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CHLORAMPHENICOL PHARMACOKINETICS AND METABOLISM
IN DOGS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Kamel F. Khazal, B.V.M.&S., M.S.

The Ohio State University

1986

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To My Parents, Sisters and Brothers
ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Thomas E. Powers for his guidance and contribution as major adviser to the present investigation, as well as his helpful advice and encouragement throughout my education career at The Ohio State University; to Dr. Jean D. Powers for her advice and encouragement throughout the study; to Dr. Thomas E. Chapman for his support, encouragement and advice to make this manuscript possible. Special thanks and appreciation to Dr. Dale E. Sharp for his interest and invaluable advice and encouragement to make this work possible. I wish to express my thanks to the Department of Pharmacology, College of Medicine, The Ohio State University, especially Dr. Nicholas Gerber and his staff for allowing me to use their equipment and materials to perform this study. I thank all of the members, faculty and staff, of the Department of Veterinary Physiology and Pharmacology for their support and encouragement throughout my stay at The Ohio State University. I wish to express my sincere thanks to the State of Ohio Canine Research Fund Program for the financial support of this investigation. Lastly, I wish to thank the staff of the Campus Chemical Instrument Center; Dr. Charles Cottrell for performing the nuclear magnetic resonance (NMR), Mr. C. Richard Weisenberger for performing the Fab mass spectroscopy, and Mr. David Chang for performing the El and CI mass spectroscopy.
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Comparison of Pharmacokinetic Parameters of Chloramphenicol after Analysis by Gas Chromatography, Colorimetric and Bioassay Methods.

FIELDS OF STUDY

Major Field: Clinical Pharmacology, Pharmacokinetics, and Metabolism
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CHAPTER 1
INTRODUCTION

Chloramphenicol is a broad-spectrum antibiotic, first isolated from *Streptomyces venezuelae* in 1947 by Burkholder. Chloramphenicol has unique physical and chemical properties which are important in its tissue disposition, tissue penetrability, spectrum against bacteria and toxicity.

Since its discovery, chloramphenicol has been used for treatment of bacterial infections in humans and animals. It has been used for treatment of *Salmonella*, *Haemophilus influenza* and *Bacillus fragilis* infections in humans. Chloramphenicol is used successfully in veterinary medicine for treatment of *Streptococcus pneumoniae*, group A beta hemolytic Streptococci, Enterococci, *Neisseria* species, *Haemophilus* species, *Corynebacterium*, *Pasteurella multocida*, *Brucella*, *Bordetella*, *Listeria*, *Bacillus fragilis*, mycoplasma, rickettsia and chlamydia infections in dogs, cats and horses.

Because chloramphenicol causes aplastic anemia in man, the United States Food and Drug Administration (FDA) prohibited its use in food-producing animals. Despite the prohibition by the FDA, chloramphenicol is used for treatment of sensitive bacterial infection in selected food-producing animals which are used for breeding purposes only.

Although chloramphenicol may be used as a first choice antibiotic for treatment of certain infection in dogs, limited data are available in the literature about its metabolism and pharmacokinetics in dogs. Basic data on the metabolism of chloramphenicol in dogs
was reported by Glazko, et al., in 1950. However, the chemical techniques he used in the isolation and identification of these metabolites are presently outdated. The metabolites were grouped as aromatic amines, nitro compounds and chloramphenicol conjugates. Moreover, the pharmacokinetic parameters of chloramphenicol in dogs were estimated from serum samples analyzed by a colorimetric method that measures intact chloramphenicol (CAP) and its metabolites. The use of this method resulted in overestimation of serum CAP levels, long elimination half-lives and low body clearance. These parameters resulted in underestimation of dosage regimens of chloramphenicol in the dog, dosage regimens used in the dog were at longer intervals which resulted in the plasma active chloramphenicol level falling below the MIC of the organism being treated. However, these pulse regimens were often effective clinically, which may be partly related to the marked post antibiotic effect (PAE) that chloramphenicol often exhibits. The colorimetric method was compared to specific gas chromatographic and microbiological methods which measure intact chloramphenicol in a study of CAP kinetics in dogs. The elimination half-life of chloramphenicol determined by the colorimetric method was about twice as long as the elimination half-lives which were estimated by gas chromatographic and microbiological methods. There were no differences in the volume of distribution; whereas, the clearance as estimated by the colorimetric method was one-half the clearance measured by gas chromatographic and microbiological methods.

Because published pharmacokinetic parameters of CAP in dogs were estimated from concentrations in serum samples analyzed by colorimetric methods, measuring both intact CAP and its metabolites, our knowledge of CAP pharmacokinetics, metabolism, and dosage regimens in the dog is equivocal. Additional work using methods that accurately measure intact CAP, and separately its metabolites, is essential
to establish a correct dosage regimen and complete our understanding of CAP metabolism in dogs. To these ends the objectives of the present study were:

1. To reevaluate the pharmacokinetic parameters of chloramphenicol after using specific HPLC and radiochemical methods to measure chloramphenicol in the serum of dogs.

2. To estimate the amount of intact chloramphenicol in serum of dogs after intravenous administration of $^{14}$C-chloramphenicol.

3. To quantitate chloramphenicol and its metabolites in dogs' urine by HPLC.

4. To identify the metabolites of chloramphenicol in the urine of dogs using HPLC, mass spectroscopy and nuclear magnetic resonance (NMR) techniques.
Chloramphenicol was isolated from *Streptomyces venezuelae*, originating from soil in Venezuela, by Burkholder in 1947. Its chemical structure was described by Ehrich in 1947 and it was chemically synthesized in 1949 by Rebstock.

Chloramphenicol is D(-)-threo-2-dichloroacetamido-1-p-nitrophenyl-1,3-propanediol. Its empirical formula is $C_{11}H_{12}Cl_2N_2O_5$. The molecular weight is 323.14. Four stereo-isomers occur, but only the D(-)-threo isomer is antibacterially active. It forms fine, white to grayish-white or yellowish-white, needle-like crystals or elongated plates with a melting point of 150.5-151.5°C. The optical rotation, according to the FDA specifications, in absolute ethanol at 20°C is $20 \pm 1.5$, and at 25°C is $18.5 \pm 1.5$.

Chloramphenicol in solution absorbs ultraviolet light over a broad range, with a maximum near 278 nm and a minimum of 240 nm. The absorption peak is due to the p-nitrophenyl group which provides both a distinguishing characteristic of the antibiotic and a useful method for analysis of chloramphenicol.

Chloramphenicol is somewhat soluble in water (2.5 mg/ml at 25°C); very soluble in methanol, ethanol, butanol, acetone, ethyl ether, ethyl acetate, and propylene glycol. It is insoluble in benzene and petroleum ether. Aqueous solutions have a pH of 5.5 and are extremely stable.

Three compounds of chloramphenicol are usually used in clinical practice: chloramphenicol USP, chloramphenicol sodium succinate USP, and chloramphenicol palmitate USP. Chloramphenicol palmitate is given orally in therapy since it lacks the
bitter taste of the parent compound. However, Chloramycetin\textsuperscript{R} (Parke, Davis and Company, trademark of CAP) tablets and capsules consisting only of chloramphenical USP, have been used in veterinary medicine. Both chloramphenicol sodium succinate USP and chloramphenicol USP are frequently administered parenterally.\textsuperscript{25}

The metabolism of chloramphenicol was first studied by Glazko, \textit{et al.} 1950,\textsuperscript{13} in the human, dog, and rat. After administration of 1.5 gm of chloramphenicol orally to human volunteers, 5 to 10 percent of the administered dose was excreted unchanged in the urine as determined by microbiological assay. However, with the colorimetric assay, 75 to 90 percent of administered dose was recovered from the urine in 24 hours. The major portion exhibited no antibiotic activity and represented inactive nitrogen containing degradation products of chloramphenicol.

Over a period of 24 hours after administration of 50 mg/kg of chloramphenicol to a male dog, 7.6 percent of the dose was recovered as active chloramphenicol in the urine, while the total nitro compound in the urine was 67.8 percent of the dose.

A series of white female rats, weighing 160-190 gm, were given 100 mg/kg CAP body weight subcutaneously in 50 percent propylene glycol solution containing 10 mg/ml CAP. After a period of 8-12 hours, 76.8 percent of the administered dose was excreted through the feces; whereas, 17.3 percent of the administered dose was recovered in the urine.

In another study, Glazko, 1966,\textsuperscript{14} separated chloramphenicol metabolites in urine specimens from 6 humans using paper chromatography. Three major nitrogen containing compounds were identified, unchanged drug, amino diol hydrolysis product and chloramphenicol glucuronide conjugate which was found in the highest concentration of the three.

VanDerLee, \textit{et al.} 1982,\textsuperscript{44} studied chloramphenicol degradation in 4 dairy cows after IV and IM administration. A dose of 5 gm of chloramphenicol was injected IV in
2 cows and the other two cows received the dose by intramammary infusion.

Chloramphenicol and chloramphenicol degradation products were isolated on a reverse phase HPLC column after acidic and alkaline extraction of urine, bile, liver and kidney. In urine, chloramphenicol concentration was 5.6 ug/ml of urine. Two metabolites of chloramphenicol, chloramphenicol glucuronide and p-nitrobenzaldehyde, were detected in trace amounts in the urine. In bile, chloramphenicol concentrations ranged between 0.07-0.14 ug/ml, but no degradation products were detected. Only a trace of p-nitrobenzoic acid was detected in liver. In the kidney neither chloramphenicol nor its degradation products were detected.

Bories, et al. 1983,3 used titrated chloramphenicol to study metabolism in rats, humans and goats using simple and ion-pair high performance liquid chromatography. The metabolic profile of chloramphenicol in rat urine after 24 hours of IM injection of 80uC[3H] CAP mixed with 20 mg nonradioactive. Chloramphenicol showed seven radioactive spots after thin layer chromatographic analysis: chloramphenicol-oxamic acid, chloramphenicol, chloramphenicol-glucuronide, chloramphenicol-base, chloramphenicol-alcohol, chloramphenicol-acetylarylamine and chloramphenicol-arylamine. Except for chloramphenicol-glucuronide, the structure of all compounds were confirmed by using CI or EI mass spectroscopy after trimethylsilation. Chloramphenicol glucuronide was identified by hydrolysis with beta-glucuronidase which led to formation of tritiated chloramphenicol. Quantitation of chloramphenicol and the other metabolites was performed using HPLC method. Seventeen percent of the injected dose was recovered in the urine. Chloramphenicol represented 11.8 percent, chloramphenicol-oxamic acid represented 15.4 percent, chloramphenicol-glucuronide represented 7.8 percent, chloramphenicol-base represented 2.5 percent, chloramphenicol-alcohol represented 9.4 percent, chloramphenicol-acetylarylamine represented 19.1 percent and chloramphenicol-arylamine represented 4.3 percent of the recovered dose.
The separation and identification of chloramphenicol metabolites in man and goat were not fully completed in the same study. However, after oral administration of 500 mg of nonradioactive chloramphenicol to one human volunteer, the urine profile showed the presence of chloramphenicol-oxamic acid, chloramphenicol-glucuronide and chloramphenicol-base. After IM administration of three goats with 600 uCi$^3$H-chloramphenicol mixed with 20 mg/kg body weight nonradioactive chloramphenicol, the HPLC profile of the urine showed fractions that had similar retention times as chloramphenicol-glucuronide, chloramphenicol, chloramphenicol-oxamic acid, chloramphenicol base, chloramphenicol acetylarylamine, and chloramphenicol base.

Disposition and metabolism of chloramphenicol in trout was studied by Cravedi, et al. 1985. The urinary and fecal excretion, tissue distribution and metabolism of $^3$H-chloramphenicol were measured in rainbow trout, after a single 50 mg/kg intragastric dose. The major route of excretion of tritium was fecal (64.3 percent of the dose), with 16 percent recovered in the urine in five days. Radioactivity was widely distributed in trout tissues and organs, the highest concentrations being in the bile and intestine. At 48 hours after dosing, the radioactivity remaining in the liver, the muscle and the perigastric adipose tissue was as chloramphenicol-derived compounds bound to tissues. In addition to unchanged chloramphenicol (4.3 percent dose after 96 hours), the other metabolites excreted in the urine were chloramphenicol-base (5.2 percent), chloramphenicol glycolic acid (4.0 percent), and chloramphenicol glucuronide (1.8 percent).

Several investigators have attributed the aplastic anemia and bone marrow depression produced by CAP in humans and animals to the metabolic degradation products of chloramphenicol. Krishna, in 1974, studied the covalent binding of chloramphenicol in vitro by incubation $^{14}$C-chloramphenicol with rat liver microsomes, NADH and NADPH, and its generating system. Three mechanisms of
covalent binding were proposed. In one mechanism chloramphenicol is converted to an arene oxide in 2-3 positions of the benzene ring. This would require oxygen, NADPH and cytochrome P-450 system. The arene oxide either reacts covalently with tissue macromolecules or with water to form dihydrodiol. In the second proposed mechanism the dichloroacetamide group is converted to a free radical by cytochrome P-450 and NADPH. The free radical either binds tissue to macromolecules or reacts with glutathione to form the monochloro derivative of chloramphenicol. In the third mechanism the dichloroacetamide side chain of chloramphenicol may be hydroxylated to form NHCOCl which then may react covalently with tissue macromolecules.

The mechanism of the metabolic activity of chloramphenicol in vivo was studied by Pohl et al. 1979. A mixture of [1,2-14C]dichloro-acetamide and 1-3H-benzylic derivative of chloramphenicol was given intraperitoneally to rats. Liver, kidney, lung and bone marrow were examined for radioactivity binding. 14C-labeled chloramphenicol bound significantly more than the 3H-labeled, indicating that only the dichloroacetamide side chain of chloramphenicol was bound.

Reduction of the nitro group of chloramphenicol by human liver tissue was studied by Salem et al. 1979. The reduction was potentitated by NADPH and abolished by boiling the liver homogenate. All livers examined showed nitroreductase activity. However, the levels varied between livers, by as much as sevenfold. This study demonstrated the ability of human liver to reduce the chloramphenicol nitro group and supports the hypothesis that the intermediate reactions leading to nitro reduction are responsible for the aplastic anemia.

Morris et al. described a reductive dechlorination pathway for chloramphenicol metabolism by rat liver microsomes. A mixture of (1-3H, 14C) chloramphenicol was incubated anaerobically with phenobarbital treated rat liver microsomes. The study postulated a metabolic pathway of chloramphenicol metabolism started with reductive
dechlorination of chloramphenicol and formation of radical anion intermediates which either form deschlorochloramphenicol or covalently bind to microsomal protein or lipid.

The oxidative metabolism of chloramphenicol was studied in rat liver microsomes. After aerobic incubation of a mixture of $[^{14}\text{C}]$ and $[^{3}\text{H}]$ chloramphenicol with phenobarbital treated rat liver microsomes for 30 minutes, several metabolites were identified using HPLC, and mass spectroscopy. The metabolites include chloramphenicol aldehyde, p-nitrobenzyl alcohol, N-(2-oxyethyl)dichloroacetamide, and N-(2-hydroxyethyl) dichloracetamide.23

The effects of reduced derivative of chloramphenicol on DNA was studied by Skolimowski, et al.41 Electrolytic reduction of chloramphenicol was carried out in the presence of DNA which was isolated from E. coli. The reduction products thus generated caused DNA damage which was dependent upon reduction of the nitro group. The damage was characterized by helix destabilization and strand breakage. The study proposed that nitro radical anion, the one electron reduction product of chloramphenicol, rather than nitro derivatives was the toxic agent responsible for DNA damage related to aplastic anemia.

A comparative study of the cellular transport of chloramphenicol and its nitroso derivative was tested in Raji cells, a transformed human lymphoblastoid cell line. Both compounds were concentrated threefold inside the cell, as compared to the extracellular concentration. The uptake of the nitroso derivative was similar to that of chloramphenicol, and was found to be rapid and temperature independent. From that finding the study concluded that the toxicity is not due to preferential uptake of the nitroso compound but due to the difference in covalent binding of chloramphenicol and the nitroso derivative to Raji cells and human bone marrow cells. The nitroso derivative was bound 15-fold greater than chloramphenicol. The increased binding of nitroso-chloramphenicol to human bone marrow cells attests to the greater reactivity of the p-
substituted aromatic nitroso group and is consistent with the postulate that reduction products of the nitro group of chloramphenicol may be responsible for chloramphenicol-induced aplastic anemia.28

The distribution of chloramphenicol in tissues and extracellular fluids was studied in dogs.47 Chloramphenicol was given orally at a dose level of 50 mg/kg to 16 Greyhound dogs. The animals were killed 1.5, 3, 6, and 12 hours after administration of the drug. Blood, cerebrospinal fluid, aqueous humor, and tissue from brain, kidney, liver, lung, lymph node, muscle, pancreas and spleen were collected for colorimetric analysis of chloramphenicol. The distribution of chloramphenicol in the body was not uniform. Except in brain, in all tissue samples the levels of chloramphenicol were higher than the concentration in blood. The highest mean values were found at the 1.5 hour sampling in all tissues other than the brain, which showed a concentration lower than that of serum.

The distribution of $^{14}$C-chloramphenicol was studied in neonatal pigs using whole body autoradiography.2 Twelve neonatal pigs were given 70 uCi $^{14}$C-chloramphenicol intravenously. In the lung, liver, adrenal cortex, kidney, heart, pancreas, thyroid, spleen and skeletal muscle the amounts of radioactivity became greater than that of the blood a short time after injection and remained higher than the blood up to 8 hours after dosing. After 4 to 8 hours the brain radioactivity was also higher than that of the blood. The amount of radioactivity in the bone marrow remained lower than that of the blood during the whole experiment.

The pharmacokinetics of chloramphenicol in 6 nonlactating Holstein cows were determined by an HPLC method following administration of single IV and subcutaneous 50 mg/kg doses of chloramphenicol.1 After IV administration chloramphenicol showed a biological half-life of 4.3 hours and a volume of distribution of the central
compartment of 0.44 L/kg. After subcutaneous administration, the same parameters measured 4.2 and 0.5 L/kg, respectively.

The pharmacokinetic behavior of chloramphenicol in healthy and chemically liver-damaged mini-pigs was studied after IV injection of 10 mg/kg body weight. The elimination half-life and clearance of chloramphenicol in healthy pigs was 93 minutes and 9.6 ml/min/kg, respectively; whereas, the same parameters in liver-damaged pigs were 167 minutes and 4.2 ml/min/kg, respectively. These findings are very important in assessment of dosage regimen and withdrawal time in liver-damaged animals. Chloramphenicol was assayed in this study using a specific radioimmunoassay.

The concentrations of chloramphenicol and its water soluble metabolites in the plasma of 6 healthy cows were measured by colorimetric methods after IM administration of 20 mg/kg of chloramphenicol. Free chloramphenicol showed a level of 1.7 ug/ml 7.3 hours after injection and a bioavailability of 63 percent. The absorption half-life of the main part of the available chloramphenicol was 10.2 hours. On the other hand, the elimination half-life of the unchanged drug was 10.2 hours.

After IV administration of chloramphenicol at a dose level of 25 mg/kg in a group of calves with age range between 1-270 days, chloramphenicol showed an elimination half-life of 7.5, 6, 4, 3.5, and 2.5 hours in calves of age 1, 7, 14, 28, and 270 days respectively. Despite the slow elimination of chloramphenicol in these calves, there was little or no toxic effect on bone marrow cells.

A colorimetric method was used to evaluate clearance of chloramphenicol in infants by Burckart, et al. Chloramphenicol-sodium-succinate was infused intravenously for a period of 20 minutes at a dose level of 25 mg/kg to infants 3-24 months of age. Serum samples were collected prior to administration and at 1, 2, 4, and 6 hours after infusion. Peak serum concentration of chloramphenicol ranged from 20.9
to 94.0 µg/ml, and occurred from 1 to 4 hours after infusion. Clearances ranged from 0.058 to 0.236 L/kg/hr.

The pharmacokinetic parameters of chloramphenicol were estimated in 42 patients with liver disease and in 8 healthy volunteers. Chloramphenicol was injected intravenously at dose level of 20 mg/kg. Serum samples were analyzed by colorimetric method for determination of chloramphenicol. The half-life of chloramphenicol ranged between 7.5 to 11.5 hours in liver diseased patients; as compared to 4.6 hours in healthy volunteers. The clearance and apparent volume of distribution in liver diseased patients were lower than those of healthy volunteers.

The plasma concentration of chloramphenicol and the kinetics of its disappearance were determined in dogs, cats, swine, goats, and ponies. Plasma half-life values ranged from 0.9 hours in ponies to 5.1 hours in cats. The half-life in dogs was found to be 4.2 hours with a peak plasma concentration of 12.4 µg/ml immediately following intravenous injection. Apparent specific volume of distribution ranged from 1.02 L/kg in ponies to 2.36 L/kg in cats. Dogs were determined to have a volume of distribution of 1.77 L/kg. Chloramphenicol concentration in the plasma was estimated by the colorimetric method of Glazko.

The pharmacokinetic parameters of chloramphenicol were measured in cattle and horses after estimation of chloramphenicol concentrations by the modified chemical method of Oh-Ishi which measures both chloramphenicol and its metabolites. In cattle, the half-life and apparent volume of distribution were 3.5 hours and 0.814 L/kg, respectively, after intravenous administration of 20 mg/kg body weight. The parameters were estimated in horses after administration of the same amount intravenously. The half-life was one hour, and the apparent volume of distribution was 1.82 L/kg.

The half-life of chloramphenicol in calves showed age dependent changes when levels in the serum were estimated chemically. The beta phase half-life of
chloramphenicol injected intravenously at the dose level of 30 mg/kg in calves ranged from 11.7 hours in one-day-old calves to 4.9 hours in 10- to 12-week-old calves. In adults it was 4.4 hours.

The pharmacokinetic parameters of chloramphenicol were estimated in 11 buffalo calves (Bubalus bubalis). Chloramphenicol was given intravenously in two dose levels (10 and 20 mg/kg body weight), and serum samples were assayed chemically for chloramphenicol. The median half-lives of elimination of chloramphenicol were 2.95 and 2.94 hours for 10 and 20 mg/kg, respectively. The corresponding median values of specific volume of distribution were 1.2 and 0.9 L/kg, respectively. The study suggested that the kinetics of this drug are not dose dependent.

Mercer et al. (1978) conducted a study in young swine to determine the pharmacokinetics of chloramphenicol in plasma, organs and body fluids. The elimination half-life in plasma after administration of 22 mg/kg body weight of chloramphenicol intravenously was 2.66 hours, whereas the volume of distribution and body clearance were 1.39 L/kg and 6.64 ml/kg/min, respectively. The elimination half-life of most major organs ranged from 2.0 to 5.0 hours. A fluorometric method was used to find drug concentration which measures both intact chloramphenicol and its metabolites.
CHAPTER 3
MATERIALS AND METHODS

Chemicals

Chloramphenicol (D(-)threo-1-4-nitrophenyl-2-dichloroacetamide 1,3-propanediol and Thiamphenicol (D(-)threo-1-(4-methylsulphonylphenyl)-2-dichloroacetamide 1,3-propanediol) were purchased from Aldrich Chemical Co. (Milwaukee, WI). $^{14}$C-chloramphenicol (D(-)threo-1-4-nitrophenyl-2-dichloro[1',2'-$^{14}$C]acetamide-1,3-propanediol) 57.8 mCi/mmol was obtained from New England Nuclear Corporation (Boston, MA). HPLC-grade methanol, acetonitrile, and dichloromethane were obtained from Fisher Scientific (Pittsburgh, PA). All other solvents and reagents were reagent grade obtained from different commercial sources. Solvents were used without further purification. Glycolic acid chloramphenicol (D-threo-2-(2-hydroxyacetamido)-1-(4-nitrophenyl)-1,3-propanediol) and reduced base (D-threo-2-amino-1-(4-aminophenyl)-1,3-propanediol) were kindly donated by Dr. M. L. Black of the Warner-Lambert Company (Ann Arbor, MI).

Equipment

A model 1084B liquid chromatograph equipped with a Model 79875A variable-wavelength detector, a Model 79850B LC terminal, a Model 79841A automatic injection and a Model 79842A autosampler, all from Hewlett-Packard, were used for all HPLC analyses. The liquid chromatograph was equipped with a flow-through liquid scintillation detector (Radiomatic, Tampa, FL). A 25 cm x 4.6 mm I.D. Nucleosil C$_{18}$ column with 5 um spherical particles was used (Alltech Assoc., Deerfield, IL) for all analyses.
Nuclear Magnetic Resonance (NMR):

NMR spectra were obtained at 11.75 T (500 MHz) on a Bricker AM-500 spectrometer. Samples were dissolved in either D_2O, acetone d_6, or methanol d_4. Residual water was suppressed using a presaturation pulse sequence. Samples were referenced to either internal or external tetramethylsilane (TMS).

For the assignment of the resonances of CAP-glucuronide, a combination of two dimensional techniques was employed. Two dimensional J-resolved spectra were obtained from CAP-glucuronide isolated from the horse, as well as metabolite F3. Since this was inadequate for complete assignment of all resonances, $^{13}$C-$^1$H correlation spectra were obtained on the CAP-glucuronide isolated from the horse. To assist in assignment of $^{13}$C resonances CH and CH$_2$ spectra were obtained using the distortionless enhancement by polarization transfer (DEPT) technique. $^{13}$C techniques could not be employed on F3 due to the limited amount of material, so resonances were assigned by analogy from the glucuronide obtained from the horse.

Chemical Ionization (CI) Mass-Spectroscopy:

CI mass-spectra were obtained using a Finnigan Model 4021-C mass spectrometer equipped with an Incos Data System. CI was used at 45 eV and CH$_4$ (methane) was used as reagent gas. Samples purified previously by HPLC were introduced into the mass analyzer by direct probe.

Fast Atom Bombardment (FAB) Mass-Spectroscopy:

FAB mass-spectra were obtained using a Karatos, M-S-30 (Ramsey, NJ). The mass spectra were obtained using fast atom bombardment (FAB). The glucuronide, dissolved in water, was added to either a glycerol matrix or a matrix consisting of 1:1 dithiothreitol and dithioerythritol ("magic bullet"). Molecular ion peaks consisted of a cluster representing chloramphenicol-glucuronic acid (CAGA) + Na$^+$, chloramphenicol-glucuronide (CAG) sodium salt + Na$^+$, CAG potassium salt + Na$^+$, and CAG potassium
salt + K⁺. The appearance of M⁺ alkali metal peak is common in FAB spectra from biological sources.

**Liquid Scintillation Counting**

An aliquot (10-30 µL) of the sample to be counted (urine, feces, stock solutions, eluted fraction and organic solvent extract) was transferred to a glass scintillation vial to which 10 ml of scintillation cocktail (Formula 963) in the case of organic samples or Atomlite® in the case of aqueous solutions was added. The content was vortexed and radioactivity was counted for 10 minutes in a Beckman Model 7000 liquid scintillation counter (Smith, Kline and Beckman, Philadelphia, PA). Sample counting efficiency was determined by the H-number technique of external standardization, which was verified by adding known amounts of [¹⁴C] toluene (New England Nuclear) to samples and recounting them.

The radioactivity of the samples, which were injected directly on HPLC column, was counted using the flow through liquid scintillation detector. ScintiVerse LC (Fisher Scientific) was used as cocktail with a flow rate of 5 ml/minute.

**Experimental Animals and Samples Collection:**

Three female, healthy beagles were maintained on a diet of commercial dog food (Quaker Oats Co., Chicago, IL). The animals were allowed water and food ad libitum.

The jugular vein of each animal was catheterized with polyethylene tubing for collection of blood samples. The cephalic vein of each animal was catheterized with a 2-inch indwelling catheter (Monoject) for injection of the drug. The urinary bladder was catheterized with a No. 8 French Foley catheter for collection of urine.

In the first experiment, 211 uCi of [¹⁴C]-chloramphenicol with initial specific activity of 57.8 uCi/mmol was mixed with 725 mg nonradioactive chloramphenicol dissolved in 9 ml absolute ethanol and injected in a single bolus intravenously at a dose
of 50 mg/kg. The final injection solution contained $^{14}\text{C}$-chloramphenicol with a specific activity of 0.29 uCi/mg.$^3$

Blood samples for estimation of pharmacokinetic parameters were collected prior to injection and at 1, 2, 3, 6, 9, 12, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, 180, 240, 300, 360, and 480 minutes after injection. Serum samples were separated and kept frozen at -20°C until time of analysis.

Urine sample were collected prior to and at 1, 2, 3, 4, 5, 6, 7, 8, 12, 14, 16, 18, 24, 30, 36, 42, and 48 hours after injection. The volume of each urine sample was measured and an aliquot of 10 ul was transferred to a scintillation vial to determine the radioactivity. The rest of the sample was frozen immediately using dry ice-acetone and kept frozen at -20°C until time of analysis.

In the second experiment, 98 uCi of $^{14}\text{C}$-chloramphenicol with initial specific activity of 57.8 uCi/mmol was mixed with 658 mg nonradioactive chloramphenicol dissolved in 4.5 ml absolute ethanol and injected as a single bolus intravenously at a dose of 50 mg/kg. Specific activity of the CAP was 0.15 uCi/mg.

Blood samples were collected prior to and at 1, 2, 3, 6, 9, 12, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, 210, 270, 330, 390, and 480 minutes after injection. Serum samples were separated and kept frozen until time of analysis.

Urine samples were collected 0, 2, 4, 6, 8, 12, 18, 24, 31, 38, 48, 55, 62, and 72 hours after injection.

In the third experiment, 93 uCi of $^{14}\text{C}$-chloramphenicol with a specific activity of 57.8 uCi/mmol was mixed with 635 mg nonradioactive chloramphenicol dissolved in 4.3 ml of absolute ethanol and injected as a single bolus intravenously at a dose of 50 mg/kg. Specific activity of chloramphenicol was .146 uCi/mg.
Blood samples were collected 0, 1, 2, 6, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, 180, 240, 300, 360, and 480 minutes after injection. Urine samples were collected 0, 2, 4, 6, 8, 12, 18, 24, 31, 38, 48, 55, 62, and 72 hours after injection.

In all three experiments, the final specific activity of the injection solution was measured by counting 3 aliquots (2 ul each) by liquid scintillation counting from each solution prior the IV injection. To ensure complete injection of the dose the vial which contained the injection solution was washed twice with 3 ml normal saline and injected IV.

During the third experiment feces were collected at 5, 25, 48, 72, 76, 77 and 96 hours after injection. Feces were homogenized in (Warner commercial blender) by adding 1-2 liters of distilled water. (The volume of the distilled water used to homogenize the feces depended on the amount of feces collected). The final volume of the fecal homogenate was measured and an aliquot of 0.5 ml was transferred to a glass scintillation vial and bleached with 30% hydrogen peroxide. The vials were kept frozen at -20°C overnight prior to counting. Total radioactivity was measured by liquid scintillation counting.

Chloramphenicol-glucuronide Isolation and Identification

CAP-glucuronide was isolated using a modification of the method described by Glazko, et al., 1950. A 20 gm dose of CAP dissolved in a mixture of propylene glycol and absolute ethanol was injected intravenously into a 500 kg mare. Urine was collected for 8 hours and kept frozen at -20°C. A total of two liters of urine were collected. One liter of urine was concentrated to 500 ml using a rotoevaporator at room temperature. The concentrated urine was filtered through Whitman No. 1 filter papers. The salt content of the filtrate was decreased by freezing the mixture until an icy slush formed and removing the solids by centrifugation. The pH of the concentrate was adjusted to 6.7, placed in a 500 ml separatory funnel and an equal volume of n-butanol
was added. An eight plate counter-current extraction series was run using 500 ml separatory funnels. At the end of the run, petroleum ether was added to each funnel equivalent to two-thirds the volume of the organic phase. After shaking, the aqueous layer from each funnel was pooled and concentrated by rotoevaporation. The pH was adjusted 8.0 (using solid sodium bicarbonate), the solution frozen and the solids removed by centrifugation. After readjustment of the pH to 6.7 (using 0.1 N HCl), the solution was subjected to a second counter-current extraction as described earlier. The resulting aqueous layers were lyophilized to remove the water and redissolved in absolute ethanol. The addition of petroleum ether resulted in the appearance of a precipitate which was isolated by filtration through Whitman No. 1 filter paper, washed with excess petroleum ether, and dried. The material was again dissolved in absolute ethanol and reprecipitated with petroleum ether. The final preparation was a firm yellowish-white powder which was extremely soluble in water.

The final preparation was identified as chloramphenicol-glucuronide by the following criteria:

a. Melting point: The compound decomposed without giving a sharp melting point. This is characteristic of CAP-glucuronide.

b. Ultraviolet absorption: The isolated compound showed a maximum absorption peak at 273 nm.

c. Mass spectroscopy: Fast atom bombardment mass spectroscopy was used for confirmation of chloramphenicol-glucuronide structure.

d. Nuclear magnetic resonance (NMR) was used to confirm the structure of chloramphenicol-glucuronide.

e. Enzymatic hydrolysis: A 10 mg portion of the putative CAP-glucuronide was dissolved in 2 ml distilled water. A 1 ml aliquot was transferred to a 15 ml glass centrifuge tube containing 1 ml of 1000 unit/ml of beta-glucuronidase enzyme
(Sigma) and 3 ml of acetate buffer at pH 5.0. The mixture was incubated for 24 hours in water bath at 37°C. One ml of the mixture was extracted with methylene chloride and analysis for chloramphenicol was performed by HPLC, which showed only one peak coeluted with standard chloramphenicol.

**Metabolic Profile of Chloramphenicol in Dog Urine**

A one ml aliquot of each urine sample was filtered through a 0.2 micron filter cartridge into a 1 ml HPLC vial. The percentage of radioactivity recovered was 97 ± 2 percent. The recovery percentage was estimated by counting a 10 ul aliquot of urine before and after filtration using liquid scintillation counting. An aliquot of 20-50 ul was injected directly onto a C18 HPLC column.

The mobile phase consisted of: A) phosphate buffer (5 mM) pH 2.5 and B) methanol acetonitrile (50:50). The flow rate was 1.2 ml/min and a linear gradient (0.9 percent B/min) over 50 minutes starting at 10 percent B. The UV absorbance was monitored at 266 nm. ScintiVerse LC (Fisher Scientific) was used as a scintillation cocktail to measure the radioactivity using the flow-through liquid scintillation detector and a cocktail flow-rate of 5 ml/minute.

**Isolation of Urinary Metabolites of CAP**

After filtration through a 0.2 micron filter cartridge, urine was injected onto a C18 column in 100 ul portion. Chromatographic conditions were the same as used for profiling urine. Fractions of the effluent were collected every 0.5 minutes for metabolite identification. The radioactivity in each fraction was determined by liquid scintillation counting of 30 ul aliquots. Each fraction containing radioactivity was dried by lyophilization.
Identification of Urinary Metabolites of Chloramphenicol:

Fraction 5 (F5):

F5 was isolated on an HPLC column using a gradient mobile phase of phosphate buffer and 50:50 acetonitrile:methanol. An aliquot of the isolated fraction was injected on an HPLC column using the same conditions which were used for isolation. The fraction showed only one peak on both UV and radiochemical detectors. The fraction was reisolated using (36/64) 50:50 acetonitrile:methanol/triple distilled water. The reisolation was necessary to remove phosphate which interferes with the identification by mass-spectroscopy. The fraction was transferred to a capillary tube for identification by CI-mass spectroscopy.

Fraction 4 (F4):

F4 was isolated on an HPLC column using the same gradient mobile phase which was used for isolation of F5. An aliquot of this isolated fraction was injected on an HPLC column using the same conditions which were used for isolation. The fraction showed only one peak on both UV and radiochemical detectors. The purified fraction was transferred to a 1 ml vial and evaporated to dryness under a gentle stream of nitrogen and submitted to fast atom bombardment spectroscopy (FAB). For FAB, it is unnecessary to remove phosphate.

Fraction 3 (F3):

F3 was isolated on an HPLC column using the same gradient mobile phase which was used for isolation of F5. An aliquot of the isolated fraction was injected on an HPLC column using the same conditions which were used for isolation. The fraction showed only one peak on both UV and radiochemical detectors. The fraction was reisolated using the same gradient except triple distilled water was used instead of buffer. The fraction was transferred to a capillary tube for CI-mass spectroscopy. The mass spectrum failed to show any peak attributable to chloramphenicol. A second
isolation was performed and the fraction was identified by nuclear magnetic resonance (NMR) after dissolving in dutorated methanol.

**Fraction 2 (F2):**

As shown in Figure 4, F2 contained more than one radioactive peak. The fraction was isolated using the same gradient mobile phase which was used for isolation of F5. The fraction was reisolated using the same gradient except triple distilled water was used instead of buffer. For further resolution of the radioactive peaks of F2, an isocratic mobile phase consisting of (5/95) 50:50 acetonitrile:methanol/triple distilled water was used. Two main subfractions were isolated F2A and F2B. An aliquot of each fraction was injected using the same isocratic mobile phase which was used to separate the subfractions. Each fraction showed only one peak on both UV and radiochemical detectors. F2A was submitted for CI FAB mass spectroscopy and NMR for identification. F2B was submitted to CI-mass spectroscopy and NMR for identification.

**Fraction 1 (F1):**

Fraction 1 contained 2-3 radioactive peaks and eluted with a retention time of 2-3 minutes. All the isolation procedures which were used to isolate the other fractions failed to resolve the subfraction of F1. Even a mobile phase consisting only of triple distilled water failed to separate the subfraction.

**Chloramphenicol Analysis in Serum**

Chloramphenicol concentrations in serum were estimated using both HPLC and a specific radiochemical assay.

1. **HPLC Method**

A chloramphenicol standard solution of 1 µg/ul was prepared by dissolving 50.0 mg of chloramphenicol in methanol to a final volume of 50 ml. Thiamphenicol was used as internal standard and was prepared by dissolving 50 mg of thiamphenicol in methanol to a final volume of 50 ml. Both standard solutions and a 0.1 M phosphate
buffer (pH, 7.0) were stored in sealed containers protected from light at 4°C. Aliquots of 0, 0.5, 1, 2, 4, 8, 16, 32 and 64 ul of chloramphenicol standard solution were transferred to the appropriately labeled 50 ml glass centrifuge tubes. Ten ul of internal standard solution was added to each tube. The methanol in each tube was evaporated to dryness under nitrogen. One ml of serum was then added to each tube and mixed well by vortex. For determination of chloramphenicol in the unknown samples, 1 ml of serum was transferred into 50 ml glass centrifuge tubes containing 10 ug of internal standard. To both standard and unknown samples 1 ml of 0.1 M phosphate buffer (pH 7.0) and 10 ml of methylene chloride were added. The tubes were capped with polyethylene plugs (Protective Closures Co., Buffalo, NY), shaken for 10 minutes on a mechanical shaker (Eberbach Corp., Ann Arbor, MI) and centrifuged at 2500 Xg for 10 minutes in an IEC Model K centrifuge (International Equipment Corp., Needham Heights, MA). Eight ml of the organic phase were transferred to 15 ml glass centrifuge tubes and evaporated under nitrogen. The residues in each tube were reconstituted in 200 ul of the mobile phase which consisted of 64 percent triple distilled water and 36 percent of 50:50 acetonitrile: methanol. An aliquot of 20 ul of the reconstituted solution from each tubes was injected on C18 HPLC column. Detection was by ultraviolet (UV) absorbance at a wavelength of 225 nm for the internal standard and 278 nm for chloramphenicol. The flow rate of the mobile phase was 1.2 m/min. The efficiency of extraction of chloramphenicol from serum with dichloromethane was 100 percent.

2. Specific Radiochemical Assay

The 14C-chloramphenicol in serum samples was measured by a specific radiochemical assay. A 1.0 ml aliquot of serum was transferred to a 50 ml glass centrifuge tube. Then, 1 ml of 0.1 M phosphate buffer (pH 7.0) and 10 ml of dichloromethane was added. The tubes were capped and shaken for 10 minutes and then centrifuged for 10 minutes. Eight ml of the organic phase was transferred to
scintillation vials and allowed to evaporate overnight. The residue was reconstituted using 10 ml of scintillation cocktail (Formula 963) and counted by liquid scintillation. Dichloromethane extract residues counted with 92 percent efficiency.

**Pharmacokinetic Analysis**

The data were described by a two compartment model. This model is described mathematically by a double exponential:

\[ Y = Ae^{\alpha t} + Be^{\beta t} \]

When \( Y \) = concentration of antibiotic, \( t \) = time of sampling, \( A \) and \( B \) are the intercepts of the distribution and elimination phases, respectively, and \( \alpha \) and \( \beta \) are the corresponding slopes of the two phases.

Sampling times and corresponding concentrations of chloramphenicol were entered into a statistical program and the regression coefficients for the time-concentration curve were estimated (\( A \) and \( \alpha \), the regression coefficients for the distribution phase of the curve; \( B \) and \( \beta \), the regression coefficients for the elimination phase of the curve). The pharmacokinetic parameters were estimated as follows:

\[ t \frac{1}{2} \alpha = \frac{0.693}{|\alpha|} \]  

When \( t \frac{1}{2} \alpha \) = the half-life of distribution of the drug, \( \alpha \) = the slope of the distribution phase.

\[ t \frac{1}{2} \beta = \frac{0.693}{|\beta|} \]  

When \( t \frac{1}{2} \beta \) = the half-life of elimination of the drug, \( \beta \) = the slope of the elimination.

\[ VDA = \frac{\text{Dose}}{|\beta| \times \text{AUC}} \]  

When \( VDA \) = area volume of distribution, \( \text{AUC} \) = total area under the curve.

\[ BC = |\beta| \times VDA \]  

When \( BC \) = total body clearance.
CHAPTER 4
RESULTS

Pharmacokinetic Studies

The concentration-time profiles of chloramphenicol in serum after IV administration of 50 mg/kg in 3 dogs are given in Figures 1, 2, and 3. A summary of the pharmacokinetic parameters for chloramphenicol analyzed by HPLC and radiochemical methods is presented in Table 1. When chloramphenicol concentrations were determined by HPLC in 3 dogs, the disposition half-lives ($t_{1/2d}$) were 2.5, 0.8 and 1.1 minutes, the elimination half-lives ($t_{1/2e}$) were 105, 63, and 64 minutes, the body clearances (BC) were 5.2, 7.4, and 5.0 ml/min/kg, and the area volumes of distribution (VDA) were 0.7, 0.7, and 0.5 L/kg. After the radiochemical assay of chloramphenicol of the same serum samples of the 3 dogs, the $t_{1/2a}$ were 1.9, 0.7, and 0.8 minutes, the $t_{1/2b}$ 88, 61, and 64 minutes, BC were 5.6, 7.4, and 5.0 ml/min/kg, VDA were 0.61, 0.6, and 0.5 L/kg.

Chloramphenicol Metabolites in Urine:

The urinary profile of chloramphenicol metabolites contained 5 major detectable peaks of radioactivity, fractions (F) 1-5 as shown in Figure 4. The peaks eluted with retention times of 27 (F5), 24 (F4), 20 (F3), 9 (F2), and 3 (F1) minutes. As shown in Figure 4, F5, 4, and 3 are well separated peaks, whereas F1 and 2 are a collection of 2-3 unresolved peaks. The amount of each fraction (in milligrams) excreted in each collection time of urine from 3 dogs is shown in Figures 5, 6, and 7. F1, 2, 3, 4, 5 comprised 10.8, 16.2, 5, 56, and 12 percent of the excreted radioactivity in the urine of 3 dogs. F5 showed a molecular ion of m/z 323 after CI mass spectroscopy, a maximum
Figure 1. Semilogarithmic plot of chloramphenicol concentrations in serum versus time obtained after administration of a single dose of 50 mg/kg as IV bolus to Dog 1.
Figure 2. Semilogarithmic plot of chloramphenicol concentrations in serum versus time obtained after administration of a single dose of 50 mg/kg as IV bolus to Dog 2.
Figure 3. Semilogarithmic plot of chloramphenicol concentrations in serum versus time obtained after administration of a single dose of 50 mg/kg as IV bolus to Dog 3.
### TABLE 1

Pharmacokinetic Parameters of Chloramphenicol after Analysis with HPLC and Radiochemical Methods

<table>
<thead>
<tr>
<th>Dog 1</th>
<th>Dog 2</th>
<th>Dog 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HPLC</td>
<td>RCA</td>
</tr>
<tr>
<td>$t_{1/2}^\alpha$ (min)</td>
<td>2.5</td>
<td>1.9</td>
</tr>
<tr>
<td>$t_{1/2}^\beta$ (min)</td>
<td>105.0</td>
<td>88.0</td>
</tr>
<tr>
<td>VDA (L/kg)</td>
<td>0.7</td>
<td>0.61</td>
</tr>
<tr>
<td>BC (ml/min/kg)</td>
<td>5.2</td>
<td>5.6</td>
</tr>
</tbody>
</table>
Figure 4. The metabolic profile of chloramphenicol in dog urine.
Figure 5. Quantity of each fraction excreted in urine samples collected from Dog 1.
Figure 6. Quantity of each fraction excreted in urine samples collected from Dog 2.
Figure 7. Quantity of each fraction excreted in urine samples collected from Dog 3.
ultraviolet (UV) absorption of 278 nm, and HPLC reaction time of 25 minutes. All those criteria matched those of authentic chloramphenicol (Figures 8 and 9).

After fast atom bombardment mass spectroscopy (FAB), F4 showed molecular ions of 521, 543, 559 and 575, which represented chloramphenicol-glucuronic acid plus sodium ion, chloramphenicol-glucuronic sodium salt plus potassium ion, and chloramphenicol-glucuronide potassium plus potassium, respectively (Figures 10 and 11). It also showed a maximum UV absorption of 278 nm and HPLC retention time of 24 minutes. The fast atom bombardment mass spectrum, UV absorption, and HPLC retention time of F4 matched those of chloramphenicol-glucuronide isolated from the horse.

F3 was identified by proton-nuclear magnetic resonance, Spectroscopy, \(^1\)HNMR and FAB. The chemical shift of the protons on 2 and 6, of 3 and 5 carbons (C) of the aromatic ring were 8.1 and 7.6 PPM respectively. The proton of C\(_2\) of the side chain (dichloroacetamide chain) showed a chemical shift of 6.2 PPM. The protons of C\(_1\) and C\(_2\) of chloramphenicol showed chemical shifts of 5.2 and 4.3 PPM. The protons of C\(_3\) of chloramphenicol showed chemical shifts of 4.1 and 3.7.

The sugar protons on C\(_1\), C\(_2\), C\(_3\), C\(_4\), and C\(_5\) showed chemical shifts of 4.4, 3.3, 3.4, 3.5 and 3.8, respectively (Figures 12, 13, and 14). The \(^1\)HNMR spectrum of F3 is similar to that of chloramphenicol-glucuronide isolated from the horse, yet it eluted on HPLC with different retention time (20 minutes). This indicates that F3 is the enantiomer of chloramphenicol-gluconide [(L+) threo isomer].

F2 was separated into two subfractions, F2B and F2A. F2B showed, after CI mass spectroscopy, a molecular ion of (m + 1)271 which matched that of standard chloramphenicol glycolic acid (Figures 15, 16, and 17). F2A failed to show a molecular ion after CI mass spectroscopy. However, after \(^1\)HNMR analysis the fraction showed a similar spectrum to chloramphenicol except that the signal of the proton of the side
Figure 8. CI mass-spectrum of standard chloramphenicol.
Figure 9. CI mass-spectrum of F5.
Figure 10. FAB mass spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 11. FAB-mass spectrum of F4.
Figure 12. Proton magnetic resonance ($^1$H NMR) spectrum of chloramphenicol.
Figure 13. Proton magnetic resonance (1H NMR) spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 14. Proton magnetic resonance (1H NMR) spectrum of F3.
Figure 15. CI-mass spectrum of standard glycolic acid-chloramphenicol.
Figure 16.  CI-mass spectrum of F$_2$B isolated from the horse.
Figure 17. Proton magnetic resonance ($^1$HMR) spectrum of $F_2B$ isolated from dog urine.
chain (dichloracetamide chain) was absent. Due to the fact that the fraction (F2A) (Figure 18) was radioactive and the proton on C2 of the side chain was absent the compound might be chloramphenicol-oxamic acid were the structure of the side chain NHCOOH. However, after FAB analysis the fraction showed a molecular ion (m + 1) of 361 (Figures 18 and 19).

F1 contained 3 subfractions which were inseparable on an HPLC column, even using only distilled water as a mobile phase. The HNMR (Figure 20) showed a mixture of unidentified signals which indicated presence of a mixture of compounds. Fraction one (F1) may only [14C] attached to some endogenous compound such as lipid or bile salts.

The Quantity of the Radioactivity Excreted in Urine and Feces

In order to account for the radioactivity injected in the dogs, urine was collected from dogs in the first and second experiments. Urine and feces were collected from the dogs in the third experiment. The urinary recovery was 60 percent, 50.2 percent, and 66.2 percent of the injected dose to the dogs in experiments one, two and three, respectively, after 48 hours in the first and 72 hours after injection in the second and third experiments.

The radioactivity excreted in the feces was 14.8 percent of the injected dose in the third experiment, 96 hours after injection. The fecal profile on HPLC showed only two radioactive peaks which matched the retention time of F1 and F4.
Figure 18. Proton magnetic resonance (\(^1\)H NMR) spectrum of F2A isolated from dog urine.
Figure 19. FAB-mass spectrum of F2A isolated from dog urine.
Figure 20. Proton magnetic resonance ($^1$HMNR) spectrum of Fl isolated from dog urine
CHAPTER 5
DISCUSSION

The pharmacokinetic parameters estimated from serum samples analyzed by HPLC and specific radiochemical method fell within the range of the same parameters estimated in another study by the author using the different but specific methods of gas chromatography and bioassay for estimation of chloramphenicol. Although the number of animals which was used in these studies did not allow the use of nonparametric statistical methods for comparison of the pharmacokinetic parameters estimated by HPLC and those estimated by the radiochemical method, the parameters are similar. The application of specific radiochemical method for analysis of chloramphenicol allows a fast and accurate method for estimation of chloramphenicol in biological samples.

The pharmacokinetic parameters of chloramphenicol have been studied in different animal species using the colorimetric assay method of Glazko, et al. This method of analysis has resulted in the reporting of falsely high half-lives of the drug. For example, in dogs the elimination half-life of chloramphenicol has been reported to be 252 minutes. In one study, where the pharmacokinetic parameters of chloramphenicol were estimated with the colorimetric method and gas chromatography, the median half-lives of chloramphenicol after the colorimetric method and gas chromatography were 225 and 94 minutes respectively. In the present study the median half-life was 64 minutes after HPLC and radiochemical method.

Calculation of the dosage regimen to maintain a minimum serum concentration of 5 ug/ml based on data generated using the colorimetric method yields a dose of 50
mg/kg every twelve hours. The present study shows that such a regimen is inadequate to maintain the recommended minimum inhibitory concentration (MIC) of 5 μg/ml and that a regimen of 50 mg/kg every six hours is necessary. The use of a dosage regimen that fails to maintain an adequate MIC may contribute to therapeutic failure and the development of chloramphenicol resistant bacteria.

Metabolic studies showed that chloramphenicol is biotransformed to at least three major metabolites in dogs. The proposed pathways of metabolism of chloramphenicol are:

1) 1-glucuronidation at the hydroxyl group of C₃.
2) Glucuronidation of hydroxyl group of the L(+) threo isomer of chloramphenicol.
3) Oxidative dechlorination of the dichloroacetamide side chain to form either glycolic acid chloramphenicol or oxamic acid chloramphenicol.

Chloramphenicol arylamine was not detected in the urine which indicates that it is either not a major pathway for chloramphenicol metabolism in dogs or is not excreted in the urine.

CAP-gluconurides are the major metabolic products of chloramphenicol in dogs. This has clinical importance in drug interaction with those drugs, such as phenytoin, which are metabolized mainly by glucuronidation. The usage of chloramphenicol concurrently with these drugs might lead to over-dose toxicity.

The second major pathway of chloramphenicol was oxidative dechlorination to form glycolic acid. This pathway was claimed by several investigators to be the main cause of chloramphenicol toxicity in humans, where it causes bone marrow depression and aplastic anemia. However, all their studies were in vitro when they showed that the oxamic acid and glycolic acid of chloramphenicol bound covalently to liver cells to a greater extent than did intact chloramphenicol. This might explain that toxicity of chloramphenicol is due to metabolic intermediates.
Reduction of the nitro group of chloramphenicol was not detected in the present study as a major metabolic pathway of chloramphenicol metabolism in dogs. A group of investigators reported that aplastic anemia in humans was due to the reduced metabolites of chloramphenicol. The absence of chloramphenicol reduction in dogs might explain the fact that no cases of aplastic anemia have been reported in dogs.

Intact chloramphenicol represented 12 percent of the excreted dose in the urine. The concentration was above the minimum inhibitory concentration for most sensitive microorganisms (5 μg/ml) for 6-8 hours. This finding indicates the usefulness of chloramphenicol for treatment of urinary tract infection in dogs.

From 50.2 to 66.2 percent of the injected dose was recovered in the urine and 14.8 percent was recovered in the feces. About 20 percent of the injected dose was not recovered during the 3-4 day collection period after injection. That suggests that chloramphenicol and its metabolites are bound to tissue. An in vitro study showed that chloramphenicol and its metabolites were covalently bound to liver microsomes, bone marrow cells and kidney when administered to rats.

In the present study, several new techniques were used to identify chloramphenicol and its metabolites. Chemical ionization (CI) mass spectroscopy was used to identify chloramphenicol and some of its metabolites. Electron-impact mass spectroscopy (EI) was tried to identify the compounds, but they either showed self-ionization (the chloramphenicol molecules protinated each other) or did not give a molecular ion due to fast loss of water molecules. Several investigators reported the EI mass spectra of chloramphenicol and its metabolites after derivitization with trimethylsilation.

Chloramphenicol-glucuronide was identified in the literature by hydrolysis with beta-glucuronidase. The shortcoming of this technique is the difficulty in identifying the position and the number of glucuronide molecules which are conjugated to the parent compound. In this study, chloramphenicol-glucuronide was identified by fast
atom bombardment (FAB) mass spectrometry. This technique can be used to measure the molecular ion of compounds such as CAP glucuronide, which are decomposed by EI or CI mass-spectroscopy. The structure of chloramphenicol-glucuronide was confirmed by using nuclear magnetic resonance (NMR). The position of the proton of the molecule was obtained by using proton NMR. Complete structural identification was confirmed using the two dimensional technique, the C13-H correlation spectrum and the CH, CH\textsubscript{2} spectrum as obtained by using the dimensionless enhancement by polarization technique (DEPT). These techniques assign the position and the number of glucuronide conjugation to the parent compound.\textsuperscript{16}

Complete identification of standard chloramphenicol-glucuronide structure was necessary to identify fraction three (F3). F3 showed a very similar \textsuperscript{1}HNMR spectrum to standard chloramphenicol-glucuronide but eluted with different retention time in the HPLC analysis. This finding indicates that F3 is an isomer of F4. Four possibilities exist for the structure of F3.

1. It is chloramphenicol-\textsubscript{a}-glucuronide.

2. It is a chloramphenicol-glucuronide where the glucuronide residue is attached to C\textsubscript{1} of chloramphenicol instead of C\textsubscript{3}.

3. It is chloramphenicol-glucuronide where the chloramphenicol is attached to a carbon other than carbon number 1 of the glucuronide.

4. It is the glucuronide conjugate of L(+) threo chloramphenicol.

The 500 mHz \textsuperscript{1}HNMR spectrum of F3 showed a coupling constant for the anomeric proton of 6-7 Hz. The coupling constant of \textbeta-glucuronide is 6-7 Hz while the coupling constant of an \textalpha-glucuronide is 1-2 Hz. This indicates that F3 is not chloramphenicol-\textalpha-glucuronide.
The chemical shifts of the proton on C₁ of F₃ and F₄ are identical. If one of the glucuronides was attached to C₁ and the other to C₃ the chemical shift should be greatly different. Therefore, this possibility is ruled out.

In order to confirm that chloramphenicol is conjugated to C₁ instead of C₂ of the glucuronide, β-glucuronidase hydrolysis of F₃ should be performed. However, it is known that the rearrangements of glucuronides from C₁ to C₂ occur only in ester glucuronides, but not for ether linkages such as found in chloramphenicol glucuronide.

The fourth possibility is that F₃ is the glucuronide of the enantiomer of CP. I have not proven this conclusively, but have ruled out all the other possibilities. The NMR spectra of F₃ and F₄ are nearly identical.

The close agreement of the chemical shifts of F₃ and F₄ as well as the connectivity shown on the two dimension experiments rule out different sites of attachment on both the sugar and the CP. That leaves only stereochemical differences. The possibility of stereochemical isomerizations of the sugar (i.e. α or β) is ruled out by the coupling constant of the anomeric proton. The only other possibility is that F₃ is the glucuronide of the enantiomer of chloramphenicol which either racemizes in the body from D(-)-threo isomer or it is the conjugate of an L(+) threo impurity in the standard chloramphenicol (both radioactive and nonradioactive) used in this study. Racemization of chloramphenicol in the body is a new phenomenon in chloramphenicol metabolism in any species. In order to confirm if this finding is a genuine metabolic process of chloramphenicol, more investigations are necessary. One possible experimental approach would be derivatizing the standard chloramphenicol with a chiral compound and analysis by HPLC to detect any L(+) threo isomer contamination in the standard CAP.

Further work is needed to identify remaining metabolic pathways of chloramphenicol in dogs. Subfractions of F₁ were not completely separated on reverse
phase HPLC column since they were very polar. Even the use of pure water as a mobile phase was not successful in separating the subfractions of F1.

Other than the inability to identify the subfraction of F1, this study established significant results regarding pharmacokinetics and metabolism in dogs. The pharmacokinetic parameters of chloramphenicol were estimated using HPLC and a specific radiochemical assay. The median of $t_{1/2}$ was 64 minutes after estimation with two methods. The median of the clearance and the volume of distribution were 5.0 ml/min/kg and 0.7 L/kg, respectively after estimation with the two methods. The major pathway of metabolism of chloramphenicol was glucuronidation and glycolic acid formation. Chloramphenicol metabolites were identified using CI-mass spectroscopy, fast atom bombardment mass spectroscopy and NMR spectroscopy.
SUMMARY

Chloramphenicol is a broad spectrum antibiotic used for treatment of bacterial and rickettsial infections in humans and animals. Because it has produced aplastic anemia in humans, chloramphenicol was banned by the Food and Drug Administration (FDA) for use in food producing animals. However, chloramphenicol is still used in treatment of certain infections such as meningitis and typhoid fever in humans and small and large animals.

Basic data on chloramphenicol metabolism were generated more than 30 years ago by Glazko et al. in humans, dogs, and rats using chemical techniques that are presently outdated. In the present study, chloramphenicol pharmacokinetics and metabolism were studied in dogs using high pressure liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and mass spectroscopic methods.

$^{14}$C-chloramphenicol with a specific activity of 57.8 mCi/mmol was mixed with nonradioactive chloramphenicol and injected as a single bolus intravenously at a dose level of 50 mg/kg in 3 female beagles. Blood samples for estimation of pharmacokinetic parameters were collected. Urine samples, for isolation of metabolites were collected for 48 hours. Pharmacokinetic parameters were estimated from the serum concentration of chloramphenicol measured by both high pressure liquid chromatography (HPLC) and radiochemical methods.

When chloramphenicol concentrations were determined by HPLC in 3 dogs, the disposition half-lives ($t_{1/2}$-alpha) were 2.5, 0.8 and 1.1 minutes, the elimination half-lives ($t_{1/2}$-beta) were 105, 63, and 64 minutes, the body clearances (BC) were 5.2, 7.4, and 5.0 ml/min/kg, and the area volumes of distribution (VDA) were 0.7, 0.7, and 0.5
L/kg. After the radiochemical assay of chloramphenicol of the same serum samples of
the 3 dogs, the $t_{1/2\text{-alpha}}$ were 1.9, 0.7, and 0.8 minutes, the $t_{1/2\text{-beta}}$ were 88, 61, and
64 minutes, $BC$ were 5.6, 7.4 and 5.0 ml/min/kg, VDA were 0.61, 0.6 and 0.5 L/kg.

The pharmacokinetic parameters of chloramphenicol estimated by HPLC and
radiochemical methods in the present study fell within the range of the same parameters
estimated in another study by the author using different but specific methods such as
gas chromatography and bioassay for estimation of chloramphenicol.

To study the metabolic profile of chloramphenicol in urine, an aliquot of each
urine sample was filtered through 0.2 micron filter paper and injected directly onto C18
HPLC column attached to a Hewlett-Packard HPLC equipped with ultraviolet and
radiochemical detectors. The mobile phase consisted of 50:50 methanol: acetonitrile and
5 mM phosphate buffer at pH 2.5. Initially the mobile phase was 10 percent organic, 90
percent aqueous. The organic phase increased to 55 percent over a period of 50
minutes. The urinary profile of chloramphenicol metabolites contained 5 major
detectable peaks of radioactive fractions (F) 1-5. F1, 2, 3, 4, 5, contained 10.8, 16.2, 5,
56 and 12 percent of the excreted radioactivity in the urine of the dogs.

After isolation and identification, F5 was identified as active chloramphenicol by
using chemical impact (Cl) mass spectroscopy and nuclear magnetic resonance (NMR).
The identification was additionally confirmed by comparing the Cl mass and NMR
spectra of F5 to those of authentic chloramphenicol.

F4 was identified as chloramphenicol glucuronide by comparing its fast atom
bombardment mass spectroscopic and NMR spectra to that of authentic chloramphenicol
glucuronide. F3 was identified as the enantiomer of chloramphenicol-glucuronide
isolated from the horse (L(+)-threo isomer). F2 was separated into two subfractions F2B
and F2A. F2B was identified as chloramphenicol-glycolic acid after identification with
CI mass-spectroscopy. F2A and F1 were not identified in this study. The major pathway of chloramphenicol metabolism in dogs is glucuronidation.
APPENDIX A
### TABLE 2

**Serum Concentration of Chloramphenicol after Analysis with HPLC and Radiochemical Methods**

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<th>Dog 3 RCA</th>
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### TABLE 3

Serum Concentration of Chloramphenicol after Analysis with HPLC and Radiochemical Methods

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Figure 21. Two dimensional C-COSY NMR spectrum of F3 isolated from dog urine.
Figure 22. $^{13}$C-$^1$HNMR correlation spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 23. $^{13}$C-H NMR correlation spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 24. $^{13}$C NMR spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 25. $^{13}$ CNMR spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 26. Two dimensional (COSY) NMR spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 27. Two dimensional (COSY) NMR spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 28. Two dimensional (COSY) NMR spectrum of chloramphenicol-glucuronide isolated from the horse.
Figure 29. EI-mass spectrum of standard chloramphenicol-glycolic acid.
Figure 30. EI-mass spectrum of standard chloramphenicol base.
Figure 32. EI mass-spectrum of standard chloramphenicol.
APPENDIX B
Nuclear Magnetic Resonance (NMR) Spectroscopy:

NMR spectroscopy is based upon the absorption of radio waves by certain nuclei in organic molecules when they are in a strong magnetic field. The nuclei of atoms of all elements have a spin quantum number. The important isotopes (H, $^{13}$C, $^{19}$F) have a nuclear spin of 1/2. Common isotopes of carbon ($^{12}$C) and oxygen ($^{16}$O) have a nuclear spin of 0. The most common isotope studied by NMR methods is $^1$H the proton.

The NMR Spectrum:

The sample is placed in a magnetic field and is irradiated with radio waves. When the protons flip from the parallel to the antiparallel state, the absorption of energy is detected by a power indicator. In order to obtain quantitative measurement, we need a reference point. The compound that has been chosen for this reference point is tetramethylsilane (TMS), (CH$_3$)$_4$Si, the proton of which absorbs to the far in the NMR spectrum. The absorption for most other protons is observed downfield from that of TMS. In practice, a small amount of TMS is added directly to the sample, and the peak for TMS is observed on the spectrum along with any absorption peaks from the sample compound. The difference between the position of absorption of TMS and that of a particular proton is called the chemical shift.

Chemical shifts are reported as part per million (PPM) of the applied radiofrequency. At 500 MHz, 1.0 PPM is 500 Hz downfield from the position of absorption of TMS, which is set at 0 PPM.

The intensity of the signal produced by each proton is proportional to the amount of the compound in the sample. However, an equivalent number of protons produce the same integrated area in the spectrum.

When there are protons on adjacent carbons, spin–spin coupling occurs. This splits the observed resonance into several peaks, the multiplicity and magnitude of which are dependent upon the number of adjacent protons and the chemical environment.
**CI-Mass Spectroscopy:**

Mass spectroscopy is based upon the ionization of compounds in the sample using a high energy electron beam (10-70 eV) to excite molecules from the sample in the vapor state. In CI mass spectroscopy, a reagent gas such as methane (CH$_4$) is used. The reagent gas is ionized by the electron beam and in turn the gas ionizes the compound in the sample. The resulting molecular ion represents the molecular mass of the compound plus proton. After ionization, ions are then sorted according to their mass/charge (m/e) ratio. A mass spectrum is a record of the mass and relative abundance of the ion produced.

**Fast Atom Bombardment (FAB) Mass Spectroscopy:**

This technique is suitable for identification of compounds that are nonvolatile under the high vacuum conditions of mass spectroscopy, such as glucuronide. Compounds are placed in a matrix such as glycerol and then bombarded with compressed xenon gas. Xenon displaces the compound from the matrix and the compound is ionized in the vapor phase. As in CI-mass spectroscopy, ions are sorted according to their mass/charge (m/e) ratio. A mass spectrum is a record of the mass and relative abundance of the ion produced.


27. Morris PL, Burke TR, Jr. and Pohl LR: Reductive dechlorination of chloramphenicol by rat liver microsomes. Drug Metabolism and Disposition 1:126-130, 1983.


