# PHYSIOLOGICAL AND CLASSICAL PHARMACOKINETIC MODELS OF OXYTETRACYCLINE IN CATTLE

by

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## Abstract

Oxytetracycline (OTC) is a broad-spectrum antibiotic, which is used widely to treat infectious diseases in humans and farm animals. OTC is often used both as a therapeutic agent and a growth promoter in cattle. With an increasing use of OTC in cattle, OTC residues in the edible tissues of the farm animals have become a public health concern.

Studies were conducted using the classical pharmacokinetic model approach to examine the bioequivalence of two long-acting OTC formulations and the physiologically based pharmacokinetic (PBPK) model approach to study the residues of OTC in the tissues of the cattle. Therefore, the objectives of the present study were (1) to investigate the bioequivalence of the OTC formulations after they were injected separately into groups of feedlot steers *via* the intramuscular (*i.m.*) or subcutaneous (*s.c.*) route of administration, (2) to determine OTC residues in the tissues of the feedlot steers after injecting *s.c.* the OTC formulations, and (3) to develop and validate a PBPK model of OTC for the beef cattle.

The OTC formulations were administered (20 mg/kg) separately to beef cattle either by the *i.m.* or the *s.c.* route. The time course of serum OTC concentrations could be described adequately by a classical, two-compartment pharmacokinetic model. A similar amount of OTC was absorbed systemically by the cattle after administration of the OTC formulations. These results indicated that the OTC formulations are bioequivalent in the beef cattle. OTC tissue concentrations other than those of the serum also were determined in the cattle. OTC concentrations in the tissues decreased in the order of

kidney>liver>muscle>>fat. As expected, a high level of OTC was found at the site of

injection even after 4 weeks of drug administration. Systematically absorbed OTC was

eliminated rapidly by the cattle since OTC was not detected in the tissues at 28 days post-

dosing.

A PBPK model of OTC was developed in beef cattle using the data set of the *s.c.*

administration route. The PBPK model of cattle consists of nine flow-limited

compartments each representing the kidney, muscle, liver, fat, gut, lung, blood, and

carcass. The transfer of OTC between these compartments is governed by the rates of

blood flow and the tissue:blood partitioning coefficients of OTC in specific tissues.

Elimination of OTC from the cattle is modeled by renal and hepatic clearances, which are

assumed to be first-order rate processes. The PBPK model was validated with empirical

OTC tissue concentration data from cattle given OTC by the *i.m.* route of drug

administration. Model-predicted OTC tissue concentrations were found to describe

closely those of the experimental data. Since the validated PBPK model can be used to

predict the pharmacokinetics of OTC for different species of cattle, exposure routes or

dosing regimes, it is a useful tool for developing new formulations of OTC in cattle.

# Dedication

To My Mom,

Hannelore Achenbach

For all you have done and given me to make me who I am.

## Acknowledgments

First and foremost, I want to thank Dr. Francis Law, "the wise old Bull" for taking this young calf into his herd. Not only has he taught me to slow down and learn the ways of the farm, he has put up with me wandering the fields of discovery, always knowing the right time to call me back to the task at hand.

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To Dr. He, for your entertainment and help, may Buddha always be very happy!

To Haemmorage, Virus and Madcow, the stress relief committee of Albertabeef, may the BBQ always be hot and the grillin' good.

And a big thanks to Sylvia Foran for all her wrangling duties she has done over the years

And last but not least, to Mr. D. Cooper who opened my eyes to science so long ago in high school.

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## List of Abbreviations

| | |
|---|---|
| AUC | Area under the curve |
| C | Centigrade |
| cm | Centimeter |
| EDTA | Ethylenediamine-tetraacetic acid |
| G | Gram |
| $g$ | Acceleration of gravity |
| hr | Hour |
| HPLC | High-performance liquid chromatography |
| I.U. | International unit |
| l | Litre |
| $K_{ow}$ | Octanol:water partition coefficient |
| M | Molar |
| MIC | Minimum inhibitory concentration |
| min | Minute |
| m | Meter |
| N | Normality |
| ND | Not detectable |
| NS | Not sampled |
| PSI | Pounds per square inch |
| ODE | Ordinary differential equations |
| OTC | Oxytetracycline |
| SD | Standard deviation |
| TC | Tetracycline |
| vs. | Versus |
| WP | Withdrawal period |
| w/v | Weight to volume |
| w/w | Weight to weight |

Prefixes for units of measurement:

| | |
|---|---|
| p | Pico |
| n | Nano |
| $\mu$ | Micro |
| m | Milli |
| k | Kilo |

## PBPK model parameters and abbreviations

| Parameter | Abbreviation |
|---|---|
| **i) Tissue volume** | |
| Lung | VLN |
| Liver | VL |
| Gut | VGT |
| Kidney | VK |
| Carcass | VC |
| Muscle | VM |
| **ii) Blood Flow** | |
| Lung | QLN |
| Liver | QL |
| Gut | QGT |
| Kidney | QK |
| Carcass | QC |
| Muscle | QM |
| Cardiac output | QT |
| **iii) Partition coefficients of oxytetracyline** | |
| Lung:blood | RLN |
| Liver:blood | RL |
| Gut:blood | RG |
| Kidney:blood | RK |
| Carcass:blood | RC |
| Muscle:blood | RM |
| **iv) Biochemical constants** | |
| Kidney Clearance | KK |
| Liver Clearance | KL |

# INTRODUCTION

Oxytetracycline (OTC), an antibiotic obtained from *Streptomyces*, was first

discovered in 1948. OTC belongs to the tetracycline family of antibiotics and has been

used widely to treat infectious diseases in both humans and farm animals. Figure 1 shows

the chemical structure of OTC.
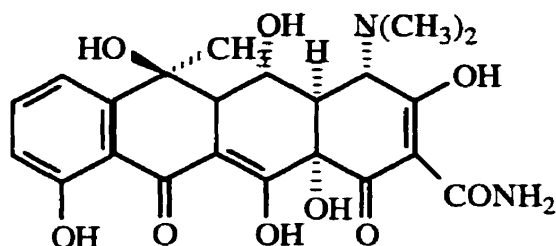


Figure 1. Chemical structure of oxytetracycline.

Pure OTC is composed of faintly yellow, odorless crystals that are slightly water-soluble.

However, OTC can form stable sodium or hydrochloride salts that are very water-soluble.

The antibacterial effects of OTC has been explained by its binding with the 70S

and 80S ribosomes, blocking the attachment of aminoacyl-transfer RNA to the

ribosomal-messenger RNA and thereby disrupting the ability of bacteria to produce

proteins (Lancini 1995). OTC is selectively toxic to the bacterial cells because

prokaryotic cells possess an active transport system for OTC whereas eukaryotic cells

actively export the antibiotic (Goodman and Gillman, 1985).

OTC is a broad-spectrum antibiotic effective for both Gram positive and Gram

negative bacteria. OTC has some side effects in humans; these include gastrointestinal

effects (vomiting, epigastric distress, nausea, diarrhea, esophageal ulceration,

hypersensitivity), urticaria, asthma, facial edema, contact dermatitis, and

photosensitization. Other side-effects of OTC in humans are teratogenic effects,

pigmentation of teeth, accumulation of OTC in the bones of infants resulting in depression of bone growth, and aggravation of pre-existing renal failure (Ginsberg and Tager, 1980).

OTC is commonly used as a therapeutic agent against a wide range of gram-positive and gram-negative bacteria such as chlamydia, rickettsiae, actinomycetes, as well as protozoa (Landoni and Errecalde, 1992). OTC is also used as a growth promoter in cattle by increasing the efficiency of feed utilization and by improving the general health of the gastrointestinal system. OTC is usually administered to cattle as medicated feed or by injection through the intraveneous (*i.v.*), intramuscular *(i.m.)* or subcutaneous (*s.c.*) route. After systemic absorption, OTC distributes rapidly into the extracellular spaces of animal tissues. It also can cross the placental and the blood-brain barriers (Riviere et al., 1990). However, little or no OTC undergoes metabolic degradation in cattle and enterohepatic recirculation of OTC is minimal (Nouws et al., 1985a). Therefore, systemic OTC is eliminated mainly unchanged in the urine and OTC renal clearance is dependent on the urine flow. (Nouws et al., 1985a). Therefore, the pharmacokinetics of OTC in cattle, are determined by a host of environmental and physiological factors such as the administration route, injection site, animal age, urine flow and the disease state of the animal (Nouws et al, 1992).

With an increasing use of OTC in the agricultural industry, OTC residues in the edible tissues of animals and the potential of developing resistant bacteria strains in these animals and humans have become a public health concern. The development of resistant bacteria strains has been difficult to detect since it is a slow and gradual process. However, once a microorganism becomes resistant to a member of the tetracycline family

it often displays cross-resistance to the others. Resistance is an inducible trait mediated by plasmids. Since OTC is taken up by an energy-dependent, active transport system, the plasmids containing the genetic code can produce nonfunctioning periplasmic protein carriers that are unable to transport tetracyclines into the prokaryotic cells (Goodman and Gillman, 1985). Resistance of Escherichia coli to tetracyclines has been observed not only in farm-raised animals such as chickens (Vazquez-Moreno, L., 1990, Tessi, M. A., 1997), swine (Nijsten-R, 1993) and cattle (Vazquez-Moreno, L., 1990), but also in veterinarians, farmers and abattoir workers who are in close contact with the farm animals (Nijsten-R, 1994; Bongers-J-H, 1995).

After treating farm animals with OTC, they must be kept for a minimum time, known as a withdrawal period (WP), before being slaughtered for human consumption. Observation of the WP ensures the edible tissues contain only low levels of OTC. In general, OTC-medicated feeds usually require a WP of less than 1 week. However, most injectable formulations of OTC in Canada require a WP of about 2-4 weeks in cattle. The following are some of the tissues and their maximum permitted levels in Canada: muscle (0.25 $\mu$g/g), liver (0.25 $\mu$g/g) and kidney (0.25 $\mu$g/g) (CFIA, 1990). These tissue residues are similar to those published by the joint Food and Agriculture Organization of the United Nations and World Health Organization Expert Committee on Food Additives; they are 0.1 $\mu$g/g in the muscle, 0.3 $\mu$/g in the liver and 0.6 $\mu$g/g in the kidney (WHO 1990).

An ideal OTC formulation for treating cattle should have the following characteristics: (a) maintenance of OTC concentrations in tissues above the minimum inhibitory concentration (MIC) for disease treatment for a long period, (b) a dosage form

which can be administered easily and does not cause tissue irritation and damage the site of administration, and (c) a short WP to minimize a delay in slaughter time and to reduce production costs. Very few OTC formulations used presently in Canada meet all these requirements. A single injection of long-acting OTC formulation is often preferred by cattle farmers over multiple injections of short-acting OTC formulation because of easiness of administration and lower cost. However, a long-acting OTC formulation causes a high OTC concentration at the injection sites (George et al., 1995) and the development of lesions at the injection sites (Banting et al., 1987, George et al. 1995). These undesirable effects persist even after the normal WP has elapsed but can be overcome by injecting the cattle in the neck *s.c.* instead of injecting in the rump *i.m.*. Therefore, there is a real need to develop a long-acting OTC formulation specifically for *s.c.* administration to beef cattle.

In the past 25 years, many OTC pharmacokinetic studies have been conducted in cattle in the form of bioequivalence studies. Bioequivalence is defined as two formulations that are equally bioavailable: that is, equal in rate and extent to which the active ingredients(s) or therapeutic ingredient(s) is (are) absorbed and become available at the site(s) of drug action. Regulatory agencies such as the US Food and Drug Administration (USFDA) and Health Canada (HC) require this information before approving a new drug formulation.

Pharmacokinetic studies have been undertaken in dairy cows of different ages using a variety of OTC dosing regimes and administration routes (Nouws & Ziv, 1978; Banting et al., 1985; Nouws et al., 1985a; Nouws et al., 1985b; Melvius et al., 1986; Landoni, M.F., 1992, Meijer, L.A., 1993). Moreover, the OTC blood concentration data

are usually fitted to a classical pharmacokinetic model (Nouws et al., 1985b., Mevius et al.,1986). Since the classical model does not include anatomical, physiological and biochemical inputs that determine the pharmacokinetic behavior of OTC in its modeling approach, it cannot be used to predict OTC concentration in a target tissue other than that of the blood compartment. Many of the limitations of classical models can be overcome by employing the physiologically-based pharmacokinetic (PBPK) models. Therefore, Olanoff and Anderson (1979, 1980) developed a PBPK model of tetracycline for the rat and Law (1992, 1998) has described the biological fates of OTC in trout and salmon with a PBPK model.

A PBPK model is a mathematical representation of a real biological system; it describes the uptake, distribution, metabolism and elimination of a chemical in an animal. Model development is based on the physicochemical characteristics of a drug and the anatomy and physiology of the animal. In order to provide a simple description for a complex biological system and its interactions with the drug, the basic unit of a PBPK model is assumed to be a tissue compartment of an animal. A compartment may represent a single organ (tissue) or a group of organs (tissues) with a similar pharmacokinetic behavior. The choice of compartments in a PBPK model usually depends on the physicochemical characteristics (e.g. tissue/protein binding, lipid solubility, and ionization) and the pharmacologic properties of the drug (e.g., mechanisms of transport and site(s) of action) (Bischoff, 1975). The following assumptions have been made on a compartment: (a) a compartment is well mixed. This means that once a chemical enters a compartment it is distributed immediately to all areas of the compartment, (b) there is always a mass balance of the chemical in a compartment

i.e., no chemical is lost or gained, and (c) chemical concentration in the blood leaving the compartment is equal to the chemical concentration in the tissue. Therefore, a mass balance equation is used to account for the influx and efflux of a drug in each compartment. The following is a typical mass balance equation:

$$dM/dt = Q_{in} \times C_{in} - Q_{out} \times C_{out} \tag{1}$$

where, M represents the mass of a drug (mg) , Q represents blood flow (ml/min), $C_{in}$ represents drug concentration in the arterial blood (mg/ml), and $C_{out}$ represents drug concentration in the venous blood (mg/ml).

Since it is impossible to determine $C_{out}$ directly, a partition coefficient (R) is used to calculate $C_{out}$ indirectly.

$$R = CM/C_{out} \qquad C_{out} = CM/R \tag{2}$$

By substituting equation (2) into equation (1), we have

$$dM/dt = Q_{in} \times C_{in} - Q_{out} \times CM/R \tag{3}$$

Since blood flow into a compartment must equal to blood flow out of the compartment, equation (3) can be rewritten as

$$dM/dt = Q \times (C_{in} - CM/R) \tag{4}$$

To find the concentration of a drug in the compartment, both sides of equation (4) are divided by the volume of the compartment

$$dC/dt = (Q/V) \times (C_{in} - CM/R). \tag{5}$$

The objectives of the present study were: (1) to investigate the bioequivalence of two long-acting OTC formulations after *i.m.* or *s.c.* route of administration to feedlot steers, (2) to determine OTC residues in the tissues of the feedlot steers after injecting the

long-acting OTC formulations *s.c.*, (3) to develop and validate a physiologically-based

pharmacokinetic (PBPK) model of long-acting OTC for the beef cattle.

## MATERIALS AND METHODS

### I. Empirical studies

Chemicals

Three long-acting liquid injectable formulations of OTC, Liquamycin LA-200®, Alamycin LA-300®, and Biomycin LA-200®, were obtained from commercial sources. These formulations contained either 200 mg/ml or 300 mg/ml of OTC. USP reference OTC standard with a chemical potency of 91.9% was purchased from Nucro Technics Inc. (Scarborough, ON). Tetracycline, the HPLC internal standard, was also purchased from Nucro Technics Inc. (Scarborough, ON). Organic solvents were of analytical or HPLC grade. All other chemicals were of analytical grade or better.

### Animals, dosing regimes, and tissue collection

Three sets of experiments were conducted to examine the bioequivalence of the long-acting OTC formulations after either *i.m.* or *s.c.* administration to cattle. This formulation was given to beef cattle at a dose of 20 mg/kg body weight. The first set of experiments was undertaken entirely by our laboratory. The last two sets of experiments were collaborative projects with Colorado Animal Research Enterprises (CARE), Ft. Collins, Colorado. The role of CARE in the project was mainly to house, dose, and collect serum and tissue samples from the cattle. The tissue samples were analyzed in our laboratory under Good Laboratory Practice (GLP). The three sets of experiments are described as follows:

Experiment 1. Bioequivalence of Liquamycin LA-200® and Alamycin LA-300®

following *i.m.* injection

Six commercial feedlot steers of similar frame and weights were used. The steers

were housed separately in a hospital pen adjacent to the handling facilities (Meadows

Feedlot, Pitt Meadows, B.C.). The pen was enclosed by 2' x 6' board fencing and was

covered by a roof. Feed was provided in wood bunks along one fence. Water was given

*ad libitum* through automatic founts. Animals were housed for one week in the pen prior

to the start of the experiment.

The study was a cross-over design consisting of two time periods (I and II) which

were separated by a 28 day withdrawal period (WP). Each animal was uniquely identified

through the use of an ear tag and assigned a random number. The six feedlot steers

weighed 372±16.8 kg and 420 ± 17.8 kg at the beginning of period I and period II,

respectively. During the period I study, the first group of 3 animals was injected with

Liquamycin LA-200®, the second group of 3 animals, Alamycin LA-300®. During period

II, the first three steers received Alamycin LA-300®, the second group received

Liquamycin LA-200®.

After weighing the feedlot steers, each animal received an individually calculated

dose from an OTC formulation, which was delivered by deep *i.m.* injection at 20 mg/kg

body weight. Injections were delivered into the neck area using a 15-ml syringe

equipped with a 5 cm, 16-gauge needle. The injection site was located approximately 10

cm cranial to the shoulder blade and approximately midway between the dorsal midline

and the lateral processes of the cervical vertebrae. No more than 15 ml of Liquamycin

LA-200® or 10 ml of Alamycin LA-300® was administered to a single injection site.

Where multiple injection sites were required, each successive site was at least 5 cm apart and cranial to the previous one. The last site received the remaining amount required to yield the full dose. Injections for Liquamycin LA-200® were administered on the left side of the neck whereas Alamycin LA-300® injections were administered on the right side. New syringes and needles were used for each *i.m.* injection.

Blood samples were collected from each animal just prior to the administration of a OTC formulation (0 hr), then at 0.5, 1, 2, 4, 8, 12, 24, 48, 72, 96 and 120 hrs post-injection. Blood samples (5-10 ml) were collected in duplicate by direct venipucture into vacutainer serum vials (Becton Dickinson, Clarkson, ON). Sample vials were clearly marked with the animal's identification and sample collection time. Blood samples were allowed to clot and serum was removed after centrifugation (2500 $g$, 15 min). The serum samples were put into marked vials and frozen at $-20^{\circ}$C until analysis.

<u>Experiment 2. Bioequivalence of Liquamycin LA-200® and Biomycin LA-200® following *s.c.* administration</u>

Animal pretreatment was conducted at CARE Inc. (Fort Collins, CO). Briefly, a stock of 28 (14 heifers and 14 steers) mixed breed (Hereford x Angus x Gelbvieh x Saler) beef calves were purchased for the study. All calves were identified by duplicate, uniquely numbered ear tags and were kept in a group in an open-air drylot with *ad libitum* access to water through automatic founts. Calves were gradually transferred from grass hay to alfalfa hay in freestanding metal bunks and received feed twice a day to-appetite feedings through the acclimation (27 days) and study.

From the stock of 28 calves, 20 healthy calves were chosen one day prior to treatment. The 20 calves were sorted by sex (10 steers and 10 heifers) and divided into

two groups each containing 10 animals (5 steers and 5 heifers). The study was a cross-over design (period I and period II) with each group receiving the other OTC formulation after a 28 day WP.

Each animal was weighed and received an individually calculated dose of either Biomycin LA-200® (T1) or Liquamycin LA-200® (T2) delivered by *s.c.* injection at 20 mg/kg body weight. Treatments were administered using a 12 ml syringe fitted with a 3 cm, 18 gauge needle. New syringes and needles were used for each injection. No more than 10 ml was given into any one-injection site. There were three (3) injection sites per animal; one located on the right-side neck (injection site #1) and two located on the left-side neck (injection sites #2 and #3). Injection sites #1 and #2 each received the full 10 ml dosage and injection site #3 received the residual amount required to yield the full dosage. Subcutaneous injection sites #1 (right-side) and #2 (left-side) were given in a location defined by palpating the midpoint of the longitudinal axis of the spine of the scapula, progressing cranially on a vector aligned with the caudal ramus of the mandible to a point ~ 15 cm from the original scapular spine reference point. Injection #3 (left-side) was given in an area ~30 cm from the original scapular reference point. All injections were given by retracting the loose skin of the neck and injecting into the created space.

Blood samples were collected just prior to drug administration (0 hr) and at 0.5, 1, 2, 4, 6, 8, 10, 16, 24, 36, 48, 60, 72, 96, and 120 hrs post administration.
Blood samples were acquired by direct venipucture into vacuseal vials to a minimum of 5 ml. Blood samples were allowed to clot and serum was removed after centrifugation and divided equally into four subsets. Each serum sample consisted of a 5 ml resealable

sample tube labeled with study number, animal number, withdrawal interval, and replicate ID (A, B, C, D). Serum samples from individual animals were bagged together in plastic resealable bags that were labeled with period time and animal number and frozen at $-20^0$ C.

Frozen serum samples were packed in dry ice and transported *via* Fedex International Priority overnight service to the Department of Biological Sciences, Simon Fraser University on the dates below. Replicate A - Period 1 samples were received on August 1, 1996, examined for sample integrity and GLP compliant labeling and placed immediately in a freezer at less than -20°C. Replicate A - Period 2 samples were received on August 15, 1996 and examined and frozen as above. Another set of serum samples (Replicate B) was retained at CARE Inc. and was transported as described above to Department of Biological Sciences, Simon Fraser University, on August 28, 1996. These samples were immediately examined and placed in the freezer as above. The remaining sets of samples were retained at CARE, Inc.

Experiment 3. OTC residue depletion in edible tissues of steers and heifers following *s.c.* administration

A group of 36 (18 heifers and 18 steers) mixed breed (Hereford x Angus x Gelbvieh x Saler) beef calves weighing about 253 kg were purchased from a local livestock producer for the study. One month prior to the study all calves were identified in duplicate by numbered ear tags and were kept in a group in an open-air drylot with *ad libitum* access to water through automatic founts. Calves were gradually transferred from grass hay to alfalfa hay in freestanding metal bunks and received feed twice a day to-appetite feedings through the acclimation (27 days) and study.

From the stock of 36 calves, 26 healthy calves were chosen one day prior to treatment. Two calves (one heifer and one steer) were randomly selected and assigned to the control group (T1). The remaining 24 calves were sorted by sex (12 steers and 12 heifers) and blocked by body weight into six groups containing four animals of two heifers and two steers. Treatments (T2-T7), which defined withdrawal internals, were randomly assigned to the six groups.

Each animal was weighed and received an individually calculated dose of either Liquamycin ® LA-200 ® (T2 –T7) delivered by s.c. injection at 20 mg/kg body weight or sterile saline (T1) administered s.c. at 1 ml/10 kg body weight. Treatments were administered using a 12 ml syringe fitted with a 3 cm, 18 gauge needle. New syringes and needles were used for each injection. No more than 10 ml was given into any one-injection site. There were three injection sites per animal; one located on the right-side neck (injection site #1) and two located on the left-side neck (injection sites #2 and #3). Injection sites #1 and #2 each received the full ml dosage and injection site #3 received the residual amount required to yield the full dosage. S.c. injection sites #1 (right-side) and #2 (left-side) were given in a location defined by palpating the midpoint of the longitudinal axis of the spine of the scapula, progressing cranially on a vector aligned with the caudal ramus of the mandible to a point ~15 cm from the original scapular spine reference point. Injection site #3 (left-side) was given in an area ~30 cm from the original scapular reference point. Each injection site was shaved (15 cm diameter circle) prior to dose administration for purposes of location identification at the time of tissue collection. All injections were given by retracting the loose skin of the neck and injecting

into the created space. Needle insertion occurred within the shaved area and ~3 cm from the top of the shaved circle.

At the designated post-dose interval (4, 10, 16, 22, 28, 35 days), calves of respective groups were humanely euthanized (electrocution followed by immediate exsanguination). The following tissues were collected from each animal in the following manner:

➢ Muscle was taken from the left leg approximately midway between the tuber schii and the point caudal to the stifle joint, representing the biceps femoris, semimembranous and semitendinous muscle.

➢ Fat was collected from the kidney area (peritoneal) and, when necessary to obtain sufficient amounts, from the abdominal cavity.

➢ Liver was collected in its entirety and the gall bladder was excised with care so it would not rupture.

➢ Kidneys (both) were removed from the carcass and from their capsules, and were trimmed of adhering fat.

➢ Injection site tissue was that tissue underlying the shaved area of each of the three neck injection sites. Each of the three injection sites was individually collected as a 15 cm diameter, 2.5 cm thick sample located directly below the shaved injection area.

Frozen tissue samples were packed in dry ice and transported *via* Fedex International Priority overnight service to the Department of Biological Sciences, Simon Fraser University on the dates below. Replicate A (4, 10 and 16 days post-injection) was received on September 14, 1996, examined for sample integrity and GLP compliant labeling and placed immediately in a freezer at less than -20°C. The second half of

replicate A (22, 28 and 35 days post injection) was received on October 2, 1996 and examined and frozen as above. Another back up set of samples (Replicate B) was retained at CARE, Inc. and was transported as described above to the Department of Biological Sciences, Simon Fraser University, on October 23, 1996. These samples were immediately examined and placed in the freezer as above. The remaining sets of samples were retained at CARE, Inc.

Tissue samples were processed (except for fat and injection sites) by rinsing with clean tap water and patted dry with paper towels at Simon Fraser University. All samples, except fat, from each animal were blended separately in a meat grinder and the homogenate mixed well. Four sub-samples (up to ~125 g each, and at least 15 g each) from each of the tissue specimens per animal were collected into labeled plastic resealable bags that were placed inside a second resealable bag. Each bag was labeled with the study number, animal number, tissue identity, withdrawal interval, date of collection and replicate ID (A, B, C, D).

**Preparation of Standard solution and McIlvaine buffer**

The USP reference standard of OTC had a chemical potency of 91.9% in its base form, the potency was multiplied by 1.08 to obtain the equivalent hydrochloride potency of 99.25%. Stock OTC solutions of 100 µg/ml were prepared in methanol. They were further diluted with methanol to concentrations of 0.1, 0.5, 1.0, 5.0 and 10.0 µg/ml before being used to prepare a HPLC calibration curve. All stock and calibration solutions were stored in the dark at $-20°C$ and were discarded after 72 hours.

McIlvaine/0.1 M disodium EDTA buffer was used to extract OTC from the tissue samples. The buffer was prepared as follows: Dibasic sodium phosphate (17.76 g) and citric acid monohydrate (21.01 g) were dissolved in 625 ml and 1000 ml of distilled water, respectively. After these solutions were mixed together, disodium EDTA (60.49 g) was added. The pH of the solution was adjusted to $4.0 \pm 0.1$ using orthophosphoric acid.

## Extraction of OTC from serum and tissue samples

(i) Extraction of serum

Aliquots of serum (1 ml) were spiked with the tetracycline internal standard (1 µg) and diluted with 10 ml McIlvaine/0.1 M disodium EDTA buffer. The samples were shaken for 10 min, centrifuged at 1500 g for 10 min and the supernatants were set aside. The pellets were washed with another 10 ml of the buffer, mixed and centrifuged as above. The supernatants from the two separate extraction steps were pooled. The centrifuge tube was washed with 5 ml of buffer followed by 10 ml of water and the wash solutions were combined with the supernatants before being passed through a Bond Elut C-18 column (Varian, Harbor City, CA) that were pre-conditioned with 10 ml methanol and 10 ml distilled water in a Baker-10 SPE system (J.T. Baker Inc., Phillipsburg, N.J.). The colums were allowed to run dry. OTC was eluted from the columns with either 4 ml or 8 ml of methanol into graduated receiving tubes. The eluates were evaporated down to less than 1 ml in a RC 10-22 Jouan Concentrator Evaporator (Jouan, Inc. Winchester, VA). After evaporation, the volumes of the eluates were made up to 1 ml with methanol, vortexed and centrifuged at 1500 g for 10 min. The supernatants were analyzed by HPLC.

(ii) <u>Extraction of liver, kidney, muscle and fat</u>

Tissues (about 1 g) were weighed and homogenized in 5 ml McIlvaine/0.1 M

disodium EDTA buffer with a Polytron homogenizer (Brinkman Co., Rexdale, ON). An

additional 5 ml of buffer was used to wash the homogenizer blades and then added to the

homogenate. The samples were shaken for 10 min, centrifuged at 1500 g for 15 min and

the supernatants were collected. The pellets were washed with another 10 ml of buffer,

shaken and centrifuged as above. The supernatants from the two separate extractions

were pooled and passed through a glass funnel containing a Whatmann glass microfibre

filter (GF/A 90 mm). The filtrates were then passed through the Bond Elut C-18 columns

that were pre-conditioned with 10 ml methanol and 10 ml distilled water in a Baker-10

SPE system (J.T. Baker Inc., Phillipsburg, N.J.). The funnel/filter/tube assemblies were

washed with 10 ml of buffer followed by two 10 ml washes of water and the wash

solutions were also passed through the columns. The columns were allowed to run dry.

OTC was eluted from the columns with 8 ml of methanol into graduated receiving tubes.

The eluates were evaporated to volumes of less than 1 ml in a RC 10-22 Jouan

Concentrator Evaporator. After evaporation, the elutes were made up to 1 ml with

methanol, vortexed and centrifuged at 1500 g for 10 min. Aliquots of the solution were

analysed by HPLC.

(iii) <u>Extraction of injection site tissues</u>

Extraction of OTC from the injection site was performed similar to section (ii). If

the OTC concentration in the eluate was slightly outside the range of the calibration

standards, an aliquot of the Baker SPE system eluate would be analysed directly by

HPLC without volume reduction. In addition to the regular OTC concentration standards

in the calibration curve, a 20 µg/ml and a 50 µg/ml standards were added to the

calibration curve If the OTC concentration in the eluate was too high or too low, the

eluate would be diluted either with methanol or concentrated by evaporation as required.

**HPLC Analysis**

A 50µl aliquot of the elute was injected directly into a HP 1090 HPLC (Hewlett

Packard Co. Vandal, PA) equipped with a diode-array UV detector set at a wavelength of

355 nm. The HPLC column was a 250 x 4.6 mm i.d. 7 µm Zorbax ODS C18 column

(Phenomenex, Torance, CA). The guard column was a 30 x 4.6 i.d. 7 µm Zorbax ODS

C18 column (Phenomenex, Torance, CA). The mobile phase consisted of a solution

prepared from 5 g of diammoniumhydrophosphate, 5 ml diethylamine and 60 ml N, N-

dimethyl formamide in 800 ml of water and 200 ml of acetonitrile. The pH of the solution

was adjusted to 2.5 using orthophosphoric acid. The flow rate of the mobile phase was 1

ml/min (Nordlander et al, 1987).

**In Vitro tissue:blood partion coefficients of OTC**

OTC tissue:blood partition coefficients were determined according to the method

of Jepson et al. (1994). Briefly, lung, liver, kidney, and muscle samples (1 gm) were

minced with a pair of scissors and placed in separately tared, 30-ml disposable glass

scintillation vials. A total of 10 vials were used for each tissue. Either 1 µg/ml or 10

µg/ml of OTC was added to five vials of each tissue and the vials capped. Blood samples

(1 ml) containing 1 µg/ml or 10 µg/ml of OTC were also prepared in tared, 30-ml

disposable glass scintillation vials. Reference solutions containing the chemical solutions

without blood or tissue mince were also prepared. The scintillation vials containing the

tissue and chemical mixture were incubated at 30°C for 24 hr. At the conclusion of the

incubation, the vials were removed and centrifuged at 1500 g for 10 min. The supernatant was filtered with a Millipore low binding cellulose filter unit with a 10,000 nominal molecular weight cutoff (Millipore Ultrafree-PF, Millipore Corp., Bedford, MA). About 2 ml of the supernatant was put into each filter unit, which was pressurized with $N_2$ to 32 psi. The first drop of filtrate was discarded. The remaining filtrate was collected into a small glass vial. About 1 ml of the filtrate was removed from the vial and extracted for OTC as described above. Tissue:saline partition coefficients were calculated by the following equations:

$$P_{t/s} = C_t/C_s = (AMT_t/V_t)/C_s = ((C_r V_r - C_s V_s)/V_t)/C_s$$

$$C_s = (C_{s,f})(C_{r,u}/C_{r,f})$$

Where $C_t$, OTC concentration ($\mu g/g$) in tissue; $C_s$ OTC concentration ($\mu g/g$) in the saline fraction; $AMT_t$, the amount of OTC ($\mu g$) in the tissue; $C_r$, the OTC concentration ($\mu g/g$) in the reference solution; $V_r$, the volume (ml) of the reference solution; $V_s$, the volume (ml) of the sample solution; $V_t$, the volume (ml) of tissue; $C_{s,f}$, the OTC concentration ($\mu g/g$) in the saline filtrate; $C_{r,u}$, the OTC concentration ($\mu g/g$) in the unfiltered reference solution; $C_{r,f}$, the OTC concentration ($\mu g/g$) in the filtrate reference solution. Tissue:blood partition coefficients were determined by dividing the tissue:saline partition coefficients by the blood:saline coefficient.

## II. Fitting of serum OTC concentration-time data to classical pharmacokinetic models

The serum OTC concentration *vs.* time curves from cattle after *i.m.* or *s.c.* administration were fitted by the following two-compartment pharmacokinetic model:

$$Cb = \frac{K_a D}{Vd_1}\left[\left(\frac{K_{21}-\alpha}{(K_a-\alpha)(\beta-\alpha)}\right)e^{-\alpha t} + \left(\frac{K_{21}-\beta}{(K_a-\beta)(\alpha-\beta)}\right)e^{-\beta t} - \left(\frac{K_{21}-K_a}{(\alpha-K_a)(K_a-\beta)}\right)e^{-k_a t}\right]$$

Where Cb represents the concentration in the blood, Vd the volume of distribution, and Ka the first-order rate constants describing the absorption of OTC from the blood. The parameters of the equation were estimated by nonlinear, least-square regression analysis using PCNONLIN (Metzler and Weiner, 1986). The statistical weighting factor of the least-square procedure was the inverse of the observed blood concentration. The overall goodness of fit was determined by comparing the sum of the squared deviations and by scatter of the actual data points around the fitted function. The parameters estimated from the nonlinear regression analysis were used to calculate the secondary parameters such as clearance, elimination half-life and area under the blood curve (AUC).

## III. PBPK model development and validation

## Model Conceptualization

The PBPK model of OTC in the cattle (Figure 2) consists of nine compartments

each representing a major organ or a group of organs. These include the kidney, muscle,

liver, fat, gut, carcass, lung, and blood. The gut compartment represents the highly

perfused organs such as the stomach, intestine and esophagus. The carcass compartment

represents the remaining organs and tissues not identified specifically in the model. All

compartments are connected by the circulatory system with the lung receiving 100% of

the blood flow and the liver receiving blood from the portal vein and the hepatic artery.

The transfer of chemical between the compartments is governed by blood flow rates and

tissue:blood partitioning. Therefore, all compartments in the model are flow-limited

compartments. Each tissue compartment is assumed to be "well-mixed"; OTC

concentration in tissue compartments is assumed to be homogenous. OTC concentration

in the efferent blood is assumed to equal those of the tissue compartment. Elimination of

OTC is modeled by renal and hepatic clearances, which are assumed to be first-order rate

processes.

Figure 2 Schematic representation of the PBPK model for cattle.

For symbols and abbreviations refer to Table 1.
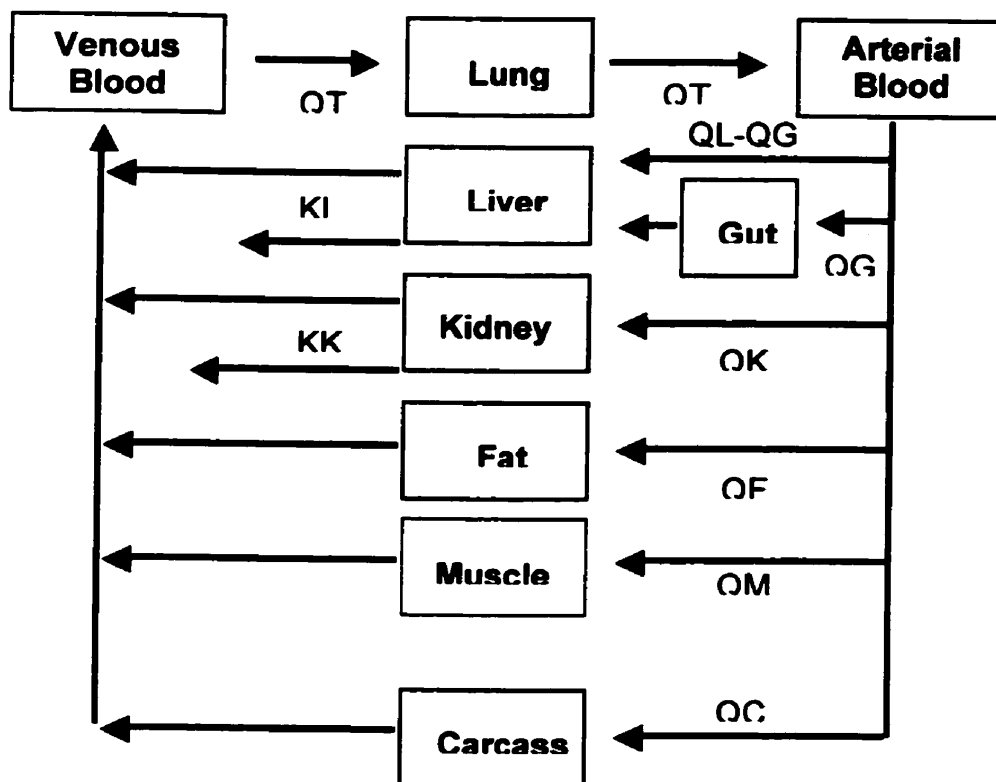
**Model Parameterization**

Table 1 shows the anatomical, physiological, and physico-biochemical parameters used to drive the PBPK model. These parameters were obtained either from the literature or were determined experimentally in our laboratory. A few of the model parameters were optimized by matching model predictions to observed data simultaneously.

Anatomical parameters

Organ volumes for blood, lungs, liver, kidney, muscle and gut were determined by the allometric approach of Mordenti (1986) using the relationship $Y = aW^b$, where Y represents organ size, W represents body weight, and a and b are the scaling coefficient. The blood, lungs, liver, kidney, muscle and gut were assigned volumes equal to 8.00, 0.93, 3.00, 0.26, 45.00 and 8.5% of a 253 kg cattle, respectively. Fat was assigned 20% of the body weight (Ellanberger et al, 1950). The remaining tissues were grouped together as the carcass compartment; it was about 14.31% of the cattle's body weight.

Physiological Parameters

Reported cardiac output (QT) of steer ranged from 36800 l/d – 47232 l/d (Ruckebusch et al, 1991, Hélène and Amory, 1994, and Huntington 1990). A QT of 40,000 l/d was used in the present study after initial model optimization with the experimental data. Blood flows to the gut and the liver were assigned 30% and 35% of QT, respectively (Hungtington et al. 1989, 1989; Whitt et al. 1996). Kidney blood flow was 11.25% of QT (Loeffler, 1986). Muscle blood flow was 45% of QT based on a study in the hindquarter of cattle (Eisemann et al., 1987; Lescoat et al., 1996). No information was available for blood flow in fat. It was assigned a value of 2% QT after optimizing the

Table 1 Input parameters of the OTC PBPK model in beef cattle

| Parameter | Abbreviation | Value |
|---|---|---|
| **Tissue volume % BW** | | |
| Lung | VLN | 0.93 |
| Liver | VL | 3.00 |
| Gut | VGT | 8.50 |
| Kidney | VK | 0.26 |
| Carcass | VC | 14.31 |
| Fat | VF | 20.00 |
| Muscle | VM | 45.00 |
| | | |
| **Blood flow % cardiac output** | | |
| Lung | QLN | 100 |
| Liver | QL | 35 |
| Gut | QGT | 30 |
| Kidney | QK | 11 |
| Carcass | QC | 7 |
| Fat | QF | 2 |
| Muscle | QM | 45 |
| Cardiac output (l/day) | QT | 40000 |
| **Partion coefficients of OTC** | | |
| Lung:blood | RLN | 2.3 |
| Liver:blood | RL | 4.0 |
| Gut:blood | RGT | 1.4 |
| Kidney:blood | RK | 8.0 |
| Carcass:blood | RC | 0.9 |
| Fat:blood | RF | 0.1 |
| Muscle:blood | RM | 0.9 |
| **Biochemical constants** | | |
| Kidney clearance (ml/day/g) | KK | 320 |
| Liver clearance (ml/day/g) | KL | 33 |

model with experimental data. The remaining blood flow (6.75%) was assigned to the carcass.

## Biochemical parameters

Elimination of OTC by the kidney and liver was assumed to occur by first-order kinetics. The elimination of OTC occurs mostly through the kidney and is dependent on the glomerulus filtration rate and urine flow (Nouws et al., 1985). Elimination of OTC from the liver is approximately 10% of kidney clearance; the level of enterohepatic recycling is minimal or nonexistent (*Ibid*).

## Physico-biochemical parameters.

Initial estimates of OTC tissue:blood partition coefficients were obtained either from the *in vitro* tissue binding study conducted at our laboratory or from the *in vivo* OTC tissue distribution study of Landoni and Errecalde (1992). These values were optimized during model development using the empirical OTC tissue concentration data.

## OTC absorption from the injection site.

Absorption of OTC from the injection site was modeled with two different rate equations: The first equation represents the fast uptake of OTC by the blood, which occurs almost immediately after injection. The second equation represents a slower uptake of OTC by the blood after the carrier solvent of the formulation is absorbed from the injection site (Mevius et al., 1986).

## Computer model

The conceptual PBPK model was translated into mass balance differential and algebraic equations, which described the movement of OTC in the cattle.

The computer program consists of two major components:

(i)     *Spreadsheet component*

The spreadsheet contains different "work" areas which have different functions i.e., model control, physiological parameters, dosing parameters, model print times and output, and model fit. Several of these functions are described briefly as follows: (a) model control is responsible for the length of model simulation, the method of solving ordinary differential equations (ODE), and the time step used in solving ODE, (b) the print control area defines if model output is printed repeatedly after a set time unit or at specific times, (c) the physiological parameter area contains the physiological and biochemical parameters, and (d) dosing area contains the amount of OTC injected and the time of injection. Therefore, each spreadsheet component contains the variables needed by the macros to solve the algebraic and mass balance differential equations (MBE). The spreadsheet also receives outputs from the MBE and compares graphically the model outputs with the experimental results. The spreadsheet also controls the macros through the use of "buttons" that are linked to the macros.

(ii)     *Macros component*

Macros consist of small sections of computer code written in Excel® Visual Basic (Microsoft, Redmond, WA) which handle different tasks or algorithms. They are responsible for solving the MBE and transferring the model output of tissue OTC concentration to the spreadsheet and in analyzing the closeness of the model output to the experimental results.

Solving mass balance differential and algebraic equations

The set of MBD equations representing the OTC PBPK model of cattle were solved first using the Euler method (Johanson, 1988) of approximation because of its

simplicity. When the model ran properly, the MBD equations were solved by the midpoint method and the Runge-Kutta method (Flanders, 1984) to improve the speed and accuracy of the results. These techniques were written in Visual Basic for Excel® so that they could be linked to the spreadsheet allowing for easy parameter entry and graphing of results.

Model optimization

The term model optimization is the length of time the PBPK model required to complete a single run. A run is defined as solving the tissue concentration from time zero to some predetermined stop time. Optimization occurs in two steps, making the code more efficient and by increasing the time step while keeping the accuracy the same:

*Step 1.* Improving code efficiency. This was accomplished by reducing all equations to their simplest form by combining and solving the constants so as to leave an equation that contained only variables and combined constants. Therefore, the constants were only solved once at the beginning of a run and not at each individual time step. For example, if both QT and VGL are constants in the differential equation, dCGL = (QT/VGL * (CBV - CGL / RGL)), they can be combined and solved as

$$QTVGL_{[const]} = QT/VGL$$

$$dCGL = (QTVGL_{[const]} * (CBV - CGL / RGL))$$

*Step 2.* Increase the time step. Different techniques have been used to solve the differential equations by increasing the amount of time between determination, time step, but maintaining the same accuracy. The following are some of the approaches used in

this study:

(i) Euler Method

The amount of OTC in a specific tissue at a new time point can be estimated by the amount at the start of the integration interval plus the product of the slope given by the differential equation and the integration step size ($\Delta t$):

New value = old value + (slope x $\Delta t$)
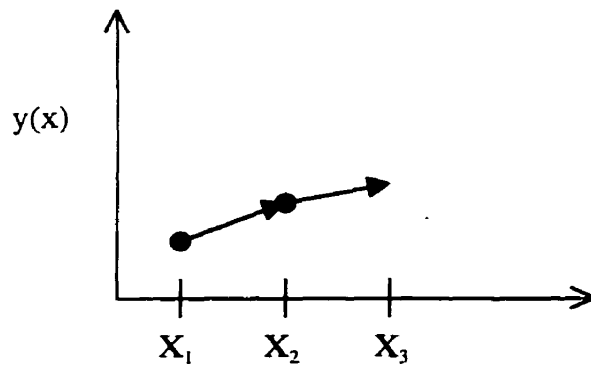


(ii) Midpoint Method

The midpoint method involves using the amount of OTC at the start of the integration interval at each step to find the amount half-way across the integration interval. Then using the midpoint amount or derivative to calculate the amount in the full width of the interval. This method provides the second-order accuracy.
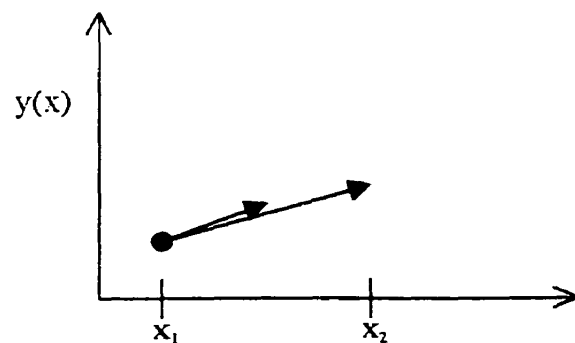
(iii) Runge-Kutta Method

In each step, the derivative is evaluated four times: once at the initial point, twice at the trial midpoints, and once at a trial endpoint. The final function value is calculated from these derivatives. This method provides the fourth-order accuracy.
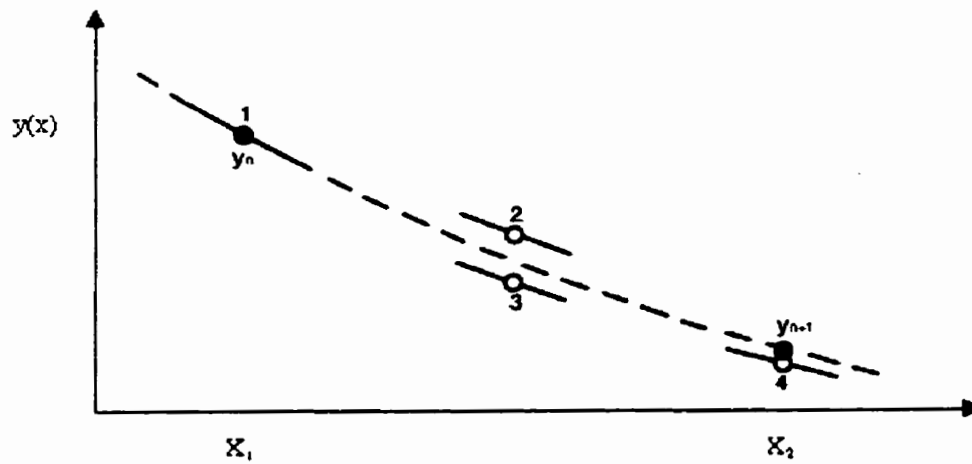
## Model Testing and Validation

### Structural validity

A model is structurally valid if it reproduces real system behavior in a way that can be interpreted as being reflective of the operating characteristics of the real system (Ziegler, 1976). The following procedure was used to test the structure of the model: OTC concentration was set to zero and the model was run. This procedure was used to test the stability of the model and to ensure that there was no OTC in the tissue compartments due to errors in writing equations representing the PBPK model. The model was then run first with a low concentration and then a high concentration to see if the concentrations in the tissue compartments would increase. Next organ volumes were adjusted to confirm that OTC concentration would be reduced from an increase in organ volume and a decrease in organ volume would lead to an increase in OTC concentration. This was followed by adjusting the blood flows to the different organs to confirm that an increase in blood flow to an organ result in an increase in OTC concentration in the tissue. Partition coefficients were also tested in a similar manner to assure an increase or decrease in partition coefficient of a compartment would result in an increase or decrease in OTC concentration in the compartment. Lastly the uptake and elimination rates were adjusted to see if an increase resulted in either a greater uptake or elimination. The hepatic and renal clearances were adjusted separately and in combination to ensure they remained within the physiological ranges.

### Goodness of model fit

Three different techniques were used to examine whether the outputs of the PBPK model described closely the experimental OTC concentrations in the tissues:

*1. Visual inspection.*

It was used to narrow down the model inputs to their approximate ranges. This was accomplished by "eye balling" the closeness/discrepancy between model simulations and experimental data.

*2. Log likelihood.*

Log likelihood is a statistical approach that has been used to compare modeled and experimental results of PBPK models. It is a measure of goodness of fit. The best fit is accomplished by using the log likelihood function.

$$LL = \sum_{i=1}^{N} \left[ -\frac{n_i}{2} \bullet \ln\left( 1 + \frac{(y_i - \hat{y}_i)^2}{S_i^2} \right) \right]$$

Where N is the number of mean experimental points used; $n_i$ and $S^2$ are, respectively, the number of experimental repetitions and the variance (with $n_i$ degrees of freedom) for each data point; $y_i$ is the experimental data point value and y hat$_i$ the corresponding model-predicted value. The loglikelihood is similar to a measure of the sum of squared deviates, weighted by the variance of each experimental data point. The benefit of using log likelihood is that it takes into consideration all experimental data points instead of just the mean of the experimental data points.

*3. Standard Error*

This method is based on different error statistics: mean error, mean percent error, mean square error, mean absolute error, and mean absolute percent error. The first two measure predictive bias and should be close to zero. The other three measure predictive accuracy and should be as small as possible.

PBPK Model Validation

A dataset was used initially to formulate and estimate the PBPK model. When the model

matched the data closely, the model became replicate valid and corresponded to the

econometric notions of goodness of fit and the statistical notions of distributional

similarity (Power, M., 1992). Once this is accomplished, the model had to undergo the

predictive validity test which examined its ability to match another data set different from

that used in model development. In the present study, two different data sets were used

in the predictive validity test: (1) the time course OTC serum concentration following

*i.m.* administration of 20 mg/kg Liquamycin LA-200 [R], (2) the time course of serum

concentration following *i.m.* administration of a 20 mg/kg OTC formulation (Meijer, L.

A. et al, 1993).

## RESULTS

### Chromatographic separation of OTC

Figure 3 shows a typical HPLC elution profile of OTC. The retention times ($R_t$) of OTC and tetracycline were 5.8 min and 6.8 min, respectively. The OTC peak was not detected in the tissue and methanol blanks. The method detection limits (MDL) and extraction recoveries of OTC from various tissues are shown in Table 2. The MDL were equal to or less than 0.1 µg/g. Recoveries of spiked OTC from the tissues were better than 81%. OTC in the tissues was not found to degrade significantly over a 6-month storage period.

### Bioequivalence studies using the classical pharmacokinetic approach

<u>Intramuscular Injection</u>

Tables 3 and 4 summarize the time course of OTC concentrations in the serum of steers after receiving 20 mg/kg Liquamycin LA 200® or 20 mg/kg Alamycin LA 300® *i.m.* Figure 4 and 5 depict the semilogarithmic plot of the experimental data and the "best fit" curves of a two compartment pharmacokinetic model. The serum concentration-time curves displayed a rapid absorption phase and an slower elimination phase with peak OTC serum concentration occurring at around 8 to 12 hr post-dosing. The terminal elimination half-lives of Liquamycin LA 200® and Alamycin LA 300® formulations were 25.98 h and 31.38 h, respectively (Table 5).

The serum OTC concentration *vs.* time curve of Liquamycin LA-200® was superimposed on that of Alamycin LA-300® (Fig. 6). Although serum OTC concentrations appeared to rise at a similar rate for both formulations, Liquamycin LA-200® attained a higher maximum concentration and peaked at a later time than Alamycin

Figure 3 A typical HPLC elution profile of OTC and Tetracycline (TC - internal

standard) from a muscle tissue extract. A) blank, B) muscle, peaks I and II represent OTC

and TC respectively.
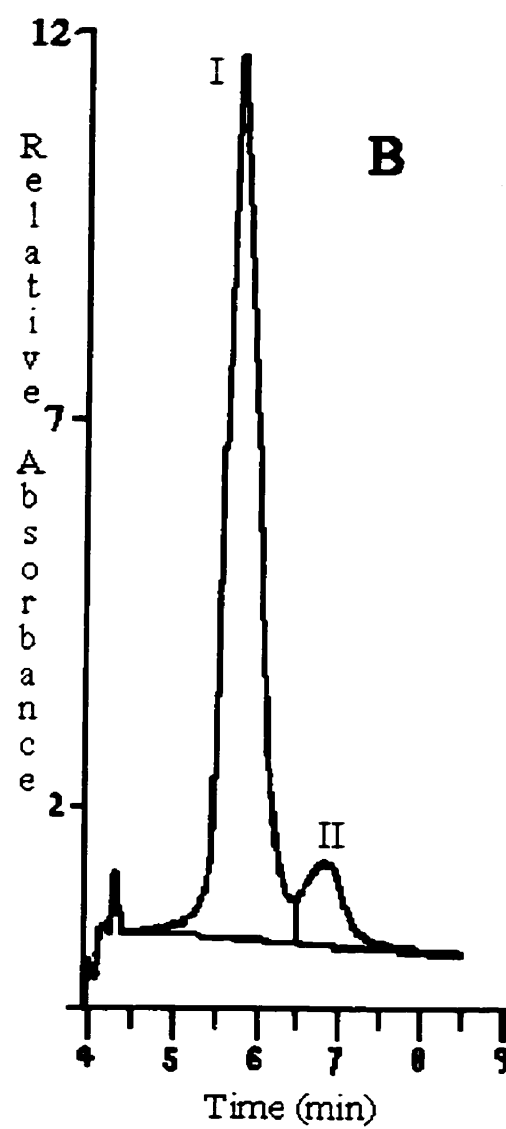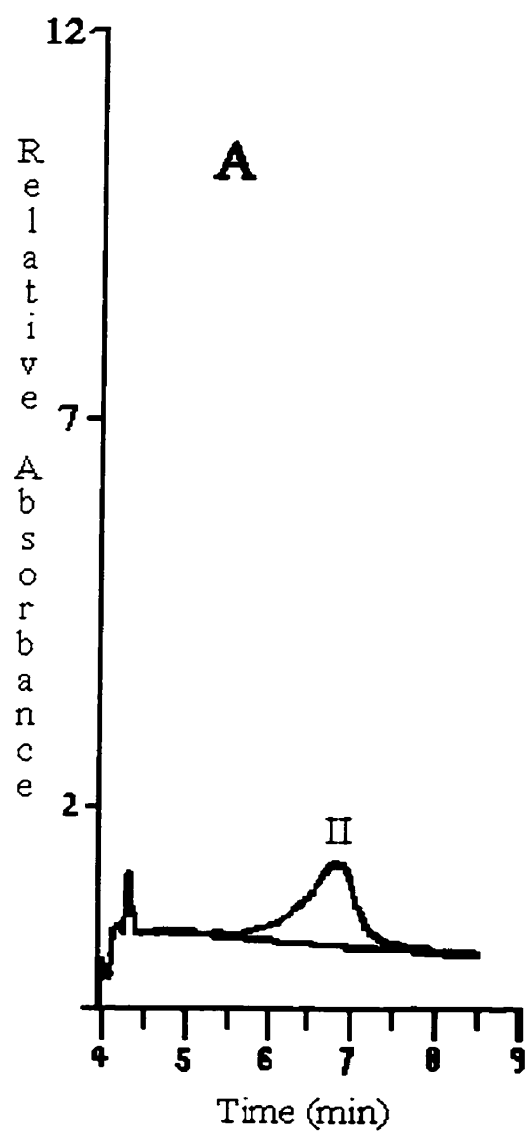
Table 2 Extraction recoveries and method detection limits of beef cattle tissue

| Tissue | % Recovery | MDL |
|--------|-----------|-----|
| | | Concentration (µg/g) |
| Serum | 98 ± 15% | 0.0910 |
| Liver | 85 ± 8% | 0.0647 |
| Kidney | 81 ±3% | 0.1004 |
| Muscle* | 95 ± 8% | 0.0837 |
| Fat | 94 ± 7% | 0.0973 |

*The muscle MDL was also used for the injection sites.

Table 3 Time course of OTC concentration in the serum (µg/ml) of steers following *i.m.* injection of 20 mg/kg Liquamycin LA-200®

| Steer ID | Post-Dosing Time (hr) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 4 | 8 | 12 | 24 | 48 | 72 | 96 | 120 |
| 51 | 2.3434 | 2.5607 | 3.4304 | 4.2822 | 5.2528 | 5.4103 | 4.5832 | 2.2131 | 1.1140 | 0.6467 | 0.4409 |
| 52 | 2.5148 | 5.2294 | 6.3538 | 6.9005 | 6.9713 | 7.3420 | 5.3160 | 1.9741 | 0.9070 | 0.4116 | 0.3540 |
| 53 | 3.2641 | 4.6170 | 4.0120 | 5.3434 | 5.3404 | 5.1037 | 3.4064 | 1.5129 | 0.7468 | 0.4375 | 0.3201 |
| 54 | 2.5723 | 2.7167 | 4.2446 | 4.5240 | 4.7389 | 4.7142 | 3.7461 | 1.5508 | 0.7768 | 0.5651 | 0.6988 |
| 55 | 2.5520 | 3.9233 | 6.5096 | 5.5372 | 5.5559 | 5.3140 | 3.8047 | 1.0156 | 0.6238 | 0.3003 | 0.2794 |
| 56 | 4.1247 | 6.3944 | 6.3192 | 5.0200 | 4.6441 | 4.5481 | 3.7687 | 1.9058 | 1.1841 | 0.5193 | 0.7420 |
| Mean | 2.8952 | 4.2402 | 5.1449 | 5.2679 | 5.4172 | 5.4054 | 4.1042 | 1.6954 | 0.8921 | 0.4801 | 0.4725 |
| SD | 0.6810 | 1.4835 | 1.3955 | 0.9306 | 0.8397 | 1.0062 | 0.7093 | 0.4257 | 0.2196 | 0.1228 | 0.1997 |

Table 4 Time course of OTC concentration in the serum (μg/ml) of steers following *i.m.* injection of 20 mg/kg Alamycin LA-300[®]

| Steer ID | Post-Dosing Time (hr) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0.5 | 1 | 2 | 4 | 8 | 12 | 24 | 48 | 72 | 96 | 120 |
| 51 | 2.2137 | 3.3111 | 4.8890 | 4.9428 | 5.2100 | 4.0428 | 3.4692 | 2.1660 | 1.0807 | 0.6430 | 0.3970 |
| 52 | 3.1732 | 4.3795 | 6.2023 | 3.2440 | 4.6038 | 4.4105 | 3.2789 | 1.7601 | 0.9026 | 0.5316 | 0.3804 |
| 53 | 1.4268 | 3.1106 | 3.5754 | 4.9289 | 4.6164 | 4.4502 | 3.2809 | 2.0755 | 0.9839 | 0.6128 | 0.4747 |
| 54 | 1.5768 | 2.3599 | 3.4547 | 3.8562 | 4.2870 | 3.9069 | 3.0716 | 1.2832 | 0.8691 | 0.4614 | 0.6395 |
| 55 | 2.8297 | 6.1649 | 5.9570 | 6.0063 | 5.3640 | 4.5737 | 3.7013 | 1.7174 | 1.0167 | 0.5898 | 0.4743 |
| 56 | 4.5858 | 5.1188 | 5.9205 | 5.9909 | 6.3686 | 5.6195 | 4.0915 | 1.7267 | 1.0392 | 1.1260 | 0.5380 |
| Mean | 2.6343 | 4.0742 | 4.9998 | 4.8282 | 5.0750 | 4.5006 | 3.4822 | 1.7882 | 0.9820 | 0.6608 | 0.4840 |
| SD | 1.1739 | 1.4146 | 1.2360 | 1.1151 | 0.7521 | 0.6050 | 0.3660 | 0.3130 | 0.0816 | 0.2369 | 0.0955 |

Figure 4 Time course of serum OTC concentration in steers after *i.m.* injection of

Liquamycin LA 200®

Each point represents the mean serum OTC concentration of six steers. The bars

represent standard deviation of the experimental data. The solid line represents the "best

fitted" curve of a two-compartment pharmacokinetic model
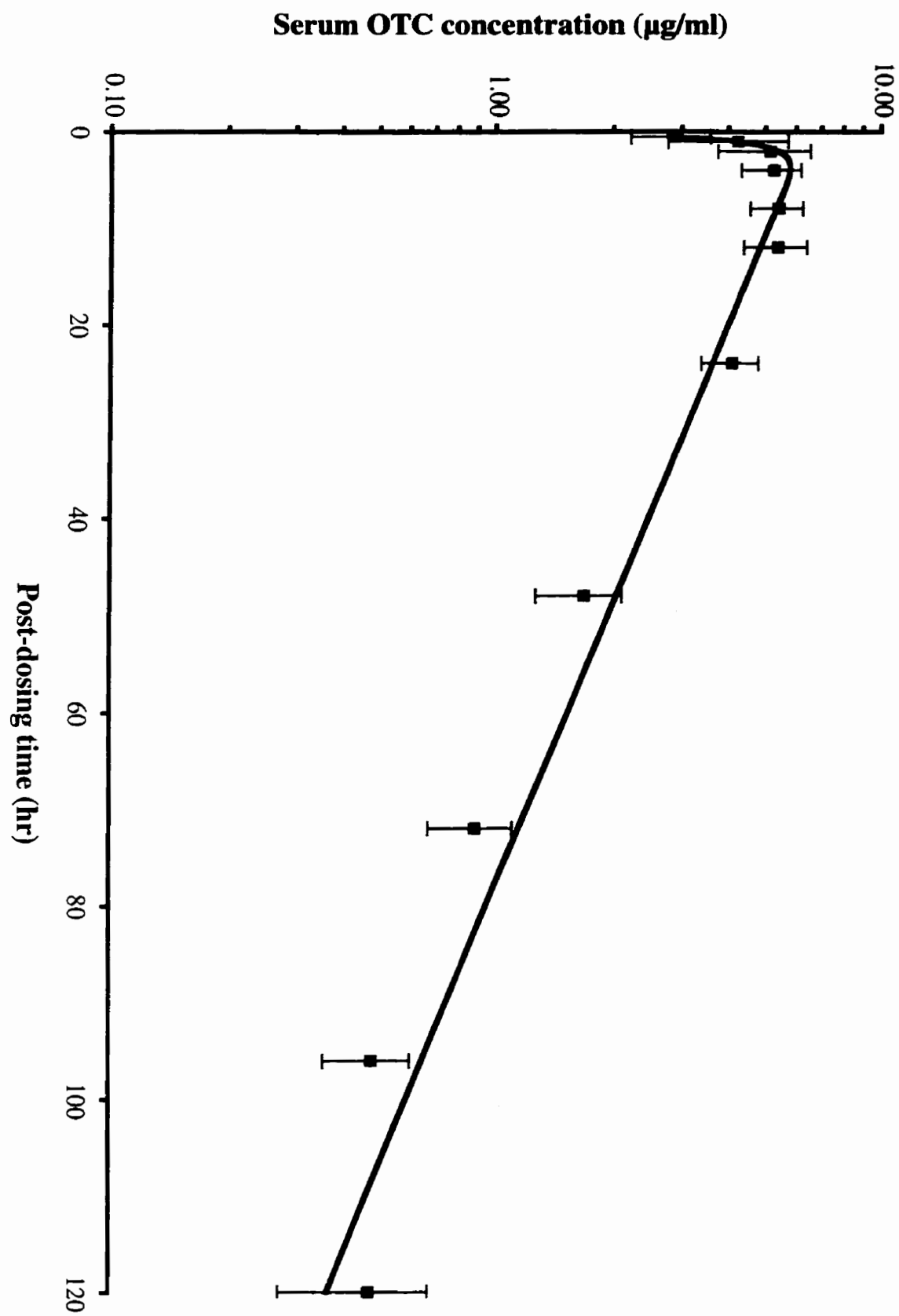
Figure 5 Time course of serum OTC concentration in steers after *i.m.* injection of

Alamycin LA-300®

Each point represents the mean serum OTC concentration of six steers. The bars

represent standard deviation of the experimental data. The solid line represents the "best-

fitted" curve of a two-compartment pharmacokinetic model
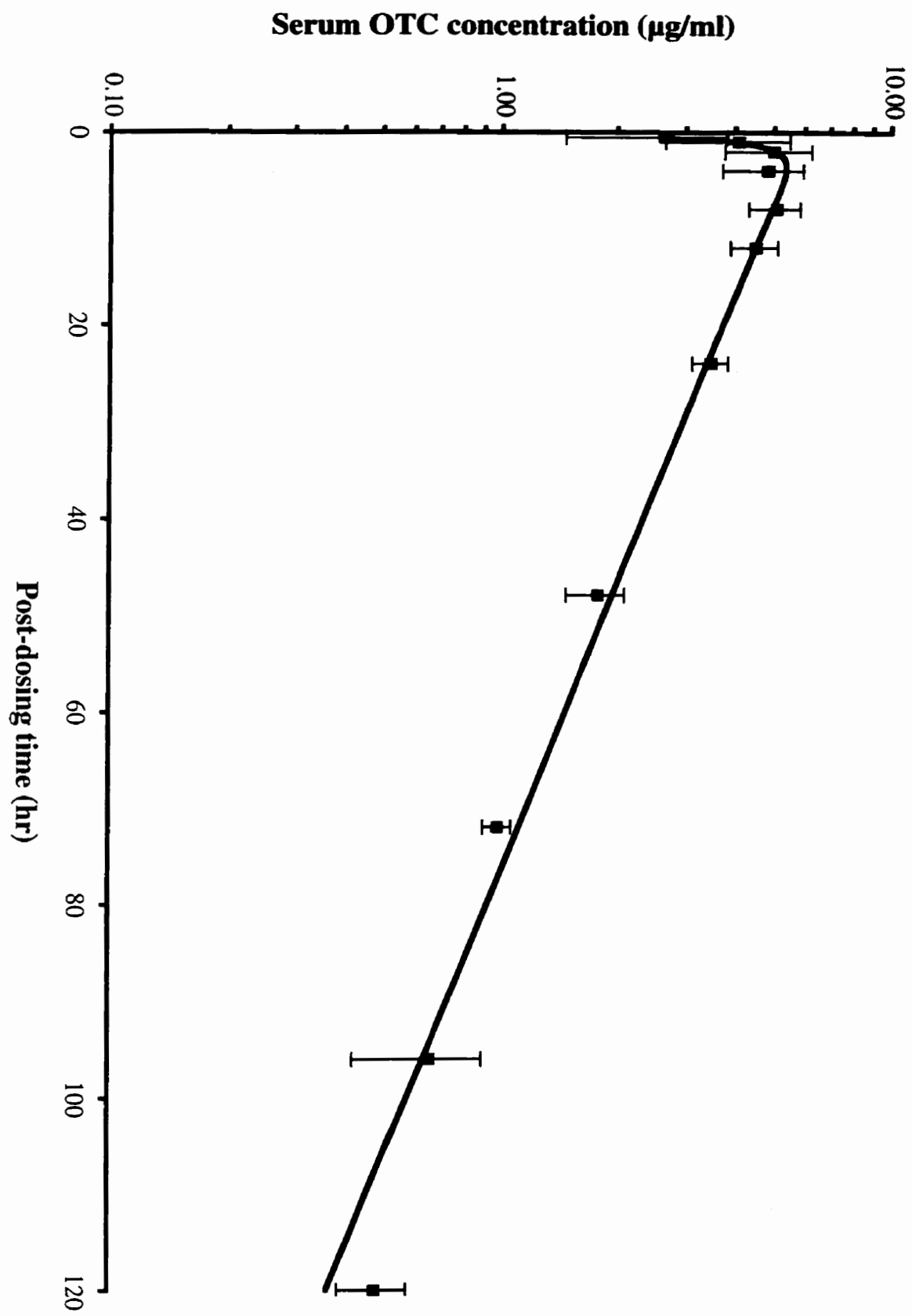
Table 5 Pharmacokinetic parameters derived from the serum OTC concentration *vs.* time curves of steers injected *i.m.* with Liquamycin LA-200® or Alamycin LA-300®

| Derived parameters | Liquamycin LA-200® | Alamycin LA-300® |
|---|---|---|
| A ($\mu$g/ml) | 7.34 | 5.96 |
| B ($\mu$g/ml) | 1.70E-04 | 4.18E-02 |
| Alpha (hr$^{-1}$) | 2.67E-02 | 2.33E-02 |
| Beta (hr$^{-1}$) | 4.62E-04 | 1.36E-03 |
| Alpha-HL (hr) | 25.93 | 29.75 |
| Beta-HL(hr) | 1501.94 | 509.29 |
| $K_{01}$ (hr-1) | 0.85 | 1.09 |
| $K_{10}$ (hr-1) | 2.67E-02 | 2.21E-02 |
| $K_{12}$ (hr-1) | 5.08E-05 | 4.03E-03 |
| $K_{21}$ (hr-1) | 4.62E-04 | 4.65E-02 |
| AUC $_{120hr}$ ($\mu$g·hr/ml) | 256.73 | 239.74 |
| $K_{10}$-HL (hr) | 25.98 | 31.38 |
| $K_{01}$-HL(hr) | 0.82 | 0.63 |
| Tmax (hr) | 4.91 | 3.48 |
| Cmax ($\mu$g/ml) | 6.20 | 5.52 |

Figure 6 Superimposition of Liquamycin LA-200® and Alamycin LA-300® serum OTC

concentration *vs.* time curves

Dashed line represents the two-compartment pharmacokinetic model simulation of

Liquamycin LA-200®. Solid line represents the two-compartment pharmacokinetic model

simulation of Alamycin LA-200®.
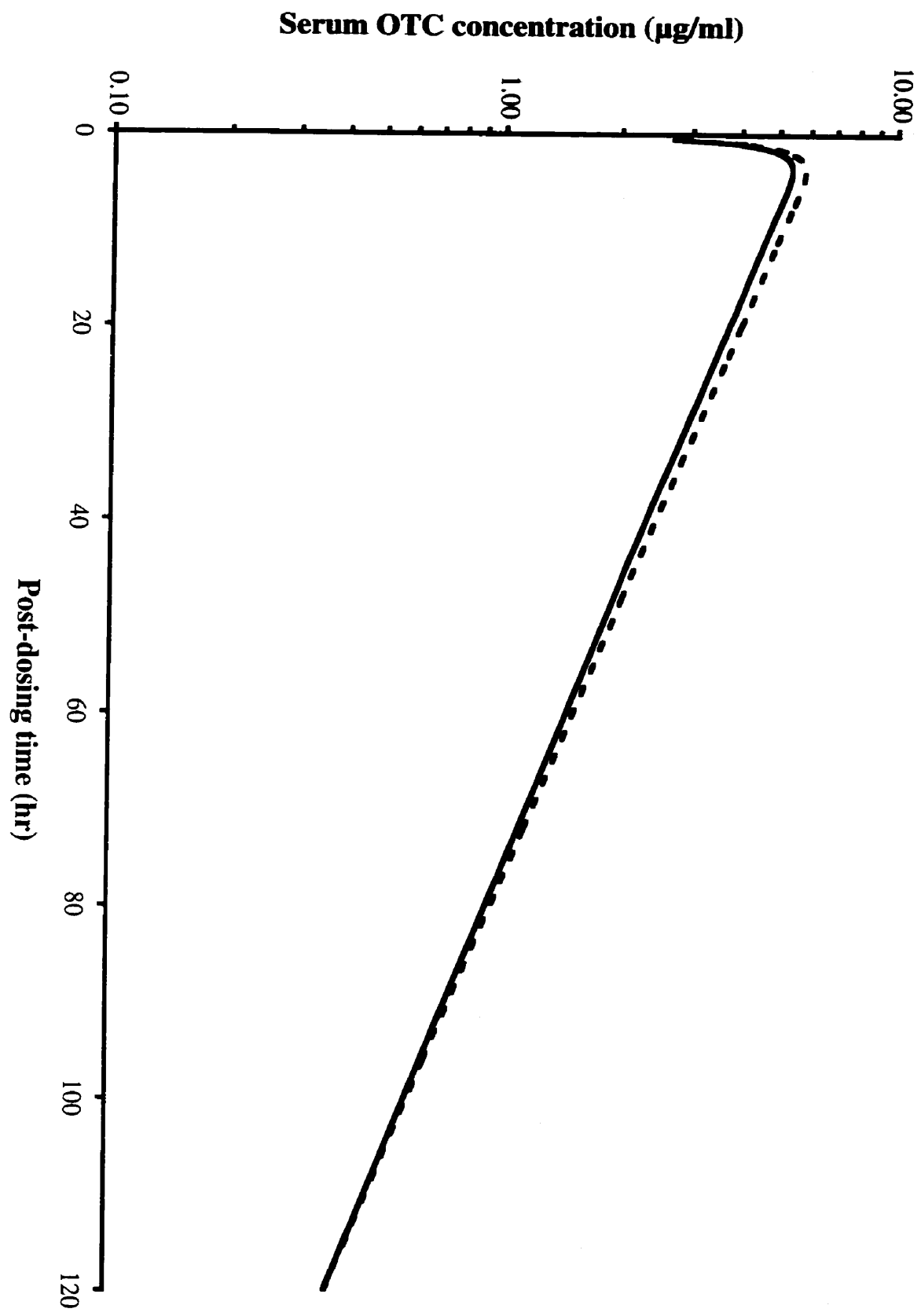
LA–300. The area under the curve (AUC) of these formulations were not found to be significantly different (p >0.05) (Table 5) indicating the amount of OTC absorbed by the steers are similar and indicate that they are bioequivalent.

Subcutaneous Injection

Tables 6 and 7 summarize the time course of OTC concentrations in the serum of steers after receiving 20 mg/kg of Liquamycin LA 200® and Biomycin LA 200® s.c., respectively. Figure 7 and 8 depict the semilog plot of the data and the "best-fit" curve of the two compartment pharmacokinetic model. Both serum concentration-time curves displayed a rapid absorption phase and a slower elimination phase with the peak OTC serum concentration occurring at around 8 to 12 h post-dosing. The terminal elimination half-lives of Liquamycin LA 200® and Biomycin LA- 200® were 60.12 h and 19.65 h, respectively (Table 8).

Liquamycin LA-200® data were superimposed on those of Biomycin LA-200 ® (Figure 9). As shown by the initial uptake phases, Biomycin LA-200® was absorbed by the steers at a more rapid rate than Liquamycin LA-200® was. Although it attained a much higher maximum concentration, it showed a slightly faster terminal elimination rate than Liquamycin LA-200®. Also, peak OTC concentration in the serum occurred at an earlier time for Biomycin LA-200® than Liquamycin LA-200®. However, the AUC blood concentration vs time curves of these formulations were not found to be significantly (p<0.05) different indicating similar amounts of OTC absorbed by the steers.

OTC concentration in the blood of steers and heifers were not found to be significantly different (P<0.05) following the administration of different OTC formulations.

Table 6 Time course of OTC concentration in the serum (µg/ml) of cattle following *s.c.* injection of 20 mg/kg Liquamycin LA-200®

| Calf ID | 0 | 0.5 | 1 | 2 | 4 | 6 | 8 | 10 | 16 | 24 | 36 | 48 | 60 | 72 | 96 | 120 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 532 | 0 | 2.4625 | 3.7673 | 4.9988 | 4.7606 | 8.2034 | 7.1972 | 6.0380 | 4.2179 | 3.1924 | 1.6013 | 1.3158 | 0.5628 | 0.3999 | 0.2911 | 0.5170 |
| 535 | 0 | 1.5421 | 2.7649 | 5.1303 | 6.6997 | 6.5883 | 6.7481 | 5.2655 | 3.6655 | 2.9900 | 1.7242 | 1.1335 | 0.5506 | 0.3962 | 0.3979 | 0.4331 |
| 536 | 0 | 1.1897 | 2.3387 | 4.6140 | 5.7792 | 5.1979 | 5.5074 | 5.2737 | 6.0709 | 4.6301 | 2.2402 | 1.2298 | 0.7675 | 0.4420 | 0.2030 | 0.4028 |
| 537 | 0 | 0.9600 | 1.0782 | 2.1654 | 4.3226 | 5.1396 | 5.3558 | 5.8402 | 4.9928 | 3.7986 | 2.4715 | 1.9298 | 1.2130 | 0.8836 | 0.6664 | 0.5705 |
| 543 | 0 | 0.9740 | 2.0253 | 2.7806 | 4.5747 | 7.2933 | 5.4771 | 5.1858 | 7.1052 | 6.2491 | 2.4501 | 1.7514 | 1.0060 | 0.7156 | 0.6067 | 0.5198 |
| 544 | 0 | 1.0684 | 1.4362 | 2.9144 | 5.5951 | 4.5425 | 5.2250 | 4.4370 | 4.6367 | 3.2823 | 2.2139 | 1.3621 | 1.3425 | 0.8316 | 0.6943 | 0.4792 |
| 547 | 0 | 2.0274 | 3.8952 | 4.4473 | 8.6273 | 9.2746 | 8.2769 | 6.6084 | 4.9329 | 4.2520 | 1.8312 | 0.9294 | 0.5479 | 0.3820 | 0.3337 | 0.5291 |
| 550 | 0 | 1.0201 | 2.5404 | 4.3113 | 8.1338 | 7.2470 | 7.4041 | 5.9332 | 3.7680 | 4.7104 | 2.2589 | 1.1656 | 0.6187 | 0.4803 | 0.5055 | 0.4991 |
| 554 | 0 | 1.2267 | 2.6164 | 3.9323 | 7.1766 | 6.9057 | 6.7452 | 5.5113 | 3.4650 | 4.1403 | 1.9356 | 1.1577 | 0.5992 | 0.5102 | 0.4764 | 0.5292 |
| 556 | 0 | 2.4760 | 3.5881 | 3.8595 | 4.7146 | 6.8412 | 5.0516 | 6.2060 | 5.1008 | 4.4454 | 1.8562 | 1.1060 | 0.7095 | 0.5976 | 0.7962 | 0.4271 |
| 561 | 0 | 1.0633 | 2.7179 | 5.2582 | 5.5498 | 4.8730 | 6.5187 | 5.5915 | 6.3018 | 3.6035 | 2.3355 | 1.1311 | 0.7292 | 0.4826 | 0.4987 | 0.4336 |
| 573 | 0 | 0.9524 | 1.6680 | 2.7939 | 6.0283 | 6.1833 | 6.0434 | 6.4096 | 4.7858 | 3.5414 | 2.4118 | 1.4782 | 1.0052 | 0.6603 | 0.5304 | 0.5496 |
| 579 | 0 | 1.4488 | 2.5138 | 3.1274 | 6.3610 | 5.9650 | 4.8250 | 4.8720 | 3.8390 | 3.3024 | 1.4721 | 0.9817 | 0.5585 | 0.7308 | 0.3685 | 0.3270 |
| 580 | 0 | 1.3474 | 2.3026 | 5.2344 | 5.8894 | 5.6964 | 5.9262 | 3.4845 | 5.3079 | 4.2150 | 2.1787 | 1.5889 | 0.7721 | 0.6287 | 0.5573 | 0.4438 |
| 582 | 0 | 1.3531 | 2.0552 | 3.6201 | 7.9915 | 8.9755 | 7.1880 | 6.4508 | 4.3415 | 4.3904 | 2.4167 | 1.3652 | 0.8134 | 0.8348 | 0.6336 | 0.4474 |
| 585 | 0 | 1.5715 | 3.1117 | 8.1188 | 6.2574 | 7.9142 | 6.5700 | 6.1810 | 6.9357 | 4.7167 | 3.4893 | 1.6623 | 0.8488 | 0.5498 | 0.3491 | 0.3781 |
| 601 | 0 | NA | 3.4370 | 4.9434 | 6.9457 | 6.1859 | 5.5197 | 5.0950 | 5.2495 | 4.4559 | 2.5725 | 1.2135 | 0.6866 | 0.7851 | 0.4696 | 0.4262 |
| 606 | 0 | 1.6496 | 2.3036 | 3.6468 | 6.3699 | 5.8659 | 5.4493 | 4.5963 | 4.6994 | 3.6852 | 2.0303 | 1.3538 | 0.8592 | 1.0140 | 0.6139 | 0.5941 |
| 610 | 0 | 0.8523 | 1.7939 | 2.0339 | 2.9640 | 3.5202 | 3.6059 | 7.4972 | 3.9019 | 2.6922 | 2.7645 | 1.8855 | 1.1659 | 0.8285 | 0.6243 | 0.5846 |
| 612 | 0 | 1.2478 | 1.8783 | 4.8591 | 4.8567 | 9.2521 | 8.3868 | 6.6144 | 5.9693 | 5.6029 | 3.5635 | 1.6512 | 1.1639 | 0.8489 | 0.4461 | 0.5963 |
| Mean | 0.0 | 1.3912 | 2.4916 | 4.1395 | 5.9799 | 6.5833 | 6.1511 | 5.6546 | 4.9644 | 4.0948 | 2.2909 | 1.3696 | 0.8260 | 0.6501 | 0.5031 | 0.4844 |
| SD | 0.0 | 0.4795 | 0.7738 | 1.3892 | 1.4049 | 1.5907 | 1.1929 | 0.9191 | 1.0669 | 0.8719 | 0.5397 | 0.2912 | 0.2460 | 0.1901 | 0.1503 | 0.0762 |

Table 7. Time course of OTC concentration in the serum (µg/ml) of cattle following *s.c.* injection of 20 mg/kg Biomycin LA-200®

| Calf ID | 0 | 0.5 | 1 | 2 | 4 | 6 | 8 | 10 | 16 | 24 | 36 | 48 | 60 | 72 | 96 | 120 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *Postdosing time (h)* | | | | | | | |
| 532 | 0 | 1.9575 | 5.0259 | 7.4326 | 7.1613 | 4.9872 | 6.0715 | 4.6203 | 4.2281 | 3.4452 | 1.8692 | 1.5411 | 0.8977 | 0.7341 | 0.4470 | 0.4026 |
| 535 | 0 | 1.3652 | 5.4790 | 6.0661 | 10.5345 | 5.8002 | 6.2687 | 5.7215 | 4.9813 | 4.7060 | 2.2761 | 1.7655 | 1.1935 | 0.6976 | 0.5021 | 0.2406 |
| 536 | 0 | 4.1197 | 9.1417 | 7.9165 | 7.3444 | 6.0643 | 5.6192 | 5.2755 | 3.9836 | 4.6719 | 1.6557 | 0.8722 | 0.5570 | 0.4104 | 0.3873 | 0.5091 |
| 537 | 0 | 4.3326 | 6.9839 | 6.8501 | 12.1928 | 7.7122 | 7.5842 | 5.5341 | 4.7294 | 3.6377 | 2.0949 | 0.9862 | 0.8446 | 0.5637 | 0.5737 | 0.4850 |
| 543 | 0 | 2.5094 | 4.2280 | 7.2085 | 7.5101 | 6.0971 | 5.7007 | 5.1049 | 4.8752 | 3.5533 | 1.8723 | 1.2291 | 0.7084 | 0.6013 | 0.5440 | 0.5692 |
| 544 | 0 | 3.4041 | 6.5721 | 7.0851 | 9.1341 | 7.5656 | 6.8755 | 6.0907 | 5.7653 | 3.1302 | 1.4486 | 1.0110 | 0.5137 | 0.4034 | 0.2658 | 0.4654 |
| 547 | 0 | 3.7487 | 6.4649 | 7.4760 | 7.5282 | 7.3116 | 5.9563 | 6.7926 | 4.7989 | 5.3036 | 2.5608 | 1.3578 | 1.0749 | 0.6692 | 0.4952 | 0.5033 |
| 550 | 0 | 3.5291 | 7.9174 | 12.4231 | 9.0460 | 8.2251 | 8.7180 | 7.0145 | 7.0563 | 4.6018 | 2.3360 | 1.4693 | 1.1240 | 0.7528 | 0.4276 | 0.4645 |
| 554 | 0 | 1.8414 | 4.6367 | 7.0885 | 6.0778 | 5.0115 | 5.9873 | 4.7510 | 4.2752 | 3.7546 | 2.4255 | 1.4793 | 1.5637 | 0.8045 | 0.5322 | 0.5067 |
| 556 | 0 | 2.6509 | 6.5565 | 11.7131 | 7.7506 | 7.5171 | 6.0001 | 6.0853 | 5.2113 | 3.9892 | 2.6701 | 1.2867 | 1.0396 | 0.7857 | 0.5480 | 0.4359 |
| 561 | 0 | 3.3767 | 8.4168 | 6.4974 | 7.9625 | 8.1908 | 6.0589 | 5.1878 | 3.6649 | 3.4920 | 1.7922 | 1.1685 | 0.5629 | 0.3760 | 0.4571 | 0.3537 |
| 573 | 0 | 2.8262 | 4.7699 | 5.7334 | 6.8467 | 5.7354 | 4.5788 | 4.9356 | 4.2001 | 3.7839 | 1.5766 | 1.1971 | 0.7265 | 0.6089 | 0.4758 | 0.4435 |
| 579 | 0 | 2.5487 | 4.5005 | 6.1158 | 6.4934 | 6.5348 | 4.9896 | 6.5722 | 4.3380 | 2.5764 | 2.3406 | 1.1835 | 0.6725 | 0.6042 | 0.2809 | 0.4738 |
| 580 | 0 | 4.7149 | 8.1070 | 6.7934 | 9.7074 | 9.4397 | 5.9465 | 5.5102 | 5.0386 | 3.1306 | 1.4970 | 0.7618 | 0.3985 | 0.3538 | 0.3242 | 0.3515 |
| 582 | 0 | 3.3068 | 6.1720 | 6.8521 | 6.4147 | 7.3719 | 6.2180 | 11.2964 | 5.2612 | 3.3158 | 2.0630 | 1.3715 | 0.8902 | 0.5571 | 0.4081 | 0.4283 |
| 585 | 0 | 3.3081 | 5.9012 | 7.7417 | 10.2947 | 7.3273 | 8.3080 | 5.7756 | 3.9063 | 5.4632 | 2.2234 | 1.1636 | 0.8653 | 0.6392 | 0.5225 | 0.3722 |
| 601 | 0 | 2.1747 | 3.3111 | 8.4295 | 6.1504 | 6.2017 | 5.9499 | 6.0193 | 5.2171 | 3.5096 | 4.4101 | 2.1058 | 1.1764 | 0.8595 | 0.6147 | 0.4932 |
| 606 | 0 | 1.6393 | 4.8468 | 9.5508 | 7.2523 | 6.5786 | 6.5500 | 7.8856 | 3.2108 | 2.9313 | 2.8536 | 1.8307 | 1.5406 | 0.9804 | 0.7224 | 0.5312 |
| 610 | 0 | 5.5197 | 7.6083 | 10.5058 | 9.6356 | 8.0097 | 6.3339 | 4.2217 | 4.6704 | 2.9061 | 1.3693 | 1.0279 | 0.5616 | 0.4746 | 0.3988 | 0.3679 |
| 612 | 0 | 2.6423 | 5.2784 | 6.8124 | 10.7742 | 6.8355 | 6.1824 | 4.5882 | 4.9255 | 3.3772 | 2.0634 | 1.2199 | 0.9580 | 0.6693 | 0.6741 | 0.3886 |
| Mean | 0.00 | 3.0758 | 6.0959 | 7.8146 | 8.2906 | 6.9259 | 6.2949 | 5.9491 | 4.7169 | 3.7640 | 2.1699 | 1.3014 | 0.8935 | 0.6273 | 0.4801 | 0.4393 |
| SD | 0.00 | 1.0735 | 1.5753 | 1.8452 | 1.7576 | 1.1473 | 0.9763 | 1.5539 | 0.8281 | 0.7953 | 0.6730 | 0.3305 | 0.3233 | 0.1686 | 0.1196 | 0.0776 |

Figure 7 Time course of OTC concentration in the serum of cattle after *s.c.* administration

of Biomycin LA-200®

Each point represents the mean serum OTC concentration of twenty cattle. The bars

represent standard deviation of the experimental data. Solid line represents the "best-

fitted" curve of a two-compartment pharmacokinetic model

**Serum OTC Concentration (µg/ml)**

**Post-dosing time (hr)**
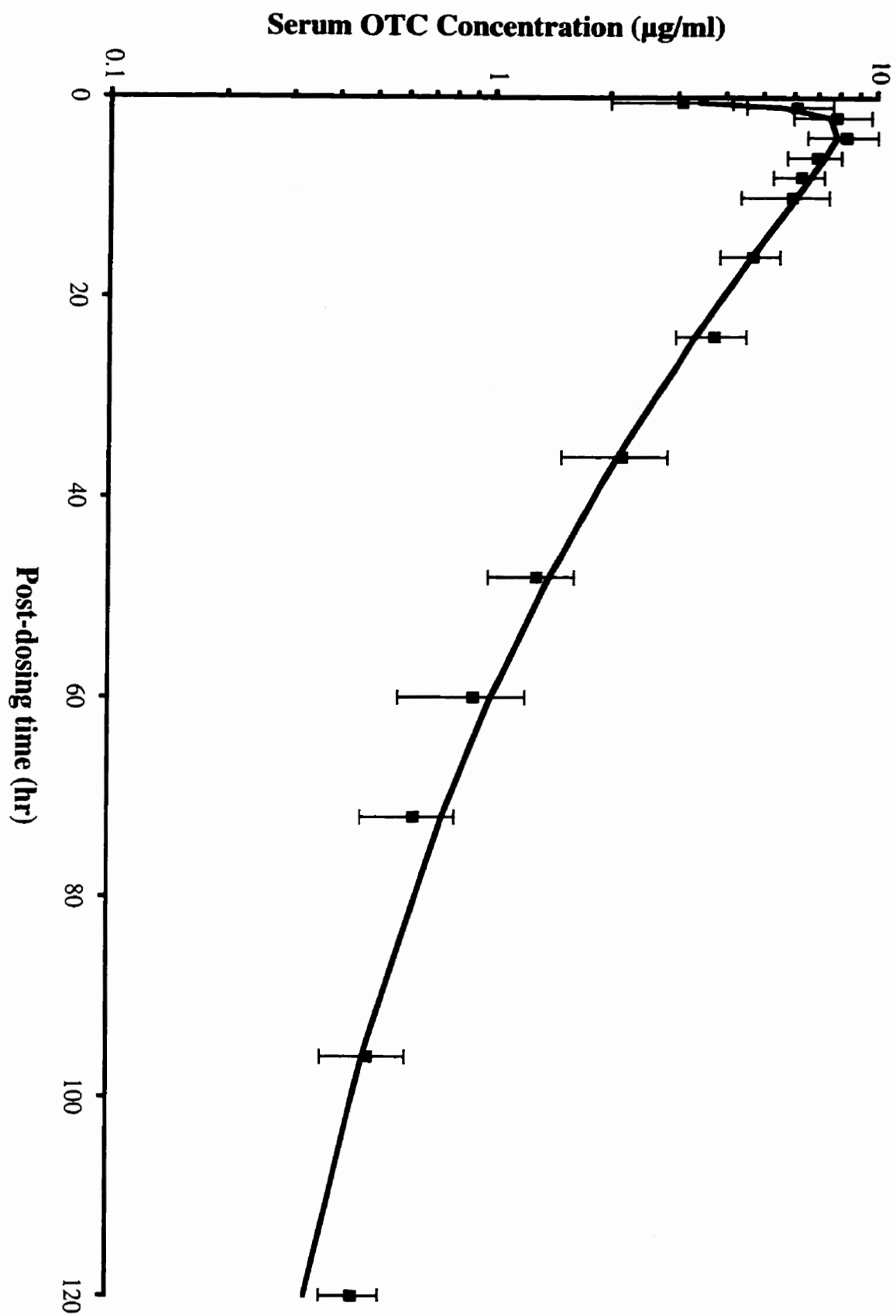
Figure 8 Time course of OTC concentrations in the serum of cattle after *s.c.*

administration of Liquamycin LA-200®

Each point represents the mean serum OTC concentration of twenty cattle. The bars

represent standard deviation of the experimental data. Solid line represents the "best-

fitted" curve of a two-compartment pharmacokinetic model
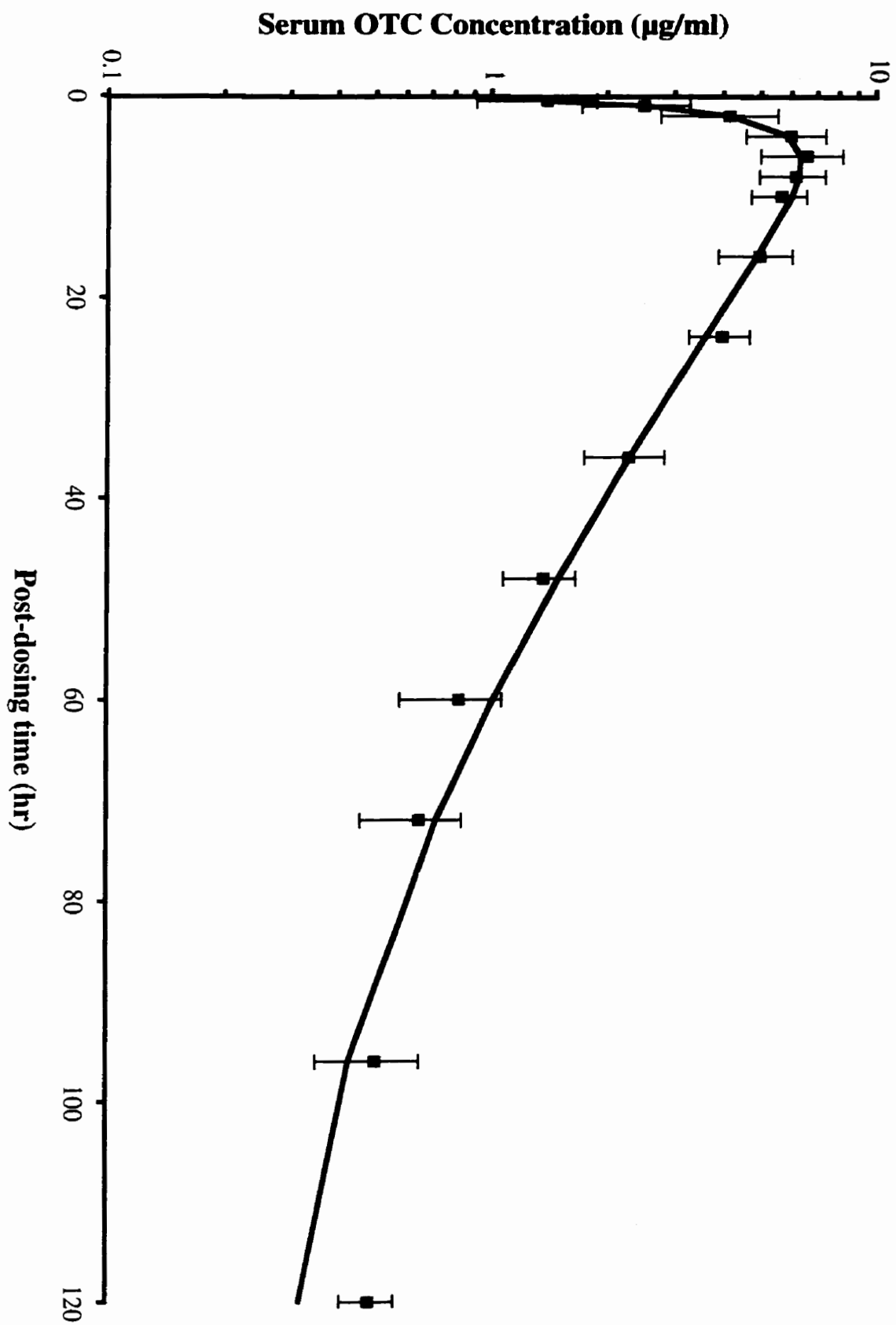
Figure 9 Superimposition of Liquamycin LA-200® and Biomycin LA-200® serum OTC

concentration *vs* time curves

Dashed line represents the two-compartment pharmacokinetic model simulation of

Liquamycin LA-200®; smooth line represents the two-compartment pharmacokinetic

model simulation of Biomycin LA-200®.

**Serum OTC Concentration (µg/ml)**



Post-dosing time (hr)
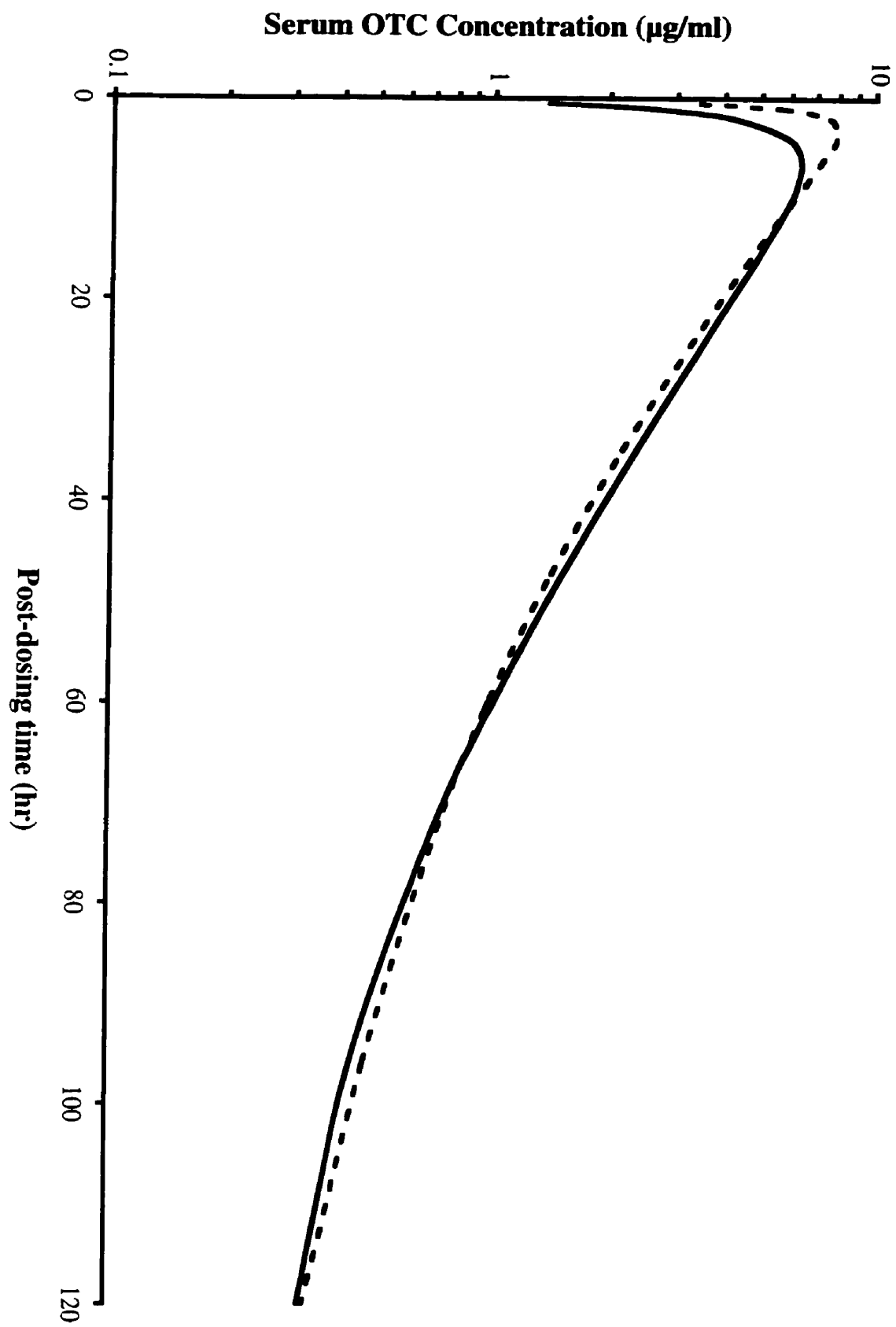
Table 8 Pharmacokinetic parameters derived from the serum OTC concentration *vs.* time
curves of steers injected *s.c.* with Liquamycin LA-200® or Biomycin LA-200®

| Derived parameters | Liquamycin LA-200® | Biomycin LA-200® |
|---|---|---|
| A ($\mu$g/ml) | 8.98 | 8.43 |
| B ($\mu$g/ml) | 0.27 | 1.17 |
| Alpha (hr-1) | 0.04 | 0.05 |
| Beta (hr-1) | 5.20E-04 | 1.00E-02 |
| Alpha-HL (hr) | 16.77 | 13.41 |
| Beta-HL(hr) | 1.33E+03 | 6.18E+01 |
| $K_{01}$ (hr-1) | 0.38 | 1.04 |
| $K_{10}$ (hr-1) | 1.10E-02 | 3.50E-02 |
| $K_{12}$ (hr-1) | 2.84E-02 | 1.10E-02 |
| $K_{21}$ (hr-1) | 1.86E-03 | 1.64E-02 |
| $AUC_{120hr}$ ($\mu$g·hr/ml) | 223.05 | 231.92 |
| $K_{10}$-HL (hr) | 60.12 | 19.65 |
| $K_{01}$-HL(hr) | 1.82 | 0.66 |
| Tmax (hr) | 6.64 | 3.12 |
| Cmax ($\mu$g/ml) | 6.35 | 7.96 |

## PBPK MODEL DEVELOPMENT

### Time course of OTC tissue concentration

Table 9 and figure 10 show the time course of OTC concentration in serum, liver, kidney, muscle and fat. All tissues showed a rapid uptake of OTC within the first few hours of OTC administration. The highest OTC concentration was observed in the kidney at day 4 post-dosing. Moreover, OTC concentration in the kidney was about 2x and 4x higher than that of the liver and the muscle, respectively. In general, OTC concentrations decreased in the order of kidney > liver > muscle >> fat. After reaching peak concentrations, OTC concentrations in the tissues declined rapidly with time. OTC was not detectable in the muscle and fat at day 16 post-dosing.

Figure 11 shows the time course of OTC concentrations at the injection sites. The data was quite variable. At 35 days post-dosing, OTC concentration at the injection sites were below 0.1 µg/g.

Tissue:blood partition coefficients

Table 10 shows the tissue:blood partition coefficients of OTC in cattle tissues. The computer optimized partition coefficients are also included for comparison. *In vitro* tissue:blood partition coefficients represent the mean of two studies each with a different OTC concentration. The tissue:blood partition coefficients decreased in the order of lung > kidney > liver > muscle.

A comparison of PBPK model prediction and empirical results

Figure 10 compare model-predicted OTC tissue concentrations with the empirical data of cattle following *s.c.* administration of 20 mg/kg Liquamycin LA-200®. The goodness of fit indicator, log likelihood (LL) are close to the experimental mean values

(Table 11), with only the muscle being slightly above a mean plus one SD LL value

indicating good fit between model predicted and experimental data. Similarly, the

different error statistics of the model prediction and experimental values are small or very

close to zero (Table 11) Again, these indicate the model-predicted tissue OTC

concentrations agreed quite well with that of the empirical data.

Table 9. Time course of OTC concentration in the tissues of steers following *s.c.* injection of 20 mg/kg Liquamycin LA-200®

| Sampling time and sample number | Liver conc. ((g/g) | Kidney conc. ((g/g) | Muscle conc. ((g/g) | Fat conc. ((g/g) | Inj Site #1 conc. ((g/g) | Inj site #2 conc. ((g/g) | Inj site #3 conc. ((g/g) |
|---|---|---|---|---|---|---|---|
| **4 day** | | | | | | | |
| 650 | 1.6963 | 3.1319 | 0.2430 | 0.0973 | 451.5741 | 353.9032 | 401.3640 |
| 656 | 1.5008 | 3.5227 | 0.2227 | 0.0486 | 1310.0365 | 575.5270 | 250.8495 |
| 672 | 2.2105 | 4.6346 | 0.3716 | 0.0486 | 541.9206 | 500.2981 | 1315.3911 |
| 674 | 1.5322 | 2.8233 | 0.2798 | 0.0486 | 610.6712 | 66.4799 | 109.4260 |
| Mean | 1.7350 | 3.5281 | 0.2793 | 0.0608 | 728.5506 | 374.0521 | 519.2577 |
| SD | 0.3284 | 0.7912 | 0.0659 | 0.0243 | 393.0938 | 224.7494 | 543.9768 |
| **10 day** | | | | | | | |
| 654 | 0.6698 | 1.2781 | 0.1378 | ND | 147.8089 | 289.1882 | 150.1262 |
| 667 | 0.8406 | 2.2202 | 0.1731 | 0.0973 | 414.6722 | 248.4704 | 232.7382 |
| 668 | 0.3069 | 0.6131 | 0.0837 | 0.0486 | 36.4766 | 19.9776 | 19.2135 |
| 678 | 0.1909 | 0.6399 | 0.0837 | ND | 309.7675 | 176.1197 | 19.0548 |
| Mean | 0.5021 | 1.1878 | 0.1196 | 0.0365 | 227.1813 | 183.4390 | 105.2832 |
| SD | 0.3042 | 0.7537 | 0.0439 | 0.0466 | 167.9698 | 118.5822 | 105.0381 |
| **16 day** | | | | | | | |
| 657 | 0.2204 | 0.4801 | ND | ND | 368.7861 | 68.3458 | 6.3695 |
| 671 | 0.2050 | 0.2815 | ND | ND | 48.4774 | 34.5076 | 12.5033 |
| 675 | 0.2475 | 0.4180 | ND | ND | 9.7492 | 230.5915 | 56.0800 |
| 676 | 0.2499 | 0.3430 | ND | ND | 181.9636 | 43.5607 | 2.4640 |
| Mean | 0.2307 | 0.3806 | ND | ND | 152.2441 | 94.2514 | 19.3542 |
| SD | 0.0217 | 0.0867 | ND | ND | 162.1172 | 92.0119 | 24.8301 |
| **22 day** | | | | | | | |
| 653 | 0.1490 | 0.2085 | ND | ND | 7.6357 | 66.2698 | 0.1458 |
| 661 | 0.1395 | 0.2062 | ND | ND | 11.2929 | 4.4621 | 0.9700 |
| 666 | 0.0647 | 0.2113 | ND | ND | 3.6633 | 1.2404 | 0.2560 |
| 673 | ND | ND | ND | ND | 0.3114 | 6.6052 | 0.1395 |
| Mean | 0.0883 | 0.1565 | ND | ND | 6.2180 | 19.6444 | 0.3778 |
| SD | 0.0699 | 0.1044 | ND | ND | 5.2839 | 31.1617 | 0.3984 |
| **28 day** | | | | | | | |
| 663 | ND | 0.0502 | ND | ND | 0.2432 | 8.5556 | 0.3796 |
| 664 | 0.1361 | 0.2571 | ND | ND | 92.0926 | 11.9516 | 7.5354 |
| 669 | ND | ND | ND | ND | 0.9421 | 1.0670 | 0.0379 |
| 683 | ND | ND | ND | ND | 0.5560 | 0.1542 | 0.0886 |
| Mean | 0.0340 | 0.0768 | ND | ND | 25.5405 | 5.4321 | 2.0104 |
| SD | 0.0681 | 0.1225 | ND | ND | 42.8020 | 5.7495 | 3.6864 |
| **35 day** | | | | | | | |
| 658 | ND | ND | ND | ND | 0.2072 | 0.0287 | 0.2451 |
| 660 | ND | ND | ND | ND | 0.0842 | 0.1313 | 0.0440 |
| 680 | ND | ND | ND | ND | 0.0986 | 0.3508 | 0.0370 |
| 684 | ND | ND | ND | ND | 0.1599 | 0.1337 | 0.1113 |
| Mean | ND | ND | ND | ND | 0.1355 | 0.1611 | 0.1093 |
| SD | ND | ND | ND | ND | 0.0745 | 0.1356 | 0.0965 |

Table 10 Tissue:blood partition coefficients of OTC in beef cattle

| Tissue | *In vitro* partition coefficients | Computer optimized partition coefficients |
|---|---|---|
| Lung | 7.74 | 2.3 |
| Liver | 5.36 | 4.0 |
| Kidney | 5.87 | 8.0 |
| Muscle | 0.86 | 0.88 |
| fat | -- | 0.08 |

Figure 10 Model-predicted *vs.* observed OTC concentration in the tissues of cattle after

*s.c.* injection of 20 mg/kg Liquamycin-LA-200®

Each point represents the mean tissue OTC concentration (n=4) except the serum OTC

concentration (n=20). The bars represent standard deviation of the experimental data.

Solid line represents model-predicted tissue OTC concentration. a) liver; b) kidney; c)

muscle; d) fat; e)serum

b)

63

c)

OTC concentration (µg/g) in the muscle

Post-dosing time (Days)

64

(e)

66
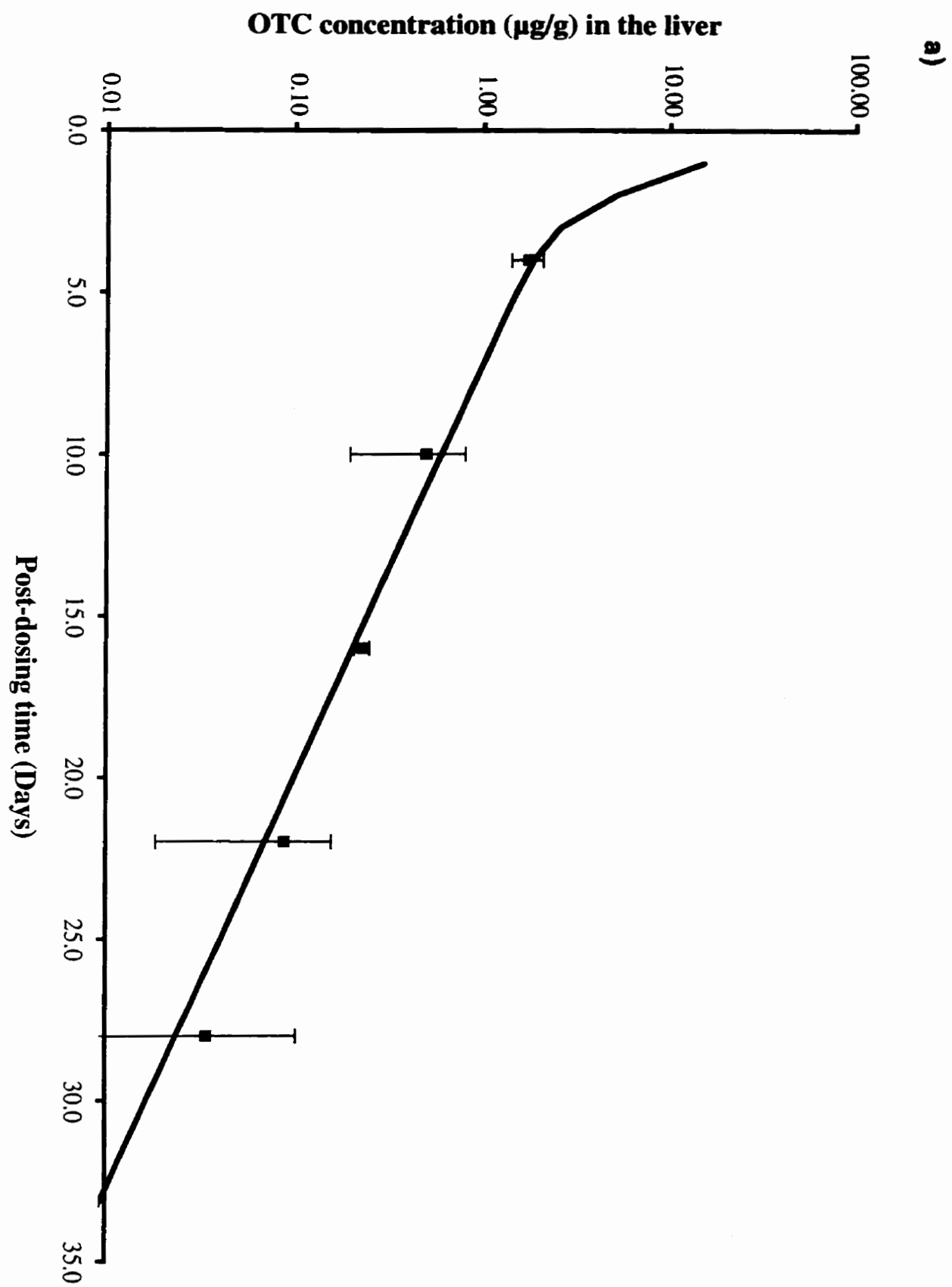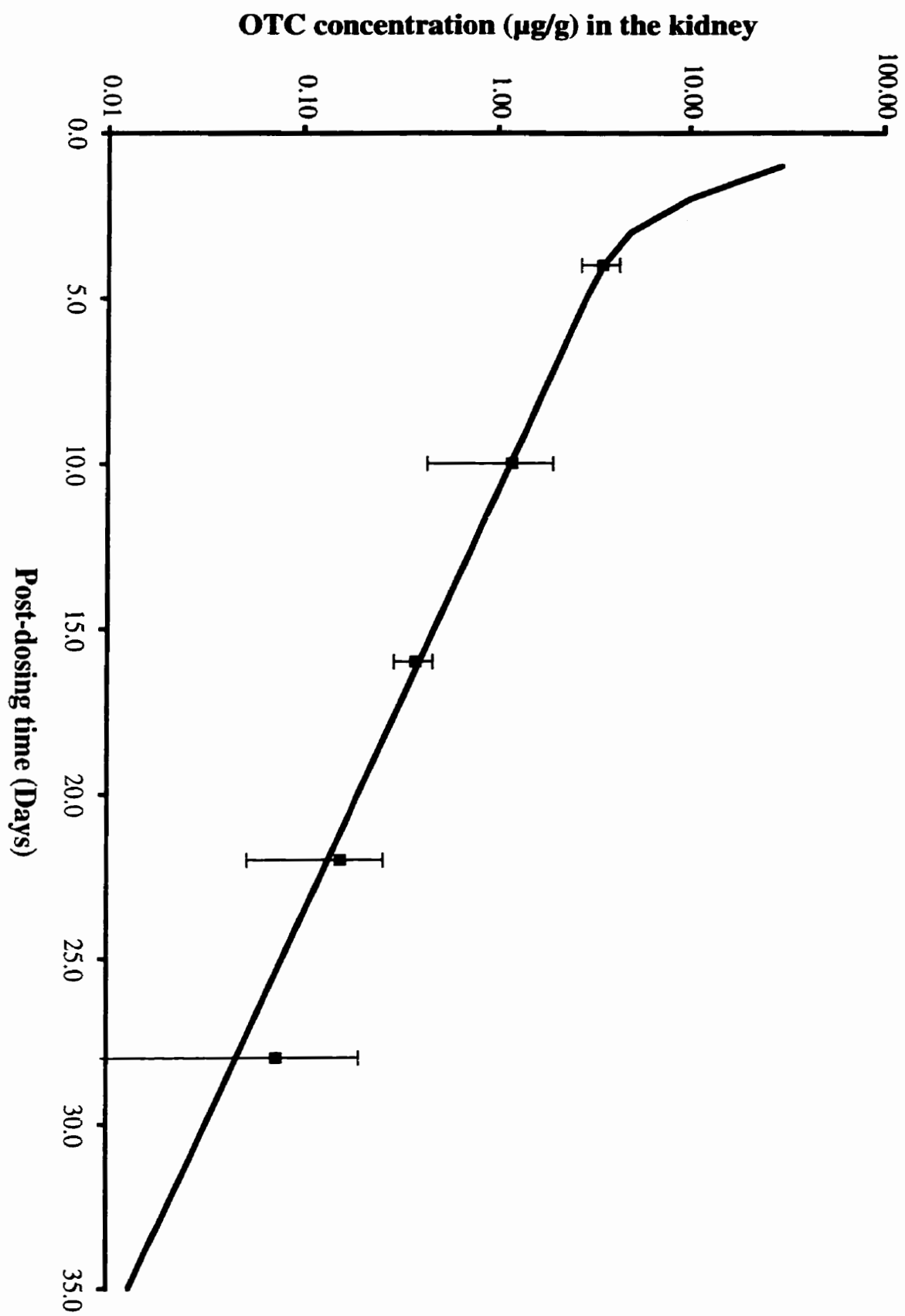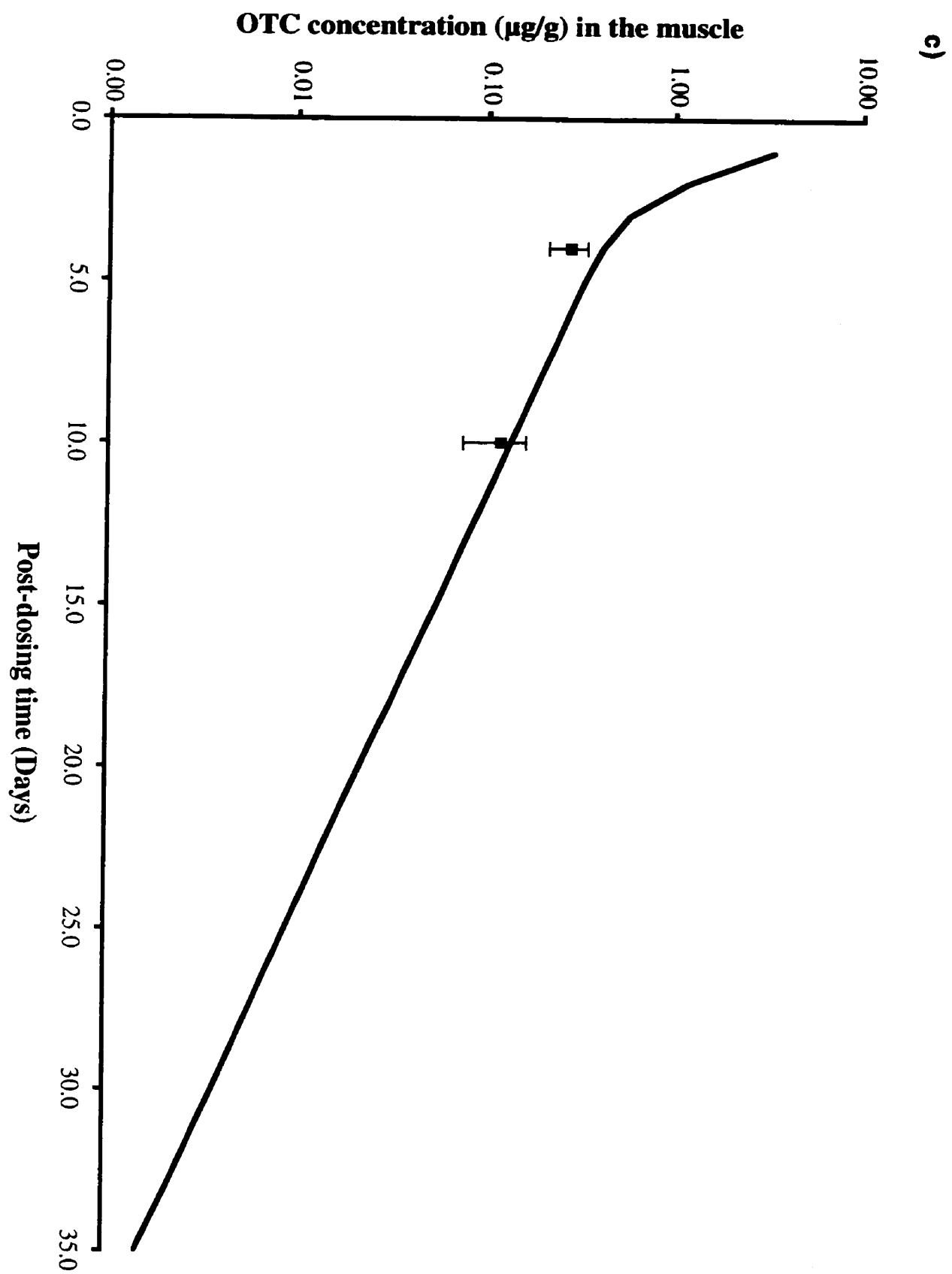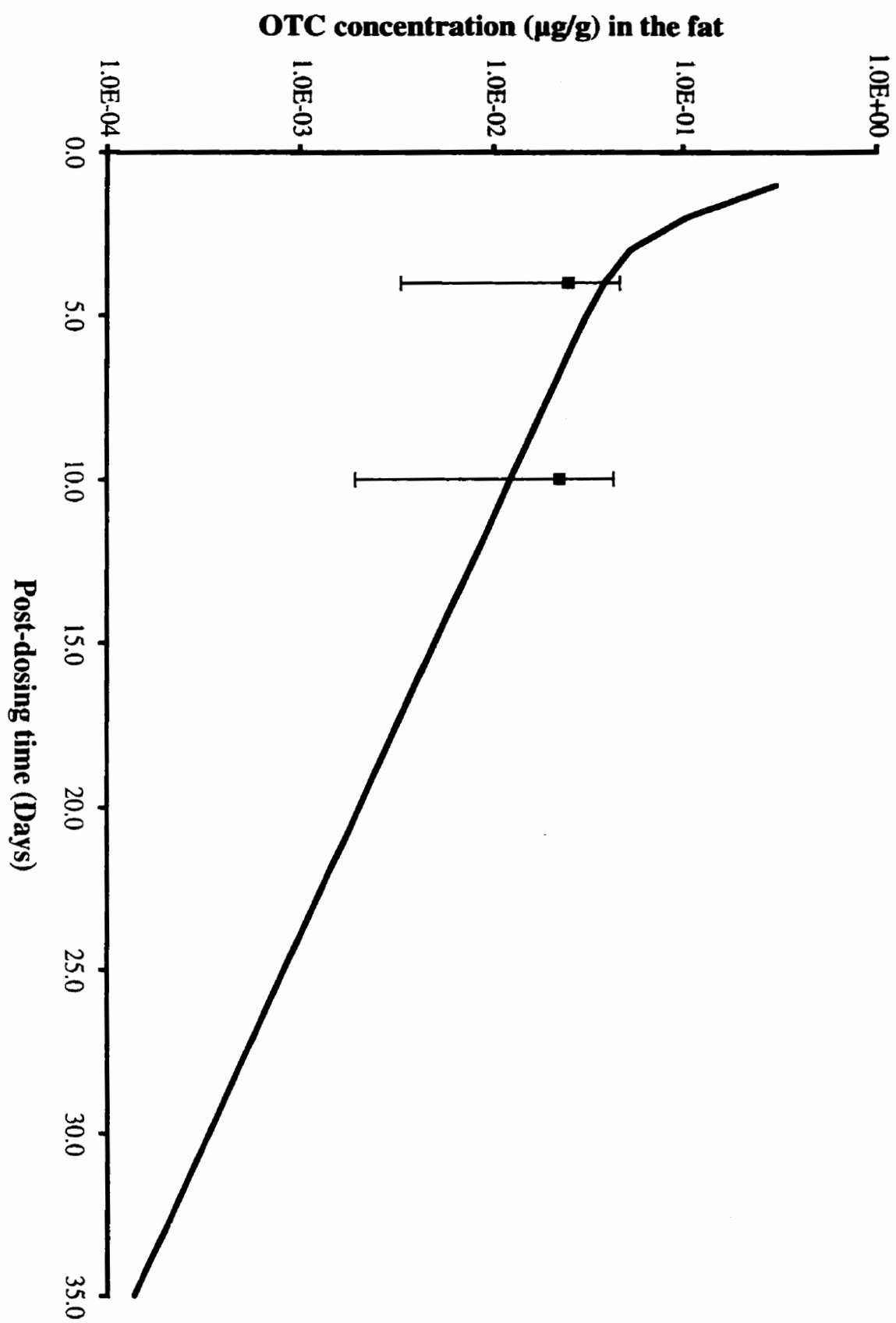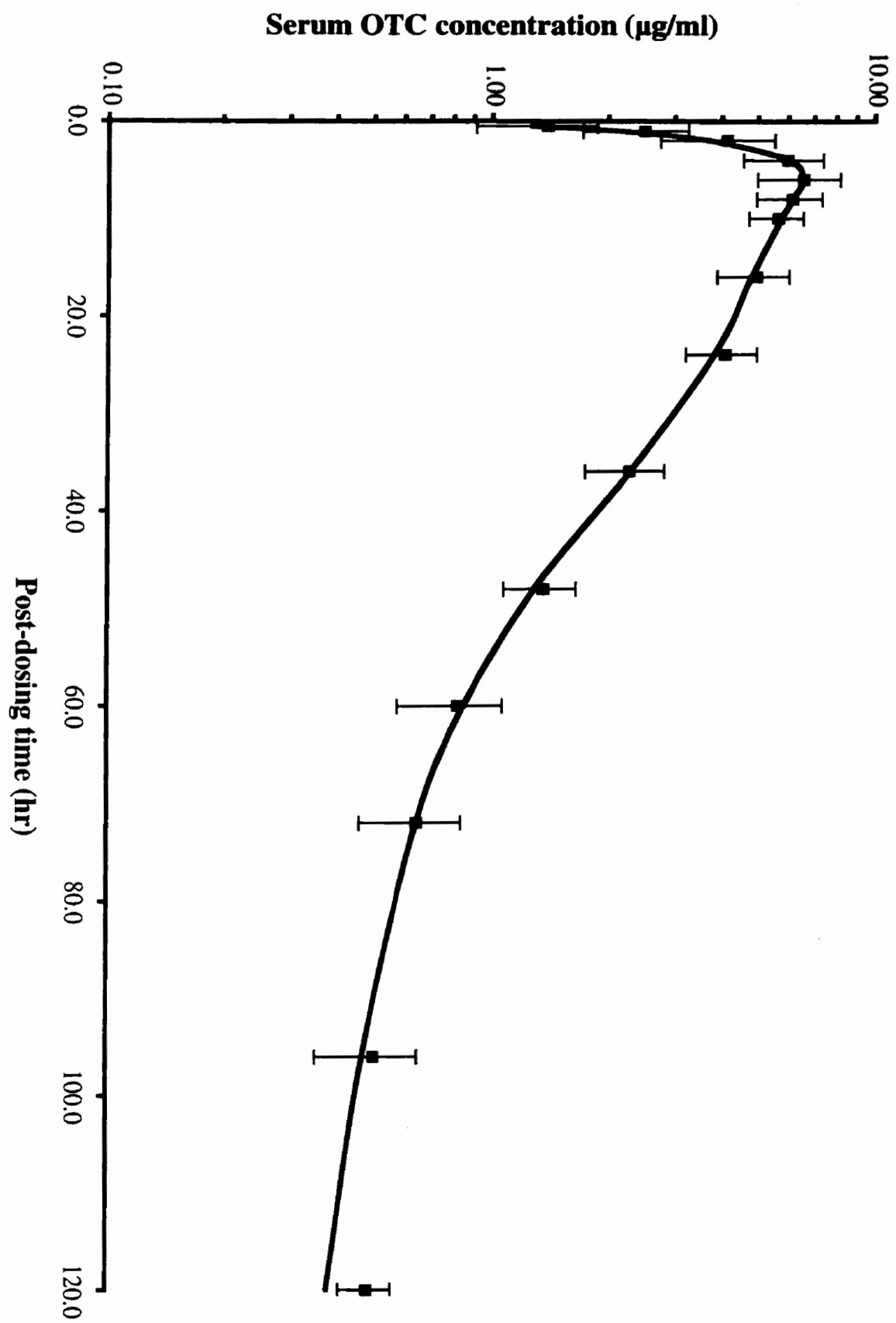
Figure 11. Time course of OTC concentrations at the injection sites of beef cattle after

*s.c.* administration of 20 mg/kg Liquamycin LA-200®

The column represents the mean OTC concentration at the injection sites (N=4). The

bars represent standard deviation of the experimental data.
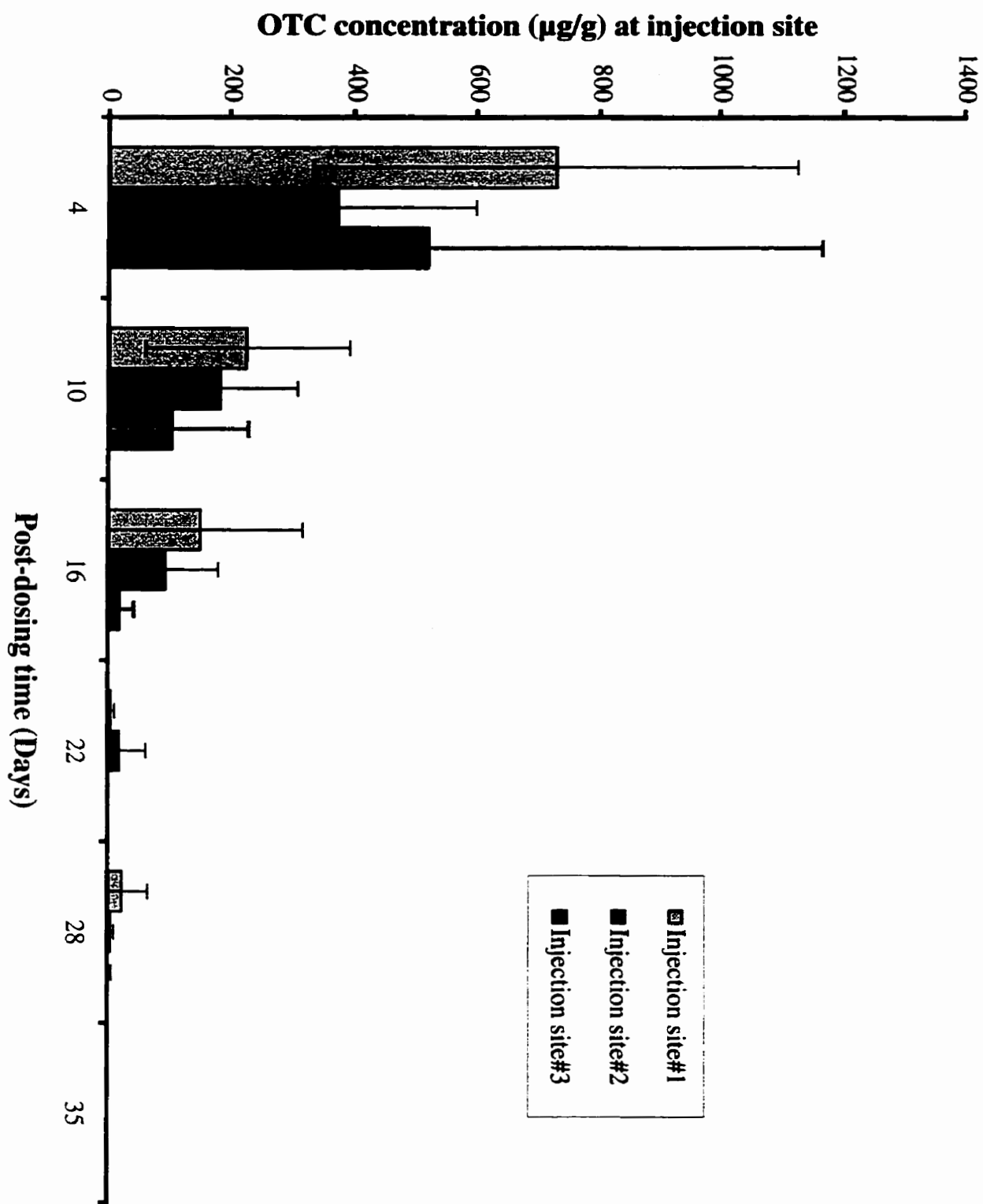
Table 11 Liquamycin® s.c. Fitting results

## Mean Error Fitting Results

| Tissue | Mean error | Mean percent error | Mean square error | Mean absolute error | Mean absolute percent error |
|--------|-----------|-------------------|------------------|--------------------|----------------------------|
| Blood | -0.080 | -13.323 | 0.026 | 0.099 | 14.796 |
| Liver | 0.034 | -6.196 | 0.005 | 0.054 | 19.940 |
| Kidney | 0.006 | -8.934 | 0.001 | 0.030 | 16.688 |
| Muscle | 0.080 | 14.351 | 0.009 | 0.080 | 10.314 |
| Fat | 0.002 | 2.008 | 1.319E-04 | 0.011 | 22.593 |

## Log Likelihood Fitting Results

| Tissue | Model | Exp. Mean* | Exp. Mean +1sd* |
|--------|-------|-----------|-----------------|
| Blood | -253.820 | -235.490 | -395.330 |
| Liver | -48.257 | -40.380 | -64.632 |
| Kidney | -41.964 | -39.613 | -62.216 |
| Muscle | -41.95 | -20.548 | -32.206 |
| Fat | -18.468 | -20.963 | -31.206 |

* The log liklihood values for both the mean and mean +1SD of experimental values are included since LL does not give an absolute value only a value which can be used to compare the fitting of data relative to another set of data.

**PBPK model validation with OTC tissue Concentration data from the _i.m._ route of administration.**

Figure 12 compares model-predicted OTC serum concentrations with those of the empirical data of cattle following _i.m._ administration of 20 mg/kg Liquamycin LA-200®.


**PBPK model validation with OTC tissue Concentration data from the _i.m._ route of administration from the literature (Meijer et al. (1993).**


Figure 13 compared model-predicted OTC serum concentrations with those of the empirical data of cattle following _i.m._ administration of 20 mg/kg OTC from Meijer et al. (1993) which was used to validate the model. Table 12 shows the mean error and log likelihood results used to compare the fitting of model predicted to empirical data. In summary, these results show that the PBPK model fits the empirical data of the _i.m._ route to the same level or extent or better than as the original _s.c._ data used to develop the PBPK model.

Figure 12 Validation of **PBPK** model of **OTC** with serum concentration data from steers

injected with 20 mg/kg Liquamycin **LA-200®** *i.m*

Each point represents the mean serum OTC (N=6). The bars represent standard deviation

of the experimental data. Solid line represents model-predicted OTC concentration.

Serum OTC concentration (μg/ml)

Post-dosing time (hr)
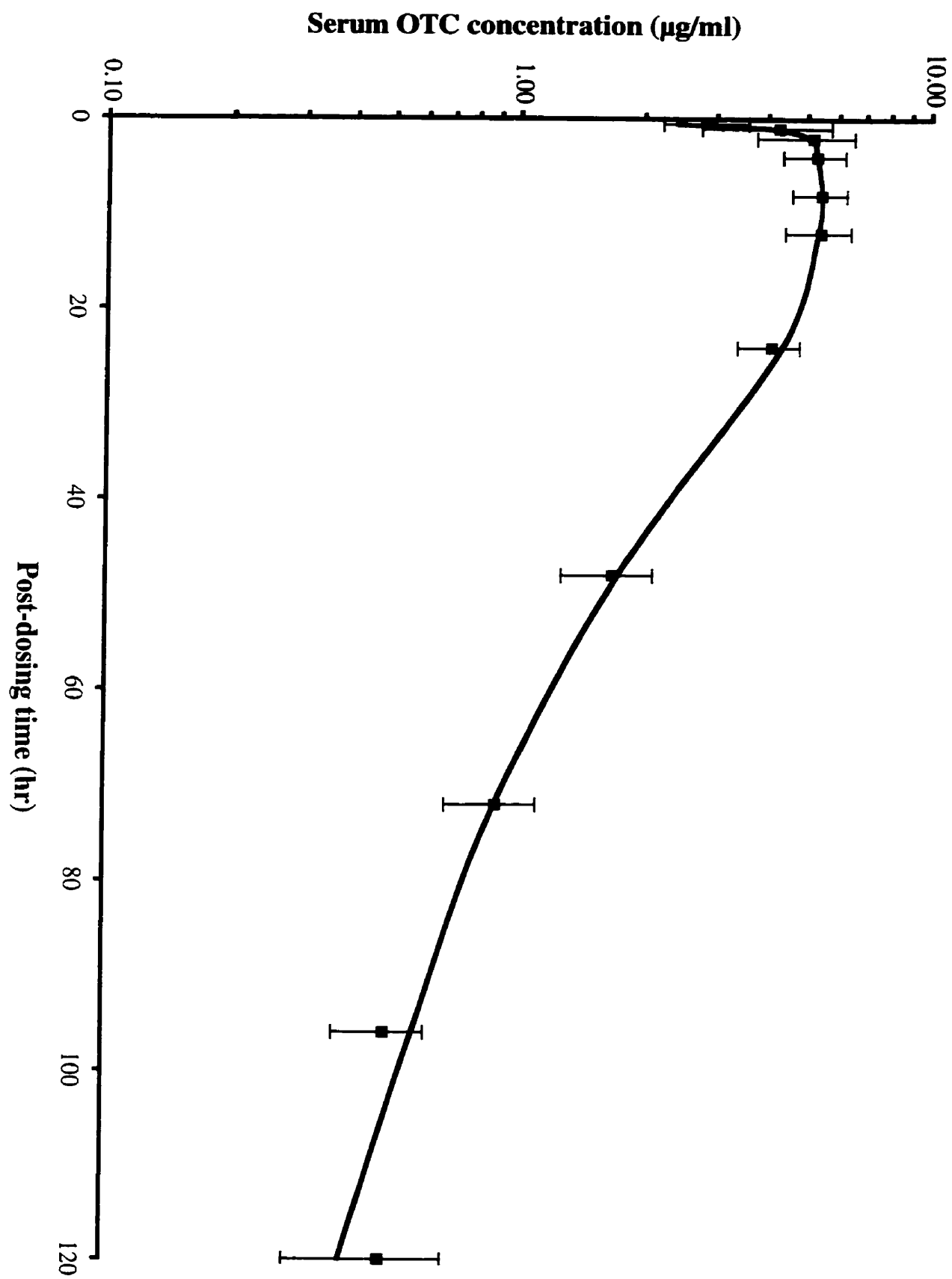
Figure 13 Validation of **PBPK** model with **OTC** data reported by Meijer et al. (1993).

Each point represents the mean serum OTC concentration of five veal caves. Solid line

represents model-predicted OTC concentration.

**Serum OTC concentration (µg/ml)**

**Post-dosing time (hr)**
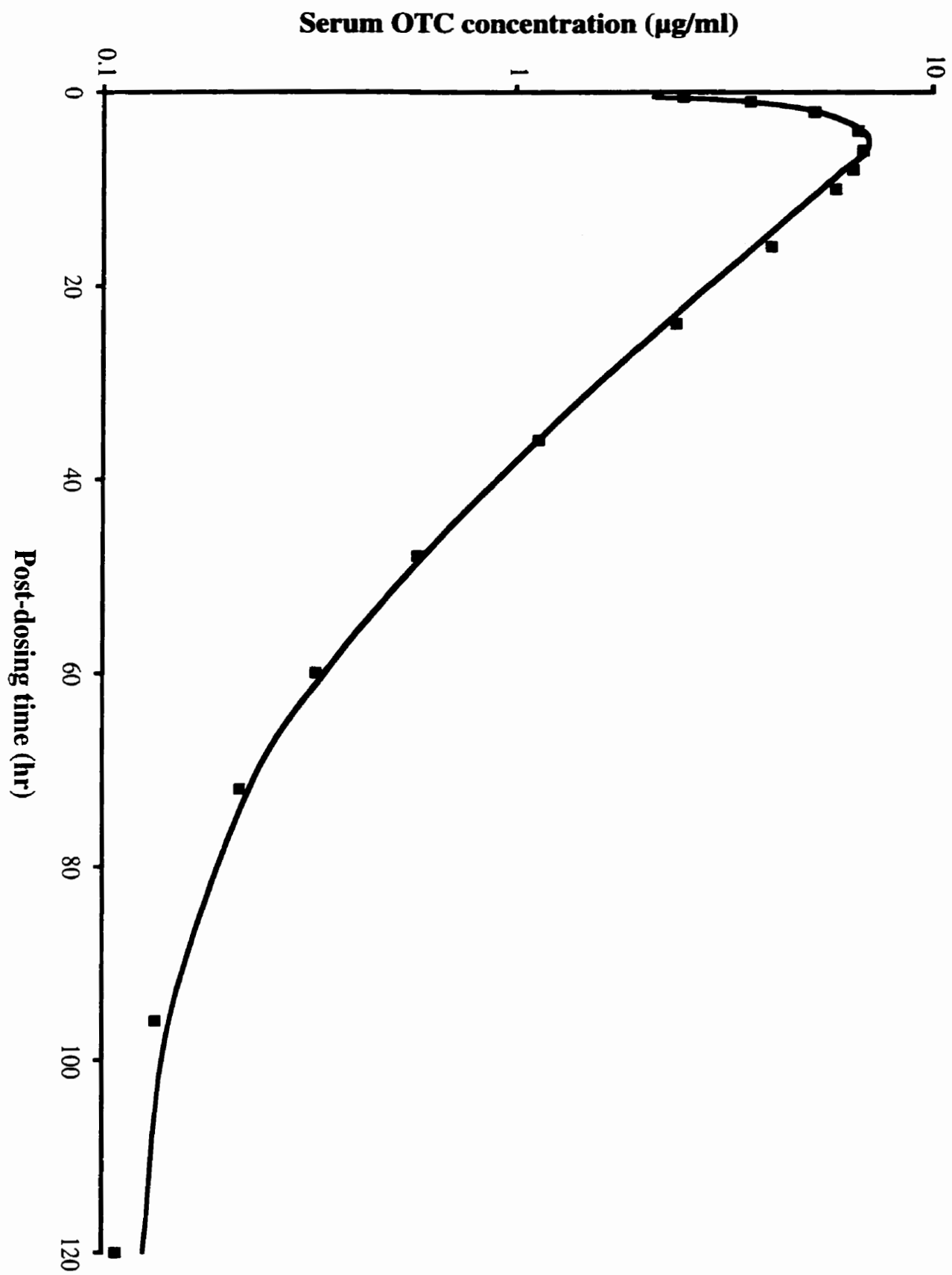
Table 12 Liquamycin® and Validation *i.m* Fitting results

## Mean Error Fitting Results

| Tissue | Mean error | Mean percent error | Mean square error | Mean absolute error | Mean absolute percent error |
|---|---|---|---|---|---|
| Liquamycin® i.m. Fitting results | | | | | |
| blood | -0.050 | -3.787 | 0.033 | 0.027 | 7.183 |
| | | | | | |
| Validation i.m. Fitting results | | | | | |
| blood | -0.077 | 0.515 | 0.044 | 0.095 | 10.877 |

## Log Likelihood Fitting Results

| Tissue | Model | Exp. Mean* | Exp. Mean +1sd* |
|---|---|---|---|
| Liquamycin® i.m. Fitting results | | | |
| Blood | -103.220 | -117.620 | -205.110 |

* The log likelihood values for both the mean and mean +1SD of experimental values are included since LL does not give an absolute value only a value which can be used to compare the fitting of data relative to another set of data.

## DISCUSSION

Previous OTC pharmacokinetic studies have been conducted mainly in the dairy cow. However, beef cattle are examined in the present study because little or no information is available on the pharmacokinetics of OTC in this species after the *s.c* route of administration. The main difference between dairy cattle and beef cattle is in the production of milk. Since the milk contains a large amount of protein which is capable of binding with OTC (Nouws et al., 1985b) and is a major route of OTC elimination, the pharmacokinetics of OTC in dairy cattle are expected to be different to that of beef cattle. As with the dairy cattle (Nouws & Ziv, 1978; Banting et al., 1985; Nouws et al., 1985a; Nouws et al., 1985b; Mevius et al., 1986; Landoni, M.F., 1992, Meijer, L.A., 1993), beef cattle absorbs OTC readily following either *i.m.* or *s.c.* route of administration. Also, similar to the results of the dairy cow (Mawhinney et al., 1996., George, M.H. et al., 1995), the *i.m.* or *s.c* route of OTC administration results in large but variable amounts of OTC residues in the site of injection (Figure 11). An explanation for the persistence of OTC at the injection site is not readily available but is probably related to local tissue irritation due to the spreading of injected OTC between the muscles (after *i.m.* injection) or under the skin (after *s.c.* injection), the precipitation of OTC at the injection site after depletion of the carrier solvent, and the injection volume (Mevius et al., 1986).

The therapeutic OTC concentration in the blood of cattle to control *Bordetella bronchiseptica, Corynebacterium pyogenes, Erysipelothrix rhusiopathiae*, and *Staphylococcus* spp. infections is about 1 µg/ml (Pfizer Liquamycin LA-200® product insert). As shown in Figs 6 and 10, the clinically effective 1 µg/ml OTC concentration in the serum has been achieved for a duration of 79 hr and 69 hr after *i.m.* (Liquamycin LA-200® and Alamycin LA-300®) and *s.c.* (Liquamycin LA-200® and Biomycin LA-200®)

routes of administration, respectively. Therefore, both OTC formulations appear to be effective for at least 3 days after OTC administration.

A comparison of the pharmacokinetic parameters of Liquamycin LA-200® after different routes of administration (Table 5 and Table 8) shows that OTC is absorbed after *i.m.* injection at a rate (K01) two-fold faster than the *s.c.* route. However, OTC penetrates the "deep" compartment ($K_{12}$) at a faster rate after *s.c.* administration compare to *i.m.* administration. As a result, a higher maximum blood concentration (*Cmax*) and a longer $t_{1/2}$ of elimination have been observed in the *s.c.* route. These results confirm those reported by Banting and Fanneau de la Horie (1987) after dairy cow was given an aqueous solution of OTC in propylene glycol.

The terminal elimination half-lives ($t_{1/2}$) of OTC are 25.98 hr and 31.38 hr after *i.m.* administration of Liquamycin LA-200® and Alamycin LA-300® to beef cattle, respectively (Table 5). This is comparable to the 25.38 hr $t_{1/2}$ of a previous Liquamycin LA-200® study (Ames and Patterson, 1985). Mevius et al. (1986) also have reported a similar $t_{1/2}$ (30.26 ± 8.12 hr) for five different long-acting OTC formulations in cattle.

A classical dilemma in pharmacology and toxicology is how the dose administered relates to the dose delivered to the target site. Serum concentration of OTC may be misleading since the concentration of any given substance in serum may not be representative of its concentration in other tissues. Hitherto, the bioequivalance studies of the different OTC formulations have been examined using the classical pharmacokinetic model approach (Table 5 and Table 8). The classical pharmacokinetic model approach examines mainly the time course of OTC concentrations in the serum of beef cattle because blood is a fluid that reaches many tissues and is convenient to sample. However, serum is but one small physiological compartment in the cattle and the

concentration of OTC in the plasma may not reflect the concentration of OTC in other body tissues or fluids. Therefore, the time course of OTC concentrations in various tissues of the beef cattle was also studied after the *i.m.* and the *s.c.* routes of administration.

Results of tissue distribution study show that the highly vascular and well-perfused organs such as the kidney and the liver have higher OTC concentrations than the muscle, a slowly perfused tissue. The fat tissue has the lowest OTC concentration among all tissues examined probably due to its poor vascularization. These results are consistent with the assumption of the PBPK model that OTC distribution in the tissues of beef cattle is determined mainly by the blood flow to the tissues. The assumption of a flow-limited PBPK model of OTC also is consistent with the variability in OTC tissue concentration data, which may be due to individual difference in the blood flow of the cattle as a result of different eating habits, water intake, and exercise (Huntington, et. al., 1990, Whitt, J. et. al., 1996). Overall, these results are consistent with previous dairy cattle and beef cattle studies in which high OTC concentrations are observed in the liver and kidney (Landoni and Errecalde, 1992; Nouws and Ziv, 1978). These results also show that *s.c.* injection of OTC is a viable alternative to *i.m.* route of administration since both administration routes are bioequivalent in OTC and the *s.c.* administration route avoids the disadvantage of having a high OTC concentration at the injection site.

The PBPK model of OTC is developed based on the OTC tissue concentration data of *s.c.* administered Liquamycin LA-200® to the beef cattle. The model structure follows closely that of the salmonids (Law et al. 1991) except that the gill compartment of the salmonid PBPK model was replaced by a lung compartment and a fat compartment was added to the original model structure. The physiological parameters of the salmonid

model also were modified since ambient temperature had less effect on the physiology of cattle than that of trout or salmon. The tissue:blood partition coefficients of the cattle and salmonid models were either very similar or within a factor of one. This is expected since OTC is absorbed into the extracellular space, minimally metabolized, and eliminated primarily by renal glomerular filtration. In summary, the main differences between the fish and cattle PBPK models are the uptake route and the physiology of the animal species.

As shown in Figs 12 and 13, model-predicted OTC concentrations in the cattle tissues are in good agreement with the empirical OTC tissue concentrations. The only exception is the concentration of OTC in the muscle, which is overestimated by the model. An explanation for the discrepancy between the modeled and experimental muscle concentrations is not readily available but this is also seen in other OTC PBPK models (Law et al, 1991). Perhaps, it is related to protein bindings, a greater affiliation to either white or red muscle fibers, or shunting of blood to this tissue due to exercise (Law et al, 1991).

OTC uptake from the injection site after *s.c.* injection was modeled initially using a first-order rate equation. However, this resulted in a rapid decline of OTC concentrations in the blood and at the injection site. This problem can be resolved by reducing the uptake rate after a time period. The two-step uptake process is consistent with the finding that OTC is rapidly taken up initially with the carrier solvent and then more slowly from the OTC depots (Mevius et al. 1986; Nouws et al. 1985b).

Implementation of the PBPK model of OTC requires a large number of physiological and biochemical data; these were obtained from the literature or by allometric scaling. A preliminary PBPK model of OTC for the beef cattle was

constructed before initiating the experimental studies. This helped tremendously in planning the sampling time and duration for the experimental studies.

The PBPK model assumes that OTC is eliminated from the beef cattle mainly by renal and hepatic clearances. Urinary excretion of OTC is assumed to be first-order and is related to the glomerular filtration rate of cattle. Nouws et al (1985a) and Nouws et al (1986) have reported that OTC elimination is closely related to the amount of urine excreted and that about 90% of the absorbed OTC is eliminated by the kidney. Only 10% of the dose is eliminated by the liver. The PBPK model predicts that about 80% of the administered dose is eliminated at 72 hr post-injection and is in agreement with the findings of Nouws et al., (1985a) and Mevius et al., (1986).

OTC tissue:blood partition coefficients especially those of the liver and kidney are higher in the *in vitro* studies than those used in the model (Table 10). This is expected since these are excretory organs and the *in vitro* method used to determine the tissue:blood partition coefficients does not account for OTC clearance from these organs. In addition, the *in vitro* procedure can provide only information on the binding of OTC to the protein and/or membrane of the cells. Nevertheless, the model-optimized tissue:blood partition coefficients are very similar to those reported previously in cattle (Landoni and Errecalde, 1992, Toutain and Raynaud, 1983).

The PBPK model of beef cattle can be used to predict OTC pharmacokinetics for a different cattle species, exposure route and dosing regime. This can be implemented easily by replacing the blood flows, tissue volumes, mass transfer coefficients and elimination rate constants of the beef cattle PBPK model with the values of a specific species or administration route. For example, in adapting the model for *i.m.* injection, the initial uptake rate was doubled for a shorter duration than the *s.c.* uptake rate, keeping all

the physiological and biochemical parameters unchanged. An increase in the uptake rate for the *i.m.* administration route is in agreement with the results of the classical pharmacokinetic model approach, in which the rate of OTC uptake (K) for *i.m.* and *s.c.* routes of administration are 0.85 h and 0.38 h, respectively (Table 5 and Table 8).

The PBPK model predicts closely the concentration of OTC in the tissue ofhealthy cattle after either *i.m* or *s.c.* administration. The model also can be used to predict OTC deposition in the tissue of diseased cattle if the physiological conditions in these animals are known. For example, Nouws and Ziv (1978) have found that drug residues persisted longer by a factor of two to three and four to five in muscle and kidney, respectively, in diseased animals when compare to healthy animals. In cases of nephritis and endocarditis, kidney drug levels might persist even longer than five times. The PBPK model can be used to predict the persistence of drugs in the tissue of these animals.

The cattle PBPK model was developed and implemented on a personal computer using Excel® and both the Euler and Runge-Kutta methods of solving the ODEs. In contrast, the salmonid PBPK model (Law 1991) was implemented on a main frame computer using ACSL (Mitchell and Gauthier Associates Inc. Concord, MA) and the Gear method of ODE solving. Two different types of computer programs generally have been used to solve the differential and algebraic equations that describe a PBPK model: (a) commercial modeling software such as ACSL® for Windows®; ScoP® (National Biomedical Simulation Resource, Duke University Medical Center, Durham, NC); STELLA® (High Performance Systems, Inc., Hanover, NH) or writing a custom program (Dong 1994). While the commercial software is a powerful tool, it is expensive and difficult to use and is not designed solely for PBPK modeling. (b) Custom programs, while often are free, are usually poorly documented. They are hard to use since they are

written in complex programming languages such as C or Pascal, need a compiler to work and usually have unfinished or missing components such as the ability to plot out the data. While Excel has been used before in PBPK model development (Johanson and Nasland, 1988; Haddad et al., 1996), the reported techniques are either outdated and do not work with the latest version of Excel or are slow and cumbersome. The approach used in this study has combined Excel and Visual Basic to develop and implement the PBPK model. This approach is cost effective, easily adaptable to other PBPK models (Namdari, 1998., Eickhoff, in press), and is a middle ground between commercial and custom solutions. The resulting PBPK model allows the user to choose the method of solving differential equations, the time step involved, and the outputs results at the user's defined times. Another benefit of using Excel in PBPK model development is its simple customizable interface, cross platform compatibility, easy access to charting and statistical analysis through Excel Plugins and easy Monte Carlo analysis using the Crystal Ball® Package (Decisioneering, Denver, CO).

Results of the sensitivity analysis indicate that the kidney parameters, RK, VK, and KK are the most sensitive parameters of the beef cattle PBPK model. RL and Ka are also sensitive parameters of the model but they are of less importance. These pharmacokinetic parameters control the uptake and elimination of OTC in the PBPK model of beef cattle. The kidney parameters of (RK, VK, and KK) are the most sensitive parameters of the PBPK model since it is the most important organ in OTC elimination (Nouws et al, 1985a). The uptake parameter, Ka also is a sensitive model parameter since it is responsible for the uptake of OTC by the beef cattle. Overall, the sensitive parameters in the model are those that are associated with either the uptake or elimination of OTC.

## Summary and Conclusion

A PBPK model was developed to describe the disposition of oxytetracycline (OTC) in beef cattle tissues. The model closely simulated the time course of OTC concentrations in the tissues of the beef cattle following either *i.m.* or *s.c.* route of administration. These results demonstrate that the PBPK model is a very useful tool to study the time course of OTC residues in the target organs of beef cattle. Based on the prediction of a validated PBPK model of OTC, a withdrawal period for cattle tissues bound for human consumption can be easily derived.

## Future Model Direction

Future model direction should deal with two important areas:

a) Monte Carlo Simulation. Monte Carlo simulations move the PBPK model output from a deterministic result into a stochastic result by assigning distributions to some or all of the input parameters such as tissue volume or blood flow. This not only allows a more realistic view of a population, it also gives us a range of possible outcomes.

b) Interspecies relationship. OTC is an ideal drug to examine an interspecies PBPK since the drug has been studied in many animal species and its efficacy is dependent on the concentration of OTC present in the extracellular fluid. Hence, it is expected that the pharmacodynamic differences between different animal species would be small (Riviere J. E, et al., 1997). In addition, since OTC is minimally metabolized and is dependant on renal glomerular filtration for its excretion in animals, the excretion rates of OTC in different animals can be easily estimated from the renal glomerular filtration rate of the animal species.

**Appendix A**   Individually fitted Parmacokinetic Parameters

Table A-1 Pharmacokinetic parameters derived from the serum OTC concentration *vs.* time curve of steers following *i.m.* injection of 20 mg/kg Liquamycin LA-200®

| Steer No. | T1/2 ($\alpha$) | T 1/2 ($\beta$) | AUC | Tmax | Cmax |
|-----------|---------|----------|---------|-------|-------|
| 51 | 1.8563 | 31.347 | 294.081 | 8.046 | 5.443 |
| 52 | 1.0630 | 24.5608 | 313.718 | 5.034 | 7.681 |
| 53 | 0.4834 | 28.5114 | 239.109 | 2.892 | 5.418 |
| 54 | 0.9160 | 30.0359 | 241.054 | 4.757 | 4.984 |
| 55 | 0.7206 | 21.5437 | 218.325 | 3.654 | 6.245 |
| 56 | 0.2481 | 29.6866 | 260.496 | 1.727 | 5.842 |

Table A-2 Pharmacokinetic parameters derived from the serum OTC concentration *vs.* time curve of steers following *i.m.* injection of 20 mg/kg Alamycin LA-300®

| Steer No. | T1/2 ($\alpha$) | T 1/2 ($\beta$) | AUC | Tmax | Cmax |
|-----------|---------|----------|---------|-------|-------|
| 51 | 0.7663 | 31.8077 | 259.400 | 4.221 | 5.156 |
| 52 | 0.3406 | 31.3718 | 236.854 | 2.247 | 4.980 |
| 53 | 0.9853 | 31.9147 | 250.168 | 5.101 | 4.863 |
| 54 | 1.056 | 29.3976 | 206.237 | 5.256 | 4.296 |
| 55 | 0.4084 | 26.3475 | 245.480 | 2.494 | 6.048 |
| 56 | 0.3922 | 30.6743 | 297.900 | 2.499 | 6.362 |

**Appendix B**  Time course of tissue concentrations

Table B-1 Time course of OTC concentration in the serum of steers following *s.c.* injection of 20 mg/kg Liquamycin LA-200®

| Calf ID | 0 | 0.5 | 1 | 2 | 4 | 6 | 8 | 10 | 16 | 24 | 36 | 48 | 60 | 72 | 96 | 120 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | *Time (Hr)* | | | | | | | |
| 532 | 0 | 2.4625 | 3.7673 | 4.9988 | 4.7606 | 8.2034 | 7.1972 | 6.0380 | 4.2179 | 3.1924 | 1.6013 | 1.3158 | 0.5628 | 0.3999 | 0.2911 | 0.5170 |
| 535 | 0 | 1.5421 | 2.7649 | 5.1303 | 6.6997 | 6.5883 | 6.7481 | 5.2655 | 3.6655 | 2.9900 | 1.7242 | 1.1335 | 0.5506 | 0.3962 | 0.3979 | 0.4331 |
| 536 | 0 | 1.1897 | 2.3387 | 4.6140 | 5.7792 | 5.1979 | 5.5074 | 5.2737 | 6.0709 | 4.6301 | 2.2402 | 1.2298 | 0.7675 | 0.4420 | 0.2030 | 0.4028 |
| 537 | 0 | 0.9600 | 1.0782 | 2.1654 | 4.3226 | 5.1396 | 5.3558 | 5.8402 | 4.9928 | 3.7986 | 2.4715 | 1.9298 | 1.2130 | 0.8836 | 0.6664 | 0.5705 |
| 543 | 0 | 0.9740 | 2.0253 | 2.7806 | 4.5747 | 7.2933 | 5.4771 | 5.1858 | 7.1052 | 6.2491 | 2.4501 | 1.7514 | 1.0060 | 0.7156 | 0.6067 | 0.5198 |
| 544 | 0 | 1.0684 | 1.4362 | 2.9144 | 5.5951 | 4.5425 | 5.2250 | 4.4370 | 4.6367 | 3.2823 | 2.2139 | 1.3621 | 1.3425 | 0.8316 | 0.6943 | 0.4792 |
| 547 | 0 | 2.0274 | 3.8952 | 4.4473 | 8.6273 | 9.2746 | 8.2769 | 6.6084 | 4.9329 | 4.2520 | 1.8312 | 0.9294 | 0.5479 | 0.3820 | 0.3337 | 0.5291 |
| 550 | 0 | 1.0201 | 2.5404 | 4.3113 | 8.1338 | 7.2470 | 7.4041 | 5.9332 | 3.7680 | 4.7104 | 2.2589 | 1.1656 | 0.6187 | 0.4803 | 0.5055 | 0.4991 |
| 554 | 0 | 1.2267 | 2.6164 | 3.9323 | 7.1766 | 6.9057 | 6.7452 | 5.5113 | 3.4650 | 4.1403 | 1.9356 | 1.1577 | 0.5992 | 0.5102 | 0.4764 | 0.5292 |
| 556 | 0 | 2.4760 | 3.5881 | 3.8595 | 4.7146 | 6.8412 | 5.0516 | 6.2060 | 5.1008 | 4.4454 | 1.8562 | 1.1060 | 0.7095 | 0.5976 | 0.7962 | 0.4271 |
| 561 | 0 | 1.0633 | 2.7179 | 5.2582 | 5.5498 | 4.8730 | 6.5187 | 5.5915 | 6.3018 | 3.6035 | 2.3355 | 1.1311 | 0.7292 | 0.4826 | 0.4987 | 0.4336 |
| 573 | 0 | 0.9524 | 1.6680 | 2.7939 | 6.0283 | 6.1833 | 6.0434 | 6.4096 | 4.7858 | 3.5414 | 2.4118 | 1.4782 | 1.0052 | 0.6603 | 0.5304 | 0.5496 |
| 579 | 0 | 1.4488 | 2.5138 | 3.1274 | 6.3610 | 5.9650 | 4.8250 | 4.8720 | 3.8390 | 3.3024 | 1.4721 | 0.9817 | 0.5585 | 0.7308 | 0.3685 | 0.3270 |
| 580 | 0 | 1.3474 | 2.3026 | 5.2344 | 5.8894 | 5.6964 | 5.9262 | 3.4845 | 5.3079 | 4.2150 | 2.1787 | 1.5889 | 0.7721 | 0.6287 | 0.5573 | 0.4438 |
| 582 | 0 | 1.3531 | 2.0552 | 3.6201 | 7.9915 | 8.9755 | 7.1880 | 6.4508 | 4.3415 | 4.3904 | 2.4167 | 1.3652 | 0.8134 | 0.8348 | 0.6336 | 0.4474 |
| 585 | 0 | 1.5715 | 3.1117 | 8.1188 | 6.2574 | 7.9142 | 6.5700 | 6.1810 | 6.9357 | 4.7167 | 3.4893 | 1.6623 | 0.8488 | 0.5498 | 0.3491 | 0.3781 |
| 601 | 0 | NA | 3.4370 | 4.9434 | 6.9457 | 6.1859 | 5.5197 | 5.0950 | 5.2495 | 4.4559 | 2.5725 | 1.2135 | 0.6866 | 0.7851 | 0.4696 | 0.4262 |
| 606 | 0 | 1.6496 | 2.3036 | 3.6468 | 6.3699 | 5.8659 | 5.4493 | 4.5963 | 4.6994 | 3.6852 | 2.0303 | 1.3538 | 0.8592 | 1.0140 | 0.6139 | 0.5941 |
| 610 | 0 | 0.8523 | 1.7939 | 2.0339 | 2.9640 | 3.5202 | 3.6059 | 7.4972 | 3.9019 | 2.6922 | 2.7645 | 1.8855 | 1.1659 | 0.8285 | 0.6243 | 0.5846 |
| 612 | 0 | 1.2478 | 1.8783 | 4.8591 | 4.8567 | 9.2521 | 8.3868 | 6.6144 | 5.9693 | 5.6029 | 3.5635 | 1.6512 | 1.1639 | 0.8489 | 0.4461 | 0.5963 |
| Mean | 0.0 | 1.3912 | 2.4916 | 4.1395 | 5.9799 | 6.5833 | 6.1511 | 5.6546 | 4.9644 | 4.0948 | 2.2909 | 1.3696 | 0.8260 | 0.6501 | 0.5031 | 0.4844 |
| SD | 0.0 | 0.4795 | 0.7738 | 1.3892 | 1.4049 | 1.5907 | 1.1929 | 0.9191 | 1.0669 | 0.8719 | 0.5397 | 0.2912 | 0.2460 | 0.1901 | 0.1503 | 0.0762 |

Table B-2. Time course of OTC concentration in the serum of steers following *i.m.* injection of 20 mg/kg Biomycin LA-200®

| | | | | | | | Time (h) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Calf ID | 0 | 0.5 | 1 | 2 | 4 | 6 | 8 | 10 | 16 | 24 | 36 | 48 | 60 | 72 | 96 | 120 |
| 532 | 0 | 1.9575 | 5.0259 | 7.4326 | 7.1613 | 4.9872 | 6.0715 | 4.6203 | 4.2281 | 3.4452 | 1.8692 | 1.5411 | 0.8977 | 0.7341 | 0.4470 | 0.4026 |
| 535 | 0 | 1.3652 | 5.4790 | 6.0661 | 10.5345 | 5.8002 | 6.2687 | 5.7215 | 4.9813 | 4.7060 | 2.2761 | 1.7655 | 1.1935 | 0.6976 | 0.5021 | 0.2406 |
| 536 | 0 | 4.1197 | 9.1417 | 7.9165 | 7.3444 | 6.0643 | 5.6192 | 5.2755 | 3.9836 | 4.6719 | 1.6557 | 0.8722 | 0.5570 | 0.4104 | 0.3873 | 0.5091 |
| 537 | 0 | 4.3326 | 6.9839 | 6.8501 | 12.1928 | 7.7122 | 7.5842 | 5.5341 | 4.7294 | 3.6377 | 2.0949 | 0.9862 | 0.8446 | 0.5637 | 0.5737 | 0.4850 |
| 543 | 0 | 2.5094 | 4.2280 | 7.2085 | 7.5101 | 6.0971 | 5.7007 | 5.1049 | 4.8752 | 3.5533 | 1.8723 | 1.2291 | 0.7084 | 0.6013 | 0.5440 | 0.5692 |
| 544 | 0 | 3.4041 | 6.5721 | 7.0851 | 9.1341 | 7.5656 | 6.8755 | 6.0907 | 5.7653 | 3.1302 | 1.4486 | 1.0110 | 0.5137 | 0.4034 | 0.2658 | 0.4654 |
| 547 | 0 | 3.7487 | 6.4649 | 7.4760 | 7.5282 | 7.3116 | 5.9563 | 6.7926 | 4.7989 | 5.3036 | 2.5608 | 1.3578 | 1.0749 | 0.6692 | 0.4952 | 0.5033 |
| 550 | 0 | 3.5291 | 7.9174 | 12.4231 | 9.0460 | 8.2251 | 8.7180 | 7.0145 | 7.0563 | 4.6018 | 2.3360 | 1.4693 | 1.1240 | 0.7528 | 0.4276 | 0.4645 |
| 554 | 0 | 1.8414 | 4.6367 | 7.0885 | 6.0778 | 5.0115 | 5.9873 | 4.7510 | 4.2752 | 3.7546 | 2.4255 | 1.4793 | 1.5637 | 0.8045 | 0.5322 | 0.5067 |
| 556 | 0 | 2.6509 | 6.5565 | 11.7131 | 7.7506 | 7.5171 | 6.0001 | 6.0853 | 5.2113 | 3.9892 | 2.6701 | 1.2867 | 1.0396 | 0.7857 | 0.5480 | 0.4359 |
| 561 | 0 | 3.3767 | 8.4168 | 6.4974 | 7.9625 | 8.1908 | 6.0589 | 5.1878 | 3.6649 | 3.4920 | 1.7922 | 1.1685 | 0.5629 | 0.3760 | 0.4571 | 0.3537 |
| 573 | 0 | 2.8262 | 4.7699 | 5.7334 | 6.8467 | 5.7354 | 4.5788 | 4.9356 | 4.2001 | 3.7839 | 1.5766 | 1.1971 | 0.7265 | 0.6089 | 0.4758 | 0.4435 |
| 579 | 0 | 2.5487 | 4.5005 | 6.1158 | 6.4934 | 6.5348 | 4.9896 | 6.5722 | 4.3380 | 2.5764 | 2.3406 | 1.1835 | 0.6725 | 0.6042 | 0.2809 | 0.4738 |
| 580 | 0 | 4.7149 | 8.1070 | 6.7934 | 9.7074 | 9.4397 | 5.9465 | 5.5102 | 5.0386 | 3.1306 | 1.4970 | 0.7618 | 0.3985 | 0.3538 | 0.3242 | 0.3515 |
| 582 | 0 | 3.3068 | 6.1720 | 6.8521 | 6.4147 | 7.3719 | 6.2180 | 11.2964 | 5.2612 | 3.3158 | 2.0630 | 1.3715 | 0.8902 | 0.5571 | 0.4081 | 0.4283 |
| 585 | 0 | 3.3081 | 5.9012 | 7.7417 | 10.2947 | 7.3273 | 8.3080 | 5.7756 | 3.9063 | 5.4632 | 2.2234 | 1.1636 | 0.8653 | 0.6392 | 0.5225 | 0.3722 |
| 601 | 0 | 2.1747 | 3.3111 | 8.4295 | 6.1504 | 6.2017 | 5.9499 | 6.0193 | 5.2171 | 3.5096 | 4.4101 | 2.1058 | 1.1764 | 0.8595 | 0.6147 | 0.4932 |
| 606 | 0 | 1.6393 | 4.8468 | 9.5508 | 7.2523 | 6.5786 | 6.5500 | 7.8856 | 3.2108 | 2.9313 | 2.8536 | 1.8307 | 1.5406 | 0.9804 | 0.7224 | 0.5312 |
| 610 | 0 | 5.5197 | 7.6083 | 10.5058 | 9.6356 | 8.0097 | 6.3339 | 4.2217 | 4.6704 | 2.9061 | 1.3693 | 1.0279 | 0.5616 | 0.4746 | 0.3988 | 0.3679 |
| 612 | 0 | 2.6423 | 5.2784 | 6.8124 | 10.7742 | 6.8355 | 6.1824 | 4.5882 | 4.9255 | 3.3772 | 2.0634 | 1.2199 | 0.9580 | 0.6693 | 0.6741 | 0.3886 |
| Mean | 0.00 | 3.0758 | 6.0959 | 7.8146 | 8.2906 | 6.9259 | 6.2949 | 5.9491 | 4.7169 | 3.7640 | 2.1699 | 1.3014 | 0.8935 | 0.6273 | 0.4801 | 0.4393 |
| SD | 0.00 | 1.0735 | 1.5753 | 1.8452 | 1.7576 | 1.1473 | 0.9763 | 1.5539 | 0.8281 | 0.7953 | 0.6730 | 0.3305 | 0.3233 | 0.1686 | 0.1196 | 0.0776 |

**Appendix C   Method validation**

1. Methodology

Tissues were assayed for OTC using high pressure liquid chromatography as described in Materials and Methods.

2. External Calibration

Calibration Standards: A calibration curve was prepared using 3-5 concentrations of the OTC standard, that were expected to be found in the tissue samples. The lowest concentration was at or near the Method Detection Limit. At minimum, a 3-point calibration was performed each time an instrument was set up for analysis, after each major equipment change or disruption, and when routine calibration check exceeded specific control limits.

A response factor (RF) was calculated for each calibration standard according to the following formula:

$$RF = \frac{At}{Ct} \quad \text{where}$$

At = integrated peak area of OTC

Ct = concentration of OTC

The mean value of all the RF's from different days was designated $\overline{RFotc}$ (Mean Response Factor for OTC). It was used to calculate the concentration of OTC in the tissues according to the following formula

$$\mu g \text{ OTC/g tissue} = \frac{At \times Ve}{\overline{RFotc} \times W \times R}$$

A$_t$ is the integrated peak area for the sample

V$_e$ is the volume of final sample extract

W is the weight of sample extracted (gm)

R is the tissue specific recovery determined from the mean of the spiked matrix samples

The criterion of acceptance is that the Relative Standard Deviation (see #7) must be ≤20%.

## 3. Calibration Verification

One mid-range standard was selected  from the initial calibration standards and used to verify calibration.  This was the calibration check sample. At minimum, a calibration check sample was analyzed after initial calibration or recalibration, after approximately 10 unknown samples and at the end of the sample analysis. The criteria of acceptance is that the percent difference between the $\overline{RFotc}$ and the RF of the calibration check sample must be <30%.

## 4. Determination of Method Detection Limit (MDL)

MDL is defined as the minimum concentration of OTC in a tissue matrix that can be measured and reported with 99 percent confidence that the concentration is greater than zero.  The MDL is determined by multiplying the n-1 degrees of freedom of the one-sided 99 percent Student's t-statistic ($t_{0.99}$) by the standard deviation obtained from a minimum of seven replicate analyses of a spiked matrix sample containing the analyte of interest at a concentration 3-5 fold of the estimated MDL. The criterion of acceptance for the MDL of OTC in each of the tissue matrix was set at > 0.1 µg/g.

## 5. Limit of detection (LOD)

The LOD is the response value of the HPLC detector that is 3 times the standard

deviation of the background response to the mean background response.

## 6. Limit of Quantitation (LOQ)

The LOQ is the response value of the HPLC detector that is 10 times the standard deviation of the background response to the mean background response.

7. The Relative Standard Deviation (RSD) is defined as the quotient of the standard deviation (SD) divided by the mean (m) and is expressed in %.

$$RSD = 100 \ SD/m$$

8. The Relative Percent Difference (RPD) is used to assess the precision between duplicates

$$RPD = 100\{(x_1-x_2)/[(x_1+x_2)/2]\}$$

$x_1$ - First number of comparison

$x_2$ - Second number of comparison

Quality Assurance Data

Table C-1. Method detection limit in serum

| Concentration* (μg/ml) | |
|---|---|
| | 0.3766 |
| | 0.4182 |
| | 0.4001 |
| | 0.4426 |
| | 0.3529 |
| | 0.4061 |
| | 0.3879 |
| Mean | 0.3978 |
| SD | 0.0291 |
| t-statistic** | 3.14 |
| | |
| MDL= | 0.0912 μg/ml |

*Originally spiked with 0.5μg/ml
**t-statistic- represents the 99% confidence level of 6 degrees of freedom of a one-sided $t$ distribution.

Table C-2 Percent serum recovery of OTC from serum

|  | Concentration* (µg/ml) |
|---|---|
|  | 1.1403 |
|  | 0.9547 |
|  | 0.8680 |
|  | 0.9807 |
|  | 0.7737 |
|  | 1.1844 |
| Mean | 0.9836 |
| SD | 0.1570 |

*Spiked at a concentration of 1 µg/ml OTC

Table C-3 Blanks and matrix spikes

| Date | Serum Blank | Methanol Blank | Spike Recovery |
|---|---|---|---|
| 19-Aug-96 | NPD* | NPD | 100% |
| 20-Aug-96 | NPD | NPD | 106% |
|  |  |  | 107% |
| 21-Aug-96 | NPD | NPD | 74% |
|  |  |  | 81% |
| 22-Aug-96 | NPD | NPD | 76% |
|  |  |  | 85% |
| 28-Aug-96 | NPD | NPD | 71% |
|  |  |  | 79% |
| 29-Aug-96 | NPD | NPD | 73% |
|  |  |  | 81% |
| 30-Aug-96 | NPD | NPD | 83% |
|  |  |  | 97% |
| 31-Aug-96 | NPD | NPD | 92% |
|  |  |  | 100% |
| 4-Sep-96 | NPD | NPD | 91% |
|  |  |  | 88% |
| 5-Sep-96 | NPD | NPD | 91% |
|  |  |  | 80% |
| 6-Sep-96 | NPD | NPD | 87% |
|  |  |  | 84% |
| 10-Sep-96 | NPD | NPD | 161%** |
|  |  |  | 121% |
| 11-Sep-96 | NPD | NPD | 128% |
|  |  |  | 134% |
| 13-Sep-96 | NPD | NPD | 117% |
|  |  |  | 122% |
| 14-Sep-96 | NPD | NPD | 63% |
|  |  |  | 99% |
|  |  | Mean | 0.9550 |
|  |  | SD | 0.2193 |

* Non Detectable
** The high value is due to low recovery of the Tetracycline

Table C-4. Serum study $\overline{RF_{otc}}$ and RSD

|  |  | RSD |
| --- | --- | --- |
| Period one | 1.0989 | 22% |
| Period two | 1.0753 | 27% |
| Study | 1.0854 | 25% |

Table C-5. Calibration data used to calculate $\overline{RF_{otc}}$ and RSD

| Concentration μg/ml | OTC Peak area | TC Peak area | RRF | Concentration μg/ml | OTC Peak area | TC Peak area | RRF |
| --- | --- | --- | --- | --- | --- | --- | --- |
| 0.1 | 2.8751 | 27.7986 | 1.0343 | 1 | 38.9594 | 34.6667 | 1.1238 |
| 0.1 | 4.202 | 33.1453 | 1.2678 | 1 | 36.6553 | 39.8499 | 0.9198 |
| 0.1 | 5.7056 | 28.254 | 2.0194 | 1 | 45.0122 | 41.8125 | 1.0765 |
| 0.1 | 4.1958 | 37.0009 | 1.1340 | 1 | 41.72 | 39.1786 | 1.0649 |
| 0.1 | 4.4344 | 28.4468 | 1.5588 | 1 | 44.4578 | 39.1307 | 1.1361 |
| 0.1 | 5.335 | 33.3708 | 1.5987 | 1 | 44.2942 | 43.8538 | 1.0100 |
| 0.1 | 5.3311 | 35.5516 | 1.4995 | 1 | 39.2575 | 40.9876 | 0.9578 |
| 0.1 | 4.6001 | 33.8922 | 1.3573 | 3 | 122.18 | 37.6628 | 1.0813 |
| 0.1 | 6.1061 | 37.9585 | 1.6086 | 5 | 201.14 | 47.177 | 0.8527 |
| 0.1 | 5.0289 | 34.3862 | 1.4625 | 5 | 196.38 | 37.9663 | 1.0345 |
| 0.1 | 5.5858 | 31.1145 | 1.7952 | 5 | 197.28 | 39.3608 | 1.0024 |
| 0.1 | 3.5104 | 37.3205 | 0.9406 | 5 | 205.5 | 40.1193 | 1.0244 |
| 0.1 | 4.3219 | 37.3919 | 1.1558 | 5 | 201.34 | 47.4612 | 0.8484 |
| 0.1 | 2.9358 | 31.3028 | 0.9379 | 5 | 206.88 | 52.3849 | 0.7898 |
| 0.2 | 8.4684 | 31.5238 | 1.3432 | 7 | 278.17 | 44.7426 | 0.8882 |
| 0.2 | 8.495 | 34.2891 | 1.2387 | 10 | 419.59 | 49.4417 | 0.8487 |
| 0.5 | 20.8753 | 31.4785 | 1.3263 | 10 | 419.27 | 41.4415 | 1.0117 |
| 0.5 | 21.846 | 35.7593 | 1.2218 | 10 | 399.73 | 46.6227 | 0.8574 |
| 0.5 | 17.2475 | 31.9788 | 1.0787 | 10 | 427.54 | 50.5896 | 0.8451 |
| 0.5 | 19.8084 | 34.4274 | 1.1507 | 10 | 414.99 | 48.9686 | 0.8475 |
| 0.5 | 21.0884 | 37.3699 | 1.1286 | 10 | 422.69 | 43.2075 | 0.9783 |
| 1 | 40.3985 | 38.9777 | 1.0365 | 10 | 405.58 | 41.669 | 0.9733 |
| 1 | 39.1652 | 32.7826 | 1.1947 | 10 | 388.12 | 41.7958 | 0.9286 |
| 1 | 36.9824 | 30.6093 | 1.2082 | 10 | 400.57 | 44.6924 | 0.8963 |
| 1 | 38.579 | 31.6162 | 1.2202 | 10 | 415.65 | 55.5646 | 0.7480 |
| 1 | 37.7941 | 38.1733 | 0.9901 | 10 | 423.92 | 50.0191 | 0.8475 |
| 1 | 41.2873 | 36.3362 | 1.1363 | 10 | 415.32 | 54.5082 | 0.7619 |
| 1 | 41.1541 | 33.4745 | 1.2294 | 10 | 431.94 | 66.6632 | 0.6479 |
| 1 | 38.0171 | 30.9494 | 1.2284 | 10 | 424.98 | 59.8483 | 0.7101 |
| 1 | 39.004 | 32.2878 | 1.2080 | 10 | 441.14 | 58.5755 | 0.7531 |
| 1 | 35.0364 | 30.4804 | 1.1495 | 10 | 439.22 | 63.3391 | 0.6934 |
|  |  |  |  | 10 | 433.18 | 56.7455 | 0.7634 |

## Table C-6 Calibration check standards data

| Date | RRF | % difference from | Date | RRF | % difference from | Date | RRF | % difference from |
|---|---|---|---|---|---|---|---|---|
| 8/19/96 | 0.9957 | 9% | 8/30/96 | 1.2551 | 14% | 9/5/96 | 1.0539 | 3% |
| | 1.0029 | 8% | | 1.2030 | 10% | | 1.0025 | 8% |
| | 1.0707 | 1% | | 1.1441 | 5% | | 1.0581 | 3% |
| | 1.0716 | 1% | | 1.1900 | 9% | | 1.0540 | 3% |
| | 1.0466 | 4% | | 1.1321 | 4% | | 1.0468 | 4% |
| | 0.9670 | 12% | | 1.0085 | 7% | | 1.0030 | 8% |
| | 0.9908 | 9% | | 1.1050 | 2% | | 0.9992 | 8% |
| 8/20/96 | 1.0897 | 0% | | 1.0916 | 1% | 9/6/96 | 0.9974 | 8% |
| | 1.0646 | 2% | 8/31/96 | 1.2564 | 15% | | 1.1120 | 2% |
| | 0.9816 | 10% | | 1.3855 | 24% | | 0.9411 | 14% |
| | 0.9054 | 18% | | 1.1780 | 8% | | 0.8682 | 22% |
| | 0.8984 | 19% | | 1.2322 | 13% | | 0.9168 | 17% |
| | 1.0950 | 1% | | 1.0730 | 1% | | 0.8790 | 21% |
| 8/21/96 | 1.2492 | 14% | | 1.0847 | 0% | | 0.8671 | 22% |
| | 1.1926 | 9% | | 1.0894 | 0% | 9/10/96 | 0.9301 | 15% |
| | 1.2013 | 10% | 9/1/96 | 1.2922 | 17% | | 0.8842 | 20% |
| | 1.0978 | 1% | | 1.1050 | 2% | | 0.9291 | 16% |
| | 1.1569 | 6% | | 1.1872 | 9% | 9/11/96 | 1.0953 | 1% |
| | 1.1710 | 8% | 9/3/96 | 1.1656 | 7% | | 0.9995 | 8% |
| | 1.0282 | 5% | 9/3/96 | 1.1566 | 6% | | 1.0400 | 4% |
| 8/22/96 | 0.9687 | 11% | | 1.1544 | 6% | | 1.0882 | 0% |
| | 1.0317 | 5% | | 1.2340 | 13% | | 0.9697 | 11% |
| | 0.9225 | 16% | 9/4/96 | 1.3547 | 22% | 9/12/96 | 1.0779 | 1% |
| | 1.0138 | 7% | | 1.1439 | 5% | | 1.1604 | 7% |
| 8/28/96 | 1.0069 | 8% | | 1.1252 | 4% | 9/13/96 | 0.9806 | 10% |
| | 1.0062 | 8% | 9/4/96 | 1.2586 | 15% | | 1.0680 | 2% |
| | 1.0564 | 3% | | 1.0832 | 0% | | 1.0708 | 1% |
| | 0.9793 | 10% | | 1.1480 | 6% | | 1.0318 | 5% |
| 8/29/96 | 1.0655 | 2% | | 1.1329 | 4% | | 1.0408 | 4% |
| | 1.0981 | 1% | | 1.0257 | 6% | 9/14/96 | 1.0481 | 4% |
| | 1.1384 | 5% | | 1.1121 | 2% | | 1.0610 | 2% |
| | 1.0249 | 6% | | 1.0963 | 1% | 9/14/96 | 1.0469 | 4% |
| | 1.0902 | 0% | | | | | 0.9331 | 15% |
| | 1.0318 | 5% | | | | | 0.9361 | 15% |

**Table C-7. Serum stability**

| Number of days in storage | Concentration* µg/ml | % Change |
|---|---|---|
| 6 | 0.9569 | -1% |
| 9 | 0.8566 | -4% |
| 16 | 0.9198 | -11% |
| 18 | 0.9861 | -14% |
| 26 | 0.8868 | -3% |
| 26 | 0.9380 | -8% |
| 26 | 1.1099 | 1% |
| 27 | 1.0139 | -8% |
| 27 | 0.9231 | -6% |
| 33 | 0.9682 | 11% |
| 43 | 0.8970 | -10% |
| 43 | 1.1146 | 11% |
| 43 | 0.8948 | -11% |
| Mean | 0.9589 | |
| SD | 0.0805 | |

*Samples were initially spiked at 1 µg/ml*

Table C-8. Method detection limits of tissues

| | Liver | | Kidney | | Muscle | | Fat |
|---|---|---|---|---|---|---|---|
| | Concentration* (µg/g) | | Concentration (µg/g) | | Concentration (µg/g) | | Concentration (µg/g) |
| | 0.4988 | | 0.4884 | | 0.4533 | | 0.5082 |
| | 0.4893 | | 0.4754 | | 0.4387 | | 0.4209 |
| | 0.5168 | | 0.5039 | | 0.4432 | | 0.4328 |
| | 0.5304 | | 0.4941 | | 0.4856 | | 0.4442 |
| | 0.5207 | | 0.4617 | | 0.4373 | | 0.4252 |
| | 0.4836 | | 0.5612 | | 0.4676 | | 0.4589 |
| | 0.5370 | | 0.4833 | | 0.5077 | | 0.4728 |
| mean | 0.5109 | mean | 0.4954 | mean | 0.4619 | mean | 0.4519 |
| SD | 0.0206 | SD | 0.0320 | SD | 0.0267 | SD | 0.0310 |
| t-statistic** | 3.14 | t-statistic | 3.14 | t-statistic | 3.14 | t-statistic | 3.14 |
| MDL= | 0.0647 | MDL= | 0.1004 | MDL= | 0.0837 | MDL= | 0.0973 |

*Originally spiked with 0.5µg/g*
**t-statistic- represents the 99% confidence level of 6 degrees of freedom of a one-sided *t* distribution.

# Spike Recovery and Tissue Blanks Results

Table C-9. Spike recoveries and tissue blanks - Liver, kidney, muscle, fat

| Liver | Spike Recovery | Tissue Blanks | Kidney | Spike Recovery | Tissue Blanks |
|---|---|---|---|---|---|
| 31-Jan-97 | 81% | NPD | 1-Feb-97 | 83% | NPD |
| | 80% | NPD | | 80% | NPD |
| | 81% | | | 85% | |
| | 85% | | | 78% | |
| | 99% | | | 79% | |
| 4-Feb-97 | 79% | | 4-Feb-97 | 81% | |
| 8-Feb-97 | 93% | NPD | | | |
| Mean | 85% | | Mean | 81% | |
| SD | 8% | | SD | 3% | |
| Muscle | Spike Recovery | Tissue Blanks | Fat | Spike Recovery | Tissue Blanks |
| 2-Feb-97 | 87% | NPD | 3-Feb-97 | 80% | NPD |
| | 86% | NPD | | 97% | NPD |
| | 106% | | | 97% | |
| | 94% | | | 98% | |
| | 96% | | | 92% | |
| 4-Feb-97 | 103% | | 4-Feb-97 | 99% | |
| Mean | 95% | | Mean | 94% | |
| SD | 8% | | SD | 7% | |

Table C-10. Spike recoveries and tissue blanks -Injection sites

| Injection Site | Spike Recovery | Tissue blanks |
|---|---|---|
| 10-Oct-96 | 85% | NPD |
|  | 85% | NPD |
|  | 100% |  |
|  | 86% |  |
|  | 89% |  |
| Mean | 89% |  |
| SD | 6% |  |
| 15-Oct-96 | 78% | NPD |
|  | 75% | NPD |
|  | 90% |  |
|  | 86% |  |
|  | 93% |  |
| Mean | 84% |  |
| SD | 8% |  |
| 16-Oct-96 | 74% | NPD |
|  | 87% | NPD |
|  | 110% |  |
|  | 80% |  |
|  | 103% |  |
| Mean | 91% |  |
| SD | 15% |  |
| Overall Mean | 88% |  |
| Overall SD | 10% |  |

Table C-11. Methanol blanks

| Date | Methanol Blank |
|---|---|
| 17-Sept-96 | NPD |
| 10-Oct-96 | NPD |
| 15-Oct-96 | NPD |
| 16-Oct-96 | NPD |
| 17-Oct-96 | NPD |
| 18-Oct-96 | NPD |
| 31-Oct-96 | NPD |
| 31-Jan-97 | NPD |
| 1-Feb-97 | NPD |
| 2-Feb-97 | NPD |
| 3-Feb-97 | NPD |
| 4-Feb-97 | NPD |
| 8-Feb-97 | NPD |

*NPD indicates no peak was detected.

Table C-12. Mean retention factor and relative standard deviation

| Method used | $\overline{RF_{otc}}$ | RSD |
|---|---|---|
| OTC external | 40.2081 | 15% |

Table C-13. Calibration data to calculate $\overline{RF_{otc}}$ and RSD

| Concentration µg/g. | Peak area | RF | Concentration µg/g | Peak area | RF |
|---|---|---|---|---|---|
| 0.1 | 4.1539 | 41.5390 | 5 | 199.61 | 39.9220 |
| 0.1 | 2.6899 | 26.8990 | 5 | 188.35 | 37.6700 |
| 0.1 | 3.9312 | 39.3120 | 5 | 183.71 | 36.7420 |
| 0.1 | 3.4277 | 34.2770 | 5 | 191.2 | 38.2400 |
| 0.1 | 4.1830 | 41.8300 | 10 | 408.15 | 40.8150 |
| 0.1 | 4.1467 | 41.4670 | 10 | 439.17 | 43.9170 |
| 0.1 | 5.0690 | 50.6900 | 10 | 364.25 | 36.4250 |
| 0.1 | 3.7651 | 37.6510 | 10 | 411.61 | 41.1610 |
| 0.1 | 5.0099 | 50.0990 | 10 | 400.21 | 40.0210 |
| 0.1 | 6.3624 | 63.6240 | 10 | 439.21 | 43.9210 |
| 0.5 | 17.1601 | 34.3202 | 10 | 457.15 | 45.7150 |
| 0.5 | 17.8550 | 35.7100 | 10 | 397.46 | 39.7460 |
| 0.5 | 16.6514 | 33.3028 | 10 | 374.93 | 37.4930 |
| 0.5 | 17.9208 | 35.8416 | 10 | 431.65 | 43.1650 |
| 1 | 42.8304 | 42.8304 | 10 | 443.64 | 44.3640 |
| 1 | 44.5924 | 44.5924 | 20 | 862.19 | 43.1095 |
| 1 | 33.3874 | 33.3874 | 20 | 786.21 | 39.3105 |
| 1 | 32.9800 | 32.9800 | 20 | 751.86 | 37.5930 |
| 1 | 40.5460 | 40.5460 | 20 | 844.17 | 42.2085 |
| 1 | 41.9724 | 41.9724 | 20 | 887.68 | 44.3840 |
| 1 | 33.3827 | 33.3827 | 50 | 2283.95 | 45.6790 |
| 1 | 29.0430 | 29.0430 | 50 | 2011.82 | 40.2364 |
| 1 | 33.9843 | 33.9843 | 50 | 1904.77 | 38.0954 |
| 1 | 40.7077 | 40.7077 | 50 | 2176.56 | 43.5312 |
| 1 | 46.7456 | 46.7456 | | | |

## Table C-14. Calibration check standards

| Date | RF | % difference from $\overline{RFotc}$ t | Date | RF | % difference from $\overline{RFotc}$ |
|---|---|---|---|---|---|
| 10-Oct-96 | 34.6608 | 14.8% | 29-Jan-97 | 39.5359 | 1.7% |
| | 35.1342 | 13.5% | | 43.4039 | 7.6% |
| | 36.8468 | 8.7% | | 38.2089 | 5.1% |
| | 35.9786 | 11.1% | | 34.1556 | 16.3% |
| | 39.1628 | 2.6% | | 41.9088 | 4.1% |
| | 38.0303 | 5.6% | 1-Feb-97 | 40.3053 | 0.2% |
| | 37.2478 | 7.6% | | 32.7157 | 20.5% |
| 11-Oct-96 | 37.0430 | 8.2% | | 34.4805 | 15.3% |
| | 35.1598 | 13.4% | | 32.9493 | 19.8% |
| | 35.4051 | 12.7% | | 36.3345 | 10.1% |
| | 35.7728 | 11.7% | | 43.8110 | 8.6% |
| | 36.3739 | 10.0% | 2-Feb-97 | 30.1118 | 28.7% |
| 15-Oct-96 | 34.0860 | 16.5% | | 39.6635 | 1.4% |
| | 33.9111 | 17.0% | | 39.2546 | 2.4% |
| | 33.3471 | 18.7% | | 44.9048 | 11.0% |
| | 31.1310 | 25.4% | | 37.0108 | 8.3% |
| | 40.4916 | 0.7% | | 37.9456 | 5.8% |
| | 38.4783 | 4.4% | 3-Feb-97 | 37.7062 | 6.4% |
| | 43.0929 | 6.9% | | 37.5120 | 6.9% |
| 16-Oct-96 | 31.7069 | 23.6% | | 38.0749 | 5.5% |
| | 40.1807 | 0.1% | | 41.1624 | 2.3% |
| | 36.2897 | 10.2% | | 34.6351 | 14.9% |
| | 32.0699 | 22.5% | | 37.4889 | 7.0% |
| 18-Oct-96 | 45.1599 | 11.6% | 4-Feb-97 | 36.8189 | 8.8% |
| | 44.2329 | 9.5% | | 38.4172 | 4.6% |
| | | | 8-Feb-97 | 42.5050 | 5.6% |
| | | | | 44.9526 | 11.1% |

Table C-15. Relative percent difference between duplicates

Liver, Kidney, Fat, Muscle

| Calf ID | Liver | Kidney | Muscle | Fat |
|---------|-------|--------|--------|-----|
| **4 day** | | | | |
| 650 | 10.77 | 5.67 | 1.67 | 0.00[a] |
| 656 | 6.68 | 13.24 | 28.39 | - |
| 672 | 27.53 | 2.15 | 1.84 | - |
| 674 | 3.99 | 14.86 | 7.10 | - |
| **10 day** | | | | |
| 654 | 14.48 | 3.74 | 9.37 | - |
| 667 | 15.67 | 0.78 | 6.17 | 0.00[a] |
| 668 | 2.77 | 16.30 | 0.00[a] | - |
| 678 | 24.81 | 6.26 | 0.00[a] | - |
| **16 day** | | | | |
| 657 | 11.02 | 16.21 | - | - |
| 671 | 13.44 | 5.32 | - | - |
| 675 | 24.04 | 10.92 | - | - |
| 676 | 2.81 | 3.20 | - | - |
| **22 day** | | | | |
| 653 | 20.98 | 20.95 | - | - |
| 661 | 12.62 | 2.39 | - | - |
| 666 | 0.00[a] | 19.89 | - | - |
| 673 | - | - | - | - |
| **28 day** | | | | |
| 663 | - | - | - | - |
| 664 | 15.05 | 3.11 | - | - |
| 669 | - | - | - | - |
| 683 | - | - | - | - |
| **35 day** | | | | |
| 658 | - | - | - | - |
| 660 | - | - | - | - |
| 680 | - | - | - | - |
| 684 | - | - | - | - |

(a) RPD is shown as zero since both tissue concentrations were below the MDL.
- Indicates either both samples were NPD or one was NPD and the other was below the MDL

**Appendix D** Mass balance differential and algebraic equations describing the PBPK model of OTC in cattle:

Lung: $\quad$ VLN(dCGL/dt) $= (QT * (CBV - CGL / RGL))$

Arterial Blood: $\quad$ VB(dCBA/dt) $= (QT * (CGL / RGL - CBA))$

RInjsite: $\quad$ -KA * InJsite

DInjsite: $\quad$ RInjsite * dt

Muscle: $\quad$ VEM(dCEM/dt) $= (QM * (CBA - CM/RM)$

Gut: $\quad$ VGT(dCGT/dt) $= (QGT * (CBA - CGT / RGT))$

Liver: $\quad$ VL(dCL/dt) $= (QL/QGT/VL * CBA + QGTVLRGT * CGT -$

$\quad$ QL/VL/RL * CL – KL/RL * CL)

Carcass: $\quad$ VC(dCC/dt) $= QC * (CBA - CC / RCA)$

Kidney $\quad$ VK(dCK/dt) $= ((QK * (CBA - CK / RK)) - (VMX/RK * CK) / (KM +$

$\quad$ CK / RK))

Fat: $\quad$ VF(dCF/dt) $= (QF) * (CBA - CF / RF)$

Venous Blood: $\quad$ VB(dCBV/dt) $= (QL/RL * CL + QM * CEM + QC/RCA * CC +$

$\quad$ QK/RK * CK + CF * QF/RF - QT * CBV) + (-RInjsite / VB)

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