THE DISPOSITION OF GENTAMICIN IN EQUINE PLASMA, SYNOVIAL FLUID AND LYMPH

A THESIS

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ABSTRACT

Although it is easy to monitor blood concentrations of antimicrobials most bacterial infections occur in extravascular sites, more specifically within the interstitial fluid. It is very difficult to sample interstitial fluid and many different methods have been used. Reports of the relationship between blood and interstitial concentrations of antibiotics have varied depending on the tissue/tissue fluid sampling technique used. The sampling of tissue fluid for antimicrobial studies in horses has been limited. Most studies have measured antibiotic concentrations in readily accessible body fluids such as urine, peritoneal fluid and synovial fluid. The relationship between these fluids and interstitial fluid in the horse is not known.

The disposition of gentamicin in equine plasma, synovial fluid and in peripheral lymph was studied. A lymph vessel (dorsal digital lymph trunk) on the medial aspect of the distal hindlimb was selected for the disposition study. To better define the relationship between synovial fluid and tissue concentrations of an antimicrobial it was shown that this vessel had a contribution to its lymph derived from the synovium of the fetlock joint. Very high concentrations of gentamicin were retrieved in the lymph collected from the cannulated vessel after intra-articular injection (150mg dose). The mean maximum lymph gentamicin concentration was approximately 50 μg/ml and the time to reach this, approximately 1.7 h after joint injection. The mean synovial fluid concentration 0.25 h following injection was 7244 $\pm$ 660 μg/ml and disappearance from the synovial fluid was consistent with first order kinetics with a mean disappearance half-life (harmonic mean) of 0.99 (0.83-1.22) h.

A technique for chronic cannulation of the dorsal digital lymph trunk was developed. Two Trials were conducted and in the first (Trial A) the disposition of gentamicin in plasma and lymph was studied after intravenous injection (2.2 mg/kg). In Trial B the disposition of gentamicin in plasma, synovial fluid and lymph was studied. Kinetic parameters were similar to other reported studies. There was no significant difference in kinetic parameters

between trials. The disposition curves for all three fluids were similar. Mean maximum lymph concentrations were approximately 4.6 μg/ml and were 40% of the plasma concentrations 15 minutes after injection. These were achieved approximately 1.35 h after injection. The maximum concentration of gentamicin in synovial fluid (2.86 $\pm$ 0.45 μg/ml) was significantly less than in lymph. Three hours after injection plasma, synovial fluid and lymph concentrations were very similar and it was concluded that a sample of any one would be a good index of the others at this time. The relationship between synovial fluid and tissue fluid 3-8 h after injection was less clear with marked divergence of the disposition curves. Gentamicin was more slowly eliminated from lymph than plasma but a parallel relationship between the two fluids was observed 3-8 h after injection, with a mean lymph:plasma ratio of approximately 1.6. It was concluded that plasma concentrations were a good index of tissue fluid concentrations.

Maximum lymph concentrations of gentamicin after intravenous injection were 10 times less than after intra-articular injection. The presence of very high concentrations in lymph derived from the synovium of a joint after intra-articular injection suggest that subsynovial interstitial fluid concentrations are also this high and therefore that intra-articular injection may have some therapeutic advantage over systemic injection.

Lymph cannulation in the horse appears to be a viable technique for antimicrobial disposition studies.

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CHAPTER I INTRODUCTION

I_1 GENERAL INTRODUCTION

Ever since antimicrobial drugs became available, researchers have persistently endeavoured to more accurately define the distribution of these drugs into blood and tissues. More than forty years ago Florey *et al* (1946) observed that penicillin administered to battle casualties was still detectable in infected wound exudates several hours after it had disappeared from the blood stream. Weinstein (1951) emphasised the problem of the distribution of antibiotic between blood and tissue fluids. He stated "there is little doubt that the tissue level of penicillin is related to the blood concentration but the degree of relationship, particularly as it involves the duration of antibacterial effect, is not settled." Other investigators (Calnan *et al* 1972, Ziv *et al* 1982) have agreed with this reasoning and Bengtsson *et al* (1989) added " knowledge of the antibacterial concentration that can be expected at a site of infection, which is usually outside the bloodstream, would enable the clinician to optimize the therapy by choosing the most appropriate drug, dosage, dosage interval and mode of administration".

Despite the consistent efforts of researchers, the concentration of an antimicrobial drug expected to be present at sites of infection is still being extrapolated from its concentration in blood. In recent years however, considerable effort has been expended in trying to relate the level of antimicrobial drug in blood to that at sites of infection (Barza 1981). This is because prediction of the tissue levels of antimicrobial drugs from blood concentrations would be more accurate if the exact relationship between the levels in the two sites was known. In order to define this relationship it is necessary to sample tissue fluids from sites of infection. Because bacterial pathogens reside in the interstitial fluid spaces (except in the case of septicaemias and infections involving cells of haematopoietic and lymphatic origin - Clarke 1989), interstitial fluid is the tissue fluid that most investigators attempt to sample. Furthermore, systemically administered antimicrobial drugs must first pass through the endothelial lining of blood capillaries and into the interstitial fluid of the tissues before reaching sites of infection.

However, direct sampling of the interstitial fluid is extremely difficult because of the nature of the interstitial space. This space is a three dimensional lattice of minute clefts interspersed between tissue cells and traversing capillaries (Bergan 1981). Collagen fibre bundles and proteoglycan filaments comprise the solid structures which form these clefts. Of the fluid found in the interstitium almost all is entrapped in the spaces among the proteoglycans forming a gel, the so called 'tissue gel'. Under normal circumstances, the amount of free fluid present is negligible although occasionally small rivulets of free fluid and free fluid vesicles are present (Guyton 1991).

To overcome the problems associated with direct sampling of interstitial fluid, a number of methods have been devised to increase the volume of fluid available for sampling and these are discussed below.

I_2 METHODS USED TO DETERMINE THE CONCENTRATION OF ANTIMICROBIAL DRUGS IN TISSUE FLUIDS

Examples of the different methods that have been used to determine the concentration of antimicrobial drugs in tissue fluids include tissue homogenates (e.g. Ercoli *et al* 1948, Schacter 1948, Baxter and McKellar 1990, Brown *et al* 1990), tissue chamber fluid (e.g. Chisolm 1973, Eickenberg 1978, Bengtsson 1989, Clarke 1989), lymph (e.g. Schacter 1948, Verwey and Williams 1962a,b, Cockett *et al* 1966,1967, Chisholm *et al* 1968, Lithander *et al* 1968, Acred *et al* 1970, Bergan *et al* 1973, Andersson *et al* 1976, Roberts *et al* 1979, Walstad *et al* 1983, Cohen *et al* 1984, Franklin *et al* 1986 and May *et al* 1987), fibrin clots (e.g. Weinstein 1951, Barza and Weinstein *et al* 1974 I,II,III), skin windows (e.g. Gillet and Wise 1978, Tan *et al* 1981 Clarke 1989), skin blisters (e.g. Schreiner *et al* 1981, Ryan *et al* 1982), subcutaneously implanted cotton threads (e.g. Ryan *et al* 1982, Renneberg and Walder 1988), subcutaneous discs (e.g. Barza and Weinstein 1974,I), and in extravascular fluids in body cavities (e.g. Beech *et al* 1977, Stover *et al* 1981, Brown *et al* 1982). A brief description of each of these is presented in Table I.

Table I. **Methods Used to Determine the Concentration of Antimicrobial Drugs in Tissue Fluids.**

Method	Methodology	Anatomical Site and Experimental Subjects	Characteristics of Tissue Fluid
Tissue Homogenate	Various tissues collected at intervals following antimicrobial administration, then homogenised, centrifuged and the tissue fluid extracted.	Various, including - kidney, liver, muscle. Many species including - cat, sheep, rat.	Heterogeneous in composition and may consist of interstitial fluid, lymph, cell cytoplasm and blood. Useful for tissue residue studies (Clarke 1989), adequate volumes of tissue fluid available for analysis.
Tissue Chamber	Hollow perforated devices implanted surgically in different tissues. The fluid which accumulates within chambers is sampled following antimicrobial administration.	Subcutaneous, peritoneum, kidney and prostate. Many species including - horse, dog, rabbit and man.	Volume : 0.2-2 ml. Protein content : 40-90% of serum. Cell content: * early 65% neutrophils, rbc $5x10^4$, wbc $7x10^3$. * late 35% neutrophils, rbc $5x10^4$, wbc $2x10^3$. * early = 2-4 weeks. late = 1-6 months (Bergeron 1986).
Lymph Collection	Surgical cannulation of lymph vessels. Continuous flow allows collection of lymph over various time intervals following antimicrobial administration.	Thoracic duct, kidney, extremity. Many species including - dog, man and sheep.	Volume of flow rates vary from: - 0.2 ml/h peripheral lymph. - up to 26 ml/h thoracic lymph. Protein content: Peripheral 20-25% serum (Barza 1981). Thoracic 50-70% serum (Yoffey and Courtice 1970). Cell content: 80-90% lymphocytes wcc .2-.7$x10^3$/ml peripheral (Smith 1970).

Table I (Contd.) **Methods Used to Determine the Concentration of Antimicrobial Drugs in Tissue Fluids.**

Method	Methodology	Anatomical Site and Experimental Subjects	Characteristics of Tissue Fluid
Fibrin Clots	Human fibrinogen reconstituted to a 2% solution, combined with bovine thrombin and the resultant clots incubated. Implanted surgically and then removed at various times following antimicrobial administration.	Subcutaneous, rabbit.	Clots dissolved with trypsin and antimicrobial content assayed. Volume: 2 ml. Protein Content: < 10% serum. Few wbc on surface of clots (Bergeron 1986).
Skin Windows	Skin abraded by high speed burr. Following antimicrobial administration paper discs applied to area at various times.	Dermis, forearm. Man.	Volume 0.02 ml. Protein content: < 10% serum. Cell content : few.
Skin Chambers	Plastic chamber applied directly over abraded skin. Filled with saline. Following antimicrobial administration, saline removed at intervals and chamber refilled.	Dermis, limb. Man, cattle.	Volume 0.2 ml. Protein content : 2-10% serum. Cell content : extravasation of blood.
Skin Blisters	1. Suction blister: induced by negative pressure for 1.5-2hrs. 2. Cantharidin blister: induced by application of cantharidin plaster for up to 18 hrs. Fluid aspirated by needle at intervals following antimicrobial administration.	Forearm, man. Forearm, man.	1. Volume: 0.01-0.15 ml. Protein content: 10-60% serum. Cell content: 0-1000 wbc/ml at 24 h. 2. Volume: 0.5 ml. Protein content: 70-80% serum. Cell content: 4000 wbc/ml at 2 h 90% leucocytes.

Table I (contd.) **Methods Used to Determine the Concentration of Antimicrobial Drugs in Tissue Fluids.**

Method	Methodology	Anatomical Site and Experimental Subjects	Characteristics of Tissue Fluid
Cotton Thread	Surgically implanted one or many per experimental subject. Removed at intervals following antimicrobial administration.	Subcutaneously or under muscle fascia in skin or flank. Mice, man.	Volume: 2μl/cm. Protein content: 35% serum. Cell content: 30-60% infiltration by neutrophils.
Paper Discs	Surgically implanted and removed at intervals following antimicrobial administration.	Subcutaneously in flank. Rabbit.	Volume: 0.03 ml. Protein content: $<10\%$ serum. Cell content: blood contamination.
Body Cavity Fluid	Aspirated from various body cavities at intervals following antimicrobial administration.	Peritoneal, pleural and synovial fluid cavities. All species.	Volume: Variable. Protein content: $<20\%$ serum. Cell content: low.

In all of these methods, it is not strictly interstitial fluid that is being examined but rather fluids which are thought to be in equilibrium with 'true' interstitial fluid. The precise relationship between these fluids and interstitial fluid is however not known because the exact composition of the latter fluid has not yet been determined (Bergan 1981, Bergeron 1986).

I_3 OBJECTIVE AND PURPOSE OF THE STUDY

The objective of the present study was to investigate the disposition of an antimicrobial agent in plasma, synovial fluid and tissue fluid. The purpose being, to provide information about the drug concentrations expected at these sites and, to allow for the examination of the following hypothesis: "the synovial fluid concentration of an antimicrobial agent is a useful index of its concentration in tissue fluid". For reasons explained above, a knowledge of the tissue concentration of an antimicrobial at the site of infection is important. Furthermore, if this hypothesis was shown to be valid (for a number of antimicrobial agents) then the relatively simple method of sampling synovial fluid could be employed to estimate the tissue fluid concentrations of antimicrobial drugs. This would obviate the need for more invasive methods such as lymph and tissue chamber fluid collection. The tissue fluid selected was peripheral lymph and the aminoglycoside, gentamicin, was the antimicrobial agent studied. This was administered by intravenous injection and appropriate pharmacokinetic methods were utilised.

To understand the factors affecting the selection of fluids studied and the relationship between the concentration of gentamicin found in each, some understanding of the following is required: the factors governing the pharmacokinetics and penetration of antimicrobial drugs from serum into tissue fluids; the pertinent anatomy and physiology of the lymphatic vessel system; the lymphatic drainage, microanatomy and physiology of synovium; and the pharmacology and pharmacokinetics of gentamicin. Therefore, a review of some aspects of each of these subjects is presented below.

I_4 THE FACTORS GOVERNING THE PHARMACOKINETICS AND PENETRATION OF ANTIMICROBIAL DRUGS FROM SERUM INTO TISSUE FLUIDS

Following entry into the blood stream, an antimicrobial agent may distribute throughout the body and pass into the tissue fluids. Passage into the extravascular space occurs by three possible routes: (i) diffusion through capillary pores, (ii) bulk flow through capillary pores and (iii) permeation across capillary membranes. Once in the extravascular fluids, drugs are distributed primarily by diffusion. They may be taken into cells or leave the extravascular spaces by back diffusion through capillary pores, by permeation through capillary membranes, or by diffusion or bulk flow through the lymphatics (Barza 1981). The factors affecting the pharmacokinetics and penetration of these drugs into tissues and tissue fluids have been extensively reviewed by a number of authors (Barza 1981, Bergan 1981, Bergeron 1986, Ryan 1986 and Clarke 1989). These factors can be divided into two, the physicochemical properties of the particular drug and, the structure and geometry of the tissue/tissue fluid compartment the drug is transferring into.

Physicochemical Properties

The molecular size, lipid solubility and pKa are all physicochemical properties of antimicrobials which determine the ease with which these drugs can cross membranes, capillary pores or enter specialised sites (e.g. CNS). Furthermore, the degree of protein binding that each drug exhibits in serum/plasma, determines the amount of drug available for transfer. This is because it is only the unbound or freely diffusible drug which can readily traverse the capillary membrane. Although these properties are important considerations in understanding how antimicrobials may differ to each other in their abilities to penetrate into tissue fluids, many researchers now believe that the rate of entry and elimination from these fluids, is more dependent on the structure and geometry of the tissue/tissue fluid compartment into which the drug transfers.

Structure and Geometry of the Tissue/Tissue Fluid Compartment

Conflicting results abound in the literature with regards to the tissue penetration of an antimicrobial when measured using different tissue fluid sampling methods. For example, the concentration time profile for tissue chamber fluid levels of cefuroxime administered parenterally to mice (Ryan 1978), was totally different to that for subcutaneous tissue fluid. Tissue chamber fluid concentrations peaked at approximately 1 hour post injection, at a time when practically all the antibiotic had disappeared from subcutaneous tissue fluid.

Researchers have attempted to explain some of the inconsistencies between various methods by focussing on the structure and geometry of the tissue/tissue fluid compartment that each method samples. It seems that such factors as the presence or absence of barriers to drug diffusion, the volume of the fluid examined, and the surface area to volume ratio of the fluid into which the antimicrobial distributes, are all important in determining the drug kinetics observed for a specific tissue fluid sampling method. When considering these factors two groups of methods used to sample tissue fluids emerge: Those which

1. Directly sample natural tissue components:
    - lymph
    - tissue homogenates
    - skin windows (dermabrasion)
    - subcutaneous tissue fluid
    - wound exudates
    - body cavity fluids

or

2. Sample created artificial compartments:
    - skin blisters (suction, cantharadin)
    - skin chambers
    - fibrin clots
    - tissue chambers

The penetration of antimicrobial drugs into the fluids in these two groups is quite different. It appears that, (apart from body fluid sampling) in the fluids sampled from natural tissue components, there is rapid (dynamic- or pseudo-)* equilibration of drug between intravascular and extravascular sites. These fluids most closely represent interstitial fluids and rapid equilibration is due to short diffusional distances and an absence of penetration barriers (Clarke 1989). Thus, in these methods the tissue fluid antimicrobial concentrations tend to closely parallel serum concentrations.

In comparison, the penetration of drugs into and the elimination from, the fluids of the artificial compartment methods, is slow (Bergeron 1986). These sample fluids have relatively large volumes and long diffusional pathways and/or penetration barriers (Clarke 1989). Barza (1981) emphasised that it was the volume of the fluid pool being sampled that greatly effected the distance an antimicrobial drug must diffuse to equilibrate between the serum and the centre of collection. He thus described blister fluid, synovial fluid, skin chamber fluid and the fibrin clot method as examples in which there was a 'small reservoir' of fluid, generally containing 1ml or less. Equilibration of drug between serum and this tissue fluid takes longer than for the rapidly equilibrating methods outlined above.

'Large reservoirs', are typified by the fluid sampled from the peritoneal and pleural cavities. These fluid cavities usually contain more than a few millilitres of fluid (especially when inflamed) and antibiotic must diffuse over comparatively long distances to reach equilibrium.

Recently, in a review of the literature Ryan (1985,1986) suggested that the major determinant of the antimicrobial kinetics of tissue penetration is the surface area to volume ratio of the particular tissue/tissue fluid compartment. Hence, for methods in which natural tissue components are sampled the SA/V ratio is very high and so tissue fluid

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* Although the terms "equilibration" and "equilibrium" are used throughout the text, it should be noted that intravascular and extravascular concentrations never reach a true equilibrium because drug is constantly being excreted. More correctly, a "pseudo" or "dynamic" equilibrium is reached.

concentrations of antimicrobials tend to parallel those in serum: thus serum concentrations of these drugs would be good indicators of the expected concentrations in the tissues. Conversely, for methods in which fluids from artificial compartments are sampled a low SA/V ratio exists and the rate of entry and elimination of antimicrobials into these fluids is slow and do not reflect the concentrations of drug in serum very well.

All these concepts are in keeping with the fact that the distribution of these drugs into such tissues obeys Fick's Law of diffusion (Appendix I). In its simplified form, this law basically states that the concentration of antimicrobial achieved in an interstitial compartment is proportional to the diffusible surface area and inversely proportional to the volume of the compartment.

I_5 METHODS USED TO DETERMINE THE CONCENTRATION OF ANTIMICROBIALS IN TISSUE FLUIDS OF THE HORSE

Reports of studies measuring antimicrobial agents in tissue fluids in horses have been few. In the past, the distribution and elimination (= disposition) of antimicrobial drugs has been studied in serum and plasma together with their penetration into fluids of various body cavities and in urine (eg. Beech *et al* 1977, Beech *et al* 1979, Stover *et al* 1981, Brown *et al* 1982). Typically, synovial fluid, and peritoneal fluid have been the body cavity fluids sampled. In recent times however, researchers have attempted to sample tissue fluid by using such methods as implanted tissue chambers, tissue homogenates and in the present study, lymph. Because of this, a summary of the features of each method is outlined below.

Tissue Chambers

Tissue chambers are hollow perforated devices and have usually been implanted at subcutaneous sites around the body, within the peritoneal cavity, and in various organs such as the prostate and kidney. First used by Guyton in 1963 to investigate interstitial

fluid pressure, they have since been used widely in different animals to study antimicrobial tissue penetration as well as *in vivo* efficacy of antimicrobial agents, growth characteristics of various microbes, and pathophysiological mechanisms of inflammation (Clarke 1989).

In the horse, Higgins and Lees (1984) used tissue chambers to study acute inflammation, and more recently Short (1987) studied the tissue fluid concentrations of cepharin sodium using these devices.

Perforated, polypropylene golf balls, steel springs, silicon tubing and thermoplastic are just some of the objects and materials used. After implantation, the chambers fill with blood and inflammatory exudate, and after some weeks become invested with a fibrous vascularised capsule. The fluid that accumulates within the cavity eventually equilibrates with the interstitium and is believed to represent interstitial fluid. These tissue chambers provide comparatively large volumes of tissue fluid that can be conveniently sampled for pharmacological investigations.

Some investigators have criticised the tissue chamber technique (Ryan 1978, Bergan 1981). Ryan believes the thick fibrous capsule that surrounds implanted tissue chambers specifically impedes the passage of antibiotic into, and out of, these devices. Bergan considered that tissue chamber fluid was not representative of interstitial fluid for two reasons. Firstly, the composition of chamber fluid, particularly the protein concentration, was highly variable and related to the length of time after implantation. Secondly, the immediate fluid balance that occurs between capillaries and interstitial fluid in normal tissues (and hence between serum and tissue concentrations of antibiotics) could not be duplicated using these artificial devices. Bergan further stated that, using this technique, different research groups obtained widely varying results for the tissue penetration of antimicrobial agents. There was variation in penetration regardless of the level of antimicrobial binding to serum proteins, type of tissue chamber or antimicrobial. Variation was even found when data was compared between different research groups that studied identical agents.

One important explanation for these differences was the variation in chamber size used by researchers. Differences in surface area to volume ratios could affect available area for drug diffusion. Another source of variation, particularly for individual animals or within laboratories, is the uncontrollable way that the tissue chambers are closed off by fibrous tissue with time. Repetitive needle sampling of tissue chambers resulting in blood contamination could also be a source of variation and error using this method. Bergan concluded, "due to the scattered nature of results, tissue chambers do not permit one to make clear conclusions regarding antibiotic tissue penetration".

Tissue Homogenates

Tissue homgenates have been used to study the penetration of various antimicrobial agents into the equine endometrium (e.g. Haddad *et al* 1985a, Pedersoli *et al* 1985, Brown *et al* 1989). Snyder and Pascoe (1985) measured the concentration of gentamicin in tissue biopsied from small intestine and large colon in anaesthetised horses after intravenous administration.

The analysis of different tissues at intervals after antimicrobial administration, would seem a logical method of determining if adequate levels of drug were present. However, a number of researchers have criticised this method (Chisholm *et al* 1973, Gillett and Wise 1978, Bergan 1981 and Clarke 1989). Bergan emphasised that tissue homogenates may consist of a number of fluids e.g. interstitial fluid, lymph, cell cytoplasm and special tissue components such as bone, secretions, urine, bile and even blood. As a consequence, the accurate interpretation of data from this source was difficult. To correct for the contributions of the various tissue components in homogenates an equation and complex calculations have been proposed (Bergan 1981). This would allow for the estimation of interstitial fluid concentrations of antimicrobials. Researchers, however, seldom make these corrections.

In addition, other problems exist, such as those involving standardization of technique, binding to tissue components and uncertainties involving enzymatic inactivation of antimicrobials. For these reasons, this technique is likely to be highly unreliable.

Lymph

The penetration of antimicrobial agents into lymph has not been reported in the horse. However, researchers have been using this fluid for antimicrobial studies in other species for some time, in fact more than forty years ago Schachter (1948) compared thoracic lymph concentrations of penicillin with those in blood, in the anaesthetised dog. He found that lymph levels of penicillin exceeded those in blood within a few hours of intravenous or intramuscular injection and persisted for much longer. This was the first time lymph was used to study the penetration of antimicrobials into tissue fluid and since then, many investigators have used lymph concentrations of these substances as an indication of interstitial fluid levels (as cited above).

The basic premise on which all these studies are founded is that lymph is a suitable representative of interstitial fluid. In fact, some workers believe that lymph and interstitial fluid have identical composition (Drinker and Yoffey 1941, Yoffey and Courtice 1970, Courtice 1971). If this was so, the protein concentration of interstitial fluid would be the same as that found in the lymph inside the initial lymph capillaries. Moreover, concentration of protein would not occur along the length of the larger lymph vessels. This has been refuted by Rusznyak *et al* (1967), Casely-Smith (1972, 1977), Rodbard and Feldman (1975), Szabo and Magyar (1978), and others. From electron microscopic studies Casley-Smith proposed that an osmotic differential across the lymph capillary membrane is responsible for the formation of lymph. The maintenance of this differential is due to the concentrating of protein within the initial lymphatic capillaries, by the movement of water out into the tissue fluid.

Szabo and Magyar (1978) compared the concentration of various enzymes in peripheral lymph and in "tissue fluid" (collected from subcutaneous implanted cotton wicks) from the limbs of rabbits. Protein concentrations in the subcutaneous tissue fluid were markedly higher than in lymph draining the same region for both normal and pathological conditions. Based on these findings it was proposed that there are two compartments within the interstitium that relate true interstitial fluid with plasma and lymph. Compartment one represents the circulation of the plasma filtrate across the walls of the blood capillaries which is then directly drained by the lymphatic capillaries. Compartment two is the true interstitial fluid which contains the extravascular protein pool and protein molecules derived from cells. It is a stationary compartment not drained by the lymphatic capillaries but in dynamic equilibrium with the fluid from the first compartment.

However, several other studies support the contention that the protein concentrations in interstitial fluid and lymph are identical. Microflurometric analyses of frozen sections of lung revealed similar concentrations of albumin in the extravascular spaces and lymphatic lumen. In another study, microsampling of subcutaneous tissue fluid showed that the interstitial fluid/lymph concentration ratio for albumin, γ-globulin and transferrin was 1 (Granger *et al* 1984).

Regardless of the intricacies of this debate, many researchers believe that lymph generally reflects the interstitial environment from which it is derived. Chen *et al* 1976 argue that "since we measure an average functional capillary pressure, tissue pressure, etc., then lymph fluid must reflect some average tissue fluid in a steady state". Furthermore, peripheral lymph, that is, lymph draining the extremities, is thought to be a very reliable source of tissue fluid for antimicrobial studies (Bergan 1981, Clarke 1989). In contrast, thoracic duct lymph or central lymph, although used in earlier antimicrobial penetration studies, is not representative of normal interstitial fluid. It has a high protein content and receives large contributions from organs which have a capacity to metabolise, excrete and accumulate drugs, e.g. the liver, kidneys and the gastrointestinal tract (Verwey *et al* 1965).

I_6 ANATOMY AND PHYSIOLOGY OF THE LYMPHATIC VESSEL SYSTEM

The lymphatic system can be divided into two anatomical components
(1) the cellular component, and
(2) the vascular component

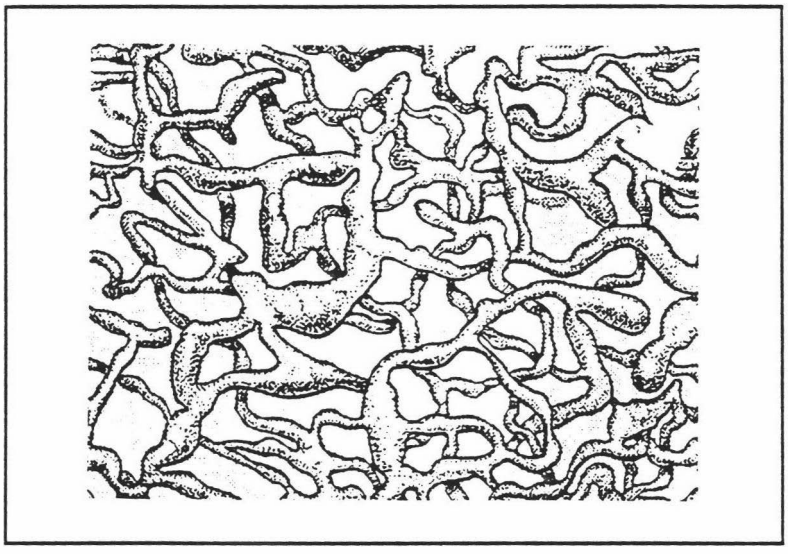
The cellular component is comprised of the lymphatic tissue in lymphatic organs. Its function is defence. In the present study, only the vascular component is of interest, and so the anatomy of this system is presented below.

Anatomy of the Lymphatic Vessel System

The lymphatic vessel system forms a tubular system which closely parallels the blood vessels. There are, however, some areas of the body in which lymphatic vessels have not been found. These include the brain, spinal cord, eye, bone, epidermis and in the epithelial lining of the mucosal surfaces (Gnepp 1984). However, even these tissues have minute interstitial channels called prelymphatics through which interstitial fluid can flow. Eventually, this fluid flows into lymphatic vessels but in the central nervous system, it flows firstly into the cerebrospinal fluid and then directly into the blood (Guyton 1991).

Lymphatic vessels consist of several different structural segments, all designed to provide centripetal drainage of the lymph from the tissues (Nickel *et al* 1981). At the distal end of the lymphatic vessel system are the lymph capillaries. These form networks of vessels which terminate in blind processes (Figure 1).

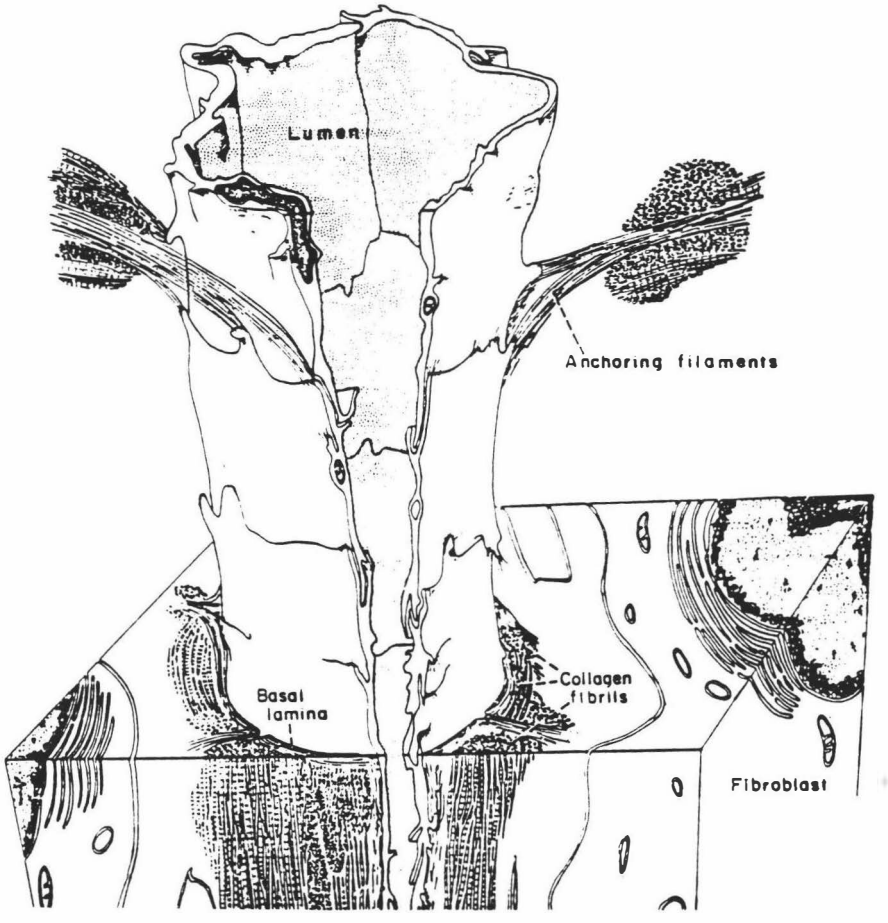
Figure 1. Diagrammatic Illustration of Lymph Capillary Network.



(adapted from Nickel *et al* 1981)

The walls of the capillaries are one cell thick and the cell boundaries irregularly notched. The intercellular space is large (9 nm) and the basement membrane is incomplete. A striking feature of the lymph capillaries are the reticulum fibres (also known as anchoring filaments) that attach the capillary wall to the surrounding connective tissue. Figure 2 shows a three-dimensional interpretative diagram of a lymphatic capillary that was reconstructed from collated electron micrographs. Illustrated is the three-dimensional relationship between the lymphatic capillary and the surrounding connective tissue. It appears that the lymphatic anchoring filaments originate from the endothelial cells and extend among the collagen bundles, elastic fibres, and cells of the adjoining tissue area, thus providing a firm connection between the lymphatic capillary wall and the surrounding connective tissue. The importance of these fibres in the formation of lymph is discussed under "The Formation of Lymph".

Figure 2. Diagrammatic Illustration of a Lymph Capillary.



(adapted from Gnepp 1984).

Lymph flows from the lymph capillaries into the collecting vessels which propel the lymph into the great veins cranial to the heart. These vessels progressively increase in diameter. Their endothelial cells tend to have fewer open junctions, a more prominent basement membrane exists, and three distinct wall layers (intima, media and adventitia) become apparent. Nickel *et al* (1981) has conveniently divided the collecting vessels into two. The first are the conducting vessels. These have the same wall structure as the lymph capillaries but are of greater calibre and possess bicuspid valves. The conducting vessels move lymph from the capillaries to the second of the collecting vessels, the transport vessels. The transport vessels have a muscular wall present in the media (which provides the means for independent movement of lymph) and numerous regularly distributed valves. Each valve segment is known as a lymphangion. The lymphangion consists of a pair of valves and the adjacent length of vessel, which has a lining endothelium and a cuff of muscle tissue. There is no muscular tissue at the insertion of the valves and so each lymphangion acts as an independently contracting unit. The lymph transport vessels run parallel to each other and have extensive lateral intercommunications. They link the lymph drainage area to the regional lymph nodes.

The first comprehensive descriptions of the lymph vascular system of the limbs of the horse were reported by a German anatomist Herman Baum (1920, 1927). Following Baum, and using a variety of techniques, Rossoff (1946), Laue (1987) and Meyer (1988) also studied the distribution of these vessels. In the present study, the lymphatic vessel system of the fore and hindlimbs of the horse was investigated and specifically the lymphatic drainage of the fetlock joint. Some of the techniques performed by the above researchers were used. A review of the ways that these and other researchers have studied these lymphatic vessel systems over the years, is presented below.

Investigation of the Anatomy of the Lymphatic Vessel System

Generally, in order for the lymphatic vessels to be seen, they must first be injected with some visible material. Natural injection occurs postprandially, as in lacteals which absorb lipids from the small intestine. In fact it was these 'milky veins' that Aspelli saw in the mesentery of a well fed dog in 1622 which marked the discovery of the lymphatic system.

Over the centuries, different techniques have been devised for making lymph vessels visible. Because these vessels have a very efficient valve system, retrograde injection of materials is not usually possible and so orthograde injections have to be made. Researchers achieved this by inserting a cannula into large lymph trunks and injecting such diverse materials as water (coloured or uncoloured), air, milk, and mercury mixed with both lead and zinc. This technique is known as direct lymphography.

Late last century it was realised that direct injections of materials into lymph vessels was not necessary and that, by injecting materials into interstitial tissue, the adjacent lymph vessels could be outlined. This technique is known as indirect lymphography (Rusznyak *et al* 1967). Injection agents used in this procedure include Prussian blue, coloured gelatin carmine mixed with mercury, various aqueous dyes (Trypan blue, Evans blue, Pontamine sky blue, Patent blue violet) and particulate suspensions such as Indian ink (Yoffey and Courtice 1970).

Iodinated radiographic contrast agents injected directly into surgically exposed lymph vessels (direct lymphangiography) have allowed clinicians to investigate problems associated with lymphoedema in man (Kinnmonth 1952) and in the horse (Fackleman *et al* 1974) and to identify tumour metastasis to lymph nodes (Yoffey and Courtice 1970). Indirect lymphangiography with newer aqueous iodinated radiographic contrast agents has also been used in the horse (Meyer 1988).

Light and electron microscopy have been used extensively to investigate the lymphatic system, e.g. Casley-Smith (1969), Leak and Burke (1968 a,b).

The mechanisms of lymph production and the way in which the lymph vascular system transports lymph from the periphery to the heart, is presented below.

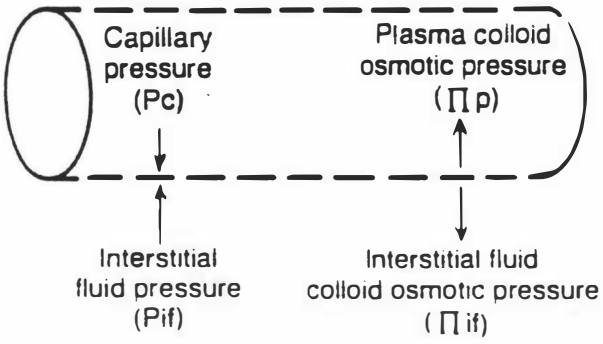
The Formation of Lymph

Lymph is basically excess interstitial fluid that flows into lymphatic vessels. This excess interstitial fluid results from the continual exchange of fluid that occurs across the capillaries, between the blood and the extravascular matrix. This fluid takes nutrients to, and receives cellular excreta from, the tissues.

Four pressures determine the movement of fluid through the capillary membrane (Guyton 1991), (Figure 3).

1. The capillary hydrostatic pressure (Pc) - which tends to move fluid outward through the capillary membrane.
2. The interstitial fluid hydrostatic pressure (Pif) - which tends to move fluid inward through the capillary membrane when Pif is positive and outward when Pif is negative.
3. The plasma colloid oncotic pressure (πp) - which tends to cause osmosis of fluid inward through the membrane.
4. The interstitial fluid colloid oncotic osmotic pressure (πif) - which tends to cause osmosis of fluid outward through the membrane.

Figure 3. Pressures Determining the Movement of Fluid Across the Capillary Membrane.



The algebraic sum of these four pressures determines the net rate of fluid movement across the capillary membrane (Q) and is described by the Starling Equation:

$$Q = K[(Pc - Pif) - \sigma(\pi p - \pi if)]$$

Where K is the capillary filtration coefficient, which determines the rate of fluid movement across the capillary for a given net driving force. It is dependent on the surface area available for fluid exchange and also on the size of the channels within the endothelial barrier. The symbol σ is the protein reflection coefficient and represents the permeability of the capillary membrane to protein. The equation signifies that the net transvascular flow is proportional to the difference in hydrostatic and protein osmotic pressures across the capillary barrier (Moss and Rice 1984).

Because the hydrostatic force at the arterial end of the capillary bed is greater than in the interstitial tissue there is a net filtration force moving fluid into the tissues. A lower hydrostatic force at the venous end of the capillary bed assists in moving fluid from the

interstitium into the blood. Under normal circumstances, there is a slight imbalance between these two processes and a net filtration pressure of approximately 0.3 mm Hg causes fluid to accumulate in the interstitial spaces (Guyton 1991). As fluid filters from the arterial end of the capillaries, small amounts of protein accompany it. However, even though much of this fluid is reabsorbed by the venous capillaries most of the protein remains in the interstitial fluid. The lymphatic capillaries absorb this excess fluid and protein and return it to the circulation. If this protein is not returned to the circulation, the oncotic osmotic pressure within the interstitial fluid increases and oedema results. In fact, by far the most important function of the lymphatic vessel system is to prevent this occurrence by removing the accumulated protein from the tissues.

The mechanisms by which excess interstitial fluid becomes lymph are not entirely clear. Apparently, when the lymph capillaries are empty they are totally collapsed. It is believed that as the volume of fluid in the interstitial space increases, the associated increase in tissue pressure pulls the reticulum fibres taut and in doing so opens up the interendothelial spaces allowing entry of fluid (Nickel *et al* 1981). The fluid entering the lymph capillaries in this way washes any accumulated tissue proteins with it.

Two theories have been proposed for the force responsible for the actual transfer of interstitial fluid into the lymph capillaries. The most popular theory favours the role of translymphatic hydrostatic pressure. The other proposes a transmural protein osmotic differential as being the main factor involved (Granger *et al* 1984). Once the lymph fluid is formed inside the lymph capillaries it is then propelled along the lymph vasculature. The propulsion of the lymph and thus the lymph flow rate, is determined by two main factors: (1) the lymphatic pump and (2) interstitial fluid pressure.

The Mechanisms of Lymph Propulsion and Factors Affecting Lymph Flow Rate

(1) Effect of the Lymphatic Pump

The propulsion of lymph due to the pumping action of the lymphatic vessels occurs by intrinsic and extrinsic mechanisms.

Intrinsic pumping is effected by the lymphangions of the transport vessels. The lymph flows into the lymphangion which then distends between the valves due to relaxation of the muscular cuff. The increase in pressure forces the inlet valve to close shut and stimulates the muscle cuff to contract. The lymph is thus propelled into the next segment via the outlet valve, which is forced open.

It is now thought that the lymph capillaries may also contribute to the intrinsic lymphatic pump by active contraction. Although the lymphatic capillaries have no smooth muscle the endothelial cells do contain contractile actomyosin filaments (Guyton 1991).

In addition to the fluid motion that is caused by intrinsic contraction of the lymph vessel walls any extrinsic factor that compresses the lymph vessel can also cause pumping. These factors include muscle contractions, movement of parts of the body, arterial pulsations and compression of the tissue by objects outside the body.

(2) Effect of Interstitial Pressure on Lymph Flow

The interstitial fluid pressure (IFP) in loose connective tissue is negative, approximately -3.0 mm Hg. This is due to the suction caused by the pumping action of the lymphatic vessel system. In response to an increase in IFP towards atmospheric levels there is a concomitant increase in lymph flow rate (up to 20-fold) which plateaus when the IFP approaches 2mm Hg. The IFP acts to increase lymph flow rate by increasing the amount of interstitial fluid that enters the lymph capillaries.

The negativity of the subcutaneous IFP acts as a safety factor against oedema formation. Any factor which acts to increase IFP will increase lymph flow and these include elevated capillary pressure, decreased plasma protein colloid osmotic pressure, increased interstitial protein and increased permeability of the blood capillaries.

In summary, the approximate rate of lymph flow can be determined by the product of the activity of the lymphatic pump and the interstitial fluid pressure (Guyton 1991).

I, MICROANATOMY AND PHYSIOLOGY OF THE SYNOVIUM

Microanatomy of the Synovium and Formation of Synovial Fluid

It is generally accepted that the synovial cavity is actually an enlarged tissue space and not a true body cavity such as the peritoneal or pleural cavities (Bauer 1940, Davies 1945, Simpkin and Pizzorno 1974). The synovium which lines the synovial cavity, is not a true membrane since it lacks a basement membrane and does not have a continuous endothelium. In fact, it is formed from mesenchyme and has been regarded as a connective tissue structure since 1866 (Van Sickle and Kincaid 1978). The synovial membrane is characterised by an incomplete intimal layer of synovial cells (synoviocytes) 1-4 cells thick, overlying a subintimal layer of connective tissue (Van Sickle and Kincaid 1978). Light and electron microscopic studies have revealed that the synoviocytes are loosely arranged in parallel fashion, embedded in ground substance rather than lying on the membrane surface. The synoviocytes are separated by intercellular spaces filled with this ground substance so that the interstitial matrix is in direct continuity with the joint space (Lever and Ford 1958, Barland *et al* 1962, Johansson and Renjno 1976).

The synovial membrane is highly vascularised. The blood vessels within the joint capsule and synovial membrane are found in three or four layers. The innermost or subintimal layer, serves the synovial villi and folds. These capillaries are only one cell removed from the synovial cavity. The next layer (located deep to the preceding) contains a large number

of veins and lymphatic capillaries. These lymphatic capillaries, although abundant, are not as numerous or close to the synovial cavity as the blood vessels. Larger arterial and venous vessels are found in the subsynovial and fibrous layers of the capsule (Davies 1945, Van Sickle and Kincaid 1978, Liew and Dick 1981).

The synovial fluid, which fills the synovial cavity, is a dialysate of blood plasma to which hyaluronic acid has been added by the synoviocytes as the dialysate diffuses through the connective tissue surrounding the synovial cavity (Bauer 1940, Yoffey and Courtice 1970, McCarty 1989).

The Drainage of Synovial Cavities by the Lymphatic Vessel System

According to Bauer (1940) it was Heuter in 1866 who first observed lymphatic vessels in synovial tissue. A decade later, Von Mosengeil proposed that well defined stomata linked the articular cavity with the lymphatic vessel system. This was disputed by Tillmanns who was unable to demonstrate lymphatic channels in synovial tissue after intra-articular injections of various substances. He did, however, identify a subintimal and deeper lymphatic plexus by injecting silver metals directly into the synovial membrane. In the early 1900's Baum also used intra-articular injections, experimenting on various animals. In 1920 he successfully delineated lymphatic vessels draining equine joints by intra-articularly injecting "Prussian Blue oil pigment many times diluted with turpentine and ether". He used freshly killed near term foetuses and, after intra-articular injection, the joints were flexed for 5-10 minutes which apparently was long enough to visualise emerging lymphatic vessels.

However, it was not until 1946 that the actual lymphatic drainage of synovial joints was established (Davies 1946). This researcher injected Mandarin Black Ink (50%) into the synovial tissues and joints of cattle (metatarso and metacarpophalangeal). After extending and flexing these joints for 30 to 60 minutes the animals were slaughtered and these areas studied grossly and then histologically. Davies found that intra-articular injection failed to

delineate the lymph vessels of the synovial membrane but ink was generally found in the larger collecting lymph trunks. Stab injections directly into the synovial membrane however, were successful, demonstrating histological evidence of a superficial lymphatic vessel plexus. Collecting trunks (in groups of 2-3) from this superficial plexus passed, with the main blood vessels, towards the flexor aspect of the joint. Here they anastomosed with lymphatic vessels associated with the periosteum where it underlies the synovial membrane (forming a deep plexus, as described by Tillmanns) and also with lymphatic vessels in the periosteum overlying the metatarsal or metacarpal bones.

Permeability of the Synovium and Transynovial Exchange of Proteins and Small Molecules

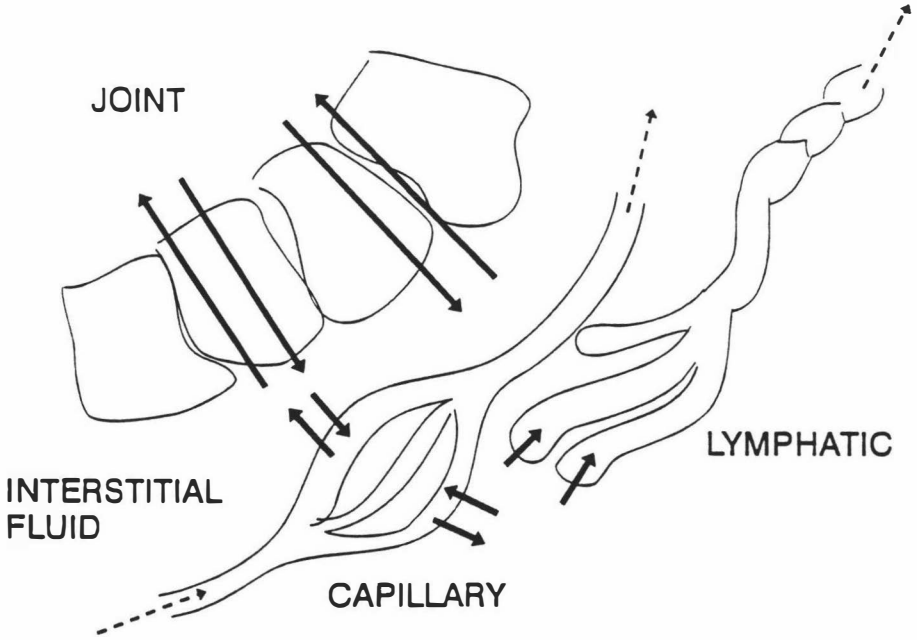
The subsynovial lymphatic vessels appear to play a major role in the removal of proteins from joints (Bauer *et al* 1933, Wallis *et al* 1987).

The interstitium and accompanying lymphatic capillaries of the synovium, also have a part to play in the transynovial exchange of small molecules. Simpkin and co-workers, whilst studying the transynovial exchange of small molecules in humans, viewed the synovium as a permeable barrier through which solutes passed from the blood to synovial fluid and vice-versa. They conceptually subdivided the permeability of this barrier into two processes: (1) passage through the microvascular endothelial wall and (2) diffusion through the interstitial, intercellular spaces. These authors proposed that molecules leave the blood vessels by filtration at the arteriolar end of the microvasculature and also by diffusion throughout the length of the microvessel. The same molecules return to the blood stream by back diffusion, by reabsorption (at the venular end) or by lymphatic drainage. Further exchange occurs continuously between the synovial interstitial space and synovial fluid (Simpkin and Pizzorno 1974, Simkin and Nilson 1981).

I_8 THE RELATIONSHIP BETWEEN SYNOVIAL FLUID AND THE LYMPH DERIVED FROM THE SYNOVIUM

In view of the above, there appears to be a very close anatomical and physiological relationship between synovial fluid and the lymph derived from the synovium. Because of this, it would be expected that the exchange of substances between the blood, synovial fluid and lymphatic capillaries would be the same as for any other connective tissue fluid (Yoffey and Courtice 1970). Figure 4 diagrammatically illustrates the possible exchange of an antimicrobial agent between plasma, synovial fluid and lymph. Drug particles leaving the capillary bed diffuse down a concentration gradient into the interstitial fluid. From the interstitial fluid drug can diffuse in and out of synovial fluid, back into plasma or into lymphatic capillaries.

Figure 4. Diagrammatic Illustration of the Exchange of an Antimicrobial Agent Between Plasma, Synovial Fluid and Lymph.



Given these possible exchanges, the fact that lymph derived from a particular tissue generally reflects the average composition of the interstitial fluid from that tissue and, that these two fluids are in dynamic equilibrium; it would be reasonable to expect that lymph derived from the interstitial fluid of the synovium, also reflects or is in dynamic equilibrium with synovial fluid.

I_9 HYPOTHESIS

With this in mind, and assuming that the antimicrobial concentration of a drug in synovial fluid is related to the concentration of the drug in the lymph derived from the synovium of the same joint, the following hypothesis was proposed: "the synovial fluid concentration of an antimicrobial agent is useful as an index of its concentration in tissue fluid".

Gentamicin was the drug investigated in the present study and so a brief review of its general pharmacological properties, disposition and pharmacokinetics, therapeutic use and toxicity, is discussed below.

I_{10} GENTAMICIN: PHARMACOLOGY AND PHARMACOKINETICS

Pharmacological Properties

Gentamicin is a member of the aminoglycoside family of antibiotics. These antibiotics all have the same basic structure being composed of amino sugars connected by glycoside linkages. The molecular weights of aminoglycosides range from 400-500 g/mol. Because of the large number of amino groups, these molecules are basic polycations with pK_as of 7.2-8.8 and have increased activity at alkaline pH (however, Stover *et al* (1985) report a pH of 3 for a commercial preparation of gentamicin). Penetration of gentamicin (and all the aminoglycosides) into bacteria depends partially on diffusion but also is dependent on an oxygen requiring transport process. For this reason, anaerobic bacteria are resistent to the aminoglycosides and these drugs also have limited activity against facultative bacteria

under anaerobic conditions. The oxygen dependent transport is an electron transport system, a proton motive force which causes the bacterial cytoplasm to be negatively charged with respect to the periplasm and external environment. The positively charged aminoglycosides are thereby attracted electrostatically into the bacterial cytoplasm. This proton motive force can be uncoupled by 2,4-dinitrophenol and carbonyl cyanide *m*-chlorophenol. The effectiveness of aminoglycosides can also be reduced by divalent cations (eg Ca^{2+} and Mg^{2+}), hyperosmolality and decreased pH. In these cases the proton gradient is destroyed but in a different manner than above. After entry into the bacterium, aminoglycosides bind to the 30s ribosomal subunits, causing misreading of the genetic code of the mRNA template which results in faulty or inhibited protein synthesis and although not certain, it seems that this is the mechanism that makes these drugs bacteriocidal.

Gentamicin shares many physicochemical, pharmacologic and toxic properties as well as similar antibacterial spectra, with the other members of this family. These properties include high water and low lipid solubility. Consequently these antibiotics are minimally absorbed when given orally, cross the lipid membranes of most cells poorly, and have difficulty in penetrating the blood brain barrier, prostate gland and the eye. The aminoglycosides are minimally bound to plasma proteins (less than 20%), and this has a minimal influence on the distribution of these drugs from the vascular compartment. These drugs are excreted largely unchanged by glomerular filtration (Brumbaugh 1987, Sande and Mandell 1990, Brown and Riviere 1991).

Disposition and Pharmacokinetics of Gentamicin

The use of gentamicin is limited by the narrow range between therapeutic and toxic doses (Riviere 1982, Wilson 1983). Therefore, dosage regimens based on kinetic studies are essential for effective and safe therapy with this drug (Haddad *et al* 1985b). Numerous studies have been conducted to investigate the disposition and pharmacokinetics of gentamicin in horses, ponies and foals. The range of pharmacokinetic data collected is

presented in Table II.

The disposition kinetics of gentamicin in equine serum/plasma have generally been analysed using compartmental methods, and best described employing a two compartment open model approach (Baggot 1977). In recent publications however, a single compartment model best described the plasma gentamicin concentration time data from anaesthetised horses (Snyder *et al* 1985, Smith *et al* 1988). Noncompartmental methods were used to analyse the gentamicin concentration time data in the three fluids collected in this study. Some aspects of the theory and mechanics of both compartmental and noncompartmental methods together with the various equations used, are presented in Appendix I.

The absorption of gentamicin after an intramuscular dose is rapid. Peak serum levels occur within 30-60 minutes (Pedersoli *et al* 1980) and 87% of the administered dose is absorbed into the blood (Haddad *et al* 1985b). The apparent volume of distribution for gentamicin is approximately equal to the volume of the extracellular fluid, 20-25% of the body weight (Wilson *et al* 1983, Sande and Mandell 1990) although is not confined to this fluid compartment (Baggot 1977). The apparent elimination half-life of the drug is short 1.53-4.28 hours (Table II), hence the recommendation of dosing usually three times daily. The total body clearance, which is the volume of blood totally cleared of the drug by all elimination processes per unit time, is reported to range from 0.83-2.18 ml/min/kg (Table II). Because gentamicin is not metabolised, this parameter is directly related to glomerular filtration rate and hence decreased total body clearance rates together with elevated plasma elimination half-lives, are evident in animals with renal dysfunction (Sojka and Brown (1986).

Table II. **Gentamicin Pharmacokinetics in Horses and Foals.**

Horses		Pharmacokinetic Parameter						
Dose	**Route**	Peak concentration Cp^o (μg/ml)	Rate constants (h^{-1}) α	β	Apparent elimination half-life $t_{½\beta\ (b)}$	Volume of distribution L/Kg	Total Body Clearance Cl_B (ml/min/kg)	**Reference**
5 mg/kg	i.v	30.6	3.75	0.275	2.54	0.167 (Vc) 0.243 (Vd) 0.254 (Vd)	1.15	Pedersoli *et al* 1980
3 mg/kg	i.v	38.09	6.97	0.4176	1.66	0.079 (Vc) 0.173 (Vd) 0.202 (Vd)	1.41	Wilson *et al* 1983

iv = intravenous
im = intramuscular
Cp^0 = concentration in plasma at time = 0
$Vd_{(b)}$ = Volume of distribution (extrapolation method)

α = distribution rate constant
β = elimination rate constant
$t_{½(\beta)}$ = apparent elimination half-life

Vc = Volume of central compartment
$Vd_{[ss]}$ = Volume of distribution steady state
$Vd_{[area]}$ = Volume of distribution (area method)
Cl_B = Total Body Clearance

Table II (contd.) **Gentamicin Pharmacokinetics in Horses and Foals.**

Horses		Pharmacokinetic Parameter						Reference
Dose	Route	Peak concentration C_p^o (μg/ml)	Rate constants (h^{-1}) α	β	Apparent elimination half-life $t_{1/2\beta}$ (h)	Volume of distribution L/Kg	Total body Clearance Cl_B (ml/min/kg)	
5 mg/kg	i.v & i.m (ponies)	-	5.89	0.38	1.82	0.116 (Vc) 0.188 (Vd) 0.197 (Vd)	1.27	Haddad *et al* 1985b * = combined intravenous and intramuscular kinetics
5 mg/kg	i.m.+ (ponies)	-	-	0.33	2.13	0.31 (Vd)	1.40	Haddad *et al* 1985a + = steady state conditions
2.2 mg/kg	i.v. (anaesthetised horses)	-	-	-	2.2	0.227 ($Vd_{(B)}$)	1.12	Snyder *et al* 1986

Table II (contd.) **Gentamicin Pharmacokinetics in Horses and Foals.**

Horses		Pharmacokinetic Parameter						Reference
Dose	Route	Peak concentration Cp^{o} ($\mu g/ml$)	Rate constants (h^{-1}) α	β	Apparent elimination half-life $t_{1/2\beta\,(h)}$	Volume of distribution L/Kg	Total Body Clearance Cl_B (ml/min/kg)	
2.2 mg/kg	i.v. (gentamicin)	23.23	0.72	0.19	4.03	0.1 (Vc) 0.15 (Vd)	0.83	Bowman *et al* 1986
	(gentamicin & ampicillin)	26.57	2.14	2.14	3.02	0.11 (Vc) 0.16(Vd)	0.91	
1.76-2.2 mg/kg (horses and foals with sepsis)	i.m.	-	-	-	3.36 (mean horses and foals)	0.344 (Vd) foals 0.184 (Vd) horses	1.53 (horses and foals) 0.6 (horses and foals with increased SUN or SC*)	Sojka & Brown 1986 * SUN = serum urea nitrogen SC = serum creatinine
2.2 mg/kg	i.v.	-	-	-	1.53	0.297 ($Vd_{[B]}$)	2.18	Lloyd *et al* 1988

Table II (contd.) **Gentamicin Pharmacokinetics in Horses and Foals.**

Horses		Pharmacokinetic Parameter						Reference
Dose	Route	Peak concentration Cp^o (μg/ml)	Rate constants (h^{-1}) α	Rate constants (h^{-1}) β	Apparent elimination half-life $t_{½\beta\,(\lambda)}$	Volume of distribution L/Kg	Total Body Clearance Cl_B (ml/min/kg)	
4 mg/kg	i.v. (control unanaesthetised)	-	-	-	2.01	0.261 ($Vd_{[B]}$)	1.54	Smith *et al* 1988
	i.v. (anaesthetised)	-	-	-	4.03	0.248 ($Vd_{[B]}$)	0.81	
4 mg/kg	i.v. 40 d post partum mares	-	-	-	1.09	0.07 (Vc) 0.169 ($Vd_{[area]}$) 0.155 ($Vd_{[ss]}$)	1.69	Cummings *et al* 1990

Table II (contd.) **Gentamicin Pharmacokinetics in Horses and Foals.**

Foals		Pharmacokinetic Parameter						Reference
Dose	Route	Peak concentration Cp^{o} (μg/ml)	Rate constants (h^{-1}) α	Rate constants (h^{-1}) β	Apparent elimination half-life $t_{½\beta\,(h)}$	Volume of distribution L/Kg	Total Body Clearance Cl_B (ml/min/kg)	
4.5 mg/kg	i.v	34.5	0.0275	0.0037	3.23	0.152 (Vc) foals 0.306 (Vd)	1.65	Riviere *et al* 1983
2 mg/kg (1 month)	i.m	28.2	-	-	4.28	0.428 (Vd)	1.20	Baggot *et al* 1986
2 mg/kg (3 month)	i.m	34.9	-	-	3.69	0.324 (Vd)	1.02	
4 mg/kg (1 month)	i.m	86.2	-	-	3.07	0.243 (Vd)	0.93	
4 mg/kg (3 month)	i.m	84.2	-	-	2.87	0.115 (Vd)	0.46	
2 mg/kg 2 weeks	i.m	-	-	-	2.81	0.516 (Vd) 0.335 (Vd)	2.15	Gronwall *et al* 1988

Table II (contd.) **Gentamicin Pharmacokinetics in Horses and Foals.**

Foals		Pharmacokinetic Parameter						Reference
Dose	Route	Peak concentration Cp° (μg/ml)	Rate constants (h^{-1}) α	Rate constants (h^{-1}) β	Apparent elimination half-life $t_{\frac{1}{2}\beta(el)}$	Volume of distribution L/Kg	Total Body Clearance Cl_B (ml/min/kg)	
Neonatal foals 4 mg/kg Day 1	i.v	39.52			2.12	0.111(Vc) 0.318(Vd) 0.306(Vd)	1.75	Cummings *et al* 1990
Day 5	i.v	22.32			1.51	0.194(Vc) 0.382(Vd) 0.350(Vd)	2.98	
Day 10	i.v	26.61			1.69	0.196(Vc) 0.395(Vd) 0.344(Vd)	2.60	
Day 15	i.v	33.72			1.77	0.122(Vc) 0.364(Vd) 0.325(Vd)	2.40	
Day 30	i.v	38.33			1.01	0.109(Vc) 0.323(Vd) 0.279(Vd)	3.66	

I_{11} GENTAMICIN: THERAPEUTIC USE IN THE HORSE

Gentamicin is primarily used to treat infections caused by aerobic gram-negative bacteria. In the horse, gentamicin has traditionally been the drug of choice for gram-negative bacterial sepsis caused by *Escherischia coli, Klebsiella, Salmonella, Enterobacter* and *Proteus* species (Sojka and Brown 1986).

A therapeutic range of serum concentrations between 1 and 10 μg/ml has been proposed by these authors. In this range the risk of nephrotoxicity is reduced and throughout most of the dosage interval the recommended minimum inhibitory concentration (2 μg/ml) Adamson 1985) is exceeded. A lower limit of 1 μg/ml was set because in humans nephrotoxicity has been more closely associated with consistently elevated trough concentrations, especially trough concentrations of > 2 μg/ml Ristuccia (1984). Sojka and Brown (1986) used this range to individualise dosage regimens for horses with bacterial sepsis by measuring peak and trough gentamicin concentrations. They found that 9 out of 12 horses required dosage adjustment to optimise antibiotic therapy.

The entry and elimination of gentamicin into synovial fluid was investigated in the present study. Synovial fluid peak concentrations of gentamicin are generally 4-5 times lower than serum peak concentrations but last slightly longer (Beech *et al* 1977, Brown *et al* 1982, Bowman *et al* 1986 and Lloyd *et al* 1988). Although these levels may exceed the minimum inhibitory concentration of the common organisms that cause septic arthritis, the effective duration of synovial fluid concentrations has been questioned by some researchers (Beech *et al* 1977, Lloyd *et al* 1988). Recently, it was found that intra-articular gentamicin, although causing a mild synovitis, was the most effective method of eliminating sensitive bacteria from the antebrachiocarpal joint (Lloyd *et al* 1988, 1990). Some authors consider that intra-articular antibiotic therapy with aminoglycosides has improved the success rate of treatment of septic joint disease (Schneider *et al* 1992).

Toxicity

The toxic effects of gentamicin include 1) non-depolarising neuromuscular blockade, 2) cardiovascular depression, 3) nephrotoxicity 4) inhibition of gut motility and 5) ototoxicity, although the latter has not been recorded in the horse (Hinchcliff *et al* 1986). Of these effects, nephrotoxicity is by far the most important in the horse.

The nephrotoxicity is related to the uptake and accumulation of the aminoglycoside in the renal cortex, and particularly in the proximal tubular cells. Proximal tubules reabsorb about 3-5% of a gentamicin dose by an active process. Within these cells gentamicin interferes with lysosomal, mitochondrial and sodium/potassium ATPase function. Alteration to renal cellular function ultimately leads to proximal tubular necrosis and, amongst other things, a reduction in renal blood flow and diminished glomerular filtration rate. Early indicators of nephrotoxicity include elevations in urinary enzyme activity (enzymuria), with elevated urinary gamma-glutamyltranspeptidase (GGT) to urinary creatinine and protein ratios, followed by proteinuria, cylindiuria, haematuria and decreased concentrating ability. Elevations in serum urea nitrogen and serum creatinine levels occur later (Riviere *et al* 1982 , Edwards *et al* 1989, Hinchcliff *et al* 1986,1989). Gentamicin-induced nephrotoxicity appears to be reversible so long as the drug is removed at the first signs of toxicity (Riviere 1982).

Some authors believe that young horses (2-3 months) may be more susceptible to gentamicin-induced nephrotoxicity than adults. In foals given one half, one, and two times the normal adult dose rate of gentamicin for 14 days, histopathological evidence of nephrotoxicity invariably occurred (Riviere *et al* 1983). However, only two foals developed elevated clinicopathological parameters.

The susceptibility of adult horses to gentamicin induced nephrotoxicity apparently varies. In ponies receiving 20mg/kg of gentamicin intravenously for 14 days, 2 of 7 treated animals developed acute renal failure and were euthanased. The remaining animals

showed no clinical abnormalities, however, enzymuria and proteinuria, followed by elevations in serum concentrations of urea and creatinine above those of control animals, indicated the presence of nephrotoxicity. In two more recent reports involving aminoglycoside-induced nephrotoxicity, in which gentamicin (Bertone 1990) and neomycin (Edwards *et al* 1989) were administered at normal dose rates to horses for 14 and 10 days respectively, evidence of renal tubular damage was detected by an elevation of urinary GGT activity and the appearance of granular casts and renal epithelial cells in the urine. Plasma creatinine concentrations however, remained within the normal range. It is apparent from these studies that a range of nephrotoxic damage occurs, as indicated by clinicopathological parameters. This range varies between subtle elevations in enzymes to marked renal dysfunction with alterations in glomerular filtration rate. The decision as to what constitutes enough renal damage to warrant withdrawal of aminoglycoside therapy is difficult to make (Edwards *et al* 1989). However, there are a number of risk factors suspected to predispose to clinically significant nephrotoxicity during gentamicin therapy and these include pre-existing renal damage, dehydration, sepsis, fever and concurrent therapy with other nephrotoxic drugs (Hinchcliff *et al* 1986). Therefore, it has been recommended that horses being treated with gentamicin (and all other aminoglycosides) have their renal function evaluated before and during treatment. Monitoring of serum creatinine and trough concentrations of gentamicin (for evidence of drug accumulation) combined with urinalysis, will enable detection of early renal damage. At the first signs of this, gentamicin therapy should be discontinued (Riviere *et al* 1982, Hinchcliff *et al* 1986, Baggot and Prescott 1987).

I_{12} STUDY DESIGN

The three investigations that follow were designed and carried out in an attempt to examine the stated hypothesis. The first two investigations provided information necessary to complete the final investigation. The findings of Investigation 1, "Anatomical Identification of an Appropriate Lymph Vessel", resulted in the selection of a peripheral lymph vessel to catheterise for the sampling of lymph. The relationship between synovial fluid and lymph concentrations of an antimicrobial was better defined by showing that the selected lymph vessel drained the synovium of a joint. Investigation 2 proved that the selected lymph vessel drained the synovium of the fetlock joint of the hindlimb. Finally, Investigation 3, "Determination of the Disposition of Gentamicin in Equine Plasma, Synovial Fluid and Lymph" directly addressed the hypothesis " the synovial fluid concentration of an antimicrobial agent is useful as an index of its concentration in tissue fluid".

CHAPTER II INVESTIGATION 1

ANATOMICAL IDENTIFICATION OF AN APPROPRIATE LYMPH VESSEL

II_1 INTRODUCTION

The techniques used by researchers to study the lymphatic vessel system and specifically the lymphatic drainage of synovial joints, have been outlined. In this investigation, some of these techniques were used to identify an appropriate lymph vessel. This vessel was to be used for the ultimate purpose of studying the relationship between the disposition of an antimicrobial in peripheral lymph and synovial fluid. Such a vessel had to:

a) be anatomically consistent in position in every horse

b) be readily accessible and of sufficient size so that it was easy to identify and cannulate and,

c) drain a convenient joint.

II_2 MATERIALS AND METHODS

Animals

Twelve horses were used in this investigation (Appendix II, Table 1). These horses were euthanased for various reasons, unrelated to abnormality of the cardiovascular system, or of the integumentary or musculoskeletal systems of the limbs studied.

Injection of Dyes

Lymph vessels of the distal limbs were delineated by subcutaneous and intra-articular injection of dyes capable of being absorbed into lymph. The dyes used and their sites of injection are shown in Tables 1,2 and 3 in Appendix II. After euthanasia, the arrangement of subcutaneous lymph vessels distal to the carpus and the tarsus was investigated by dissection.

a) *Subcutaneous Injection Technique*

The nature of the horse determined if a nose twitch, sedation with intravenous xylazine[1] (dose rate 0.5 mg/kg), or any restraint at all, was required. Subcutaneous dye was injected through a 23 gauge x 25 mm needle (without skin preparation) at single or multiple sites on the pastern, approximately half way between the coronet and the fetlock joint.

The volume of dye injected was 1-2 ml and 10% Patent Blue Violet (PBV) was used in all horses except horse 1 (Evans Blue) and horse 9 (Congo Red). In horse 2 the concentration of PBV used on the right forelimb was 5%.

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1 Rompun^r solution 10% Bayer.

b) *Intra-articular Injection Technique*

In all horses a nose twitch was applied and the foot to be injected held off the ground. The injections sites were prepared by firstly shaving the hair around the site and then depositing 0.5-1 ml of local anaesthetic (2% lignocaine[2]) subcutaneously. Dye was injected into the lateral palmar/plantar recess of the fetlock joint through an 18 gauge x 44 mm "over the needle" catheter or an 18 gauge x 40 mm needle.

Three ml of dye was usually injected except in horse 6 where 2 ml was used and in horse 11 where 6 ml was used. Congo Red dye was injected in all horses except horse 5 (PBV) and horse 11 (India Ink).

c) *Timing of Injections*

Three horses received dye injections at the time of euthanasia (Horses 4,5 and 12). Eight other horses were injected with dye(s), walked, and after a variable time (5 minutes to 7.5 hours) euthanased. The remaining horse (9) was injected and then briskly exercised by walking and trotting for twenty minutes, prior to euthanasia.

d) *Euthanasia of Horses*

Euthanasia was achieved in 10 horses by the intravenous injection of a lethal dose of pentobarbitone[3] followed by exsanguination. The other 2 horses (being slaughtered for pet food) were firstly stunned, and then exsanguinated.

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2 Nopaine Ethical Agents Ltd, Parnell, Auckland.

3 Pentobarb 500 South Island Chemicals Ltd, 53 Lunns Rd Christchurch N.Z.

In all cases, once the horse was recumbent and before exsanguination, a 45 cm length of Esmarch's bandage[4] (8 cm wide) was placed very tightly proximal to the carpus or tarsus and secured with a pair of large artery forceps.

In the 3 horses receiving dye injections at the time of euthanasia, recumbency was first induced with pentobarbitone. The limbs were injected, then the tourniquet applied, and finally the horse was exsanguinated.

Each injected leg was removed from the carcass by transection through the mid radius or mid tibia, just proximal to the tourniquet. At the same time, the cubital lymph nodes in the forelimb and the deep inguinal lymph nodes in the hindlimb were inspected for discoloration with dye.

Dissection

Dissection of the transected limbs, with the tourniquet still applied, began within 2 hours of euthanasia.

An incision was made along the entire length of the dorsal aspect of the limb. The skin was separated from the underlying subcutaneous tissues using a scalpel, care being taken to avoid blood vessels or any dye filled lymph vessels. Where necessary, blood vessels were divided between mosquito forceps to prevent staining of the specimen.

The subcutaneous tissues and injection sites were carefully scrutinised for dye filled vessels. A conscientious effort was made to trace the course of these vessels proximally, distally, and into deeper tissues by bluntly dissecting vessel associated fascia and fat.

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4 Aesculap, Kempthorne Medical Supplies, Box 1234 Auckland.

After intra-articular injection, the site of arthrocentesis was carefully observed for signs of leakage or periarticular injection. The fetlock joint was then incised, and the joint space inspected for the presence of dye to confirm that the dye had been injected into the joint.

The position of all lymph vessels, and their relationship to other structures, was recorded in writing and with drawings. Observations regarding the effectiveness of each dye, dye combination and injection technique were also recorded. Photographs of selected specimens were taken for illustrative purposes.

II_3 RESULTS

Injection of Dyes

a) *Subcutaneous Injection Technique*

The horses showed no untoward reaction to the subcutaneous injections.

b) *Intra-articular Injection Technique*

Horses showed no signs of pain or lameness after intra-articular injection with Congo Red. The horse in which 6 ml of India Ink was used showed some immediate discomfort. Within 30 minutes the injected joint was warm and the horse pointed its toe, was reluctant to bear weight, but was only mildly lame at the trot. These signs diminished 6 hours after the intravenous injection of 2 g of phenylbutazone[5].

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5 Nabudone P^r: Troy Laboratories Pty. Limited, distributed by Ethical Agents, Wiri, South Auckland.

Needle injection of the fetlock joint was easier than catheter injection, however the latter resulted in less haemorrhage and ensured no leakage of dye from the puncture site (because of the plugged catheter, which remained in the joint).

Dissection

Lymph vessels were detected at dissection by the uptake into their lumina of the various dyes used, and by their characteristic 'string of pearls' appearance, imparted by the regularly placed valves present along their length (Figure 3). All dissected lymph vessels were positioned between the skin and deeper structures. Inspection of the cubital lymph nodes in the forelimb and the deep inguinal lymph nodes in the hind limb, for the presence of dye, confirmed the effectiveness of dye uptake into the lymph vessel system. The tourniquet maintained the lymph vessels in a dilated state for the duration of the dissection and when released, the main trunks immediately collapsed.

The largest (most obvious) lymph vessels were seen proximal to the fetlock and were approximately 1-2 mm in diameter. The distance between successive valves in these vessels was approximately 5 mm.

a) *Volume of Dye Used*

The volume of subcutaneously administered dye which resulted in the best anatomic specimens was 1 ml. Larger volumes were more difficult to inject and resulted in a pool of dye at the injection site as well as greater spread throughout the subcutaneous tissues. This obscured anatomical detail, especially in the pastern and distal fetlock regions.

b) *Timing of Injections*

Dye injections made at the time of euthanasia were as effective in delineating lymph vessels as were injections made prior to euthanasia.

c) *Subcutaneous Injections*

Subcutaneous injections of Patent Blue Violet (5% W/V or 10% W/V concentration), resulted in superior anatomical specimens. This dye diffused from its deposition site, throughout the subcutaneous tissues into an area encompassing most of the pastern (Figure 1). Dye was absorbed into lymph vessels on all occasions and no differences were apparent between the two concentrations used. Although blue when injected, the dye filled lymph vessels often appeared blue-green or green in appearance, and this was more evident in specimens observed some time after dissection.

Injected Evans Blue (3% W/V or 2.5% W/V diluted in serum) however, remained confined to the site of deposition and did not diffuse through the subcutaneous tissues or infiltrate any lymph vessels. In the one horse which received a subcutaneous injection of Congo Red (0.5% saline serum), no red dye was evident in the lymph vessels at dissection.

d) *Intra-articular Injections*

The intra-articular administered dye did not appear in lymph vessels. However, uncoloured lymph vessels were often present in these limbs and could be detected due to their characteristic appearance and typical anatomical positioning, which was identical to that seen in limbs that had been injected subcutaneously only. However, on two occasions, when some periarticular leakage occurred at the time of intra-articular deposition, some dye appeared in the lymph vessels.

In only one horse were no lymph vessels (coloured or uncoloured) detectable at dissection. This horse received intra-articular and subcutaneous injections of Congo Red and was exercised briskly by walking and trotting for 0.5 hours prior to euthanasia.

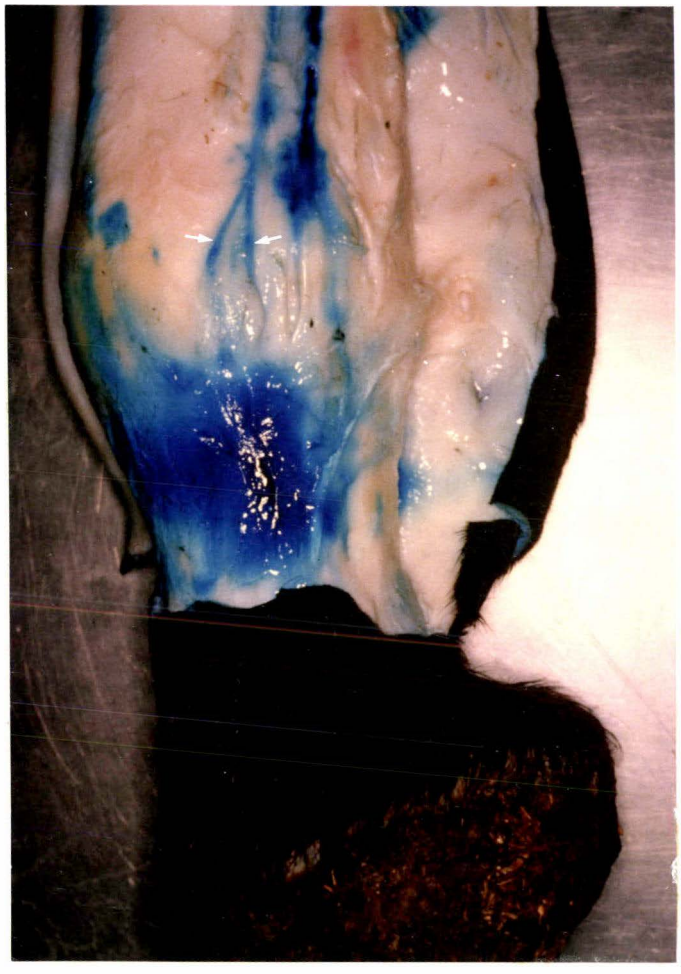

Figure 1. Dissection Specimen Showing Subcutaneous Dye Deposition Site. Note lymphatic tributaries (white arrows).

e) *Distribution of Lymph Vessels*

Tributaries of the larger lymph vessels could be seen emerging from the point of dye deposition at the mid pastern level. At this position the vessels were smaller in diameter and less distinct, possibly because they were partially collapsed or less filled with dye. In the forelimb, the arrangement of lateral and medial lymph vessels conformed to a general pattern with minor variations in the number and the position of individual vessels. In the two horses in which hind limbs were examined only the medial side of the limb was investigated and a similar pattern was observed in each.

The following lymph vessel patterns were observed:

i) Forelimb:

a) *Lateral Aspect* - Typically, 1-3 lymph trunks (usually 2) with their origins traced from the pastern region, coursed proximally over the abaxial surface of the lateral proximal sesamoid bone. Above the fetlock they were found in the groove between the suspensory ligament and the deep digital flexor tendon. These vessels were invested in fascia and accompanied the lateral palmar vein, artery and nerve in this region. The lymph vessels were seen to be either dorsolateral, or palmarolateral to these structures.

In most horses, 2 of the lateral lymph trunks joined to form a single trunk immediately distal to the carpus. This vessel coursed proximopalmarly, to the medial side.

In 1 horse, a solitary lymph vessel originating from the pastern terminated in the fetlock flexor tendon sheath, filling it with green dye.

In 2 horses, 1-3 lymph vessels originating from the pastern coursed proximally and then turned medially, over the apex of the lateral proximal sesamoid bone, passing between the suspensory ligament and the deep digital flexor tendon to join the lymph vasculature on

the medial side.

b) *Medial Aspect* - A similar pattern was observed on the medial aspect of the forelimb although the lymph vessels were more numerous, and on reaching the proximal metacarpus these vessels usually passed beneath the flexor retinaculum on the palmar aspect of the carpus.

Typically, there were 3-5 lymph vessels with their origins in the pastern region. These vessels coursed proximally over the abaxial surface of the medial proximal sesamoid bone toward the metacarpus, where they were found to be closely associated with and either dorsal or palmar to, the medial palmar vein, artery and nerve in the groove between the suspensory ligament and the deep digital flexor. Most of these vessels then passed beneath the flexor retinaculum (on the palmar aspect of the carpus) as they continued proximally, although occasionally a vessel would pass over this structure.

ii) Hindlimb:

Subcutaneous lymph vessels were delineated in the 3 hindlimbs examined.

In the horse in which only 1 hindlimb was examined (11), a single large lymph vessel whose origin was traced to the pastern, coursed the entire length of the hindlimb. The vessel was found to be closely associated (joined by common fascia) to the dorsal aspect of the common dorsal digital II vein in the metatarsus, and the medial saphenous vein in the tibial region, before it entered the deep inguinal lymph node. No other lymph vessels were observed.

In the other horse, 3-4 small thin lymph vessel tributaries emerged from the deposition of subcutaneous dye on the medial aspect of the pastern and merged to form 2 lymph trunks, one dorsal to, and the other plantar to, the common dorsal digital vein (Figure 2). These vessels were closely associated with the vein and remained in this position for the entire

length of the metatarsus. Approximately 10 cm from the fetlock joint, a lymph vessel from the skin was seen to merge with the plantar lymph trunk (Figure 3). A similar pattern was observed in the other leg of this horse.

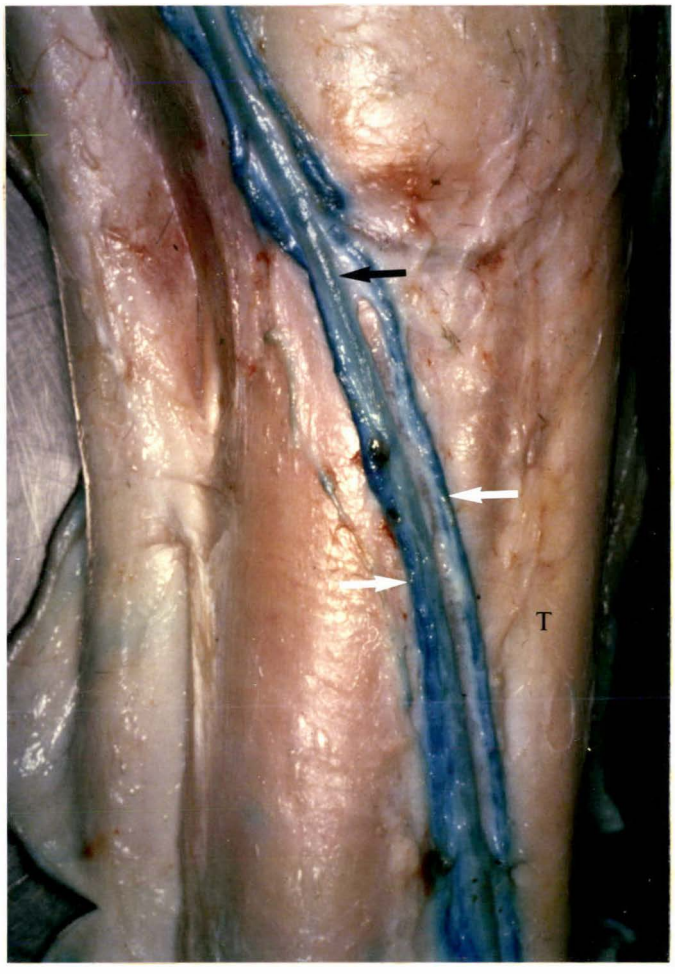

Figure 2. Dissection Specimen Showing Subcutaneous Lymph Trunks on the Medial Aspect of the Metatarsus.

The photograph is oriented proximal to distal with the skin reflected dorsally (to the left of the picture). Two lymph trunks can be seen (white arrows) dorsal and plantar to the common dorsal digital vein (black arrow).

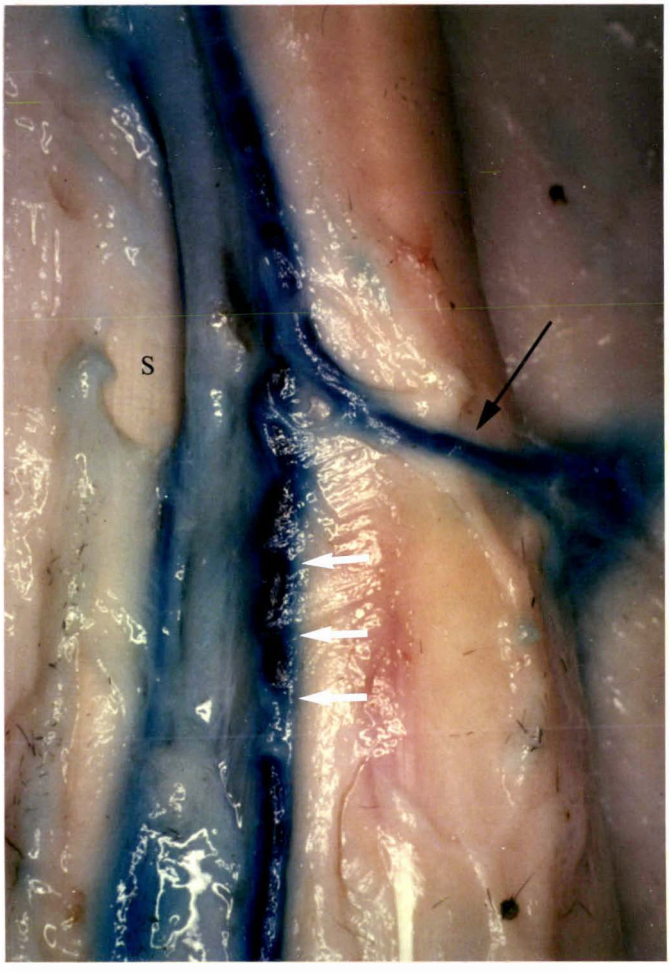

Figure 3. Dissection Specimen Showing a Lymphatic Tributary from the Skin Joining a Plantar Lymph Trunk on the Medial Aspect of the Distal Metatarsus.

The photograph is oriented proximal to distal with the reflected skin surface plantar (to the right of the picture). Note the lymphatic tributary (black arrow) and lymphatic valves (white arrows). The vessels are medial to the deep digital flexor tendon and just plantar to the suspensory ligament (S).

II_4 DISCUSSION

The general arrangement and position of lymph vessels identified in the subcutaneous tissues in the distal hindlimbs of horses in this investigation were similar to those described by others (Baum 1920, Rossoff 1946, Fackleman *et al* 1974, Laue 1987). However, using intra-articular injections of dye, lymph vessels draining the fetlock joint could not be identified. This difficulty was not encountered by Baum (1920) in his studies on the horse nor by researchers using other species (Rynearson 1931, Davies 1946, Levick 1980, Turner 1980). It was, however, similar to the experiences of Laue (1987) who used techniques comparable to those of Baum.

The reasons for the inability to delineate lymph vessels by intra-articular injection in the present study are unclear although the technique used differed from that of Baum (1920) and others, and may explain the different result. For example Baum used dead horses which were very young (near term foetuses), and mixed his dyes with highly lipid soluble substances (turpentine and ether). It is possible that death prior to injection of dye may facilitate transfer of dye out of the joint by increasing synovial permeability. Also, the lipid soluble substances may have assisted absorption of the dye. In this study, a protein bound preparation was used (Congo Red) in order to aid absorption of dye from the joint into lymph. It was assumed that because the principle route of protein removal from the joint is thought to be via lymphatic pathways (Yoffey and Courtice 1970), such a formulation would enable joint draining vessels to be marked. The reason(s) why this solution did not work are unknown.

Certainly, many inconsistencies are evident in the literature with regards to the success or failure of various methods. For example, Turner (1980) using India Ink, was able to delineate joint draining lymph trunks in the porcine forelimb after intra-articular injection under anaesthesia followed by dissection four hours later. Rynearson (1931) on the other hand, some 49 years earlier, could not achieve the same result in dogs using the same material and a similar technique. He pointed out that the formulation was all important and

described joint draining lymphatics if Trypan Blue (colloid) or Ultra-marine Blue (suspensoid) were used instead. Laue (1987) gave no reasons for the apparent failure of Berlin Blau to delineate joint draining vessels in horse limbs. This was despite the use of a rigorous technique which involved injecting 8-10 ml of dye in distal joints of the forelimb and flexion and extension for a period of 20-30 minutes. Even Baum (1920) reported that not all injections were successful and that the lymph vessels draining joints in adult limbs were more difficult to delineate.

Although the intra-articular technique used in the present study could not directly identify lymph vessels draining joints, the subcutaneous injection readily delineated vessels which Baum described as draining the joints of the distal limb.

In the forelimb, there were many lymph vessels demonstrated. However, this limb was considered inappropriate for studying antimicrobial disposition because of the difficulty in confidently identifying exactly the same joint draining vessel in each horse used. In contrast to the forelimb, the hindlimb contained one or two lymph trunks which had all the attributes required for the intended disposition study. The vessels were consistently recognisable, of sufficiently large calibre to allow cannulation and had been shown by Baum (1920) to drain the fetlock joint. To determine that these vessels did in fact drain this joint as indicated by this researcher, the following investigation was conducted.

CHAPTER III INVESTIGATION 2

THE ABSORPTION OF GENTAMICIN FROM SYNOVIAL FLUID INTO LYMPH

III_1 INTRODUCTION

The common dorsal digital lymph trunk identified in Investigation 1 satisfied the criteria required to study the disposition of an antimicrobial agent in tissue fluid. However, in order to relate antimicrobial concentrations in the lymph in this vessel to those in synovial fluid, it remained to be proven that a contribution to this lymph, was derived from the synovium of a joint. To achieve this gentamicin was injected directly into the adjacent fetlock joint after cannulation of the above lymph vessel. The lymph collected was then tested for the presence of the drug.

III_2 MATERIALS AND METHODS

Experimental Animals

Four horses were used in this investigation (Appendix III, Table 1). One of these horses (number 4) was used on two occasions, one week apart. All the horses were to be euthanased for various reasons unrelated to abnormality of the cardiovascular system or of the integumentary or musculoskeletal systems of the hindlimbs involved. Each horse was examined physically and weighed. A blood sample was collected to determine haemoglobin, total protein and fibrinogen concentrations; packed cell volume, total and differential white cell numbers; creatinine kinase (CK), alkaline phosphatase (AP), aspartate transferase (AST), gamma glutamyl-transpeptidase concentrations (GGT); blood urea nitrogen and creatinine concentration.

Ivermectin[1] was given orally to each horse at a dose rate of 200 μg/kg to remove internal parasites at least one week prior to the start of the experiments.

In this study the lymph vessel selected in the previous investigation was cannulated and the method of cannula preparation and surgical implantation is outlined below.

Lymph Vessel Cannulation

One metre lengths of polyvinyl chloride tubing[2] (OD = 0.96 mm, ID = 0.45 mm) were prepared as outlined in Appendix III. The surgical implantation of lymph vessel cannulae was performed on anaesthetised horses in lateral recumbency.

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1 Eqvalan^r MSD Agvet, Wiri, Auckland.

2 Dural Plastics and Engineering, Dural N.S.W 2158 Australia.

Each horse had food withheld for 12-24 hours prior to surgery but had free access to water. Thirty minutes prior to induction they were premedicated with intramuscular acetylpromazine[3] at a dose rate of 0.05 mg/kg bodyweight(bwt).

Anaesthesia was induced by intravenously injecting half the required dose of a 10% solution of glyceryl guaiacolate[4] delivered at a dose rate of 100 mg/kg followed by a bolus intravenous injection of thiopentone sodium[5] (5.5 mg/kg bwt). The remainder of the glyceryl guaiacolate was then given. Horses were maintained with a halothane[6] and oxygen mixture delivered through a large animal circle system.

Immediately after anaesthetic induction, a tourniquet was placed around the distal tibia of the appropriate limb to facilitate lymph vessel recognition and cannulation (Figure 1). Following tourniquet application, 1 ml of a sterile 10% W/V Patent Blue Violet solution was subcutaneously injected in one site on the medial side of the mid-pastern.

The horse was positioned in lateral recumbency on the surgery table. The upper hindlimb was pulled cranially or caudally and secured with a soft rope around the pastern. The skin of the lower hindlimb was clipped and shaved between the fetlock and tarsometatarsal joints, prepared for aseptic surgery and draped appropriately.

A cruciate incision was made in the skin over the common dorsal digital vein 8-10 cm distal to the tarsometatarsal joint. The resultant skin flaps were reflected and held in that position with monofilament polypropylene[7] stay sutures.

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3 ACP, Techvet Laboratories Ltd., NZ.

4 Giafen, Parnell Laboratories Ltd., NZ.

5 Thiovet, Techvet Laboratories Ltd., NZ.

6 Fluothane[r] ICI NZ Ltd.

7 O Prolene Ethicon[r] Somerville, New Jersey USA.

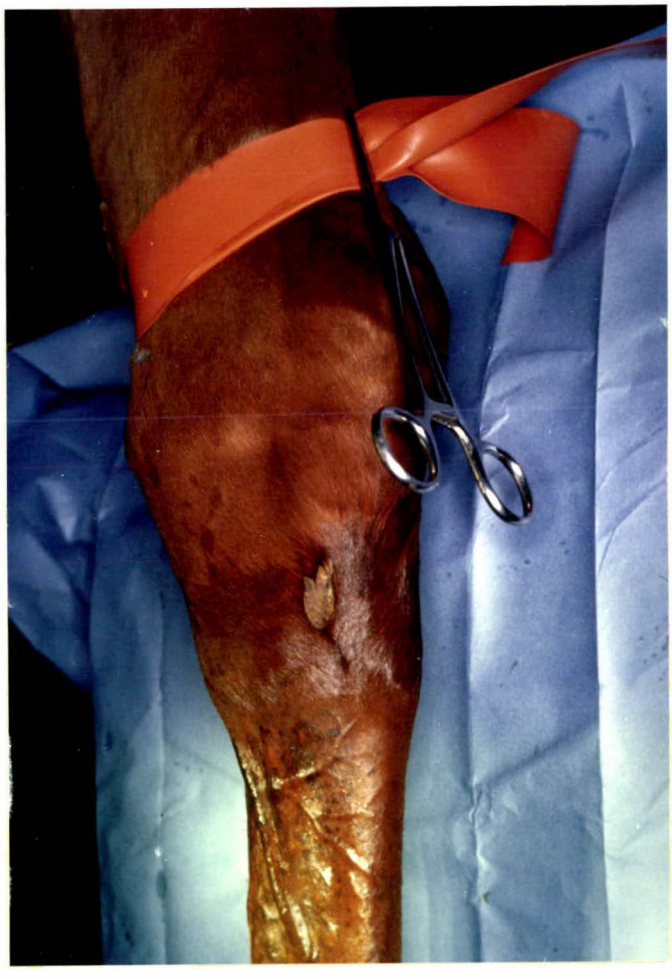

Figure 1. **Tourniquet Placement Around the Distal Tibia to Facilitate Lymph Vessel Recognition and Cannulation.**

After blunt dissection through the subcutaneous fascia, a search was made for the blue (dye filled) lymph trunk (Figure 2). Once located, a few drops of warm 1% lignocaine were placed over the lymph vessel to prevent vasospasm. Gentle sharp and blunt dissection with fine iris scissors was then used to remove loose connective tissue from around the lymph vessel and the common dorsal digital vein.

Three ligatures of 4-0 polyglactin 910[8] were placed around the lymph vessel. The most proximal ligature was tied off and used to elevate the vessel and, at the same time, cause lymph vessel engorgement. A longitudinal incision 2-3 mm long was made distally in the exposed surface of the vessel, using a curved number 12 scalpel. The escape of blue coloured lymph from the lumen of the vessel indicated a full thickness incision.

An 8 cm blunt 18 gauge spinal needle stylet was used as an obturator. This was introduced into the incised vessel and advanced distally about 30-40 mm. The characteristic "popping" of the lymph vessel valves during this procedure confirmed the correct placement of the instrument within the vessel lumen. Once positioned, it was moved back and forth within the vessel lumen to destroy these valves and facilitate cannulation.

The obturator was removed and the bevelled end of a heparin-filled[9] cannula immediately introduced into the lymph vessel by grasping the end with mosquito forceps and gently guiding the tip through the incision. The cannula was advanced distally for a distance of approximately 20 mm.

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8 Vycryl Ethicon[r] Somerville, New Jersey USA.

9 Heparin Saline i.u/ml, Leo Pharmaceutical Products Ballerup, Denmark.

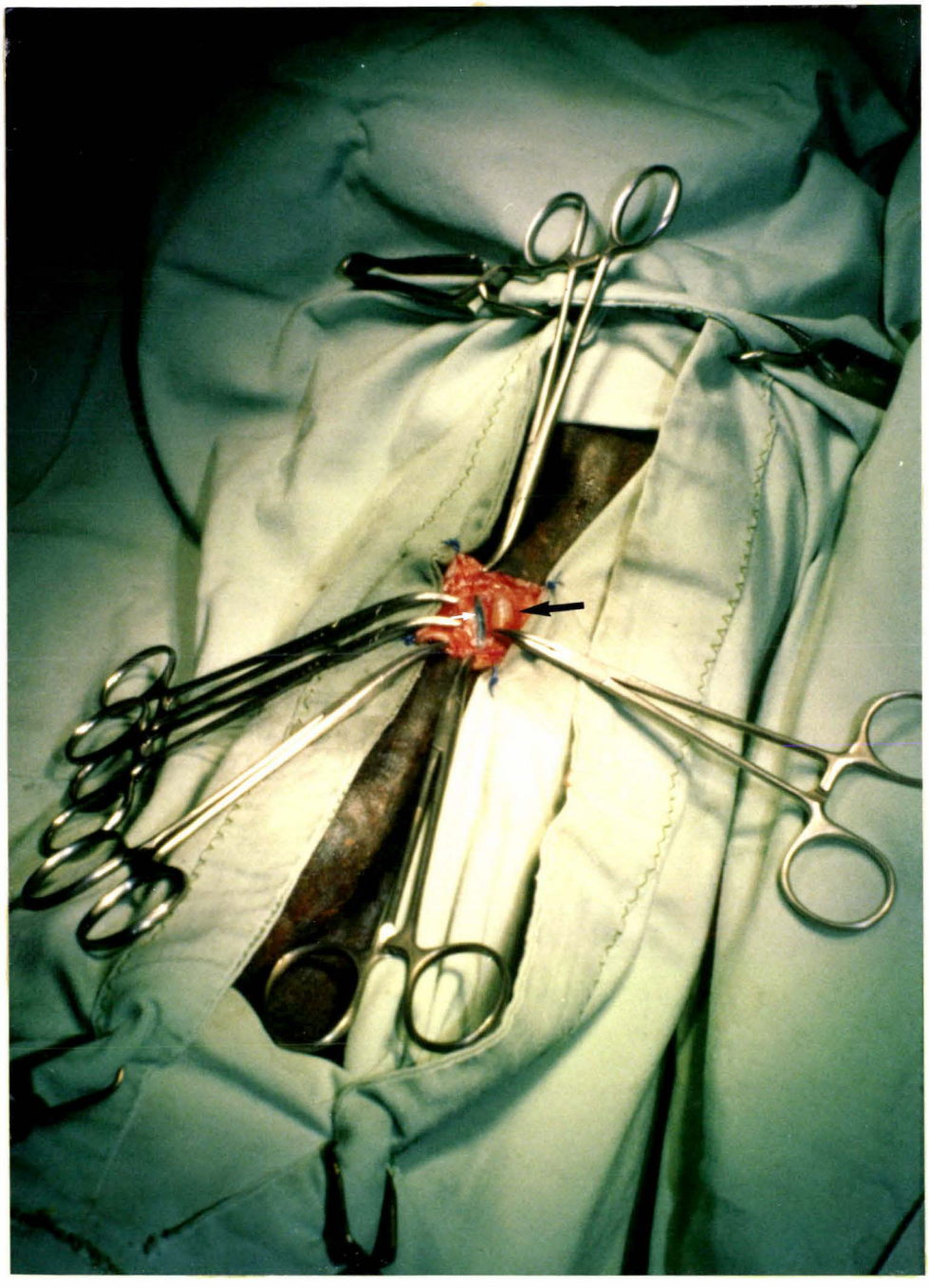

Figure 2. Common Dorsal Digital Vein and Accompanying Lymph Trunk.

Photograph is oriented proximal to distal. The skin flaps resulting from the cruciate incision have been sutured to the drape. The blue coloured dorsal digital lymph trunk is readily apparent (white arrow) dorsal to the commom dorsal digital vein (black arrow).

Correct positioning and patency of the cannula were checked by massaging the distal limb through the drapes. The flow of blue lymph into the cannula showed that cannulation was successful. The distal two ligatures were tied around the cannulated vessel whilst the most proximal ligature was tied directly onto the cannula.

A 14 gauge 5 cm needle was then introduced through the skin just proximal to the cruciate incision and the free end of the cannula threaded through the needle. The needle was then removed and the cannula pulled gently out through the skin, leaving its distal portion in the lymph vessel lumen. The cannula was then coiled once, to produce a single flat loop between the exit point from the lymph vessel and the exit point through the skin. The skin incision was closed with a cruciate suture using monofilament polypropylene, together with extra simple interrupted sutures along each limb of the incision, as required. The loop of cannula was thus left in a subcutaneous position.

Excess cannula was excised, leaving 250-300 mm protruding beyond the skin and a 3 ml heparin rinsed syringe with the plunger drawn was threaded on to the end of the cannula and taped to the leg.

Experimental Procedure

The sequence of steps that were performed in the experiments in this investigation are outlined below and illustrated in Figure 3. Catheter and cannula placement is illustrated in Figure 4.

1) After successful cannulation of the medial common dorsal digital lymph trunk, a 16 gauge x 83 mm catheter was placed into the adjacent medial common dorsal digital vein. A three way stopcock[10] was fitted to the end of this catheter to enable blood sample collection, and to allow for heparinisation of the catheter between samples.

2) A 20 gauge x 32 mm catheter was then placed into the medial plantar recess of the metatarsophalangeal joint.

3) A 14 gauge x 83 mm catheter was also placed into a jugular vein and was fitted with a three way stopcock as in 1) above.

4) Blood (3 ml) and synovial fluid (0.4 ml) samples were collected at the commencement of the experiment, prior to joint injection, from the jugular vein, common dorsal digital vein (local blood) and fetlock joint respectively.

5) Three ml of 50 mg/ml gentamicin solution were then injected into the fetlock joint through the needle of the implanted catheter. Simultaneously, the heparinised 3 ml syringe attached to the free end of the lymph trunk cannula was replaced with a new syringe. The volume of the collected lymph was measured and recorded (this was the zero time sample for lymph). The lymph flow rate was also recorded. This was calculated by dividing the collected lymph volume by the time interval over which the lymph was collected. In this

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[10] Pharmaseal American Hospital Supply Corporation, Valencia, CA 91355 - 8900, USA.

case, the time interval was from the completion of lymph trunk cannulation to the time of intra-articular injection. For the rest of the experiment the lymph collection time interval was the period between sampling.

6) Jugular and local venous blood, synovial fluid and lymph samples were then collected at post injection times (PIT) 0.25, 0.5, 1, 1.5, and 2 hours*.

Three ml blood were collected by syringe at each sampling time from the jugular and the common dorsal digital veins. In each case, 1 ml of blood was aspirated, then discarded and, with a new syringe, a further 3 ml of blood were collected and placed into an EDTA tube. The catheter lumen was then refilled with heparin.

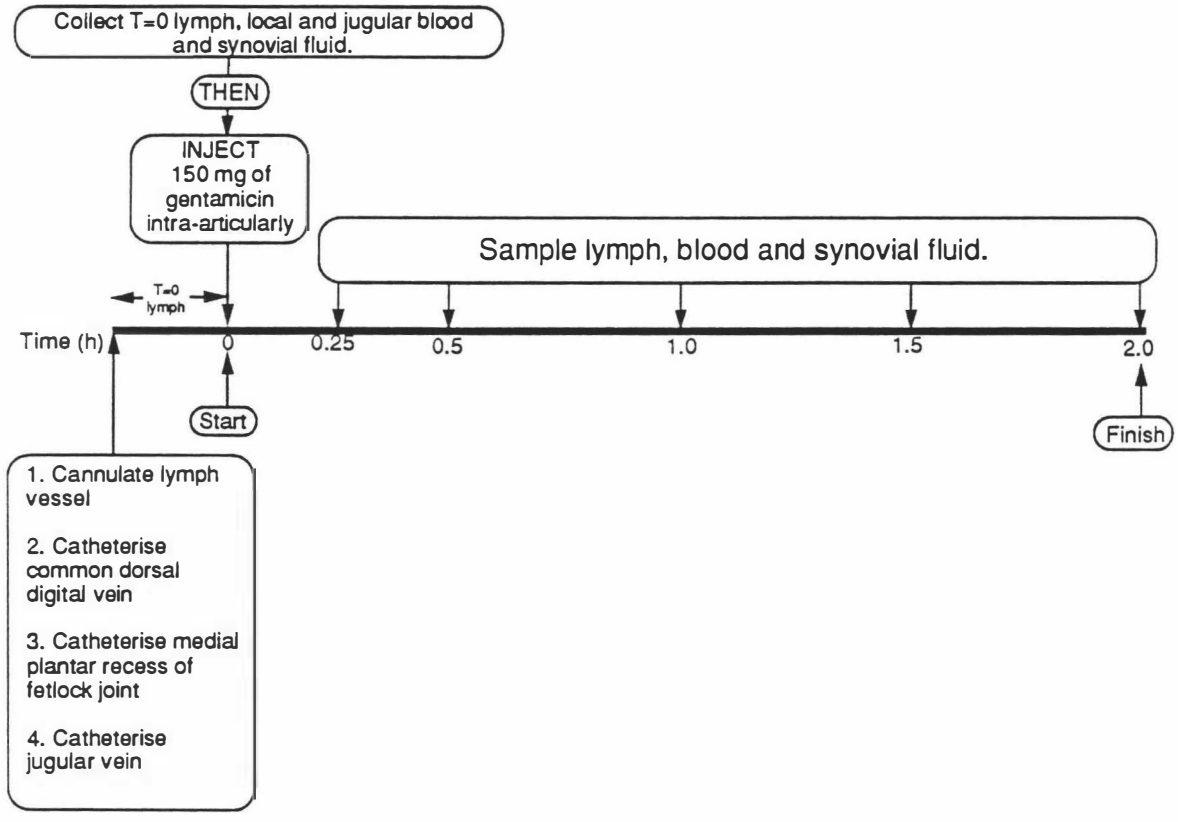
After injection of gentamicin into the fetlock joint, 0.2-0.4 ml of synovial fluid was aspirated via a new catheter needle at each subsequent sampling time.

At each sampling a new heparinised 3 ml syringe with the plunger drawn was threaded onto the free end of the lymph cannula and attached to the leg with tape. The volume of lymph collected at each sampling was recorded and the lymph flow rate calculated. These samples represented the total lymph flow for the cannulated vessel, between sampling times.

In order to promote lymph flow, the fetlock joint was flexed and the pastern massaged for 10-15 seconds at approximately five minute intervals throughout the experiment.

* Samples were collected over 4 hours for Horse 3 (second experiment).

Figure 3. Sequence of Steps Followed in the Determination of the Disappearance of Gentamicin from Synovial Fluid after Intra-Articular Injection.



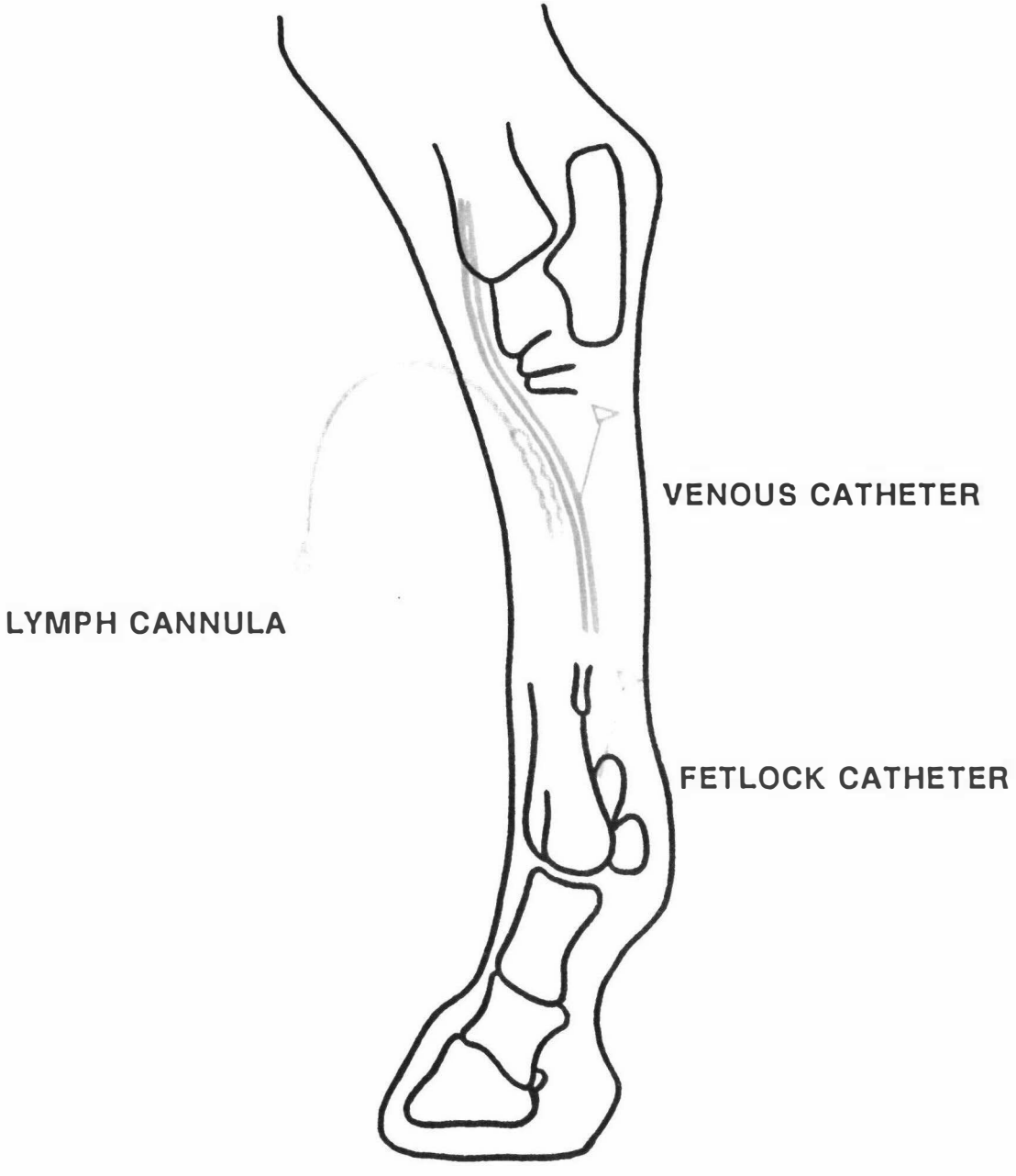


Figure 4. Catheter and Cannula Placement for the Determination of the Disappearance of Gentamicin from Synovial Fluid after Intra-Articular Injection. Medial Aspect of the Distal Hindlimb.

Analysis of Samples

a) Jugular and local venous blood samples were allowed to stand for approximately 2 hours and then centrifuged for 5-10 minutes at 1200 g and the plasma separated. Any blood contaminated synovial fluid was centrifuged also and the supernatant aspirated.

b) Jugular and local venous plasma, synovial fluid, and lymph samples were frozen to -20^{0}C and stored for less than two weeks before gentamicin analysis. These analyses were performed using the technique of Fluorescence Polarization Immunoassay (FPIA) on a TDX[11] analyser with a lower detection limit for gentamicin of 0.2 μg/ml. The possible interference with the assay of gentamicin due to the presence of dye in the lymph samples was checked and no interference was found.

Pharmacokinetic and Statistical Analysis

The pharmacokinetic and statistical analyses performed on the data in this investigation are detailed in Appendix III.

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11 TDx Abbot Laboratories, Diagnostics Division, Irving,Texas 75015, USA.

III_3 RESULTS

Surgery

Approximately 45 to 60 minutes was required for lymph vessel cannulation and, in each horse, a blue dye filled lymph trunk was easily located adjacent to the dorsal common digital vein. In one horse, two lymph trunks were evident, one dorsal and one plantar to the vein and in this instance the dorsal vessel was cannulated.

Lymph vessel constriction (usually the result of excessive handling or dissection of surrounding fascia) and haemorrhage, were the main difficulties encountered during surgery. Bleeding from peripheral veins was controlled by meticulous attention to haemostasis.

Lymph Flow Rates

Lymph flowed freely from the end of the implanted cannula and remained blue for the duration of the experiment. Regular massage of the pastern and flexion of the fetlock ensured a steady rate of flow. The individual horse and mean lymph flow rates recorded during the two hours are presented in Table I. There was no significant difference in mean lymph flow rate between sampling intervals. The overall mean hourly flow rate was 1.45 ± 0.18 ml/h.

Table I. Lymph Flow Rates (ml/h).

Sampling interval (h)	Horses					Mean (ml/h ± sem)
	1	2	3	4	3*	
Before Injection	**2.12**	**0.86**	**0.9**	**1**	**2.06**	**1.39 ± 0.29**
0 - 0.25	1.2	0.8	2	-	3.6	1.9 ± 0.62
0.25 - 0.5	1.6	0.8	1.2	2.4	3.2	1.84 ± 0.43
0.5 - 1	0.8	0.6	0.4	2	1.4	1.04 ± 0.29
1 - 1.5	2	0.5	1.3	1.2	0.4	1.08 ± 0.29
1.5 - 2	1.2	0.6	0.8	2	2.8	1.48 ± 0.41

* Horse 3 was used twice, one week apart.

Gentamicin Concentrations

The mean concentration of gentamicin in jugular and local plasma, lymph and synovial fluid following intra-articular gentamicin injection, is presented in Table II (raw data Appendix III, Tables 2-4) and the mean concentration of gentamicin in plasma and lymph presented graphically in Figure 5. The relationship between lymph flow rate and lymph gentamicin concentration is presented as a scattergraph in Figure 6 and the correlation coefficient for these two variables was -0.26. However, this was not significantly different from 0 ($p > 0.05$).

Table II. **Mean (± sem) Concentration of Gentamicin (μg/ml) in Jugular and Local Plasma, Lymph and Synovial Fluid after Intra-Articular Injection (n=5).**

	Body Fluid			
Time (h)	**Jugular plasma**	**Local plasma**	**Lymph**	**Synovial fluid**
0	0	0	0	0
0.125	-	-	0.34 ± 0.12	-
0.25	0.83 ± 0.26	2.59 ± 0.71	-	7244 ± 660
0.375	-	-	7.14 ± 5.28	-
0.5	0.89 ± 0.23	1.81 ± 0.43	-	5090 ± 759
0.75	-	-	24.15 ± 8.33	-
1	0.85 ± 0.22	1.85 ± 0.46	-	3400 ± 564
1.25	-	-	43.36 ± 10.73	-
1.5	0.88 ± 0.18	1.52 ± 0.45	-	2260 ± 262
1.75	-	-	42.84 ± 13.01	-
2	0.81 ± 0.17	1.85 ± 0.30	-	1682 ± 156

Figure 5. **Semilogarithmic Plot of Mean (± sem) Gentamicin Concentration (μg/ml) in Lymph and Plasma after Intra-Articular Injection.**

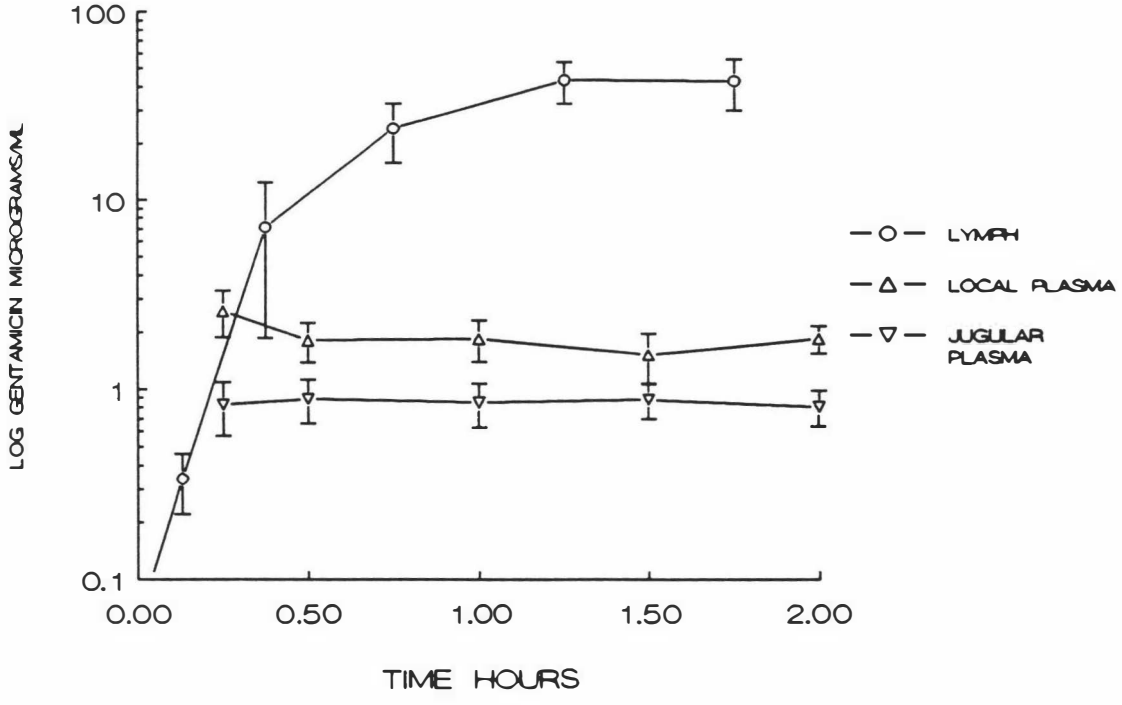
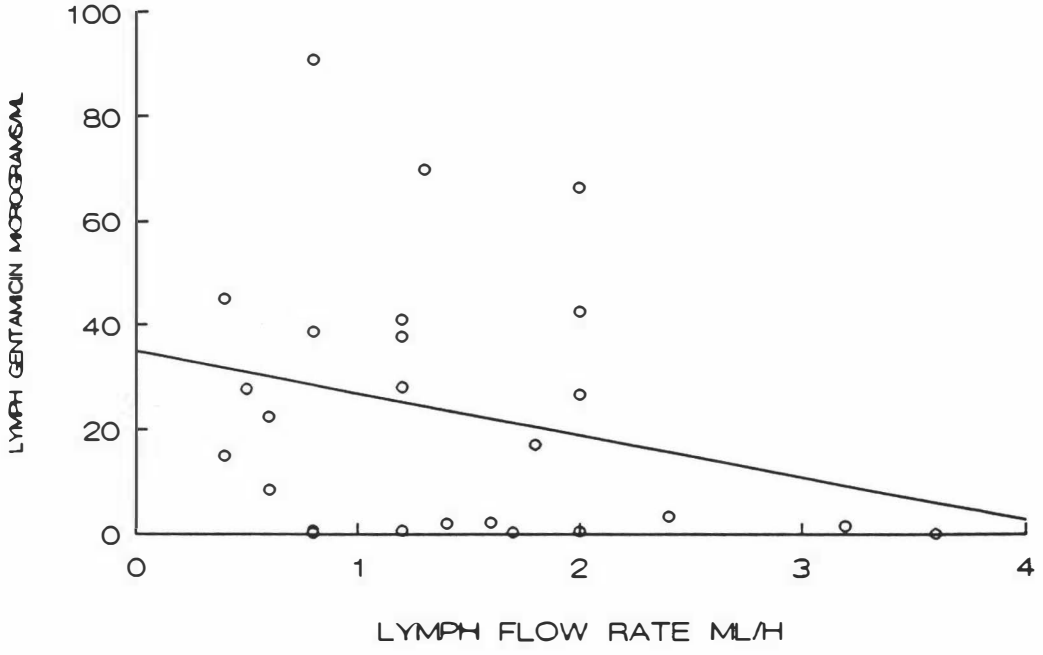


Figure 6. Relationship Between Lymph Flow Rates and Gentamicin Concentration in Collected Lymph.



Gentamicin concentrations quickly reached steady state levels (within 0.5 hours) in local and jugular plasma, indicating rapid absorption of antibiotic into the blood stream had taken place. These levels then remained constant for the duration of the experiment. The concentration of gentamicin in local plasma exceeded that of jugular plasma at all times and was for the two hour period 2.37 $\pm$ 0.3 times higher (Table III).

Gentamicin was present in samples collected between 0 and 0.25 hours following injection. However, the rate of increase of gentamicin in lymph was much slower than that in plasma, with the mean time to reach maximum concentration being 1.7 $\pm$ 0.23 h after joint injection. The maximum concentration of drug in lymph varied markedly between horses (range 19.9-90.9 μg/ml) and the mean was 49.48 $\pm$ 13.01 μg/ml (median 42.6), which was approximately 20 times greater than the mean maximum concentration achieved in local plasma (mean 2.63 $\pm$ 0.7 μg/ml, median 1.9)

Absorption and Disappearance of Gentamicin

Pharmacokinetic data describing the absorption of gentamicin into blood and lymph and its disappearance from the fetlock joint is shown in Table III.

Table III. Pharmacokinetic Data for Gentamicin Absorption and Disappearance from the Fetlock Joint after Intra-articular Injection (n=5).

Parameter	Horse Number					
	1	2	3	4	3*	Mean (± sem)
$t_{1/2}d$ (h) (0.5-2 h)	2.46	0.80	0.67	0.90	1.26	0.99 (0.83-1.22)
Ratio L/J	2.44	1.49	2.16	2.41	3.34	2.37 (± 0.3)
TG (μg)	107.3	16.4	99.5	94.2	29.5	69.4 (± 19.2)

* Horse 3 was used twice one week apart.

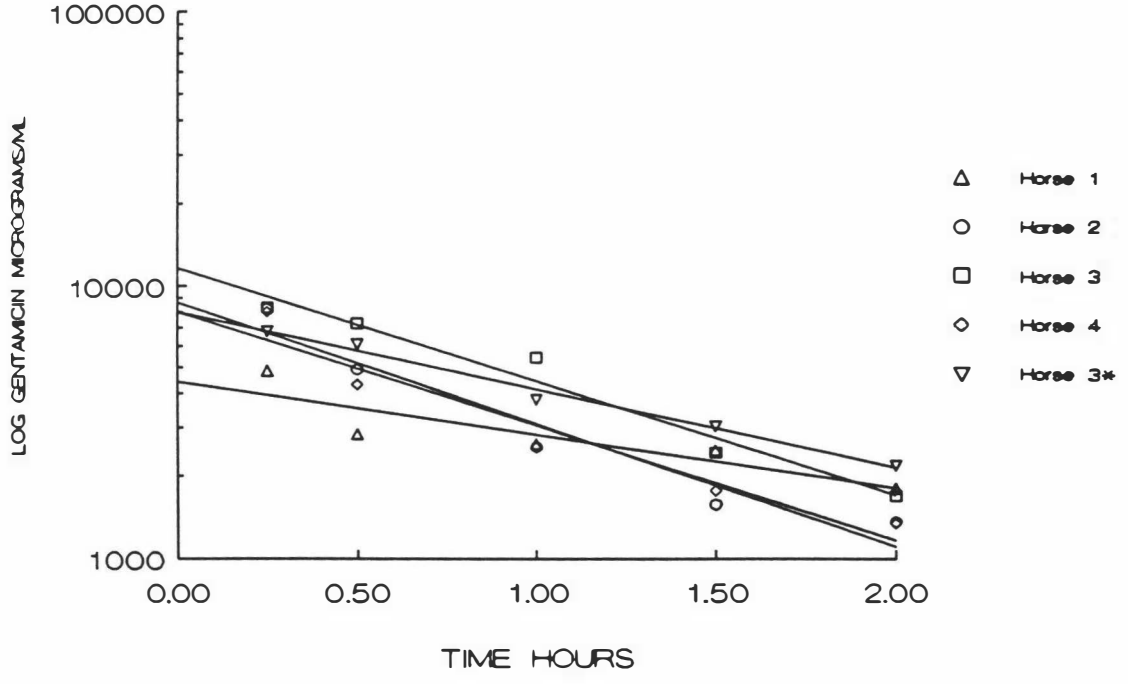
$t_{1/2}d$ = the disappearance half-life of gentamicin from the joint (diffusion into the surrounding tissues, blood and lymph absorption and synovial fluid sampling) between 0.5-2 hours.

Ratio L/J = ratio of local to jugular plasma concentrations of gentamicin.

TG = Total amount of gentamicin absorbed into the lymph vessel examined.

The disappearance of gentamicin from the joint appeared to be biphasic, with an initial distribution/mixing phase occurring within the first 0.5 hours (horse 6 had a longer distribution phase of 1 hour). Following this distribution/mixing phase a log-linear decrease in synovial fluid gentamicin concentration was demonstrated (Figure 7).

Figure 7. Semilogarithmic Plot of Mean (± sem) Gentamicin Concentration in Synovial Fluid after Intra-Articular Injection.



* Horse 3 used twice, one week apart

Horse 1 $r^2 = 0.8$
Horse 2 $r^2 = 0.94$
Horse 3 $r^2 = 0.97$
Horse 4 $r^2 = 0.93$
Horse 3 $r^2 = 0.99$

This signified that a single exponential function would adequately describe the kinetics of drug disappearance from the joint (Baggot 1977, Ekman *et al* 1981). The mean disappearance half-life (harmonic mean) was 0.99 (0.83-1.22) hours.

The contribution by the lymphatic vessel that was cannulated, to the removal of gentamicin from the fetlock joint, was small. The mean total amount of gentamicin transported via this vessel was only 69.4 $\pm$ 19.2 μg or 0.07% of the mean total amount of gentamicin removed from the joint by all sources (lymph, blood and sampling).

III$_4$ DISCUSSION

The aim of this investigation was to determine whether the common dorsal digital lymph trunk had a contribution to its lymph derived from the synovium of the fetlock joint, as reported by Baum (1920). The presence of high concentrations of gentamicin in lymph collected from this vessel following direct injection into the fetlock joint is consistent with Baum's finding. These results obtained from living animals support physiologically the anatomical observations made by Baum some 65 years ago.

However, before accepting this conclusion it is necessary to rule out routes other than transynovial absorption of drug into subsynovial lymphatic capillaries that could account for the presence of gentamicin in the sampled lymph One possible alternative could have been the absorption of drug into lymphatic capillaries in periarticular tissues due to accidental periarticular injection and or leakage. Such an injection would introduce the drug into the tissues surrounding the fetlock joint and from there absorption into tributaries of the common dorsal digital lymph trunk. Another possibility was the absorption of gentamicin into venous capillaries draining joint tissues and then into lymph, either by redistribution to the subcutaneous tissues or perhaps, via local lymphaticovenous anastomoses.

Periarticular injection was unlikely because synovial fluid was aspirated prior to all injections and leakage was prevented by retaining the catheter in the joint. Furthermore, if this had occurred then much higher concentrations of gentamicin would have been expected in both local plasma and lymph, and lower concentrations than those observed in synovial fluid. Given the large plasma volume of the horse, and the fact that lymph gentamicin concentrations were still rising (and to very much higher levels) when both jugular and local plasma levels were constant, it is highly improbable that there was any significant redistribution of drug by the systemic circulation. Lymphaticovenous anastomoses are known to exist, but are thought to be of significance only in pathological states or when lymphatic pressures rise greatly above normal (Gnepp 1984).

Steady state concentrations of gentamicin were achieved in local blood 15 minutes after joint injection. To precisely characterise the kinetics of antibiotic absorption into local blood more frequent sampling within this period would have been necessary. Furthermore, it is evident that "true" local blood was not in fact sampled, since the concentration of gentamicin in samples collected from the common dorsal digital vein were on average only 2.67 times higher than those collected from the jugular. This ratio is lower than would be expected and indicates that some degree of dilution was occurring. Despite these circumstances, the rapid equilibration of gentamicin in plasma combined with the very small mass of gentamicin that was recovered in lymph, indicate that vascular absorption of gentamicin from the fetlock joint following intra-articular injection appears more important than lymphatic absorption. This is consistent with the fact that gentamicin is of relatively small molecular size (molecular weight 472), and that the transynovial absorption of small molecules (Simpkin and Pizzorno 1974) and fluid, such as Ringers solution (Levick 1980) appears to be predominantly via subsynovial venous capillaries. In addition, the principal function of lymphatic drainage of joints is thought to be the absorption of protein and large particulate matter (Key 1926, Yoffey and Courtice 1970).

Although the concentration of gentamicin retrieved in lymph was a lot higher than that in local plasma (approximately 20 times), the rate of increase was slow in comparison. This slow rate of increase could have been due to a slow penetration of drug into lymph, a slow lymph flow or both.

Differences in penetration rate between the two fluids could be explained by the fact that subintimal blood capillaries are both closer and more numerous (and therefore have a larger surface area) than the subintimal lymphatic capillaries, with respect to the synovial cavity (Davies 1945, Van sickle 1978). In obeyance with Fick's Law (Appendix I), the rate of diffusion from synovial fluid would thus be faster into blood compared to lymph.

Very low lymph flow rates at the end of the experiment could have resulted in high gentamicin levels at this time. However, there was no significant difference between the mean lymph flow rate at each sampling interval. To see if lower lymph flows were associated with higher concentrations of gentamicin a correlation analysis was done on these two variables. A small and statistically insignificant correlation between lymph flow rate and lymph gentamicin concentration (-0.26) was found, suggesting that the influence of lymph flow on gentamicin concentration was at best minimal. However, the correlation analysis was performed on all the lymph concentration data collected over the two hour period and because the lymph concentration changes with time, it is possible that this could have had a confounding effect. Correlation analysis between flow rate and gentamicin concentration at each sampling time would have negated such an effect but too few data pairs were available for this.

Although fluctuations in lymph flow rate do not appear strongly associated with changes in lymph gentamicin concentration, an overall low lymph flow rate (combined with the effect of cannula deadspace - discussed more fully in Chapter V) could none the less have resulted in an apparently slow lymph penetration and possibly drug accumulation. Despite the fact that lymph flow rate is known to be very low in anaesthetised animals the overall flow rate (1.45 $\pm$ 0.18ml/h) was reasonably high when compared to that reported in anaesthetised horses whose limbs were not manipulated, mean 0.41 ml/h (Robinson *et al* 1975), and from that recorded from a similar lymph vessel in humans during sleeping, 0.24 $\pm$ 0.06 ml/h or when standing, 0.41 $\pm$ 0.21 ml/h (Olszewski 1977). In summary, it is difficult to say exactly what effect lymph flow rate had on gentamicin concentrations in this preparation. Further investigation involving high and low lymph flow rates would be required to resolve the problem.

However, the most logical explanation for the high concentrations of gentamicin in lymph is the very large concentration gradient that would be created between the synovial fluid and the subsynovial interstitium, following the intra-articular injection.

Once it was established that the lymph vessels studied drained the synovium of the fetlock joint, the relationship between the synovial fluid concentration of gentamicin and the tissue fluid concentration (as represented by peripheral lymph) could then be investigated.

CHAPTER IV INVESTIGATION 3

DETERMINATION OF THE DISPOSITION OF GENTAMICIN IN EQUINE PLASMA, SYNOVIAL FLUID AND LYMPH

IV_1 INTRODUCTION

It was established from the results of the last two investigations that specific lymph vessels drained the synovium of the hind limb fetlock joint in the horse. With this information it was possible to examine the following hypothesis: " the synovial fluid concentration of an antimicrobial agent is useful as an index of its concentration in tissue fluid".

For this purpose two trials were conducted. The first (Trial A) commenced the day following lymph cannula implantation. The second (Trial B) commenced not less than 48 hours after cannulation of the lymph vessel and immediately following joint catheterisation.

IV_2 MATERIALS AND METHODS

The materials and methods adopted in both trials are described below.

Animals

The same five horses were used in both trials (Appendix IV, Table 1). The experimental horses were examined and given ivermectin orally as described in Chapter III. In addition, each horse was examined for evidence of disease or lameness associated with the hindlimb fetlock joints. This included palpation of these joints for evidence of heat, pain or effusion, and observation for lameness at the walk, trot, and after a 1 minute flexion test of each fetlock joint. Lateral and dorso-plantar radiographs of the fetlock joints of both hindlimbs in each horse were taken to exclude radiographic abnormality.

The horses were kept on straw bedding in 3 x 3 m square stalls and fed hay, maize, oats and bran mash. Water was supplied *ad libitum*. To prepare for the trials, the horses were kept in these surroundings and their hind limbs handled and bandaged several times during the week immediately prior to the study.

Anaesthesia and Surgery

Premedication and induction of general anaesthesia in horses 3, 4 and 5 was by the thiopentone sodium-glyceryl guiacolate technique previously described. Induction of general anaesthesia in horses 1 and 2 was achieved using a combination of intravenous xylazine at a dose rate of 1.1 mg/kg followed 5-10 minutes later by intravenous ketamine[1] at a dose rate of 2.2 mg/kg. No premedication was used in these horses. All horses were maintained on a mixture of halothane and oxygen.

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1 Ketavet 100 Delta Veterinary Laboratories Pty Ltd, Hornsby NSW 2077, Australia.

Each horse had the common dorsal digital lymph trunk of a hindlimb aseptically cannulated with a sterile polyvinyl chloride cannula as described in the previous chapter.

The cannula was carefully bandaged into place prior to recovery, in the following manner:

1) The wound was sprayed with povidone iodine[2].

2) A piece of paraffin gauze was placed over the incision.

3) A gauze bandage was wrapped loosely around the limb, covering the incision and the first 5 cm of the protruding cannula. The bandage was then secured to itself with tape.

4) The free end of the cannula was then led to the lateral side of the mid metatarsus, and in the process, formed a semicircular arc (about 6 cm in diameter) the apex of which lay flat on the dorsal surface of the proximal metatarsus.

5) A single layer of cotton wool extending the length of the metatarsus was wrapped around the leg.

6) Finally, an elastic bandage[3] was loosely wrapped around the cotton wool layer. The free end of the cannula emerged through an incision made in the elastic bandage and the cotton wool, about halfway down the metatarsus on the lateral side.

7) A 20 ml heparin-rinsed syringe with the plunger drawn was threaded over the end of the cannula and then taped to the lateral side of the leg with sellotape.

To prevent disruption of the implanted cannula during anaesthetic recovery, horses were assisted when attempting to stand. After recovery, each horse was confined in a box for the duration of the experiment. In Trial A, horse 2 was housed in a partitioned stall which allowed no walking.

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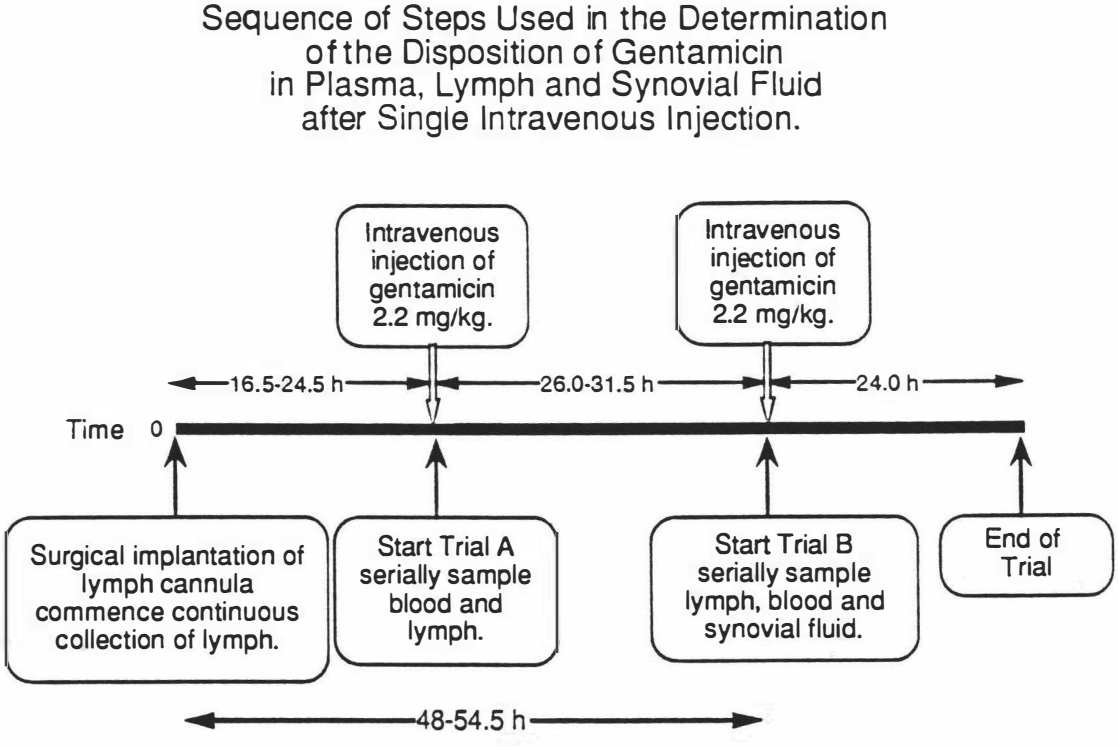
2 Biocil Ethical Agents, Parnell, Auckland.

3 Vetwrap[r] 4 inch roll, St. Paul, MN 5514.

Prior to the commencement of each trial, free flowing lymph was collected into heparinised syringes attached to the lower limb. To do this, the plunger of each syringe was cut flush with the barrel (so as not to contact the ground or annoy the horse). The syringe nozzle was then threaded onto to the free end of the implanted cannula and the syringe affixed to the limb with velcro or sellotape (Figure 2). Syringes were changed at approximately 2 hour intervals during the day and 8 hour intervals at night. A shortened, 60 ml syringe was used overnight and was replaced each morning. Figure 1 shows the sequence of procedures used in these Trials.

Figure 1. **Sequence of Procedures Used in the Determination of the Disposition of Gentamicin in Plasma, Synovial Fluid and Lymph after Intravenous Injection.**



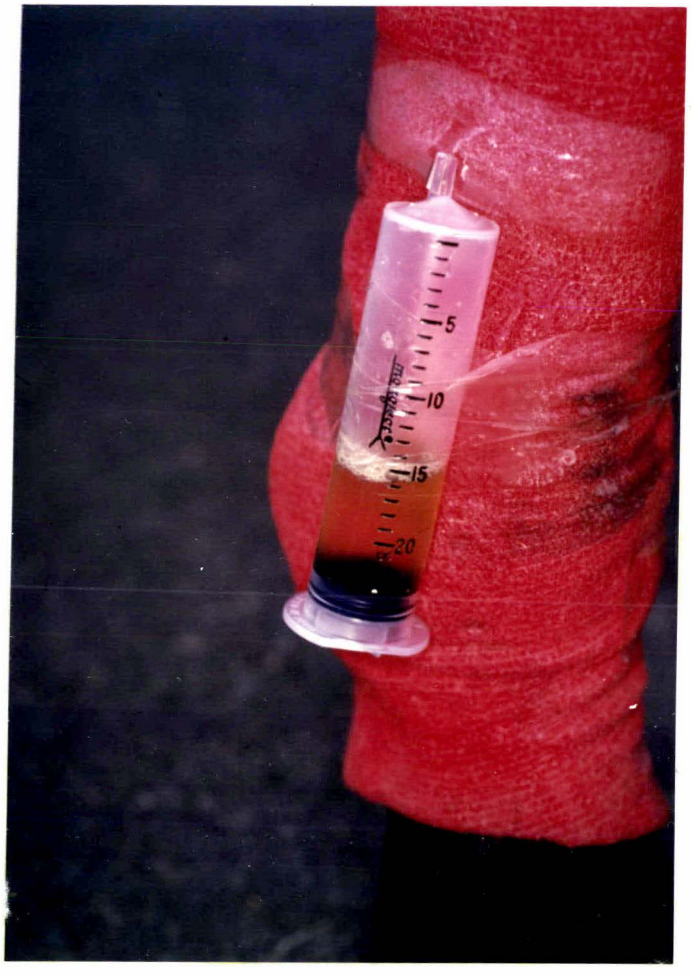


Figure 2. **Lymph Collection Syringe Threaded on to Lymph Cannula and Attached by Sellotape to the Hindlimb of a Horse.**

Experimental Procedure: Trial A.

In Trial A, the disposition of gentamicin in venous blood and lymph was studied as follows:

(i) A 14 gauge x 83 mm catheter was placed retrograde into an aseptically prepared jugular vein. A butterfly tab, made with tape was attached to the hub of the catheter and fixed to the skin of the neck with two simple interrupted (polypropylene) sutures. A three way stopcock was attached to the catheter hub and the catheter was heparinised with 1 ml of heparin between collections.

(ii) Blood and lymph samples were collected prior to the intravenous injection of gentamicin.

(iii) Using an 18 gauge x 44 mm catheter each horse then received an intravenous injection of gentamicin at a dose rate of 2.2 mg/kg into the contralateral jugular vein.

(iv) Samples of blood were then collected sequentially at post injection times 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 hours.

(v) Lymph collection syringes were changed and lymph volume and lymph flow rate recorded at post injection intervals 0-0.25, 0.25-0.5, 0.5-1, 1-2, 2-3, 3-4, 4-6, 6-8, 8-12 hours and at variable intervals between 12-24 hours.

If a clot formed on the end of the lymph cannula it was gently removed. When lymph flow was very low or cannula blockage occurred, the horse was briefly walked around its box or for a distance of 100-200 m outside its box. If flow halted completely, heparin was back flushed through the cannula using a 23 gauge x 25 mm needle and a 3 ml syringe.

A record was kept of the behaviour of each horse, including appetite, rectal temperature, pulse, respiratory rate, amount and type of voluntary movement and changes in posture. The amount of forced exercise was also recorded.

Analysis of Samples

a) Jugular venous blood and lymph samples were processed for gentamicin concentration at each sampling time/interval, in the same manner described in Chapter III.

b) Lymph protein concentration was determined by the method of Lowry using bovine serum albumin as the standard (Lowry *et al* 1951). Total and differential cell count was performed three times within the first 12 hours following surgery and then at approximately 12 hour intervals throughout the trial period.

Total cell count was performed by diluting 100 μl of sample 1:1 with 10% crystal violet in isotonic saline and counting the number of cells in the middle 25 squares of an haemocytometer[4]. Differential cell counts were performed on a stained[5] smear of centrifuged lymph. At least 100 cells were counted.

Experimental Procedure: Trial B

In Trial B, the disposition of gentamicin was studied in venous blood, synovial fluid and lymph. The same protocol was followed as in A, with the addition that synovial fluid was sampled (Figure 2). The jugular venous catheter used for blood sampling in Trial A was removed and a new catheter placed in the opposite vein at the commencement of Trial B.

The procedure for the sampling of lymph and blood was identical to that performed in Trial A. For the sampling of synovial fluid, a 20 gauge x 32 mm catheter was placed into the lateral plantar recess of the fetlock joint of the limb containing the cannulated lymph

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4 Spencer^r Improved Neubauer Haemocytometer.

5 Diffquick^r Stain. Baxter Health Care Corporation, Miami Florida USA.

vessel. Joint catheterisation was achieved by first cutting a rectangular "window" out of the distal end of the bandage that protected the lymph cannula.

The skin over the lateral plantar recess of the fetlock joint was then aseptically prepared and a bleb of 2% lidocaine placed subcutaneously at the puncture site. The horse was restrained with a nose twitch and the catheter was slowly introduced through the skin over the joint recess some 2 cm distal to the end of the splint bone and almost vertically to the skin surface. This ensured that the end of the catheter was seated just dorsal to the lateral proximal sesamoid and plantar to the lateral condyle of the distal metatarsus.

A piece of tape 1 cm wide was wrapped once only, around the circumference of the catheter hub leaving a long end free. A drop of glue was then applied between the catheter hub and the skin of the horse resulting in attachment of the catheter to the skin. The free end of the tape was then wrapped 1-2 times around the entire distal metatarsus, to firmly support the catheter hub. A single piece of reusable bandage was then wrapped around the fetlock to loosely protect the implanted catheter. Figure 3 shows the catheterised joint.

Figure 3. Distal Hindlimb of a Horse with a Catheterised Fetlock Joint and an Implanted Lymph Cannula.

Note that a "window" has been cut in the bandage (open arrow). The joint catheter is "plugged" (white arrow) and the lymph collection syringe can be seen on the dorsolateral aspect of the limb (closed arrow).

Prior to intravenous injection of gentamicin, 0.4 ml of synovial fluid was collected, the catheter needle removed and the catheter hub capped with a catheter plug. Synovial fluid sampling was attempted at 0.5, 1, 2, 4, 8, and 12 hours after gentamicin injection. Sampling through the implanted catheter was achieved either with, or without, a needle. Initially, a new 20 gauge catheter needle was used to sample 0.2 ml of synovial fluid directly from the joint through the catheter lumen. The end of this needle protruded approximately 2 mm past the end of the catheter ensuring that only synovial fluid within the joint was sampled. However, an alternative method was also used to avoid haemorrhage associated with arthrocentesis using the needle. No needle was used with this method and 0.1 ml of synovial fluid (the volume of the catheter) was first aspirated and then discarded. A 0.2 ml sample of synovial fluid was then collected.

Four hours after the injection the implanted catheter was removed and synovial fluid sampling was then performed with an 18 gauge x 40 mm needle. This occurred in all horses except horse 5 in which the catheter was used for all sampling.

Sample Analysis

a) All samples were processed exactly the same way as in Trial A.
In addition synovial fluid was analysed as described below.

b) Synovial fluid samples collected prior to and 12 hours after gentamicin administration were divided in two. One was analysed without centrifugation, for protein concentration (Lowry method as described above) erythrocyte, total and differential white cell counts. The other half of these samples together with the samples collected 0.5,1,2,4 and 8 hours post injection, were centrifuged for five minutes at 1200g. The supernatants were then frozen and stored as described, prior to gentamicin analysis.

Data Analysis and Statistical Analysis

The disposition curves describing the decline in plasma gentamicin concentration, with respect to time, were analysed separately for each horse. Compartmental analysis (Baggot 1977) was first performed on the data using a non-linear least squares computer programme[6] to fit both a one compartment and a two compartment model. However, final data analysis was performed using noncompartmental methods (Ritschel 1986) and the reasons for this are outlined in the results section (IV_3).

Accordingly, the following parameters were estimated (i) the area under the plasma concentration-time curve from time zero to infinity ($AUC_{0-\infty}$), was calculated as follows $AUC_{0-\infty} = AUC_{0-t} + C_t/\lambda_z$, where $AUC_{0-\infty}$ is the AUC from time zero to time t and C_t is the last measurable concentration of drug in plasma at time t. λ_z is the terminal rate constant and this was obtained by non-weighted least squares linear regression (pocket calculator) using the last 4-6 points selected from the terminal phase of a semilogarithmic plot of gentamicin concentration vs time (Table 8, Appendix IV). AUC_{0-t} was estimated using the trapezoidal method. (ii) the area under the first moment curve (AUMC) was calculated as $AUMC_{0-\infty} = AUMC_{0-t} + (C_t.t)/\lambda_z + C_t/(\lambda_z)^2$, where C_t is the last measurable plasma concentration in plasma at time t. $AUMC_{0-t}$ was calculated by the linear trapezoidal method. (iii) the mean residence time (MRT) was calculated as MRT = $AUMC_{0-\infty}/AUC_{0-\infty}$. (iv) the apparent volume of distribution ($V_{d(area)}$) was calculated as $V_{d(area)}$ = dose/$AUC_{0-\infty}$ x λ_z and (v) the apparent volume of distribution at steady state (V_{ss}) was calculated as V_{ss} = dose x $AUMC_{0-\infty}/(AUC_{0-\infty})^2$. (viii) Total Body Clearance (Cl_B) was calculated as Cl_B = dose/AUC. (vii) an estimate of the elimination half-life ($t_{1/2}$) of gentamicin in plasma was calculated as $t_{1/2} = \ln 2/\lambda_z$.

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6 PC Nonlin. Copyright 1984,1986 Statistical Consultants Carl M Metzler, and Daniel L. Weiner.

Because gentamicin in lymph was measured over an interval, all concentrations were referred to the mid-point of the interval for analyses and graphical presentation. The AUC, λ_z, AUMC, MRT and $t_{1/2}$ were calculated in the same manner for the lymph disposition data. The percentage penetration of gentamicin into lymph was calculated from the ratios of the AUC's for each fluid - AUC_L (AUC lymph)/AUC_P (AUC plasma) x 100% (Bergan 1981). Also estimated for the lymph data was the maximum concentration ($C_L$max) and the time taken to reach this level ($T_L$max). Not enough data points were available to accurately describe the elimination phase of the synovial fluid gentamicin disposition curves for each horse, therefore only the maximum concentration (C_s max) and the time taken to reach this level (T_s max) were estimated.

A Wilk's Shapiro test (1965)[7] was used to determine if estimated and derived pharmacokinetic parameters, Cmax and Tmax data were normally distributed. This test is a "goodness of fit" test which compares the mean and variance of the sample in question, to a sample of equal size which is known to be normally distributed. Distribution was considered not normal if $p < 0.05$. For variables that were normally distributed, Student's t test for paired data (Rosner 1990) was used to make the following statistical comparisons: (a) all mean pharmacokinetic parameters between Trial A and Trial B, and (b) mean maximum concentration of gentamicin between lymph ($C_L$max) and synovial fluid ($C_s$max) in Trial B. The elimination half-life estimates of gentamicin in plasma and lymph in Trial A and in Trial B were found to be not normally distributed. However, the inverse of the half lives was found to be normally distributed and these were averaged to produce the "harmonic mean". The standard error for the harmonic mean is not symmetrical, and therefore was reported as a range. The Student's t test for paired data was used to compare the harmonic mean elimination half-life of gentamicin in plasma to that for lymph (Trial A and Trial B). In all cases, differences were considered significant if $p < 0.05$.

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7 SAS, Statistical Analysis System, SAS Institute Inc.
Cary, NC USA.

Because the mid-point of the lymph collection interval and the plasma sample times were not the same, direct statistical comparison of the measured gentamicin concentrations was not possible. Therefore, non-weighted least squares linear regression analysis was used in the following way. The log plasma gentamicin concentration time data for each horse (obtained from the semilogarithmic plots of plasma and lymph gentamicin concentration vs time) measured over the terminal elimination phase (1.5-10 h) were used. Non-weighted least squares linear regression analysis[8] yielded the equation, predicted gentamicin concentrations and 95% confidence interval, for the line which best described this data. Separate analyses were performed for Trials A and B.

The predicted plasma and lymph gentamicin concentrations for both Trials were compared by inspecting the regression lines for overlap of the 95% confidence intervals. Comparison of the predicted concentrations (3-8 h after injection) was also made by calculation of lymph:plasma ratios.

Because of a small number of data points, it was not possible to accurately determine the regression line for the terminal phase of a semilogarithmic plot of synovial fluid gentamicin vs time using the same technique. Differences in gentamicin concentrations between synovial fluid and plasma, and synovial fluid and lymph were therefore documented by calculating ratios for the interval 2-8 h following injection. Due to the differences in sampling times, actual measured synovial gentamicin concentrations and the predicted lymph and plasma gentamicin concentrations described above, were used in these calculations.

Details of the techniques of compartmental and noncompartmental pharmacokinetic analysis, together with further explanation of some of the equations used, is presented in Appendix I.

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[8] SAS, Statistical Analysis System, SAS Institute Inc.
Cary, NC USA.

IV_3 RESULTS

Lymph vessel cannulation was successfully performed in 8 horses. In 2 horses the lymph cannulae became dislodged in the immediate post operative period whilst recovering from anaesthesia. In one other horse, cannulation was successful but the horse broke a leg during recovery, necessitating euthanasia. Five horses were therefore available for lymph collection and lymph was collected continuously until either cannula failure (3 horses) or termination of the trials (4 days).

Lymph Collection and Lymph Flow Rates

The colour of the collected lymph gradually changed over a 24 hour period from blue, through green to yellow/straw (Figure 4). Orange coloured lymph was indicative of the presence of an increased amount of red blood cells. In one horse (5), when the lymph no longer contained blue dye it stayed orange in colour for the remainder of the study period. The four other horses had yellow/straw coloured lymph for Trial A but in two of these horses (1 and 3), the lymph changed to orange in Trial B (after fetlock joint catheterisation). In all horses, lymph collected after joint catheterisation formed an obvious red dot of sedimented red blood cells at the bottom of the sample tubes.

Lymph flow rates for individuals and, mean lymph flow rates recorded for each sampling period (Trials A and B) are presented in Tables I and II. A plot of mean lymph flow rate for each sampling period vs time, for both trials, is presented in Figure 5.

Lymph flow rates varied widely, ranging from as little as 0.13 ml/h to as much as 28 ml/h. Such variability was apparent for individuals throughout a trial, between individuals and between trials.

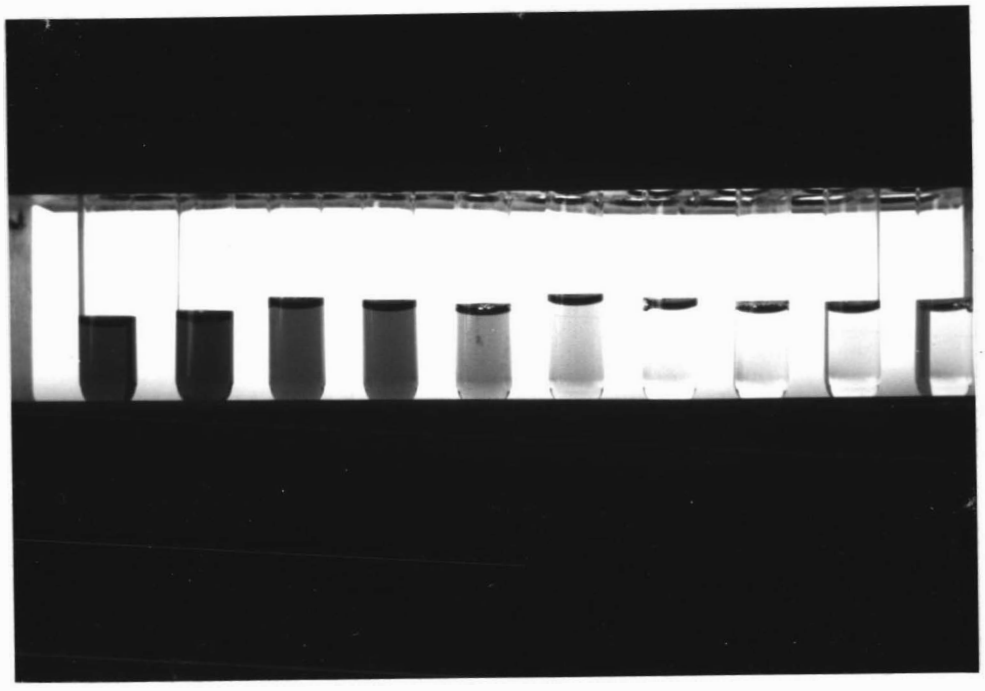


Figure 4. **Lymph Samples Collected Over a 24 Hour Period.**

Table I. **Individual and Mean (± sem) Lymph Flow Rates Recorded for Each Sampling Interval in Trial A.**

	Lymph Flow Rate ml/h Trial A					
Sampling Interval (h)	**H_1**	**H_2**	**H_3**	**H_4**	**H_5**	**Mean (±sem)**
Prior to 0	3.89	1.55	3.91	5.93	2.57	3.57 ± 0.74
0-0.25	10.4	4.8	12	12	4.8	8.8 ±1.66
0.5-1	8	0.8	8.8	2	3.2	3.36 ±1.52
1-2	7.2	0.8	9	11	5	6.6 ±1.76
2-3	7	0.2	9.6	9.6	4.6	6.2 ±1.77
3-4	5	0.7	8.6	10.2	4	5.7 ±1.68
4-6	4.7	0.25	7.7	10	3.7	5.27 ±1.68
6-8	1.6	0.8	6.5	10	1.5	4.08 ±1.8
8-12	2.64	0.24	4.75	9.25	3.5	4.08 ±1.49
12-24	2.64	ns	1.03	4.76	2.01	2.61 ±0.79

ns = no sample

Table II. **Individual and Mean (± sem) Lymph Flow Rates ml/h Recorded for Each Sampling Interval in Trial B.**

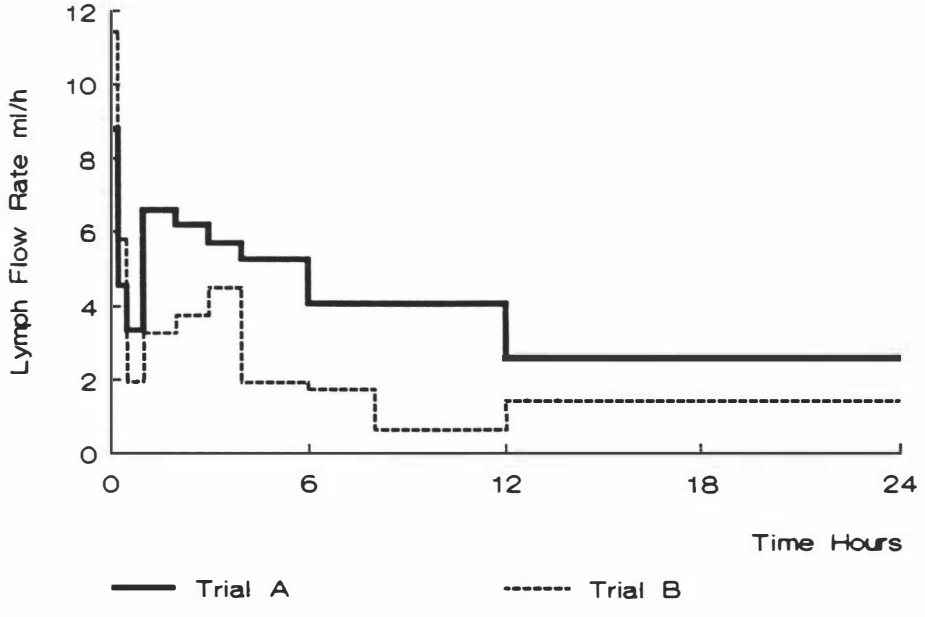
	Lymph Flow Rate ml/h Trial B					
Sampling Interval (h)	**H_1**	**H_2**	**H_3**	**H_4**	**H_5**	**Mean (±sem)**
Prior to 0	3.3	0.59	1.27	9.1	4.33	3.72 ±1.5
0-0.25	8.4	2	2	28	16.8	11.44 ±4.95
0.25-0.5	4	0.6	2	12	10.4	5.8 ±2.28
0.5-1	3.8	0.2	0.8	3.7	1.2	1.94 ±0.76
1-2	3.8	0.9	1.2	8	2.5	3.28 ±1.29
2-3	2.15	0.5	1.2	9	5.9	3.75 ±1.44
3-4	2.15	0.35	2	7.8	10.2	4.5 ±1.9
4-6	1.7	0.2	1.3	2.5	4	1.94 ±0.64
6-8	0.4	0.55	0.3	3.5	4	1.75 ±0.82
8-12	ns	0.35	0.4	0.13	1.75	0.66 ±0.37
12-24	ns	0.95	1.9	ns	ns	1.43 ±0.48

ns = no sample

It was observed that increased activity associated with recovery from anaesthesia, walking, massage of the pastern, compression of the bandages and general handling all transiently increased the lymph flow rate, whilst recumbency, tight bandages and progressive clot formation all reduced lymph flow rates.

Experiments usually began in the morning between 9 am and 12 pm and flow rates were higher at this time gradually decreasing over the trial (Figure 5).

Figure 5. Mean Lymph Flow Rates for Trials A and B.



Horses were most active in the earlier part of the day in association with cleaning out their stalls, feeding and in response to stimulation from outside their boxes (noise, other horses etc.). This activity involved moving to and from the feed bin and moving around their boxes. The unusually high flow rates recorded after the start of each trial (at time intervals 0-0.25 and 0.25-0.5 h) reflect the increased amount of activity at this time. At this time the horses received intravenous injections and in Trial B, fetlock joint catheterisation had been performed. Reduced flow rates were observed towards the end of a trial (not

necessarily associated with night time sampling) and for Trial B compared to Trial A. These trends are seen in the profiles of mean lymph flow rate vs time, for which the two trials were similar (Figure 5). Over the 24 hour trial periods the mean lymph flow rates ranged from 0.66 $\pm$ 0.37 - 11.44 $\pm$ 4.95 ml/h.

Lymph Composition

Mean protein concentration and total and differential white cell counts (referred to the mid-point of the lymph collection interval) for lymph at various intervals are presented in Figures 6,7 and 8 (raw data in Appendix IV, Table 2-4).

There was marked variability amongst individual horses with respect to lymph protein concentration and total white cell count over the experimental period. This is reflected in the mean values depicted in Figures 6 and 7.

Figure 6. **Lymph Protein (g/L) at Various Intervals after Surgery (mean ± sem).**

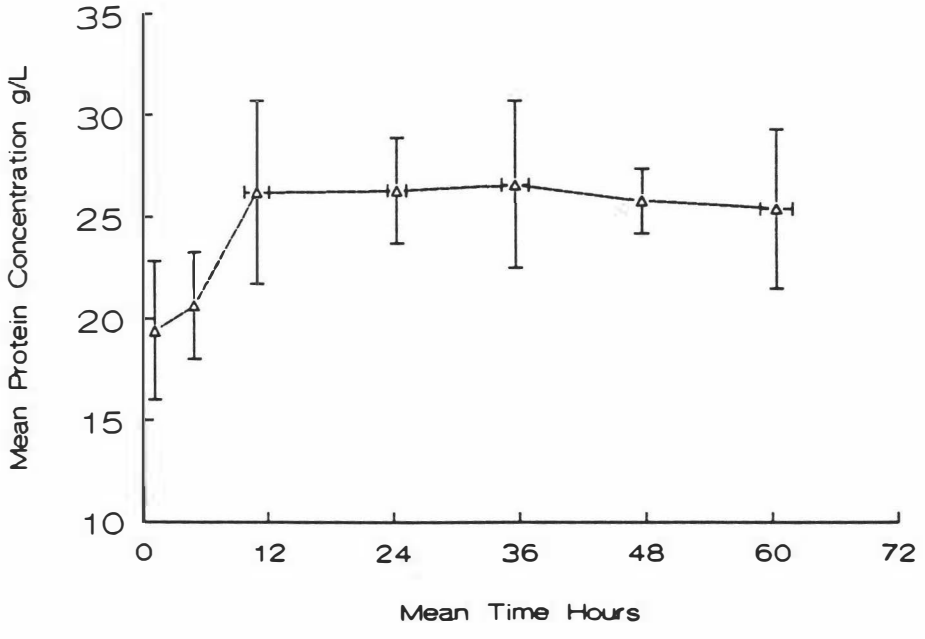
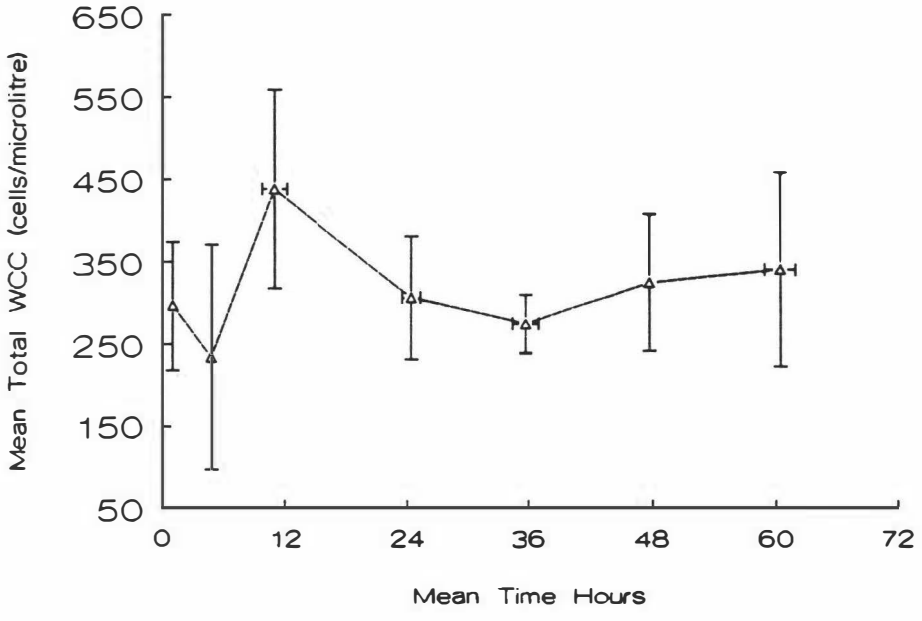


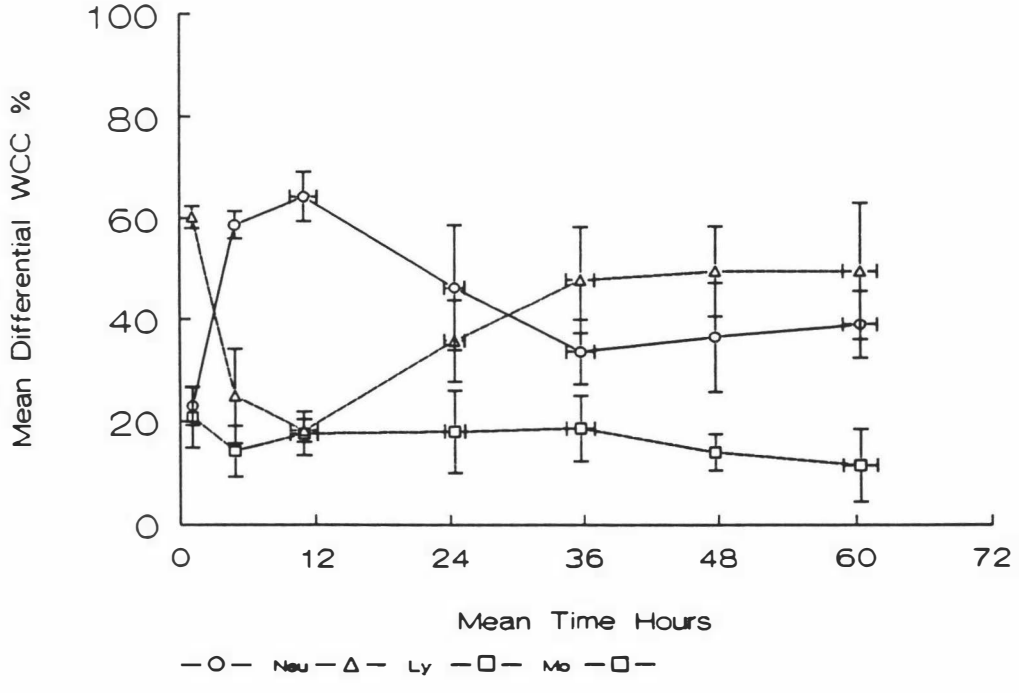
Figure 7. **Lymph Total White Cell Count (cells/microlitre) at Various Intervals after Surgery (mean ± sem).**



There was however, a trend towards lower protein concentration immediately post surgery. The mean lymph protein levels were 30-41% of mean pre-experimental plasma protein concentration (64.6 ± 1.5 g/l). Total lymph white cell count tended to be lower post surgery and were highest approximately 12 hours following anaesthetic recovery, remaining relatively constant for the duration of the experimental period.

There were marked deviations in the lymph differential white cell count throughout the postoperative period. Initially, lymphocytes predominated (60% of the differential) over neutrophils (20%). The reverse situation was seen 5-11 hours after surgery. By the start of Trial B the differential was becoming similar to that immediately post surgery (Figure 8). Monocyte numbers remained relatively constant throughout the postoperative period.

Figure 8. **Lymph Differential White Cell Count (%) at Various Intervals after Surgery (mean ± sem).**



Synovial Fluid

The protein concentration, mean white cell count and differential for post injection time zero and 12 hours respectively, are presented in Table II. There was a large increase in both protein concentration and white cell count. The differential count also changed over this time, from predominantly lymphocytes to neutrophils.

Table II. Synovial Fluid Parameters for Horses in Trial B, Measured at 0 and 12 hours after Joint Catheterisation (mean ± sem).

Parameters		Time after Joint Catheterisation (h)	
		T = 0 (n = 4)	*T = 12 (n = 3)*
Protein g/L		8.8 ± 2	40.8 ± 3.2
Total Leucocytes		466 ± 128	13290 ± 8008
Differential %	neutrophils	26.5 ± 9.5	76.7 ± 14.4
	lymphocytes	57 ± 6.2	12.7 ± 10.2
	monocytes	15 ± 5.8	10.7 ± 4.3

Disposition of Gentamicin in Plasma, Synovial Fluid and Lymph

The disposition curves describing the decline in the concentration of gentamicin in plasma were analysed using both compartmental and noncompartmental methods. Separate analyses were performed for each horse. Scrutiny of the semi-logarithmic plots of the plasma disposition curves, and of the compartment-model analyses (R^2 values, residual trends, standard deviation of the y estimates and pharmacokinetic parameters), revealed that different models fit the disposition data (Appendix IV). The one compartment model was appropriate for 7 disposition curves and the two compartment model for 3 disposition curves. It appeared that in most instances too few data points were available to fully describe both the distribution and elimination phases of the disposition curve necessary for the two-compartment model approach. Because the noncompartmental methods do not require the assumption of a specific compartment model (Gibaldi and Perrier 1982) and, to allow comparison between all horses, this method was used for plasma disposition data analysis.

The disposition curves describing the penetration and decline of gentamicin in lymph and synovial fluid were similar in appearance and in the majority of horses the penetration phase was followed by a monoexponential elimination phase (individual horse plasma, lymph and synovial fluid gentamicin disposition curves are presented in Appendix III).

The mean concentration of gentamicin in plasma and lymph (Trial A) and in plasma, lymph and synovial fluid (Trial B), is presented graphically (semilogarithmic plot) in Figures 9 and 10 (raw data - Appendix IV). Individual horse and mean disposition curve pharmacokinetic parameters for gentamicin in plasma, lymph and synovial fluid for Trials A and B are shown in Table III.

Figure 9. **Trial A: Disposition of Gentamicin (μg/ml) in Plasma and Lymph (mean ± sem).**

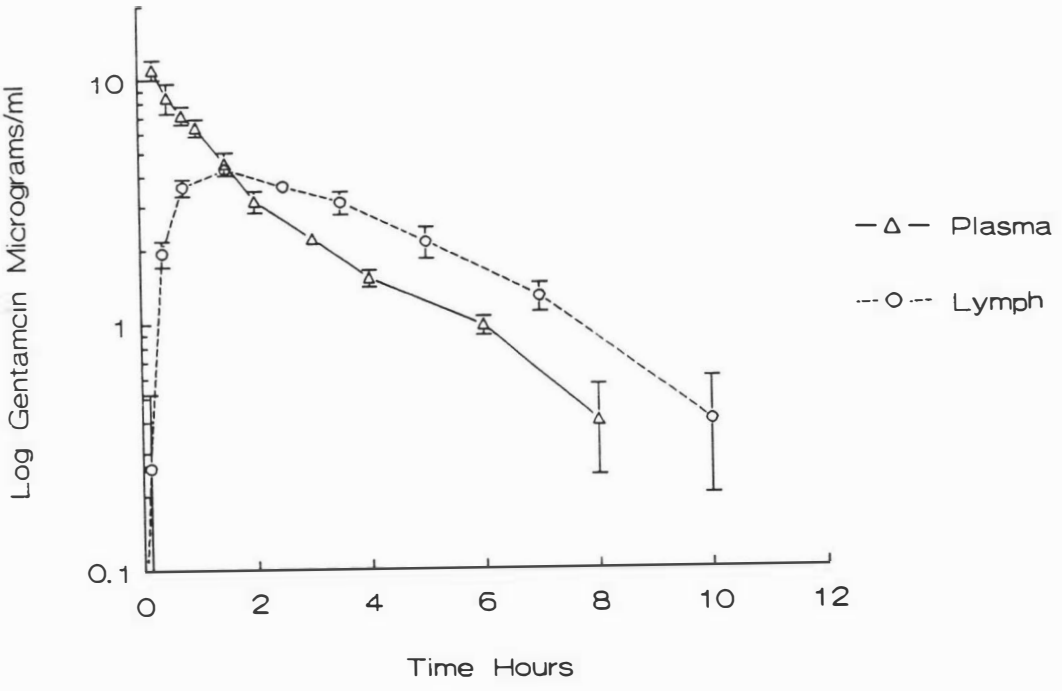


Figure 10. **Trial B: Disposition of Gentamicin (μg/ml) in Plasma, Synovial Fluid and Lymph (mean ± sem).**

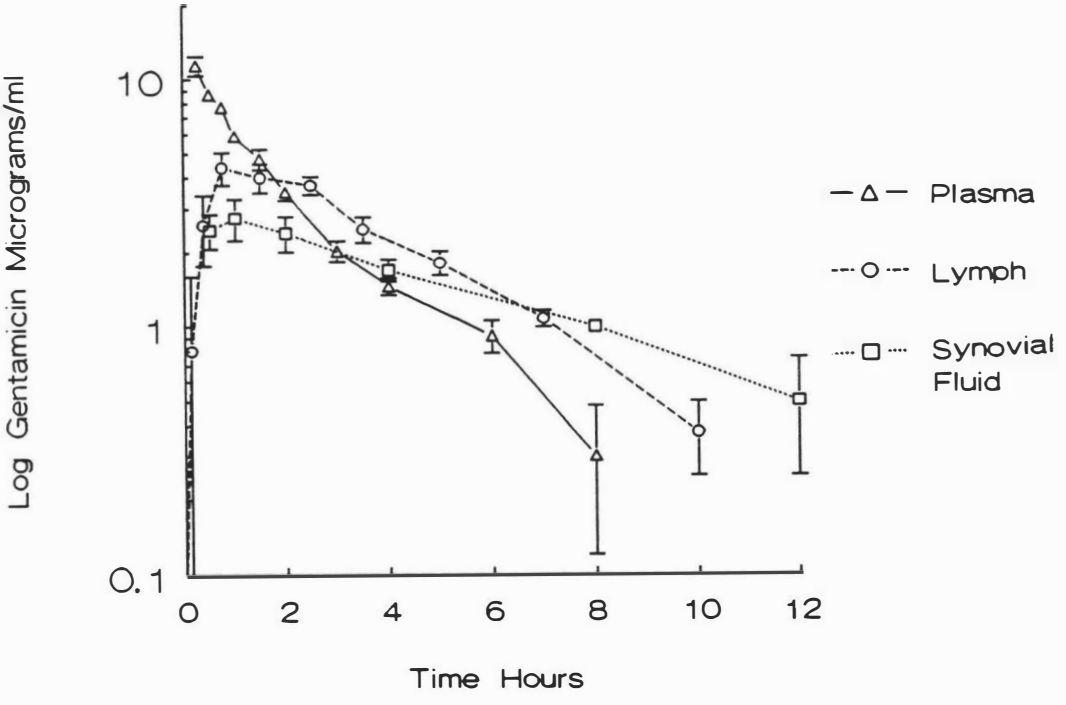


Table III. Pharmacokinetic Parameters for the Disposition of Gentamicin in Plasma, Lymph and Synovial Fluid.

Plasma		H_1		H_2		H_3		H_4		H_5		Mean ± sem	
Parameter	Unit	A	B	A	B	A	B	A	B	A	B	A	B
λ_z	h^{-1}	0.286	0.418	0.21	0.536	0.297	0.401	0.459	0.42	0.345	0.199	0.319 ±0.04	0.395 ±0.06
$t_{1/2}$	h	2.42	1.66	3.3	1.29	2.33	1.73	1.51	1.65	2.01	3.48	2.17* (1.92-2.5)	1.75* (1.54-2.04)
MRT	h	3.13	2.69	4.09	2.11	2.78	2.35	2.1	2.05	2.27	3.66	2.87 ±0.28	2.57 ±0.29
$AUC_{0-\infty}$	hμg/ml	25.51	25.17	21.37	27.27	23.15	21.41	21.23	19.97	27.48	24.82	23.75 ±1.21	23.73 ±1.33
$AUMC_{0-\infty}$	$\mu g/ml/h^2$	79.72	67.59	87.44	57.58	69.34	50.31	44.7	40.86	62.5	90.8	67.74 ±7.42	61.43 ±8.6
Cl_B	ml/kg/min	1.44	1.46	1.72	1.35	1.59	1.71	1.73	1.84	1.33	1.48	1.56 ±0.08	1.57 ±0.09
$V_{(darea)}$	L/kg	0.30	0.21	0.49	0.15	0.32	0.26	0.23	0.26	0.23	0.45	0.31 ±0.05	0.27 ±0.05
V_{SS}	L/kg	0.27	0.24	0.42	0.17	0.26	0.24	0.22	0.23	0.18	0.32	0.27 ±0.04	0.24 ±0.02

Table III
(contd.)

Lymph		H_1		H_2		H_3		H_4		H_5		Mean±sem	
Parameter	Unit	A	B	A	B	A	B	A	B	A	B	A	B
λ_Z	h^{-1}	0.111	0.202	0.216	0.284	0.251	0.267	0.287	0.124	0.289	0.214	0.232 ±0.032	0.218 ±0.03
$t_{1/2}$	h	5.97	3.43	3.21	2.44	2.76	2.6	2.42	5.59	2.4	3.24	3.03* (2.63-3.57)	3.13* (2.78-3.57)
MRT	h	7.25	6.19	5.56	4.33	4.96	4.21	4.08	7.18	4.06	4.4	5.18 ±0.59	5.26 ±0.6
$AUC_{0-\infty}$	hμg/ml	37.1	26.37	32.36	24.55	20.82	21.81	18.97	24.74	25.27	22.18	26.90 ±3.44	23.93 ±0.85
$AUMC_{0-\infty}$	$\mu gh^2/ml$	268.83	163.26	179.87	106.28	103.36	91.82	77.31	177.6	102.7	96.29	146.41 ±35.1	127.04 ±18
T_L max	μg/ml	4	3.4	4.5	5.1	3.7	5.1	3.9	4.5	5.3	6.8	4.28 ±0.29	4.98 ±0.55
C_L max	h	1.5	1.5	1.5	1.5	1.5	1.5	1.5	0.75	1.5	0.75	1.5	1.2 ±0.18
AUC_L/AUC_P		1.45	1.05	1.51	0.9	0.9	1.02	0.9	1.24	0.92	0.89	1.14 ±0.14	1.02 ±0.06

Table III
(Contd.)

Synovial Fluid		A	B	A	B	A	B	A	B	A	B	A	B
C_s max	μg/ml	ND	1.5	ND	4	ND	2.5	ND	2.6	ND	3.7	ND	2.86 ±0.45
T_s max	h	ND	4	ND	1	ND	1	ND	0.5	ND	1	ND	1.5 ±0.63

*= Harmonic mean and range of one sem.
ND = Not done

The means of the kinetic parameters describing the disposition of gentamicin in plasma and lymph were not significantly different between the two Trials. Following intravenous injection gentamicin levels in plasma declined exponentially (Figures 9 and 10). Fifteen minutes post injection the mean plasma concentration was 11.04 $\pm$ 1.02 μg/ml and 11.42 $\pm$ 1.01 μg/ml in Trials A and B respectively. Gentamicin was not detectable 12 hours following injection. The mean elimination half lives (harmonic means) for gentamicin in lymph (Trials A and B), were 1.4 and 1.8 times longer than those for plasma, 3.03 (2.63-3.57) h and 3.13 (2.78-3.57) h vs 2.17 (1.92-2.5) h and 1.75 (1.54-2.04) h. These differences were significant for Trial B only ($p < 0.05$). The mean residence times for gentamicin in lymph (Trials A and B), were 1.8 and 2.1 times those for plasma, 5.18 $\pm$ 0.59 h and 5.26 $\pm$ 0.6 h vs 2.87 $\pm$ 0.36 h and 2.57 $\pm$ 0.29 h. These differences were significant ($p<0.05$). The mean plasma Cl_B was 1.56 ml/kg/min in Trial A and 1.57 ml/kg/min in Trial B. The mean $V_{d(area)}$ and V_{SS} for gentamicin in plasma was 0.31 $\pm$ 0.05 L/kg and 0.27 $\pm$ 0.04 L/kg for Trial A and 0.27 $\pm$ 0.05 L/kg and 0.24 $\pm$ 0.02 L/kg for Trial B.

The penetration of gentamicin into lymph (Trials A and B) and synovial fluid (Trial B) was rapid (Figures 9 and 10), occurring at approximately the same rate and gentamicin was present in samples of lymph collected as early as 7.5 minutes after injection. Gentamicin concentrations reached their maximum in lymph ($T_L$max) after 1.5 h (Trial A) and 1.2 $\pm$ 0.18 h (Trial B) respectively, whilst maximum levels were achieved in synovial fluid ($T_s$max) at a similar time (1.5 $\pm$ 0.63 h). The mean maximum gentamicin concentration in lymph ($C_L$max) was 4.28 $\pm$ 0.29 μg/ml and 4.98 $\pm$ 0.55 μg/ml for Trials A and B respectively and these were approximately 40% of the 15 minute post injection concentrations recorded in plasma. The mean maximum gentamicin concentration in synovial fluid ($C_s$max) - 2.86 $\pm$ 0.45 μg/ml was only 25% of the 15 minute injection concentration recorded in plasma and was significantly less than the $C_L$max of 4.98 $\pm$ 0.55 μg/ml ($p<0.001$).

Mean lymph concentrations of gentamicin exceeded those in plasma at approximately 1.5-2 hours post injection. Levels remained slightly higher and paralleled those in plasma for the duration of the elimination phase (8-10 hours) - Figures 9 and 10. The mean concentration of gentamicin in synovial fluid exceeded that of plasma at approximately 3 hours. Over the first 2-4 hours post injection, synovial fluid and plasma levels were similar with the mean plasma:synovial fluid ratio over this period of 1.25 $\pm$ 0.2.

Initially, the mean synovial fluid levels paralleled those of lymph, and the mean lymph:synovial fluid ratio over the first 2-4 hours post injection was 1.54 $\pm$ 0.2. However, at approximately 6.5 hours post injection the two curves crossed and between 8 and 12 hours the two were markedly divergent (Figure 10).

A plot of the lymph and plasma gentamicin concentrations for the elimination phase of the disposition curve, together with upper and lower 95% confidence intervals (computed from the non-weighted least squares linear regression lines) are presented in Figures 11 (Trial A) and 12 (Trial B). The 95% confidence intervals for lymph and plasma overlapped at 2 h post injection in Trial A and between 2-3 h post injection in Trial B. The mean lymph:plasma ratio over the following 3-8 h was 1.7 $\pm$ 0.06 for Trial A and 1.54 $\pm$ 0.07 for Trial B.

Figure 11. **95% Confidence Intervals for the Elimination Phase of the Disposition of Gentamicin in Plasma and Lymph (Trial A).**

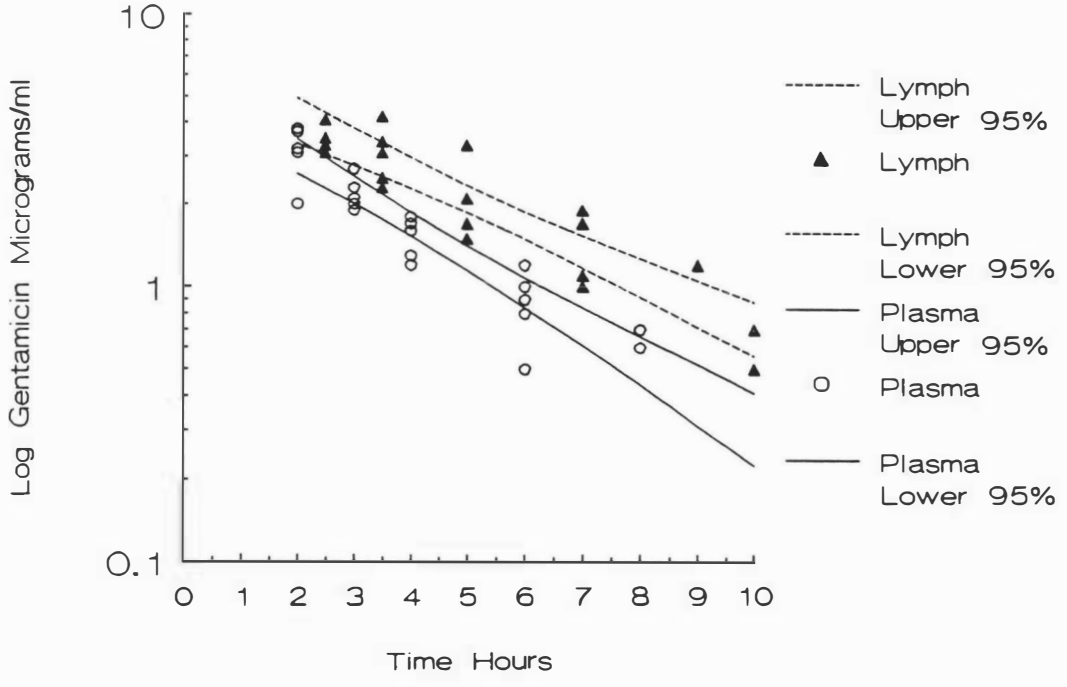
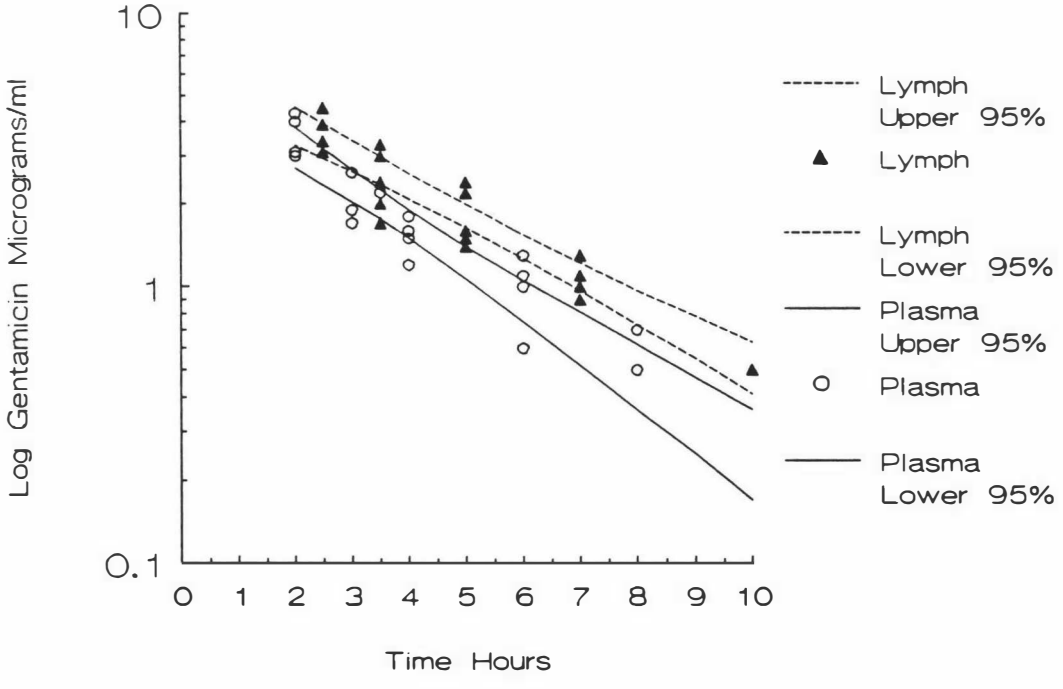


Figure 12. **95% Confidence Intervals for the Elimination Phase of the Disposition of Gentamicin in Plasma and lymph (Trial B).**



The mean ratio AUC_L/AUC_P (an indication of the penetrability of gentamicin into lymph - Appendix I) was close to unity, being 1.14 ± 0.14 for Trial A and 1.02 ± 0.06.

IV_4 DISCUSSION

A discussion and assessment of the techniques used in this investigation, including lymph cannula patency and maintenance, joint catheterisation and experimental design is presented in Chapter V. The results of this investigation are discussed below under the following headings: lymph flow rate and composition, disposition of gentamicin in plasma, disposition of gentamicin in lymph, disposition of gentamicin in synovial fluid, synovial fluid as an indicator of the tissue fluid concentration of gentamicin and plasma as an indicator of the tissue fluid concentration of gentamicin.

Lymph Flow Rate and Composition

Lymph flow rates in unanaesthetised horses in this investigation were generally much higher (2-8 times) than those recorded for anaesthetised horses in Investigation II, and this agrees with the findings of others (Yoffey and Courtice 1970, Quin and Shannon 1975). The range of mean lymph flow rates recorded at each sampling period over the 24 hour Trials was 0.66 ± 0.37 - 11.44 ± 4.95 ml/h. Lymph flow rates have not been reported for the distal limb of the unanaesthetised horse, but in other species such as sheep, ranged from 1-8 ml/h and in man, from 0.01 ml/h - 0.78 ml/h (Smith *et al* 1970, Engeset *et al* 1973, 1977, Olszewski *et al* 1981).

Lymph flow rate in conscious animals (including man) can be very variable and is affected by many factors (Engeset *et al* 1977, Olszewski *et al* 1977, 1981, Guyton 1991). As detailed earlier (Chapter I) these include both intrinsic and extrinsic factors. Intrinsic mediated lymph flow is a function of the autonomous contractions of the lymph vessels themselves, and could not be assessed in this study, nor is there any information in the literature quantifying this in the horse. Extrinsic factors include all things which cause compression of the lymphatic drainage area or lymph vessel wall. The other important determinant of lymph flow rate is tissue fluid formation. Inflammation, changes in regional

blood flow or ambient temperature and, changes in the Starling forces operating on the microvasculature can all increase or decrease the rate of tissue fluid formation (Yoffey and Courtice 1970, Hurley 1984).

Probably the most important influences on lymph flow rate were increased tissue formation due to post-surgical inflammation and extrinsic factors such as activity level and bandage compression. It is known that inflammation can cause a 10-20 fold increase in lymph flow (Casely-Smith 1973) whilst the effects of activity level and bandage compression have been described above. Outflow interference caused by problems with cannula patency and the effects of joint catheterisation were also important and are discussed further in Chapter V.

The combination of these factors and marked individual variation meant that lymph flow rates were difficult to control, making statistical analysis of differences in lymph flow rate within individual animals, between animals and over time, not meaningfully possible. However, of considerable importance was the observation that despite the marked variability in lymph flow rate between horses, lymph gentamicin concentrations were very similar.

Lymph composition, including protein concentration, total white cell count and differential were measured at various intervals after lymph cannula implantation. When evaluating this data it is important to consider what effect anaesthesia and surgery had on lymph composition. Quin and Shannon (1975) showed that there were notable differences in lymph composition between samples collected from the afferent and efferent lymphatic ducts of the popliteal lymph node during anaesthesia and cannula implantation and those collected at various times after cannulation (in unanaesthetised sheep). Protein concentrations were higher during anaesthesia and lymph flow rates were lower (as mentioned above) compared to those recorded 1-3 days post surgery. White cell concentration and differential were also affected by these procedures (especially in afferent lymph). Immediately after cannulation the total white cell count and differential in afferent lymph was similar to that measured 3 days later. However, within 12 hours there was an

increase in total white cells with a large influx of neutrophils, signifying an inflammatory response stimulated in the drainage area of the cannulated vessel. High levels of neutrophils persisted for 2-3 days. Conversely, anaesthesia and surgery had little effect on these indices in efferent lymph.

Measurements of protein concentration, total white cell count and differential were very variable in this investigation and therefore only trends were described. Protein concentration tended to be lower immediately post-surgery and after 12 hours remained relatively constant throughout the Trial period with a mean of 26.03 $\pm$ 1.48 g/L. This was approximately 40% of the plasma protein concentration (pre-experimental levels). This value is slightly lower than other reports of lymph protein concentration in the distal limb of the horse 31.1 g/L (range 18.9-46.3)(Robinson *et al* 1975), 31.8 $\pm$ 0.74 g/L (Allen *et al* 1988) however these were recorded in anaesthetised animals. Protein concentration was not recorded in the anaesthetised horse in this investigation and so direct comparisons were not possible. However, this value was similar to lymph protein concentrations in conscious men (12-40 g/L at 3-7 days after cannulation), conscious sheep (30 g/L 6 hours after surgery and 15 g/L 3 days after cannulation) (Olszewski *et al* 1977, Quin and Shannon 1975). Protein concentrations measured from "interstitial fluid" models and from peripheral lymph are usually quoted as 20-25% of those in plasma (Barza 1981). In this investigation lymph protein concentrations were closer to 40% of those in plasma, possibly due to inflammation. However, recent studies indicate that the distal limb and digital blood vessels may be more "leaky" compared to other species causing lymph/plasma ratios of protein to be higher (Robinson *et al* 1975, Allen *et al* 1988).

The total white cell count in lymph was similar to that reported for man and sheep (Engeset *et al* 1973, Smith *et al* 1970, Quin and Shannon *et al* 1975). The lymph total white cell count tended to be lower just after cannulation (Figure 7), in agreement with the report of Engeset *et al* 1973. The count then appeared to rise and peak 12 hours after cannulation, with numbers being constant thereafter and possibly starting to increase

towards the end of the experimental period. Although variability was high (differences should therefore be interpreted with caution) the general pattern was very similar to that reported by Quin and Shannon (1975) in sheep. It could be speculated that joint catheterisation may have caused the total white cell count to appear to rise towards the end of the study period since this procedure caused blood contamination of the lymph and mild inflammation of the fetlock joint.

The change in lymph white cell differential in this investigation was also very similar to that described by Quin and Shannon, with marked neutrophil elevation within 12 hours following lymph vessel cannulation. The percentage of neutrophils was declining towards the end of Trial A (34%) but appeared to rise slightly towards the end of Trial B, again possibly due to fetlock joint catheterisation (Figure 8). Peripheral lymph usually contains 70-90% lymphocytes with the remainder being macrophages and monocytes and very few neutrophils (Engeset *et al* 1973, Smith *et al* 1970). However, throughout the trial period of this investigation the differential showed that neutrophils were close to 40% and lymphocytes were close to 50%. This probably indicated mild inflammation at the lymph drainage site or from the cannulated vessel.

Disposition of Gentamicin in Plasma

The disposition of gentamicin in equine plasma is well documented and the pharmacokinetic parameters reported for a number of studies were presented in Table II, Chapter I. When compared to these studies the antibiotic concentrations, disposition curve profiles and pharmacokinetic parameters described in this investigation agree favourably.

The salient findings from this study were that mean plasma gentamicin concentrations were approximately 11 μg/ml fifteen minutes after an intravenous injection of gentamicin (at a dose of 2.2 mg/kg) and these declined in exponential fashion such that antibiotic was not detected 12 hours after injection. The mean elimination half-life was short (approximately 2 hours) and the mean volume of distribution of approximately 0.29 L/kg ($V_{d(area)}$) was

comparable with the size of the extracellular fluid compartment (20-25% of body weight). Gentamicin is exclusively excreted via glomerular filtration in an unchanged form and the figure for total body clearance (Cl_B) of 1.57 ml/kg/min found in this study is similar to that reported for glomerular filtration in the horse (1.5 $\pm$ 1.7 ml/kg/min Riviere *et al* 1983).

The only parameter that was somewhat higher than most other reports was the mean volume of distribution ($V_{d(area)}$). This was the result of two unusually large calculated values of 0.49 L/kg and 0.45 L/kg for Horse 2, Trial A and Horse 5, Trial B respectively. The magnitude of these two values could be artifactual. The value for $V_{d(area)}$ is inversely proportional to the terminal elimination slope. When the terminal elimination phase is long and the value of the elimination rate constant k_{el} (in this instance λ_z), small, (as was the case for these two animals) estimates of $V_{d(area)}$ may become artificially enlarged (Brown and Riviere 1991). Alternatively, increased retention within the tissues (due to tissue binding) could be possible, and this is known to increase the apparent volume of distribution (Baggot 1977). In the case of horse 2, tissue retention of antibiotic may have been a possibility because the AUC_L/AUC_P was greater than unity (Schentag 1984). However, the exact reason why these two horses had long elimination phases is not known.

Disposition of Gentamicin in Peripheral Lymph

The disposition of antibiotics into various types of lymph (peripheral, thoracic and renal) has been described, but in only a few studies was the disposition of gentamicin into peripheral lymph investigated. The results of all these investigations, including the current study, have been similar. In summary, it appears that antibiotics penetrate into peripheral lymph rapidly and reach peak levels within the first 2 hours. These peak levels are attained up to 1 hour behind those in serum/plasma if administration has been intramuscularly or per os. Once dynamic equilibration has occurred lymph levels tend to be higher and are maintained longer (over the latter 1/2 - 2/3 of the dosage interval) in lymph than in

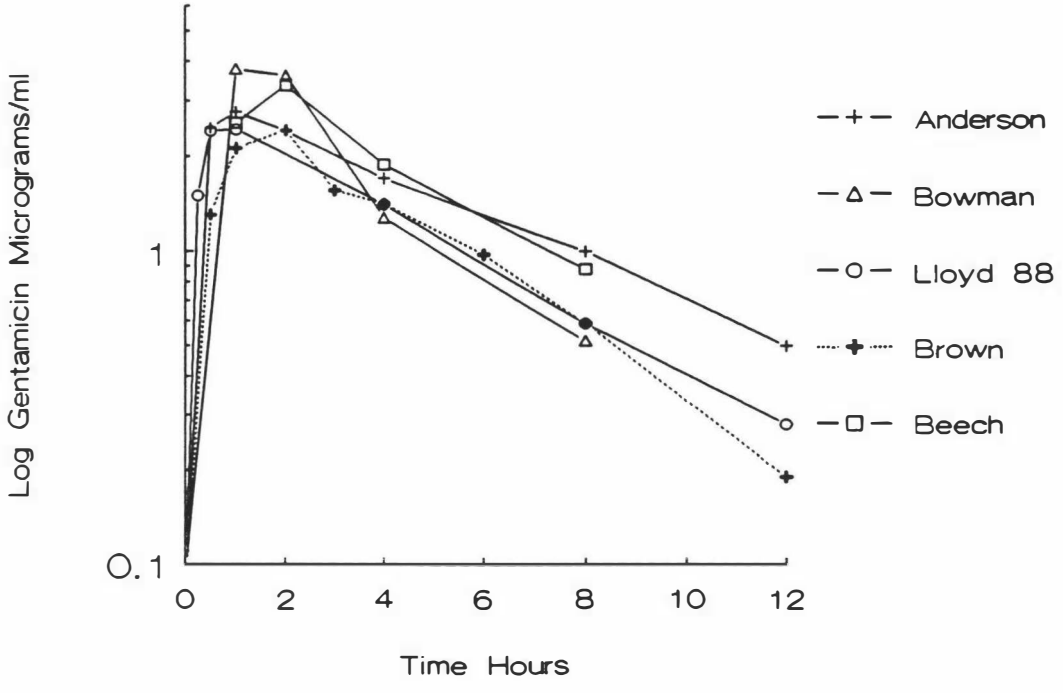
serum/plasma with the two curves being approximately parallel. Furthermore, the actual concentration of antibiotic in peripheral lymph seems to be inversely related to the binding of antibiotic to serum proteins. With serum protein binding of < 80% the ratio of concentrations of antibiotic (peripheral lymph:serum) can be expected to be greater than or equal to 60% (Schacter 1948, Chisolm *et al* 1968, Acred *et al* 1970, Bergan *et al* 1973, Bergan *et al* 1981, Roberts *et al* 1977, Walstad *et al* 1983, Cohen *et al* 1984, Franklin *et al* 1986 and May *et al* 1987).

Disposition of Gentamicin in Synovial Fluid

The disposition of gentamicin in synovial fluid was similar to that reported by others who employed the same or similar doses of gentamicin (Beech *et al* 1977, Brown *et al* 1982, Bowman 1986, Lloyd *et al* 1988). A comparison between the synovial fluid concentration vs time disposition profiles reported in each of these studies and in the present study, is shown in Figure 13.

Inspection of the plasma and synovial fluid disposition curves in each of these studies demonstrated that penetration of gentamicin into synovial fluid was rapid and peak concentrations were reached between 1-2 hours following intravenous or intramuscular injection. Peak levels ranged between 2.41 and 3.46 μg/ml and thereafter, elimination of antibiotic was relatively constant. Plasma and synovial fluid curves intersected between 3-6 hours after administration. Over the next 6-9 h synovial fluid concentrations remained higher, paralleling those in serum/plasma. However, some divergence of the two curves appears to occur at the end of the sampling period. In addition, the present investigation enabled a comparison between synovial fluid and tissue fluid (lymph) concentrations of gentamicin to be made. This showed that the disposition curves were similar and that the elimination parts of these curves intersected at just after 6 hours following intravenous injection.

Figure 13. **Comparison of the Disposition of Gentamicin in Synovial Fluid in 5 Studies.**



Synovial Fluid as Index of the Tissue Concentration of Gentamicin

The objective of this study was to investigate the hypothesis "the synovial fluid concentration of an antimicrobial agent is useful as an index of its concentration in tissue fluid". Discrepancies within the literature as regards the relationship between serum/plasma concentrations of antibiotics and those in various measures of "tissue fluid" motivated the idea of looking at synovial fluid (which is easy to collect in the horse) as a possible alternative for prediction of tissue fluid antibiotic levels. This relationship was studied by measuring the levels of gentamicin in free flowing lymph collected from a cannulated lymph vessel which, in an earlier investigation, had been shown to contribute to the lymphatic drainage of the synovium of the fetlock joint.

As discussed previously (Chapter I), there are good theoretical reasons why lymph (derived from synovium) and synovial fluid concentrations of an antibiotic, could be in related. The possibility that such a relationship exists was supported by the results of this investigation, which showed that the disposition of gentamicin in synovial fluid and peripheral lymph had similar kinetic profiles. The two curves were parallel over the initial part of the elimination phase, with lymph concentrations approximately 50% higher 2-4 hours post injection. Over the same period plasma levels were also very similar to synovial fluid levels, being on average 25% higher. As mentioned earlier, direct statistical comparison between lymph and synovial fluid concentrations of gentamicin was not possible. However, with these ratios kept in mind and the fact that between 2-3 hours post injection, there was essentially no difference between plasma and lymph concentrations of antibiotic; it could be concluded that a sample of synovial fluid taken 3 hours post injection would be a good index of the concentration of gentamicin in normal tissues within the distal hindlimb, as characterised by this technique. In fact, it can be seen that a sample of one of these three fluids would be a good index of any of the other two.

The relationship between the concentration of gentamicin in synovial fluid and lymph over the latter phase of the disposition curves (4-12 hours post injection) appears to be less clear. At approximately four hours post injection there was some divergence of the synovial fluid and lymph curves, becoming more marked over the last 4 hours of the sampling period. Similarly, there was a marked divergence between the plasma and synovial fluid curves over the last 8 hours post injection with the plasma:synovial fluid ration becoming smaller. The elimination slopes of the lymph and plasma curves remain fairly parallel and the observed divergence was due to the relatively slower elimination of gentamicin from the joint (indicated by the "flatter" curve in Figure 10).

"Small reservoir models" (Chapter I) have kinetic profiles that are typified by slower drug entry and elimination compared to "interstitial fluid models" (Ryan 1978, Barza 1981). Therefore, the observed divergence between lymph and synovial fluid may have been expected. However, many studies also report a close parallelism between the antibiotic

concentration in plasma and synovial fluid during the entire sampling period, contrary to the results of this investigation (Stover *et al* 1981, Beech *et al* 1979, Bengtsson *et al* 1989). Similarly, for other therapeutic agents such as antirheumatic drugs, a close parallelism between serum and synovial fluid concentrations following equilibration has been observed (Wallis and Simpkin 1983). These authors proposed that a general disposition pattern exists for these drugs such that initially serum levels exceed those in synovial fluid and a diffusion gradient is established which favours the diffusion of drug into the joint. Just after the equilibration point, the concentration gradient is reversed and elimination of drug from synovial fluid occurs, with the result that synovial fluid and serum concentrations of drug decline in parallel at a fixed ratio.

The slower elimination rate of gentamicin from the joint compared to plasma and lymph, could be due to error or artifact. In this investigation the last 2 sampling points (8 and 12 hours post injection) determined the shape of the elimination phase. Only 5 of 10 possible synovial fluid samples were collected at these time points because of difficulties in arthrocentesis. Therefore, the accuracy of the determination of this part of the curve was reduced. Furthermore, repeated sampling could have resulted in dilutional errors.

Alternatively, an increase in permeability of the synovial membrane (induced by inflammation/haemorrhage) may have resulted in an increase in the volume of synovial fluid within the joint. A reduced surface area to volume ratio might then have been responsible for a slower elimination of gentamicin (Bengtsson *et al* 1989). The effects of tissue binding, cellular binding could also result in retarded antibiotic elimination from the joint (Dan *et al* 1981, Nouws 1991).

It appears that other investigators have found similar gentamicin elimination patterns from synovial fluid (Figure 13). Furthermore, the elimination of kanamycin, another aminoglycoside, from normal and inflamed joints was also similar to these studies (Firth *et al* 1988). Methodology was similar in all these studies.

For these reasons, the precise nature of the relationship between synovial fluid concentrations of gentamicin and tissue fluid concentrations 4-12 hours post injection was not determined.

Perhaps the most important finding of this investigation was that plasma concentrations of gentamicin, were similar to those in tissue fluid. Furthermore, the pattern of elimination of gentamicin from plasma and lymph paralleled each other quite closely. The significance of this relationship is discussed below.

Plasma as an Index of the Tissue Concentration of Gentamicin

That a relationship existed between the concentration of gentamicin in plasma and peripheral lymph was supported by the fact that the concentration vs time disposition profiles were very similar (Figures 10 and 11). Furthermore, the 95% confidence intervals for each of the fluids overlapped 2-3 hours post injection and over the following 3-8 hours the lymph concentrations (although higher, with an average plasma:lymph ratio of approximately 1.6) paralleled those in plasma.

In addition, the mean penetration ratio (AUC_L/AUC_P) for both Trials was close to 1. This signified that the total amount of drug in each compartment was similar and this agrees with the report of Bergan (1985). Based on these observations it could be concluded that plasma samples taken between 2-3 hours post injection would be good estimates of concentrations within normal tissues in the distal hindlimb, as characterised using this technique. After this period (3-8 hours post injection) plasma samples would tend to underestimate tissue fluid levels by 54-70%. A knowledge of this relationship is important clinically because it means that plasma based pharmacokinetic data could be used with confidence when establishing dosage regimens. The observed relationship suggests that blood levels of gentamicin would be predictive of equal or possibly higher tissue levels.

These findings are similar to those of others that investigated the relationship between gentamicin in blood and lymph. Concentrations of gentamicin in serum and thoracic lymph, renal capsular and renal hilar lymph were compared following intra-muscular injection in dogs by Chisolm *et al* (1968). They concluded that following equilibrium (2 1/2 - 3 hours) there was no difference between drug concentrations in all the fluids and that "after 2 hours the plasma level is representative of the level in the body fluids, and this may be used for therapeutic purposes". Peripheral lymph concentrations of gentamicin were examined in humans in a manner similar to the present study (Bergan *et al* 1973). The results showed that 3 1/2 hours following intramuscular injection serum and lymph concentrations were of the same order, with the mean elimination half-life of gentamicin in lymph being approximately 2.1 hours. These workers concluded that serum concentrations of gentamicin taken at this time would be acceptable estimates of concentrations in extracellular tissue fluid.

In comparison, a study that measured the tissue levels of gentamicin in equine bowel using tissue homogenates (Snyder *et al* 1986), showed that whilst concentrations found in biopsies of the small intestine and large colon paralleled those in serum they were only 10-25 % and 50% of serum concentrations respectively. This study would thus indicate that the concentration of gentamicin in serum was not an accurate estimate of tissue levels. It is possible that the distribution of gentamicin to the gut and the musculoskeletal tissues of the distal hindlimbs are different. However, the assay of tissue samples for antibiotics is very complex and large errors are possible if the composite nature of tissue samples is ignored i.e. dilutional effects may cause underestimation of antibiotic concentrations due to measured drug levels being a mean of the concentration within an aggregate of blood, lymph, interstitial and intracellular fluid (Cars and Ogren 1985).

CHAPTER V CRITICAL EVALUATION OF TECHNIQUES AND EXPERIMENTAL DESIGN

General Study Design

Three investigations were conducted in this study. The findings of each investigation were used in the design of the next so that ultimately the hypothesis "that the synovial fluid concentration of an antimicrobial drug is useful as an index of its concentration in tissue fluid" could be studied. To enable investigation of this hypothesis it was necessary to a) choose a method to sample "tissue fluid", b) show that a potential relationship existed between this sample of tissue fluid and synovial fluid and, c) evaluate such a relationship by determining the disposition of a selected antimicrobial agent in synovial fluid and concurrently in the chosen "tissue fluid".

Peripheral lymph was chosen as an estimate of "tissue fluid" and this was sampled by lymph vessel cannulation. The reasons for choice of this method are detailed in Chapter I. To show that a potential relationship between peripheral lymph and synovial fluid existed it was necessary to find a lymph vessel that drained the synovium of a joint. This was performed in Investigations 1 and 2. Finally, in Investigation 3 the disposition of gentamicin was determined in the synovial fluid of a hindlimb fetlock joint and concurrently in lymph collected from a lymph vessel which had been shown to have a contribution to its supply by the synovium of this joint.

Critical evaluation of aspects of the techniques and design of each of these investigations is presented below.

Investigation 1: Anatomical Identification of an Appropriate Lymph Vessel

The purpose of this investigation was not to study the lymph vasculature of the lower limb in the horse but specifically to identify a lymph vessel draining a joint. The available literature indicated that the most suitable joint to investigate was the fetlock joint of the hind limb. The number of lymph vessels draining this particular joint were fewer and their distribution less complex than other joints such as the carpal or tarsal joints. The normal fetlock joint is relatively more difficult to puncture and results in more haemorrhagic samples compared to the above mentioned joints. This created problems in subsequent investigations.

The use of dead material for the intra-articular injection of dyes (capable of being absorbed into lymph) was avoided so that a more physiological process could be documented. Success may have been achieved if direct synovial membrane injection had been performed (Davies 1946). Experimentation with different formulations and increasing the interval between intra-articular injection and euthanasia may have given better results. However, the testing of such combinations would have taken a lot of time and many horses. Most importantly, subcutaneous dye injections performed in this investigation identified an appropriate lymph vessel (accompanying the common dorsal digital II vein in the hindlimb) which Baum (1920) had shown anatomically drained the fetlock joint. Given the failure of intra-articular injections it was decided to simply inject antibiotic in the hindlimb fetlock joint and determine if it could be retrieved from this vessel. This was the idea behind Investigation 2.

Investigation 2: The Absorption of Gentamicin from Synovial Fluid into Lymph

Only 4 horses were used in this investigation with one being used twice. With such small numbers and the experiments being conducted on anaesthetised animals, interpretation of results and extrapolation to unanaesthetised animals needs to be done with some caution.

Lymph vessel cannulation was performed in this and the third investigation, and aspects of the surgical techniques used are discussed here. Techniques for the cannulation of lymph vessels are numerous (e.g. Yoffey and Courtice 1970, Hecker 1974) and all authors agree that gentle atraumatic technique and experience are both very important for success. With increasing numbers of cannulations both experience and better technique were gained. Haemorrhage at the surgical site (contributed by the tourniquet used to promoted lymph flow and lymph vessel recognition), excessive dissection around lymph vessels, rough handling, excessive damage to the intima after passing of the obturator and inadvertent threading of the cannula beneath the adventitia of the lymph vessel wall, all resulted in difficult, if not impossible cannulations, due to spasm of the lymph vessels.

Massage of the pastern and flexion of the fetlock joint was performed regularly during the experiments of this investigation. It could be argued that such manipulations artificially increased lymph flow rates and distorted the kinetics of drug disposition. However, it is well known that lymph flow in anaesthetised animals is very low and so to avoid problems with clotting, ensure a steady flow, and provide enough sample for analysis these manipulations were performed. It seems that this procedure is acceptable for studies of this kind (Roberts *et al* 1981).

Despite this massage, in some animals lymph flow was slow and the rate of increase of gentamicin in lymph slow (relative to plasma). Discussion of the possible reasons for this slow rise in lymph concentrations was presented in Chapter III. Of worthy mention is the importance of lymph cannula length and hence volume. The length of the cannula was about 25-40 cm and the volume of the cannula was approximately 0.1-0.15 ml. Therefore, in those samples in which the volumes of lymph collected were not much greater than the volume of the catheter, some delay between the time the lymph was formed and the time it reached the collection vial could be encountered. In most cases the collected lymph volume was at least twice the volume of the catheter, at most therefore, the lag this cannula deadspace would have caused was 15 minutes. The conclusions of this investigation were not affected by this discrepancy.

Finally, one major criticism could be that the experimental period was too short. Lymph levels only reached a peak at the end of the 2 hour sampling period. At this time synovial fluid and lymph concentrations were approaching a dynamic equilibration and elimination from lymph about to begin. The relationship between synovial fluid and lymph concentrations of gentamicin after **intra-articular injection** could then have been better evaluated had the decline in lymph levels been followed for an extended period.

There are a number of reasons why an experimental period of 2 hours was chosen. Firstly, few horses were available and to ensure maximal use of those that were, both limbs of each horse were to be used (this was done in one horse). This dictated that recovery surgery was necessary and therefore anaesthesia time was limited due to the risk of post anaesthetic complications. It eventuated that in some cases surgical cannulation of one limb failed, and the other limb was cannulated immediately, or at a following surgery. In some of these cases a longer experimental period could have been an option. In fact in one horse, antibiotic was collected for up to 4 hours, but little further information was obtained. A period of 2 hours was chosen because in other antibiotic studies using lymph cannulation, peak levels were achieved within 1-2 hours and since the aim of the investigation was to confirm the presence of gentamicin within the suspected joint draining lymph vessel, this time period was thought to be sufficient. The findings of this investigation on aspects of the penetration of gentamicin into lymph after intra-articular injection, although incomplete, have important clinical implications and have provoked many questions.

The results of Investigations 1 and 2 showed that the selected lymph vessel (common dorsal digital II) had a contribution to its supply of lymph from the synovium of the fetlock joint. The lymph in this vessel is derived from skin, subcutaneous tissues and probably tendon and ligament as well as joint. The exact proportion of lymph derived from joint sources was not determined and according to Yoffey and Courtice (1970) is not known. These authors however consider that the lymph sampled at this position in the leg would have a high proportion of lymph from joints and muscles with increasing exercise.

Since there is no muscle in the lower limb of the horse the proportion of joint derived lymph in this vessel could be high. However, because the total contribution was not determined, the **exact** relationship between peripheral lymph and synovial fluid that was evaluated in Investigation 3 could not be established.

Investigation 3: Determination of the Disposition of Gentamicin in Equine Plasma, Synovial Fluid and Lymph.

In this investigation the disposition of gentamicin in plasma, synovial fluid and lymph was determined and compared. Difficulties were encountered with sampling procedures and techniques. Problems also occurred with data analysis and study design. These difficulties and problems are discussed below.

The maintenance of a patent lymph cannula in the conscious horse demanded constant 24 hour vigilance. Complete cessation of flow or reductions in flow were usually the result of a clot within the cannula, either at the external end or more seriously at the internal end. The causes of clot formation were multiple. Early after surgery, blood clots were often seen but later clots formed due to reduced flow (such as when an animal lay down) or blocked flow (such as when there was a kink or a syringe had filled to capacity). These external clots could usually be milked out of the cannula or grasped. Internal clots not able to be back flushed (a limited option) were the source of a reduced lumen capacity and hence reduced lymph flow. Presumably flow rates were also negatively influenced when cannula tips were positioned close to lymph vessel valves or were associated with a clot within the lymph vessel. The inherent thrombogenicity of cannula material differs and other materials such as silicon rubber may have resulted in fewer clotting problems. In fact, in a lymph study that was performed after the present investigation (Anderson 1991), silicone rubber cannulas were used with much fewer clotting problems. One final factor most certainly influenced the composition and flow rate of lymph, was the catheterisation of the fetlock joint in Trial B.

Increased numbers of red cells and inflammatory products released into the lymph following catheterisation would have predisposed to clot formation. However, an increased lymph flow rate would have been expected at the same time due to increased tissue fluid formation.

Finally, the apparent rates of penetration and elimination of gentamicin in lymph might have also been influenced by alterations in flow rate as described above. Individual variability in lymph flow rate was also marked. Despite this the disposition of gentamicin in lymph between horses was very similar.

The serial sampling of synovial fluid from equine joints for various studies has always been a problem and it was known that it would be a limiting factor in the present study. Multiple arthrocentesis usually results in joint haemmorrhage and inflammation as well as removal of synovial fluid. Haemmorrhage may interfere with accurate drug assay, inflammation may alter the disposition of drugs and the sequential removal of synovial fluid may result in dilutional errors (Bowman *et al* 1986, Lloyd *et al* 1988). Arthrocentesis of different joints has been used to avoid some of these problems. However, there is growing evidence that the concentration of antibiotic in one joint may be quite different than the concurrent level in another joint (Bowman *et al* 1986, Bengtsson *et al* 1989, Firth *et al* 1988). Chronic catheterisation of the antebrachiocarpal joint in the horse has been successful (Lloyd *et al* 1988, Lloyd *et al* 1988). In this investigation a catheter was placed into the plantar pouch of the fetlock joint in a similar manner to avoid some of the problems described above. However, the horses in Lloyd *et al's* studies were cross tied and confined for 24 hours. It was not possible to so severely limit the activity of the horses in this study due to the need for adequate lymph flow rates to prevent clotting. The catheters however caused some inflammation and immediately after catheterisation were a source of irritation for most horses. To try to limit this inflammation the catheters were removed after 4 hours and the final samples collected by arthrocentesis. This worked well in some horses but in some cases the last few samples were very haemmorrhagic and only

5 of 10 samples were available for the last two sample points.

Hence, the accuracy of the last part of the synovial fluid disposition curve may be in doubt. The overall inflammation of the joint was moderate but represents an uncontrolled source of variability in the results. Despite these problems the gentamicin concentrations measured in this study, although slightly higher, were very similar to other studies.

Difficulties associated with the pharmacokinetic analysis of the plasma gentamicin data have been mentioned in Chapter IV. Another problem that was faced during statistical analysis was related to sampling interval. The principal aim of Investigation 3 was to relate the concentration of antibiotic in synovial fluid and in tissue fluid (peripheral lymph). It became apparent after sample collection and drug analysis that direct statistical comparison of drug concentrations between the three fluids was not possible. The primary reason for this was that lymph, by necessity, was sampled continuously. Therefore, although samples were collected at the same time as those for plasma and synovial fluid, lymph concentrations of gentamicin had to be referred to the mid point of the collection interval. To circumvent this problem regression analysis was used as described in Chapter IV. Unfortunately however, too few concentration time points were available to employ the same technique on the synovial fluid data. This meant that confidence intervals could be used to statistically compare lymph and plasma concentrations of gentamicin, but inferences about the relationship between these two fluids and synovial fluid were restricted to non-statistical graphical and concentration ratio comparisons. Changes to the sampling times and more synovial fluid concentration time data would have avoided this problem.

As outlined, the objective of the final investigation was the determination of the disposition of gentamicin in plasma, synovial fluid and lymph. This was the subject of Trial B, conducted not less than 48 hours after surgery. Another trial,(A), was conducted the day following surgery. This study design was chosen because of the known effect that anaesthesia and surgery may have on gentamicin pharmacokinetics (Smith *et al* 1988) and

on lymph flow rate and composition (Quin and Shannon 1975). To avoid these disturbances a 2 day period was nominated before the start of Trial B. However, because it was unknown how long the lymph cannulae would remain functional and to get maximal use of the cannulation and the research animals, Trial A was conducted the following day after surgery when the horses had adequately recovered.

The confounding effect of inflammation and the possibility of drug residues were two problems associated with this study design. The lymph white cell differential at the start of Trial A (46% neutrophils, 36% lymphocytes) indicated moderate inflammation of the lymph sample. This may have influenced the disposition of gentamicin in lymph, in particular, the penetration rate and the peak levels attained. However, the disposition of gentamicin in lymph in Trial A and Trial B were not significantly different. Unfortunately, the inflammation of the lymph drainage site was still apparent after 48 hours because the lymph white cell differential was 39 % neutrophils and 49.5 % lymphocytes. Therefore, it must be concluded that both studies were performed under conditions associated with moderate inflammation. The variability this would have on the results and the inability to control such an effect should be kept in mind. Such an influence may explain why lymph concentrations were higher than plasma concentrations over the elimination phase of the disposition curves. The disposition of gentamicin into lymph of uninflammed tissues may be different. However, the findings and conclusions of these experiments are similar to those which used the technique of lymph cannulation and did not encounter significant inflammation (Bergan *et al* 1979). Furthermore, the results and conclusions from the present study are not markedly different from those of Roberts *et al* (1981) who looked specifically at the concentration of antibiotics in peripheral lymph from inflamed vs non-inflamed tissues. In another more recent lymph study (Anderson 1991) in which cannulae were kept in place for long periods (up to 10 days), the time it took for the lymph white cell differential to approach normal (70-90 % lymphocytes) was very variable but averaged 3-4 days, indicating that a longer waiting period is required.

Persistence of gentamicin within renal tissues due to back to back dosing in the two trials could have had an influence on the pharmacokinetic analyses of plasma gentamicin. It is known that there is a prolonged slow phase of elimination of gentamicin from blood. To document this blood samples must be obtained for longer than 24 hours and drug assay must be sensitive enough to detect small concentrations of drug. The use of incomplete pharmacokinetic models which ignore this process could cause the half-life to appear to change with multiple dosing (Brown and Riviere 1991). Two doses of gentamicin were given to the same horses in this investigation 24 hours apart. This represents a washout period of over 5 elimination half-lives, which is apparently acceptable in drug studies (Whittem 1992). However, the gamma phase was ignored and it is highly probable that some accumulation of gentamicin (binding to renal and other tissues) was starting to occur after the first dose. However, there was no difference in any pharmacokinetic parameter between the two trials and the influence that the gamma phase had on estimation of the pharmacokinetic parameters in this investigation is likely to be small.

CHAPTER VI GENERAL DISCUSSION AND CONCLUSIONS

The specific findings of the individual investigations have been discussed separately. Experimental techniques and design were also critically evaluated. In this chapter, a general discussion relating the important findings of all three investigations, together with conclusions and therapeutic implications, is presented below.

Absorption of Gentamicin into Lymph Following Intra-articular Injection

A large lymph vessel which accompanied the common dorsal digital vein II on the medial aspect of the metatarsus was found to have a contribution to its lymph supply derived from the synovium of the fetlock joint. The verification of this relationship was the primary objective of Investigation 2 and this was accomplished by direct injection of antibiotic into the fetlock joint and retrieval in the collected lymph. The exact proportion derived from this site was however, not determined. Although not the primary objective of Investigation 2, collection of some pharmacokinetic data describing the disappearance of gentamicin from the fetlock joint of the anaesthetised horse was possible. This data has not been reported in the literature previously and information regarding the pharmacokinetics and clinical efficacy of intra-articular antibiotic administration in horses is limited. Therefore, some discussion and comparison of the results of Investigation 2 and this information follows.

It was found that gentamicin penetrated into lymph in high concentrations but at a much slower rate compared to local or systemic plasma. The mean maximum concentration of gentamicin in lymph was approximately 50 μg/ml and the time to reach this concentration approximately 1.7 hours after intra-articular injection. The total amount of gentamicin removed from the joint in the first two hours by this single lymph vessel was very tiny compared to that removed by venous absorption. It was concluded therefore, that lymphatic removal is probably only a very minor route for the exit of gentamicin from the fetlock joint.

The mean maximum concentration of gentamicin measured in lymph after intra-articular injection was approximately 10 times higher than that reached in lymph after an intravenous injection (4.28 $\pm$ 0.29 μg/ml (Trial A) and 4.98 $\pm$ 0.55 μg/ml (Trial B)). Since peripheral lymph concentrations of antibiotics reflect those in interstitial fluid (Bergan 1981, Barza 1981) and because the causative organisms of infectious arthritis localise in the synovial membrane (Bertone *et al* 1987, O'Meara and Bartal 1988, Smith 1990) this finding would indicate a possible therapeutic advantage of intra-articular injection over systemic injection.

However, intra-articular therapy for the treatment of septic joint disease in horses has generally be regarded as being of little value and even harmful. The reasons cited have been that most antibiotics appear to achieve therapeutic concentrations in synovial fluid following systemic administration, that antibiotics delivered intra-articularly may induce a synovitis and lastly, antibiotics delivered this way are thought to be absorbed rapidly (McIlwraith 1983, Orsini 1984).

In the case of therapy with gentamicin, some of these objections have recently been challenged. Firstly, gentamicin disposition studies have shown that following intramuscular or intravenous injection, synovial fluid concentrations barely exceed the recommended minimum inhibitory concentration of 2 μg/ml[1] and do so for only a few hours (Figure 13). Hence, the duration of therapeutic effect has been questioned when the drug is administered systemically (Lloyd *et al* 1988, Lloyd *et al* 1990). Secondly, recent studies on the use of intra-articular gentamicin have indicated that the synovitis induced by this drug is mild and that drug concentrations remain above therapeutic concentrations for up to 24 hours. Finally, intra-articular therapy was shown to be more effective at eliminating sensitive bacteria from the antebrachiocarpal joint compared to systemic administration (Lloyd *et al* 1988, Lloyd *et al* 1990).

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[1] Some authors have proposed a reference minimum inhibitory concentration of 2 μg/ml for use in the clinical setting when calculating dosage regimens. Such a level is likely to be effective in the treatment of most common infections caused by susceptible bacteria in horses (Adamson *et al* 1985, Lloyd *et al* 1988).

The mean peak concentration of gentamicin in hindlimb fetlock synovial fluid 0.25 hours after intra-articular injection (Investigation 2) was 7224 $\pm$ 660 μg/ml and this was higher than reported by Lloyd *et al* (1988) (1828 $\pm$ 240 μg/ml and 2680 $\pm$ 1069 μg/ml) at the same time for buffered and unbuffered gentamicin injected intra-articularly into normal antebrachiocarpal joints, but similar to that recorded in the same joints following intra-articular and intravenous injection of gentamicin (5720 $\pm$ 3930 μg/ml). The mean disappearance half-life (harmonic mean) following intra-articular injection of gentamicin into the fetlock joint recorded in the present study was 0.99 (0.83-1.22) h, compared to approximately 4 h for Lloyd *et al's* study. In another study by Lloyd *et al* (1990) in which anteobrachiocarpal joints were first inoculated with *Escherichia coli* and then 24 hours later injected intra-articularly with gentamicin, mean peak concentrations 0.25 h after injection were similar but lower than those of the present study (4745 $\pm$ 1161 μg/ml and 6190 $\pm$ 1326 μg/ml for unbuffered and buffered gentamicin respectively). The mean disappearance half-lives of gentamicin for these joints were just over 5 hours. Of clinical importance, Lloyd *et al* (1988,1990) also reported that synovial fluid levels of gentamicin remained high (greater than M.I.C.) for up to 24 hours.

Differences in the peak concentrations achieved in the synovial fluid of the present study and those mentioned above are possibly related to the volume and geometry of the joint itself (fetlock vs antebrachiocarpal joint), joint movement (anaesthetised vs unanaesthetised), inflammation effects, differences in the distribution/mixing of the initial dose within the joint fluid and even drug assay. The marked difference between disappearance half-lives is probably an artifact due to the short sampling period used in the present study.

Despite the limitations of Investigation 2 and the differences alluded to above, it is clear from all these studies that very high synovial fluid concentrations of antibiotic are achieved following intra-articular injection compared to intravenous injection. The results of Investigation 2 showed that lymph concentrations of gentamicin could also be high. Since it is the high concentration gradient between synovial fluid and the subsynovial interstitium

that drives the diffusion of antibiotic into lymph, the prolonged high levels of antibiotic in synovial fluid reported by Lloyd *et al* 1988 and Lloyd *et al* 1990, could possibly also maintain prolonged high levels of antibiotic in the subsynovial interstitial fluid. Further investigation would be needed to confirm this.

Intra-articular therapy would probably be most beneficial early in the course of a joint infection. Inflammatory changes which could alter the diffusion properties of the synovial membrane and subsynovial interstitium (e.g. membrane thickness and cross sectional area) would be minimal at this time. Furthermore, given the nephrotoxic nature of gentamicin, intra-articular therapy may be of most use in animals with preexisting dehydration, impaired renal function or endotoxaemia (Lloyd *et al* 1988, Lloyd *et al* 1990). In such cases antibiotic therapy of a single joint by local injection at 24 hour intervals could be instituted with or without some form of lavage or drainage of the joint. This would avoid multiple systemic injections at 8 hourly intervals which do not result in such high tissue concentrations; but which may induce nephrotoxicity.

Disposition of Gentamicin in Plasma, Synovial Fluid and Lymph Following Single Intravenous Injection

The disposition of gentamicin in plasma, synovial fluid and in lymph after single intravenous injection was examined in Investigation 3. Although unable to be examined statistically, it was concluded that 3 hours after intravenous gentamicin injection, that a sample of synovial fluid was a good estimate of the concurrent tissue fluid concentration. Thereafter the relationship between the concentrations of antibiotic in these two fluids was less clear and these findings have been discussed in detail in Chapter IV.

A close parallelism of the elimination phases of the disposition curves for plasma and lymph 2-8 h post injection was also observed. Plasma and lymph concentrations were not essentially different 2-3 h after intravenous gentamicin injection and it was concluded that a sample of plasma taken at this time, would be a good estimate of the concurrent tissue

fluid concentration. Over the remainder of the usual dosage interval (from 3-8 h after injection) it appears that plasma samples could underestimate tissue fluid concentrations between 54 and 70%.

However, these findings and conclusions, specifically relate to gentamicin and the experimental design and techniques of this study. Plasma protein binding, molecular size and lipid solubility differ between antibiotics and can markedly alter drug disposition. Possibly lymph/plasma/synovial fluid relationships would also be different. The relationship between synovial fluid and lymph draining another part of the body could be different. Further, the relationship between gentamicin concentrations in plasma and from lymph draining specialised tissues or organs could vary. For example, the capillary beds of the central nervous system, ocular vitreous humor and the prostate gland are non-porous and may have tight junctions. Lipid solubility therefore becomes very important for passage of drugs into the interstitial fluids of these organs (Barza 1981). Differences in blood flow between tissues could alter such a relationship.

These investigations of "tissue fluid" concentrations of antibiotic in peripheral lymph add new information to, and complement, established equine disposition data for gentamicin in blood and synovial fluid. In practical terms it could be concluded that plasma concentrations of gentamicin adequately predict the "average tissue fluid" concentrations of the various tissues of the distal hindlimb. Plasma based pharmacokinetic and *in vitro* MIC data could be used confidently to design dosage regimens aimed at providing adequate tissue fluid concentrations of gentamicin in these tissues.

Disposition of Gentamicin in Equine Peripheral Lymph

The disposition of antibiotics in lymph in the horse has not been reported. A brief discussion of the findings of this study and the relevance of this technique as an estimate of the tissue penetration of antimicrobials, is presented below.

Gentamicin penetrated into lymph relatively quickly. However, the mean maximum concentrations of 4.28 $\pm$ 0.29 μg/ml (Trial A) and 4.98 $\pm$ 0.55 μg/ml (Trial B) were not achieved until 1.5 h and 1.2 $\pm$ 0.18 h post injection respectively. These levels were approximately 40% of those recorded in plasma 15 minutes after intravenous injection. After reaching peak concentrations elimination was consistent with first order kinetics.

The lymph and plasma disposition curves were similar in appearance but gentamicin was eliminated more slowly from lymph than plasma. Although the elimination half-life was only significantly longer for lymph compared to plasma in Trial B, the mean residence time (MRT - represents the time for 63.2% of the administered dose to be eliminated) for gentamicin in lymph was significantly longer than that in plasma in both trials. This general pattern of disposition is the same as described for many studies of peripheral lymph and for the disposition of gentamicin in peripheral lymph in other species (Chisolm 1968, Bergan *et al* 1973, Bergan *et al* 1979, Walstad *et al* 1983, Bergan 1985, Franklin *et al* 1986).

However, in other studies in which gentamicin disposition has been evaluated using different estimates of "tissue fluid" such as tissue cages or tissue homogenates, different patterns have been seen (Ryan 1978, Dan *et al* 1981). Ryan 1985 and Ryan *et al* 1986 has offered an explanation for this and suggested how peripheral lymph disposition data may be interpreted in a clinical sense. These authors propose that the surface area to volume ratio (SA/V) for a particular model or tissue fluid will determine the pattern of antimicrobial disposition observed. Thus methods/techniques/models with small volumes of tissue fluid such as skin abrasions, subcutaneous fluid collected on paper discs or cotton threads and peripheral lymph, have a SA/V ratio of $>$ 60 and antimicrobial concentration vs time profiles which reflect concurrent serum levels. These profiles are characterised by peak concentrations in tissues which are attained quickly, are generally $>$ 50 % peak serum concentrations, and have elimination half-lives similar to serum half-lives. Conversely, methods/techniques/models with large volumes of fluid such as tissue cages, skin blisters or fibrin clots, have a SA/V $<$ 10, bear little relationship to concurrent serum

concentrations and, have antimicrobial concentration vs time profiles characterised by peak concentrations in tissues that are reached slowly, are generally < 50% serum concentrations and have extended elimination half-lives.

Peripheral lymph thus would be representative of a high SA/V "tissue fluid" but in this, and other investigations, peak tissue levels were <50 % blood levels and time to reach these levels was quite long (approximately 1.3 hours in the present study). Ryan 1985 and Ryan *et al* 1986 believe that cannula deadspace and slow lymph flow rates explain this discrepancy.

It seems that the geometry of the tissue/tissue fluid is thus important and should be considered when comparing the results of tissue fluid antimicrobial disposition studies. Ryan 1985 and Ryan *et al* 1986 further speculate that in most clinical situations involving prophylaxis and treatment of early infection in non-specialised tissues (before there is a marked change in the SA/V relationships), extracellular fluid concentrations of antibiotics (such as those estimated in peripheral lymph) will be closely reflected by concentrations in serum. The results of the present study would support this contention.

Finally, it is concluded that lymph vessel cannulation was a viable technique for the study of the disposition of gentamicin in equine peripheral lymph and could be used for the study of the disposition of many antimicrobial drugs. A period of greater than 48 hours after surgery would be required to obtain samples of lymph with little or no evidence of technique related inflammation, and to reduce the variability in lymph flow rate and composition.

APPENDIX I

Ficks' Law of Diffusion

Distribution of an antimicrobial drug into non-specialised tissues is a passive process and obeys Fick's Law of Diffusion (equation 1).

$$A_t = (qK/d)(C_p - C_i) \qquad (1)$$

where A is the total amount of drug diffusion into an interstitial compartment, q is the diffusible surface area, K is the diffusion coefficient, d is the distance, and C_p and C_i are the concentrations of antimicrobial in the plasma and interstitial compartments, respectively.

If the situation is simplified to consider an interstitial focus exposed to a constant concentration of diffusible drug then it can be shown that:

$$A_t = 2qC(Kt/\pi)^{1/2} \qquad (2)$$

where C is the constant concentration at the boundary of the focus and t is time. Rearrangement of this equation gives:

$$C_i = (2qC/V_i)(Kt/\pi)^{1/2} \qquad (3)$$

where C_i is the concentration within the focus and V_i is the volume of the focus (Bergan 1981, Clarke 1989). Equation 3 predicts that the concentration of antimicrobial should be linearly related to the square root of time. Bergan (1981) has shown this relationship to be true for the diffusion of ampicillin and mecillinam into peripheral lymph in man.

Pharmacokinetic Analysis

The goal in drug therapy is to select an optimal dosage of a drug. By optimal we mean one that will be effective, non-toxic, and in veterinary medicine not result in prolonged tissue residues in food producing animals. This goal is approached by using clinical pharmacokinetics. Basically clinical pharmacokinetics is the science of dosage regimen design, which includes selection of the drug, formulation, dosage, dosage interval and mode of administration. To construct a dosage regimen the concentration vs time profile of a drug is determined by repeatedly measuring the plasma/serum concentration of the drug at predetermined time intervals after administration of a specific dose. The changes in drug concentration are then mathematically described (pharmacokinetic analysis). The pharmacokinetic equations and parameters that are derived by this process are then used to calculate the optimal dose and dosage interval of a drug that will hopefully provide effective "target" tissue concentrations.

The principles behind the use of two types of analysis of drug concentration data, namely, compartmental and noncompartmental pharmacokinetics, and the equations used in each, are briefly outlined below.

Compartmental Pharmacokinetic Analysis

A common approach, developed to explain the way in which drug concentrations change within the body (i.e. their pharmacokinetic behaviour) is to view the body as a system of distribution compartments. In this approach the body is mathematically modelled as a series of interconnecting compartments from which drugs are distributed and eliminated. These compartments are mathematical entities used for predicting drug concentrations and do not strictly have a corresponding physiological area in the body. There are three basic commonly used compartment models, (1) One-Compartment open, (2) Two-Compartment open and (3) Three-Compartment open.

Only the one and two compartment models will be described and to begin with the two compartment model is explained. The theory and equations presented below has been well reviewed in a number of publications (Baggot 1977, Baggot 1978, Gibaldi and Perrier 1982, Riviere 1988).

In the two compartment "open" model (where a route of elimination is present), it is assumed that a drug entering the body distributes instantaneously and homogeneously into the first compartment, termed the "central compartment". The central compartment probably represents the blood plasma and the extracellular fluid of highly perfused organs, such as lungs liver and kidneys. It then distributes (more slowly) to other fluid compartments collectively designated the "peripheral compartment", until a dynamic equilibrium is reached between both compartments (Figure 1).

Figure 1. Diagrammatic Illustration of the Two-Compartment Open Model

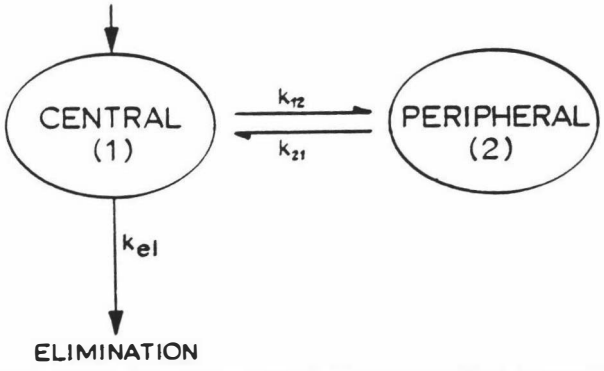


Figure 6–1 Schematic diagram of the two-compartment open model. The dose of drug is introduced into the central compartment, where it distributes instantaneously. Distribution between central and peripheral compartments takes place more slowly; k_{12} and k_{21} are first-order rate constants for drug transfer between the two compartments. Elimination, which comprises biotransformation and excretion, is assumed to occur exclusively from the central compartment; k_{el} is the first-order rate constant for drug elimination from the central compartment.

(Figure 6-1, from Baggot 1977, p145)

Distribution between the central and peripheral compartments is described by the constants k_{12} and k_{21}, which are first order rate constants for drug transfer between the two compartments. "First order" means that the rate at which a drug is removed from a compartment is proportional to the drug concentration in it. Elimination, which comprises biotransformation and excretion, is assumed to occur exclusively from the central compartment and k_{el} is the first order rate constant for drug elimination from the central compartment.

The rate of change in concentration of a drug (following intravenous injection) described by the two-compartment open model can be calculated in terms of these rate constants:

$$-dC_c/dt = (k_{12} + k_{el})C_c - k_{21}C_p \qquad (4)$$

where C_c is the concentration of drug in the central compartment and C_p is the concentration of drug in the peripheral compartment *t*. Solution of this differential equation yields the biexponential expression:

$$C_c = Ae^{-\alpha t} + Be^{-\beta t} \qquad (5)$$

If the central compartment is considered to be the plasma then C_c becomes C_P

and

$$C_P = Ae^{-\alpha t} + Be^{-\beta t} \qquad (5a)$$

and this equation describes the curve that is generated when the concentration vs time profile for such a drug is plotted on semilogarithmic axes (Figure 2), where:

C_P = the plasma concentration at time t,

A = the plasma drug concentration intercept at time = 0 of the distribution phase of the disposition curve.

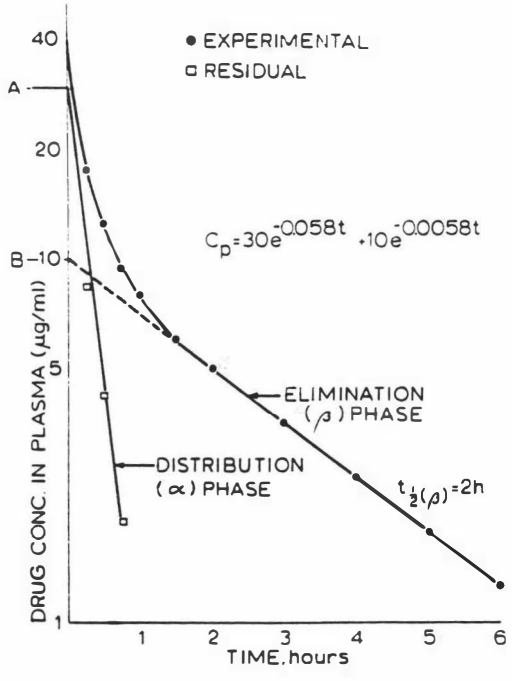
α = the apparent first order disappearance rate constant for the distribution component of the disposition curve. This obtained from the slope of the residual data points.

B = the plasma concentration at time = 0 extrapolated from the elimination phase of the exponential curve.

β = the apparent first order disappearance rate constant, obtained from the slope of the elimination phase of the semilogarithmic plot of the plasma drug concentration versus time.

e = the base of the natural logarithm (ln).

Figure 2. **Semilogarithmic Graph of a Plasma Disposition Curve.**



(Figure 6-2, from Baggot 1977, p146)

The initial steep decline in plasma drug concentration is due mainly to distribution from the central to the peripheral compartment. Once "pseudo-distribution" equilibrium is established the rate of decline of drug concentration is reduced and determined mainly by irreversible elimination from the central compartment. This is represented by the terminal part of the semilogarithmic plot and is called the ß elimination phase. Accurate determination of equation which best fits the disposition curve is extremely important, since all parameters are derived from this information. Approximate estimates for the coefficients and exponents of equation 5 can be made by resolving the disposition curve into a distribution, or α phase and the elimination, or ß phase. Least squares linear regression analysis is used to find the best fit line which describes the elimination phase and to derive B and ß (Figure 2). The values for A and α are obtained by linear regression analysis of what are called the "residual data points". The residual data points are obtained by subtracting the extrapolated portion of the ß phase form the experimental data and represent the distribution or α phase. This method of generating the distribution phase is in this manner is known as the "method of residuals" or the "feathering technique".

The most common method of accurately determining the equation for the best fit line of the disposition curve (and the method used in this study) is to use a curve fitting computer programme such as NONLIN[1]. This programme utilises a weighted nonlinear least squares regression analysis to calculate lines that best fit the concentration time data. Initial estimates of the coefficients and exponents are first required and these are derived from the semilogarithmic plot of plasma drug concentration versus time and the method of residuals described above. Using these initial parameter estimates the programme fits curves to the data in a sequential manner. Each line is statistically compared to the one before until finally, the line which best fits the data, is determined.

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1 PC NONLIN. Copyright 1984, 1986 Statistical Consultants, Inc., Carl Metzler, and Daniel L. Weiner.

The final coefficients and exponents of the equation for this line are then used to calculate the major pharmacokinetic parameters used in describing the disposition of any drug. The definition and derivation of each of these parameters are described below.

1) Apparent Volume of Distribution

The apparent volume of distribution (V_d) is defined as that volume of fluid into which the drug appears to distribute in order to account for the observed plasma concentration. Although not a physiological volume it is a quantitative estimate of the extent of drug distribution. Volume of distribution is a proportionality constant relating plasma/serum concentration to dose.

Various volume of distribution estimates can be computed (equations 6 and 7)

$$V_C = \text{dose}/C_P^0 = \text{dose}/(A+B) \qquad (6)$$

where V_C is the volume of the central compartment and C_P^0 is the plasma concentration at time equals zero and A and B are defined above.

$$V_{d(\text{area})} = \text{dose}/\text{AUC} \cdot ß \qquad (7)$$

where $V_{d(\text{area})}$ is the volume of distribution at pseudo-distribution equilibrium calculated by the area method. AUC is the area under the curve calculated as

$$\text{AUC} = A/\alpha + B/ß \qquad (8)$$

Where α and ß are defined as above. The AUC can also be calculated by the trapezoid method (p 152). The volume of distribution is used to calculate the required dose to give a desired plasma concentration.

2) Clearance

Total Body Clearance (Cl_B) is defined as that volume of plasma which the body must "clear" of drug per unit time in order to account for the total amount of drug disappearing from plasma via all routes of elimination. This factor relates the amount of drug eliminated from the body per unit time to the plasma concentration. Total Body Clearance is the sum of the clearances of each organ of elimination and changes in exact proportion with k_{el} unlike the hybrid parameter, half-life, which is influenced by drug distribution, biotransformation and renal excretion.

$$Cl_B = \text{dose/AUC} \qquad (9)$$

3) Elimination Half-life

The elimination half-life ($t_{1/2}$) describes the rate of drug elimination, being the time taken for the plasma concentration of a drug to be reduced by 50% during the elimination phase of the disposition curve, and is calculated from equation 12:

$$t_{1/2} = \ln 2/ß \quad \text{(all models)} \qquad (10)$$

in the case of the one-compartment open model

$$Cl_B = k_{el} \times V_d \qquad (11)$$

and therefore

$$t_{1/2} = (\ln 2 \times V_d)/Cl_B \qquad (12)$$

where Cl_B, k_{el}, and V_d are defined above.

In disease states it is primarily k_{el} that is altered and this may make $t_{1/2}$ an unreliable indicator of drug indicator of drug elimination. However, the $t_{1/2}$ is a useful clinical parameter of drug disposition because it gives an estimate of the duration of drug effects in the body, since multiples of the $t_{1/2}$ give the fraction of the original dose remaining. eg after 5 $t_{1/2}$'s approximately 97% of the drug has been eliminated. This can be calculated from equation 13.

$$FE = 1-(e^{k_{el}\,t}) \qquad (13)$$

where FE = fraction eliminated.

"One-Compartment Open Model"

In the simple case of the one-compartment open model, the equilibration between the central and peripheral compartment is extremely rapid and the disposition kinetics of the drug may be adequately described by assuming the body to behave as one homogeneous distribution compartment. It is also assumed that changes in plasma concentrations reflect quantitatively changes occurring in tissue drug levels. The change in the plasma concentration of the drug over time can be described by a monoexponential equation:

$$C_P(t) = C_P(0)e^{-k_{el}\,t} \qquad (14)$$

Where:

$C_P(t)$ = the plasma concentration at time t,

$C_P(0)$ = the plasma concentration at time = 0 back extrapolated from the elimination phase of the disposition curve,

k_{el} = the apparent first order disappearance rate constant, obtained from the slope of the elimination phase of the semilogarithmic plot of plasma concentration versus time.

NonCompartmental Pharmacokinetic Analysis

In recent years, analysis of drug disposition data using noncompartmental (or compartment model independent) methods, has gained increasing attention and application. The reason for this is that in reality, the body is comprised of millions of compartments, but only a few compartments are characterised in even the most sophisticated kinetic multicompartment model. Furthermore, for practical application in the clinical setting, it is not possible to obtain a large enough number of blood samples to properly describe a multicompartmental concentration-time course for a drug (Ritschel 1986).

Noncompartmental methods do not require the assumption of a specific compartmental model for either drug or metabolite but it is assumed that all dispositional processes may be described by first order kinetics with elimination occurring from the rapidly equilibrating or central compartment (Gibaldi and Perrier 1982, Perrier and Mayersohn 1982). Analysis is based on statistical moment theory and the calculation of the area under various concentration time curves. Basically, the time course for plasma concentration (C_P) following a single dose of drug can be regarded as a statistical distribution curve. Regardless of route of drug administration the first three moments (zero to second) are defined as follows:

$$AUC = \int_0^\infty C_P \, dt \qquad (15)$$

$$MRT = \int_0^\infty tC_P \, dt \Big/ \int_0^\infty C_P \, dt = AUMC/AUC \qquad (16)$$

$$VRT = \int_0^\infty t^2C_P \, dt \Big/ \int_0^\infty C_P \, dt = \int (t - MRT)^2 \, C_P \, dt \,/\, AUC \qquad (17)$$

where C_P and t are defined above.

AUC, MRT, and VRT are termed the zero, first and second moment, respectively, of the drug concentration vs time curve. AUC is the area under the statistical zero moment (plot of concentration vs time) from zero to infinity, and this is a measure of the total amount of drug in the body. MRT is the mean residence time and represents the time for 63.2% of the administered dose to be eliminated. The area under a plot of the product of concentration and time vs time, from zero to infinity, is known as the area under the first moment curve or AUMC. The AUMC gives the "centre of gravity" of the area under the curve. VRT is the variance of the mean residence time of a drug in the body (Gibaldi and Perrier 1982).

The primary parameters derived from these area-estimates are the volume of distribution terms $V_{(darea)}$, V_{ss} and whole body clearance CL_B. These parameters are called "robust" since they are determined via the AUC and hence are not very sensitive to small changes in concentration time data (Ritschel 1986).

The first moment of the blood drug concentration time curve, MRT, is the statistical moment analogy to the half-life ($t_{1/2}$). For a drug that is described by a one-compartment model after intravenous administration:

$$MRT = 1/k_{el} \qquad (18)$$

and therefore

$$t_{1/2} = \ln(2) \times MRT \qquad (19)$$

where k_{el} is the elimination rate constant.

In most instances, for a drug that requires multicomparment characterization, the effective half-life is also calculated by equation 10 (Gibaldi and Perrier 1982). Alternatively the $t_{1/2}$ has been calculated by dividing 0.693 by λ. Where λ is the first order elimination rate constant determined by linear regression of the terminal (ie post-absorption, post-distribution) concentration time data (Gibaldi and Mayerson 1982, Ritschel 1986, Wright

et al 1991, Kinzig *et al* 1992).

The following calculations are used to compute and derive the $AUC_{0-\infty}$, $AUMC_{0-\infty}$, MRT, CL_B, V_λ, V_{ss}. Where the terminal disposition rate is called λ_Z, regardless of model and the apparent volume of distribution is called V_Z (determined by model-independent area method). This nomenclature is used to underline the fact that the procedure is model-independent (Ristchel 1986).

Total Area Under the Curve AUC

$AUC_{0-\infty}$ is determined in two steps using the linear trapezoidal rule from t=0 to the last sampling time t(x):

$$AUC_{(0\rightarrow t(x))} = \Sigma\,[1/2(C(n) + C(n+1))] \cdot [t(n+1) - t(n)]\ [(\mu g/ml)h] \qquad (20)$$

where C(n) is the concentration of drug and n=0,1,2.....x. C(x) is the last measurable concentration.

and for the remaining area $AUC_{(t(x)\rightarrow\infty)}$:

$$AUC_{(t(x)\rightarrow\infty)} = C(x)/\lambda_Z\ \ [(\mu g/ml)h] \qquad (21)$$

where λ_Z is the elimination rate constant and is calculated as described above

The total AUC is:

$$AUC_{0-\infty} = AUC_{(0\rightarrow t(x))} + AUC_{(t(x)\rightarrow\infty)}\ [(\mu g/ml)h] \qquad (22)$$

in the case of intravenous administration, C(0) is unknown and therefore the $AUC_{0\rightarrow 1}$ is calculated as C(1) x [t(1) - t(0)]. $AUC_{1\rightarrow t(x))}$ is then calculated as in (20).

Area Under the First Moment Curve AUMC

This again is done in two steps, namely for t=0 to t(x):

$$AUMC_{(0\rightarrow t(x))} = \Sigma\,[1/2\{(t(n)\cdot C(n)) + (t(n+1)\cdot C(n+1))\}]\cdot(t(n+1) - t(n)) \quad [(\mu g/ml)h^2] \tag{23}$$

$$AUMC_{(t(x)\rightarrow\infty)} = t(x)\cdot C(x)/\lambda_Z + C(x)/(\lambda_Z)^2 \quad [(\mu g/ml)h^2]$$

the total AUMC is:

$$AUMC_{0\rightarrow\infty} = AUMC_{(0\rightarrow t(x))} + AUMC_{(t(x)\rightarrow\infty)} \quad [(\mu g/ml)h^2] \tag{24}$$

Mean Residence Time MRT

$$MRT = AUMC/AUC \quad [h] \tag{25}$$

Total Body Clearance CL_B

For intravenous bolus:

$$CL_B = D/AUC \quad [ml/h] \tag{26}$$

where D = dose [mg] or if dose per kg then CL_B is ml/kg/h.

Apparent Volume of Distribution V_Z and Apparent Volume of Distribution at Steady State V_{ss}.

For intravenous bolus:

$$V_Z = D/(AUC\cdot\lambda_Z) \quad [L] \tag{27}$$

For intravenous bolus:

$$V_{ss} = D\cdot AUMC/(AUC)^2 \quad [L] \tag{28}$$

APPENDIX II

Table 1. **Experimental Horses and Subcutaneous Injection Sites Used in Investigation 1.**

Horse	Age (yrs)	Breed	Sex	Limbs Used* Left	Limbs Used* Right	Sites Injected		Palmar/ Plantar
1	aged	A	F	+	+	+	+	+
2	2.5	TB	M	+	+	+	+	+
3	7	XB	M	+	-	+	+	+
4	0.5	TB	F	-	+	+	+	+
5	3	STB	M	+	-	+	+	+
6	aged	TB	M	-	+	+	+	-
7	aged	TB	F	-	+	+	+	-
8	aged	XB	M	-	-	-	-	-
9	5	TB	M	-	+	+	+	-
10	aged	XB	M	+	-	+	-	-
11	aged	TB	M	-	+(H)	+	-	-
12	2.5	STB	M	+(H)	+(H)	+	-	-

F = Female TB = Thoroughbred XB = Crossbred
M = Male A = Arab STB = Standardbred

* Forelimbs only were injected, except in horses 11 and 12, in which hindlimbs only were injected.

APPENDIX II

Table 2. **Intra-Articular Injections Performed in Investigation 1.**

Horse	Fetlock Joint Injected		Dye Used
1	LF	RF	CR
2	LF	RF	CR
3	-	RF	CR
4	LF	-	CR
5	-	RF	PBV
6	LF	-	CR
7	LF	-	CR
8	LF	RF	CR
9	LF	-	CR
10	-	-	-
11	LF	-	II
12	-	-	-

LF = Left forelimb
RF = Right forelimb
LH = Left hindlimb
CR = Congo Red
PBV = Patent Blue Violet
II = India Ink

APPENDIX II

Table 3. **Dyes Used in Investigation 1.**

Dye	Comments	Method of Formulation
Patent Blue Violet (PBV)	Water soluble. Essentially non-protein bound. Almost exclusively absorbed into lymphatic vessel system. High tissue diffusibility. Molecular weight 1158.	1. 10% w/v - add isotonic saline to stock PBV powder[1] 2. 5% w/v - as above
Evans Blue (EB)	Water soluble. Highly protein bound. Low tissue diffusibility. Molecular weight 960.	1. 3% w/v - add isotonic saline to stock EB powder[2] 2. 2.5% w/v - dilute with equine serum.
Congo Red (CR)	Water soluble. Quantitatively bound to protein (albumin) at 0.5% w/v concentration (Rusznyak *et al* 1967).	1. 1% w/v - add isotonic saline to stock CR powder[3] 2. 0.5% w/v - dilute 1% concentration with an equal quantity of equine serum.
India Ink[4] (II)	Particulate. Removed from synovial cavity by macrophage ingestion and absorption into lymph (Key 1926).	use undiluted

1. Gurr. Biological Stain 02285, Hopkin and Williams, Chadwell Heath Essex, England.
2. Evans Blay E.Merck, Darmstadt, Federal Republic of Germany. Distributed Smith Biolab, Auckland.
3. Georget Gurr Ltd. London, S.W.6 London.
4. Super Black India Ink, Hunt Manufacturing Ltd, Statesville. N.C. 28677 U.S.A.

APPENDIX III

Table 1. **Experimental Animals Used in Investigation 2.**

Experiment number	Horse	Weight kg	Age (yrs)	Breed	Sex
1	1	420	Aged	Pony	F
2	2	470	8-9	TB	F
3	*3	430	3	TB	M
4	4	504	6	TB	MC
5	*3	430	3	TB	M

* = Right leg used in Experiment 3
Left leg used in Experiment 5

TB = Thoroughbred F = Female M = Male MC = Male castrate

Preparation of a Lymph Cannula

(i) Each piece of tubing was washed in Pyroneg[1] solution and its lumen flushed with 1-2 ml of the solution 2-3 times using a 23 gauge needle and syringe.

(ii) The tubing was left to soak in Pyroneg solution overnight.

(iii) The tubing was rinsed in distilled water and flushed in the same manner as in (i) above.

(iv) The tubing was flushed with air.

(v) Absorbent paper was used to blot the outer surface of the tubing, which was then left to dry in an incubator (at 45°C) overnight.

(vi) Each piece of washed tubing was ethylene oxide sterilised to produce a lymph vessel cannula.

(vii) Each cannula was not used for at least three days after sterilisation.

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1 Wallace, Distributed by Smithbiolab NZ, Ltd.

APPENDIX III

Table 2. Jugular and Local Plasma Concentrations of Gentamicin (μg/ml) after Intra-Articular Injection.

Time (h)	Jugular Plasma					Local Plasma				
	H_1	H_2	H_3	H_4	H_3*	H_1	H_2	H_3	H_4	H_3
0.25	1.5	1.26	0.8	0.6	0	5.24	1.93	2.9	1.6	1.3
0.5	1.61	1.06	1	0.5	0.3	3.45	1.4	1.5	1.7	1
1	1.6	1.03	0.7	0.5	0.4	3.64	1.52	1.2	1.2	1.7
1.5	1.41	1.02	0.8	0.7	0.4	3.25	1.53	1.1	0.9	0.8
2	1.43	0.9	0.7	0.5	0.5	2.94	1.48	1.8	1.15	1.9
3	ND	ND	ND	ND	0.9	ND	ND	ND	ND	1.5
4	ND	ND	ND	ND	1	ND	ND	ND	ND	1.6

ND = not done
* = Horse 3 used twice, one week apart.

APPENDIX III

Table 3. **Lymph Concentrations of Gentamicin (μg/ml) after Intra-Articular Injection.**

Time (h)	Lymph				
	H_1	H_2	H_3	H_4	H_3^*
0.125	0.68	0.2	0.5	0.3	0
0.375	2.11	0.67	28.2	3.2	1.5
0.75	38.7	8.37	44.9	26.8	2
1.25	66.2	27.8	69.9	37.9	15
1.75	41	22.6	90.9	42.6	17.1
2.5	ND	ND	ND	ND	19.9
3.5	ND	ND	ND	ND	8.4

ND = not done
* = Horse 3 used twice, one week apart.

Table 4. **Synovial Fluid Concentrations of Gentamicin (μg/ml) after Intra-Articular Injection.**

Time (h)	Synovial Fluid				
	H_1	H_2	H_3	H_4	H_3^*
0.25	4850	8230	8330	8030	6780
0.5	2840	4910	7290	4320	6090
1	2630	2560	5450	2560	3800
1.5	2480	1580	2440	1770	3030
2	1810	1360	1700	1350	2190
3	ND	ND	ND	ND	1320
4	ND	ND	ND	ND	1120

ND = not done
* = Horse 3 used twice, one week apart.

APPENDIX III

Pharmacokinetic and Statistical Analyses Performed in Investigation II

Pharmacokinetic Analysis

a) *Lymph*

The total amount of gentamicin removed from the fetlock joint via lymphatic absorption after intra-articular injection (TG), was calculated as the sum of all the gentamicin collected at each sampling interval (equation 2).

Total gentamicin collected per sampling interval.	=	total volume of lymph collected per sampling interval (ml) x concentration of gentamicin in lymph (μg/ml).	(2)

The percentage of the total amount of gentamicin removed by all sources (sampling, blood and lymph) that was due to lymphatic absorption, was calculated as TG/TOT x 100%. Where TOT is the total amount of gentamicin removed by all sources and is calculated from equation 3.

$$TOT = D \times R \qquad (3)$$

where D is the intra-articular dose (150 mg) and R is percentage of gentamicin removed from the joint over the 2 hour sampling period and is calculated from equation 4.

$$R = 2 \times 50\%/t_{1/2}d \qquad (4)$$

b) *Synovial Fluid*

The elimination of gentamicin following distribution/mixing, from the fetlock joint was described by the monoexponential equation:

$$A(t) = A_0 e^{-k_{el}t} \quad (5)$$

Where A represents the concentration of gentamicin at time = t and 0 respectively and kd is the disappearance rate constant.

The half-life for the disappearance of gentamicin from the fetlock joint ($T_{1/2}d$) by all routes ie blood, lymph and sampling, was calculated from:

$$T_{1/2}d = \ln 2/k_d \quad (6)$$

c) *Plasma*

The ratio of local/jugular gentamicin concentration was calculated for each horse by dividing the mean local plasma gentamicin concentration by the mean jugular plasma gentamicin concentration over the 2 hour period.

Statistical Analysis

A one way Analysis of Variance was used to test the null hypothesis that there was no difference in mean lymph flow rate between sampling intervals.

This was computed using a data analysis programme Panacea[2] and a p-value less than 0.05 was interpreted as indicating that a significant difference in mean lymph flow rate between at least two sampling intervals, existed.

To quantify the relationship between lymph flow rate and lymph gentamicin concentration, the correlation coefficient relating these two variables was computed (Panacea). A one sample student's t-test was used to test the null hypothesis that the estimated correlation coefficient was equal to zero (Rosner 1990). A p-value of less than 0.05 was interpreted as indicating that the correlation between lymph flow rate and lymph gentamicin concentration was not equal to zero.

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Department of Agriculture, University of Reading,
Berkshire, England.

APPENDIX IV

Table 1. Experimental Animals Used in Investigation 3.

Exp. No.	Horse ID	Weight	Age (yrs)	Breed	Sex
1	1	486	6	TB	MC
2	2	505	3	TB	M
3	3	408	2	TB	F
4	4	477	4	TB	MC
5	5	432	4	TB	MC

TB=Thoroughbred
F=Female
M=Male
MC=Male castrate

Table 2. **Lymph Composition: Lymph Protein Concentration (g/L) for Horses in Investigation 3 at Various Intervals after Surgery.**

	Lymph Protein Concentration g/L					
Mean (± sem) Mid-point of Sampling Interval (h) Post Surgery	H_1	H_2	H_3	H_4	H_5	Mean (± sem)
1.1 ± 0.2	13	30.6	17.5	25	11	19.4 ± 3.4
4.9 ± 0.5	15.5	27.5	23.5	14	22.7	20.64 ± 2.6
11 ± 1.2	ND	36	30.3	15.5	23	26.2 ± 4.5
24.4 ± 0.9	23	26.6	29.2	18.5	34	26.3 ± 2.6
35.7 ± 1.3	19.2	25.5	16.7	34.2	37.5	26.6 ± 4.1
47.7 ± 0.7	26	24.7	20.5	27.7	30.2	25.8 ± 1.6
60.4 ± 1.5	17.1	22.5	32.4	18.5	36.5	25.4 ± 3.9

ND = not done

Table 3. **Lymph Composition: Lymph Total White Cell Count (per/μl) for Horses in Investigation 3 at Various Intervals after Surgery.**

	Total White Cell Count					
Mean (±sem) Mid-point of Sampling Interval (h) Post Surgery	H_1	H_2	H_3	H_4	H_5	Mean (± sem)
1.1 ± 0.2	155	209	291	544	195	296 ± 78
4.9 ± 0.5	367	98	82	267	356	234 ± 61
11 ± 1.2	189	304	893	396	409	438 ± 120
24.4 ± 0.9	194	38	527	236	273	307 ± 75
35.7 ± 1.3	312	32	349	257	182	275 ± 36
47.7 ± 0.7	247	566	96	456	262	325 ± 83
60.4 ± 1.5	118	293	799	242	256	341 ± 118

Table 4. **Lymph Composition: Lymph Differential White Cell Count (%) for Horses in Investigation 3 at Various Intervals after Surgery.**

Mean (± sem) Mid-point of Sampling Interval (h) Post Surgery	Lymph Differential Cell Count (%)																	
	H_1			H_2			H_3			H_4			H_5			Mean % (± sem)		
	N	L	M	N	L	M	N	L	M	N	L	M	N	L	M	N	L	M
1.1 ± 0.2	20	60	20	35	55	10	16	66	18	28	64	8	16	56	28	23 ± 4	60 ± 2	17 ± 4
4.9 ± 0.5	74	18	8	91	6	3	12	64	23	66	26	8	60	11	29	61 ± 13	25 ± 10	14 ± 5
11 ± 1.2	74	18	8	46	24	30	63	14	23	69	22	9	69	13	18	64 ± 5	18 ± 2	18 ± 4
24.4 ± 0.9	34	28	38	55	40	5	20	56	24	76	19	5	ND	ND	ND	46 ± 12	36 ± 8	18 ± 8
35.7 ± 1.3	18	74	8	28	70	2	28	44	28	39	24	37	55	27	18	34 ± 12	48 ± 11	19 ± 6
47.7 ± 0.7	58	38	4	10	74	16	ND	ND	ND	49	35	16	29	51	20	37 ± 11	50 ± 9	14 ± 4
60.4 ± 1.5	28	72	0	28	72	0	54	18	28	ND	ND	ND	46	36	18	39 ± 7	50 ± 14	12 ± 7

N = Neutrophils L = Lymphocytes M = Monocytes ND = not done

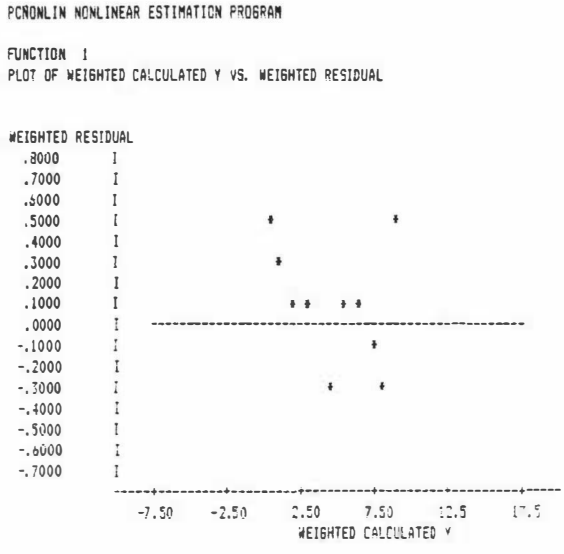
Results of Compartmental Pharmacokinetic Analysis

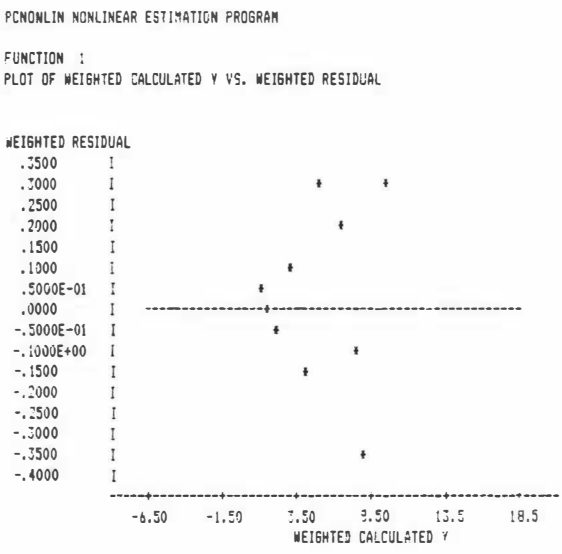
As described previously the plasma gentamicin concentration vs time data was initially analysed using non-linear regression analysis (NONLIN). The semilogarithmic plots, r^2 values, standard deviation of the estimated values, residual trends and pharmacokinetic parameters were inspected to choose the most appropriate model that fit the data (Haddad *et al* 1985, Pedersoli *et al* 1980). Some examples of the results of this analysis are presented below.

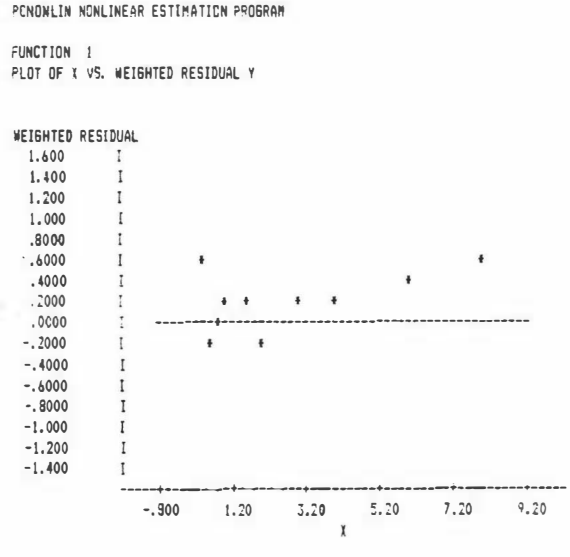
Example 1. Horse 1 Trial A.

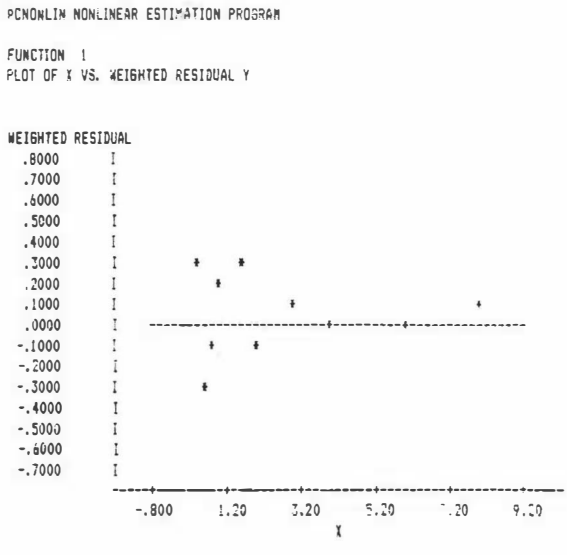
The one compartment model appeared to describe the disposition data the best in this case. The r^2 values for the one and two compartment models were very similar (0.992 and 0.996 respectively) as were the standard deviations of the y estimates. However, the residual trends for the one compartment model showed random scatter (evidence of a good model fit). In comparison the residual trends for the two compartment model exhibited a non-constant variance type pattern (evidence of a less appropriate fit) (Figure 1). Examination of the semilogarithmic plot (Figure 4) revealed no obvious distribution phase. Finally, the two-compartment fit calculated a $t_{1/2}$ for the distribution phase of 1.1 h and a $t_{1/2}$ for the elimination phase of 4.17 h. Both these parameters are extremely long for this drug.

Figure 1. **Comparison of the Residual Plots for the One-Compartment and Two-Compartment Model Fits for the Disposition Data for Horse 1, Trial A.**









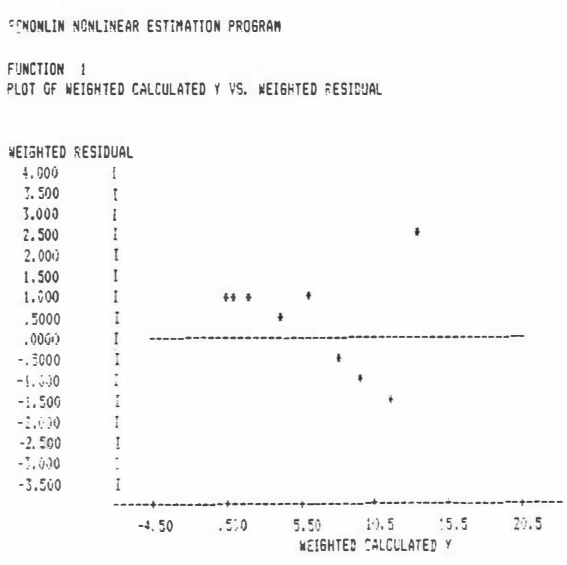
ONE COMPARTMENT

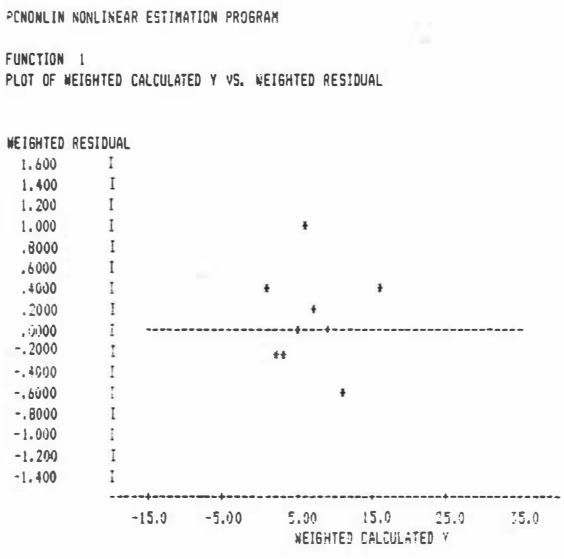
TWO COMPARTMENT

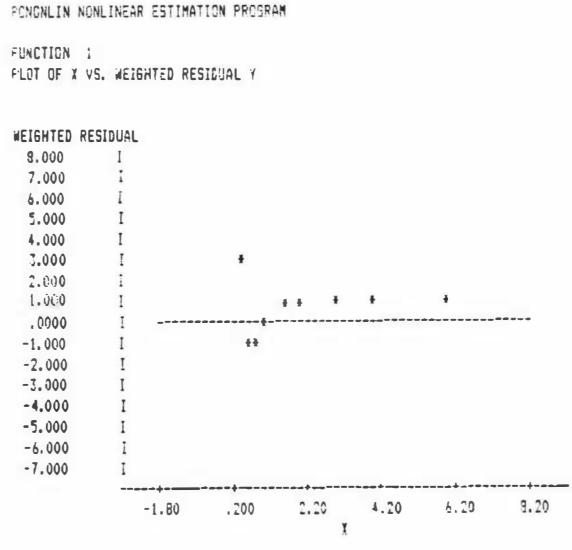
Example 2. Horse 2 Trial B.

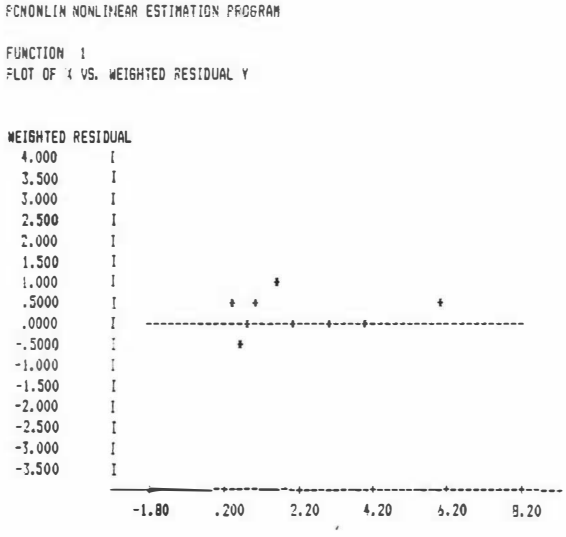
The two-compartment model appeared to describe the disposition data the best in this case. The r^2 value of 0.99 for the two-compartment model was greater than for the one-compartment model (0.93) and the standard deviation of the y estimates was less. The residual trends for the two-compartment model showed more of a random scatter pattern c.f. the non-constant variance type pattern exhibited for the one-compartment model (Figure 2). It was possible to discern a distributive phase of the semilogarithmic plot (Figure 10) disposition.

Figure 2. **Comparison of the Residual Plots for the One-Compartment and Two-Compartment Model Fits for the Disposition Data for Horse 2, Trial B.**









ONE COMPARTMENT

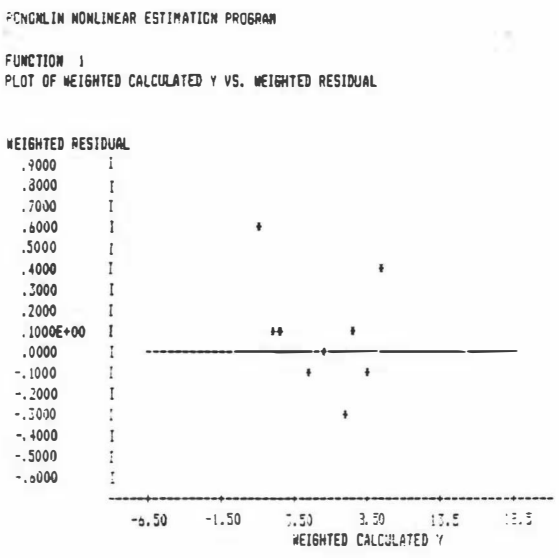
TWO COMPARTMENT

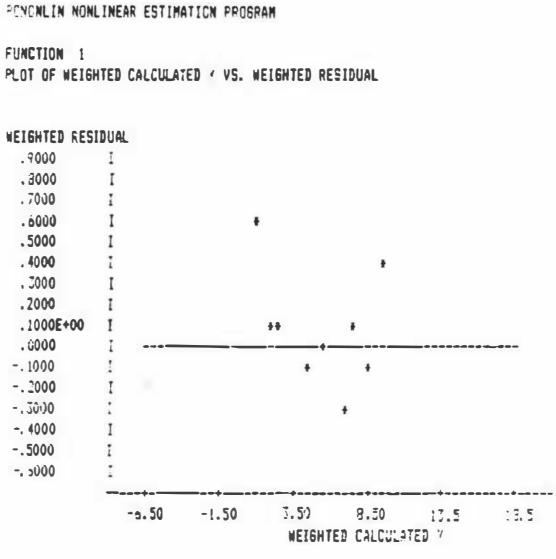
Example 3. Horse 3 Trial B.

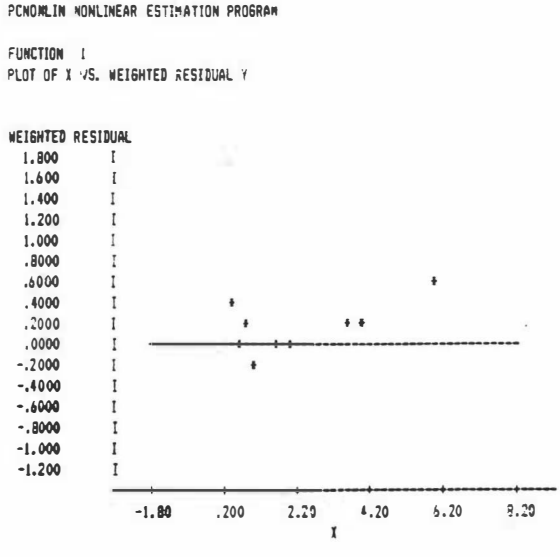
The one-compartment model appeared to describe the disposition data the best in this case. The r^2 values and the standard deviation of the y estimates were very similar for both models. However, residual trends for the one-compartment fit were more random in nature compared to the two-compartment fit (Figure 3) and the semilogarithmic plot showed no obvious distribution phase (Figure 11). Finally, although an equation the fit the disposition curve very closely was generated by fitting the two-compartment model, the estimated parameters for the $t_{1/2}$ of distribution and of elimination were ridiculously long (1.2 h and 23 h respectively). Such an inaccurate estimation could be the result of insufficient sampling early after drug injection. It seems that due to the rapid distribution of gentamicin, frequent sampling is required to be able to clearly separate the distribution and elimination phases (Wilson *et al* 1983).

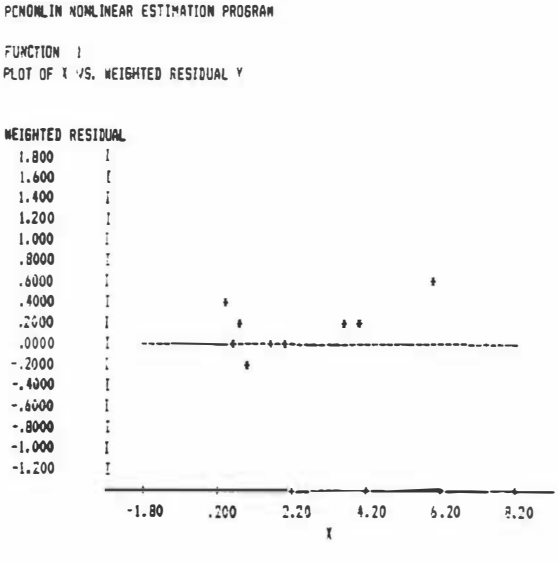
Noncompartmental pharmacokinetic analysis was ultimately used to circumvent some of the problems outlined above (p90).

Figure 3. **Comparison of the Residual Plots for the One-Compartment and Two-Compartment Model Fits for the Disposition Data for Horse 3, Trial B.**









ONE COMPARTMENT **TWO COMPARTMENT**

Figure 4. **Trial A: Disposition of Gentamicin (μg/ml) in Plasma and Lymph for Horse 1.**

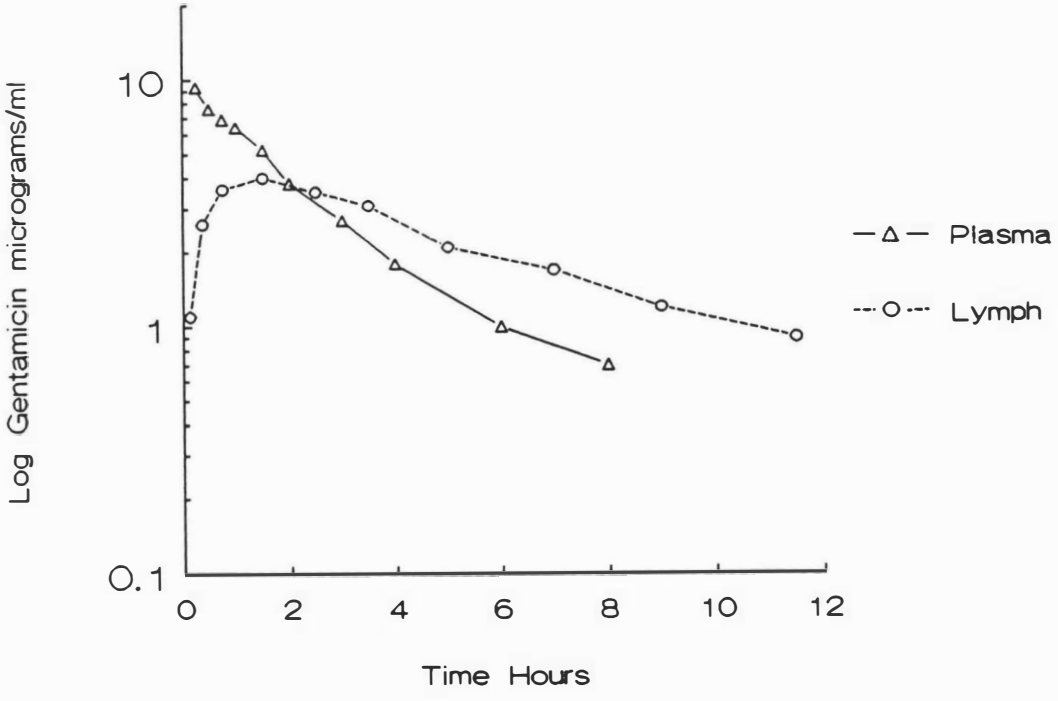


Figure 5. **Trial A: Disposition of Gentamicin (μg/ml) in Plasma and Lymph for Horse 2.**

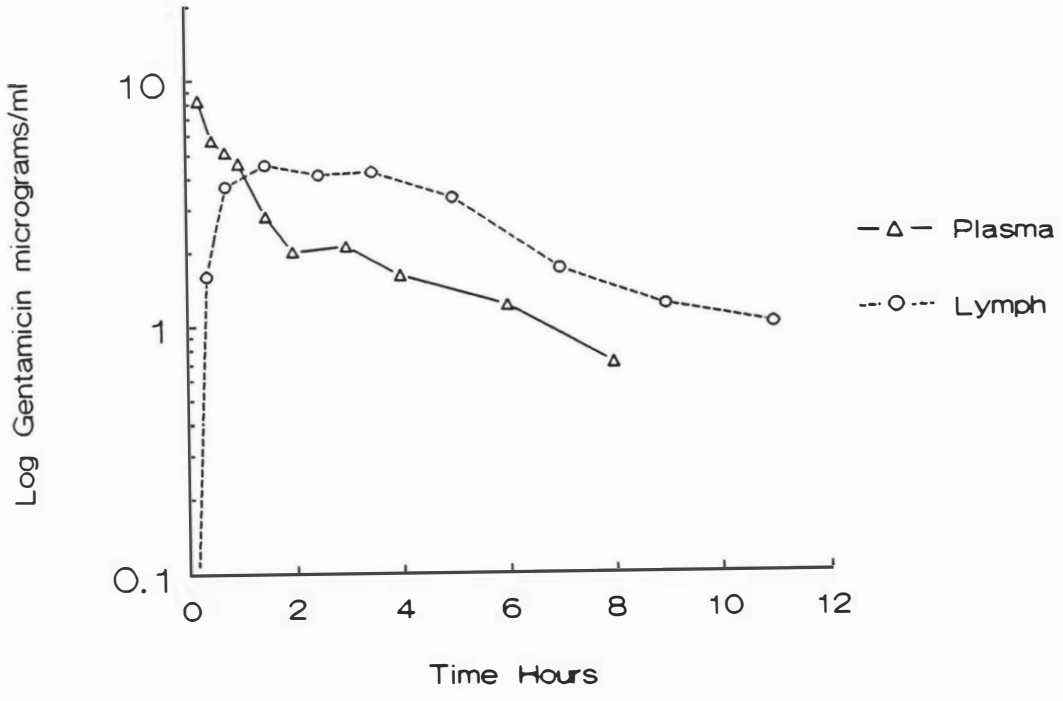


Figure 6. **Trial A: Disposition of Gentamicin (μg/ml) in Plasma and Lymph for Horse 3.**

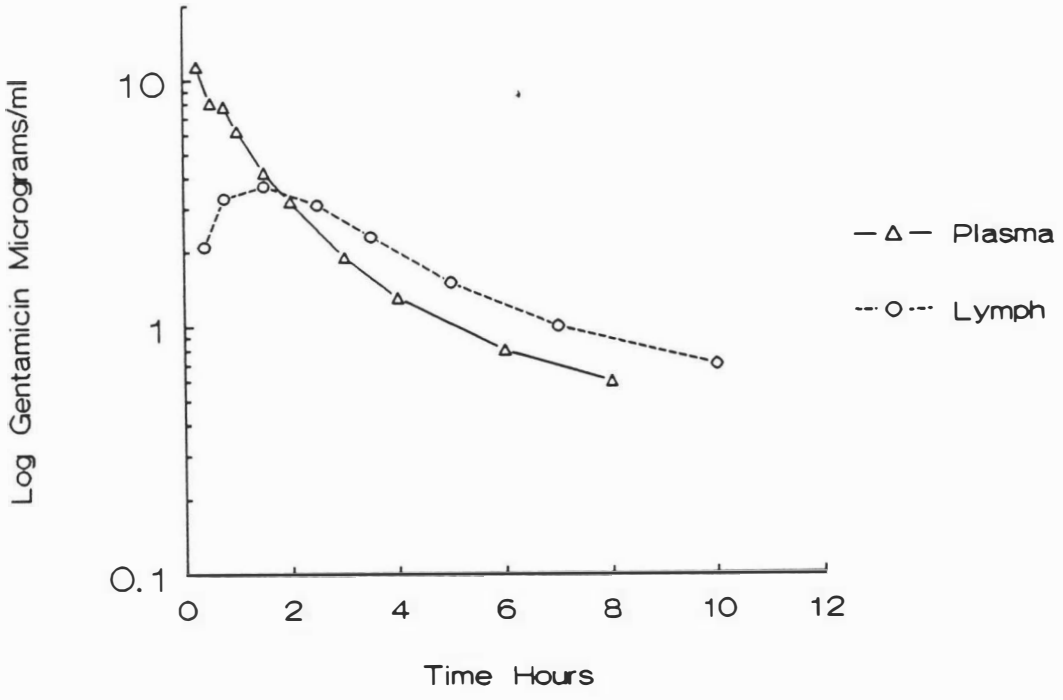


Figure 7. **Trial A: Disposition of Gentamicin (μg/ml) in Plasma and Lymph for Horse 4.**

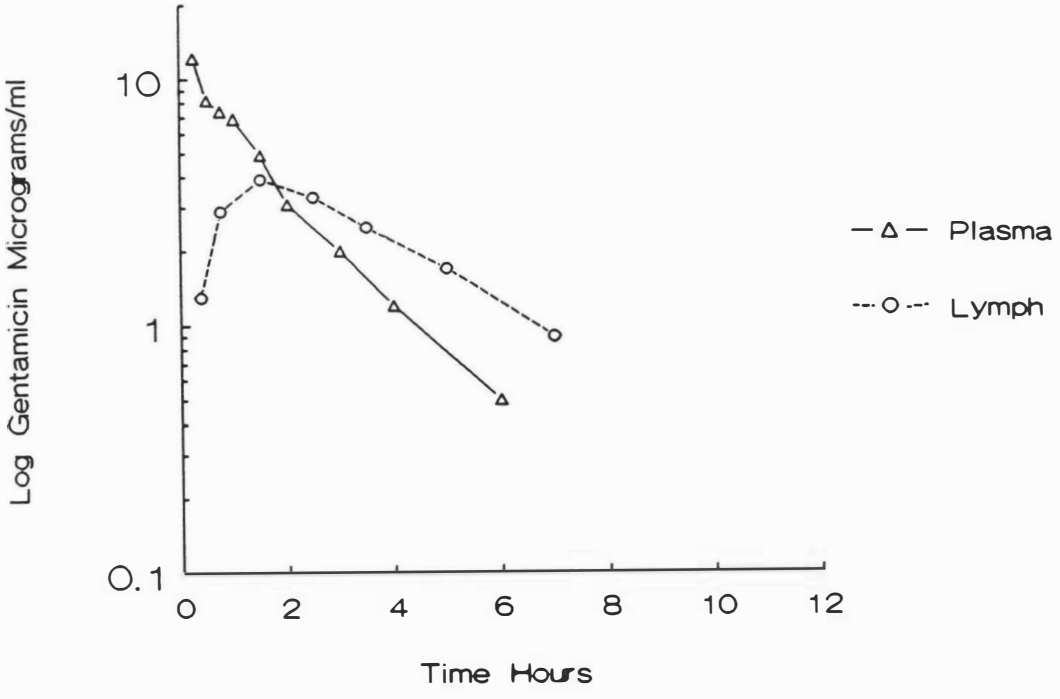


Figure 8. **Trial A: Disposition of Gentamicin (μg/ml) in Plasma and Lymph for Horse 5.**

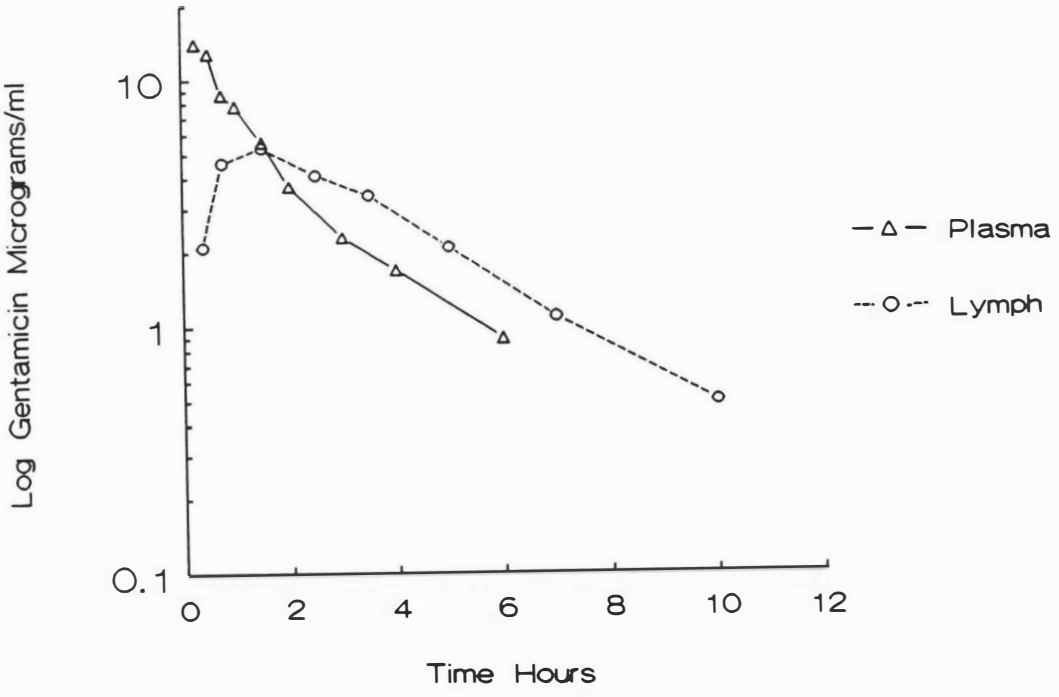


Figure 9. **Trial B: Disposition of Gentamicin (μg/ml) in Plasma, Synovial Fluid and Lymph for Horse 1.**

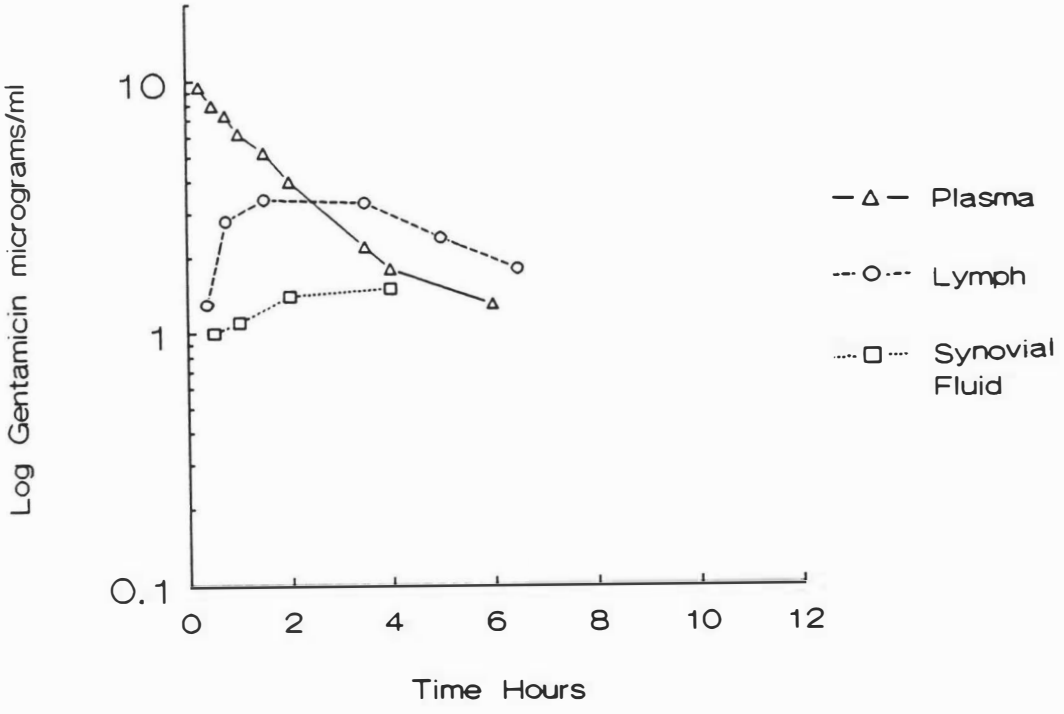


Figure 10. **Trial B: Disposition of Gentamicin (μg/ml) in Plasma, Synovial Fluid and Lymph for Horse 2.**

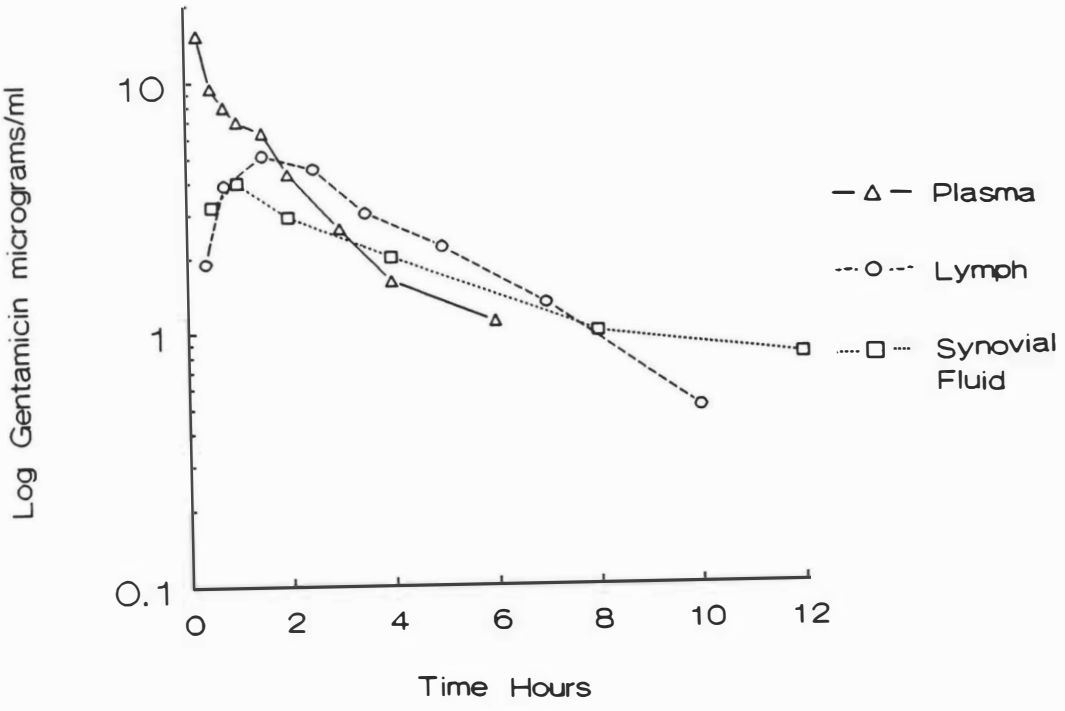


Figure 11. **Trial B: Disposition of Gentamicin (μg/ml) in Plasma, Synovial Fluid and Lymph for Horse 3.**

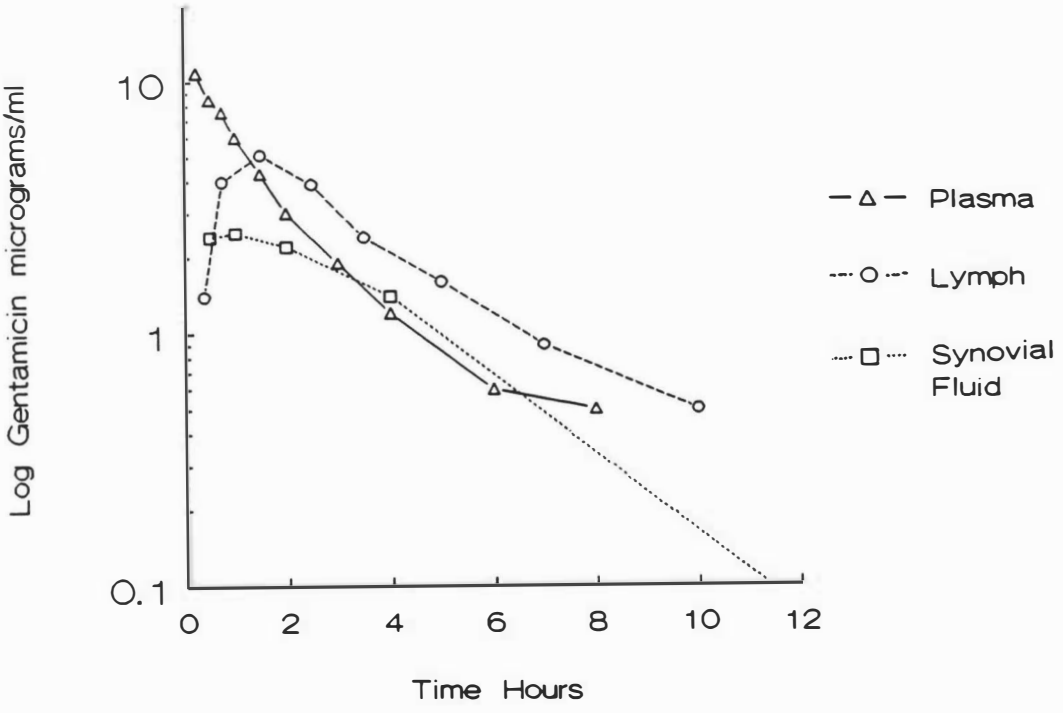


Figure 12. **Trial B: Disposition of Gentamicin (μg/ml) in Plasma, Synovial Fluid and Lymph for Horse 4.**

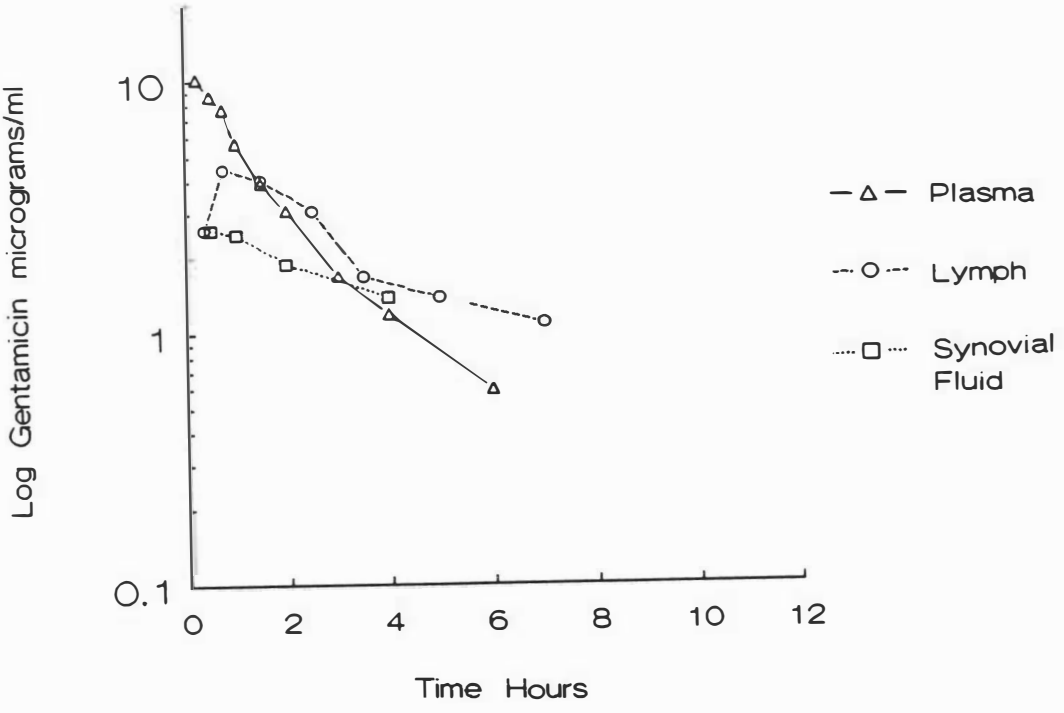


Figure 13. **Trial B: Disposition of Gentamicin (μg/ml) in Plasma, Synovial Fluid and Lymph for Horse 5.**

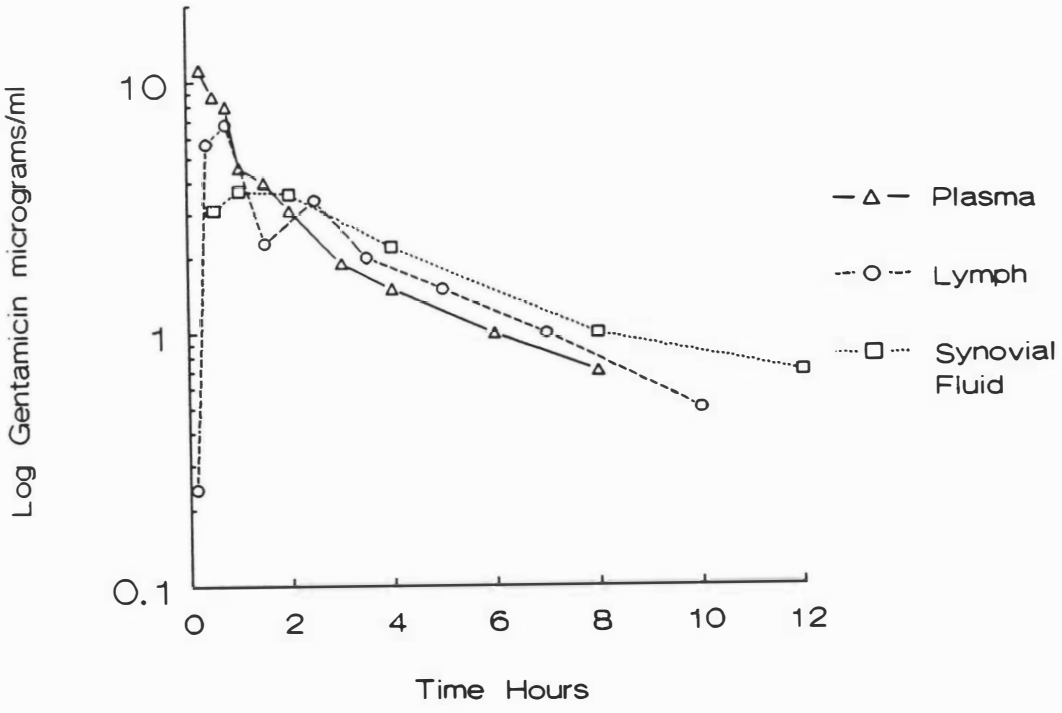


Table 5. **Plasma Concentrations of Gentamicin (μg/ml) after Intravenous Injection (2.2 mg/kg) for Trials A and B.**

Sample Time (h) Post injection	H_1		H_2		H_3		H_4		H_5		Mean(±sem)	
	A	B	A	B	A	B	A	B	A	B	A	B
0.25	9.3	9.5	8.3	15.3	11.4	10.9	12.2	10.2	14.0	11.2	11.04±1.02	11.42±1.01
0.5	7.6	8.0	5.7	9.5	8.1	8.5	8.2	8.8	12.8	8.8	8.48±1.17	8.72±0.24
0.75	6.9	7.3	5.1	8.0	7.8	7.6	7.4	7.8	8.7	8.0	7.18±0.6	7.74±0.13
1.0	6.4	6.2	4.6	7.0	6.2	6.0	6.9	5.7	7.8	4.6	6.38±0.52	5.9±0.4
1.5	5.2	5.2	2.8	6.3	4.2	4.3	4.9	4.0	5.6	4.0	4.54±0.49	4.76±0.44
2	3.8	4.0	2.0	4.3	3.2	3.0	3.1	3.1	3.1	3.1	3.16±0.32	3.5±0.27
3	2.7	--	2.1	2.6	1.9	1.9	2.0	1.7	2.3	1.9	2.2±0.4	2.03±0.19
3.5	ND	2.2	--	--	--	--	--	--	--	--	--	--
4	1.8	1.8	1.6	1.6	1.3	1.2	1.2	1.2	1.7	1.5	1.52±0.12	1.46±0.12
6	1.0	1.3	1.2	1.1	0.8	0.6	0.5	0.6	0.9	1.0	0.98±0.09	0.92±0.14
8	0.7	--	0.7	ND	0.6	0.5	ND	ND	ND	0.7	0.4±0.16	0.3±0.18
12	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
24	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

-- = not assayed
ND = not detected

Table 6. **Lymph Concentrations of Gentamicin (μg/ml) after Intravenous Injection (2.2 mg/kg) for Trials A and B.**

Sampling time (h) Mid-point of Lymph Sampling Interval	H_1		H_2		H_3		H_4		H_5		Mean(±sem)	
	A	B	A	B	A	B	A	B	A	B	A	B
0	0	0	0	0	0	0	0	0	0	0		
0.125	1.1	0	0	0	0	0	0	0	0	2.4	0.22 ±0.22	0.48 ±0.48
0.375	2.6	1.3	1.6	1.9	2.1	1.4	1.3	2.6	2.1	5.7	1.94 ±0.23	2.58 ±0.81
0.75	3.6	2.8	3.7	3.9	3.3	4.0	2.9	4.5	4.6	6.8	3.62 ±0.28	4.4 ±0.66
1.5	4.0	3.4	4.5	5.1	3.7	5.1	3.9	4.1	5.3	2.3	4.28 ±0.29	4.0 ±0.53
2.5	3.5	—	4.1	4.5	3.1	3.9	3.3	3.1	4.1	3.4	3.62 ±0.21	3.73 ±0.31
3.5	3.1	3.3	4.2	3.0	2.3	2.4	2.5	1.7	3.4	2.0	3.1 ±0.34	2.48 ±0.29
5	2.1	2.4	3.3	2.2	1.5	1.6	1.7	1.4	2.1	1.5	2.14 0.31	1.82 ±0.2
6.5	ND	1.8	—	—	—	—	—	—	—	—	—	—
7	1.7	—	1.7	1.3	1.0	0.9	0.9	1.1	1.1	1.1	1.28 ±0.17	1.08 ±0.09
9	1.2	—	1.2	—	—	—	—	—	—	—	—	—

Table 6
Contd.

10	—	—	—	0.5	0.7	0.5	ND	ND	0.5	0.5	0.4 ±0.2	0.38 ±0.13
11	—	—	1.0	---	---	---	---	---	---	---	---	---
11.5	0.9	---	---	---	---	---	---	---	---	---	---	---
13	—	—	0.5	---	---	---	---	---	---	---	---	---
14	0.8	---	---	---	---	---	---	---	---	---	---	---
14.5	ND	ND	ND	ND	---	—	—	---	—	—	---	ND
16	—	—	—	—	—	ND	---	—	ND	---	---	---
17.75	—	—	—	—	ND	---	---	---	---	---	---	---
18.5	0.5	---	---	---	---	---	---	---	---	---	---	---

--- = not assayed
ND = not detected

Table 7. **Synovial Fluid Concentrations of Gentamicin (μg/ml) after Intravenous Injection of (2.2 mg/kg) for Trial B.**

Sampling Time (h) Post injection	H_1	H_2	H_3	H_4	H_5	Mean(±sem)
0	0	0	0	0	0	0
1.5	1.0	3.2	2.4	2.6	3.1	2.46±0.4
1	1.1	4	2.5	2.5	3.7	2.76±0.52
2	1.4	2.9	2.2	1.9	3.6	2.4±0.39
4	1.5	2.0	1.4	1.4	2.2	1.7±0.17
8	--	1.0	--	--	1.0	1.0
12	--	0.8	ND	--	0.7	0.5±0.25

-- = not assayed
ND = not detected

Table 8. Elimination of Gentamicin from Plasma and Lymph. Non-weighted Least Squares Estimates of the Terminal Rate Constant (λ_Z) and Zero time Concentration ($C_P(0)$ and $C_L(0)$), Trials A and B.

Plasma		H_1		H_2		H_3		H_4		H_5		Mean±sem	
Parameter	Unit	A	B	A	B	A	B	A	B	A	B	A	B
$C_P(0)$	μg/ml	6.21	9.49	3.91	13.32	5.37	6.4	7.76	6.89	6.93	3.37	6.04 ±0.66	7.89 ±1.67
λ_Z	h^{-1}	0.286	0.418	0.21	0.536	0.297	0.401	0.459	0.42	0.345	0.199	0.319 ±0.04	0.395 ±0.056
Nos. Pts. used in Estimation		5	4	4	4	6	4	4	5	4	4		
r^2		0.98	0.95	0.98	0.99	0.94	0.99	0.99	0.98	0.99	0.99		

Lymph		H_1		H_2		H_3		H_4		H_5		Mean±sem	
Parameter	Unit	A	B	A	B	A	B	A	B	A	B	A	B
$C_L(0)$	μg/ml	3.90	6.66	8.9	8.85	5.61	6.54	6.86	2.62	8.78	4.32	6.81 ±0.96	5.80 ±1.01
λ_Z	h^{-1}	0.111	0.202	0.216	0.284	0.251	0.267	0.287	0.124	0.289	0.214	0.232 ±0.032	0.218 ±0.03
Nos. Pts. used in Estimation		7	3	6	5	4	5	4	3	5	4		
r^2		0.97	0.99	0.98	0.99	0.99	0.98	0.99	0.99	0.99	0.99		

Table 9. **Lymph:Plasma Gentamicin Ratios from 2-10 h after Intravenous Injection*.**

	Trial A			Trial B		
Time (h) Post Injection	Plasma Pred.	Lymph Pred.	L/P	Plasma Pred.	Lymph Pred.	L/P
2	3.01	4.07	1.35	3.22	3.86	1.19
3	2.26	3.26	1.44	2.34	3	1.28
4	1.69	2.61	1.54	1.69	2.33	1.38
5	1.27	2.09	1.65	1.23	1.81	1.47
6	0.95	1.68	1.77	0.89	1.4	1.57
7	0.72	1.34	1.86	0.65	1.09	1.68
8	0.54	1.01	1.87	0.47	0.86	1.83
9	0.4	0.86	2.15	0.34	0.66	1.94
10	0.3	0.69	2.3	0.25	0.51	2.04

* calculated from the predicted (pred.) plasma and lymph concentrations obtained by linear regression of the elimination phase of the disposition data from all horses.

L/P = lymph/plasma ratio
Mean L/P ratio for 3-8 h Trial A = 1.7 $\pm$ 0.06
Mean L/P ratio for 3-8 h Trial B = 1.54 $\pm$ 0.07

Table 10. **Lymph:Synovial Fluid and Plasma:Synovial Fluid Gentamicin Ratios 2-8 h after Intravenous Injection for each Horse in Trial B*.**

	H_1		H_2		H_3		H_4		H_5	
Time (h) Post Injection	**L:SF**	**P:SF**	**L:SF**	**P:SF**	**L:SF**	**P:SF**	**L:SF**	**P:SF**	**L:SF**	**P:SF**
2	3.2	2.9	1.7	1.6	1.7	1.3	1.1	1.6	0.8	0.6
4	2	1.2	1.4	0.8	1.6	0.9	1.1	0.9	0.84	0.7
8	ND	ND	0.91	0.18	ND	ND	ND	ND	0.78	0.69

L:SF = Lymph:Synovial Fluid
P:SF = Plasma:Synovial Fluid

* The lymph and plasma concentrations used to calculate the ratios were the predicted concentrations. These were obtained by linear regression analysis of the elimination phase of the disposition data from all horses. The synovial fluid concentrations are those actually measured.

The mean($\pm$sem) ratio from 2-4 h was 1.54 ± 0.2 and 1.25 ± 0.2 for L:SF and P:SF respectively.

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