMSO/H_20 in the reference cell shows one small peak around 300 nm which was enlarged by changing recorder sensitivity to 10 mV.

UV scan using 2% ethyl lactate in water, with Scan rate II (0-2A) and recorder at 100 mV shows a smaller peak around 260 nm to 300 nm and another peak occurring between 220 and 250 nm.

A wavelength of around 300 nm could be used to detect $C_{27}H_{22}N_4O_3S$ spectrophotometrically. Along with application of HPLC, an analysis of $C_{27}H_{22}N_4O_3S$ with chromatographic condition could be implemented with a good resolution. For the HPLC analysis of $C_{27}H_{22}N_4O_3S$, the detector was set at 313 nm.

Assay of Solubility Study

Chromatograms show the separation of PEG 400, butylparaben and $C_{27}H_{22}N_4O_3S$. The retention times for $C_{27}H_{22}N_4O_3S$ and butylparaben are approximately 6 and 5 minutes, respectively. Figure I.4 shows a linear relationship between area peak ratio and concentration of $C_{27}H_{22}N_4O_3S$.

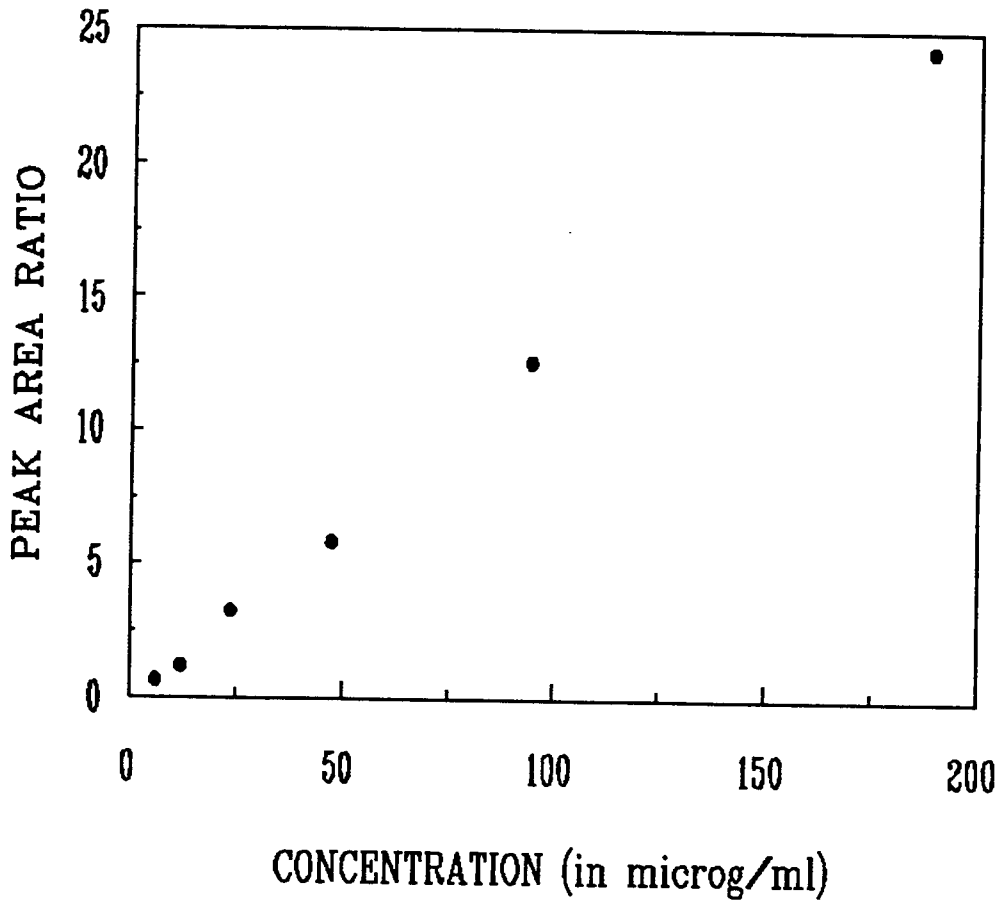

Figure I.4: Typical standard curve for $C_{27}H_{22}N_4O_3S$ concentration vs. peak area ratio in solubility study.

Table I.3. Results Showing Amount of $C_{27}H_{22}N_4O_3S$ Added and Assayed

Sample no.	Amount $C_{27}H_{22}N_4O_3S$ added (mg/ml)	Amount $C_{27}H_{22}N_4O_3S$ assayed (mg/ml)
1	41.900	36.252 ; 35.399
2	39.600	35.693 ; 34.472
3	31.800	26.303 ; 25.870
4	20.800	17.692 ; 17.878
5	15.400	12.962 ; 13.066
6	15.800	7.859 ; 7.692

From Table I.3, the solubilities or concentrations of $C_{27}H_{22}N_4O_3S$ (mg/ml) were plotted (Fig.I.5) against % PEG 400 in the cosolvent mixtures and against estimated solubility parameter (δ_1), assuming the total volume fraction of the three solvents to be unity (refer to equation 3 and 4). According to equation (1), if the second term on the right side is ignored, the ideal solubility of a drug relies only on its thermodynamic properties, i.e., heat of fusion and melting point. Unfortunately, the ideal solubility could not be predicted due to the unavailability of the thermodynamic properties. However, the plot of solubilities versus δ_1 (Fig.I.5) suggests the maximum solubility occurs when $\delta_1 \sim 11.4$. This may be due to specific interactions such as hydrogen bonding between the drug and the solvent mixtures. This interaction decreases solubility of $C_{27}H_{22}N_4O_3S$ with increasing solubility parameter (δ_1) above 11.4.

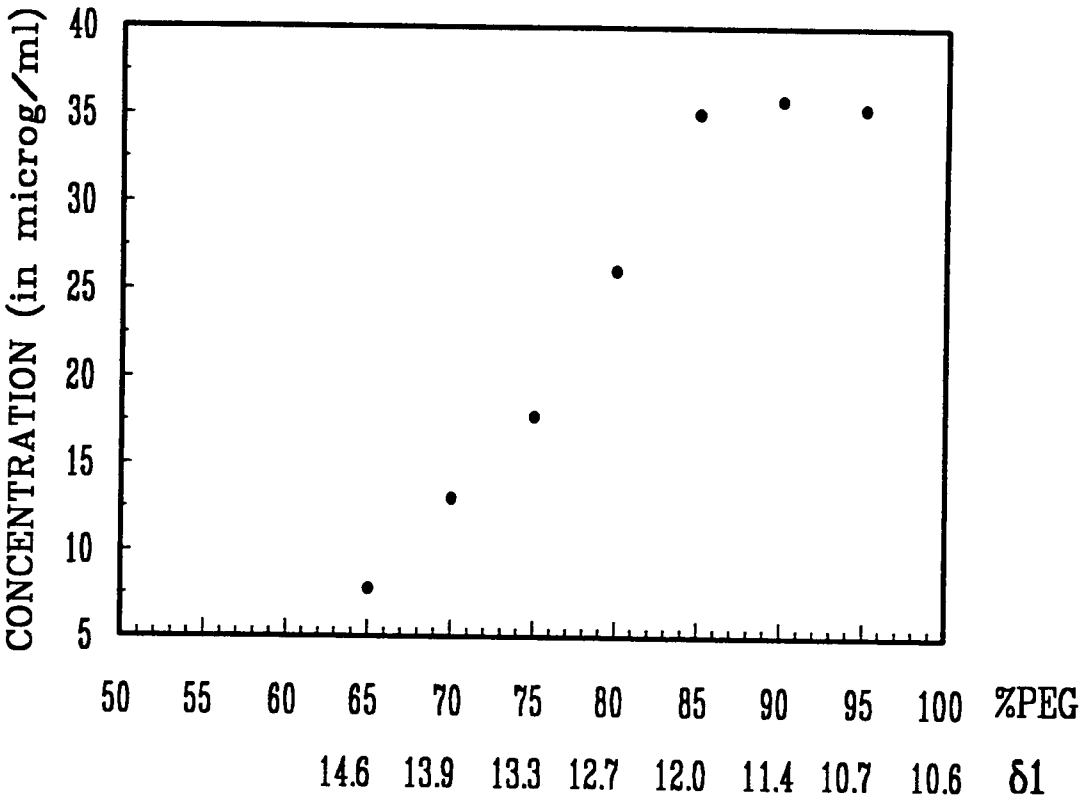

Figure I.5: Plot of $C_{27}H_{22}N_4O_3S$ solubility vs. %PEG 400, with the corresponding δ_1.

The plot of $C_{27}H_{22}N_4O_3S$ solubility, as expressed in mg/ml, versus δ_1 started to show a plateau approximately around 11.4. If it declines as δ_1 decreases, then the estimation of δ_2 by group contribution method (12.96 - 14.21) of the drug is not far from the experimental δ_2. The calculation of δ_2 was an estimation as the ΔE and ΔV of O=S=O group have not been reported. Solubility can also be expressed in mole fraction units, but mass/volume units are more practical and can avoid some confusion when considering modifying solvent ratios to increase solubility.

In the plot (Fig.I.5) of % PEG 400 used in the solvent mixtures depicted on the X - axis vs. drug solubility the ratio of PEG 400 rises as does the $C_{27}H_{22}N_4O_3S$ solubility. Solubility of drug reaches a maximum at 90 % PEG 400, 5 % ethanol, and 5% water. An exact δ_2 of $C_{27}H_{22}N_4O_3S$ could be determined by investigating another system mixture which covers the δ_1 range from about 8.5 to 14.0.

Extended Solubility Study

With the solubility parameter of $C_{27}H_{22}N_4O_3S$ experimentally determined to be around 11.4 $(Cal/cc)^{1/2}$, another investigation was set up to see the effect of replacing ethanol with N,N-dimethylacetamide (DMA) and adjusting the proportion of solvents to yield the same overall δ_1 of 11.4 $(cal/cc)^{1/2}$.

According to the solubility equation of Hildebrand-Scatchard the second term on the right side of equation (1) describes the solvent's effects on the solute's environment, or the excess free energy of regular solutions due to the intermolecular forces of the solute and solvent that are not present in ideal solutions[13] while the first term delineates the roll of the thermodynamic properties of the heat of fusion and melting point in solvation of a molecule. The approach used was to predict the cosolvent mixture of the appropriate solubility parameter to achieve maximum solubility of the drug. However, the magnitude of solubility depends upon solute-solvent interaction[14] and is not always predictable. The attempt to match the solubility parameters of solvent and solute, i.e., point of maximum of solubility of drug in PEG400+ethanol+water solvent system was attained. Substitution of a new cosolvent

system with an identical solubility parameter of PEG 400+EtOH+H_2O could have new solute-solvent interactions when the new solvent system is used, causing the total solubility of the compound as expected to be affected, resulting in either an increase or a decrease in solubility as compared to the previous PEG400+ethanol+water system.

The saturated solubility of $C_{27}H_{22}N_4O_3S$ in 25% DMA+5% water+70% PEG400 was determined by HPLC. N,N-dimethylacetamide (DMA) was eluted approximately 3.3 minutes after injecting 10 μl of a mixture of 25 μl DMA in 200 μl acetonitrile. DMA, PEG 400, and $C_{27}H_{22}N_4O_3S$ were chromatographically separated. Analysis of the diluted sample showed a very small peak of DMA, the resolution of which did not interfere with either $C_{27}H_{22}N_4O_3S$ or butylparaben. Figure I.6 illustrates a linear relationship between area peak ratio and concentration of $C_{27}H_{22}N_4O_3S$. The solubility in this new solvent system increased from 35 mg in PEG400+EtOH+water, Tab.I.3, to 72 mg/ml shown in Tab. I.4.

The average amount of $C_{27}H_{22}N_4O_3S$ assayed in the four samples was 72.5 mg/ml. The solubility of $C_{27}H_{22}N_4O_3S$ increased to almost twice in 25% DMA - 5% water + 70% PEG 400 as compared to 90% PEG 400 + 5% ethanol + 5% water. Both solvent mixtures have a solubility parameter (δ_1) of 11.4. One advantage in dissolving $C_{27}H_{22}N_4O_3S$ in this new mixture (70 % PEG 400 + 25 % DMA + 5 % water) is that the drug readily dissolves when added.

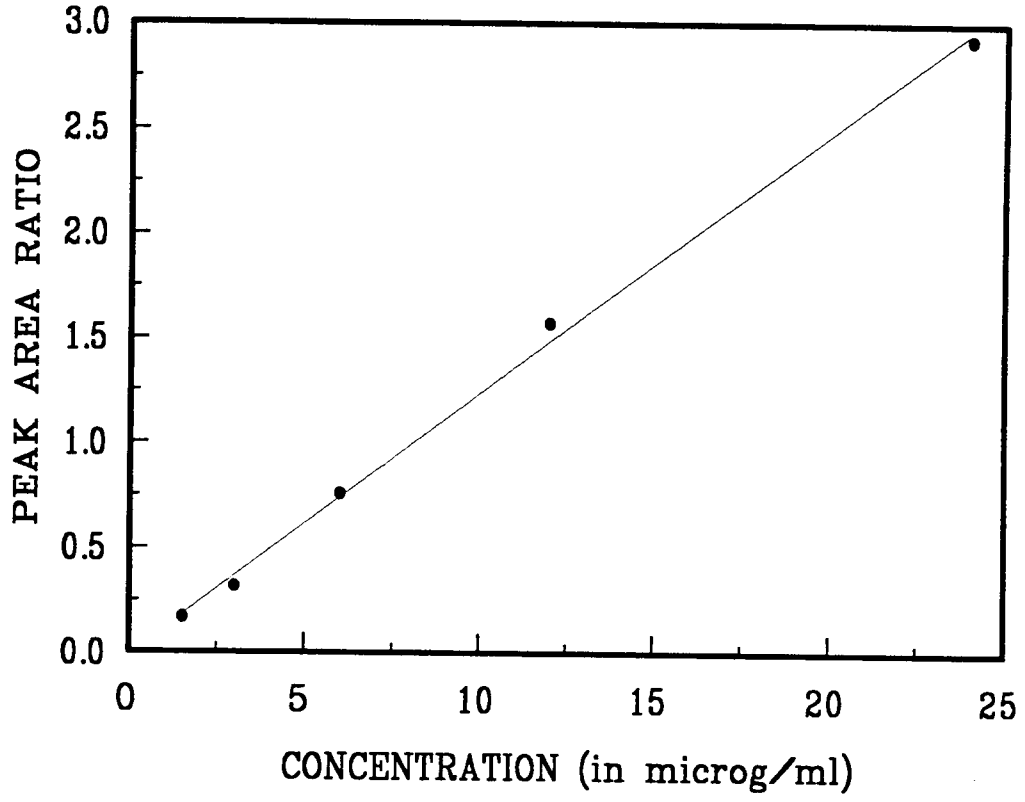

Figure I.6: Typical standard curve for $C_{27}H_{22}N_4O_3S$ concentration vs. peak area ratio in solubility study of the new solvent.

Table I.4. Results of HPLC Analysis of $C_{27}H_{22}N_4O_3S$ Solubility in the New DMA Cosolvent

Sample no.	Amount of $C_{27}H_{22}N_4O_3S$ assayed(mg/ml)
1	70.5
2	71.7
3	72.8
4	73.2

A 60 mg of $C_{27}H_{22}N_4O_3S$/kg or 4200 mg/70 kg dosage was expected to the highest dose to be administered. The required injection volume of the cosolvent system 70% PEG 400 + 25% DMA + 5% water would be about half of what is needed for the 90% PEG400 + 5% EtOH + 5% water co-solvent mixture. An injection volume of 60 ml of a 70 mg/ml solution of $C_{27}H_{22}N_4O_3S$, will be needed to be administered as an IV infusion to provide a dose of 4.2 gm of $C_{27}H_{22}N_4O_3S$ to a 70 kg person. The LD_{50} of PEG 400 administered intravenously in rats is 7.3 g/kg, and is 8.5 g/kg in mouse.[12] LD_{50} of DMA (i.v.) in rats is 2.6 g/kg, and that of ethanol (i.v.) in rats is 1.4 mg/kg.[12]

The cosolvent of DMA+PEG400+water increased the solubility of $C_{27}H_{22}N_4O_3S$ almost two-fold, therefore the injection volume would be reduced to almost half compared to PEG400+EtOH+water formula. The amount of PEG 400 to be administered into the body from the DMA + PEG 400 + water cosolvent system would also be reduced by half, i.e., approximately 42 g/70 kg. At the same time, the amount of DMA being administered would be about 15 g/70 kg, approximately 8 % of its LD_{50}. The DMA-PEG400-H_2O cosolvent has a slight decrease in viscosity for a potential improvement in "syringeability".

Hemolysis of Erythrocytes

Hemolysis was carried out in heparinized horse blood as previously described.[6,15] Hemolysis may be due to a direct interaction of the solvent(s), especially PEG, with the cell membrane.[16] 100 mg/ml of saponin solution and normal

saline solutions were used as reference solutions for 100% hemolysis and 0% hemolysis respectively. The bottom of Tab.I.5 shows the effects of mixed solvents with no drug in the cosolvent systems. In treatment A (10 ml of mixed cosolvent to 0.1 ml blood) simulates the conditions of immediate mixing of the test solutions at the site of injection with blood, while in treatment B (0.1 ml cosolvent to 2 ml blood) attempts to simulate the conditions after the test is diluted throughout the circulation system.

Measured absorbances were often below zero indicating that % hemolysis was very low in many samples, or did not occur. The absorbance below zero may be due to a slight wavelength shift due to a solvent effect, especially when there is a very high ratio of solvent to blood. In treatment B the ratio of cosolvent to blood was reduced from 1:100 to 1:20. The degree of hemolysis ranged from 0 to 34% in this treatment.

At the top of table I.5 shows results of the effect on hemolysis of red blood cells when the compound $C_{27}H_{22}N_4O_3S$ is included in the cosolvents and when $C_{27}H_{22}N_4O_3S$ is not incorporated in the cosolvent systems. $C_{27}H_{22}N_4O_3S$ dissolved in mixed solvents ruptures red blood cells. It was not evident that the hemolytic activity was caused only by the drug. In PEG 400 + EtOH + Water systems higher amounts of drug dissolved gave less % hemolysis than lower amounts of drug dissolved. $C_{27}H_{22}N_4O_3S$ dissolved in DMA+PEG 400+Water system showed 28.3% hemolysis, which was low compared to PEG 400 + ethanol + water system. The 70% PEG400 + 25% DMA + 5% water system ruptured red blood cells to a moderate degree

despite having the highest amount of $C_{27}H_{22}N_4O_3S$ dissolved (~ 70 mg/ml). It may be possible to administer the parenteral formulation of $C_{27}H_{22}N_4O_3S$ in 70% PEG 400 + 25% DMA + 5% water slowly by i.v. infusion with low or moderate degree of hemolysis resulting due to of the rapid dilution of drug that would occur in circulation of the blood stream.

Table I.5: Percentage of Hemolysis by $C_{27}H_{22}N_4O_3S$ Dissolved in Mixed Cosolvent and Cosolvents.

Test solution	%Hemolysis	
	A	B
$C_{27}H_{22}N_4O_3S$ (~ 36 mg/ml) in 90% PEG 400: 5% EtOH: 5% H_2O	100	3.8
$C_{27}H_{22}N_4O_3S$ (~ 35 mg/ml) in 85% PEG 400: 5% EtOH: 10% H_2O	100	18.9
$C_{27}H_{22}N_4O_3S$ (~ 26 mg/ml) in 80% PEG 400: 5% EtOH: 15% H_2O	100	15.1
$C_{27}H_{22}N_4O_3S$ (~ 17 mg/ml) in 75% PEG 400: 5% EtOH: 20% H_2O	100	15.1
$C_{27}H_{22}N_4O_3S$ (~ 12 mg/ml) in 70% PEG 400: 5% EtOH: 25% H_2O	100	37.7
$C_{27}H_{22}N_4O_3S$ (~ 7 mg/ml) in 65% PEG 400: 5% EtOH: 30% H_2O	100	3.8
$C_{27}H_{22}N_4O_3S$ (~ 70 mg/ml) in 25% DMA: 5% H_2O: 70% PEG 400	100	28.3
90% PEG 400: 5% EtOH: 5% H_2O	*	19.0
85% PEG 400: 5% EtOH: 10% H_2O	*	34.0
80% PEG 400: 5% EtOH: 15% H_2O	*	*
75% PEG 400: 5% EtOH: 20% H_2O	*	*
70% PEG 400: 5% EtOH: 25% H_2O	0.71	*
65% PEG 400: 5% EtOH: 30% H_2O	4.64	*
25% DMA: 5% H_2O: 70% PEG 400	*	5.0
Normal saline	*	*
Saponin 100 mg/ml	100	100

* indicates the absorbance measured was equal to or below zero. Normal saline solution as blank gives zero %hemolysis in both treatment A and B, 100 mg/ml saponin solution yields 100% hemolysis in both treatments.

Pharmacokinetic Study

The pharmacokinetics of $C_{27}H_{22}N_4O_3S$ were analyzed by RSTRIP after i.v. infusion in mice of 15, 30, and 60 mg/kg doses. The drug concentration versus time profile was best described by a biexponential equation

$$C = A_1(e^{+\lambda_1\tau} - 1)e^{-\lambda_1 t} + A_2(e^{+\lambda_2\tau} - 1)e^{-\lambda_2 t} \quad \text{Eq. 6}$$

where C is the $C_{27}H_{22}N_4O_3S$ plasma concentration, λ_1 and λ_2 are the distribution and elimination rate constants, τ is the infusion time, and t is the postinfusion time. The plasma concentrations of the three doses of $C_{27}H_{22}N_4O_3S$ are shown in Table I.6, Table I.7, and Table I.8 for doses 15 mg/kg, 30 mg/kg and 60 mg/kg, respectively.

Table I.6: $C_{27}H_{22}N_4O_3S$ Plasma Concentrations of 15 mg/kg doses

Time (min)	Rat A1 (μg/ml)	Rat A3 (μg/ml)	Rat A5 (μg/ml)	Average (μg/ml)	S.D.
1	4.0356	8.7750	-	6.4053*	3.3512*
2	3.2462	7.2125	6.1697	5.5428	2.0561
3	3.0397	4.1867	5.0457	4.0907	1.0064
5	2.1522	3.2713	3.0540	2.8258	0.5934
10	0.8131	2.4737	1.9546	1.7471	0.8495
15	0.4737	0.6976	1.1322	0.8028	0.4659
30	-	0.3657	0.4813	0.4235	0.0817

*, n = 2

Table I.7: $C_{27}H_{22}N_4O_3S$ Plasma Concentrations of 30 mg/kg doses

Time (min)	Rat B1 (μg/ml)	Rat B5 (μg/ml)	Rat B6 (μg/ml)	Average (μg/ml)	S.D.
1	13.6727	12.6567	-	13.1647[--]	0.7184[--]
2	10.0792	3.1667	15.1270	9.4576	6.0043
3	3.0040	2.6806	13.1742	6.3279	5.9299
5	2.8087	2.1705	6.1340	3.7044	2.1282
10	-	-	2.7571	2.7571[-]	-
15	2.5198	1.9030[@]	1.5180	2.0060[--]	0.5014[--]
30	0.5353	1.0638	0.9092	0.8361	0.2717

Note: [-], n = 1

[--], n =2

Table I.8: $C_{27}H_{22}N_4O_3S$ Plasma Concentrations of 60 mg/kg doses

Time (min)	Rat C1 (μg/ml)	Rat C3 (μg/ml)	Average (μg/ml)	S.D.
1	-	23.5828	23.5828*	-
2	17.9658	8.6759	13.3209	6.5689
3	11.1308	6.9773	9.0540	2.9370
5	8.2660	5.4650	6.8654	1.9808
10	3.9321	3.4991	3.7156	0.3062
15	2.9348	3.1528	3.0438	0.1541
30	2.4095	1,8770	2.1432	0.3766

Note: *, n = 1.

Figure I.7, Fig. I.8, Fig. I.9, and Fig I.10 show plasma profiles in rat A1, rat A3, rat A5, and the average plasma concentration for the 15 mg/kg dose. Figure I.11, Fig. I.12, Fig. I.13, and Fig. I.14 are the plasma profiles in rat B1, rat B5, rat B6, and the average plasma concentration for the 30 mg/kg dose. For the 60 mg/kg dose, Fig. I.15, Fig. I.16, and Fig. I.17 represent the profile in rat C1, rat C3, and the average plasma concentration.

The pharmacokinetic parameters (mean and standard deviations) for 15, 30, and 60 mg/kg doses are presented in Tab. I.9, Tab. I.10, and Tab. I.11, respectively. The plots of AUC and MRT versus dose are depicted in Fig I.18 and Fig. I.19, respectively.

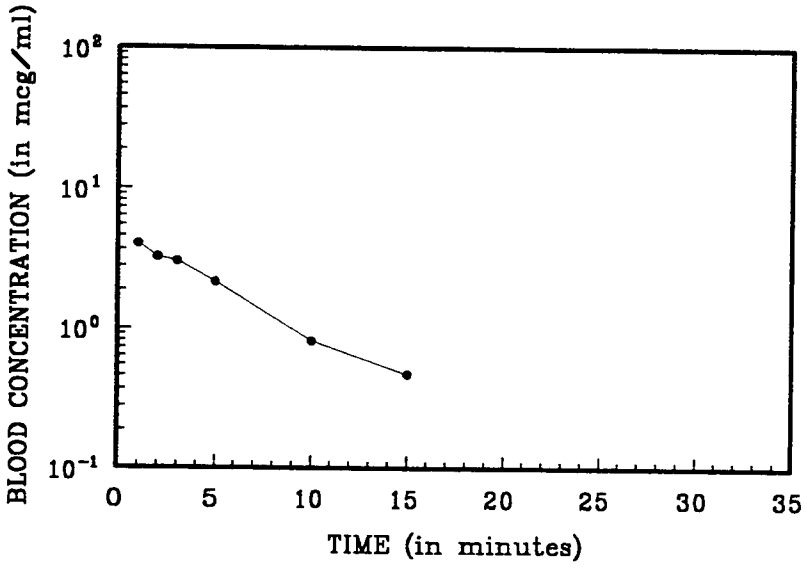

Figure I.7: Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 15 mg/kg $C_{27}H_{22}N_4O_3S$ in rat A1.

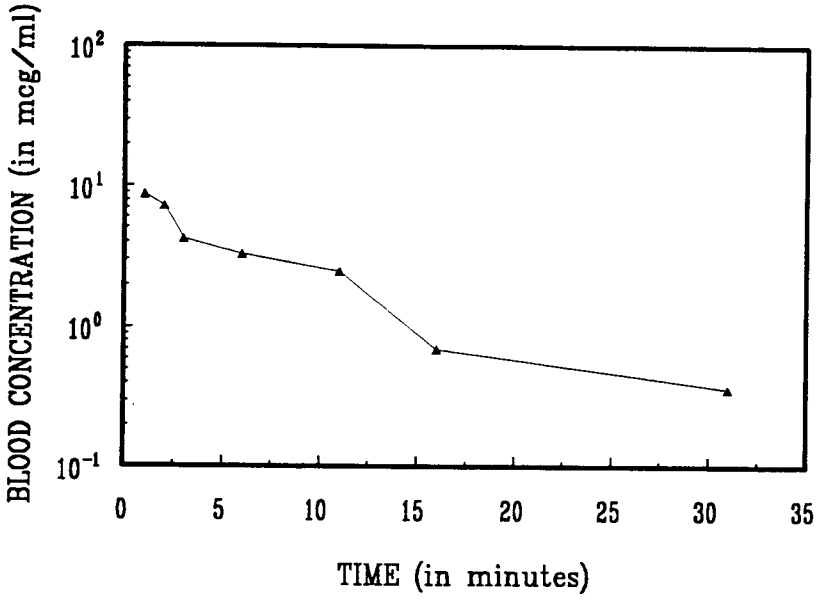

Figure I.8: Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 15 mg/kg $C_{27}H_{22}N_4O_3S$ in rat A3.

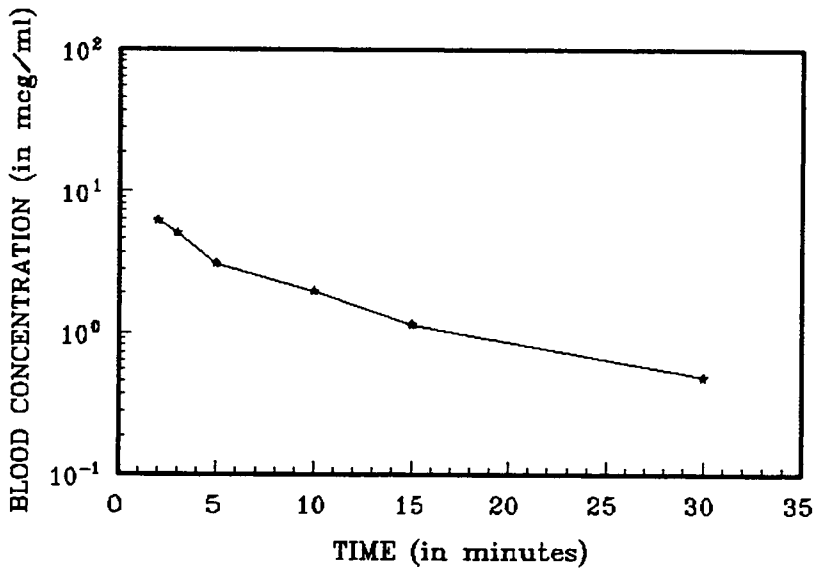

Figure I.9: Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 15 mg/kg $C_{27}H_{22}N_4O_3S$ in rat A5.

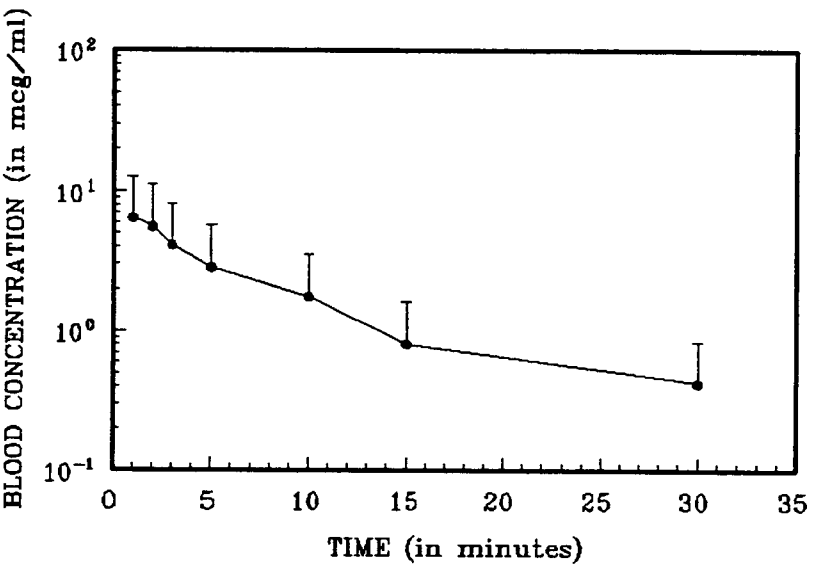

Figure I.10: Average blood concentration of $C_{27}H_{22}N_4O_3S$ vs.time after administration of 15 mg/kg dose in rat A1, A3, and A5.

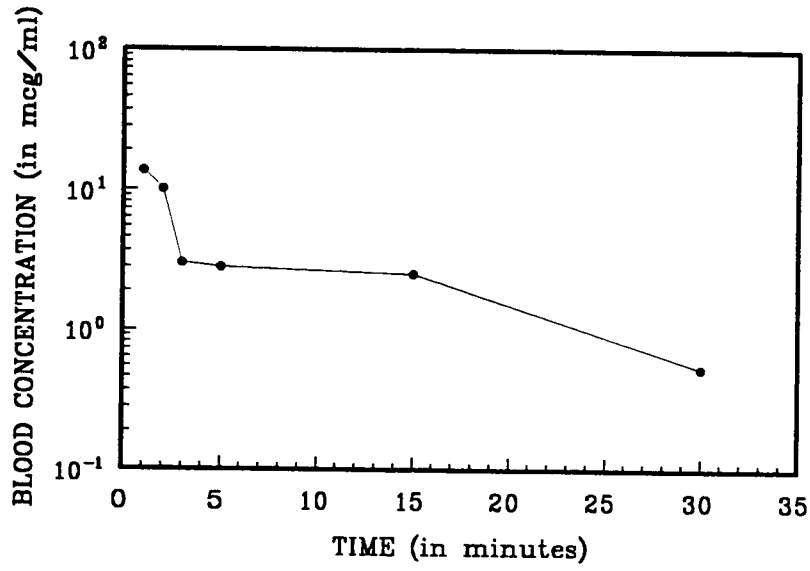

Figure I.11: Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 30 mg/kg $C_{27}H_{22}N_4O_3S$ in rat B1.

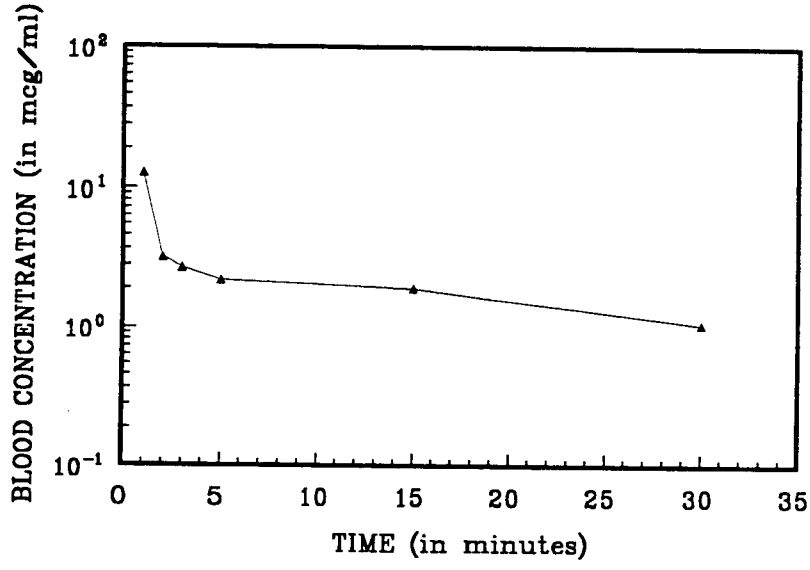

Figure I.12: Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 30 mg/kg $C_{27}H_{22}N_4O_3S$ in rat B5.

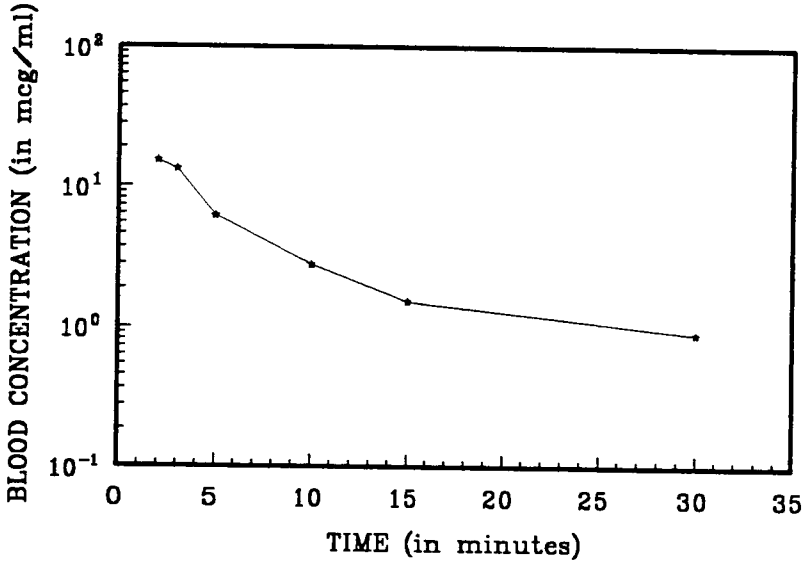

Figure I.13: Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 30 mg/kg $C_{27}H_{22}N_4O_3S$ in rat B6.

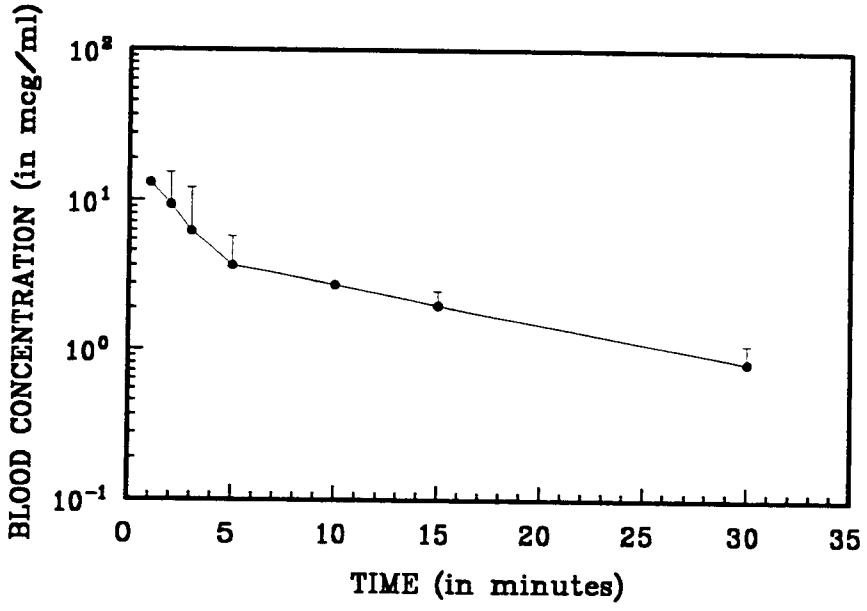

Figure I.14: Average Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 15 mg/kg dose in rat B1, B5, and B6.

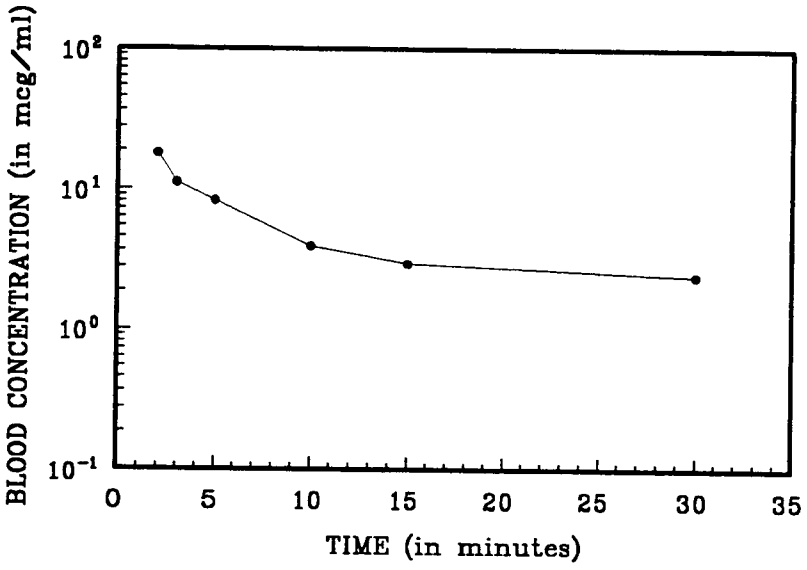

Figure I.15: Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 60 mg/kg $C_{27}H_{22}N_4O_3S$ in rat C1.

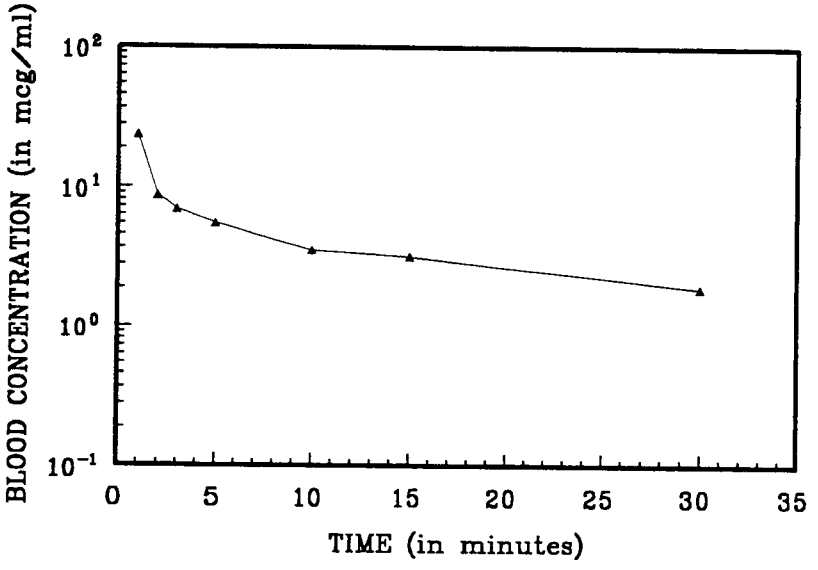

Figure I.16: Blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 60 mg/kg $C_{27}H_{22}N_4O_3S$ in rat C3.

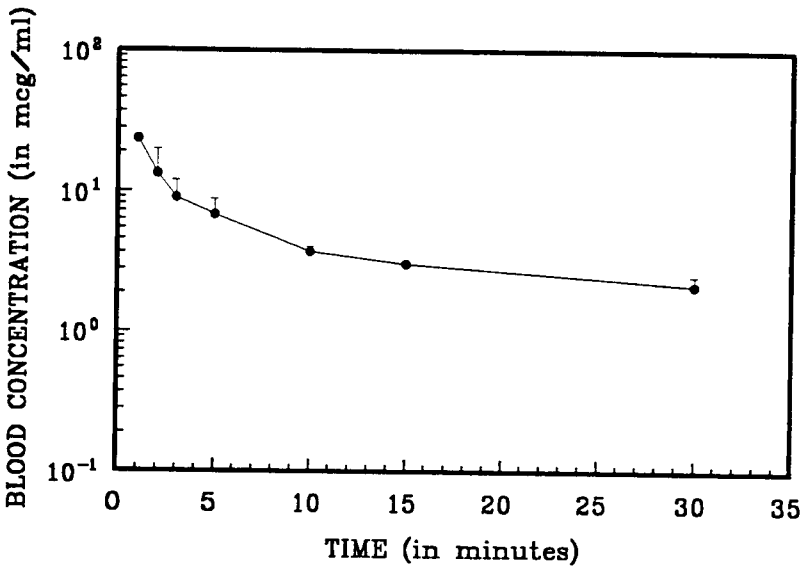

Figure I.17: Average blood concentration of $C_{27}H_{22}N_4O_3S$ vs. time after administration of 60 mg/kg dose in rat C1 and C3.

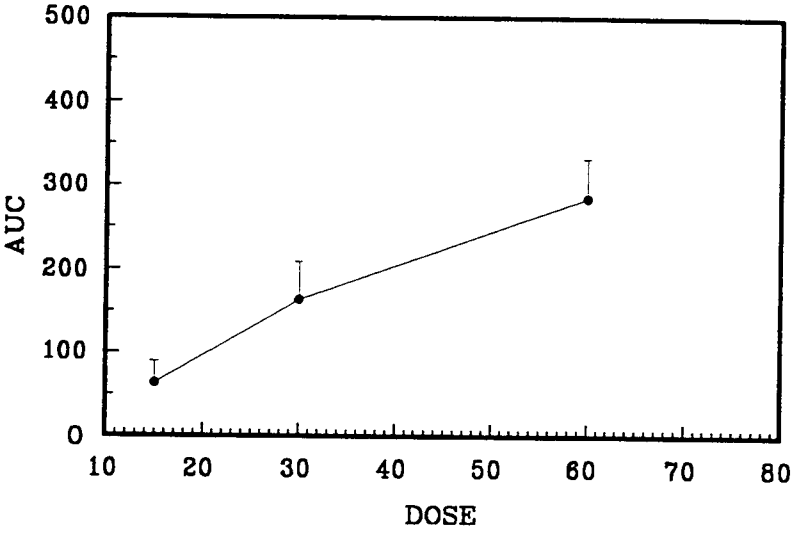

Figure I.18: Plot of area under $C_{27}H_{22}N_4O_3S$ vs. time curve vs. dose.

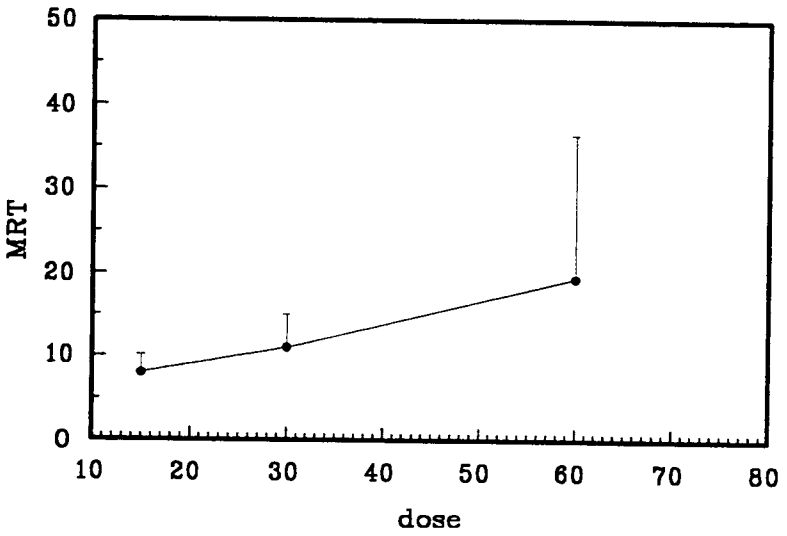

Figure I.19: Plot of mean residence time of $C_{27}H_{22}N_4O_3S$ vs. dose.

Table I.9: Pharmacokinetic Parameters of 15 mg/kg Dose

	AUC* (μg.min/ml)	MRT** (min.)	Half-life(β) (min.)	Half-life(α) (min.)
Rat 1	34.1033	6.1423	3.4631	3.2163
Rat 3	75.2519	7.1160	6.4134	0.8028
Rat 5	81.0426	10.3086	10.5426	1.5717
Average	63.4659	7.9856	6.8064	1.8636
S.D.	25.5931	2.1794	3.5560	1.2329

Note: *, total area under curve (AUC) during infusion and after infusion.

**, mean residence time (MRT) after infusion.

Table I.10: Pharmacokinetic Parameters of 30 mg/kg Dose

	AUC* (μg.min/ml)	MRT** (min.)	Half-life(β) (min.)	Half-life(α) (min.)
Rat 1	117.0119	7.2720	11.1687	0.4504
Rat 5	205.0050	10.9525	29.7063	0.2744
Rat Y	170.8995	15.0800	21.8036	1.9881
Average	164.3054	11.1015	20.8929	0.9043
S.D.	44.3656	3.9062	9.3023	0.9427

Note: *, total area under curve (AUC) during infusion and after infusion.

**, mean residence time (MRT) after infusion.

Table I.11: Pharmacokinetic Parameters of 60 mg/kg Dose

	AUC* (μg.min/ml)	MRT** (min.)	Half-life(β) (min.)	Half-life(α) (min.)
Rat 1	318.9161	31.4606	33.9595	1.5960
Rat 3	251.3250	7.4194	14.3518	0.3853
Average	285.1206	19.4400	24.1556	0.9907
S.D.	47.7941	16.9997	13.8647	0.8561

Note: *, total area under curve (AUC) of during infusion and after infusion.

**, mean residence time (MRT) after infusion.

$C_{27}H_{22}N_4O_3S$ given at three different doses showed dose-dependent pharmacokinetics as depicted in Fig. I.19, showing MRT versus doses. At each dose administered, the pharmacokinetic profiles could be fitted to biexponential equations. The drug was rapidly distributed with half-lives of distribution from one to two minutes. Elimination half-lives and MRT's increased as the dose increased and this may be due to the toxicity of the drug. Upon autopsy the physical appearance of the kidneys of treated rats were clearly damaged, appearing darker and softer in treated than in control (solvent treated only rats) rat kidneys. At the smallest dose of 15 mg/kg $C_{27}H_{22}N_4O_3S$, the rats survived the duration of the three hour experiment. A control study with solvent only injection, the rats also did not show any sign of struggling or toxicity. As doses increased to 30 mg/kg, the rats started to have difficulties in surviving, often dying after one or one and half hours. When 60 mg/kg doses were administered, all rats died within one hour.

$C_{27}H_{22}N_4O_3S$ was also given to mice as the cosolvent system. Upon autopsy no lesions were observed in any of tissues that showed discoloration in rats nor was any discoloration of tissue organs observed in mice. The cosolvent system did cause edema to occur in the lungs of the mice whether $C_{27}H_{22}N_4O_3S$ was present or not, restricting their breathing seriously. This was not observed in rats.

CONCLUSIONS

$C_{27}H_{22}N_4O_3S$ is a potent cytotoxic agent with poor solubility. By determining the solubility parameter of a cosolvent system (PEG 400+ethanol+water) which matched the drugs solubility parameter, δ_2 ~ 11.4, a relatively high solubility system developed. The cosolvent system of PEG 400 + DMA + water with a solubility parameter of 11.4 was devised. $C_{27}H_{22}N_4O_3S$ solubility was double in PEG 400 + DMA + water compared to the PEG 400 + ethanol + water system.

Hemolysis studies showed that cosolvents themselves or cosolvents with $C_{27}H_{22}N_4O_3S$ exerted a great degree of hemolysis of RBC. The extent of hemolysis could be lessened to a moderate degree if the solution is infused slowly into the system in circulation.

The solutions of $C_{27}H_{22}N_4O_3S$ in cosolvent (or the cosolvent itself) need to be slowly infused into a vein or artery, allowing dilution and distribution to occur or hemolysis will be extensive. The compound $C_{27}H_{22}N_4O_3S$ is toxic at 15, 30 and 60 mg/kg in the rats but not so in mice. The compound is rapidly distributed after administration and its biological half-life is fairly short in rats ($t_{1/2}$ - 3.6 to 10 min).

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CHAPTER II
PHARMACOKINETICS OF ANTIPEPTIC AGENTS IN LLAMA

ABSTRACT

Plasma concentration time curves for six llamas after intravenous administration of 1.5 mg/kg of ranitidine, 0.3 mg/kg, 0.4 mg/kg and 0.8 mg/kg of omeprazole and 10 μg/kg misoprostol were studied, respectively. Plasma profiles after intravenous administration of all drugs showed plasma concentrations declining in a biexponential manner. All plasma concentration time curves were well described mathematically by a two compartment open pharmacokinetic model with a rapid distribution phase. Pharmacokinetic parameters after administering ranitidine in six llamas show a mean elimination half-life of 1.57 + 0.29 hours. The mean volume of distribution (Vd) in llamas is 1.91 + 0.38 L/kg, and mean body clearance in llamas is 0.8456 + 0.0955 L/kg/hr. Ranitidine produces only a small transitory (<2 hr) decline in acid production when administered at a dose of 1.5 mg/kg intravenously.

Omeprazole showed dose-dependent pharmacokinetics. Mean half-life of 0.2 mg/kg iv omeprazole is mush shorter than that of 0.8 mg/kg and 0.4 mg/kg iv omeprazole, i.e. 0.13 to 1.38 and to 0.62 hours, respectively. The area under the curve (AUC) and mean residence time (MRT) increases with increasing dose. Clearance decreases as dose increases. Vd goes up from 0.1881 L/kg in 0.2 mg/kg dose to 0.8252 L/kg in 0.4 mg/kg dose, and finally Vd value becomes 0.3601 L/kg in 0.8 mg/kg dose.

The decline in acid production following 0.2 mg/kg intravenous omeprazole was highly variable and did not produce a clinically useful suppression of third compartment acid production. While both 0.4 mg/kg and 0.8 mg/kg omeprazole i.v. administration significantly reduced third compartment acid production. The reduction in acid production following 0.8 mg/kg omeprazole was not significantly greater than the reduction observed following 0.4 mg/kg dosage.

Misoprostol was administered intravenously. Absolute alcohol was chosen as solvent for misoprostol administration in animals. Unfortunately, two animals collapsed, which could be the result from either misoprostol or the alcohol in the solution. Unfortunately, the limitation of ultraviolet detection did not provide the sensitivity needed to quantify the amount of misoprostol in llama plasma, and answer many of the questions of misoprostol's disposition in llamas.

INTRODUCTION

Although there are some differences in the causes of gastric ulcer versus duodenal ulcer disease, intraluminal acid is a prerequisite for the development of gastroduodenal ulceration.[1] The term peptic ulcer disease will be used to describe parameters common to both disorders.[1] Gastric and duodenal ulceration is apparently related in some poorly understood way where there is a breakdown of the barrier that normally prevents irritation, an autodigestion of the mucosa by the gastric secretion.[2] The presence or absence of gastroduodenal ulceration depends on a balance between mucosal damaging (aggressive) factors and mucosal protective factors.[1,3] In the development of duodenal ulcers, aggressive factors such as acid and pepsin secretion are felt to play the primary role, while in the gastric ulcers, a decrease in protective factors such as mucous secretion or mucous synthesis may be more important.

Gastric ulceration in foals has been recognized as an incidental finding at necropsy of equine succumbing to other diseases.[4] It has been recognized for several years that foals subjected to stress and/or non-steroidal anti-inflammatory drugs are susceptible to gastric ulcers.[4-6] For llama, third compartment ulcers have been recognized recently as a significant problem.[7,8] The digestive system of llamas is quite different, in terms of anatomy and physiology, from other mammals, like human, horse, or cattle. The anatomy of the forestomach of camelids differs significantly from that of true ruminant; i.e. only three compartments contrasting with the four-

compartment stomach of the true ruminant.[7] It is questionable and unknown if llama lack significant numbers of H_2-histaminergic receptors.[9]

Traditionally, neutralization of gastric acid with antacids provided the only relief of pain of ulcers in human.[1,10] Studies of the physiological control of acid secretion have demonstrated that anticholinergic agents suppress this process. The development of antagonists acting at H_2-histaminergic receptors provided a more specific class of gastric acid secretion suppressing agents. The more recent advent of substituted benzimidazole inhibitors of the H^+, K^+ -ATPase offers a very effective means of selectively blocking the proton pump that is responsible for acid secretion by parietal cell. These are rather standard approaches in treating peptic ulcer, i.e. to minimize the aggressive factors. The other approach, besides minimization of aggressive factors, where the gastric mucosa protects itself from damage needs further investigation.

The rationale for the use of agents that reduce gastric acidity can be envisioned in terms of the physiological regulation of acid secretion. The regulation of acid secretion by the parietal cell is demonstrated in Figure II.1[1,10-11]. The stimulation of acid secretion in the parietal cell is a complex mechanism that involves activation of three specific receptors (gastrin, histamine type2, and muscarinic) stimulation of cyclic adenosine monophosphate (cAMP) which can also be stimulated by increased intracellular calcium; and activation of the final pathway of H^+ release, the hydrogen-potassium- adenosine triphosphatase (H^+, K^+, ATPase) proton pump.Histamine exerts a role on gastric acid secretion by specifically binding to the H_2 receptors on the

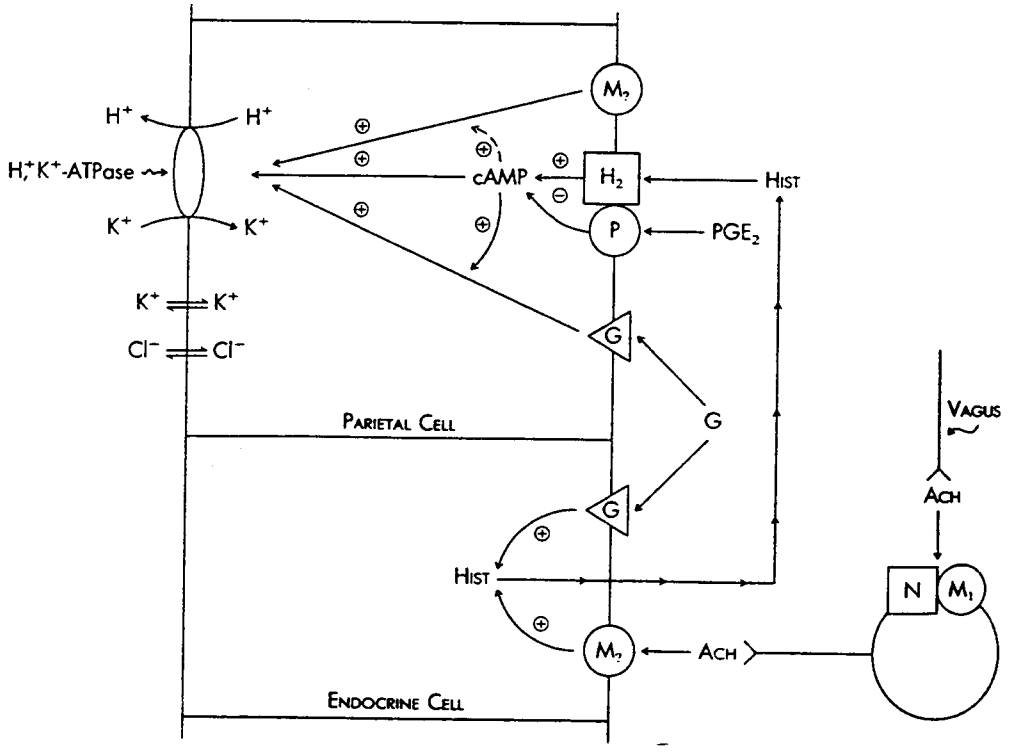

Figure II.1: Regulation of acid secretion by the parietal cell.

parietal cells. Once activated, the H_2 receptor complex activates a stimulatory G subunit (G_s) which in turn activates adenylate cyclase (AC) and a complex array of morphological and biochemical changes ensues. Although the sequence of events is not completely known, mediation by cAMP has been established. An increase in the concentration of cytolic Ca^{2+} is also involved. The most important consequence of these events is the activation of a H^+, K^+ -ATPase and its insertion into the membrane of the apical canaliculus of the parietal cell. This enzyme catalyzes the exchange of

intracellular H^+ for extracellular K^+. The human stomach is capable of producing 20 to 40 mEq of HCl per hour; this capacity accounts for the use of 960 mEq of antacid per day in many therapeutic regimens. To deliver this quantity of antacid frequent dosing of 6 to 10 times a day is necessary, but is quite difficult to do in large animals. The standard therapeutic approach to ulcer disease in human focuses on the reduction of gastric acidity by neutralization (via antacids) or decreased secretion (via H_2-receptor antagonists). In foals, the goals in treating GI ulcers are to eliminate clinical signs, promote ulcer healing, and prevent ulcer recurrence and complications. The limited information of gastric and duodenal function in horses and the lack of proven methods for treating ulcers in foals have necessitated the extrapolation of dosage regimen for drugs that are used in human. Inhibitors of the H^+, K^+ -ATPase, such as omeprazole, can virtually eliminate acid secretion achieving most of the therapeutic goals.

Stimuli for acid secretion enhances the secretion of mucus and bicarbonate, which serve to protect the gastric mucosa from damage. There is also an inhibitory G subunit (G_i) associated with the AC that is activated by some prostaglandins (PGs). Once activated by PGs, the G_i subunit decreases the AC activity and decreases the conversion of ATP to cAMP.

In llama, third compartment ulcers (TCUs) pose serious problems. The veterinary practice of reducing acid production by blocking the H_2 receptor is the routine procedure in controlling gastroduodenal ulcer disease.[7] Due to the variable presentation of TCUs and the difficulty of the accurately diagnosing ulcers, medical

treatment of colicky or 'stressed' llama routinely includes oral, intramuscular, or intravenous administration of the H_2-receptor antagonists, cimetidine and ranitidine. Cimetidine (Tagamet®) and ranitidine (Zantac®) are the two most widely used H_2-receptor antagonists to reduce gastric acid production in human.[14] They are effective in reducing gastric acid production in dog[15], cat[15], and horse.[13,16-17] However, this class of drug is not effective in reducing gastric acid in third compartment in cows due to either the absence or very low numbers of H_2 receptors on parietal cells in the third compartment (abomasum)[7]. Preliminary work in the Oregon State Veterinary Diagnostic Lab at OSU has indicated that cimetidine only has slight (marginal) effect in reducing acid production in llama. Thus, one of the objectives of this study is to examine if this class of drug is effective in lowering acid secretion in llama.

Over the past decade with the recent discovery in humans of two additional mechanisms involving the gastric acid secretion. Increasingly potent antisecretory agents have been developed. In addition new agents that primarily address the improvement of mucosal protective factors have emerged also[1,18-19], The stomach and intestines of human and many other species have a barrier that protects the mucosa from the damaging effects of acid, pepsin, bile salts, digestive enzymes, and mechanical shear force.[20] The main physiologic factors that protect the epithelium are the secretion of mucus[20], the secretion of bicarbonate[21], epithelial cell restitution[20,22], and mucosal blood flow. Stimuli for acid secretion also enhance the secretion of mucus and bicarbonate.[10] Any stimulation or perturbation of the gastrointestinal mucosa results in the release of prostaglandins. The organs of the gastrointestinal

tract, especially the stomach, synthesize high concentrations of prostaglandins.[23-24] The E-type prostaglandins, particularly E_2, are involved in mucosal defense mechanisms[23-25], inhibiting the secretion of acid and stimulating the secretion of mucus and bicarbonate.[22,25-27] The ulcerogenic properties of aspirin-like drugs that inhibit PG synthesis suggest a role for these autocoids in normal gastric function.[10] Orally administered synthetic analogs of PGs (e.g., misoprostol) have shown to be effective in decreasing acid secretion. Another therapeutic approach to reducing acidity in the stomach is to inhibit the H^+, K^+ -ATPase enzyme responsible for gastric acid production. Omeprazole has been widely used to reduce acid production in a range of species.[27] Its effectiveness in reducing gastric acid production in llama has not been proven yet.

The overall objective of this study is to examine if HCl production in the llama third compartment can be reduced by the H2-receptor antagonist, ranitidine, the H^+, K^+ -ATPase inhibitor, omeprazole, and the PGE analog, misoprostol. The results of this study can provide a rational basis for the prophylactic and therapeutic management of third compartment ulcers in llama. Besides application in llama, this information may be applicable to alpaca due to physiologic similarities.

The study is divided into 3 stages. In the first stage, intravenous administrations of the three drugs for evaluation of third compartment acid secretion, with the doses of 1.5 mg/kg ranitidine, 0.4 mg/kg omeprazole, and 10 μg/kg misoprostol. Depending on the result obtained, an investigation of oral administration(s) of ranitidine, omeprazole, and/or misoprostol follows. Based on the

drug pharmacokinetics, optimization of acid suppression in third compartment by varying the dose of the drug for therapeutic efficacy is performed last.

MATERIALS AND METHODS

Animal Preparation

Six sexually mature llamas were used in this study. All animals were medically sound although llamas with slight to moderate behavioral problems, and/or structural problems unlikely to interfere with this study were not excluded. Prior to initiation of the experiments, all animals were held in quarantine for at least 1 week, vaccinated, dewormed, given a complete examination, and any routine health care was provided as indicated. Prior to surgery, a complete blood count and a chemistry panel were run on each animal. The surgeries were performed at OSU Veterinary Teaching Hospital to isolate the third compartments of animals. All animals were monitored for two weeks postoperatively prior to the start of the study. Animals were catheterized in the jugular vein for collection of blood samples, with extension attached to the catheter.

Study Design

The experiment was divided into 3 stages of study. A pair of animals were used at a time to finish all three stages of the experiment before the next pair of animals were initiated. A washout period between each drug administration was at

least 72 hours. Following each intravenous drug administration, the catheter was slowly flushed with at least 10 ml. of heparinized normal saline solution. Replacement of another extension was made and flushed out with heparinized normal saline solution before sample collection. Animals were fasted overnight prior to drug administration. Animals were later fed 8 hours after administration.

Zantac® Injection (Glaxo, Inc., 25 mg/ml) was used to as ranitidine solution. Omeprazole sodium for Injection (Astra, Hässle, Sweden. 40 mg/10 ml) was used for iv administration, while 20 mg Prilosec® (MSD and AB ASTRA) capsule was administered orally as oral omeprazole.

Due to the unavailability of intravenous dosage form for misoprostol, absolute ethanol was chosen as the solvent for misoprostol injection, according to its solubility, to produce a concentration of 100 μg/ml. 11.6 ml and 23.7 ml of the solution was administered in animal #248 and animal #232, respectively, in order to dose the animal at 10 μg/kg. Unfortunately animal #248 collapsed to its knees, due either the misoprostol, or the adverse effect of the alcohol. Absolute ethanol was changed to a cosolvent system of 50% v/v propylene glycol and absolute ethanol as a vehicle for misoprostol injection. This solution matched misoprostol's calculated solubility parameter of 10.84 $(Cal/cc)^{1/2}$. This also reduced the quantity of ethanol being administered i.e. a 50% reduction of ethanol content. Misoprostol injection was prepared by dissolving lyophilized misoprostol (G. D. Searle & Co.) with absolute ethanol, and adding an equal volume of propylene glycol. No cloudiness or precipitation was observed during the preparation. The misoprostol in cosolvent was

then filtered through Disposable Milipore Assembly to sterilize the parenteral solution, stored, and sealed in a sterilized vial until use. The concentration of misoprostol injection prepared ranged from 48.8676 to 60 μg/ml.

Oral dosage form of misoprostol obtained was from 200 μg Cytotec® tablets (G. D. Searle &Co). 20 tablets of Cytotec tablets were weighed and ground into powder. An equivalent amount of powder was weighed containing the 200 μg of misoprostol needed. The weighed powder was wrapped as powdered drug with weighing paper and taped. The oral administration of misoprostol was performed in the same manner as the oral administration of omeprazole described below in study #2. The powdered drug was dispersed in mixture of KY® jelly-water, administered directly to stomach of the animal via the intubation tube. The tube was flushed with warm water to assure the misoprostol was administered to the stomach.

In study #1, each animal received four treatments, intravenous administration of 1.5 mg/kg of ranitidine, 0.4 mg/kg of omeprazole, and 10 μg/kg of misoprostol and normal saline solution as control treatment. The experiment was a cross-over design. All 4 treatments were given randomly to each animal. The effect of the intravenous administration on the third compartment acid secretion was evaluated in order to continue the drug to study #2 in the OSU Veterinary Teaching Hospital.

In study #2, depending on the results obtained from study #1, the effect of oral administration of 60 mg sustain-released omeprazole on acid secretion was evaluated. KY® jelly, roughly 30 ml, was mixed with warm water, and the contents of three capsules were dispersed in the mixture of water and KY® jelly. Each animal's

stomach was intubated. The dispersion of omeprazole was drawn into 50 ml syringe and was pushed through the intubation tube into the stomach. Another 240 -300 ml of warm water was used to rinse the remaining contents from the beaker, in which the omeprazole capsule contents were dispersed in KY® jelly-water mixture, and the solution delivered to flush the tube to ensure of complete delivery of capsule contents to the stomach.

Study #3 was to optimize omeprazole dosage in llama. Intravenous administration of omeprazole at the doses of 0.2 and 0.8 mg/kg, i.e. at the doses of half and at the dose of twice of that in study #1, was investigated.

Sampling Protocol

Venous blood samples were collected through the neck catheter extension and slowly drawn into 10 ml syringe. The first 4 - 5 ml of blood was discarded due to dilution of blood by the heparinized normal saline that remained in the extension. Then 10 ml blood sample was collected and transferred to a 12 ml sterile sodium heparin tube, Monoject®. Blood samples were obtained at 0, 5, 10, 15, 30, and 45 min., 1, 2, 3, 4, 6, 8, and 24 hrs. after intravenous administration of all treatments and oral administration of misoprostol. The protocol for oral administration of omeprazole was slightly modified to be at 10, 20, 30, 45, 60, and 90 min., 2 , 3, 4, 5, 6, 8, 24 hrs. The collected blood samples were centrifuged at 3000 rpm (1509xg) at 4° C for 30 min. Plasma was then separated and frozen until assayed.

Assay

Ranitidine Assay:

Ranitidine was assayed by modifying the method of Mihaly et al.[29] 120 μl of internal standard (10.8 μg/ml of beta-hydroxy propyl theophylline - SigmaChemical Co., St. Louis, MO - in water) and 50 μl of 5 M NaOH (SigmaChemical Co., St. Louis, MO) solution were added to plasma of 1 - 4 ml, depending on the expected amount of ranitidine in sample. The ranitidine plasma extraction was done twice as follows. Five ml of methylene chloride (J. T. Baker Chemical Co., Phillipsburg, NJ) was added to each thoroughly mixed plasma sample. Parafilm was used to cover the sample and later holes were poked to prevent pressure build-up during vortexing and centrifuge. The sample was vortexed gently and carefully for 45 seconds in order not to form an emulsion intentionally. The sample was centrifuged at 3000 rpm (1509xg), refrigerated at 4° C, and the methylene chloride layer (bottom layer) was withdrawn and placed in another tube. The collected methylene chloride from the two extractions was evaporated to dryness in a vacuum chamber (Lab-Line Instruments, Inc., Melrose Park, IL) without heat. Finally the sample was reconstituted with 250 μl of 15% methanol/water before injecting 100 μl into HPLC system.

Standard curves of ranitidine were prepared in the same way as unknown samples, but using standard solution of 2 μg/ml ranitidine to add to plasma to obtain 25, 50, 100, 200, 400, 600, 800, 1000, and 1200 ng/ml of ranitidine.

HPLC analyses were performed on a Water Associates Chromatography pump, a model 440 absorbance detector, WISP 710B, a reversed phase Nova Pak C_{18} column

and a precolumn (of C_{18} packing material). A 75% v/v acetonitrile (J. T. Baker Chemical Co., Phillipsburg, NJ) in dibasic ammonium phosphate (SigmaChemical Co., St. Louis, MO) (pH 8, 7 mM) was used as mobile phase at the flow rate of 0.4 ml/min. Absorbance was monitored at 313 nm. Peak height ratios were used for drug quantitation.

Omeprazole Assay:

The analytical method by Lagerstrom and Persson[30] was modified to analyze omeprazole samples. 50 μl of internal standard solution (164 μg/ml butylparaben - SigmaChemical Co., St. Louis, MO - in ethanol) and 100 μl of 1 M NaH_2PO_4 (SigmaChemical Co., St. Louis, MO) buffer (pH 6.5) were added and mixed to each plasma sample of 1 - 4 ml, depending on the amount of omeprazole expected in sample. The sample was then extracted with 5 ml of methylene chloride. Parafilm was used to cover sample and holes poked to release any pressure build-up. The sample was vortexed carefully and gently in order not to form an emulsion intentionally. After centrifugation at 3000 rpm (1509xg) for 30 min., the methylene chloride layer was collected and transferred to another tube. Another 5 ml of fresh methylene chloride was added to extracted sample for a second extraction. The methylene chloride was collected, combined with methylene chloride from the first extraction, and evaporated to dryness in a vacuum chamber. 400 μl of 20% v/v acetonitrile in phosphate buffer (pH 7.5) was added to reconstitute the dried sample. 100 μl of the solution was injected into the HPLC system.

Standard curves of omeprazole were prepared in the same manner as unknown samples by mixing known amount of omeprazole from 9.5 μg/ml omeprazole aqueous solution to obtain 46.9484, 93.8967, 187.7934, 357.5868, 467.4836, 938.9671, and 1408.4507 ng/ml of omeprazole.

HPLC analyses were carried out on a Water Associates Chromatography pump, a model 440 absorbance detector, WISP 710B, a reverse phase Nova Pak C_{18} column, and precolumn (with C_{18} packing material). A 0.6 ml/min flow rate of 30% v/v acetonitrile in phosphate buffer pH 7.5 (0.05 M) was used in the HPLC analyses. Absorbance was monitored at 313 nm. A linear relationship of peak height ratios (drug peak height/internal standard peak height) versus omeprazole plasma concentration of standard solution was used as calibration curve for determination of drug concentrations in unknown samples.

Misoprostol Analysis:

50 μl of 164 μg/ml butylparaben solution (in ethanol) and 2 ml of acetonitrile was added to 2 ml of plasma sample. The sample was centrifuged at 1509xg, at a refrigerated temperature of 4° C. The top clear supernatant solution was drawn and placed into another glass test tube, covered with piece of Kimwipe paper and rubber band. The solution was dipped into a dry ice-isopropanol solution (J. T. Baker Chemical Co., Phillipsburg, NJ) until it was frozen. The frozen solution was lyophilized in vacuum chamber (less than 0.1 mmHg) at -50°C overnight (at least 24 hours). The lyophilized sample was then extracted with 300 μl of acetonitrile three

times. After each extraction, the lyophilized sample was added to acetonitrile, centrifuged at 11,179 x g for 10 min, and the supernatant solution was drawn and dried under a vacuum chamber without heat. The sample was finally reconstituted with 300 μl of acetonitrile before 150 μl was injected into HPLC system.

Standard curves in the range from 500 ng/ml to 5000 ng/ml were prepared in the same fashion by adding known amount of misoprostol from its stock solution of 200 μg/ml (in ethanol). Then it was incubated at 37° C for 15 min. to simulate the hydrolysis of misoprostol into misoprostol acid in plasma.

The HPLC analysis of misoprostol in plasma samples was modified from the method of Terragno et al.[31] HPLC analyses were performed on a 1090M Hewlett-Packard Chromatography system with 5 μ (100 x 4.6 mm) C_{18} ODS Hypersil column along with precolumn. A mobile phase of 32.8% v/v acetonitrile in 0.017 M H_3PO_4 (J.T. Baker Chemical Co., Phillipsburg, NJ) was run at 0.6 ml/min. 150 μl injection was made and the absorbance was monitored at 192 nm. for the drug and 280 nm for internal standard.

Pharmacokinetic Analysis

Plasma concentration versus time data were analyzed by RSTRIP program to fit data. The parameters were then used to evaluate the total area under the curve by converting the postinfusion-fit parameters to i.v. bolus-fit parameters, and treating the data the same way as in the case of an i.v. bolus.[32]

RESULTS AND DISCUSSION

Plasma concentration time curves for 6 llamas each after intravenous administration of 1.5 mg/kg of ranitidine, and 0.4 mg/kg of omeprazole are shown in Figures II.2, II.3, respectively. Plasma profiles after intravenous administration of 1.5 mg/kg ranitidine show plasma concentration declining in a biexponential manner. All plasma concentration time curves were well described mathematically by a two compartment open pharmacokinetic model with a rapid distribution phase. Pharmacokinetic parameters for 1.5 mg/kg ranitidine are shown in Table II.1. All calculated pharmacokinetic parameters have been corrected for the infusion time.

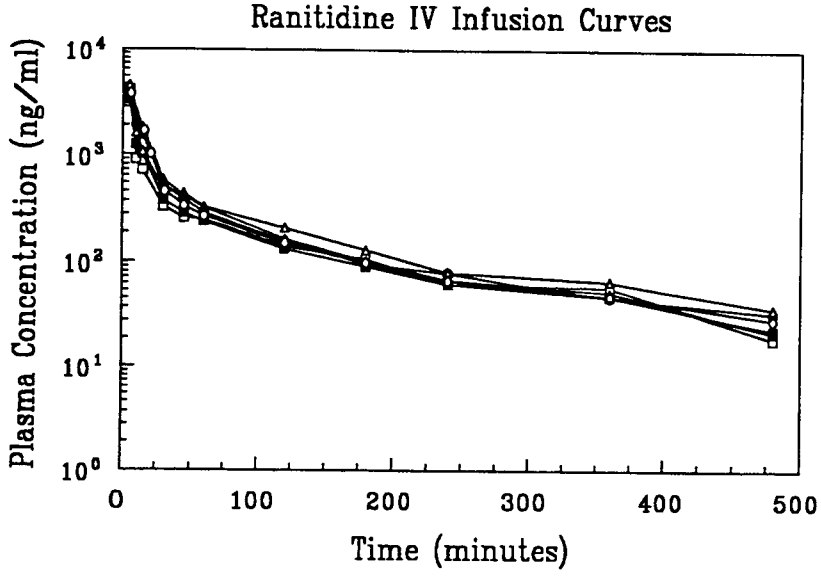

Figure II.2: Plasma concentration for ranitidine after i.v. administration (1.5 mg/kg over 1-2 min.) in 6 llamas.

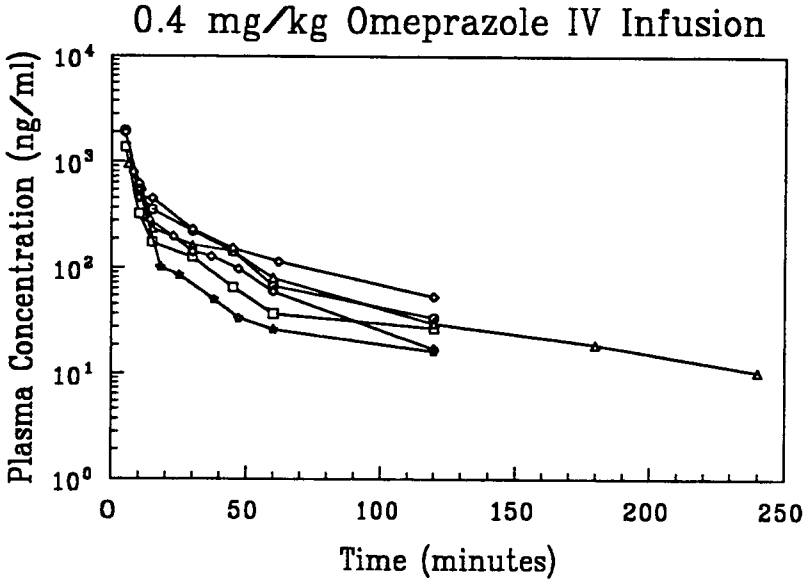

Figure II.3: Plasma concentration for omeprazole after i.v. administration (0.4 mg/kg over 1-2 minutes) in 6 llamas.

Ranitidine is a derivative belonging to dimethylaminofuran group like the other 4 main H_2-receptor antagonists.[33] Pharmacokinetic parameters after administering ranitidine in 6 llamas (Table II.1, Figure II.2) show a mean elimination half-life of 1.57 hours, as compared to the half-life of 1.73-2.42 hours in human[34-36], and 2.23 hour half-life of cimetidine in horse.[37] The mean volume of distribution (Vd) in llamas is 1.91 + 0.38 L/kg, while the Vd in healthy human is 1.2-1.64 L/kg.[34-36] Mean body clearance in llamas is 0.8456 L/kg/hr, compared to 0.5546-0.6077 L/kg/hr in human.[34-36]

Table II.1: Pharmacokinetic Parameters for Ranitidine after Intravenous Administration (1.5 mg/kg over 1 - 2 min.) in Llamas

Animal	Half-Life	AUC	MRT	Vd	Clearance
	hr	ng.hr/ml	hr	L/kg	L/kg/hr
232	1.28	1693.2452	1.11	1.6314	0.8859
248	1.28	1851.7031	0.88	1.4918	0.8101
222	1.59	1475.5420	1.41	2.3370	1.0166
272	1.62	2024.9959	1.48	1.7267	0.7407
225	1.61	1844.2746	1.35	1.8932	0.8133
226	2.06	1858.0513	1.52	2.3984	0.8073
Mean	1.57	1791.3020	1.29	1.9131	0.8456
SD	0.29	186.9985	0.25	0.3761	0.0955
%CV	18.33	10.44	19.33	19.66	11.30

In llamas ranitidine produces only a small transitory (<2 hr) decline in acid production when administered at a dose of 1.5 mg/kg intravenously. Thus, ranitidine was not included study #2 and #3.

In man, ranitidine is well absorbed, mostly eliminated unchanged in the urine, 60-85% of the dose being recovered in urine. Approximately 20-30 % of the dose is metabolized via N-oxidation, S-oxidation, and N-demethylation.[38-39] The plasma protein binding of ranitidine in human is low, ranging from 15-30 %. These low values have no major clinical or pharmacokinetic significance, so that changes in the unbound concentration of ranitidine due to altered protein binding are unimportant.[38] It has been demonstrated in literature that the H_2-receptor antagonists can be classified as a family by the similarity in their absorption and elimination.

To compare the pharmacology of H_2-receptor antagonists, the average antagonist dissociation constant (k_B) values derived from *in vitro* models and the negative logarithm of molar concentration of antagonist in the presence of which the potency of the agonist being reduced 2-fold are used. The k_B values for each antagonist obtained by the same tissue preparation of the same species agree reasonably well among different laboratories[38] , with the smaller the k_B value, the more potency. However, the values estimated from different species or different tissue preparations can be markedly different: values for ranitidine obtained with mouse stomach tissue assay are higher than that obtained with the guinea-pig right atrium assay. The average k_B value for ranitidine is 0.125×10^{-6} mol/L in the guinea-pig gastric mucosa system, 0.933×10^{-6} mol/L in mouse stomach tissue, and 0.447×10^{-6}

mol/L in human atrial pectinate muscle.[38] The average k_B value for cimetidine is $1.148x10^{-6}$ mol/L in human atrial pectinate muscle.[38] This implies that plasma concentration required to elicit a pharmacological response is lower for ranitidine than for cimetidine based on human atrial pectinate muscle assay. Thus, the therapeutic daily dose for ranitidine should be 3-fold less than for cimetidine.

Omeprazole is a substituted benzimidazole. Its absorption characteristics for both formulation and dose-dependent has shown biexponential decline after intravenous administration in human.[40]

In the 6 llamas, plasma omeprazole concentration time curves after 0.4 mg/kg intravenous administration declined biexponentially, Figure II.3. All plasma concentration time curves were well described mathematically by a two compartment open pharmacokinetic model with a rapid distribution phase. Pharmacokinetic parameters for 0.4 mg/kg omeprazole are shown in Table II.2. All calculated pharmacokinetic parameters have been corrected for the infusion time.

Pharmacokinetic parameters after administering 0.4 mg/kg omeprazole in 6 llamas (Table II.2, Figure II.3) show a mean elimination half-life of 0.62 hours, as compared to the half-life of 0.5-1.5 hours in human.[40] The mean Vd in llamas is 0.8252 L/kg, while the apparent Vd's in healthy human are 0.34 and 0.37 L/kg following intravenous 10 and 40 mg omeprazole.[40] Mean body clearance in llamas is 0.8456 L/kg/hr.

Omeprazole is rapidly distributed. The mean volume of distribution initially was 0.83 L/kg, while the apparent Vd at pseudo-equilibrium was 0.31 L/kg. This

Table II.2: Pharmacokinetic Parameters for Omeprazole after Intravenous Administration (0.4 mg/kg over 1 - 2 min.) in Llamas

Animal	Half-Life	AUC	MRT	Vd	Clearance
	hr	ng.hr/ml	hr	L/kg	L/kg/hr
232	0.45	446.1697	0.24	0.5779	0.8952
248	0.51	437.3445	0.27	0.6692	0.9146
222	0.83	429.8173	0.74	1.1107	0.9306
272	0.64	503.0046	0.66	0.7952	0.7952
225	0.47	495.3440	0.30	0.5504	0.8199
226	0.80	372.2679	0.32	1.2475	1.0745
Mean	0.62	447.3246	0.42	0.8252	0.9050
SD	0.17	47.8478	0.22	0.2904	0.0989
%CV	27.58	10.70	51.11	35.20	10.92

indicates that omeprazole undergoes limited tissue distribution with localization of a major fraction of the drug in extracellular water.[40] Autoradiographic studies in the mouse have shown that [^{14}C] omeprazole is widely distributed within 5 minutes of intravenous administration. Sixteen hours later omeprazole is virtually confined to parietal cells within gastric mucosa.[41] In rats, high levels of radioactivity were found in the liver, kidneys, duodenum, stomach, and thyroid gland, while in mice radioactivity rapidly reached high levels in the choroid plexus, liver, kidneys, gallbladder, stomach, bladder, and hair follicles. In both species, rat and mouse, only small quantities of omeprazole were observed in brain tissue which suggests that the drug and/or its metabolites can only penetrate the blood-brain barrier to a limited extent. However, radioactivity was shown in the fetuses of pregnant mice, indicating that the placental barrier was permeable to omeprazole or its metabolites.[41-42]

Omeprazole has been reported to be rapidly eliminated and almost completely metabolized in many species, including in human.[40,42-44] No unchanged drug, omeprazole, could be detected in urine samples from dog, rat , and mouse.[43] In each species at least 10 metabolites were detected in urine (pH 9) by gradient reverse phase HPLC[43].

At concentrations covering the normal therapeutic range (0.19 to 19.4 μmol/L) in human, omeprazole protein binding was calculated to be between 95-96%, mainly to albumin and α_1-acid glycoprotein.[42] The penetration into red blood cell is low as estimated by the ratio between the concentration of drug in whole blood and plasma, which is about 0.6.[42]

The effectiveness of omeprazole in inhibiting gastric acid secretion has been investigated in conscious dogs with gastric fistulae or cannulated Heidenhain pouches[45-48], in *ex vivo* canine gastric chamber[49], and in conscious guinea-pigs with cannulae surgically implanted into the antral portion of the stomach.[50] In all of these studies omeprazole, whether administered orally, intravenously, intraduodenally, or subcutaneously, inhibited basal and stimulated gastric secretion dose-dependently. It was found to be 2 - 10 times more potent than H_2-antagonist, cimetidine, depending on the route of administration and the experimental model used. The potency of omeprazole, an acid labile drug, following oral administration is generally less than its potency when given intravenously or intraduodenally. This is thought to be due to its instability at low pH, resulting in reduced systemic availability. However, its antisecretory activity does not correlate with plasma peak concentrations in animal and man.[42,51] In animal studies, omeprazole has shown to markedly inhibit acid secretion long after plasma levels have decreased below detection limits.[52] In man, omeprazole produces prolonged but reversible reduction of gastric acidity. The onset of action in man is within 1 hour of oral administration but is maximal at about 6 hour.[53] In most studies in animals and man which assessed plasma concentrations of omeprazole in relation to acid secretion inhibition have shown a correlation between antisecretory activity and area under the curve (AUC).

The 0.4 mg/kg intravenous administration of omeprazole suppresses llama third compartment acid production for more than 6 hours, i.e. reducing titrateable acidity by 50%. It did not reduce titrateable acidity by more than 80%, one of the criteria for

efficacy. This may lead to the suggestion that omeprazole was only moderately effective in reducing acid production. It should be noted that the surgical preparation did not permit collection of the entire contents of the third compartment. Following the omeprazole administration, the volume of the third compartment collected decreased substantially and the viscosity of the samples increased. Thus, it is evident that omeprazole decreases both the volume and titrateable acidity of the gastric secretion in llamas, which occurs in other species. Consequently, the absolute decrease in total acid production in the third compartment following omeprazole administration is probably more than 80% for quite a period of time. Omeprazole was continued in study #2 and #3.

In the study #2, when omeprazole was given orally, the plasma omeprazole concentration time curves in the 6 llamas are shown in Figure II.4. All plasma

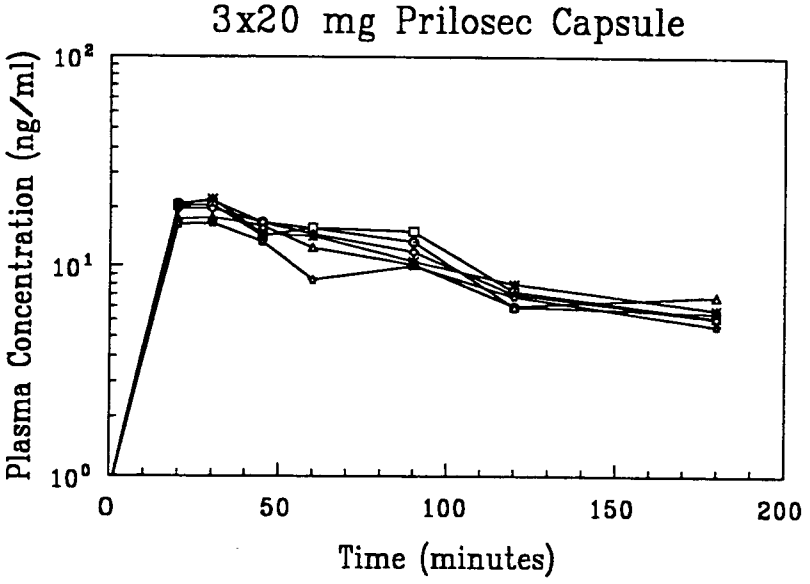

Figure II.4: Plasma concentration vs. time curves for omeprazole after oral administration (3x20 mg Prilosec Capsules) in 6 llamas.

concentration time curves were described mathematically by one compartment open pharmacokinetic model. Pharmacokinetic parameters for oral three 20 mg Prilosec® capsules (omeprazole) are shown in Table II.3. The mean apparent elimination half-life for oral administration is 1.38 hours, as compared to 0.62 hours for 0.4 mg/kg iv. This is due to the formulation of Prilosec®, a prolonged action dosage form. Omeprazole could be detected in plasma within 20 minutes of administration with the average time to peak concentration of 0.3074 hours.

Oral administration at the dosage of approximately 0.4 mg/kg produced a minimal decline in third compartment acid production. Titrateable acidity did not decline to less than 50% of basal acid secretion following oral administration of 3 x 20 mg Prilosec® capsules. Experimentation with higher oral dosage could be examined in further studies. A decline in the volume of third compartment secretions was noted starting approximately 2 hours after omeprazole administration. No adverse effects were observed. Omeprazole was selected to continue to optimization study according to the efficacy and lack of significant side effects.

Table II.3: Pharmacokinetic Parameters from Omeprazole Oral Administration in Llamas (3x20 mg Prilosec Capsules)

Animal	Half-Life (elim.) hr	Half-Life (abs.) hr	AUC ng.hr/ml	MRT hr	Cmax ng/ml	tmax hr	F
232	1.22	0.0809	43.1483	1.87	20.201	0.3393	0.0733
248	1.46	0.0510	47.4154	2.18	19.979	0.2524	0.0730
222	1.53	0.0609	42.9967	2.29	17.085	0.2947	0.0620
272	1.33	0.0791	43.4567	2.03	19.002	0.3422	0.0777
225	1.47	0.0590	38.5957	2.20	15.917	0.2852	0.0576
226	1.29	0.0764	43.5692	1.97	19.634	0.3310	0.0823
Mean	1.38	0.0679	43.1970	2.09	18.636	0.3074	0.0710
SD	0.12	0.0125	2.8005	0.16	1.742	0.0360	0.0094
%CV	8.71	18.39	6.48	7.64	9.35	11.70	13.23

In study #3, optimization of the dose for omeprazole, the omeprazole plasma concentration time curves after intravenous administration of 0.2 and 0.8 mg/kg omeprazole are shown in Figure II.5 and II.6, respectively. The 0.8 mg/kg i.v.

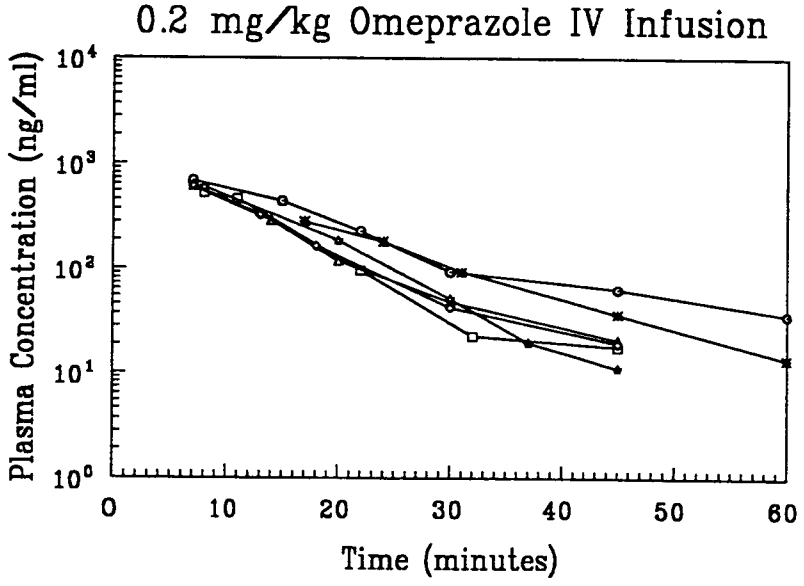

Figure II.5: Plasma concentration for omeprazole after i.v. administration (0.2 mg/kg over 1-1.75 min.) in 6 llamas.

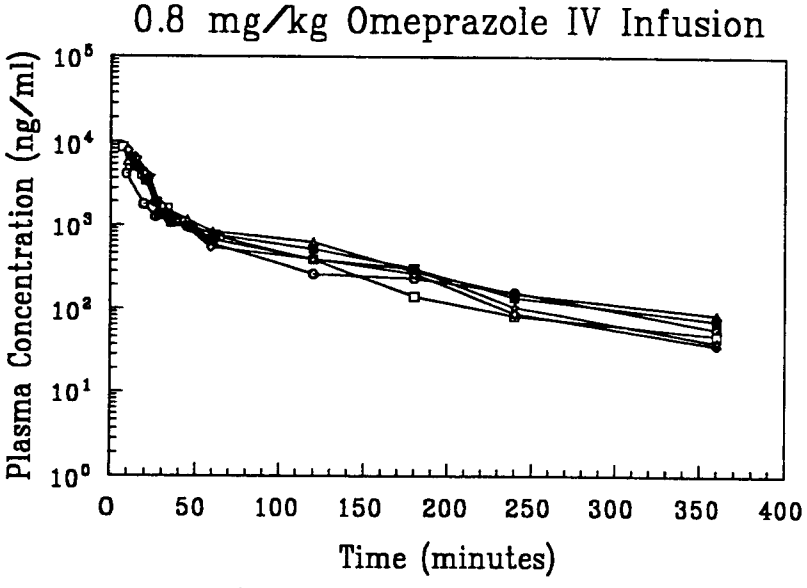

Figure II.6: Plasma concentration for omeprazole after i.v. administration (0.8 mg/kg over 3-4 min.) in 6 llamas.

omeprazole plasma concentration time curves were described mathematically by two compartment open pharmacokinetic model, while plasma concentration time curves for 0.2 mg/kg intravenous omeprazole were mathematically described by one compartment open pharmacokinetic model. Omeprazole is rapidly distributed in the systemic circulation. The distribution phase was completed by the time the early sample collections were taken in each animal resulting in a one compartment open model analysis, as only the elimination phase being evident due to the small dose of 0.2 mg/kg and the slow IV infusion of omeprazole. Pharmacokinetic parameters of the two doses are summarized in Table II.4 and II.5, respectively. As mentioned previously, omeprazole has shown dose-dependent pharmacokinetics in man, even comparing different doses of the same oral dosage form.[40] Mean half-life of 0.2 mg/kg iv omeprazole is much shorter than that of 0.8 mg/kg and 0.4 mg/kg i.v. omeprazole, i.e. 0.13 to 1.38 and to 0.62 hours, respectively. The area under the curve (AUC) and mean residence time (MRT) increases with increasing dose. Clearance decreases as dose increases. Vd goes up from 0.1881 L/kg in 0.2 mg/kg dose to 0.8252 L/kg in 0.4 mg/kg dose, and finally Vd value becomes 0.3602 L/kg in 0.8 mg/kg dose. The disagreement in Vd in different doses administered cannot be explained, further study is needed.

The decline in acid production following 0.2 mg/kg intravenous omeprazole was highly variable and did not produce a clinically useful suppression of third compartment acid production. Both 0.4 mg/kg and 0.8 mg/kg omeprazole i.v. administration significantly reduced third compartment acid production. The

Table II.4: Pharmacokinetic Parameters for Omeprazole after Intravenous Administration (0.2 mg/kg over 1 - 1.75 min.) in Llamas

Animal	Half-Life	AUC	MRT	Vd	Clearance
	hr	ng.hr/ml	hr	L/kg	L/kg/hr
232	0.17	255.4046	0.25	0.1937	0.7831
248	0.12	171.1534	0.17	0.2042	1.1685
222	0.10	179.7671	0.15	0.1662	1.1126
272	0.11	183.7773	0.15	0.1663	1.0883
225	0.12	188.7538	0.17	0.1845	1.0596
226	0.16	214.7664	0.23	0.2139	0.9312
Mean	0.13	198.9371	0.19	0.1881	1.0239
SD	0.03	31.3435	0.04	0.0196	0.1420
%CV	21.74	15.76	21.74	10.42	13.87

Table II.5: Pharmacokinetic Parameters for Omeprazole after Intravenous Administration (0.8 mg/kg over 3 - 4 min.) in Llamas

Animal	Half-Life	AUC	MRT	Vd	Clearance
	hr	ng.hr/ml	hr	L/kg	L/kg/hr
232	1.18	3007.7840	1.19	0.4520	0.2660
248	0.97	3881.1907	0.81	0.2899	0.2061
222	1.47	4584.7112	1.28	0.3696	0.1745
272	1.70	4566.4602	1.02	0.4296	0.1752
225	1.53	5664.6478	0.88	0.3121	0.1412
226	1.43	5354.2207	1.02	0.3083	0.1494
Mean	1.38	4509.8358	1.03	0.3602	0.1854
SD	0.26	970.2313	0.18	0.0682	0.0456
%CV	18.91	21.51	17.24	18.94	24.59

reduction in acid production following 0.8 mg/kg omeprazole was not significantly greater than the reduction observed following 0.4 mg/kg dosage.

Misoprostol is a synthetic analogue of natural prostaglandin E_1. It consists of 4 isomers and is a water-insoluble; a viscous, oily fluid.[54] While misoprostol is highly unstable in the pure oil state, its stability is significantly enhanced in a hydroxypropyl methylcellulose (HPMC) (1:100) dispersion.[55] It is extensively absorbed and undergoes de-esterification to misoprostol acid, its biologically active metabolite.[56-58] Unfortunately, the limitation of ultraviolet detection did not provide the sensitivity needed to quantify the amount of misoprostol in llama plasma. The standard curve for misoprostol plotting peak area ratios between misoprostol and butylparaben versus misoprostol concentration (from 500-5000 ng/ml) showed high correlation (r=98%).

In study #1, misoprostol was administered intravenously. Absolute alcohol was chosen as solvent for misoprostol administration in animals #232 and #248. Unfortunately, animal #248 collapsed, which could be the result from either misoprostol or the alcohol in the solution. An alternative solution to deliver misoprostol IV was made by formulating a cosolvent of 50% propylene glycol and absolute alcohol to match misoprostol's solubility parameter. The amount of alcohol administered was reduced by half. This cosolvent was filtered through Disposable Millipore Assembly unit. The less alcohol content made it easier to filter. The cosolvent mixture with misoprostol was administered intravenously to animals #225 and #226.

Intravenous administration of misoprostol produced signs of acute toxicity in these animals and was discontinued following administration to 4 animals. Third compartment acid production was effective suppressed for more than 6 hours. Misoprostol also reduced titrateable acidity by more than 50% for variable periods of time. It did not reduce titrateable acidity by more than 80%, as outlined as one of the criteria for efficacy. Following misoprostol administration, like omeprazole, the volume of third compartment content collected declined and the viscosity of the samples increased. Hence, the absolute decrease in total acid production in third compartment following misoprostol administration is probably more than 80% for variable periods of time as well. Misoprostol was continued into study #2.

In study #2, misoprostol was given orally. Cytotec® tablets (misoprostol) were ground into powder. The powder was weighed to give the equivalent amount of misoprostol needed. Immediately before administration, the weighed powder, being wrapped individually, was dispersed in KY® jelly and water, after oral administration of misoprostol the animals did not show any signs of adverse effects. Oral administration of misoprostol (10 μg/kg) did not significantly change third compartment acid production. Titrateable acidity did not decline to less than 50% of basal acid secretion following misoprostol oral administration. Comparing the efficacy following oral misoprostol to oral omeprazole, misoprostol reduced titrateable acidity less than omeprazole. Misoprostol was discontinued for study #3, optimization of dosage, due to its low efficacy and toxicity.

Studies in animals and humans demonstrated a biphasic elimination of misoprostol acid[59], with reported half-life in human about 1.5 hours.[56-57] After rapid de-esterification to the acid form, the active metabolite undergoes further metabolic conversion to its dinor and tetranor acid metabolite compounds.[57] The dinor metabolite possesses weak antisecretory activity, whereas the tetranor metabolite has no substantial antisecretory activity.[60] Additional metabolites of the tetranor metabolite include a PGF_1 analog and its omega-16-carboxylic acid derivative.[57] The serum protein binding of misoprostol acid is similar in young and elderly subjects (81-89%) and is independent of the concentration of misoprostol.[57] Salicylic acid at concentration of 300 μg/ml decreased the protein binding of misoprostol acid from 84% to 52%.[61] This decrease is not considered clinically important because the binding of misoprostol acid is not extensive and its elimination half-life is short.[62] Renal excretion of radiolabelled misoprostol over a seven-day period accounted for $73.2 \pm 11.3\%$ of the administered dose.[59] Most of this excretion occurred in the first 24 hours.[56-57,59,63] Total renal excretion of unchanged misoprostol and its acid metabolite amounted to less than 1% of the dose.[59]

CONCLUSION

Ranitidine pharmacokinetics in llamas when administered 0.4 mg/kg intravenously can be mathematically described by a two compartment open model, with average elimination half-life of 1.6 hours. Plasma profiles of omeprazole in llama when administered 0.4 mg/kg intravenously decline biexponentially, with very short half-life of about half an hour, 0.62 hours. Unfortunately, misoprostol pharmacokinetics could not be demonstrated due to the limited sensitivity by UV detection. Ranitidine was shown to slightly reduce acid production in llama third compartment suggesting the presence of H_2- histaminergic receptors, suggesting ranitidine could be effective in reducing acid production in the llama third compartment. 3 x 20 mg omeprazole capsules (approximately 0.4 mg/kg) and 10 μg/kg misoprostol were given orally in the study #2. Neither showed the decline of titrateable acidity to less than 50% of basal acid secretion. Misoprostol was less effective orally and signs of acute toxicity occurred when administered intravenously. Experimentation with higher oral doses of omeprazole could be clinically relevant. Varying the doses of i.v. omeprazole administration resulted in different pharmacokinetic parameters. As the dose increases, AUC and MRT increase, whereas clearance decreases as dose increases. The decline in acid production following 0.2 mg/kg i.v. omeprazole was highly variable and did not produce a clinically useful suppression of acid production. While 0.8 mg/kg i.v. omeprazole did

not significantly suppress acid production more effectively than 0.4 mg/kg i.v. omeprazole.

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