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Studies on muscle residues of the anti-bacterial veterinary medicines tilmicosin, enrofloxacin and tiamulin in livestock and the risk to consumers from ingestion of injection-sites

Jeremiah Gabriel Beechinor

A dissertation submitted for the Degree of Doctor of Philosophy

Department of Biochemistry,
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September, 2000

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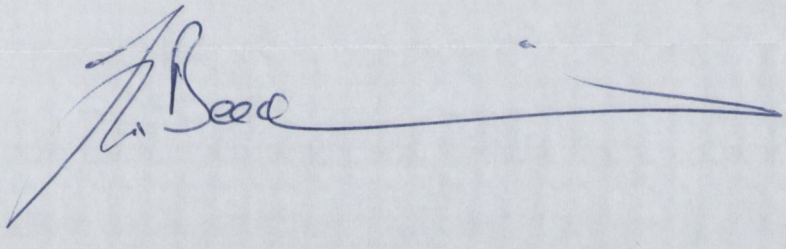
Signed 

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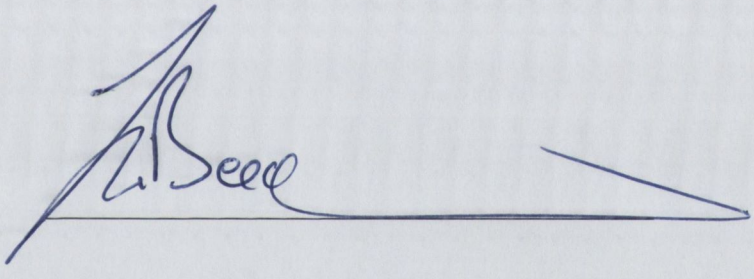

Jeremiah Gabriel BEECHINOR

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Beannacht dé oraibh go léir.

Dedicated to my parents and especially to my late father, Jerry Beechinor who led an extraordinary, inspirational life.

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LIST OF WORKS BASED ON THIS THESIS ALREADY PUBLISHED OR SUBMITTED FOR PUBLICATION

Title	Work type	Journal/Congress	Current Status
Prevalence and public health significance of blemishes in primal and subprimal cuts of Irish beef	Paper	Veterinary Record	Accepted for publication, in press
Prevalence of injection-site blemishes in primal cuts of Irish pork	Paper	Irish Veterinary Journal	Accepted for publication, in press
Prevalence of injection-site blemishes in Irish beef primal cuts	Oral presentation	European Association of Veterinary Pharmacology and Toxicology	Presented, August 1, 2000
Distribution of residues of tilmicosin in muscles of cattle	Poster	European Association of Veterinary Pharmacology and Toxicology	Presented, July 30 - August 3, 2000
Effect of cooking on residues of tiamulin in beef	Poster	European Association of Veterinary Pharmacology and Toxicology	Presented, July 30 - August 3, 2000

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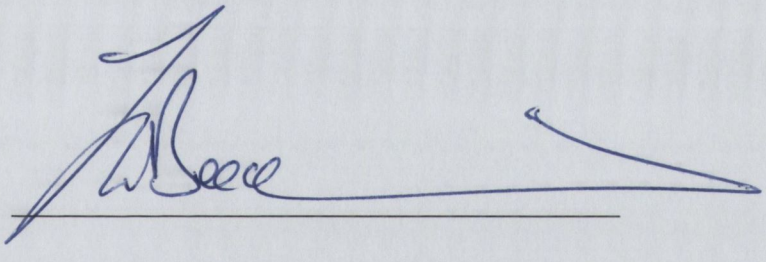


Jeremiah Gabriel BEECHINOR

Summary

Studies on incurred residues of the veterinary anti-bacterials tilmicosin, enrofloxacin and tiamulin were undertaken in cattle, sheep and pigs in order to investigate the effects of cooking on injection-site residues and to evaluate their potential hazards for public health. The studies also included a survey of more than 2500 blemishes in primal and subprimal portions of Irish beef, mutton and pork which provided information both on the extent of tissue damage and on the prevalence of residues of anti-bacterials at these sites. These data, together with published information, were used to conduct qualitative and quantitative risk assessments on consumer exposure to residues of anti-bacterials at injection-sites. The research had two significant secondary objectives: firstly to compare of the performance of the 3 analytical methods used for residue determination in each of the target species and secondly, an investigation into the distribution of the 3 drugs within the musculature of the target species.

The method of investigation for the residue analytical studies involved the development and validation of two chemically-based methods and one bioassay for analysis of muscle residues. The performance parameters established served as a basis for subsequent studies on a) the comparison of method performance in each species b) the distribution of residues within the musculature and c) the effects of cooking injection-site residues included both the effects of microwaving and boiling. The method of investigation of the blemish survey involved an analysis of blemishes in primal and subprimal meat portions in beef, mutton and pork from meat factories throughout Ireland and represents the produce of more than 11,000 cattle, 25,500 sheep and 10,500 pigs.

The studies on method development demonstrated the complexities of developing an analytical method for residue analysis in muscle with the required performance characteristics. The methods displayed essentially similar performance in all target species with comparable levels of accuracy and limits of quantification and absence

of matrix interference and may assist in the extrapolation of residue methods across the species barrier. There was marked variation in the residue concentrations found in the musculature of animals, both between individual muscles and within the same muscle. This result may have significance both for residue surveillance and for the future conduct of residue depletion studies in animals. The effect of cooking was an overall reduction in the concentration of drug residues present of from 0 – 17% for microwaving and 12 - 46% for boiling. The extent of the reduction was dependent on the drug tested and the cooking method used. The results of survey indicate that while the prevalence of blemishes in beef represent a considerable quality assurance problem for the beef industry, blemishes were not prevalent in pork and mutton. Inhibitory substances (anti-bacterials) were detected in only 0.4% of samples tested. The data generated in the risk assessment are an important contribution to the understanding of the risks to consumers of ingestion of injection-sites in meat and demonstrate that the probability of ingestion of harmful residues from injection-sites containing anti-bacterials is negligible. This information is expected to have application in defining international scientific policy on injection-site residues and the establishment of withdrawal periods for veterinary drugs.

ABBREVIATIONS

ADI	acceptable daily intake
^0C	degrees Centigrade
c.f.u	colony forming units
cm	centimetre
conc.	Concentration
CCRVDF	Codex Committee on Residues of Veterinary Drugs in Food
CV	coefficient of variation
CVMP	Committee for Veterinary Medicinal Products
DES	diethylstilboestrol
Dept.	Department
EC	European Commission
EMEA	European Medicines Evaluation Agency
EU	European Union
FAO	Food Agricultural Organisation
FEDESA	European Federation of Animal Health
g	gram
GLC	gas liquid chromatography
µg	microgram
h	hour
HCl	hydrochloric acid
HPLC	high performance liquid chromatography
H_2O	water

i.e.	that is
IEC	Irish Equine Centre
im	intramuscular
JECFA	Joint Expert Committee on Food Additives
kg	kilogram
l	litre
LAOTC	long-acting oxytetracyline
LOD	limit of detection
LOQ	limit of quantification
M	molar
MAVIS	Medicines Act Veterinary Information Service
mg	milligram
min	minute
ml	millilitre
mm	millimetre
MRL	maximum residue limit
n or No.	number
na	not analysed
n.app	not applicable
nd	not detected (concentrations below limit of detection)
ng	nanogram
nm	nanometres
nq	not quantifiable (concentrations below limit of quantification)
nz	no zone of inhibition

pg	picogram
pK_a	dissociation constant
p.s.i.	pounds per square inch
r^2	correlation coefficient
RIA	radioimmunoassay
sc	subcutaneous
SD	standard deviation
SEM	standard error of mean
SRWP	Safety of Residues Working Party
SSC	Scientific Steering Committee
$t_{½\beta}$	half-life of elimination
temp.	temperature
T_{max}	time to maximum concentration
TMDI	theoretical maximum daily intake
TVC	total viable count
UK	United Kingdom
USA	United States of America
UV	ultraviolet
Vd	volume of distribution
VICH	Veterinary International Conference on Harmonisation
W	watt
WHO	World Health Organisation
%	percent

CHAPTER 1: INTRODUCTION

1.1 Background

Veterinary medicines are an essential tool in the production of healthy animals. According to the European Association for Animal Health [FEDESA] (1995) without them the number of chickens, pigs, sheep and cattle would have to increase by 25%, 28%, 54% and 89% respectively to reach the same production level achieved today. However, the use of xenobiotics may lead to the presence of residues in animal tissues and produce. The hazards posed to consumers by veterinary drugs are well known. Huber (1971) reported that drug residues in contaminated meat could produce:

a) direct toxic effects ranging from mild sensitising responses to anaphylactic shock,
b) intoxication of the liver, haematopoietic centres and kidney,
c) indirect effects on the body's enteric flora that might become resistant to antibiotics.

Dayan (1993) reported that case of proven allergy to residues of anti-bacterial substances in food are extremely rare, whereas there have been less substantiated reports blaming antimicrobial substances in up to 50% of cases of chronic urticaria. Nicholls *et al* (1994) noted that although the general community perceives chemical residues as a major health risk, the risk of food causing illness or death because of chemical residues is low. Paige *et al* (1997) concurred with this, noting that most residues of veterinary drugs occurred in food at such low levels that they rarely posed a chronic or long-term health hazard to consumers. In order to safeguard consumers of animal tissues from residues which might affect their health, drug regulatory and food safety authorities set legally permitted maxima for edible tissues and produce of treated animals. These maximum residue limits (MRLs) are defined in European Community law (Council Regulation 2377/90) and by the world body on food standards, Codex Alimentarius, as being the maximum concentration of residue which may be legally permitted or recognised as acceptable in or on a food. The MRLs are designated in relation to the acceptable daily intake (ADI) of residues of

each particular drug and are established on the presumption of chronic (life-time) exposures to residues up to the permitted maxima. The ADI is in turn based on concentrations shown to be without adverse effects on humans or animals.

Government officials in each Member State of the EU undertake the monitoring of residues of veterinary medicines. Recent data from the UK's Veterinary Medicines Directorate (MAVIS, 2000) revealed a very low proportion of residues of veterinary medicines. Of 22,312 red meat samples analysed for the surveillance programme during 1999, no residues of antibiotics, synthetic steroids, beta-agonists or natural hormones were detected. The samples taken were mainly from the liver and kidney. In Northern Ireland, Currie *et al* (1998) reported that of 1,830 muscle samples taken from cattle and sheep between 1994 and 1996 and screened for the presence of anti-bacterials, 38 (2%) had positive results. Of the positive samples, 36 were of bovine origin and 2 were of ovine origin. The 2 ovine carcasses contained penicillin G as did 10 bovine carcasses. A further 17 bovine carcasses contained oxytetracycline, with sulphonamides detected in 4 others. A single carcass contained chlortetracycline and 4 carcasses contained antimicrobial residues that could not be identified. These data give some reassurance to the consumer on the risk of residues in foodstuffs.

Residue data for monitoring for anti-bacterial substances, including sulphonamides and quinolones, taken in the slaughterhouse in accordance with the national residue control plan for Ireland as submitted to the European Commission are outlined in Table 1-1 below.

Table 1-1 Results of analyses of meat for anti-bacterial residues in Ireland for 1998, taken from report of the Dept. of Agriculture to the European Commission

Species	Number of samples	Number positive for anti-bacterial residues	% above MRL
Bovine	1488	23	1.55
Ovine	722	0	0.00
Porcine	41070	349	0.85
All species	43280	372	0.85

Residue data taken from targeted sampling for prohibited substances, including stilbenes, stilbene derivatives, steroids, resorcyclic acid lactones, beta-agonists and compounds included in Annex IV of Council Regulation (EEC) No. 2377/90, in accordance with the national residue control plan for Ireland as submitted to the European Commission are outlined in Table 1-2 below.

Table 1-2 Results of analyses of meat for prohibited substances in Ireland for 1998, taken from the report of the Dept. of Agriculture to the European Commission

Species	Number of samples	Number positive for prohibited substances	% positive
Bovine	45724	16	0.035
Ovine	349	1	0.287
Porcine	754	0	0.000
All species	46827	17	0.036

These data are in line with those reported by Ritter (2000) who stated that the vast majority of food commodities analysed for residues of veterinary drugs had residues well within the national MRLs with most being undetected. Where residues were present at all, they were typically present in only a small proportion of the overall samples and only at very low levels.

While MRLs are used by monitoring laboratories to ensure foodstuffs are safe to eat, they are also used by regulatory authorities responsible for licensing of veterinary medicinal products as the basis for evaluating the withdrawal period of the relevant medicinal product (the time between the last administration of a veterinary medicinal product and the slaughter of the treated animal for human consumption). MRLs for muscle, fat, liver and kidney reflect the pharmacokinetic behaviour and distribution of the compound. Some classes of compounds e.g. aminoglycosides are bound to renal tissue and persist there for several months. Other compounds, e.g. imidocarb persist in hepatic tissue. Thus residues that persist above the MRL in the target tissues lead to long withdrawal periods. As drugs given parenterally must be absorbed from the injection-site and distributed to the organs and other tissues of the animal's body, the concentration of residues at the injection-site will be higher than that in other parts of the carcass for some time. Moreover, some veterinary medicines administered by intramuscular or subcutaneous injection are irritant and cause tissue reactions at the site of injection. At this site (or in the underlying muscle beneath the injection in the case of drugs given by the subcutaneous route), residues might exceed the MRL established for muscle tissue for a period of time until the injected mass is fully absorbed.

The risk for consumers who daily consume meat containing residue concentrations at the MRL is different from that of consumers who may occasionally ingest an injection-site residue at or above the MRL. Currently, within the EU, the MRL set for muscle also applies to residues present at the site of injection (CVMP, 1994). This has resulted in many veterinary medicinal products having an extremely long withdrawal period (several weeks or months duration). Nouws (1990) stated that prescribed withdrawal periods of longer than 4 weeks are impractical in that they are difficult to control effectively.

Jones (1994) noted that there was a high degree of variability in the persistence of residues at the injection-site. He stated that this might be due in part to a differing metabolic profile of the product depending on whether its residue lay in the muscle tissue itself or in the intermuscular fascia and noted that the variability appeared to

increase as time from injection to slaughter increased. Galer and Monro (1996) reported that there was only a remote chance of a consumer ingesting an injection-site. They estimated that the frequency of human exposure to injection-sites was approximately 0.01 - 0.1% or, for a daily meat eater, 1 day every 3 - 30 years. In reaching this estimate, the authors assumed that:

a) the meat 'portion' containing the injection-site comprised 0.1 - 1.0% of the edible muscular portion of the carcass,
b) the injection-sites were used indiscriminately along with other muscle to prepare meat for consumption,
c) many injection-sites were trimmed off,
d) a particular drug had limited use in a cohort of animals requiring treatment,
e) the dosage or seasonal pattern of use of a drug limited the number of injections given and the presence of the residue at slaughter,
f) few persons ate the same meat, i.e. beef, pork, lamb/mutton, repetitively, every day.

Stephany (1988) estimated the probability of a consumer ingesting an injection-site from beef during a lifetime at 1 in 21 persons, based on assumptions of the weight of an injection-site of 20g and an average daily beef consumption of 35g. Whilst the theoretical approaches taken to estimate the risk to consumers are useful, several consumers and scientific experts persist in their belief that ingestion of injection-sites is not as rare an event as that outlined above. Hoogland (2000, personal communication) stated that in a survey in the Netherlands in 1989 in 8,500 slaughter pigs, the reported incidence of injection-site blemishes rose depending on the intensity of the inspection conducted; during inspection on the slaughter line the incidence was 0.2% of the animals; in the deboning/cutting plant, injection-sites were found in 5.4% of neck-sides (2.7% of the animals) and in the butchery, injection-sites were found in 11% of the neck-sides (5.5% of the animals). He stated that in a recent survey there has been an 80% decrease in the frequency due to the implementation of integrated quality control measures. Bette (1998, personal communication) estimated that the real exposure to injection-site residues in processed meat is much smaller than in carcass 'cuts' because in addition to muscle, other edible products such as fat,

connective tissue, water and starch are often included in processed meat. Furthermore, few animals are treated with veterinary medicines shortly before slaughter; therefore there will be a diluting effect of tissues from untreated animals.

Nevertheless, the presence of injection-site blemishes has given rise to consumer concerns. George *et al* (1996) reported that injection-site lesions were a serious quality assurance problem and represented a huge economic loss to the beef industry in the USA. Moreover, the lesions were present in approximately 21.6 million meals in the USA each year. The authors stated that the true costs of injection-site lesions, in terms of customer dissatisfaction from extremely variable and undesirable eating experiences, might exceed the economic losses from trimming alone.

1.2 Injection-site blemishes and residues at the injection-site

It is important to distinguish between the presence of injection-site blemishes and the presence of residues at the injection-site. The existence of one does not necessarily imply the presence of the other. While enormous human resources go into the visual inspection of meat to ensure it is fit for human consumption and free of blemishes, Korsrud *et al* (1994) found that visual inspection of muscle surfaces did not reliably reveal all injection-site lesions in yearling beef steers which had received intramuscular or subcutaneous injections of penicillin G. The authors expressed concern that because lesions were not grossly visible at most injection sites, these sites might escape detection and pose a risk to penicillin-sensitised individuals. George *et al* (1995a), reporting on studies conducted in the USA, stated that in cattle exposed to injections of vitamins, vaccines and antibiotics at branding and weaning, lesions could persist in choice meat rounds for up to 12 months even though residues resulting from their administration would have disappeared within days or weeks. From a consumer safety point of view, only the presence of residues presents a risk, albeit that blemishes are undesirable. Residues at injection-sites present particular problems in estimating the risks for consumer safety. Hathaway (1996) reported that the presence of residues at levels above the MRL at the injection-site may have human health implications in some situations, and may constitute a threat to both

domestic and international trade in animal products intended for human consumption. Moreover, they also pose a threat to the economic return to the animal producer, when meat is rejected as unfit for human consumption. However, according to the Codex Alimentarius Committee on Residues of Veterinary Drugs in Food [CCRVDF] (2000a) for drugs used in veterinary medicines that are devoid of any potential for acute pharmacological, toxicological, or allergenic effects, residues at the injection-site are not expected to pose a risk to consumers. Notwithstanding this, the issue of residue concentrations above the MRL at an injection-site has legal implications for residue surveillance programmes. Residues at the injection-site are not exclusively limited to use of so-called long-acting products or irritant drugs (CCRVDF, 2000a) although several such products are known to induce irritation and tissue damage at the injection-site. The Joint Expert Committee on Food Additives [JECFA] (1995) reported that the concentrations of dexamethasone at the injection-site significantly exceeded levels in all other edible tissues for a period post treatment. Thus, while certain drugs (e.g. dexamethasone) may exert little tissue damage, others (e.g. certain long-acting oxytetracycline formulations), as reported by Nouws (1990), cause significant lesions with or without the presence of residues.

1.2.1 Aetiology of injection-site blemishes

Various factors influence the persistence of blemishes at the injection-site. These include both the active substance(s) in the medicinal product as well as other components of the formulation. Indeed, Bost (1994) stated that a number of factors may come together to potentiate the effect. George *et al* (1995a) found that even when an antibiotic or vaccine was administered to cattle 7.5 to 12 months previously, the injection-site blemish persisted until slaughter. In a very similar study, Heinrich *et al* (1976) reported that injection-site lesions as a proportion of total muscle tissue remained constant and might even enlarge as the animal grew, corresponding to muscle growth and leading the authors to the belief that the injection-site blemishes might originate from cow-calf (suckler) operations. Van Donkersgoed *et al* (1998) also reported that 63% of the lesions found in beef subprimals in Canada were '*mature*', occurring in calfhood. Heinrich *et al* (1996) showed that the intramuscular administration of either 2ml or 5ml clostridial vaccine at branding (usually around 2 months of age) resulted in higher incidences of injection-site blemishes (73% and

93% respectively) than did the administration of a long-acting oxytetracycline (51%). However, when these products were administered at weaning (at approximately 7 months of age) the incidences were 46%, 80% and 92% respectively. In all cases the authors reported that the quantity of trim necessary to remove the injection-site blemish was not significantly different between branding and weaning.

1.2.1.1 Volume injected

Diness (1985) showed that tissue damage was proportional to injection volumes between 0.25 and 1ml in rabbits and 0.5 and 3ml in pigs while tissue damage per injected ml in rabbits was found to be more than twice that seen in pigs. Mawhinney *et al* (1996) also found that volume had a marked effect on the concentration of oxytetracycline in injection-sites. A single injection of up to 35ml of oxytetracycline to cattle resulted in the formation of an encapsulated liquid filled cyst containing 160mg/ml of oxytetracycline with the tissue immediately surrounding the injection-site containing up to 12,000mg/kg of the anti-bacterial drug. In cattle that received 10ml oxytetracycline or less per site, tissue damage was minimal; cysts were not observed and none of the tissues collected 45 days after injection contained oxytetracycline concentrations greater than 0.1mg/kg. The use of multiple sites may assist in increasing absorption of the drug and reduce tissue damage. Van Donkersgoed *et al* (1999) in a study on groups of 10 beef calves found that animals treated with 30ml of long-acting oxytetracycline (LAOTC) in the neck appeared to have more lesions, greater trim loss and a higher level of residues compared to cattle treated with 10ml. However, contrary to expectation, there was a higher level and severity of lesions in animals which received 30ml LAOTC by hand in the thigh compared to animals similarly treated by dart gun. The authors speculated that possible reasons for this were that not all cattle that were injected by hand were properly restrained or that some of the injection volume administered by dart gun leaked out from the injection-site.

1.2.1.2 Concentration

Svendsen (1988) stated that next to the inherent properties of the molecule itself, the concentration of the drug or the solvent is the most important factor that contributes to local damage. Nouws (1984) reported that 20% formulations of oxytetracycline

caused more irritation and revealed generally higher residue levels at the injection-site than the 10% formulations.

1.2.1.3 Injection volume-concentration

Svendsen and Blom (1984), in a study in rabbits administered 10mg clopenthixol in concentrations of 0.1, 0.3, 1.0, 3.0 and 10% found that the weight of necrotic tissue was 5.7, 4.7, 4.6, 2.3 and 1.4g respectively. They concluded that a small volume of a concentrated solution caused less muscle damage than a large volume of a relatively less concentrated solution.

1.2.1.4 Nature of the active substance

After intramuscular injection of saline or sterile water to swine, Rasmussen and Svendsen (1976) reported little or no tissue reaction. By contrast, the same authors found that sulphonamide and/or trimethoprim caused macroscopic and microscopic changes, mainly appearing as areas of necrotic muscle tissue 6 days after the injection and as scar tissue 30 days after the injection. Svendsen (1988) reported a correlation between the molecular weight of a substance and muscle lesions. He suggested that this was due to the possible presence of the drug at a toxic concentration in the tissue for a longer period than with more rapidly absorbed drugs of lower molecular weight. Bost (1994) stated that penicillins and cephalosporins provoke the least irritation, while chloramphenicol and sulphonamides produce severe reactions following intramuscular administration. Tetracyclines differed in their toxicity depending on their formulation, while macrolides caused relatively severe local reactions and persisted at the site of injection. Rasmussen (1978) reported that both water-soluble and fat-soluble vitamins caused damage to muscle tissue. Manor and Sadeh (1989) found that a number of amphiphilic and lipid-soluble drugs of heterogenous pharmaceutical properties, when injected into rat anterior tibial muscles, induced acute muscle fibre necrosis. The myotoxic agents were penicillin, cloxacillin, phenobarbital, haloperidol, diazepam, hydantoin, metoclopramide, pentazocine and chlorpromazine. Injections of the water-soluble drugs aminophylline, tranexamic acid and vitamins B_6 and B_{12} produced no tissue damage. They suggested that the pathogenesis of muscle fibre necrosis involves direct damage to cell membranes by

lipid-soluble drugs and that this injury was similar to that caused by local anaesthetics.

1.2.1.5 Excipients

Rasmussen (1978) reported that vehicles containing glycerol formal or propylene glycol caused damage to muscle tissue. Nouws *et al* (1990) noted that the differences in local tissue irritation and the residue state of the carcass (including injection-site) were related to the various solvent systems used in 5 formulations of oxytetracycline tested in calves, pigs and sheep, with glycerol formal being the most irritant. Svendsen (1993) found that a number of neuroleptic drugs given intramuscularly to rabbits, which caused local muscle damage when in aqueous solution, caused considerably less or even no damage when in oily solution. Postulating the reasons for this, he noted that the local damaging effect of a drug might be elicited by damage of the sarcolemma membrane of the muscle fibres or by toxic effects upon myofibrils or the sarcoplasmic organelles. A large number of muscle fibres were exposed to aqueous solutions immediately on injection, whereas, delay in drug release from oily vehicles to the aqueous phase would result in a lower initial drug concentration. In addition, the higher viscosity of the oily vehicle would tend to prevent the oil from reaching quite as many muscle cells initially, resulting in fewer cells being exposed to the drug. Bost (1994) reported that some excipients caused greatest tissue damage including glycerol formal, vegetable oil, dimethyl acetamide and propylene glycol. Others such as polyvinylpyrrolidone seemed better tolerated with polyols and vinyl polymers being intermediate.

1.2.1.6 pH and tonicity

Bost (1994) stated that the pH did not play a role in the existence of lesions unless it was above 10 or below 4. He further stated that, in practice using commercial veterinary medicinal products, this did not occur. However, in a study on human pharmaceuticals in Japan, Oshida *et al* (1979) reported that the pH of the injectables examined was found to range widely from 1.4 to 12.8 and the osmotic ratio from 0.2 to 36. Whereas no significant correlation was found between pH of injectables and muscle lesions in treated rabbits as far as pHs between 3 and 10 were concerned, most of the products with osmotic ratio over 5 caused severe muscle lesions. Oshida

et al (1979) also found that that there was a close connection between the haemolytic potential of injectables and the severity of muscle lesions in treated rabbits.

1.2.1.7 Site and method of administration

Nouws *et al* (1990) reported that, in general, the greater the degree of tissue irritation produced by a product the greater the chance of residues persisting at the injection-site. They also reported that tissue reactions following intramuscular administration of oxytetracycline in the neck region of cattle were localised in and between the muscles indicating that injections can be deposited *intra-* as well as *inter*muscularly. Lesions following administration of drugs were not limited to the intramuscular route. Hanson (1961) found local tissue changes following both intramuscular and subcutaneous injections of oxytetracyline and chloramphenicol in rabbits. Rasmussen *et al* (1973) also found that the site of administration influenced the degree of damage produced in pigs that had been administered sulphadimidine. The extent of the tissue injuries was smallest in the injection-sites from the neck and largest in the injection-sites from the back, with injection-sites from the thigh being intermediate. However, Van Donkersgoed *et al* (1999) noted that the injection technique (administration by dart-gun or hand-held syringe) might also be important, as too was whether animals were properly restrained for the duration of the injection.

1.2.1.8 Effect of needles

Heinrich *et al* (1996) investigated the effect of using an "old" needle (one that had already been used in 4 other animals). In this study, which was conducted in cattle, the results did not indicate a clear trend. Animals that received a vaccination with "old" needles showed a higher incidence of blemishes and a higher blemish weight at slaughter 225 or 376 days later, but these parameters were not significantly different from animals treated with needles used 4 times or less. Animals that received an anti-bacterial with an "old" needle showed significantly lower incidence rates (64%) compared to animals treated with a needle used less than 5 times (76%). In a study in guinea pigs punctured 20 times in a 1-square cm surface area of the gastrocnemius muscle with an electromyographic needle 0.65mm diameter and biopsied 1, 2.5 and 6 hours and 1, 3 and 8 days later, Engel (1967) found a linear tract of muscle fibre destruction (so-called 'needle myopathy'). In the early stages (1 to 24 hours post

injection) the tracts were characterised by acute necrosis of muscle fibres, followed by phagocytosis at 1 to 3 days. By 3 to 8 days there were large vesicular muscle nuclei with prominent nucleoli surrounded by basophilic cytoplasm, indicative of muscle fibre regeneration.

1.2.1.9 Other factors

Svendsen *et al* (1998) reported that the temperature of the injection solution did not affect the local tolerance following intramuscular administration in pigs. In an earlier study in rabbits using clopenthixol and saline, Svendsen and Blom (1984) found that injection speed was not an important factor for the muscle damaging effect of intramuscular preparations. Concerning species-specific effects, Nouws (1990) reported that in sheep, tissue irritation is generally less than in calves or pigs.

1.2.2 Pathogenesis

Bowman and Rand (1980) stated that, following subcutaneous injection, the injected mass spread out through the loose connective tissue of the subcutaneous layer, or, in the case of intramusular injections, between the sheaths of muscle bundles. The drug was disseminated into the system after passage through capillary or lymphatic endothelium. The drug could induce cell death in those cells with which it comes into contact either by direct toxic effects leading to necrosis or by physiological means (apoptosis). Duchen (1999) reported that mitochondria, present within the cells, played a key role in the homeostatic mechanisms of the cell and in both necrotic and apoptotic cell death.

1.2.3 Histopathology

Svendsen (1972) investigated the histology of injection-sites following the intramuscular administration of several commercial antibiotics to cattle and swine. In animals slaughtered 6 days after treatment, 2 groups of lesions were identified. Changes in the first group were vascular and fibroblastic proliferation, regeneration in hyaline degenerated bordering muscle fibres, and regeneration in less damaged peripheral muscle fibres. In the second group, a necrotic area surrounded by a demarcating zone was found. After 21 days the injection-sites with the most severe changes consisted of a thick layer of scar tissue surrounding a smaller muscle

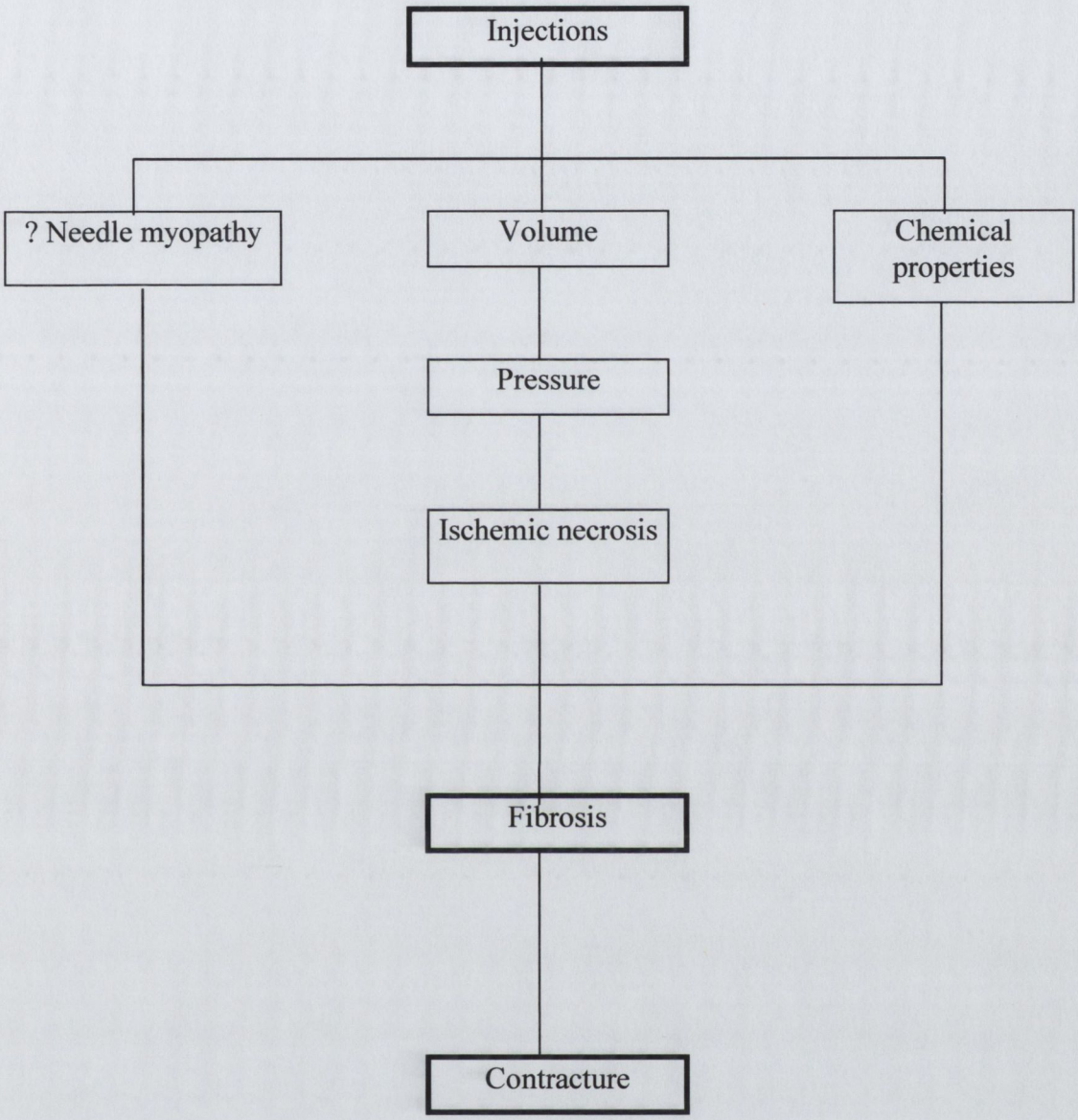
necrosis. Svendsen and Blom (1984), in a study in rabbits, stated that the injectables that caused severe muscle lesions, including necrosis, 7 days after a single intramuscular injection were antipyretics, analgesics, antibiotics and anti-allergenic agents. They noted that the experimentally-induced injuries resembled those of the muscle contracture in humans and were characterised by increased fibrosis, scar tissue, fatty infiltration and atrophy of the muscle fibre. Mikaelian *et al* (1996a) found that where muscle fibre necrosis was extensive following intramuscular administration of antibiotics in sheep, healing involved encapsulating the necrotic tissues and thus resulted in extensive scar formation. By contrast, if myodegeneration was found alone, full recovery within 21 days could be predicted. Takahashi *et al* (1989) noted that in calves given ampicillin aqueous suspension intramuscularly, the drug diffused along the long axis of the muscle but not in the radial direction and not to neighbouring muscles. Grey (1967) reported that in long-term tolerance studies with spectinomycin and lincomycin in dogs and cats in which animals received intramuscular injections into the thigh once or twice daily, 6 days per week for 3 months, the muscles remained relatively unaffected and facultative lymphatic drainage was established. Certain distinctive histological changes accompanied the response. The loose connective tissue and fatty tissue of the fascial planes were replaced by a more resistant granulation tissue. Collections of lymphocytes were organised along the drainage pathway into a prenodal lymphofollicular response. Muscle fibres that immediately bordered the drainage tract were walled away from the low-grade recurrent insult by a fibroblastic response that capped the exposed fibres. George *et al* (1995a) reported that steatosis was associated with injection-site lesions in beef animals. George *et al* (1995b) histologically examined 12 injection-site lesions taken from beef steaks. In sections taken at sites at the lesion centre the relative percentages of connective tissue, muscle and fat were 45.63, 30.00 and 19.38% respectively; at 2.54cm from the centre the relative percentages were 21.25, 52.50 and 28.75% and at 5.08cm from the centre, the percentages were 8.13, 75.63 and 15.63% respectively while for control steaks the values were 7.50, 80.83 and 11.67% respectively. They reported that the lesion centre contained dense sheets of fibroblasts with extensive collagen deposition, which was gradually replaced by adipose tissue as the distance from the lesion centre increased. At a greater distance

from the lesion centre they reported that there was a further reduction in the concentration of connective and adipose tissue, which was replaced by degenerate muscle fibres. Ossified tissues resulting from intramuscular administration of pharmaceuticals in cattle have also been cited by George *et al* (1995b).

1.2.4 Human experience

In human infants and young children, Bergeson *et al* (1982) reported that an uncommon complication of intramuscular injections in the gluteal area was muscle fibrosis. Hessels *et al* (1975) outlined the possible pathogenesis of quadriceps fibrosis in children as outlined in Figure 1-1 hereunder.

Figure 1-1 Possible pathogenesis of quadriceps fibrosis in children, taken from Hessels *et al* (1975)



Hessels *et al* (1975) postulated that the injections into muscle caused the production of adipose tissue and fibrosis in the muscle bellies; these changes being due to a direct disruption of the muscle by the needle, or by local destruction by the volume and the chemical properties of the injected material. Large volumes of fluid injected into the small and relatively inelastic fascial compartments of the infant muscles led to pressure and ischemic necrosis with subsequent fibrosis. Injection of irritating chemicals led to a chemical myositis that also progressed to fibrosis. Theodorou (1975), who found that both local trauma and irritation from the drug contributed to

the genesis of fibrosis and contracture of the quadriceps in children supported this aetiology. Histology of the muscles of affected children showed excessive fibrous tissue infiltrating the muscle fibres and burying regions of them. The muscle fibres themselves showed degeneration. Indeed, Alvarez *et al* (1980) stated that quadriceps myofibrosis is now recognised as a clinical entity in infants and young children which is characterised by alterations in the normal extensor mechanism of the knee joint resulting from fibrofatty replacement of the quadriceps muscles. More than 75% of the 2,800 cases, which they reviewed from the USA and Japan, were alleged to have been caused by injections. Alvarez *et al* (1980) also stated that even the slight trauma of an injection could cause disruption of the muscle fibres and local necrosis. The addition of compression of the muscle bundles and capillaries brought about by the volume of the injected material, combined with the ensuing local oedema and haemorrhage, could lead to more widespread ischaemic changes in the muscles. Gunn (1964) noted that the most common age of onset of contracture of the quadriceps was between 6 and 24 months, and suggested that it might be that muscles were particularly susceptible to irritants at this age or merely that the volume of the irritant was large in relation to the bulk of the muscle. Glazer *et al* (1987) found in a cross-sectional study of injection-site leakage of depot neuroleptics in 40 humans that significantly more leakage occurred after intramuscular than after subcutaneous injection. They suggested that age, sex, body mass, and injection volume might also affect leakage. Buchta (1976) stated that many types of parenteral injections had a propensity to cause muscle atrophy in children, including procaine penicillin, corticosteroids and insulin as well as immunisations. Taylor (1981) reported that nodules at the injection-site, which occur following immunisation, were due to a granulomatous reaction to metal salt adjuvant in the vaccine.

1.2.5 Prevalence of injection-site blemishes in animals

As farm sizes in North America tend to be larger and husbandry methods may differ from those in Europe, the data from both regions are evaluated separately in Sections 1.2.5.1 and 1.2.5.2 below.

1.2.5.1 Prevalence of injection-site blemishes in North America

Dexter *et al* (1994) reported that the occurrence of damaged muscle tissue resulting from intramuscular administration of animal health products represented a 'quality control' problem and an economic loss to the meat industry. George *et al* (1997) noted that quality assurance systems in the US beef industry had reduced the incidence of injection-site lesions in beef top sirloin butts from 21% to 10% in the period 1991 – 1995. Most of the blemishes identified in audits in the USA were "dry" and contained no fluid. Nevertheless, trim losses and decreased tenderness resulting from injection-site lesions in top sirloin butts, bottom rounds, and inside rounds represented a serious quality control problem. George *et al* (1996) reported that the mean weight of injection-site lesion trim in the US beef study was 152g. This translated into a huge economic loss in the US beef industry estimated by George *et al* (1997) to be $7 per steer or heifer slaughtered in 1995. Similar losses have also been reported in Canada, where lesions were found by Van Donkersgoed *et al* (1998) in 35% of outside rounds of cull cows and bulls and in 16% of top butts and 8% of yearling cattle.

1.2.5.2 Prevalence of injection-site blemishes in Europe

Published data from Europe are scarce. In Belgium, Vanoosthuyze *et al* (1994) analysed over 2000 injection-sites from cattle between 1989 and 1994 for residues of hormones. These blemishes, which the authors described as local inflammations encapsulating an oily matrix containing illegal hormone cocktails, were found on the carcasses in the slaughterhouses and usually contained high amounts (mg) of these substances. However, Mikaelian *et al* (1996b) reported that injection-site blemishes, mostly associated with the poor tissue compatibility of certain veterinary drugs, were also found in other species long after the withdrawal times of the administered veterinary medicines. Bost (1994) stated that nearly 6% of French cattle presented for emergency slaughter had injection-site lesions in the neck that had to be trimmed. These included abnormally coloured tissue (83%), abscesses (16%) and scar tissue or necrosis of the cervical ligament (1%). From a total of 1,179 necks (which were condemned) out of 286,146 cattle slaughtered, the mean weight of trimmed tissue was 21.3kg.

1.2.6 Regulatory aspects of injection-sites

The European Union and Codex Alimentarius bodies responsible for establishing standards for residues of veterinary drugs in foods of animal origin are currently reviewing policy on the importance of injection-sites in establishing withdrawal periods for animals treated with veterinary drugs. In the absence of precise information on the probability of the actual hazards involved, progress in the development of an agreed guideline has been slow and a final guideline has yet to be adopted. The prevalence on injection-site blemishes in beef may not be the same for other species. Reliance on data from beef only could lead to an overestimation of the consumer risk; beef animals are likely to receive more parenteral injections during their lives compared to other species (pigs are usually mass medicated via the food or water and fattened pigs and lambs are slaughtered at younger ages compared to cattle). Indeed, Van Dresser and Wilcke (1989) reported from an analysis of 292 field investigative reports of drug residues in food-producing animals in the USA that residues were most often found in cows. There are few studies on the prevalence of injection-site blemishes in respect of pork or mutton. The absence of European prevalence data and information from food-producing animals other than cattle has, until now, precluded a reliable estimate of the exposure of European consumers to injection-sites in meat. Moreover, the absence of data on the prevalence of residues at injection-sites has made it difficult to separate food-quality issues from food-safety issues.

1.3 Hazard characterisation of veterinary drug residues

Regarding the hazard characterisation of the residues in tissues of animals, Hathaway (1996) identified 3 groups of veterinary medicines with the potential for concern: a) those drugs with potential for acute adverse health effects to the consumer resulting from ingestion of residues at the injection-site; b) those drugs with specific potential for local irritation and c) anti-bacterial drugs. Several processes, which are routinely carried out following slaughter, may result in reduction of the quantity of residue present in the injection-site, including storage, preparation and cooking of meat. It is known that many drug substances used in veterinary medicines and in pesticides are not stable in meat or other organs during storage and cooking. Haagsma (1993) stated

that many anti-bacterial drugs that are widely used in animals show a decrease in their activity following storage, preparation and cooking. Meat is not usually consumed raw but requires preparation such as boiling, frying or other cooking methods before consumption. However, studies on what actually happens in the incurred muscle residue matrix are scarce. Wal (1979) reported that cooking techniques could lead to a thermodegradation of the residue but also to the release of residue present as protein conjugate e.g. penicilloyl-protein conjugate in milk. Rašeta *et al* (1975) reported that published data and personal studies demonstrated that cooling and freezing of meat had made no substantial influence on the biological activity of most commonly used antibiotics, although subjecting meat to a high temperature inactivated the antibiotic residue in some cases.

Slanina *et al* (1989) argued that when assessing the drug residues in foods, the ultimate goal should be to consider the amounts that really represent 'fork exposure' for a consumer. Residue limits of veterinary medicines do not take into account effects of processing. In agreement with the Food Agricultural Organisation [FAO] and World Health Organisation [WHO], (FAO/WHO, 1995), MRLs are expressed on a fresh meat basis While a change in the definition of MRLs for residues of veterinary origin from that currently expressed in fresh meat is unlikely, the processes affecting the ultimate amounts are important and should be characterised to allow a better estimate of the risk to consumers.

1.3.1 Pesticidal approach to residue intake estimates

Elkins (1989) reported that commercial food processing operations such as washing, blanching and cooking removed major portions of the pesticide residues that are currently permitted on the raw agricultural crop. Holland *et al* (1994) reported that processes involving heat or chemicals could increase volatilisation, hydrolysis or other routes of chemical degradation and thus reduce pesticide residue levels. However, the same authors also noted that drying processes might result in higher concentrations of residues due to loss of moisture, while rates of degradation and volatilisation of residues could be highly variable depending on whether the cooking process was open or closed.

In the case of residues of plant pesticides, there is international recognition of the use of processing studies in calculating consumers' intake of the drug substances and/or their relevant metabolites. This approach provides for a more realistic risk assessment than is possible by calculating the Theoretical Maximum Daily Intake (TMDI), which is calculated by multiplying the established MRL by the estimated average daily regional consumption of each food commodity and then summing the products (WHO, 1997). The TMDI is reported to be an overestimate of the true pesticide residue intake because, among other things, only a portion of a specific crop is treated with a pesticide; most treated crops contain residues well below the MRL at harvest; residues are usually reduced during storage, preparation, commercial processing, and cooking; and it is unlikely that every food for which an MRL is proposed will have been treated with the pesticide over the lifetime of the consumer.

1.3.2 Effect of cooking on residues of veterinary medicines

Already several substances used in veterinary medicinal products have been subject to cooking and heat stability experiments. O'Brien *et al* (1981) stated that the effects of cooking on the biological activity of the residues of ampicillin, chloramphenicol, oxytetracycline, streptomycin and sulphadimidine were varied. In a review article, Moats (1988) stated that ordinary cooking procedures for meat cannot be relied on to inactivate even the more heat sensitive compounds such as penicillins and tetracyclines. However, he stated that more severe heating as for canning or prolonged cooking with moist heat might inactivate the more sensitive compounds. The studies reported in the literature have certain limitations insofar as many relate to data from a single animal or from a single species, or fortified tissue rather than incurred tissue was used.

Among the substances already investigated for heat and cooking stability are clenbuterol (Rose *et al*, 1995a; Ramos *et al*, 1993), levamisole (Rose *et al*, 1995b; Hsu and Epstein, 1993), sulphadimidine (Rose *et al*, 1995c; O'Brien *et al*, 1981; Epstein *et al,* 1988), chloramphenicol (O'Brien *et al*, 1981; Costa *et al*, 1993; Epstein *et al,* 1988), ivermectin (Costa *et al*, 1994; O'Brien *et al*, 1981; Rose *et al*, 1998), oxytetracycline (Rose *et al*, 1996b; Slanina *et al*, 1989; Honikel *et al*, 1978) the quinolones oxolinic acid and flumequine (Steffenak *et al*, 1993; Steffenak *et al*,

1994), oxfendazole (Rose *et al*, 1997a), lasalocid (Rose *et al*, 1997b), ampicillin (O'Brien *et al*, 1981), neomycin (Katz and Levine, 1978), streptomycin (Inglis and Katz, 1978; O'Brien *et al*, 1981), benzyl penicillin (DePaolis *et al,* 1977; Rose *et al*, 1997c), procaine penicillin (Katz *et al*, 1978), furazolidone (McCracken and Kennedy, 1997), organophosphorous compounds (Askew *et al*, 1968) cyromazine (Epstein *et al,* 1988) and nitroxynil (Ekström and Slanina, 1982). Organophosphate compounds including chlorfenvinphos and parathion, were broken down to varying degrees during the course of cooking in vegetable mash. In the case of phorate, analysis by gas liquid chromatography (GLC) revealed the formation of a new compound, phorate sulphoxide that was more stable to hydrolysis than the sulphone or parent compound.

By contrast, Alfredsson and Ohlsson (1998) investigated the stability of sulphonamides in bovine and porcine muscle during frozen storage (-20°C) for 3 months. They reported a decline in sulphonamide levels with an average decrease of 35% in bovine and 55% in porcine meat. Parker *et al* (2000) reported that avermectin, doramectin, eprinomectin, ivermectin and moxidectin when fortified into bovine liver and stored at -20°C for 47 days led to a decline in the concentration of up to 20%.
Rose *et al* (1995a, 1995b, 1995c, 1996b, 1997a, 1997b, 1997c, 1998, 1999) conducted both *in vitro* heat stability studies in aqueous and in oily solutions as well as actual cooking experiments. The heat stability studies in aqueous and oily solutions are summarised in Tables 1-3 and 1-4 below. The data show a wide variety of responses with some drugs being stable for 6 hours and others very unstable.

Table 1-3 Stability of various drugs in hot water

Drug substance	Test conditions		Effect	Reference source
	pH	Bath temp. (^{0}C)		
Clenbuterol	Not stated	100	Stable for 5 hours	Rose *et al*, (1995a)
Levamisole	Not stated	100	Stable for 4 hours	Rose *et al*, (1995b)
Sulphadimidine	Not stated	100	Stable for 6 hours	Rose *et al*, (1995c)
Oxytetracycline	Not stated	100	Unstable $T_{1/2}$ = 2 mins	Rose *et al*, (1996b)
Oxfendazole	Not stated	100	Stable for 2 hours	Rose *et al*, (1997a)
Lasalocid	5.5	100	Stable for 3 hours	Rose *et al*, (1997b)
Lasalocid	7.0	100	Stable for 3 hours	Rose *et al*, (1997b)
Lasalocid	10.0	100	Unstable $T_{1/2}$ = 30 mins	Rose *et al*, (1997b)
Benzylpenicillin	5.5	100	$T_{1/2}$ = 40 mins	Rose *et al*, (1997c)
Benzylpenicillin	8.2	100	Unstable $T_{1/2}$ = 15 mins	Rose *et al*, (1997c)
Benzylpenicillin	Not stated	65	Stable for 20 mins	Rose *et al*, (1997c)
Dimetridazole	5.0	100	Stable for 3 hours	Rose *et al*, (1999)
Dimetridazole	8.2	100	Stable for 3 hours	Rose *et al*, (1999)
Chloramphenicol	Not stated	100	Complete degradation	Costa *et al*, (1994)

In cooking oil, all the drugs were unstable with half-lives of 5 minutes to 2 hours reported as shown in Table 1-4 hereunder.

Table 1-4 Stability of various veterinary drugs in cooking oil

Drug substance	Oil temp. (^{0}C)	Effect	Reference source
Clenbuterol	260	Unstable $T_{1/2}$ = 5 mins	Rose *et al*, (1995a)
Levamisole	260	Unstable $T_{1/2}$ = 5 mins	Rose *et al*, (1995b)
Sulphadimidine	180	Unstable $T_{1/2}$ = 2 hours	Rose *et al*, (1995c)
Oxytetracycline	180	Unstable $T_{1/2}$ = 8 mins	Rose *et al*, (1996b)
Oxfendazole	150	Unstable $T_{1/2}$ = 15 mins	Rose *et al*, (1997a)
Oxfendazole	180	Unstable $T_{1/2}$ = 6 mins	Rose *et al*, (1997a)
Lasalocid	180	Unstable $T_{1/2}$ = 15 mins	Rose *et al*, (1997b)
Benzylpenicillin	140	Unstable $T_{1/2}$ = 45 mins	Rose *et al*, (1997c)
Benzylpenicillin	180	Unstable $T_{1/2}$ = 20 mins	Rose *et al*, (1997c)
Dimetridazole	170	Unstable $T_{1/2}$ = 2.5 hours	Rose *et al*, (1999)

The approach taken by Rose *et al* (1995a, 1995b, 1995c, 1996b, 1997a, 1997b, 1997c, 1998, 1999) to the heat stability experiments in aqueous media and in oil had the advantage of ensuring comparable and reproducible data on the various drugs. Moreover, *in vitro* heating experiments might allow for more precise estimation of stability compared to cooking of incurred residues, due to the possible lack of homogeneity of the residue within the tissue sample. Similar type stability studies are routinely conducted on active ingredients in pharmaceutical preparations to predict the stability of a product under defined conditions (Cartwright, 1989). The rates of most chemical reactions increase enormously as the temperature is raised (Stevens, 1961). The relationship is expressed by the Arrhenius equation given hereunder.

Equation 1-1 Arrhenius equation

$$k = Ae^{\frac{-\Delta Ha}{RT}}$$

where k = first order reaction rate constant

A= frequency factor

ΔHa = activation energy; the energy a molecule must acquire in excess of that possessed in its initial state in order to react to form products

R= gas constant

T= absolute temperature

This equation is intended for use where constant temperatures are applied to a drug substance for relatively long periods of time. However, for predictive cooking experiments, short-term exposure to high temperatures is the norm. Indeed, Pope (1980) noted that deviations from the Arrhenius equation were possible due to solvent effects, mechanistic effects and formulation deviations. Solvent effects e.g. vaporisation have been taken into account to some extent by Rose *et al* (1995a, 1995b, 1995c, 1996b, 1997a, 1997b, 1997c, 1998, 1999) by solubilising the drug substance in aqueous media (sometimes at different pHs) and oily media. Nevertheless, moisture loss caused by heat might compensate for loss of the active substance due to degradation. Mechanistic effects have also been considered by applying temperatures that simulate actual cooking temperatures. Again, however there are some limitations; different mechanisms for the degradation of a substance might exist at different temperatures leading to non-linearity of response. Formulation deviations are not of direct application to studies on residues of veterinary drugs. However, it is worth noting some similarities to those described by Pope (1980):

a) some ingredients might melt, changing the liquid composition and viscosity of the system. Drug degradation in the heated formulation might follow a completely different mechanism dependent upon composition and viscosity change,

b) solubilities of components might change with temperature. If a drug substance partitioned between 2 solvents in a set manner at room temperature, the

respective concentrations might change with change in temperature. If concentration were an inherent factor modifying rate, the reaction rate would be different. If degradation occurred only in 1 phase of a 2-phase system (depending upon the partition ratio) the amount of drug subjected to degradative processes might change, hence invalidating the Arrhenius prediction,

c) heating could cause transition of hydrates. Different hydrates of the same chemical species would have potentially different solid-state stabilities resulting in change in viscosity or degree of flocculation.

Non-isothermal studies might also be conducted on pharmaceutical preparations. Pope (1980) stated that compared to the isothermal approach, these methods were a compromise, reducing experimental effort but at the same time decreasing the accuracy of the final estimates of stability.

The principal findings of the available literature concerning cooking studies on drugs are outlined in Tables 1-5 to 1-8 below. The data show a lack of agreement with usually only a single animal providing the incurred residue samples. Taking an arbitrary figure of 50% or greater as indicative of a significant reduction in residue concentration post cooking, it is noted that tetracyclines, penicillins and chloramphenicol were generally unstable, while the parastiticides compounds tested as well as clenbuterol, lasaloid and several of the anti-bacterials tested were relatively stable. Recently, Van Egmond *et al* (2000) investigated the stability of several anti-bacterials in fortified pork in a simulated high temperature denaturing process involving sequential pasteurisation at a temperature of 80°C for 15 minutes, sterilisation at 134°C (3 bar) for 20 minutes and drying at 100°C for 4 hours. During the different stages of this process, samples were taken and analysed for antimicrobial activity by means of a bioassay. The remaining activity after the full destruction process was as follows: lincomycin 80%, flumequine 69%, enrofloxacin 68%, neomycin 46%, tylosin 44%, sulfamethazine 38% and spiramycin 15%. Penicillin, amoxycillin, ampicillin, cloxacillin, oxytetracycline, doxycycline, colistin,

dihydrostreptomycin and sulfamethaoxazole were fully degraded (less than 10% remaining activity) after the sterilisation step.

Table 1-5 Effects of cooking on incurred residues of sulphonamides, tetracyclines and penicillins in muscle tissue

Drug substance	Species	No. of animals	Cooking method	Net conc. change after cooking (%)	Reference source
Sulphadimidine	Porcine	1	Microwaved for 7.5 mins at 700W	No effect	Rose *et al*, (1995c)
Sulphadimidine	Porcine	1	Boiled/casseroled for 1 hour	No effect	Rose *et al*, (1995c)
Sulphadimidine	Porcine	1	Grilled for 9 mins	-30	Rose *et al*, (1995c)
Sulphadimidine	Porcine	2	Heated at 122^0C for 95 mins	No effect	Epstein *et al,* (1988)
Sulphadimidine	Bovine	2	Roasted at 190^0C for 45 mins	No effect	O'Brien *et al,* (1981)
Oxytetracycline	Bovine	1	Microwaved for 7 mins at 700W	-80	Rose *et al*, (1996b)
Oxytetracycline	Bovine	1	Boiled in bag for 20 mins.	-78	Rose *et al*, (1996b)
Oxytetracycline	Bovine	2	Roasted at 190^0C for 45 mins	-25 approximately	O'Brien *et al,* (1981)
Ampicillin	Bovine	2	Roasted at 190^0C for 45 mins	-60	O'Brien *et al,* (1981)
Benzylpenicillin	Bovine	1	Boiled for 13 mins	No effect	Rose *et al*, (1997c)
Benzylpenicillin	Bovine	1	Microwaved at 700W for 3 mins	-59	Rose *et al*, (1997c)

Table 1-6 Effects of cooking on incurred residues of other anti-bacterials in muscle tissue

Drug substance	Species	No. of animals	Cooking method	Net conc. change after cooking (%)	Reference source
Chloramphenicol	Rabbits	9	Boiled for 60 mins	-92	Costa *et al*, (1993)
Chloramphenicol	Rabbits	9	Roasted for 30 mins	No effect	Costa *et al*, (1993)
Chloramphenicol	Porcine	1	Cured and heated at 122^{0}C for 95 mins	-100	Epstein *et al,* (1988)
Chloramphenicol	Bovine	2	Roasted at 190^{0}C for 45 mins	-30	O'Brien *et al,* (1981)
Oxolinic acid	Salmon	4	Boiled (time not given)	No effect	Steffenak *et al*, (1993)
Oxolinic acid	Salmon	4	Baked at 200^{0}C	No effect	Steffenak *et al*, (1993)
Furazolidone	Porcine	1	Grilled for 23 mins	No effect	McCracken and Kennedy, (1997)
Furazolidone	Porcine	1	Microwaved for 3 mins	No effect	McCracken and Kennedy, (1997)
Dimetridazole	Chicken	Not stated	Boiled for 60 mins	No effect	Rose *et al*, (1999)
Dimetridazole	Chicken	Not stated	Microwaved for 5 mins	-27	Rose *et al*, (1999)

Table 1-7 Effects of cooking on incurred residues of certain antiparasitic drugs in muscle tissue

Drug substance	Species	No. of animals	Cooking method	Net conc. change after cooking (%)	Reference source
Levamisole	Porcine	1	Microwaved for 4.5 mins at 700W	No effect	Rose *et al*, (1995b)
Levamisole	Porcine	1	Boiled for 40 mins	No effect	Rose *et al*, (1995b)
Levamisole	Porcine	1	Grilled for 9 mins	No effect	Rose *et al*, (1995b)
Levamisole	Porcine	1	Broiled for 8 mins	-26	Hsu *et al*, (1993)
Levamisole	Porcine	1	Microwaved for 2 mins	-4	Hsu *et al*, (1993)
Ivermectin	Bovine	1	Boiled mince for 9 mins	-45	Slanina *et al,* (1989)
Ivermectin	Bovine	1	Microwaved at 700W until well done	No effect	Rose *et al*, (1998)
Ivermectin	Bovine	1	Boiled mince for 20 mins	No effect	Rose *et al*, (1998)
Ivermectin	Porcine	1	Microwaved at 700W until well done	No effect	Rose *et al*, (1998)
Ivermectin	Salmon	1	Microwaved at 700W until well done	No effect	Rose *et al*, (1998)
Cyromazine	Bovine	2	Cured and heated at 122^0C for 95 mins	-35	Epstein *et al,* (1988)
Oxfendazole	Bovine	1	Various	Not consistent	Rose *et al*, (1997a)

Table 1-8 Effects of cooking on residues of clenbuterol and certain feed additives in muscle tissue

Drug substance	Species	Status	Number of animals used	Cooking method	Net conc. change after cooking (%)	Reference source
Clenbuterol	Bovine	Incurrred	1	Boiled for 40 mins	No effect	Rose *et al*, (1995a)
Clenbuterol	Bovine	Incurred	1	Grilled for 6 mins	No effect	Rose *et al*, (1995a)
Clenbuterol	Meat	Fortified sample	n. app*	Boiled for 25 mins	-22	Ramos *et al*, (1993)
Clenbuterol	Meat	Fortified sample	n. app*	Grilled for 10 mins	-32	Ramos *et al*, (1993)
Oleandomycin	Chicken extract	Fortified sample	n. app*	Heated to 100^0C for 3 hours	-25	Pilet and Toma, (1969)
Lasalocid	Chicken	Incurred	1	Boiled for 5 mins	No effect	Rose *et al*, (1997b)
Lasalocid	Chicken	Incurred	1	Microwaved for 7 mins at 700W	No effect	Rose *et al*, (1997b)

*not applicable

It is difficult to conclude on the overall effect of cooking of incurred residues of veterinary drugs as reported in the literature due to the wide differences in the experimental designs and test conditions and sometimes contradictory findings. While the effects seen may be due to real effects of cooking, some may be a result of random error due to small sample sizes and analytical or methodological error. Even when very comprehensive cooking methods were employed, such as those described by Rose *et al* (1995a, 1995b, 1995c, 1996b, 1997a, 1997b, 1997c, 1998, 1999), including microwaving, grilling, roasting, frying and boiling, the results obtained could be broken down into 2 main effects:

a) that of wet heat (boiling in water),
b) that of dry heat (microwaving).

The variations on these basic cooking methods did not result in major differences in drug stability compared to those achieved using boiling and microwaving alone.

In respect of cooking methods employed by other research workers, it is noted that even when a drug was stable during cooking, it might exude from the tissue with juices into the surrounding medium (e.g. gravy) during the cooking process. Moreover, it would be difficult to design cooking methods that would reflect all possible cooking processes e.g. cooking in alcohol or with spices. Thus, for the purposes of comparison and logic, the experimental protocol chosen for the test drugs followed both the *in vitro* heat stability experiments and the effects of dry and wet heat.

The application of residue reduction principles, such as those taken by the Codex Committee on Plant Protection, to edible tissues of animal origin may not receive universal acceptance as some individuals eat raw or lightly cooked meat. Nevertheless, it is a valid concept in risk assessment, particularly in the context of risk to consumers of ingestion of injection-sites containing residues of veterinary medicinal products. It is also a worthwhile area of research in the context of establishing withdrawal periods for veterinary medicinal products based on the principles of hazard characterisation.

1.4 Distribution of drugs in tissue

In a review of the pharmacokinetics of antibiotics, Cars (1991) observed that tissue levels of antibiotics represented the means of different concentrations in the major tissue compartments: interstitial fluid, cells and blood capillaries. Non-lipophilic drugs, which did not penetrate cells, were confined to the extracellular fluid volume, which constituted approximately 20% of the tissue. Antibiotic molecules had to leave the circulation and be distributed to extravascular sites. Only non-protein bound drug was available for rapid diffusion. The concentration gradient was the driving force for diffusion through the capillary wall. The diffusion of small molecules such as antibiotics occurred mainly through the intercellular pores, with some (lipid-soluble) drugs passing through the capillary endothelial cells. Many antibiotics were dissociated at physiological pH. The ionised form was not lipid-soluble and, consequently, the degree of ionisation, the lipid-solubility of the non-ionised fraction and differences in pH between the 2 compartments influenced the distribution of the drug. A fraction of the drug was bound to plasma proteins in the interstitial fluid while more penetrated or accumulated in cells unless there was an active transport mechanism operating. Different mechanisms of penetration might operate in different types of cells and the intracellular pH may vary between cells. Indeed, Bager and Petersen (1983) found differences in pH values between different sites of the *longissimus dorsi* muscle in both sheep and cattle. They noted that the differences found were probably caused by both real randomly distributed differences between actual muscle fibre tissue as well as a randomly distributed error associated with sampling, homogenisation and measurement of the pH values. The geometry of the tissue fluid compartment (ratio of surface area to volume) was recognised as a most important determinant of antibiotic pharmacokinetics.

Thus, important factors that can affect the site and extent of distribution of a drug in the living animal include blood flow to tissues, lipid solubility, regional differences in pH, the degree of binding to extracellular substances and intracellular uptake. Following its absorption into the blood stream, a drug was redistributed into the tissues, diffusing along its concentration gradient from the intravascular system or was selectively absorbed (concentrated) and/or bound to tissues by energy-dependent

processes following which it was metabolised and eliminated. Mungall and Docktor (1983) stated that for those drugs that were protein bound to plasma (and tissue) proteins, a reversible equilibrium existed between protein and unbound drug on the one hand and drug-protein complexes on the other. Within this equilibrium, drug molecules were constantly binding and unbinding at an extremely rapid rate. The distribution of protein-bound drugs followed the same pattern as its carrier protein. Bowman and Rand (1980) reported that in the case of drugs having pK_a values within a unit or so of the pH of the plasma (pH 7.4), changes in plasma pH occurring in acidosis or alkalosis could exert a considerable effect on the distribution of the drug between extracellular and intracellular water, since intracellular buffering capacity was greater than that of extracellular water and did not change to the same extent. Distribution occurred right down to the cellular level where the unbound or 'free' drug was credited with the pharmacological activity seen. Bowman and Rand (1980) also stated that the rate of reaching equilibrium distributions between drug in the blood and in tissue depended largely on the rate of blood flow through the tissue, with muscle being much less rapidly perfused than lung, liver and kidney. Ritschel (1976) reported that the capillary network was the principal place of exchange and interchange of biological fluid, but also with intracellular fluid. The microcirculatory system was controlled by homeostatic mechanisms. Ritshcel (1976) also stated that whole body fluid was divisible into 3 compartments being intravasal fluid, interstitial fluid and intracellular fluid. The volume of the compartments could change during the course of the day with any change resulting in change to the drug concentration. The fluid passing into the interstitial fluid might further pass into cells, or might move back into the blood steam or pass into the lymph system. Cars (1989) in a study in rabbits with ampicillin, dicloxacillin and cefuroxime reported that the anti-bacterials were not homogenously distributed in muscle, but were mainly confined to the extracellular fluid volume. He stated that whole tissue levels could therefore underestimate the actual anti-bacterial concentrations in the tissue fluid where the bacteria causing soft tissue infections were most likely to be found.

Following slaughter, different mechanisms affect the drug redistribution process. Pounder and Jones (1990) noted that these included the depletion of energy-dependent processes that might concentrate the drug in specific tissues, cell death,

changes in drug-binding proteins, changes in the permeability of inter-tissue barriers after death, *post-mortem* changes in pH and ionic strength of intra- and extracellular fluids which allowed the drug to redistribute down concentration gradients. Furthermore, Richardson (2000) reported that *post-mortem* catabolism of unstable drugs also occurred and the twin processes of redistribution, which might increase drug concentrations and degradation, which decreased drug concentration, might occur together and at different rates. In veterinary medicine, the redistribution of a drug *post-mortem* is expected to be similar to, but different from, the human situation as animals are exsanguinated following slaughter.

1.4.1 Muscle composition and *post-mortem* changes

Walls (1960) stated that muscle tissue could be categorised into 3 types: a) smooth or involuntary muscle, b) striated voluntary or skeletal muscle, and c) striated or involuntary muscle. Smooth muscle fibres are long, spindle shaped cells containing an elongated nucleus and a cytoplasm that contains fine fibrils. Smooth muscle is found in the viscera and is usually associated with connective tissue layers. Striated muscle fibres are long, tapering multinucleated cells containing alternating light and dark bands of myofibrils. Cardiac muscle fibres give off branches and effect anastomoses with adjacent fibres. The myofibrils present in cardiac muscle are similar to those of striated muscle.

Skeletal muscle fibres are arranged into patterns by a series of connective tissue components. An entire muscle is usually surrounded by a heavy connective tissue sheath that also courses through the muscle dividing it into bundles of fibres. The connective tissue comprises principally of collagen fibres, elastic fibres, ground substance and cells. Fat cells are present in striated muscle also, either scattered singly or in groups in loose connective tissue, mainly near blood vessels.

Water is the most important constituent of meat. Ramsbottom and Strandine (1948) found that the water and fat content of 50 muscles of adult beef animals varied from 62.5% and 18.1% respectively in the intercostal muscles to 76% and 1.5% respectively in the *extensor carpi radialis.* Swift and Berman (1959) also found a variation in water content in 8 beef muscles from 70.9% in *serratus ventralis* muscle to 74.2% in the *latissimus dorsi.* They found that the fat content of these muscles

were 8.3% and 2.5% respectively. They also noted that the content of electrolytes of individual muscles varied widely and paradoxically; components which may be considered as intracellular including calcium, magnesium, potassium, phosphorous, and the bulk of the protein were found to be inversely associated with water retention, while the trend of sodium and chloride, which are mainly expected to be components of extracellular fluids, were found to be directly associated with water retention. Lawrie *et al* (1963) reported that in a group of pigs of similar age and reared under identical conditions, highly significant differences were present in the fat-free moisture content between muscles of the same and between different animal carcasses. They found that the moisture content of different muscles was not constant and, together with other criteria such as total nitrogen, connective tissue content, and ultimate pH, served to distinguish them from each other. However, the same authors also observed other differences between muscles including myoglobin, nitrogen and intramuscular fat content.

Price and Schweigert (1971) reported that considerable variation could occur in the water retaining capacity of meat both within the same muscle and between muscles of the same animal. Briskey and Wismer-Pedersen (1961) noted several reasons for this, including differences in connective tissue content and ultimately pH, differences in calcium and magnesium content and the state of contraction of muscle fibres as a result of varying degrees of shortening during onset of rigor. Ginger and Weir (1958) found wide variation in the tenderness throughout the length of individual beef *semi-membranosus* and *biceps femoris* muscles, as evidenced both by 8 taste judges and using shear strength measurements at 5 separate sites.

Price and Schweigert (1971) stated that muscle proteins were responsible for the binding of water in meat due to the folding of the protein molecules, such that the electric charged and polar side groups lay at the surface to which the water molecules were attached. They reported that a certain amount of water, about 4 - 10g per 100g protein, was bound very firmly and this probably represented a layer of water, 1 molecule in thickness, around the charged and polar groups. Another amount of water, about equal to the previous amount, represented a second layer of water molecules over the hydrophilic groups while a third amount, which was bound to

between 20 – 60g per 100g protein, represented water molecules located in a less organised fashion between the protein molecules. Price and Schweigert (1971) concluded that the amounts of water bound closely to meat proteins constituted a minor part of the 300-360g water per 100g protein in fresh meat. The major part of the water was present as apparently free molecules within the meat fibres and interlining connective tissue. Myofibrils retained water because of their 3-dimensional network of filaments, a structure that persisted even after homogenisation of meat. When a muscle fibre contracted, the actin and myosin filaments overlapped, reducing the amount of space and the water carrying capacity. At pH 5.0 - 5.1, the isoelectric point of fibrillar proteins and corresponding to the normal ionic environment of meat, the filaments were strongly bound and there was little space for water molecules. Conversely, when the pH was either above or below the isoelectric point, there was a surplus of charges on the filaments that repelled each other and therefore allowed more space for water molecules. The water binding capacity of muscle may also be influenced by the presence of salts and polyphosphates. Swift and Berman (1959) found a highly significant negative correlation coefficient between water-retaining capacity and the concentration of magnesium, calcium and potassium ions in 8 beef muscles. These ions were thought to form bridges between the filaments and thus decreased the space between them.

Alvi (1980) found that the sex of sheep had an important influence on the muscle:fat:bone ratio of various retail cuts with lower fat and higher lean content in carcasses from entire males. However, he noted that other factors such as differences in age at slaughter, plane of nutrition and breed might also affect the yield of retail cuts.

Following slaughter, muscle undergoes several compositional changes, including the formation of lactic acid from carbohydrate present. Price and Schweigert (1971) reported that when muscles go into *rigor mortis*, the combination of temperature drop and pH drop from the normal 7.4 to around 5.2 - 5.6, brought about denaturation of certain sarcoplasmic proteins, including creatine kinase, which interacted with the fibrillar proteins. The denatured proteins formed bonds between the filaments. The

integrity of the cell membrane was lost, causing a redistribution of anions and cations between the cells and the surrounding fluids.

1.4.2 Human forensic drug distribution

Pounder and Jones (1990), having examined 4 human cases of drug overdose found that *post-mortem* concentrations in blood were dramatically site-dependent. They speculated that complex physico-chemical changes occurred in the solid organs as part of the processes of autolysis and putrefaction. Using suitable extraction techniques and chemical analytical methods with internal standards in a quality controlled laboratory and using duplicate specimens, variable results were found as shown in Table 1-9 hereunder.

Table 1-9 Drug concentrations reported in human cadaver muscles, taken from Pounder and Jones (1990)

Case 1	Number	**Drug found (μg/g)**	
Tissue	of sites	Doxepin	Desmethyldoxepin
Myocardium	1	16	11
Skeletal muscle	6	7.5 - 10.8	4.1 - 6.1

Case 2		**Drug found (μg/g)**		
		Amobarbital	Secobarbital	Pentobarbital
Myocardium	1	16.1	19.4	21.5
Skeletal muscle	6	4.4 - 6.6	4.4 - 6.5	6.0 - 9.7

Case 3		**Drug found (μg/g)**		
		Clomipramine	Desmethyl-clomipramine	Flurazepam
Myocardium	1	38	14	0.24
Skeletal muscle	6	5.3 - 9.0	3.6 - 4.6	trace

Case 4		**Drug found (μg/g)**		
		Imipramine	Desipramine	Lidocaine
Myocardium	1	50	3.1 - 13	0.8
Skeletal muscle	6	16 - 24	3.8 - 6.7	1.4 - 2.9

While the authors' principal focus was to challenge the assumption that drug concentrations in *post-mortem* blood mirrored drug concentration at time of death, the data showed the considerable variation both in the range of concentrations at 6 separate muscle sites and between these sites and myocardium in each individual case, independent of the drug found. Langford *et al* (1998) evaluated the homogeneity of drug concentrations in muscle in 14 cadavers. These comprised 11 drug overdoses and 3 cases of chronic therapeutic drug use. Analyses were performed on samples from 12 muscles using standard chemical analytical techniques and instrumentation. The muscle samples were from the *pectoralis major*, sternomastoid, deltoid, biceps, triceps, brachioradialis, *rectus abdominus*, *sartorius*, *vastus lateralis*, *gastrocnemius*, *gluteus maximus* and the diaphragm. The results are given in Table 1-10 hereunder.

Table 1-10 Drug concentrations reported in human cadaver muscles, taken from Langford *et al* (1998)

Case no.	Drug	Vd (l/kg)	Diaphragm conc. (μg/g)	Mean muscle conc. (μg/g)	Range of muscle conc. (μg/g)
1	Temazepam	0.8 - 1.0	0.6	0.5	0.5 - 0.6
2	Temazepam	0.8 - 1.0	0.8	0.8	0.4 - 0.9
3	Temazepam	0.8 - 1.0	4.7	2.2	2.0 - 2.6
4	Paracetamol	0.8 - 1.0	300	203	163 - 250
5	Paracetamol	0.8 - 1.0	341	198	122 - 261
	Prothiadin	20 - 92	11.8	3.1	0.4 - 6.0
6	Prothiadin	20 - 92	51.9	17.1	14.8 - 18.5
7	Fluoxetine	20 - 42	1.1	0.3	0.03 - 0.6
	Thioridazine	18	1.3	0.6	0.3 - 1.1
8	Thioridazine	18	2.3	0.9	0.4 - 1.4
	Amitriptyline	6 - 10	12.7	4.3	1.8 - 8.3
9	Amitriptyline	6 - 10	85.0	14.8	4.9 - 31.2
10	Amitriptyline	6 - 10	24.4	10.3	8.7 - 13.1
11	Paracetamol	0.8 - 1.0	542	273	171 - 415
	Propoxyphene	16	19.8	4.0	2.0 - 7.3
12	Paracetamol	0.8 - 1.0	237	195	176 - 211
	Propoxyphene	16	9.14	3.0	2.7 -3.7
13	Paracetamol	0.8 - 1.0	253	247	225 - 274
	Propoxyphene	16	14.4	5.4	4.3 - 7.9
14	Paracetamol	0.8 - 1.0	140	101	88 - 110
	Propoxyphene	16	6.0	3.1	1.9 - 3.8

Vd = volume of distribution

There was marked within-case variability in drug concentrations with highest:lowest concentrations ranging up to 21.7. The variability was generally greater for those drugs with a high volume of distribution (Vd), but it was not always the case that

drugs with a low Vd showed little variability. There was no consistent pattern of hierarchy of muscles in terms of drug concentration, with the exception of the diaphragm, which was almost invariably higher than in other muscles. The authors speculated that this was likely to be the result of greater blood flow to the diaphragm than to other skeletal muscles so that the equilibrium between blood and diaphragm occurred more rapidly. Klöppel *et al* (1980) also found higher concentrations of intoxicating drugs in the respiratory muscles (intercostals and diaphragm) than in the skeletal muscles (*psoas major* and quadriceps) in a study of 7 cadavers. The respiratory muscles had concentrations of drugs 1 - 6 times greater than the skeletal muscles. They stated that during the acute phase of intoxication an enhanced quantity of drugs is stored in the active respiratory muscles, while the skeletal muscle showed reduced metabolism activities. Williams and Pounder (1997) also observed site-to-site variability within the leg muscle of 8 cadavers who had died from fatal overdoses. Samples were randomly taken from dissected leg muscle and homogenised prior to chemical analysis. Results were as shown in Table 1-11 hereunder.

Table 1-11 Drug concentrations reported in human cadaver muscles, taken from Williams and Pounder (1997)

Case No.	No. of samples	Drug	Vd (l/kg)	Drug concentrations in muscle			
				Mean (μg/g)	Range (μg/g)	SD (n-1)	CV
1	20	Amitriptyline	6-10	2.7	1.8-3.9	0.52	19.26
		Nortriptyline	20-57	nd	nd	nd	nd
2	10	Amitriptyline	6-10	4.54	2.8-6.1	1.01	22.14
		Nortriptyline	20-57	2.4	0.9-4.2	0.91	37.92
3	10	Paracetamol	0.8-1.0	38.6	31-47	5.30	13.76
		Amitriptyline	6-10	0.56	0.2-1.13	0.26	46.43
4	20	Promethazine	9-19	nd	nd	nd	nd
		Paracetamol	0.8-1.0	111	91-158	17.72	15.96
5	10	Paracetamol	0.8-1.0	5.81	3.9-8.5	1.50	25.82
		Temazepam	0.8	7.48	5.9-8.8	0.80	10.70
6	30	Paracetamol	0.8-1.0	51.8	28-65	10.00	19.31
		Dothiepin	20-92	1.84	0.8-4.17	0.73	39.67
7	20	Dothiepin	20-92	9.02	1.1-15.7	4.18	46.32
8	15	Carbamazepine	0.8-1.8	15.3	10.3-17	1.60	10.46
		Trazodone	0.9-1.5	1.2	0.8-1.7	0.30	25.00
		Thioridazine	18	1.8	0.3-2.9	0.90	50.00

Vd = volume of distribution

SD = standard deviation

CV = Coefficient of variation

nd = not detected

The authors concluded that skeletal muscle was not homogeneous with respect to drug concentrations in fatal overdose situations as evidenced by the coefficient of variation (CV) being greater than 10. The authors did note that drugs that had lower Vds tended to have lower CVs. They also found that there was no apparent correlation between the relative levels of one drug and another, even where the Vds were similar, and suggested that drug inhomogeneity within muscle is not solely

influenced by the Vd of a drug but might be influenced by the time lapse between consumption of the drug and death.

1.4.3 Distribution of veterinary residues

1.4.3.1 Distribution of veterinary residues between different muscles

In a study into tissue residues of the anti-bacterial ceftiofur using a validated HPLC assay, Robb *et al* (1997) reported that residue concentrations in the diaphragm of 6 cows treated intramuscularly at a dose of 2.2mg/kg bodyweight were almost double those found in the gluteal muscles (200ng/g and 109ng/g respectively). In a study on incurred residues (i.e. residues occurring in tissues following drug administration to the living animal and its subsequent slaughter) of nitroxynil in calves by Ekström and Slanina (1982), concentrations differed substantially between muscle groups; loin residue concentrations were 0.09μg/g while chuck (shoulder and neck) muscle and tongue concentrations were 0.11μg/g and 0.34μg/g respectively. In a study of bulls administered diethylstilboestrol (DES) diproprionate intramuscularly in the neck, Jansen *et al* (1989) found that residue concentrations were quite variable. There were 4 animals slaughtered at each time-point. The method of analysis was HPLC-RIA with a limit of detection of 0.1ng/g. The authors gave no explanation for the wide variation observed and inconsistency in the depletion profile shown in Table 1-12 hereunder.

Table 1-12 Diethylstilboestrol concentrations in muscle from bulls injected with diethylstilboestrol dipropionate, taken from Jansen *et al* (1989)

No. of days between treatment and slaughter	Diaphragm (ng/g) Mean ± SD	Psoas (ng/g) Mean ± SD
7	0.6 ± 0.6	0.2 ± 0.2
14	0.1 ± 0.1	0.5 ± 0.4
28	0.9 ± 1.8	1.2 ± 2.5
42	0.1 ± 0.1	0.1 ± 0.1

SD = standard deviation

In a study on incurred residues of levamisole in muscles of pigs, Rose *et al* (1995b) also reported that the distribution of residue was not homogenous; larger differences were seen between different muscle sites of the animal than within the same muscle. Incurred residues of oxytetracycline from 2 different muscles of the same bovine animal were also analysed in an experiment by Rose *et al* (1996b). They reported differences between the flank and thigh muscles analysed (210 – 282ng/g for left flank and 223 - 356ng/g for left thigh). In a study on a bull calf given nitroxynil by the subcutaneous route, Ekström and Slanina (1982) reported that 63 days following treatment, the concentrations of nitroxynil in the tongue were 3 times those of skeletal muscle (tongue: 0.34μg/g, loin: 0.09μg/g and chuck 0.11μg/g). They stated that the relatively high level of nitroxynil in the tongue was difficult to explain and more experiments were necessary to verify the finding.

1.4.3.2 Distribution of veterinary residues within the same tissue

In sub-sample analysis of raw liver and muscle in a bovine animal treated with clenbuterol, Rose *et al* (1995a) reported that, using statistical analysis, the drug was

not evenly distributed throughout the liver sample (89 – 127ng/g). While insufficient data points for statistical analysis of muscle data were available, the concentrations found were quite variable (16 – 28ng/g). In a study on a single pig given levamisole, Rose *et al* (1995b) noted that the distribution of the drug within the muscle sampled was not homogenous. Variation in the concentration of residues was seen between different sites of the same limb (86 - 104ng/g in right hind limb; 56 - 91ng/g in left fore limb). Similarly, in a study by Rose *et al* (1997a) on oxfendazole residues in cattle liver, residues were not evenly distributed, with a CV of 17% for 8 samples of incurred tissue prior to homogenisation compared to a CV of 10% after homogenisation. By contrast however, in a limited study on sulphadimidine residues in a pig, Rose *et al* (1995c) reported the drug to be evenly distributed in muscle (31 – 34μg/g). Incurred residues of oxytetracycline from 2 different muscles of the same bovine animal were also analysed in an experiment by Rose *et al* (1996b) who reported that neither of the samples used was homogenous for residues of oxytetracycline as shown in Table 1-13 hereunder.

Table 1-13 Tissue distribution of oxytetracycline in bovine muscle, taken from Rose *et al* (1996b)

Muscle location	Site	Oxytetracycline conc. (ng/g)
Left flank	Site 1	210
Left flank	Site 1 duplicate	236
Left flank	Site 2	282
Left flank	Site 2 duplicate	280
Left Thigh	Site 1	313
Left Thigh	Site 1 duplicate	316
Left Thigh	Site 2	257
Left Thigh	Site 2 duplicate	223
Left Thigh	Site 3	282
Left Thigh	Site 3 duplicate	264
Left Thigh	Site 4	350
Left Thigh	Site 4 duplicate	356

Takahashi *et al* (1989) reported that the concentration of ampicillin was remarkably different from part to part of the injected muscle layers in calves. In a study in rabbits given intramuscular injections of ampicillin and cefuroxime, Ryan and Cars (1980) reported that concentrations of antibiotic were substantially lower in whole muscle compared to concentrations in muscle tissue fluid; from this they deduced that antibiotics were not evenly distributed within the tissue. In a study in sheep treated intravenously with flumequine, Sanders and Delmas (1996) analysed 8 samples of liver, 6 different muscle groups and various pieces from the kidney cortex and medulla. Concentrations determined in the liver were more reproducible (CV < 10%) between sites than in muscle (intra-site CV = 4 - 12.5%; inter-site CV = 14 - 33%).

They attributed the higher variability in muscle by physiological factors such as blood flow or fat content. Cooper *et al* (1998) noted grossly inhomogeneous distribution of an incurred chloramphenicol residue in a pig; both within the kidney as a whole and within the individual regions of the kidney, with the kidney cortex showing a variation between 1.1pg/g and 13pg/g (CV = 101%). The authors did not offer any reason for the findings but stated that further work needed to be undertaken to determine the distribution of a wider range of analytes within the kidney and other sample types.

1.4.4 Significance for drug distribution and residue studies

It is not feasible to analyse routinely the entire muscle mass of each animal carcass for residues of veterinary drugs. Should residue concentrations differ between muscle masses, residue monitoring by government inspectors could be targeted at those muscles exhibiting slowest depletion and maximum residue concentrations, offering the highest probability of finding residues at concentrations above the maximum residue limit.

In relation to residue studies conducted to support the establishment of tissue withdrawal periods for veterinary pharmaceuticals, it may be important to know the muscle of origin, lest a muscle mass is chosen which exhibited more rapid elimination of residues compared to others. An understanding of possible sources and the magnitude of variation is important to define the statistical parameters or other methods that are used in calculation of withdrawal periods. Alternatively, it may become necessary for competent authorities that evaluate residue studies to recommend that samples for residue analysis be taken from several specified muscle groups and homogenised.

Finally, an understanding of the extent of intra-animal variation in residue concentrations in tissues may be important in the context of pharmacodynamic action and efficacy determination.

1.5 Validation of analytical methods for residues across the species barrier

Martín-Jiménez and Riviere (1998) stated that there were several complex sources of variability that determine the disposition of drugs in individuals. However, in veterinary medicine, this was further complicated by the variety of animal species to which therapeutic agents were administered. They noted that while there was great potential for the approach of population pharmacokinetics studies to address drug tissue distribution and residue avoidance, adequate strategies for its implementation had yet to be explored. In respect of the so-called *minor* species (species other than cattle, sheep, pigs and poultry that, in the context of annual meat consumption norms, form a minor part of the diet of consumers) the requirements for establishing MRLs are still regarded as being too onerous on many pharmaceutical companies (Zänker, 1999, personal communication). The Committee for Veterinary Medicinal Products [CVMP] (1997) stated that the absence of MRLs for minor species raised real problems for the animal welfare and the protection of human health. CCRVDF (2000b) also debated whether MRLs could be extrapolated from species to species (not necessarily restricted to *minor* species) so that generic food group MRLs could be established. A final decision by CCRVDF has not yet been reached. While it is appreciated that there are differences in metabolism between species that might lead to the establishment of different marker residues between species, the absence of MRLs for minor species and some major species has led to the off-label use of drugs. The CVMP (2000a) has called for a limited relaxation of the species-specific policy for MRL allocation in order to encourage the development of drugs to cover minor food-producing species and thereby diminish the risk to public health from off-label usage of drugs. Given that muscle is the major edible tissue of animals and is readily available for monitoring purposes, an investigation into the comparability of the performance of analytical methods in several species could assist in defining the minimum requirements for validation of analytical methods that are already fully validated in a major species but not fully validated in another species. CVMP has already defined the minimum validation requirements for MRLs in tissues from a major species to the corresponding minor species. CVMP (2000a) reported that only 12 drugs had MRLs established for minor species. In the interests of animal welfare, veterinary surgeons have little option but to use drugs off-label in the treatment of

minor species. Unless analytical methods are used for monitoring drug residues from these species, consumer health may be put at risk.

1.6 Overall aim of research

The primary purpose of this research was to provide scientific data on hazard characterisation of residues of certain veterinary drugs at injection-sites by means of cooking experiments together with an exposure assessment on their prevalence in Irish cattle, sheep and pigs. These data were necessary in order to conduct a risk assessment on the likelihood of consumer damage from the ingestion of injection-sites. There were 2 secondary objectives of this research: an investigation into the performance characteristics of 3 analytical methods in the determination of residues in muscle of cattle, sheep and pigs and an investigation into the distribution of residues of certain anti-bacterials in the carcass and other muscle tissues of animals.

1.6.1 Objective on comparison of performance of analytical methods in various species

This objective was to compare the performance of the analytical methods employed for residue analysis of tilmicosin, enrofloxacin and tiamulin in the muscle of cattle, sheep and pigs. For most compounds used in veterinary medicinal products in the European Union, MRLs have been established on a species-specific basis. Against this background, a comparison of the performance of 3 distinct methods (2 chemically-based and a bioassay) was undertaken in beef, mutton and pork. The details of the study are provided in Chapter 3, 'Methods Validation and Performance'.

1.6.2 Objective on distribution of residues within the musculature of animals

In Chapter 4, 'Distribution of Residues in Muscle', the distribution of residues of tilmicosin, enrofloxacin and tiamulin in cattle, sheep and pigs is investigated and the significance of the data generated evaluated. The objective was to establish whether it is possible to identify a particular muscle site for the monitoring of drug residues of anti-bacterials to increase the efficiency of residue detection, thereby increasing consumer protection.

1.6.3 Objective on risk assessment

The main aim of this part of the research was to conduct a risk assessment of the public health significance of injection-site residues of veterinary anti-bacterials in muscle of livestock. This is composed of 2 distinct phases:

a) hazard characterisation,
b) risk characterisation.

In the hazard characterisation stage, changes in the quantity of incurred marker residue of 3 commonly used veterinary drugs, due to the effect of cooking the injection-site, were measured. The anti-bacterial substances tilmicosin, enrofloxacin and tiamulin, which had not been subjected to such investigation previously, were chosen in order to compliment the published information on several anti-bacterial compounds. All 3 of the major livestock species i.e. cattle, sheep and pigs were investigated to establish whether there are any species differences in the results obtained. Additionally, in order to reflect the variety of cooking processes that may be carried out and to facilitate comparison with studies carried out on other veterinary compounds, the studies were conducted using both open and closed cooking systems as well as *in vitro* heating studies similar to those employed by other researchers. A feature of the work was the emphasis on reliability of the data generated by means of using at least 3 animals from each species for each study; by using repeated analytical samples and by using several analytical techniques including both chemical and microbiological assays for the analytical tests. The work built on the tapestry of heat stability and cooking studies on other drugs carried out previously by various workers using fortified tissues or incurred tissue from a single or a very few animals, usually from 1 or 2 species. These data are presented in Chapter 5, 'Heat Stability of Tilmicosin, Enrofloxacin and Tiamulin'.

The risk characterisation step aimed to estimate the qualitative and quantitative risks to the consumer from residues of anti-bacterials in injection-sites of Irish livestock. This risk is itself composed of 2 elements:

a) the hazard characterisation of the residue and
b) the exposure assessment of the likely intake through ingestion of injection-site blemishes from treated animals in food.

The prevalence of injection-site blemishes in primal and sub-primal cuts of Irish beef, pork and mutton is given in Chapter 6, 'The Epidemiology of Injection-site Blemishes'. This information enabled the risk characterisation between different species to be determined and the probability of consumer exposure to be estimated. While it is appreciated that this exposure assessment does not represent 'fork exposure', the blemishes ordinarily being discarded and not used for human consumption, it does indicate the extent to which injection-site blemishes are present in meat and allows for international comparison with data from North America. The approach to risk assessment follows the methodology of Wooldridge (1999) and is described in Chapter 7, 'Risk Assessment'.

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1 Outline of studies

Two separate and distinct methodological approaches were necessary: a) experimental studies on cattle, sheep and pigs to investigate primarily the effects of cooking on residues of the 3 veterinary drugs tilmicosin, enrofloxacin and tiamulin in muscle tissues and b) field studies to investigate the prevalence of injection-site lesions in Irish cattle, sheep and pigs and to monitor them for the presence of residues of anti-bacterial substances. These are considered separately in Subsections 2.2 and 2.3 hereunder. The focus of attention of both subsections was on anti-bacterial substances because of their public health importance and their widespread use in livestock.

2.2 Experimental study design

The particular anti-bacterial substances selected for investigation were tilmicosin, enrofloxacin and tiamulin. These substances were chosen on the basis of the following criteria:

a) their use in livestock,
b) the fact that cooking studies had not been previously studied or reported,
c) the likelihood of residues being present in muscle at the injection-site. All test drugs have relatively similar pharmacokinetic characteristics as can be seen from Table 2-1.

Table 2-1 Reported pharmacokinetic characteristics of test drugs in cattle

Test drug	Volume of Distribution (l/kg)	Elimination half life (hours)	Percentage protein binding	Reference
Tilmicosin	2.0	0.8 – 1.2	25 - 64	Ziv *et al* (1995)
Enrofloxacin	2.8 – 3.1	5.6 - 5.9	36 - 45	Ziv *et al* (1994) Kaartinen *et al* (1995)
Tiamulin	Not given	3.0 – 6.0	45	Ziv (1980) Ziv *et al* (1983)

It was decided to use incurred tissue samples rather than fortified samples in order to simulate actual consumer exposure to residues of the test drugs in meat. Cattle, sheep and pigs were chosen for study on the basis that these are the major livestock species. All 3 species were selected in order to compare the performance of the analytical methods used and to investigate whether the effects seen were species-specific or not. A group size of 3 or 4 animals/drug/species was chosen in order to have the minimum statistical size necessary for data analysis.

The studies were designed to produce measurable residue concentrations of each drug in excess of the MRL in the muscle at the site of injection in treated animals, while at the same time, providing quantifiable concentrations of residues in muscles remote from the injection-site. The dose of each drug was selected to be at or above the maximum recommended dosage for the target species, but within the therapeutic index for the species. Lower dosages would have reduced the probability of finding residues at concentrations above the limit of quantification (LOQ) of the analytical methods. The drugs had to be used 'off-label' in some species i.e. none was authorised for all 3 species. In advance of using the products off-label, the company responsible for the authorisation of the products were contacted to obtain background pharmacokinetics and tolerance data before the dosage was chosen. A detailed protocol for each of the studies was devised and followed. The welfare of the study animals was monitored by the responsible veterinary surgeon on whose farm the animals were treated and maintained for the study duration. The time periods for

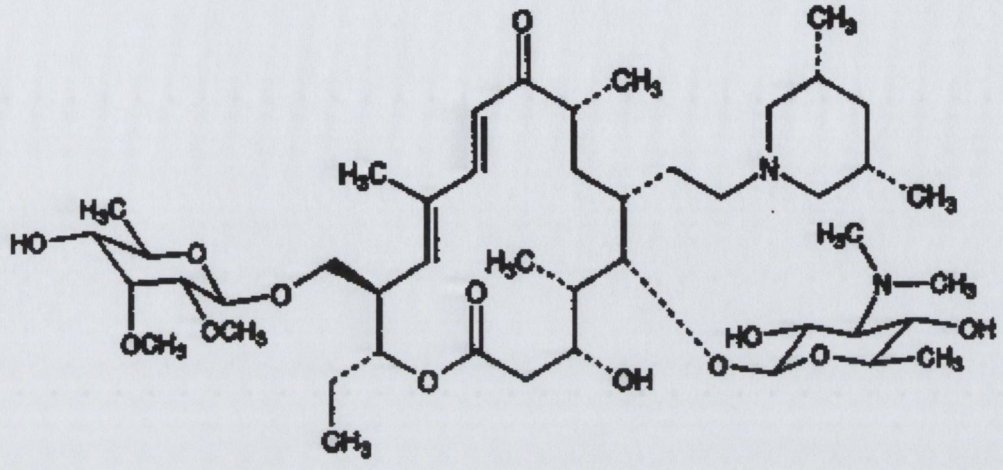
slaughter of the treated animals were selected on the basis of an estimation of the residue concentrations expected in muscle.

2.2.1 Chemistry of the test drugs

Tilmicosin is a macrolide antibiotic synthesized from tylosin which is believed to inhibit bacterial protein synthesis. It is recommended for the treatment of pneumonia in cattle and sheep following a single subcutaneous injection of 10mg/kg bodyweight. There is also a premix formulation authorised for pigs at a dosage of 8 - 20mg/kg bodyweight.

MacNeil (1997) reported that the water solubility of tilmicosin was very dependent on temperature and pH, being 566mg/ml at pH 7 and 25°C, but it was freely soluble in most organic solvents. MacNeil (1997) also reported that tilmicosin consists of 82 – 88% *cis* isomer and 12 – 18% *trans* isomer. The molecular structure of tilmicosin is given in Figure 2-1 hereunder.

Figure 2-1 Molecular structure of tilmicosin



Enrofloxacin is a synthetic fluoroquinolone antibiotic that acts on the bacterial DNA topoisomerases. It exhibits broad-spectrum activity against aerobic Gram-negative and Gram-positive bacteria and mycoplasmas and is recommended for the treatment of alimentary and respiratory infections in cattle and pigs following subcutaneous or intramuscular administration at a dose of 2.5 - 5mg/kg bodyweight. JECFA (1995b) reported that enrofloxacin was a relatively stable molecule which is primarily metabolised in the liver, with the main metabolite in all species being ciprofloxacin, probably formed by oxidative phosphorylation.

The molecular structures of enrofloxacin and ciprofloxacin are given in Figure 2-2 and Figures 2-3 hereunder.

Figure 2-2 Molecular structure of enrofloxacin

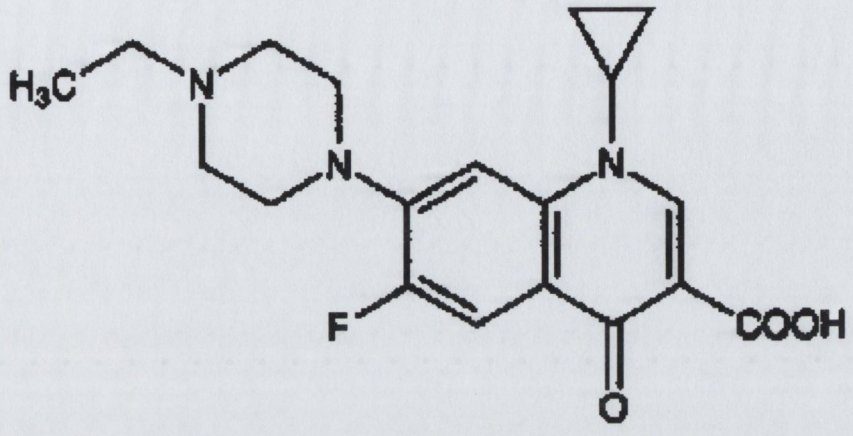
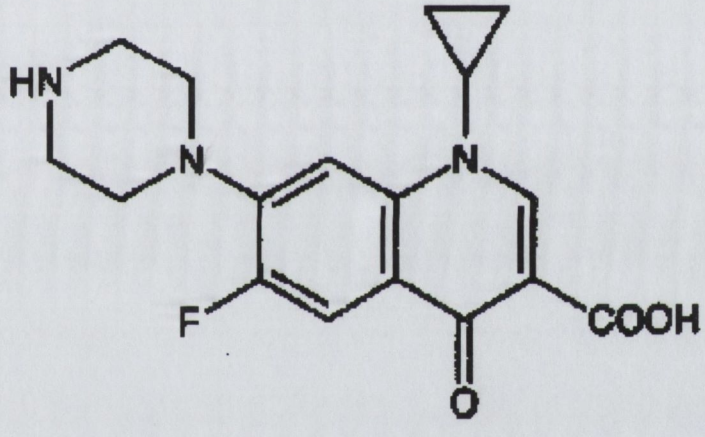


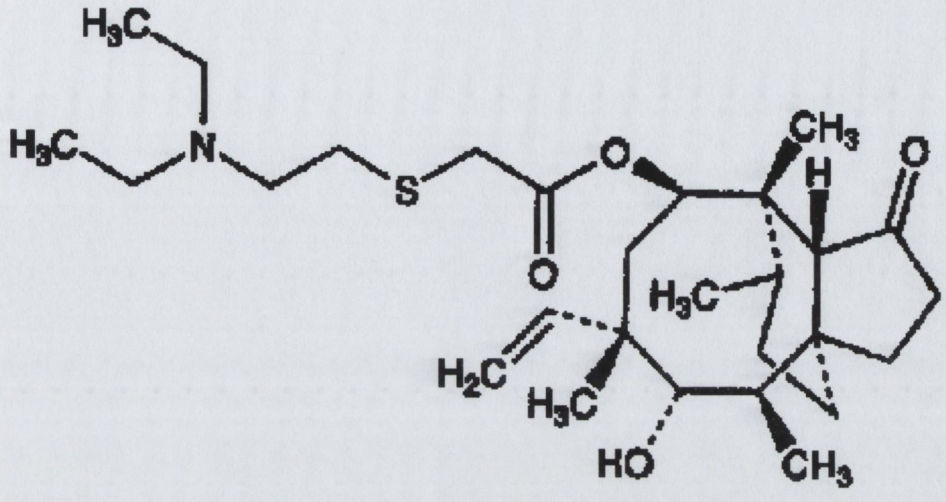
Figure 2-3 Molecular structure of ciprofloxacin



Tiamulin hydrogen fumarate is a diterpine antibiotic produced synthetically from pleuromutilin which acts by preventing bacterial protein synthesis. Goodwin (1985) reported that the drug had activity against mycoplasmas while Taylor (1980) reported activity against *Treponema hyodysenteriae.* An injectable form of the drug is authorised for the treatment of dysentery and pneumonia in swine at a dose of 10 - 15mg/kg bodyweight by intramuscular administration only.

Tiamulin is reported by Laber and Schütze (1977) to be readily soluble in water. The molecular structure of tiamulin is shown in Figure 2-4 hereunder.

Figure 2-4 Molecular structure of tiamulin



2.2.2 Study animals and administration of test drugs

A total of 9 cattle, 9 sheep and 12 pigs, representative of native Irish stock, were allocated on the basis of weight into 3 treatment groups per species. The cattle and sheep drug treatment groups contained 3 animals per group while the pig drug treatment groups contained 4 animals per treatment group. The animals had received no medication in the previous 3 weeks and were maintained outdoors on grass in the case of ruminants and inside on a bed of straw and on a standard non-medicated diet of barley, corn-gluten and mineral mixture in the case of calves and pigs. The cattle were either 6-week-old milk fed calves or 4-month-old ruminants. All of the sheep were adult grazing animals of 2 years or older. Pigs were 13-weeks-old fattening animals. An unmedicated (control) pig was maintained and slaughtered together with the test pigs. Control beef and mutton was obtained from a commercial outlet.

The weighing equipment was checked for accuracy prior to the parenteral administration of the test drugs in accordance with the programme outlined by Tables 2-2 to 2-4 hereunder.

Table 2-2 Dosage and slaughter schedule of animals administered tilmicosin

Species	Mean bodyweight (kg)	Dose rate (mg/kg)	Route	Dose to slaughter interval (hours)
Cattle	87.7	10	Subcutaneous	72
Sheep	60.7	10	Subcutaneous	72
Pigs	37.3	8	Intramuscular	46

Table 2-3 Dosage and slaughter schedule of animals administered enrofloxacin

Species	Mean bodyweight (kg)	Dose rate (mg/kg)	Route	Dose to slaughter interval (hours)
Cattle	61.0	10	Intramuscular	24
Sheep	71.0	10	Intramuscular	24
Pigs	36.6	5	Intramuscular	7

Table 2-4 Dosage and slaughter schedule of animals administered tiamulin

Species	Mean bodyweight (kg)	Dose rate (mg/kg)	Route	Dose to slaughter interval (hours)
Cattle	77.3	15	Intramuscular	24
Sheep	59.7	15	Intramuscular	24
Pigs	36.1	15	Intramuscular	7

The dose was divided and given at 2 separate injection sites at either side of the animal in order to increase the yield of injection-sites from each animal. The neck

was chosen as the site of injection as this is the site normally used by veterinarians and recommended by pharmaceutical companies. The animals were kept under observation for several hours post treatment for signs of intolerance. Any clinical signs of intolerance were recorded.

Animals were slaughtered following stunning at a slaughterhouse using normal practice. Sample tissue (50 – 200g) from various carcass muscles as well as both injection-sites, heart and diaphragm were harvested from each animal. Samples were placed in polythene sampling bags and labelled, following which they were stored at –20°C prior to analysis.

2.2.3 Development of analytical methods

Each assay was developed using, as a starting point, any published information on the analysis of the drug in plasma or tissue, together with technical information on the physico-chemical characteristics of the analyte provided by the company. Further development work was then undertaken using the available laboratory equipment and analytical instrumentation. Efforts were directed towards optimising the performance of each assay by minimising the handling and transfer of samples between containers, and by ensuring optimal extraction conditions and run times for the chemically-based assays and optimal extraction and operating conditions for the bioassay. The specific developmental steps of each method are given in Subsections 2.2.3.1-2.2.3.3 below. All methods followed the same basic principle i.e. the homogenisation of muscle tissue with a suitable extractant, standing samples in the fridge or freezer, centrifugation and subsequent assay.

2.2.3.1 Tilmicosin

An analytical method for the detection of residues of tilmicosin in muscle that would have acceptable performance over the range of concentrations 300 to 3000ng/g was required. Reference to the method outlined by Coleman *et al* (1995) led to experimentation with a bioassay using antibiotic medium number 11 impregnated with *Micrococcus luteus* which had been designed for analysis of bovine serum. However, this method did not display acceptable sensitivity over the desired concentration range in muscle. In order to attain an acceptable limit of detection, efforts were then concentrated on the development of an HPLC-based method. Chan *et al* (1994) used a reversed-phase liquid chromatography with UV detection to determine tilmicosin residues in animal tissues. Using a Phenomenex C-18 Bondclone 10 micron HPLC column, a mobile phase similar to that used by Chan *et al* (1984) comprising 0.1M ammonium acetate (pH 5.0), acetonitrile and methanol (60:30:10) with a flow rate of 1.5ml/minute, an injection volume of 50µl and an ultraviolet (UV) lamp setting of 280nm, a peak at 4.44 minutes on the chromatogram trace was obtained. Under this system, a related macrolide, tylosin, showed a smaller peak at 9.78 minutes. Using a Waters Spherisorb S5P Phenyl HPLC column and the same mobile phase and detector, a tilmicosin peak occurred at 4.0 minutes with a tylosin peak (about 50% of the tilmicosin peak) at 6.4 minutes. Slowing the flow to 1ml/minute delayed the peaks to 6.3 and 9.5 minutes respectively. While no difference in the chromatographic signal was found between the 2 columns, it was decided to proceed with subsequent experiments using the Phenyl column. The main findings of the method development using various conditions on analytical standard solution made up in methanol, while employing an organic phase composed of acetonitrile:methanol 50:50 and an aqueous phase comprised of 0.1M phosphate buffer are highlighted in Table 2-5 hereunder.

Table 2-5 Effects of different mobile phases and flow rates on response to tilmicosin analyte

Mobile phase		Flow rate	pH	Observations
% Organic	% Aqueous	(ml/min)		
100	0	1	5	No peak observed
80	20	1	5	Peak at 9.16 mins
70	30	1.5	5	Peak at 6.09 mins
70	30	1.5	6.5	Peak at 5.84 mins
70	30	1.5	6.9	Peak at 5.4 mins
60	40	1.5	6.9	Peak at 6.2 mins
60	40	1	5.8	Peak at 5.67 mins

Using methanol only as the organic phase in a 60:40 ratio with phosphate buffer at pH 6.5 and using an analytical standard comprising both tilmicosin and tylosin solubilised in methanol resulted in a single peak at 4.6 minutes. Repeating this experiment using acetonitrile only as the organic phase resulted in a split peak for tylosin and a very broad peak for tilmicosin. Based on the shape and timing of peaks for tilmicosin and tylosin, the following mobile phase was used for all subsequent experiments: 300ml methanol, 300ml acetonitrile and 400 ml 0.1M sodium dihydrogen phosphate at pH 5.8. The flow rate chosen was 1.0ml/minute. The wavelength for optimal performance was set at 287nm being that used by Chan *et al* (1994) although there appeared to be little differences in response between samples analysed at 280nm and 290nm.

Applying the methodology of Chan *et al* (1994), experiments were conducted using solid-phase extraction. This was carried out using commercially available Sep-Paks which were conditioned with methanol and water. Various extractants including 0.1M phosphate buffer (pH 2.5), distilled water, or buffer followed by rinsing with distilled water, methanol and 0.01M methanolic acid (methanol containing 0.01M hydrochloric acid) were used while elutions were carried with acetonitrile, methanol or methanolic acid. Consistent linear recoveries were not always achieved and this approach was therefore abandoned. Extraction procedures were also conducted using

various organic solvents including ethyl acetate, hexane and diethyl ether, followed by drying down under nitrogen gas and resuspension in methanol or mobile phase. These methods proved very resource intensive and time consuming however and were difficult to replicate precisely. Physical conditions for the extraction were also investigated including the effect of changes in pH, the ratio of tissue to extractant, the freezing of samples to aid the precipitation of proteins and centrifugation. However, a problem of emulsification, samples not drying completely and dried samples being dirty or yellow on resuspending occurred. A further problem was the lack of reproducibility of the method and variable recoveries (50 - 85%). It is known that tilmicosin is light-sensitive and this may have been a reason for the variable results found.

Finally, a simple extraction method was developed where muscle tissue was blended in a 1:3 ratio (tissue expressed in grams; extractant in millilitres total volume) initially using methanol as the extractant, but subsequently using acetonitrile as the latter gave a better peak shape and a cleaner sample. Standing the blended muscle samples in the fridge overnight and centrifugation of the samples in a millifuge at 11,500g for 30 seconds gave rise to the cleanest samples and these could be run directly on the HPLC.

2.2.3.2 Enrofloxacin

An analytical method for the detection of residues of enrofloxacin (being parent compound and its principal metabolite, ciprofloxacin) in muscle that would have acceptable performance over the range of concentrations 250 to 2500ng/g for both compounds was the target for the development of this assay. Both enrofloxacin and ciprofloxacin are marker residues and it was important that the analytical method should be capable of detecting and quantifying both substances.

Reference to Strelevitz and Linhares (1996) led to experimentation with a 1mg/ml solution of enrofloxacin in methanol on HPLC using a Phenomenex Luna (5 micron) column and using a mobile phase comprised of 0.05M sodium dihydrogen phosphate buffer (pH 3.5) and acetonitrile (88:12). When run for 20 minutes with the UV detector set at 280nm, there was a small peak at 2.44 minutes. When enrofloxacin was diluted to 1:1000 in a mobile phase made up of 0.1M ammonium acetate:

acetonitrile: methanol (60:30:10) at pH 5.0 and with the UV detector set at 280nm, enrofloxacin showed a chromatographic peak at 4.32 minutes. A mobile phase of phosphate buffer: acetonitrile: methanol (80:15:5) resulted in a diminutive peak at 1.76 minutes which was judged not to be acceptable. Return to the original mobile phase of 0.1M ammonium acetate: acetonitrile: methanol (60:30:10) at pH 7.4 resulted in a smaller than expected peak at 8.96 minutes. Reducing the pH to 6.9 resulted in a similar sized peak at 7.04 minutes.

Regarding ciprofloxacin, following experimentation on solubility it was found that this compound was insoluble in methanol and in methanol plus water. It was also insoluble in acetonitrile and in a phosphate buffer at pH 3.5. Ciprofloxacin was soluble in 1M and 0.1 M hydrochloric acid (HCl), and following mixing, in a 0.1M solution of methanol plus hydrochloric acid. Repeating the HPLC experiment using both enrofloxacin and ciprofloxacin and a mobile phase comprised of phosphate buffer: acetonitrile: methanol (60:30:10) at pH 6.9 resulted in a peak identified as ciprofloxacin at a retention time of 3.08 minutes and enrofloxacin at 7.2 minutes. Experimentation in the use of a 0.1M ammonium acetate buffer at pH 5.0 was also carried out. In this case the mobile phase consisted of buffer: acetonitrile: methanol (70:20:10). Chromatograms showed a retention time for ciprofloxacin of 5.52 minutes and enrofloxacin of 6.93 minutes. As the peak shape of the chromatograms for both enrofloxacin and ciprofloxacin using this mobile phase was sharper than those obtained using the phosphate buffered mobile phase, subsequent runs were conducted using the ammonium acetate containing mobile phase. A change in the pH from 5.0 to 4.5 led to slightly shorter retention times with ciprofloxacin appearing at 5.2 minutes and enrofloxacin appearing at 6.16 minutes. The use of a lower pH was favoured as it appeared to give a greater in-use shelf life to the HPLC column which otherwise needed rinsing in 0.1M HCl prior to use and occasional regeneration using rinses in methanol, methanol:chloroform (1:1), chloroform, chloroform:hexane (1:1) and hexane.

Extraction of the 2 compounds from fortified muscle involved placing 1g muscle samples into 25ml plastic glass universal containers and adding a volume of 9ml of 0.1M methanolic acid to each. Tissues were homogenised using a Polytron blender.

The samples were fortified with both enrofloxacin and ciprofloxacin at concentrations between 250ng/g and 2500ng/g prior to being frozen (- 20°C) for 25 minutes to precipitate proteins. Centrifugation of the samples at 850g for 5 minutes yielded a clear supernatant that was chromatographed as before. The method displayed good linearity and recovery and was therefore chosen as the basis for experiments on validation.

An unpublished bioassay for muscle residues of enrofloxacin, which had been developed by the manufacturer, was also investigated. However, this method did not prove to be as sensitive or as useful as the chemical method, especially given the fact that the residue of enrofloxacin which occurs in tissues of treated animals is both parent compound and ciprofloxacin, both of which have antibacterial activity against the test organism. This finding concurs with that of Ellerbroek (1993) who reported that a HPLC method that he used was more sensitive than either a thin layer chromatographic or a microbiological inhibition assay.

2.2.3.3 Tiamulin

An analytical method for the detection of residues of tiamulin in muscle that would have acceptable performance over the range of concentrations 500 to 3000ng/g was required.

At the time of the conduct of the studies, neither an internationally recognised marker residue nor a MRL for residues of tiamulin was available. The only chemical assay method available was for the analysis of the residue 8α-hydroxymutilin in liver. However, this marker is a hydrolysis product of a number of metabolites formed in the liver and is unsuitable for analysis of injection-sites where parent compound is the marker residue.

A HPLC assay which used a mobile phase comprising phosphate buffer:acetonitrile:methanol (60:30:10) was attempted but not developed further because the method was poorly sensitive. A microbiological assay was developed for muscle based on a proprietary method for the assay of tiamulin in plasma (Ripley P., 1997, personal communication). Standard solutions of tiamulin in a

methanol:distilled water (1:1) over a concentration range from 10µg/ml to 1mg/ml yielded acceptable inhibition zones in the agar (14mm or greater). In order to improve the performance of this method, various parameters were examined, including the effects of varying the pH of the standard solutions on zone diameter and the effect of varying diameters and depth of agar wells. While pH effects were apparent at concentrations from 2.5 - 20.0µg/ml (zone diameters for pH 6 > pH 7 > pH 8), no marked differences were present at concentrations between 0.05 and 0.3µg/ml (500 - 3000ng/g). An agar depth of 7.5mm, which was achieved by pouring the agar into several Petri dishes and measuring the depth of the set agar with a callipers, gave optimal performance as can be seen from Table 2.6 hereunder. The data generated originate from 3 replicates per concentration tested, with the result expressed as a mean value.

Table 2-6 The effect of agar depth on the inhibitory zones of *Micrococcus luteus* in a tiamulin assay

Tiamulin conc. (µg/ml)	Diameter of zone of inhibition (mm) [mean of 3 samples]					
	Agar depth (mm)					
	5.0	6.0	7.0	7.5	8.0	9.0
0.200	11.70	10.15	11.05	13.10	12.10	13.55
0.100	10.10	9.95	10.45	11.85	10.60	11.60
0.050	9.05	9.30	9.60	10.25	9.20	9.80
0.025	7.95	nz	nz	8.45	nz	nz

nz = no zone of inhibition

A number 3 cork bore of 7.5mm diameter proved optimal to achieve an agar depth of 7.0 - 8.0mm depth. A preincubation period of at least 2 hours at < 8°C gave a better response when compared to immediate incubation in the oven although there was little difference between the results of preincubation for 1 hour in the fridge and overnight preincubation. Taking the plates from the fridge and placing them in the incubator resulted in a fall in the incubation temperature from 37°C to 31.5°C. It took approximately 7 hours for the temperature to recover.

Studies on the optimisation of dose volume were also conducted (Table 2-7). Volumes greater than 200µl proved unsuccessful as the agar depth needed to accommodate these volumes resulted in poor light transmission and difficulties in reading the inhibitory zones. The theoretical volume was calculated assuming that each agar well was a perfect cylinder. However, this assumption is not strictly correct as the well borer is slightly fluted at its cutting edge and therefore the diameter is not uniform, while the well depth is not precisely known due to the fact that the agar plates themselves are also slightly fluted. A dose volume of 150µl was selected based on the response achieved over the concentration range 0.05 - 0.2µg/ml.

Table 2-7 Effect of dose volume of tiamulin on inhibitory zones of Micrococcus luteus

Tiamulin concentration (µg/ml)	Diameter of zone of inhibition (mm) [mean of 3 samples]	
	Volume of 150µl used	Volume of 200µl used
0.200	11.75	12.80
0.100	10.65	11.20
0.050	9.50	10.35
0.025	nz	9.50

nz = no zone of inhibition

Katz *et al* (1978) reported that centrifugation improved the performance of a microbiological assay for penicillin residues in edible tissues by removing physical barriers to diffusion. Thus, all tissue samples were centrifuged prior to analysis for tiamulin residues. Whilst a technique of centrifuging tissue samples a second time in the high-speed centrifuge at 11,500g for 30 seconds showed no significant improvement in the performance of the assay compared to centrifugation at 850g only, nevertheless this additional step was incorporated in the analytical method as a precautionary measure in ensuring optimal sample clean-up.

2.2.4 Description of analytical methods for subsequent studies

2.2.4.1 Tilmicosin

A HPLC assay with UV detector was used to measure residue concentrations of tilmicosin. The following materials and methods were used:

Chemicals and reagents

Common Solvents and Solutions

a) Acetonitrile (HPLC grade), Scharlau.
b) Methanol (HPLC grade), Scharlau.
c) Water (Distilled).
d) Concentrated hydrochloric acid, Scharlau.

Buffers, Diluents and HPLC Mobile Phase Solutions

The buffer was 0.1 M sodium dihydrogen phosphate which was made up by taking 12g NaH_2PO_4 and making it up to just less than 1 litre with distilled water and adjusting the pH to 5.8 with concentrated hydrochloric acid. This was then made up to 1 litre exactly and filtered through a Whatman membrane filter with a pore size of 0.45µm. The mobile phase was made up by taking typically 600ml acetonitrile and adding 600ml methanol and 800ml phosphate buffer of pH 5.8. The solution was degassed and again brought to pH 5.8 with concentrated hydrochloric acid.

Equipment and Supplies

a) High-speed blender, Polytron.
b) Centrifuge, high speed, capable of providing 850g with 25ml containers.
c) Centrifuge, high speed, capable of providing 11,500g with 2ml containers.
d) pH meter.
e) Membrane filters, 0.45µm and filter apparatus.
f) Magnetic stirrer and Teflon-coated stirring bars.
g) Analytical balance.
h) HPLC system consisting of the following components:
   UV Spectrophotometric variable wavelength detector.
   Isocratic pump.

Liquid Chromatograph.

Auto Injector.

i) Integrator.

j) Degassing apparatus.

Procedure

A. Preparation of Tilmicosin Standard Solutions

1. Tilmicosin reference standard was a gift from Eli Lilly and Company, Indianapolis, Indiana, USA. The compound is hydroscopic and was maintained in a sealed, dark ampoule that was stored in the fridge at approximately 4°C. Under these conditions, the standard was reported by the manufacturer to be stable for 3 years.
2. Stock Standard Solution, 1000µg/ml.

   An appropriate amount of the reference standard (typically 5 to 10mg) was weighed accurately into a clean, glass septum vial. Using a calibrated pipette, methanol was added to make a 1000µg/ml solution. The stopper was tightly fastened and the solution was vortexed for 1 minute. Aluminium foil was wrapped around the vial to minimise light degradation. Stock solutions were made up fresh on the day of use.
3. Analytical Standard Solutions

   A 1000µg/ml stock standard was diluted to 100µg/ml with methanol. The 100µg/ml standard solution was diluted to 10µg/ml with methanol. These stock solutions were used to fortify the standard recovery muscle tissues as shown in Table 2-8 hereunder.

Table 2-8 Concentration of tilmicosin used to make up fortified muscle samples

Quantity of solution added (µl)	Stock solution used (µg/ml)	Conc. of tilmicosin in fortified tissue (ng/g)	Expected quantity of tilmicosin on HPLC column (ng)
0	-	0	0
50	10	150	5
100	10	300	10
20	100	600	20
40	100	1200	40
60	100	1800	60
80	100	2400	80
100	100	3000	100

For the recovery experiments, a 1:1000 dilution of the stock standard solution was also made using methanol giving a 1µg/ml solution. A 2 ml glass HPLC vial was filled with this solution. Ten, 20, 40, 60, 80 and 100µl samples of this solution were injected onto the HPLC column as shown in Table 2-9 hereunder. All samples were run in duplicate.

Table 2-9 Preparation of authentic standards of tilmicosin for recovery experiments

Quantity of solution added (µl)	Stock solution used (µg/ml)	Expected quantity of tilmicosin on HPLC column (ng)
10	1.0	10
20	1.0	20
40	1.0	40
60	1.0	60
80	1.0	80
100	1.0	100

B. *Preparation and extraction of tissue samples.*

Muscle samples were taken from the frozen state and were sliced with a scalpel or hack saw into thin pieces while still chilled and firm. Samples were prepared on Petri dishes to avoid cross contamination of samples and blades were cleaned with a tissue soaked in methanol between uses. Samples of 3.33g were weighed into 25ml plastic Sterilin containers to which 7.0ml of acetonitrile was added to give a total volume of 10ml. The samples were blended using a Polytron blender for approximately 20 seconds in the Sterilin vials until the tissue pellet was fully homogenised. Again care was taken to clean the blender between samples and it was also rinsed using distilled water at these times. The Sterilins were capped and stored either in a fridge overnight or in a freezer for 20 minutes prior to being centrifuged at 850g at 0^0C for 5 minutes. The supernatants were taken off by pipette into 2 ml conical, plastic millifuge vials and centrifuged again at 11,500g at room temperature for 2 minutes. Approximately 250µl of this supernatent was transferred by pipette to the glass inserts in the HPLC vials. A fortified set of standards including an untreated (negative) control muscle tissue sample was always prepared and run with each set of treated samples in the same way. Fortification took place at the point when samples had been homogenised.

c. *Assay Conditions*

Tilmicosin standard solutions and unknown sample solutions were injected into a HPLC using the parameters listed below:

a) Waters Spherisorb S5P Phenyl HPLC column.
b) Flow rate: 1.0ml/min.
c) Injection volume: 100µl.
d) Detector wavelength: 287nm.
e) Run time: 10.5 mins.
f) Mobile phase: acetonitrile: methanol: phosphate buffer, 6:6:8, pH 5.8.
g) Rinse: acetonitrile.

Two standard samples were chromatographed before starting a run in order to stabilise the baseline. The peak area of the tilmicosin signal in each chromatogram was determined using a peak width of 1000 and a peak threshold of 10. These values were chosen on the basis of experience. The chromatographic responses obtained from the fortified and negative control standards were used to calibrate a standard curve. As each fortified sample was injected 3 times onto the HPLC, the mean peak area was chosen for the construction of the concentration/response curve. Using a linear regression computer programme, the concentration of tilmicosin in the test samples (i.e. those of unknown concentration) was determined from each peak area obtained. Test samples were always injected twice and the mean response value used for the derivation of predicted concentrations. As the recovery of tilmicosin from fortified tissue was between 90 and 110% it was not necessary to use an internal standard.

2.2.4.2 Enrofloxacin

A HPLC assay with UV detector was used to measure residue concentrations of enrofloxacin and its major metabolite ciprofloxacin. The following materials and methods were used:

Chemicals and reagents

Common Solvents and Solutions

a) Acetonitrile (HPLC grade), Scharlau.
b) Methanol (HPLC grade), Scharlau.
c) Water (Distilled).
d) Concentrated hydrochloric acid, Scharlau.
e) Glacial acetic acid, Scharlau.

Buffers, Diluents and HPLC Mobile Phase Solutions

The buffer was a 0.1M solution of ammonium acetate that was made up by taking 7.71g ammonium acetate and making it up to slightly less than 1 litre with distilled water. The pH of the solution was adjusted to 5.0 with glacial acetic acid, following which the solution was made up to 1 litre with distilled water. This was filtered through a Whatman membrane filter with a pore size of 0.45µm. The mobile phase was made up by adding acetonitrile and methanol to the ammonium acetate buffer in a ratio 2:1:7 acetonitrile:methanol:ammonium acetate. The solution was degassed and brought to pH 5.0 with concentrated hydrochloric acid. The mobile phase was run through the HPLC column for at least 15 minutes to allow the column to equilibrate prior to sample analysis.

Equipment and Supplies

a) High speed blender, Polytron.
b) Centrifuge, high speed, capable of providing 850g with 25ml containers.
c) Centrifuge, high speed, capable of providing 11,500g with 2ml containers.
d) pH meter.
e) Membrane filters, 0.45µm and filter apparatus.
f) Magnetic stirrer and Teflon-coated stirring bars.
g) Analytical balance.
h) HPLC system consisting of the following components:
   UV Spectrophotometric variable wavelength detector.
   Isocratic pump.

Liquid Chromatograph.

Auto Injector.

i) Degassing apparatus.

j) Integrator.

Procedure

A. Preparation of enrofloxacin and ciprofloxacin standard solutions

1. Enrofloxacin and ciprofloxacin (as ciprofloxacin HCl) reference standards were a gift from the Bayer Company, Leverkusen, Germany. They were stored separately in glass vials at room temperature, in the dark and had an expiry date of 18 months from the date of receipt.
2. Stock Standard Solutions, 1000µg/ml.

   Appropriate amounts of the reference standards (typically 5 to 10mgs) were weighed accurately into clean, glass septum vials. Using calibrated pipettes, methanol was added to make a 1000µg/ml solution of enrofloxacin while a 1000µg/ml solution of ciprofloxacin was made up using a 90:10 solution of methanol:0.1M HCl. The stoppers were tightly fastened and the solutions were vortexed for 1 minute. Stock solutions were made up fresh on the day of use.
3. Analytical Standard Solutions

   Both enrofloxacin and ciprofloxacin were diluted in the same way using methanol: 0.1M hydrochoric acid 90:10 i.e. 0.01M methanol HCl. From the 1000µg/ml stock standards of enrofloxacin and ciprofloxacin, 1 in 10 dilutions in methanol were made i.e. 0.5ml of enrofloxacin plus 0.5ml ciprofloxacin made up to a total volume of 5ml in methanol. This stock solution contained 100µg/ml of each active substance. The 100µg/ml standard solution was diluted to 10µg/ml with methanol. These stock solutions were used to fortify the standard recovery muscle tissues as shown in Table 2-10 hereunder. Blank (unfortified) control tissues were made up in the same way and run alongside fortified samples.

Table 2-10 Conc. of enrofloxacin and ciprofloxacin used to make up fortified muscle samples

Quantity of solution added (µl)	Stock solution used (µg/ml)	Conc. of enrofloxacin and ciprofloxacin in fortified tissue (ng/g)	Expected quantity of enrofloxacin and ciprofloxacin on HPLC column (ng)
25	10	250	2.5
50	10	500	5.0
10	100	1000	10.0
15	100	1500	15.0
20	100	2000	20.0
25	100	2500	25.0

A 1:1000 dilution of the standard solutions of enrofloxacin and ciprofloxacin was also prepared by taking 20µl of each stock solution, adding together and making up to 20 ml with methanol, giving a 1µg/ml solution of each standard. A 1:10 solution of this was prepared again in methanol, giving a 0.1µg/ml solution of each standard. A 4ml glass HPLC vial was filled with each stock solution. Samples of these solutions were injected onto the HPLC column as shown in Table 2-11 hereunder.

Table 2-11 Preparation of authentic standards of enrofloxacin and ciprofloxacin for recovery experiments

Quantity of solution added (µl)	Stock solution used (µg/ml)	Expected quantity of enrofloxacin and ciprofloxacin on HPLC column (ng)
25	0.1	2.5
50	0.1	5.0
100	0.1	10.0
15	1.0	15.0
20	1.0	20.0
25	1.0	25.0

Each sample for was run in triplicate and separate standard curves for enrofloxacin and ciprofloxacin were constructed by plotting the mean response (peak area) obtained from each injection against the concentration used.

B. *Preparation and extraction of tissue samples.*

Muscle samples were taken from the frozen state and were sliced with a scalpel or hack saw into thin pieces while still chilled and firm. Samples were prepared on Petri dishes to avoid cross contamination of samples and blades were cleaned with a tissue soaked in methanol between uses. Tissue samples of 1g were weighed into 25ml plastic Sterilin containers to which 9.0ml of 0.01M methanol acid was added to give a total volume of approximately 10mls. The samples were blended using a Polytron blender for approximately 20 seconds in the Sterilin vials until the tissue pellet was fully homogenised. Again care was taken to clean the blender between samples and it was also rinsed using distilled water at these times. The Sterilins were capped and either placed in a fridge overnight or allowed to stand in a freezer for 1 hour prior to being centrifuged at 850g at 0°C for 5 minutes. The supernatants were taken off by pipette into 2ml conical plastic millifuge tubes and centrifuged again at 11,500g at room temperature for 2 minutes. Approximately 350µl of this supernatant was transferred by pipette to the glass inserts in the HPLC vials.

A fortified set of standards including an untreated (negative) control muscle tissue sample was always prepared and run with each set of treated samples in the same way and the data generated was used to calibrate a dose/response curve from which the unknown samples were read. Fortification took place at the point when samples had been homogenised.

c. *Assay Conditions*

Enrofloxacin and ciprofloxacin standard solutions and unknown sample solutions were injected into a HPLC using the parameters listed below:

a) Luna 5µm C_{18} HPLC column.
b) Flow rate: 1.0ml/min.
c) Injection volume: 100µl (for analysis of 'unknowns').
d) Detector wavelength: 280nm.
e) Run time: 11 mins.
f) Mobile phase: acetonitrile:methanol:ammonium acetate buffer, 2:1:7, pH 5.0.
g) Rinse: mobile phase.

At least 1 authentic standard was run in duplicate to equilibrate the column before starting the analyses proper. The peak areas of the enrofloxacin and ciprofloxacin signals in each chromatogram were determined using a peak width of 1000 and a peak threshold of 10. These values were chosen on the basis of experience. The chromatographic responses obtained from the fortified and negative control standards were used to calibrate a standard curve. As each fortified sample was injected 3 times onto the HPLC, the mean peak area was chosen for the construction of the concentration/response curve. Using a linear regression computer programme, the concentration of enrofloxacin and ciprofloxacin in the test samples (i.e. those of unknown concentration) were determined from each peak area obtained. Test samples were always injected twice onto the HPLC column and the mean response value used for the derivation of predicted concentrations. As the recovery of enrofloxacin and ciprofloxacin from fortified tissue was between 85 and 110% it was not necessary to use an internal standard.

2.2.4.3 Tiamulin

A bioassay, using *Micrococcus luteus,* was used to measure residue concentrations of tiamulin. The following materials and methods were used:

Chemicals and reagents

Common Solvents and Solutions

a) Methanol (HPLC grade), Scharlau.
b) Water (Distilled)

Materials and Test Organism

a) *Micrococcus luteus* NCIMB 8553, NCIMB.
b) Nutrient agar (Oxoid CM3), Fanin Healthcare.
c) Antibiotic medium 11(Difco # 0593), Pall Gelman.
d) Muellar-Hinton broth (Oxoid CM 405), Fanin Healthcare.

Equipment

a) High speed blender, Polytron.
b) Centrifuge, high speed, capable of providing 850gwith 25 ml containers.
c) Centrifuge, high speed, capable of providing 11,500g with 2 ml containers.
d) Analytical balance.
e) Autoclave.
f) Waterbath.
g) Incubator @ 37°C.
h) Fridge @ 4°C.
i) Glass septum vials (20ml size).
j) Vortex.
k) Graduated plastic Sterilins (25ml size).
l) Plastic conical millifuge containers (2ml).
m) 30 ml Universal containers.
n) Pipettes (50 and 100µl Hamilton syringes and micropipettes 100µl, 1ml and 5ml)
o) Pipette tips.

p) 500 ml Duran bottle.

q) 1 litre conical flask.

r) Petri dishes (90mm).

s) Cork borer (no. 3).

t) Vernier callipers.

u) Table lamp.

v) Sterile lancet.

Procedure

A. *Preparation of Tiamulin Standard Solutions*

1. Tiamulin (as tiamulin hydrofumarate) reference standard was a gift from Novartis, Basle, Switzerland. It was stored in a glass vial, in the dark at 2°C and had an expiry date of 5 years from the date of manufacture. It carried the instruction 'protect from moisture'.
2. Stock Standard Solution, 1mg/ml.

   An appropriate amount of the reference standard (typically 5 to 10 mgs) was weighed accurately into a clean, glass septum vial. Using a calibrated pipette, a methanol water solution (50:50) was added to make a 1000µg/ml solution of tiamulin. As 1.235g tiamulin hydrofumarate is equal to 1.0g tiamulin, the volume of methanol water required was calculated by dividing the theoretical volume by 1.235. The stopper was tightly fastened and the solution was vortexed for 1 minute. Stock solutions were made up freshly on the day of use.

3. Analytical Standard Solutions

A 1000µg/ml stock standard was diluted to 100µg/ml with methanol:water 50:50. This was diluted to 10µg/ml with methanol:water 50:50. Both stock solutions were used to fortify the standard recovery muscle tissues as outlined in Table 2-12 hereunder.

Table 2-12 Concentration of tiamulin used to make up fortified muscle samples

Quantity of solution added (µl)	Stock solution used (µg/ml)	Expected tiamulin conc. in fortified tissue (ng/g)
0	-	0
25	10	250
50	10	500
10	100	1000
20	100	2000
30	100	3000

A 1:1000 dilution of the stock standard solution was also made using methanol:water 50:50 giving a 1µg/ml solution. An authentic set of standard solutions was made up using the 1µg/ml solution as outlined in Table 2-13 hereunder.

Table 2-13 Preparation of authentic standards of tiamulin for recovery experiments

Quantity of 1µg/ml solution (ml)	Quantity of Methanol Water (50:50) used (ml)	Final concentration of tiamulin (µg/ml)
0	10	0.0
0.5	9.5	0.05
1.0	9.0	0.1
2.0	8.0	0.2
3.0	7.0	0.3

All samples were run in triplicate.

B. *Preparation and extraction of tissue samples.*

Muscle samples were taken from the frozen state and were sliced with a scalpel or hack saw into thin pieces while still chilled and firm. Samples were prepared on Petri dishes to avoid cross contamination of samples and blades were cleaned with a tissue soaked in methanol between uses. One gram samples were weighed into 25ml plastic Sterilin containers to which 9.0ml of methanol water (50:50) was added to give a total volume of 10mls. The samples were blended using a Polytron blender for approximately 20 seconds in the Sterilin vials until the tissue pellet was fully homogenised. Again care was taken to clean the blender between samples and it was also rinsed using distilled water at these times. The Sterilins were capped and stored either in a fridge overnight or in a freezer for 20 minutes prior to being centrifuged at 850g at 0°C for 5 minutes. The supernatants were taken off by pipette into 2ml conical, plastic millifuge vials and centrifuged again at 11,500g at room temperature for 2 minutes. The supernatants of these samples were again transferred to 30ml Universal containers that were stored in the fridge until assayed.

A fortified set of standards including an untreated (negative) control muscle tissue sample was always prepared and run with each set of treated samples in the same way. Fortification took place at the point when samples had been homogenised.

C. Assay Conditions.

Preparation of the bio-assay plates:

a) Culture maintenance agar

A 14g sample of nutrient medium (Oxoid CM3) was weighed and dissolved in 500ml of distilled water. The solution was boiled until the nutrient medium was completely dissolved. The solution was sterilised by autoclaving at 15p.s.i. for 15 minutes at 121°C. Cooling to 50°C took place in a waterbath. A volume of 7mls of the molten agar was transferred into 30ml Universal containers that were then placed at a 45° angle in order to make agar slopes.

b) Assay agar

A 15.25g sample of antibiotic medium No. 11 (Difco #0593) was weighed and dissolved in 500ml of distilled water. The solution was boiled until the medium was completely dissolved. The solution was sterilised by autoclaving at 15 p.s.i. for 15 minutes at 121°C. Cooling to 50°C took place in a waterbath and the pH was adjusted with 1 N HCl or 1 N KOH so that after sterilisation it was 8.0 ± 0.1 at 45°C.

c) Suspending fluid

A 10.5g sample of Muellar-Hinton medium (Oxoid CM 405) was weighed and dissolved in 500ml of distilled water. The solution was sterilised by autoclaving at 15 p.s.i. for 15 minutes at 121°C. Cooling to 50°C took place in a waterbath and 9mls of the broth was transferred into 30ml Universal containers.

d) Preparation of the inoculum

The test strain *Micrococcus luteus* NCIMB 8553 was supplied to the laboratory in a freeze-dried form within a glass vial. It was resuspended with 0.5ml nutrient broth on buffered peptone water and then streaked on nutrient agar slope. The agar was incubated at 37°C for 18 hours. A loopful of test strain was inoculated into 9mls of suspending fluid. The suspending fluid was then incubated at 37°C for 18 hours. A total viable count (TVC) was performed on this suspension in accordance with the standard operating procedure of the laboratory. Depending on the TVC, as much suspension was added to the assay agar (500mls approximately) to provide 1.0×10^4 c.f.u. per ml.

e) Preparation of the assay agar

The assay agar was prepared according to the specifications outlined above. After the addition of a suitable amount of bacterial suspension, approximately 40mls of this medium was poured into each Petri dish. The depth of the agar was approximately 7.5mm. Any plates with a depth outside the range 7.0 to 8.0 were not used in the assay. The agar was left to solidify at room temperature for 2

hours. Three wells of approximately 7.5mm diameter were cut in the agar equidistant from the edge of the plate using a cork-borer (No. 3).

Standard preparation

A 1 mg/ml solution of tiamulin was made up by dissolving in an equivalent volume of methanol:water (50:50) in a volumetric flask (stock solution). From this solution a 1:10 dilution and a 1:100 dilution were made using the methanol water to give working stocks of 100µg/ml and 10µg/ml that were used to fortify the muscle tissues as outlined in Table 2-14 hereunder.

Table 2-14 Concentration of tiamulin used to make up fortified muscle samples

Quantity of solution added (µl)	Stock solution used	Conc. (µg/ml)	Expected tiamulin conc. in fortified tissue (ng/g)
0	-	0	0
25	0.01	0.025	250
50	0.01	0.05	500
10	0.1	0.1	1000
20	0.1	0.2	2000
30	0.1	0.3	3000

Samples were run in triplicate.

Filling the plates

A volume of 150µl of standards and samples were pipetted into each labelled well in the agar plates. All samples and standards were dosed within 15 minutes of each other. The plates were put into a refrigerator at 4°C for 2 hours prior to incubation to allow prediffusion to take place. All plates were then incubated at 37°C in an incubator for 18 hours.

Calculation of results

The diameter of the resulting zones of inhibition was measured to the nearest 0.1mm using Vernier callipers. These responses (expresses in millimetres) obtained from the fortified and negative control standards were used to calibrate a standard curve. As each fortified sample was run on the bioassay in triplicate, the mean diameter of the zones of inhibition was chosen for the construction of the concentration/response curve. Using a linear regression computer programme, the concentration of tiamulin in the test samples (i.e. those of unknown concentration) was determined from each peak area obtained. Analyses of samples from the experiments (unknown concentrations) were conducted twice or 3 times according to the availability of a sufficient number of wells. As the recovery of tiamulin from fortified tissue was between 90 and 110% it was not necessary to use an internal standard.

2.3 Survey design

The design of the studies took into consideration the need to collect data on the prevalence of blemishes and injection-site residues of anti-bacterials in respect of cattle, sheep and pigs, the need for geographical spread in the origin of the livestock to ensure sample was truly representative and the need to include data from both export factories as well as product destined for consumption locally. It was decided to restrict the study to injection-site blemishes observed during the cutting of the carcass into primal and sub-primal cuts. The reasons for this were twofold:

a) to allow comparison with published data from North America,
b) to avoid possible conflict with governmental veterinarians who inspect carcasses and harvest blemishes for official residue surveillance purposes.

The factories and butcher shop used in the studies were chosen on the basis of the following criteria:

a) willingness to co-operate,
b) through-put of carcasses,
c) logistical considerations (proximity, availability of records).

The co-operation of some factories was not forthcoming and several difficulties were encountered before satisfactory recruitment was achieved. The participating factories included a sheep boning-hall near Dublin and another in the south of the country, a beef boning-hall in the midlands and another in the south and a single pork boning-hall in the midlands of the country. A butcher operating in central Dublin was also recruited. The reason for including data from a local butcher shop was that, up to the early to mid 1990s, factories in Ireland producing meat for export markets operated to higher standards of veterinary control and meat hygiene compared to local abattoirs and butchers which, though closer to their customers, were not subject to the same official veterinary scrutiny. Moreover, the number of blemishes in meat cuts and portions for sale might be different from that recorded at the boning-halls where the blemishes were hidden within the primal or subprimal portion and not visible on the surface or palpable and therefore overlooked.

The animals included in the survey were produced in farms throughout Ireland. The butcher's animals were either reared on his own farm or were from farms close to him and known to him personally. The butcher's cattle were heifers 18 - 22 months old and lambs/hoggets 3 - 10 months old. The pigs were typical fattened stock (less than 5-months-old) with a very few sows being included. The cattle were either steers or heifers and were less than 3-years-old; no cows were available. The author set the sampling periods with all carcasses processed during the period being included.

2.3.1 Sample collection and recording

The author or the boning-hall quality control supervisor harvested injection-site blemishes, including lesions and abscesses, from primal cuts in the boning-halls of participating factories on a regular basis during 1999 and in early 2000. The butcher recorded any blemishes in meat cuts that he prepared from meat primals and subprimals for sale to customers during 1999.

The author examined all samples grossly. Blemishes were categorised as cystic, scar or abscess according to their physical characteristics. A cyst was characterised by the presence of a fluid or blood filled sac. A scar was characterised by the presence of either clear, fibrous almost plastic-like tissue or white-fibrous or fatty-fibrous tissue. An abscess was characterised by the presence of pus. The weight of the tissue

samples, which would have been condemned as unfit for human consumption, was taken and the daily throughput of the boning-hall was recorded. Samples were excised using a butcher's knife that was sterilised between samples. The harvested samples were placed in polythene sample bags, labelled with sample number, date and factory of origin and dispatched in special freezer boxes for antibacterial screening. Upon delivery to the laboratory where the analysis took place, the samples were either processed on the day of receipt or stored at 2-8°C pending analysis within 3 days. When it was not possible to collect samples on the day of harvesting, they were stored in the freezer at –80°C for up to 6 weeks and then transported by the author to the laboratory in the frozen state. These samples were allowed to thaw in the fridge at 2-8°C pending analysis.

2.3.2 Sample analysis

Samples were analysed using the EU 4-Plate-Method developed by Bogaerts and Wolf (1980). Using this method, it was possible to differentiate between chemical families of anti-bacterial drugs but not to quantify or identify the precise drug present. The test was carried out as an agar diffusion test on 4 plates of agar media inoculated with *Bacillus subtilis* spores (at pH 6.0, 7.2 and 8.0) and *Micrococcus luteus* (at pH 8.0). Diffusion of an anti-bacterial substance could be demonstrated by the formation of zones of inhibition. This work was carried out at the Irish Equine Centre (IEC) under conditions of Good Laboratory Practice. The pivotal points in the test procedure used at the IEC were as outlined in Sections 2.3.2.1 – 2.3.2.3 hereunder.

2.3.2.1 Handling of samples

Samples were logged into the anti-bacterial residue logbook and were assigned a laboratory number. A cylindrical core 8mm in diameter was cut from the centre of each tissue sample using a cork borer. Slices of this tissue, of 2mm thickness, were placed in each of 4 bioassay plates using a forceps. A maximum of 63 samples could be analysed on the bioassay plate using an 8 X 8 template, 1 space being allocated for the anti-bacterial control. Thus each sample was analysed in each of the 4 test plates. Once all the samples to be analysed were placed in the bioassay plate, the positive anti-bacterial control disc for each plate was placed onto the agar. The test plates were incubated at 30°C for 18 to 24 hours.

2.3.2.2 Quality control

The sensitivity of the test plates was monitored by measuring the inhibition zones given by the anti-bacterial control disks that were required to be equal to, or in excess of, the minimum zone sizes given in Table 2-15 hereunder.

Table 2-15 Anti-bacterial control disks used in the EU 4-plate-test

Test plate	Anti-bacterial control disk	Minimum acceptable inhibition zone required (mm)
pH 6.0 + *B. subtilis*	Penicillin G 0.01 iu	18
pH 7.2 + *B. subtilis*	Sulphadimidine 0.5µg	18
pH 8.0 + *B. subtilis*	Streptomycin 0.5µg	22
pH 8.0 + *M. luteus*	Streptomycin 0.5µg	18

2.3.2.3 Interpretation and recording of results

When any of the test plates demonstrated a zone of inhibition greater than 2mm, the sample was considered positive. No detectable inhibition of growth was considered negative. Any responses regarded as doubtful (scattered colonies in zone of inhibition, contamination) were repeated. The zone size results were recorded on a raw data day sheet with a positive or negative result being recorded alongside the relevant sample on the residue logbook. A positive response in the bioassay plate inoculated with *Bacillus subtilis* at pH 6.0 is indicative of residues of β-lactam anti-bacterials and tetracyclines; *B.subtilis* at pH 8.0 detects, in particular, aminoglycoside residues and at pH 7.2 detects sulphonamide residues; *Micrococcus luteus* at pH 8.0 detects, in particular, β-lactam and macrolide anti-bacterial residues.

2.4 Statistical analyses and calculations

Calibration standard curves, generated in the method validation studies as well as following each sample set, were constructed on the basis of method response of the marker residue versus concentration by means of a computer-aided programme using

a least squares regression fit. The calculation of the regression line for the calibration curve is expressed as Equation 2-1 hereunder.

Equation 2-1 Calculation of regression line

$$Y = a + bX$$

where Y = the response variable (peak area for HPLC analysis, zone of inhibition for bioassay analysis),

X = the predictor variable (concentration of drug),

b = the slope of the line,

a =the intercept.

Method accuracy was expressed as a percentage of the true value (100%). When the response variable (Y) was less than the true value (X), Equation 2-2 outlined hereunder was used.

Equation 2-2 Calculation of accuracy when $X < Y$

$$100 - \left(\frac{Y - X}{X} \times 100 \right)$$

where Y = response variable (predicted concentration of drug)

X = predictor variable (concentration of drug used for fortification)

When the response variable (Y) was greater than the true value (X), Equation 2-3 outlined hereunder was used.

Equation 2-3 Calculation of accuracy when $X > Y$

$$100 - \left(\frac{X - Y}{X} \times 100 \right)$$

where Y = response variable (predicted concentration of drug)

X = predictor variable (concentration of drug used for fortification)

The coefficient of variation (CV) was calculated from the standard deviation and the mean values using Equation 2-4 hereunder. It was expressed as a percentage.

Equation 2-4 Calculation of coefficient of variation

$$\% \text{ CV} = \frac{\text{Standard Deviation}}{\text{Mean}} \text{ X } 100$$

The standard error of the mean (SEM) was calculated for those values obtained from the repeated heating experiments on the test drugs and cooking studies on subsamples of incurred residues using Equation 2-5 hereunder.

Equation 2-5 Calculation of standard error of the mean

$$SEM = \frac{\text{Standard Deviation}}{\sqrt{n}}$$

where SEM = standard error of the mean

SD = the standard deviation

n = the number of responses taken.

In the residue studies conducted, the limit of detection of the analytical method (LOD) was calculated from Equation 2-6 hereunder.

Equation 2-6 Calculation of limit of detection

$$\text{Limit of Detection} = \frac{3 \times c \times \delta}{r}$$

where c = analyte concentration

δ= standard deviation of mean response

r = mean response.

Equation 2-7 relates the limit of quantification (LOQ) to the LOD and is shown hereunder.

Equation 2-7 Calculation of limit of quantification

$$\text{Limit of Quantification} = \frac{10 \times \text{LOD}}{3}$$

The Student's test was used to analyse the variability between residue concentrations in the tissue samples in the raw state and those following cooking. The test method used a 2-tailed distribution with paired analysis (raw *versus* cooked concentrations). Values of $p < 0.05$ were classified as significant.

CHAPTER 3: METHODS VALIDATION AND PERFORMANCE

3.1 Introduction

The objective of this segment of the research was to define the performance characteristics of each of the analytical methods for the quantification of residues of tilmicosin, enrofloxacin and its marker metabolite ciprofloxacin and tiamulin in beef, mutton and pork and to compare the performance of the methods in respect of muscle tissue from each species. Information on cross-species method performance may have application in developing a better understanding of the risk assessment in extrapolation of residue data from major food species to minor food-producing species, albeit that all 3 species investigated were classified as major species.The performance of the tests on each drug was characterised by means of validation studies. Validation is a process that aims to demonstrate, by repeated analysis, that a method is fit for the purpose for which it is developed (SRWP, 1999). The characteristics are outlined in Sections 3.1.1 – 3.1.5 hereunder.

3.1.1 Specificity

Specificity is the ability of a method to distinguish between the analyte being measured and other substances that may be present in the sample being analysed (SRWP, 1999). It must be noted that the objective of developing the assays was not to have a confirmatory method (methods that provide full information enabling the analyte to be identified unequivocally) for each substance, but rather to have a method capable of meeting the requirements for detection and quantification. According to Kateman and Pijpers (1981) the term *specific* generally refers to a method that produces a response for a single analyte only; other components present in the sample do not produce an analytical signal. The term *selective* refers to a method that can discriminate between various components of a mixture without any mutual interaction of the components. While the term *selective* is more appropriate to describe this parameter, most authoritative books and regulatory guidelines (e.g. VICH, 1998b) on analytical methods use the term *specific* and it is the latter term that is used hereunder.

3.1.2 Linearity and accuracy

The linearity of a method refers to the ability of the method to produce a response (within a given range) that is directly proportional to the concentration of analyte in the sample. A high correlation coefficient indicates the linearity of response. The accuracy of the method refers to the closeness of agreement between the true value and the mean result (accuracy of the mean) that is obtained by applying the experimental procedure a large number of times.

3.1.3 Precision and reproducibility

Precision is the measure of the closeness of agreement (degree of scatter) between the results obtained by applying the analytical procedure several times under the prescribed conditions. It includes repeatability and within-laboratory reproducibility (intermediate precision). Possible variations that affect method precision include differences in room temperature and humidity, differences in equipment used, variations in materials and instrument conditions, consumables of different batches or ages, solvents, reagents and other materials of different quality.

A high level of accuracy combined with a low percentage CV and a high correlation coefficient denotes a good, reproducible assay. The minimum acceptable trueness for a quantitative method required by European Union legislation (EC, 1991) for an analyte with a concentration $\geq$10µg/kg and $\leq$100µg/kg (ng/g) is -20% to + 10%.

3.1.4 Sensitivity of methods

The limit of quantification (LOQ) corresponds to the smallest measured content of an analyte above which a determination of the analyte can be made with a specified degree of accuracy and precision. The limit of detection (LOD) and LOQ values are measures of the sensitivity and precision of an assay. The LOD refers to the lowest concentration of an analyte that a particular assay system can detect but not necessarily quantified as an exact value. LOD values may be determined by several means including visual examination, signal to noise ratio, standard deviation of the response and the slope (VICH, 1998a). The lower the LOQ and LOD, the more sensitive (and precise) is the assay system.

3.1.5 Robustness

The evaluation of robustness depends on the type of procedure under study. In the context of the studies conducted, of greatest import is the stability of the analytes.

3.2 Materials and methods

A detailed description of each of the test methods, including extraction technique, clean-up procedure and method of analysis is provided in Chapter 2. The analytical methods employed for tilmicosin and enrofloxacin were HPLC based methods while analysis of tiamulin was by means of a bioassay. As enrofloxacin is metabolized in mammals to ciprofloxacin, validation studies were also conducted for this substance. Muscle samples from each species were fortified with the test drugs over the required concentration range being 0 - 3000µg/kg for tilmicosin and tiamulin and 0 - 2500µg/kg for enrofloxacin and ciprofloxacin. Responses for the HPLC assays were calculated as peak areas while those in the bioassay were calculated as the zone diameter of inhibition. The specific characteristics mentioned in Section 3.1 above are described in Sections 3.2.1 – 3.2.5.

3.2.1 Specificity

Selectivity in the HPLC was obtained by using particular analytical equipment and chromatographic conditions, such as mobile-phase composition, pH, flow rate, column type and detector wavelength. Internal standards were not used in the assays, as there was no transfer of analyte from one phase to another. The identification of the analytes was confirmed by:

a) co-chromatography using fortified samples in the HPLC assays employed,
b) demonstration of linearity of response to the range of concentrations tested,
c) comparing the retention times of the standard substances with those obtained from tissue samples.

Specificity can seldom be fulfilled for microbiological methods that rarely differentiate between individual anti-bacterial substances. Nevertheless, the linearity of response of the assay, both using standard solutions and in the muscle matrix, allowed for an assessment of possible interference. Moreover, the specific assay

conditions serve to limit the response of the system to a limited number of anti-bacterial substances.

3.2.2 Linearity and accuracy

Linearity and accuracy was investigated over the concentration range for each drug in respect of each species tissue matrix. A total of 6 concentrations of fortified tissue were used in respect of tilmicosin, enrofloxacin and ciprofloxacin while 4 concentrations were chosen for the bioassay. A negative control (unfortified muscle) was also used in all methods. The responses were used to construct a linear regression line from which a correlation coefficient was generated. Methods recovery from the tissue matrix was evaluated by comparing the responses obtained from fortified tissue with those obtained from standard solutions of analyte run directly. As recovery was within the 85 –110% in respect of all drugs in all species, no correction factor was necessary in the calculation of accuracy. The accuracy of the analytical method was evaluated by inputting the responses obtained (y values) and reading the predicted concentration variables (x values) obtained. The accuracy of each concentration measured was calculated in relation to the true value and was expressed as 0 – 100% of this value. The overall accuracy was calculated from the mean of each of the accuracy values over the concentration range.

3.2.3 Precision and reproducibility

Repeatability tests were performed on each of the methods by assaying the same samples over the entire concentration range 4 times by the same method using the same equipment within short time intervals (within 24 hours for the chemically-based assays and within 48 hours for the bioassay). Reproducibility tests were performed on each method by fortifying control tissues with substrate standard at various different levels over the entire concentration range on each of 4 different days.

3.2.4 Sensitivity of the methods

The limits of quantification and detection were calculated using the method described in Chapter 2 and based on the standard deviation of the responses obtained. The method was chosen on the basis that it was in regular use in the laboratory and was considered to provide acceptable results over years of application.

3.2.5 Robustness

The analytical conditions were standardised for all the experiments conducted. The same equipment, reagents and analytical instrumentation were used throughout. Particular care was taken when handling tilmicosin samples, as they were known to be light sensitive. All such samples were protected from the light by means of foil wrapping to enclose sampling vials, and when necessary, by storage in a fridge. In order to investigate whether the measured analyte levels could be subject to variation due to fluctuations in time, temperature and matrix components, the stability of each of the extracted analytes was studied over several days. In practice, all samples were prepared and analysed within a few hours or days in the case of the chemical assays and within a couple of days to a week in the case of the bioassay. Storage of samples was in the fridge at 2 – 6°C.

3.3 Results

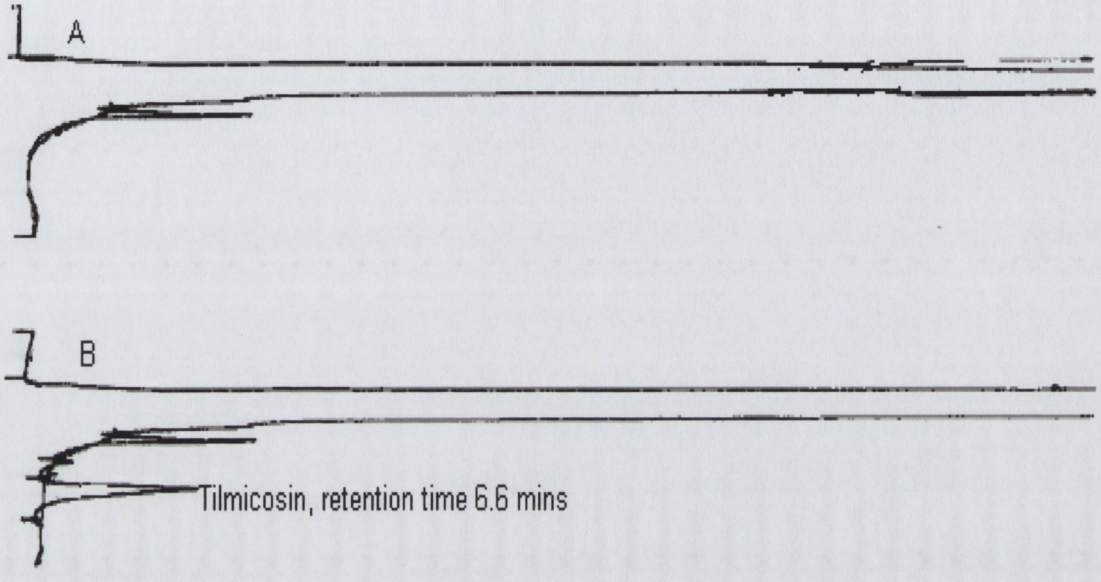
3.3.1 Tilmicosin

3.3.1.1 Specificity

The HPLC system employed in this method separated tilmicosin from impurities extracted from muscle and from related substances that may be present in tissue extract solutions as can be seen in Figures 3-1 to 3-3 hereunder.

Figure 3-1 Typical HPLC chromatogram for tilmicosin in beef muscle

A= Control (blank) beef; B= Fortified beef (2400ng/g).



While tilmicosin displayed a second signal at a retention time of approximately 2.8 minutes, this signal was not measured and did not form part of the validation of the fortified sample because it was masked by the signal from the matrix in respect of all tissues analysed. Note in respect of the numbers given for the mean peak area in the tables below, the decimal point has been arbitrarily but consistently set.

Figure 3-2 Typical HPLC chromatogram for tilmicosin in lamb muscle

A= Control (blank) sheep muscle; B= Fortified sheep muscle (2400ng/g).

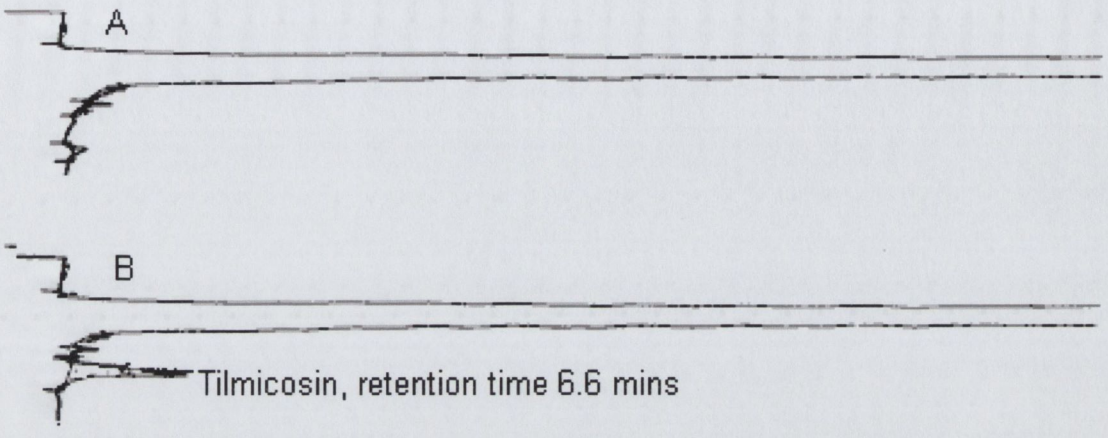
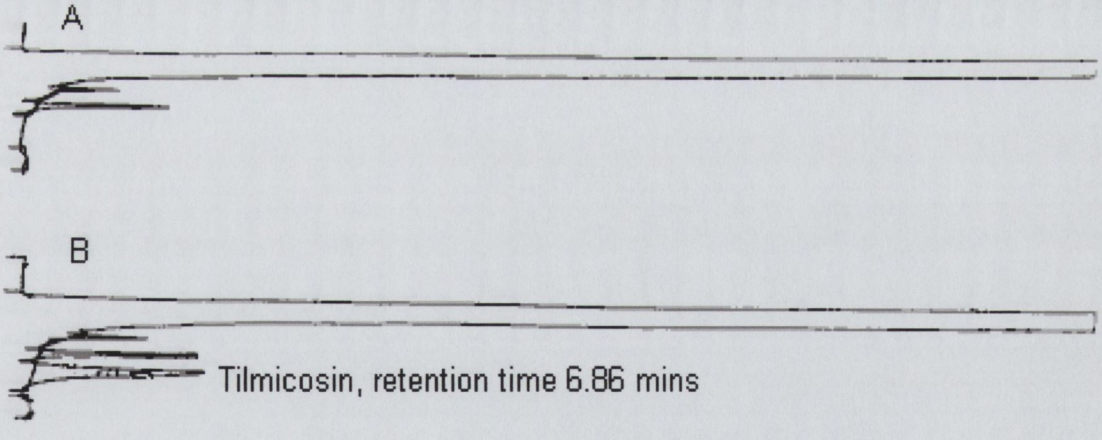


Figure 3-3 Typical HPLC chromatogram for tilmicosin in pork muscle

A= Control (blank) pork; B= Fortified pork (3000ng/g).



3.3.1.2 Linearity and accuracy

A linear response over the entire range was obtained in respect of beef as shown in Tables 3-1 to 3-4.

Table 3-1 Linearity and accuracy of cattle muscle fortified with tilmicosin (300-3000ng/g), day 1

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	29.132 ± 1.520	6.57	360.51	79.83
600.00	53.195 ± 0.496	0.93	599.30	99.88
1200.00	103.476 ± 1.361	1.32	1098.30	91.53
1800.00	177.498 ± 1.204	0.68	1832.80	98.18
2400.00	231.643 ± 0.975	0.42	2370.10	98.75
3000.00	299.046 ± 1.852	0.62	3039.00	98.70

Mean coefficient of variation (CV) = 1.76% n = 4

Mean accuracy = 94.48% Correlation coefficient = 0.9984

SD = standard deviation

Table 3-2 Linearity and accuracy of cattle muscle fortified with tilmicosin (300-3000ng/g), day 2

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	28.123 ± 2.726	9.69	320.20	93.27
600.00	54.764 ± 4.097	7.48	588.15	98.03
1200.00	114.693 ± 0.939	0.81	1190.90	99.24
1800.00	176.336 ± 1.386	0.79	1810.90	99.39
2400.00	231.124 ± 1.245	0.54	2362.00	98.42
3000.00	297.331 ± 0.560	0.19	3027.90	99.07

Mean coefficient of variation (CV) = 3.25% n = 3

Mean accuracy = 97.90% Correlation coefficient = 0.9997

SD = standard deviation

Table 3-3 Linearity and accuracy of cattle muscle fortified with tilmicosin (300-3000ng/g), day 3

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	22.462 ± 0.566	2.52	297.75	99.25
600.00	59.712 ± 0.763	1.28	664.35	89.28
1200.00	112.646 ± 0.591	0.52	1185.30	98.78
1800.00	169.487 ± 0.022	0.01	1744.70	96.93
2400.00	226.654 ± 4.085	1.80	2307.30	96.14
3000.00	307.250 ± 1.241	0.40	3100.50	96.65

Mean coefficient of variation (CV) = 1.09% n = 3

Mean accuracy = 96.17% Correlation coefficient = 0.9976

SD = standard deviation

Table 3-4 Linearity and accuracy of cattle muscle fortified with tilmicosin (300-3000ng/g), day 4

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	25.075 ± 0.479	1.91	286.33	95.44
600.00	63.635 ± 0.297	0.47	669.27	88.46
1200.00	116.604 ± 0.847	0.73	1195.30	99.61
1800.00	172.946 ± 0.774	0.45	1754.80	97.49
2400.00	226.710 ± 4.442	1.96	2288.80	95.37
3000.00	308.946 ± 0.757	0.25	3105.50	96.48

Mean coefficient of variation (CV) = 0.96% n = 3

Mean accuracy = 95.48% Correlation coefficient = 0.9972

SD = standard deviation

The data show that the method is linear and accurate in respect of cattle muscle, albeit that the accuracy at the lowest level tested (300ng/g) on day 1 was only 80%. The mean accuracy over the concentration range 300 – 3000ng/g for each of the 4 days ranged from 94 – 98%.

A linear response over the entire range was obtained in respect of mutton as shown in Tables 3-5 to 3-8.

Table 3-5 Linearity and accuracy of sheep muscle fortified with tilmicosin (300-3000ng/g), day 1

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	48.815 ± 1.880	3.85	375.38	74.87
600.00	71.498 ± 0.552	0.77	599.51	99.92
1200.00	116.879 ± 1.220	1.04	1047.90	87.33
1800.00	203.297 ± 1.251	0.61	1901.80	94.34
2400.00	245.159 ± 4.266	1.74	2315.40	96.48
3000.00	320.513 ± 4.683	1.46	3060.00	98.00

Mean coefficient of variation (CV) = 1.58% n = 4

Mean accuracy = 91.82% Correlation coefficient = 0.9955

SD = standard deviation

Table 3-6 Linearity and accuracy of sheep muscle fortified with tilmicosin (300-3000ng/g), day 2

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	46.387 ± 1.025	2.21	381.23	72.92
600.00	69.952 ± 1.115	1.59	606.72	98.88
1200.00	115.919 ± 1.302	1.12	1046.60	87.22
1800.00	201.264 ± 3.162	1.57	1863.20	96.49
2400.00	251.252 ± 4.830	1.92	2341.50	97.56
3000.00	326.421 ± 0.915	0.28	3060.80	97.97

Mean coefficient of variation (CV) = 1.45% n = 3

Mean accuracy = 91.84% Correlation coefficient = 0.9962

SD = standard deviation

Table 3-7 Linearity and accuracy of sheep muscle fortified with tilmicosin (300-3000ng/g), day 3

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	42.590 ± 5.281	12.40	380.62	73.13
600.00	61.695 ± 4.531	7.34	557.62	92.94
1200.00	119.960 ± 6.096	5.08	1097.40	91.45
1800.00	198.496 ± 8.486	4.28	1825.00	98.61
2400.00	267.525 ± 4.379	1.64	2464.50	97.31
3000.00	322.610 ± 2.853	0.88	2974.80	99.16

Mean coefficient of variation (CV) = 5.27% n = 3

Mean accuracy = 92.10% Correlation coefficient = 0.9978

SD = standard deviation

Table 3-8 Linearity and accuracy of sheep muscle fortified with tilmicosin (300-3000ng/g), day 4

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	29.067 ± 0.298	1.03	338.92	87.03
600.00	53.170 ± 0.663	1.25	575.02	95.84
1200.00	116.772 ± 0.238	0.20	1198.00	99.83
1800.00	171.857 ± 0.442	0.26	1737.60	96.53
2400.00	245.160 ± 3.799	1.55	2455.60	97.68
3000.00	300.217 ± 2.130	0.71	2994.90	99.83

Mean coefficient of variation (CV) = 0.83% n = 3

Mean accuracy = 96.12% Correlation coefficient = 0.9992

SD = standard deviation

The accuracy of the lowest concentration tested is 73 - 87%. The data show that the method is linear in sheep muscle over the range tested with an overall accuracy of 91 - 96%.

A linear response over the entire range was obtained in respect of pork as shown in Tables 3-9 to 3-12.

Table 3-9 Linearity and accuracy of pig muscle fortified with tilmicosin (300-3000ng/g), day 1

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	17.047 ± 1.756	10.30	322.92	92.36
600.00	43.587 ± 1.216	2.79	593.44	98.91
1200.00	98.785 ± 1.696	1.72	1156.10	96.33
1800.00	170.140 ± 15.54	9.13	1883.40	95.37
2400.00	209.847 ± 7.041	3.36	2288.10	95.34
3000.00	285.188 ± 32.89	11.53	3056.10	98.13

Mean coefficient of variation (CV) = 6.47% n = 3

Mean accuracy 96.07% Correlation coefficient = 0.9977

SD = standard deviation

Table 3-10 Linearity and accuracy of pig muscle fortified with tilmicosin (300-3000ng/g), day 2

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	22.101 ± 0.374	1.69	329.35	90.22
600.00	51.017 ± 0.729	1.43	608.36	98.61
1200.00	105.488 ± 1.900	1.80	1134.00	94.50
1800.00	175.479 ± 10.02	5.71	1809.30	99.48
2400.00	238.154 ± 15.84	6.65	2414.00	99.42
3000.00	299.398 ± 2.346	0.78	3005.00	99.83

Mean coefficient of variation (CV) = 3.01% n = 4

Mean accuracy = 97.01% Correlation coefficient = 0.9995

SD = standard deviation

Table 3-11 Linearity and accuracy of pig muscle fortified with tilmicosin (300-3000ng/g), day 3

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	24.899 ± 4.157	16.68	386.54	71.15
600.00	48.297 ± 0.307	0.64	618.92	96.85
1200.00	97.777 ± 0.150	0.15	1110.30	92.53
1800.00	159.651 ± 18.89	11.83	1724.80	95.82
2400.00	223.017 ± 22.69	10.18	2354.20	98.09
3000.00	298.634 ± 27.63	9.25	3105.20	96.49

Mean coefficient of variation (CV) = 8.12% n = 3

Mean accuracy = 91.82% Correlation coefficient = 0.9968

SD = standard deviation

Table 3-12 Linearity and accuracy of pig muscle fortified with tilmicosin (300-3000ng/g), day 4

Conc. Added (ng/g)	Mean ± SD Peak Area	% CV	Predicted Value (ng/g)	Accuracy (%)
300.00	22.639 ± 0.267	1.18	307.73	97.42
600.00	52.262 ± 6.379	12.21	637.57	93.74
1200.00	98.828 ± 0.344	0.35	1156.10	96.34
1800.00	153.069 ± 1.242	0.81	1760.00	97.77
2400.00	212.942 ± 1.881	0.88	2426.70	98.89
3000.00	265.502 ± 0.838	0.32	3011.90	99.60

Mean coefficient of variation (CV) = 2.63% n = 3

Mean accuracy = 97.29% Correlation coefficient = 0.9995

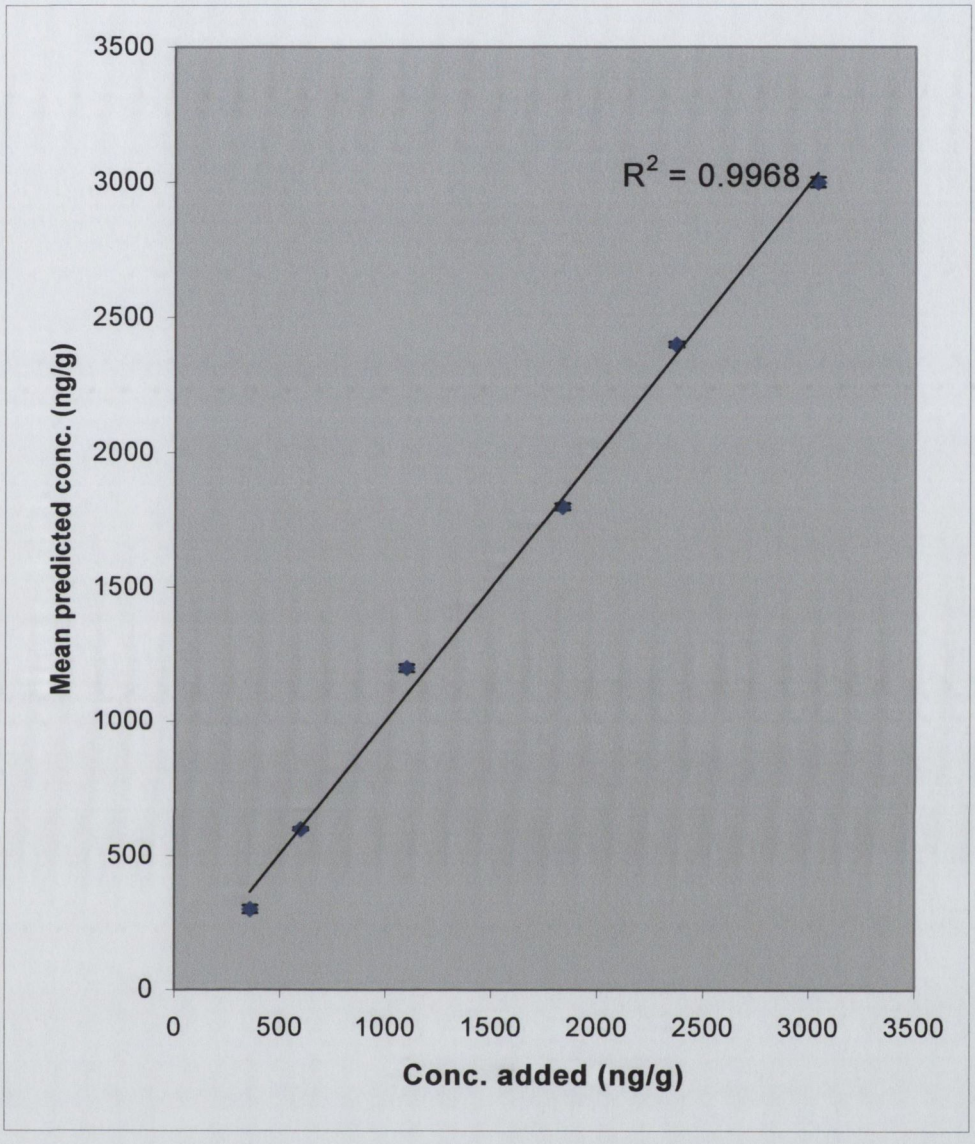
SD = standard deviation

The accuracy of the lowest concentration tested is 71 - 97%. The data show that the method is linear in sheep muscle over the range tested with an overall accuracy of 92 - 97%.

3.3.1.3 Precision and reproducibility

In respect of precision, the co-efficients of variation (CV) for each of the assays was always below the EU guideline limit of 15% being maximally 5% in the studies conducted as indicated in Figures 3–4, 3–5 and 3–6 hereunder.

Figure 3-4 Intra-assay variability for cattle muscle fortified with tilmicosin (300 – 3000ng/g)



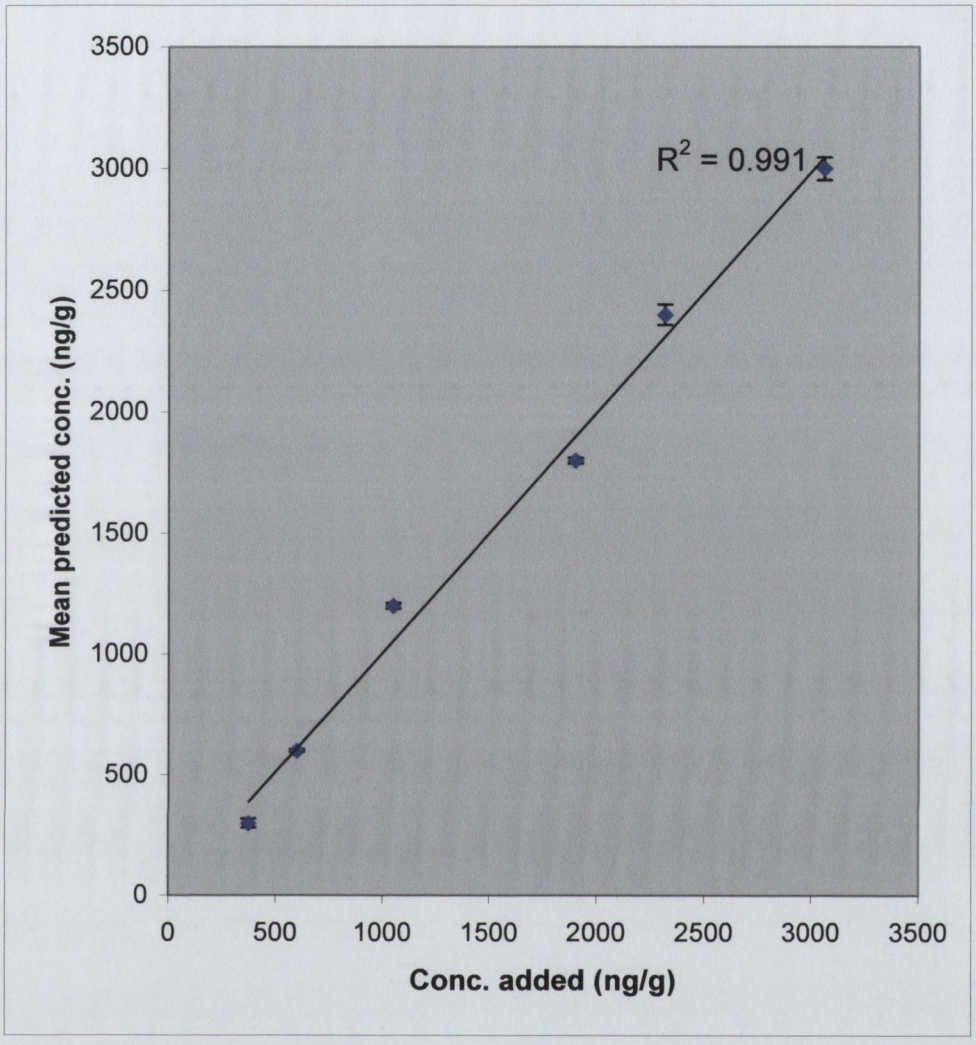
n = 4

Mean accuracy = 94.48%

Mean coefficient of variation (CV) = 1.32%,

Error bars indicate standard deviations

Figure 3-5 Intra-assay variability for sheep muscle fortified with tilmicosin (300 – 3000ng/g)



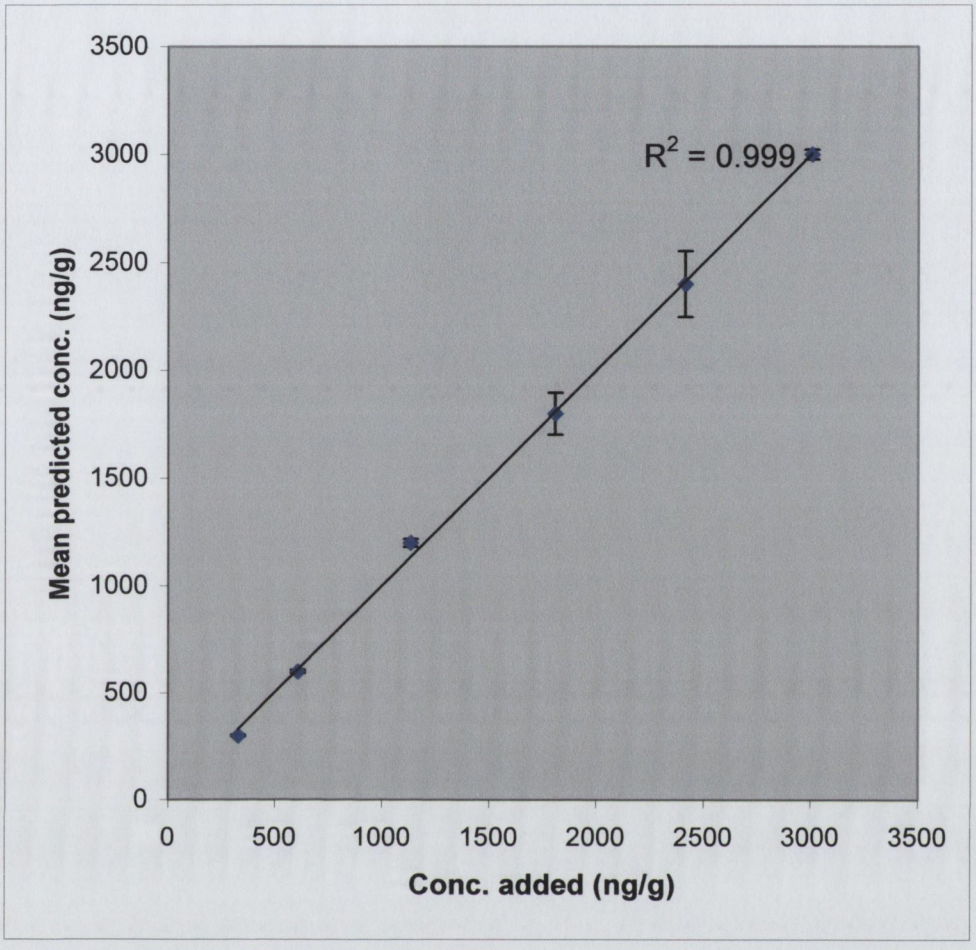
n = 4

Mean accuracy = 91.83%

Mean coefficient of variation (CV) = 1.83%

Error bars indicate standard deviations

Figure 3-6 Intra-assay variability for pig muscle fortified with tilmicosin (300 – 3000ng/g)



n = 4

Mean accuracy = 97.0%

Mean coefficient of variation (CV) = 2.72%

Error bars indicate standard deviations

In respect of reproducibility, the methods displayed acceptable performance with the CVs being less than 16% as outlined in EU guidance limits (in fact they were always less than 10%). The mean accuracy varied from 92 – 97% and the mean CVs were 3% approximately as can be seen from Tables 3–13 to 3–15 hereunder.

Table 3-13 Inter-assay variability of cattle muscle fortified with tilmicosin (300 - 3000ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
300.00	316.20 ± 25.34	8.01	94.60
600.00	620.27 ± 32.91	5.31	96.62
1200.00	1167.45 ± 35.87	3.07	97.29
1800.00	1785.82 ± 33.13	1.86	99.21
2400.00	2332.05 ± 31.07	1.33	97.17
3000.00	3068.21 ± 31.35	1.02	97.73

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 3.43%

Mean accuracy = 97.10%

day 1. Y = 0.1008x - 7.19662 Correlation coefficient = 0.998

day 2. Y = 0.0994x - 3.71227 Correlation coefficient = 1.000

day 3. Y = 0.1016x - 7.79236 Correlation coefficient = 0.998

day 4. Y = 0.1007x - 3.7574 Correlation coefficient = 0.997

Table 3-14 Inter-assay variability of sheep muscle fortified with tilmicosin (300 - 3000ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g)	± SD	% CV	Accuracy (%)
300.00	369.04	± 15.68	4.25	76.99
600.00	534.72	± 17.50	2.99	89.12
1200.00	1097.47	± 55.06	5.02	91.46
1800.00	1831.89	± 54.42	2.97	98.23
2400.00	2394.26	± 59.49	2.48	99.76
3000.00	3022.62	± 34.36	1.14	99.25

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 3.14%

Mean accuracy = 92.47%

day 1. Y = 0.1012x + 10.8239 Correlation coefficient = 0.995

day 2. Y = 0.1045x + 6.54523 Correlation coefficient = 0.996

day 3. Y = 0.1079x - 1.50581 Correlation coefficient = 0.998

day 4. Y = 0.1021x - 5.53423 Correlation coefficient = 0.999

Table 3-15 Inter-assay variability of pig muscle fortified with tilmicosin (300 - 3000ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
300.00	336.63 ± 26.71	7.93	87.79
600.00	614.57 ± 14.37	2.34	97.57
1200.00	1139.11 ± 16.91	1.48	94.93
1800.00	1794.39 ± 53.22	2.97	99.69
2400.00	2370.76 ± 49.21	2.08	98.78
3000.00	3044.54 ± 35.88	1.18	98.52

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 2.99%

Mean accuracy = 96.21%

day 1. Y = 0.0981x - 14.6337 Correlation coefficient = 0.998

day 2. Y = 0.1036x - 12.0317 Correlation coefficient = 0.999

day 3. Y = 0.1007x - 14.0211 Correlation coefficient = 0.997

day 4. Y = 0.0898x - 4.99812 Correlation coefficient = 0.999

3.3.1.4 Sensitivity

The sensitivity of the methods, in terms of the validated LOQ, is approximately 130ng/g for cattle and sheep and 210ng/g for pigs. The LOD is approximately 40ng/g for cattle and sheep and 65ng/g for pigs. The data are given in Tables 3-16 to 3–18 hereunder.

Table 3-16 LOQ and LOD of assay method for cattle muscle fortified with tilmicosin (300-3000ng/g)

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
300.00	29.132 ± 1.520	6.57	46.96	156.53
600.00	53.195 ± 0.496	0.93	16.78	55.95
1200.00	103.476 ± 1.361	1.32	47.35	157.83
1800.00	177.498 ± 1.204	0.68	36.63	122.10
2400.00	231.643 ± 0.975	0.42	30.31	101.02
3000.00	299.046 ± 1.852	0.62	55.74	185.79

SD = standard deviation n = 4

CV = coefficient of variation

Mean limit of detection (LOD) = 38.96ng/g

Mean limit of quantification (LOQ) = 129.87ng/g

Table 3-17 LOQ and LOD of assay method for sheep muscle fortified with tilmicosin (300-3000ng/g)

Conc. Added (ng/g)	Mean Area	Peak ± SD	% CV	LOD (ng/g)	LOQ. (ng/g)
300.00		29.067 ±0.298	1.03	9.23	30.77
600.00		53.170 ±0.663	1.25	22.44	74.82
1200.00		116.772 ±0.238	0.20	7.34	24.46
1800.00		171.857 ±0.442	0.26	13.89	46.29
2400.00		245.160 ±3.799	1.55	111.57	371.90
3000.00		300.217 ±2.130	0.71	63.85	212.85

SD = standard deviation n = 3

CV = coefficient of variation

Mean limit of detection (LOD) = 38.05ng/g

Mean limit of quantification (LOQ) = 126.84ng/g

Table 3-18 LOQ and LOD of assay method for pig muscle fortified with tilmicosin (300-3000ng/g)

Conc. Added (ng/g)	Mean Area	Peak ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
300.00		22.639 ± 0.267	1.18	10.61	35.38
600.00		52.262 ± 6.379	12.21	219.70	732.34
1200.00		98.828 ± 0.344	0.35	12.53	41.77
1800.00		153.069 ± 1.242	0.81	43.82	146.05
2400.00		212.942 ± 1.881	0.88	63.60	212.00
3000.00		265.502 ± 0.838	0.32	28.41	94.69

SD = standard deviation n = 3

CV = coefficient of variation

Mean limit of detection (LOD) = 63.11ng/g

Mean limit of quantification (LOQ) = 210.37ng/g

3.3.1.5 Stability

Tilmicosin is stable for at up to 16 days as can be seen from Table 3–19 hereunder.

Table 3-19 Stability of tilmicosin in fortified muscle of cattle, sheep and pig over time

Theoretical conc. (ng/g)	day 0	4	16
	Measured conc (ng/g)		
Beef			
1200.00	1162.50 (100%)	1200.09 (103%)	1200.50 (103%)
3000.00	3010.70 (100%)	3112.60 (103%)	na
Lamb			
1200.00	1154.00 (100%)	1154.20 (100%)	1120.20 (97%)
3000.00	3034.30 (100%)	3203.90 (106%)	3033.20 (100%)
Pork			
1200.00	1056.10 (100%)	1112.50 (105%)	1181.30 (112%)
3000.00	2856.30 (100%)	2776.50 (97%)	2941.60 (103%)

na = not analysed

3.3.2 Enrofloxacin

3.3.2.1 Specificity

The HPLC system employed in this method separated enrofloxacin and ciprofloxacin from impurities extracted from muscle and from related substances that may be present in tissue extract solutions as can be seen in Figures 3–7 to 3-9 hereunder.

Figure 3-7 Typical HPLC chromatogram for enrofloxacin and ciprofloxacin in beef muscle

A = Control (blank) beef; B = Fortified beef (250ng/g).

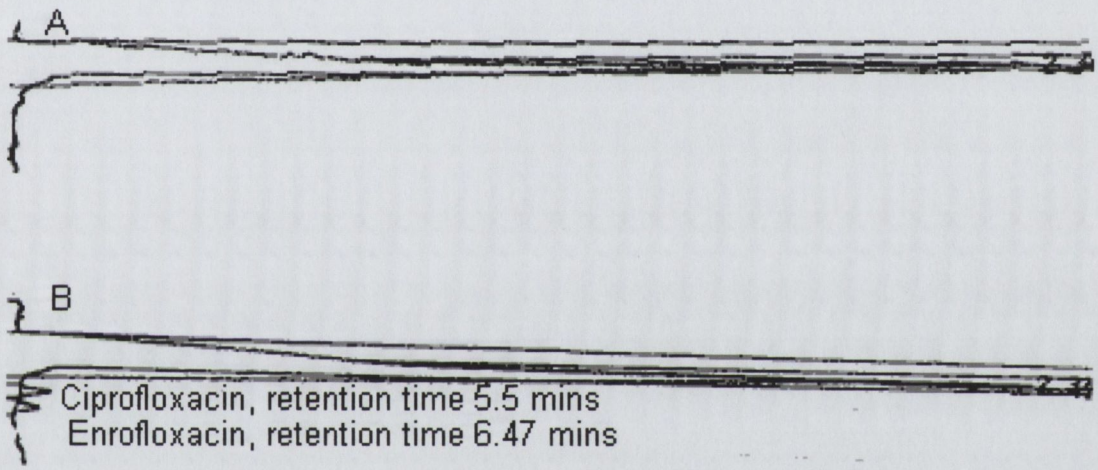


Figure 3-8 Typical HPLC chromatogram for enrofloxacin and ciprofloxacin in lamb muscle

A = Control (blank) lamb; B = Fortified lamb (500ng/g).

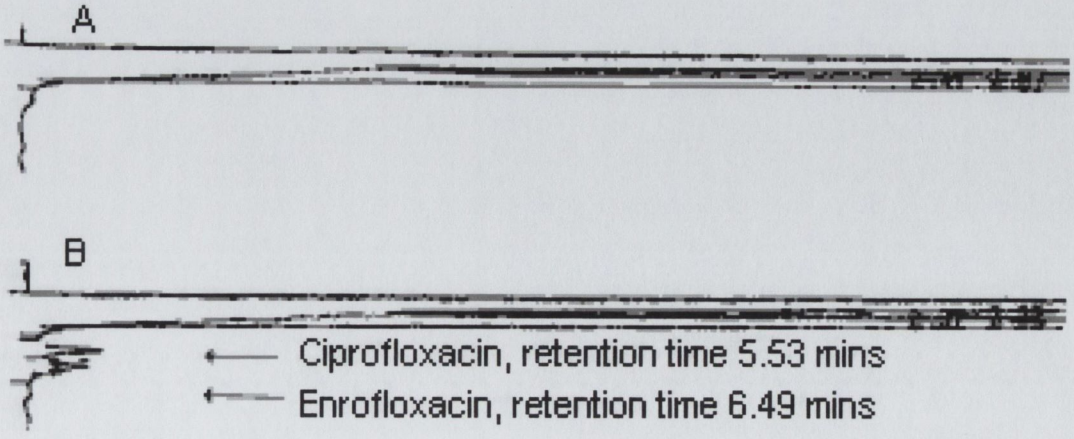
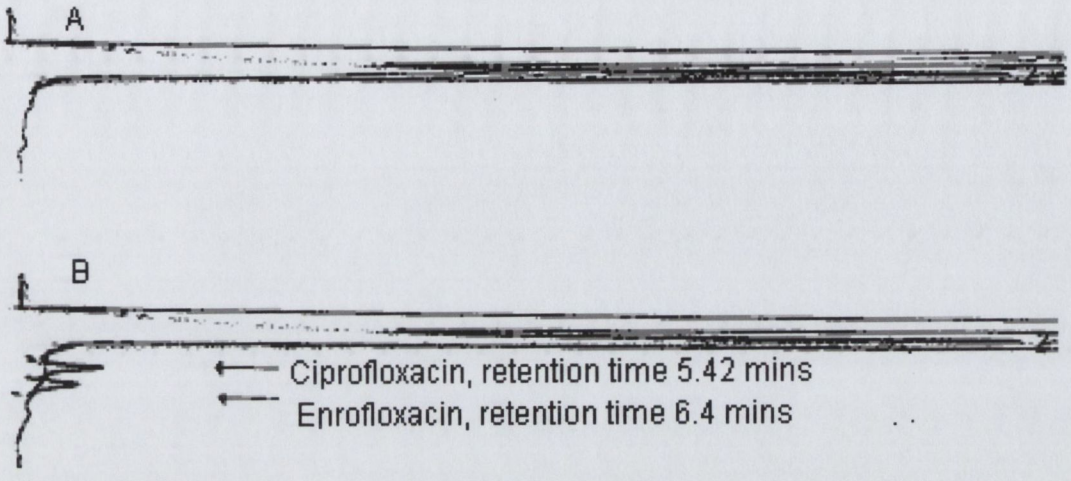


Figure 3-9 Typical HPLC chromatogram for enrofloxacin and ciprofloxacin in pork muscle

A = Control (blank) pork; B = Fortified pork (500ng/g).



3.3.2.2 Linearity and accuracy

A linear response over the entire range for beef was obtained as shown for enrofloxacin in Tables 3-20 to 3-23 and ciprofloxacin in Tables 3-24 to 3-27.

Table 3-20 Linearity and accuracy of cattle muscle fortified with enrofloxacin (250-2500ng/g), day 1

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	32.538 ± 3.411	10.48	249.48	99.79
500.00	52.250 ± 0.182	0.35	489.86	97.97
1000.00	97.660 ± 7.437	7.61	1043.60	95.64
1500.00	130.483 ± 8.969	6.87	1443.90	96.26
2000.00	178.079 ± 6.680	3.75	2024.30	98.79
2500.00	216.995 ± 7.430	3.42	2498.90	99.96

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 5.41%

Mean accuracy = 98.07% Correlation coefficient = 0.9992

Table 3-21 Linearity and accuracy of cattle muscle fortified with enrofloxacin (250-2500ng/g), day 2

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	22.554 ± 0.558	2.47	217.62	87.05
500.00	45.949 ± 0.503	1.95	490.86	98.19
1000.00	92.995 ± 1.810	1.95	1040.30	95.97
1500.00	132.751 ± 2.230	1.68	1504.70	99.69
2000.00	179.615 ± 0.295	0.16	2052.00	97.40
2500.00	213.224 ± 5.839	2.73	2444.50	97.78

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 1.82%

Mean accuracy = 96.01% Correlation coefficient = 0.9989

Table 3-22 Linearity and accuracy of cattle muscle fortified with enrofloxacin (250-2500ng/g), day 3

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	26.021 ± 2.713	10.42	226.51	90.60
500.00	47.951 ± 0.039	0.08	487.53	97.51
1000.00	93.435 ± 0.326	0.35	1028.90	97.11
1500.00	131.214 ± 6.491	4.94	1478.60	98.57
2000.00	184.414 ± 1.154	6.26	2111.80	94.41
2500.00	210.037 ± 0.835	0.39	2416.70	96.67

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 3.74%

Mean accuracy = 95.81% Correlation coefficient = 0.9972

Table 3-23 Linearity and accuracy of cattle muscle fortified with enrofloxacin (250-2500ng/g), day 4

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	26.274 ± 1.171	4.45	252.87	98.85
500.00	47.692 ± 5.167	10.83	491.55	98.31
1000.00	93.782 ± 1.435	1.53	1005.20	99.48
1500.00	134.968 ± 2.247	1.66	1464.20	97.61
2000.00	189.957 ± 8.063	4.24	2077.00	96.15
2500.00	224.259 ± 6.041	2.69	2459.20	98.37

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 4.23%

Mean accuracy = 98.13% Correlation coefficient = 0.9988

Table 3-24 Linearity and accuracy of cattle muscle fortified with ciprofloxacin (250-2500ng/g), day 1

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	19.962 ± 0.369	1.85	273.16	90.74
500.00	41.573 ± 3.572	8.59	521.03	95.79
1000.00	81.807 ± 3.208	3.92	982.49	98.25
1500.00	119.195 ± 8.201	6.88	1411.30	94.09
2000.00	174.149 ± 0.337	0.19	2041.60	97.92
2500.00	215.898 ± 8.206	3.80	2520.40	99.18

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 4.21%

Mean accuracy = 95.99% Correlation coefficient = 0.9985

Table 3-25 Linearity and accuracy of cattle muscle fortified with ciprofloxacin (250-2500ng/g), day 2

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	17.035 ± 1.704	10.00	274.52	90.19
500.00	34.116 ± 0.936	2.74	469.06	93.81
1000.00	81.637 ± 4.357	5.33	1010.30	98.97
1500.00	121.740 ± 2.794	2.29	1467.00	97.80
2000.00	172.790 ± 4.663	2.69	2048.50	97.58
2500.00	210.732 ± 0.702	0.33	2480.60	99.22

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 3.89%

Mean accuracy = 96.26% Correlation coefficient = 0.9993

Table 3-26 Linearity and accuracy of cattle muscle fortified with ciprofloxacin (250-2500ng/g), day 3

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	19.112 ± 0.142	0.74	266.46	93.42
500.00	36.767 ± 0.359	0.97	465.94	93.19
1000.00	82.372 ± 1.353	1.64	981.22	98.12
1500.00	132.120 ± 16.213	12.27	1543.30	97.11
2000.00	175.360 ± 7.575	4.32	2031.90	98.41
2500.00	213.357 ± 0.558	0.26	2461.20	98.45

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 3.37%

Mean accuracy = 96.45% Correlation coefficient = 0.9992

Table 3-27 Linearity and accuracy of cattle muscle fortified with ciprofloxacin (250-2500ng/g), day 4

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	18.404 ± 0.836	4.54	237.39	94.96
500.00	41.437 ± 3.319	8.01	487.13	97.43
1000.00	90.601 ± 3.414	3.77	1020.20	97.98
1500.00	134.310 ± 3.554	2.64	1494.10	99.61
2000.00	186.455 ± 3.455	1.85	2059.50	97.03
2500.00	222.631 ± 3.494	1.57	2451.70	98.07

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 3.73%

Mean accuracy = 97.51% Correlation coefficient = 0.9991

The data show that the method is linear and accurate in respect of cattle muscle, with accuracy values well within the EU guideline limits for both analytes. The mean accuracy for each of the 4 days ranged from 96 – 98% in respect of enrofloxacin and 96 – 97.5% in respect of ciprofloxacin. The correlation coefficients were above 0.997 in respect of enrofloxacin and above 0.998 in respect of ciprofloxacin.

A linear response over the entire range was obtained in respect of mutton was demonstrated as shown in Tables 3-28 to 3-31 for enrofloxacin and 3-32 to 3-35 for ciprofloxacin.

Table 3-28 Linearity and accuracy of sheep muscle fortified with enrofloxacin (250-2500ng/g), day 1

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	27.598 ± 9.683	35.09	298.45	80.62
500.00	40.408 ± 10.694	26.46	474.97	94.99
1000.00	77.420 ± 0.638	0.82	985.01	98.50
1500.00	115.115 ± 0.676	0.59	1504.50	99.70
2000.00	145.138 ± 0.949	0.65	1918.20	95.91
2500.00	192.363 ± 0.569	0.30	2568.90	97.24

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 10.65%

Mean accuracy = 94.49% Correlation coefficient = 09981

Table 3-29 Linearity and accuracy of sheep muscle fortified with enrofloxacin (250-2500ng/g), day 2

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	28.087 ± 9.139	32.54	299.25	80.30
500.00	36.635 ± 3.899	10.65	421.69	84.34
1000.00	79.143 ± 0.517	0.65	1030.60	96.94
1500.00	112.735 ± 2.976	2.64	1511.80	99.21
2000.00	145.171 ± 2.303	1.59	1976.40	98.82
2500.00	182.450 ± 0.314	0.17	2510.40	99.58

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 8.04%

Mean accuracy = 93.19% Correlation coefficient = 0.9986

Table 3-30 Linearity and accuracy of sheep muscle fortified with enrofloxacin (250-2500ng/g), day 3

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	23.646 ± 6.632	28.05	255.06	97.98
500.00	39.484 ± 8.973	22.73	485.75	97.15
1000.00	77.553 ± 0.219	0.28	1040.20	95.98
1500.00	108.894 ± 2.928	2.69	1496.70	99.78
2000.00	137.957 ± 0.847	0.61	1920.10	96.01
2500.00	181.354 ± 2.967	1.64	2552.20	97.91

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 9.33%

Mean accuracy = 97.47% Correlation coefficient = 0.9986

Table 3-31 Linearity and accuracy of sheep muscle fortified with enrofloxacin (250-2500ng/g), day 4

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	19.767 ± 0.596	3.02	156.23	62.49
500.00	40.040 ± 1.864	4.65	460.78	92.16
1000.00	82.866 ± 1.402	1.69	1104.10	89.59
1500.00	120.033 ± 8.101	6.75	1662.50	89.17
2000.00	138.611 ± 2.222	1.60	1941.50	97.08
2500.00	170.784 ± 2.695	1.58	2424.90	96.99

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 3.22%

Mean accuracy = 87.91% Correlation coefficient = 0.9926

Table 3-32 Linearity and accuracy of sheep muscle fortified with ciprofloxacin (250-2500ng/g), day 1

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	20.564 ± 1.666	8.10	257.96	96.82
500.00	35.856 ± 0.440	1.23	445.81	89.16
1000.00	86.018 ± 0.880	1.03	1062.00	93.80
1500.00	125.025 ± 2.777	2.22	1541.20	97.25
2000.00	155.260 ± 2.924	1.88	1912.60	95.63
2500.00	205.558 ± 1.106	0.54	2530.50	98.78

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 2.50%

Mean accuracy = 95.24% Correlation coefficient = 0.9978

Table 3-33 Linearity and accuracy of sheep muscle fortified with ciprofloxacin (250-2500ng/g), day 2

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	19.205 ± 0.376	1.96	249.76	99.90
500.00	36.044 ± 0.086	0.24	464.96	92.99
1000.00	83.057 ± 2.050	2.47	1065.80	93.42
1500.00	119.155 ± 1.219	1.02	1527.10	98.19
2000.00	147.526 ± 0.828	0.56	1889.70	94.49
2500.00	199.407 ± 0.656	0.33	2552.70	97.89

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 1.09%

Mean accuracy = 96.15 % Correlation coefficient = 0.9972

Table 3-34 Linearity and accuracy of sheep muscle fortified with ciprofloxacin (250-2500ng/g), day 3

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	19.675 ± 0.279	1.42	263.33	94.67
500.00	33.768 ± 0.910	2.69	441.91	88.38
1000.00	80.446 ± 0.564	0.70	1033.40	96.66
1500.00	116.911 ± 0.223	0.19	1495.50	99.70
2000.00	163.131 ± 28.459	17.44	2081.20	95.94
2500.00	191.029 ± 2.314	1.21	2434.70	97.39

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 3.94%

Mean accuracy = 95.46% Correlation coefficient = 0.9980

Table 3-35 Linearity and accuracy of sheep muscle fortified with ciprofloxacin (250-2500ng/g), day 4

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	21.502 ± 0.279	1.29	278.90	88.44
500.00	51.117 ± 2.829	5.53	663.06	67.39
1000.00	102.821 ± 1.806	1.76	1333.70	66.63
1500.00	139.046 ± 17.997	12.94	1803.60	79.76
2000.00	142.079 ± 3.296	2.32	1843.00	92.15
2500.00	175.341 ± 2.259	1.28	2274.40	90.98

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 4.19%

Mean accuracy = 80.89% Correlation coefficient = 0.9461

The data show that the method is linear and accurate in respect of sheep muscle, albeit that for 2 of 4 enrofloxacin results at the 250ng/g fortification level were outside the EU recommended accuracy limits. The mean accuracy for each of the 4 days ranged from 88 – 97% in respect of enrofloxacin and 81 – 96% in respect of ciprofloxacin. are given. The correlation coefficients were above 0.993 in respect of enrofloxacin and above 0.946 in respect of ciprofloxacin.

A linear response over the entire range was obtained in respect of pork as shown in Tables 3-6 to 3-39 in respect of enrofloxacin and Tables 3-40 to 3-43 in respect of ciprofloxacin.

Table 3-36 Linearity and accuracy of pig muscle fortified with enrofloxacin (250-2500ng/g), day 1

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	6.422 ± 0.368	5.72	311.06	75.58
500.00	14.069 ± 2.347	16.68	494.52	98.90
1000.00	33.378 ± 4.238	12.69	957.75	95.78
1500.00	51.647 ± 0.708	1.37	1396.04	93.07
2000.00	80.232 ± 5.584	6.96	2081.82	95.91
2500.00	98.030 ± 10.343	10.55	2508.81	99.65

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 8.99%

Mean accuracy = 93.15% Correlation coefficient = 0.997

Table 3-37 Linearity and accuracy of pig muscle fortified with enrofloxacin (250-2500ng/g), day 2

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	9.075 ± 0.664	7.31	281.58	87.37
500.00	17.135 ± 3.182	18.57	457.87	91.57
1000.00	39.333 ± 4.111	10.45	943.39	94.34
1500.00	68.637 ± 3.143	4.58	1584.33	94.38
2000.00	88.902 ± 2.423	2.72	2027.57	98.62
2500.00	108.456 ± 10.437	9.62	2455.26	98.21

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 8.88%

Mean accuracy = 94.08% Correlation coefficient = 0.998

Table 3-38 Linearity and accuracy of pig muscle fortified with enrofloxacin (250-2500ng/g), day 3

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	7.873 ± 0.567	7.20	260.76	95.70
500.00	21.620 ± 7.130	32.98	505.87	98.83
1000.00	47.930 ± 3.993	8.33	974.96	97.50
1500.00	78.552 ± 5.694	7.25	1520.93	98.60
2000.00	103.255 ± 23.085	22.36	1961.37	98.07
2500.00	134.930 ± 11.602	8.60	2526.12	98.96

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 14.45 %

Mean accuracy = 97.94% Correlation coefficient = 1.000

Table 3-39 Linearity and accuracy of pig muscle fortified with enrofloxacin (250-2500ng/g), day 4

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	10.931 ± 1.547	14.16	303.40	78.64
500.00	22.771 ± 0.675	2.96	510.33	97.93
1000.00	45.383 ± 2.185	4.82	905.52	90.55
1500.00	78.833 ± 2.412	3.06	1490.14	99.34
2000.00	109.238 ± 3.938	3.60	2021.53	98.92
2500.00	137.706 ± 2.805	2.04	2519.07	99.24

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 5.11%

Mean accuracy = 94.10% Correlation coefficient = 0.998

Table 3-40 Linearity and accuracy of pig muscle fortified with ciprofloxacin (250-2500ng/g), day 1

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	8.286 ± 0.832	10.04	238.02	95.21
500.00	19.352 ± 2.016	10.42	508.57	98.29
1000.00	40.817 ± 1.255	3.07	1033.34	96.67
1500.00	59.254 ± 2.565	4.33	1484.09	98.94
2000.00	78.368 ± 0.770	0.98	1951.42	97.57
2500.00	102.221 ± 9.018	8.82	2534.56	98.62

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 6.28%

Mean accuracy 97.55% Correlation coefficient = 0.999

Table 3-41 Linearity and accuracy of pig muscle fortified with ciprofloxacin (250-2500ng/g), day 2

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	11.949 ± 3.092	25.88	239.72	95.89
500.00	22.901 ± 5.845	25.52	497.45	99.49
1000.00	42.327 ± 2.474	5.84	954.59	95.46
1500.00	68.809 ± 0.126	0.18	1577.77	94.82
2000.00	88.331 ± 3.002	3.39	2037.18	98.14
2500.00	105.589 ± 5.621	5.32	2443.30	97.73

SD = standard deviation n = 3

Mean CV = 11.02%

Mean accuracy = 96.92% Correlation coefficient = 0.998

Table 3-42 Linearity and accuracy of pig muscle fortified with ciprofloxacin (250-2500ng/g), day 3

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	9.466 ± 1.450	15.31	283.61	86.56
500.00	26.135 ± 2.854	10.92	604.49	79.10
1000.00	40.750 ± 1.818	4.46	885.85	88.59
1500.00	70.407 ± 1.494	2.12	1456.77	97.11
2000.00	91.337 ± 4.105	4.49	1859.69	92.98
2500.00	132.888 ± 4.729	3.56	2659.58	93.62

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 6.81%

Mean accuracy = 89.66% Correlation coefficient = 0.991

Table 3-43 Linearity and accuracy of pig muscle fortified with ciprofloxacin (250-2500ng/g), day 4

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
250.00	11.821 ± 0.316	2.68	279.83	88.07
500.00	25.237 ± 1.355	5.37	516.69	96.66
1000.00	48.407 ± 3.496	7.22	925.78	92.58
1500.00	82.038 ± 1.465	1.79	1519.55	98.70
2000.00	108.263 ± 2.568	2.37	1982.57	99.13
2500.00	139.018 ± 4.212	3.03	2525.58	98.98

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 3.74%

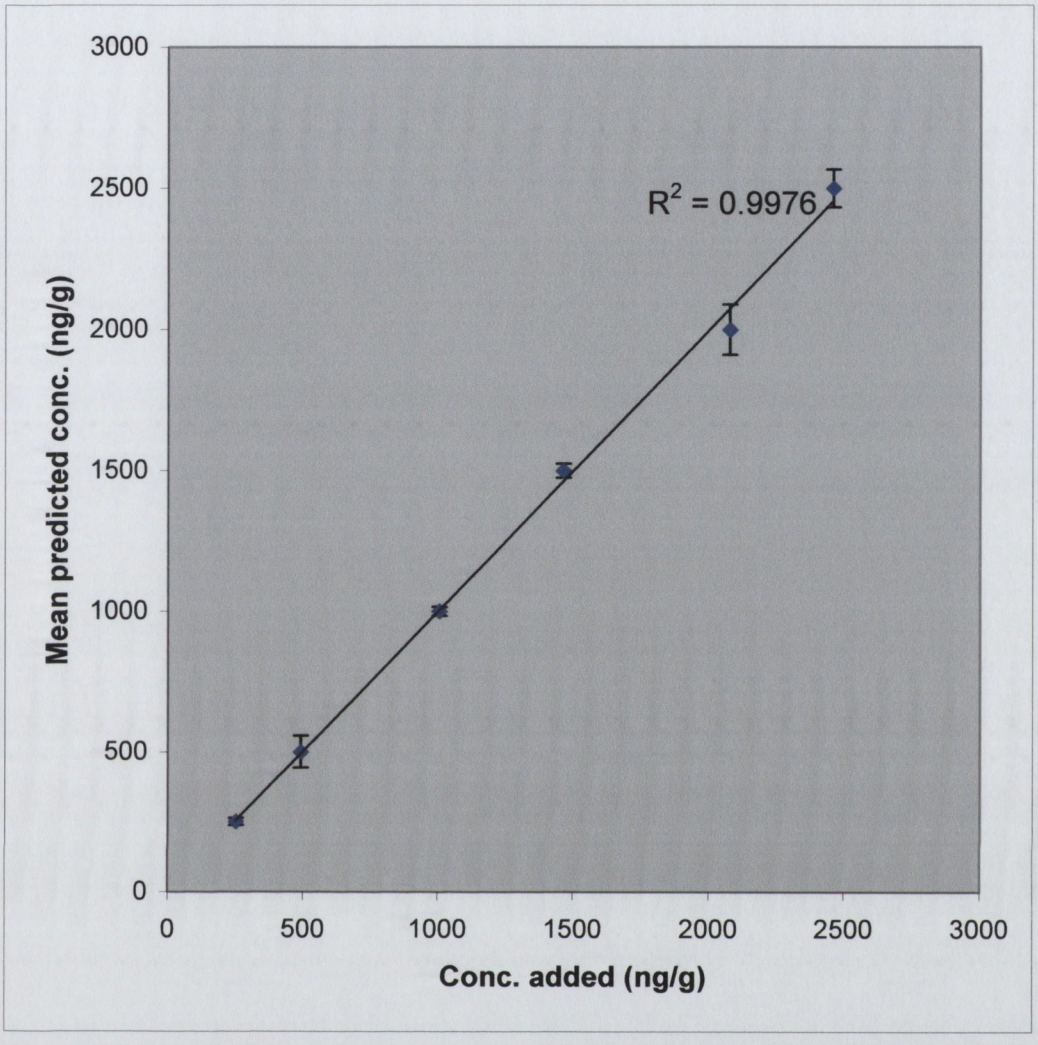
Mean accuracy = 95.69% Correlation coefficient = 0.999

The data show that the method is linear and accurate in respect of pig muscle, albeit that for 3 of 4 enrofloxacin results and 2 of 4 ciprofloxacin results at the 250ng/g fortification level were outside the EU recommended accuracy limits. The mean accuracy for each of the 4 days ranged from 90 – 98% in respect of enrofloxacin and 94 – 98% in respect of ciprofloxacin. The correlation coefficients were above 0.997 in respect of enrofloxacin and above 0.991 in respect of ciprofloxacin.

3.3.2.3 Precision and reproducibility

Concerning precision, the co-efficients of variation (CV) for each of the assays was high in respect of enrofloxacin at the lowest fortification level (Figures 3–10, 3–12 and 3-14 hereunder) but was low in respect of ciprofloxacin (Figures 3-11, 3-13 and 3-15 hereunder). However, the mean intra-assay CV for enrofloxacin in respect of all 3 species ranged from 4 – 13%, which is within the EU recommended limit of 15%. The mean intra-assay CV in respect of ciprofloxacin ranged from 2 – 3% in each species.

Figure 3-10 Intra-assay variability for cattle muscle fortified with enrofloxacin (250 – 2500ng/g)



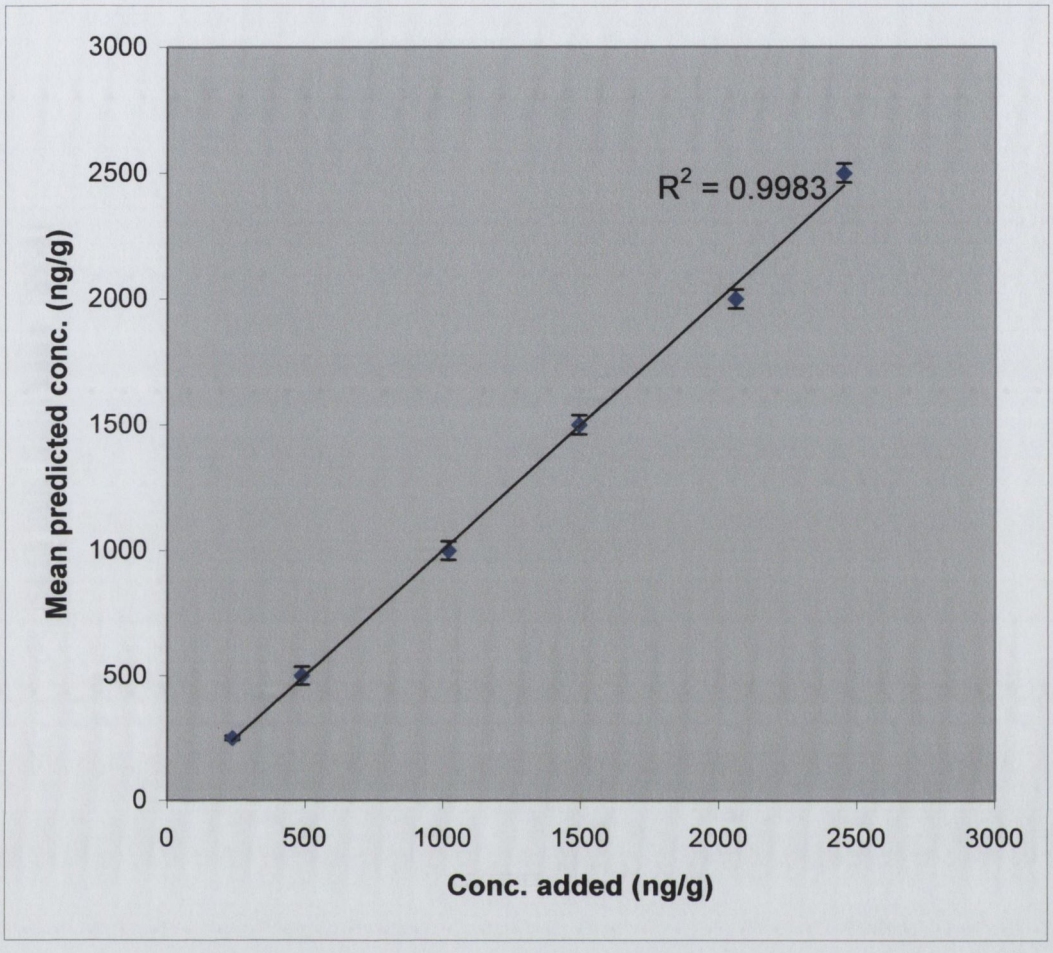
n = 4;

Mean accuracy = 98.13%

Mean coefficient of variation (CV) = 4.54%

Error bars indicate standard deviations

Figure 3-11 Intra-assay variability for cattle muscle fortified with ciprofloxacin (250 – 2500ng/g)



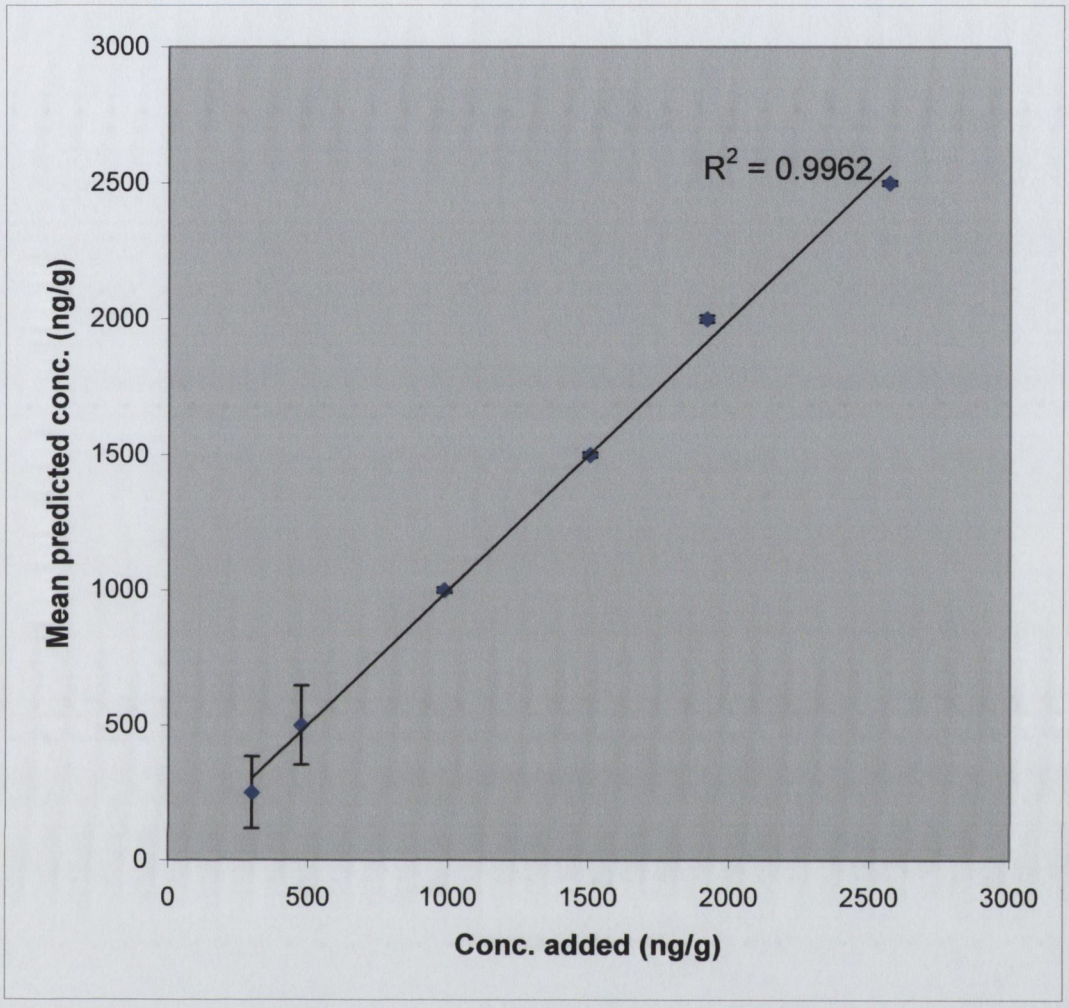
n = 4

Mean accuracy = 97.51%

Mean coefficient of variation (CV) = 3.46%

Error bars indicate standard deviations

Figure 3-12 Intra-assay variability for sheep muscle fortified with enrofloxacin (250 – 2500ng/g)



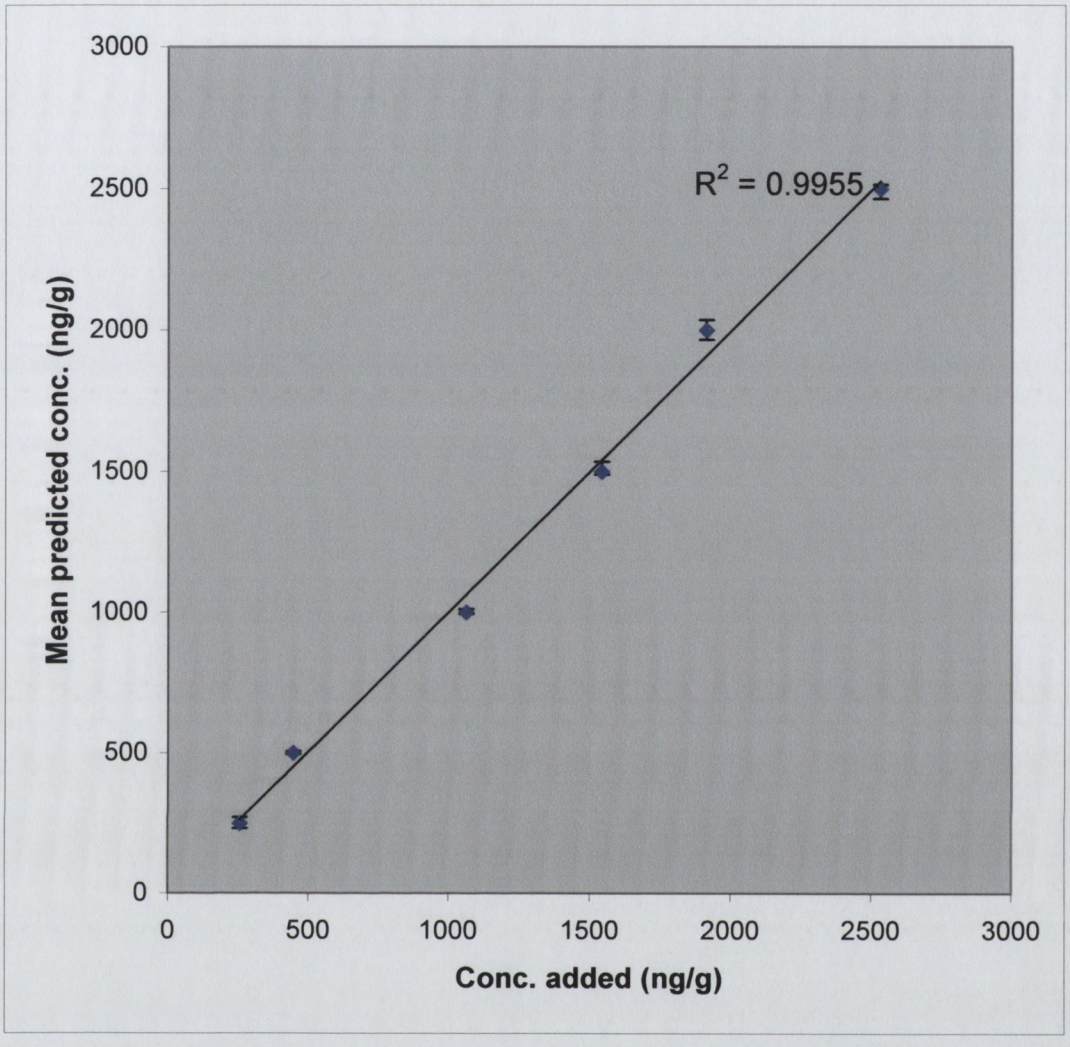
n = 4

Mean accuracy = 94.49%

Mean coefficient of variation (CV) = 13.04%

Error bars indicate standard deviations

Figure 3-13 Intra-assay variability for sheep muscle fortified with ciprofloxacin (250 – 2500ng/g)



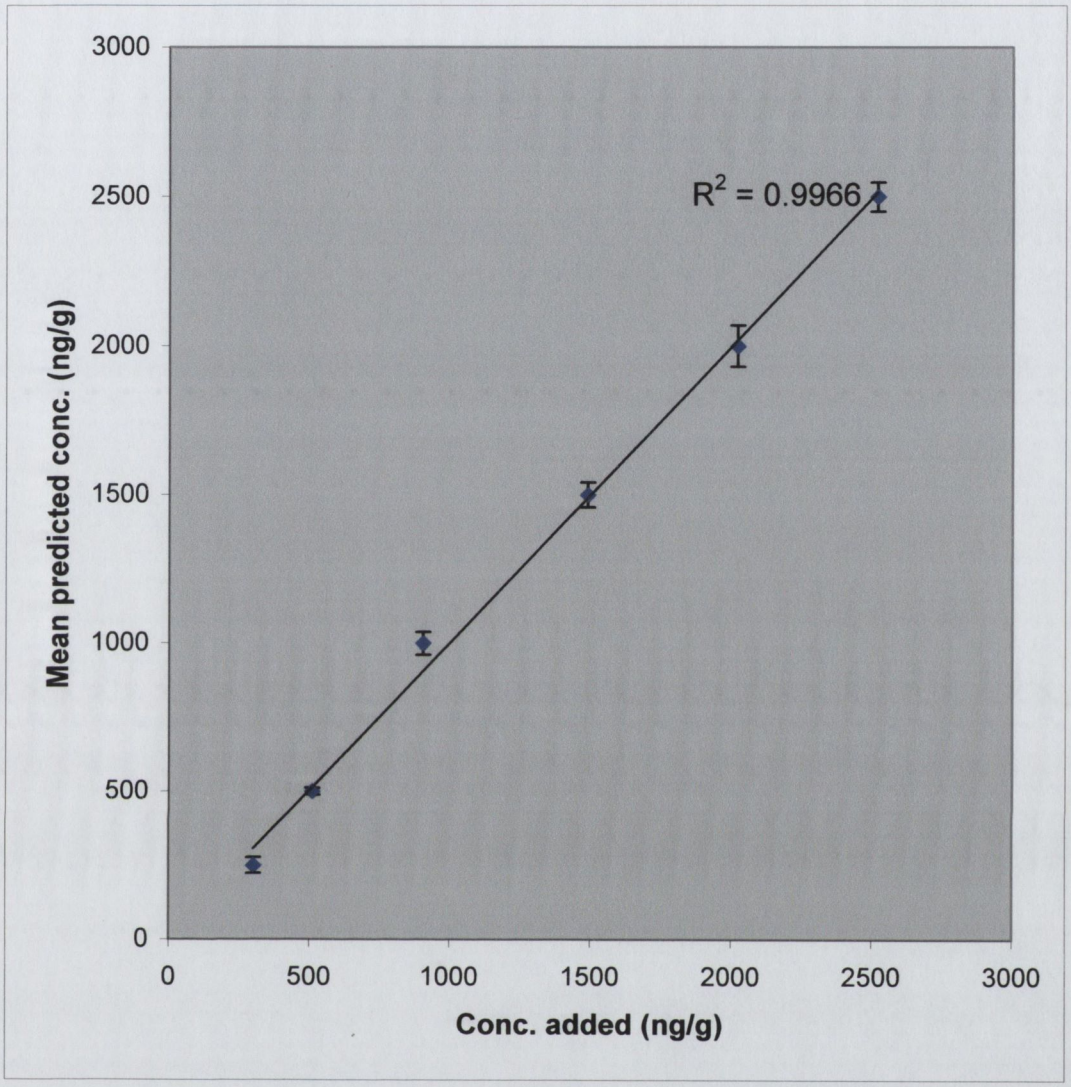
n = 4

Mean accuracy = 95.24%

Mean coefficient of variation (CV) = 2.47%

Error bars indicate standard deviations

Figure 3-14 Intra-assay variability for pig muscle fortified with enrofloxacin (250 – 2500ng/g)



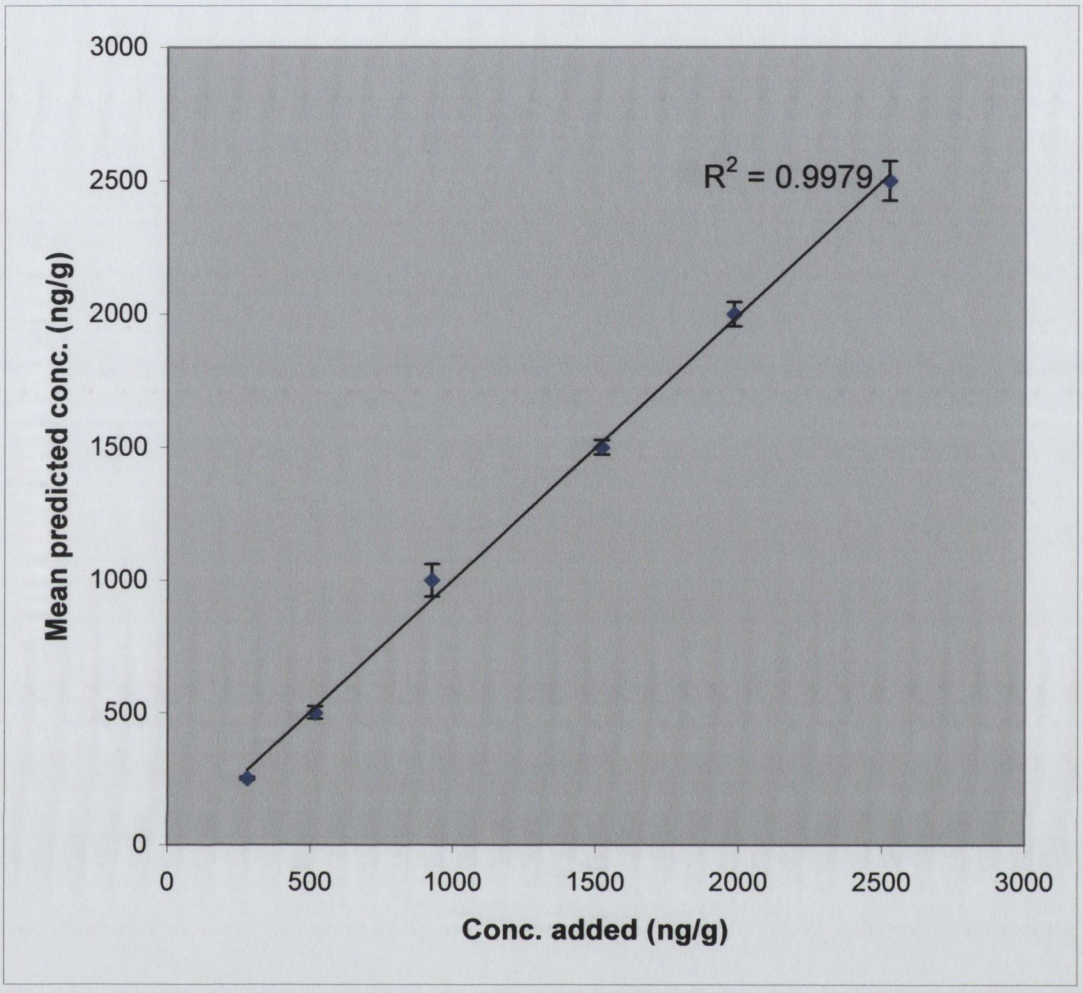
n = 4

Mean accuracy = 94.11%

Mean coefficient of variation (CV) = 3.94%

Error bars indicate standard deviations

Figure 3-15 Intra-assay variability for pig muscle fortified with ciprofloxacin (250 – 2500ng/g)



n = 4

Mean accuracy = 95.68%

Mean coefficient of variation (CV) = 3.38%

Error bars indicate standard deviations

Concerning reproducibility, the methods displayed acceptable performance with the CVs being less than 16% as outlined in EU guidance limits (in fact they were always less than 10%). The mean accuracy varied from 92 – 97% and the mean CVs were 3% approximately as can be seen from Tables 3–44 to 3–49 hereunder.

Table 3-44 Inter-assay variability of cattle muscle fortified with enrofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
250.00	236.62 ± 13.36	5.65	94.65
500.00	489.95 ± 1.36	0.28	97.99
1000.00	1029.51 ± 13.47	1.31	97.05
1500.00	1472.82 ± 19.79	1.34	98.19
2000.00	2066.26 ± 28.81	1.39	96.69
2500.00	2454.85 ± 26.51	1.08	98.19

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 1.84%

Mean accuracy = 97.13%

day 1. $Y = 0.082x + 12.080$ Correlation coefficient = 0.999

day 2. $Y = 0.086x + 3.9221$ Correlation coefficient = 0.999

day 3. $Y = 0.084x + 6.9907$ Correlation coefficient = 0.997

day 4. $Y = 0.089x + 3.5837$ Correlation coefficient = 0.999

Table 3-45 Inter-assay variability of cattle muscle fortified with ciprofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
250.00	262.84 ± 13.41	5.10	94.87
500.00	485.74 ± 19.61	4.04	97.15
1000.00	998.70 ± 15.35	1.54	99.87
1500.00	1478.91 ± 42.65	2.88	98.59
2000.00	2045.34 ± 8.99	0.44	97.73
2500.00	2478.47 ± 23.57	0.95	99.14

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 2.49%

Mean accuracy = 97.56%

day 1. Y = 0.0872x - 3.8545 Correlation coefficient = 0.999

day 2. Y = 0.0878x - 7.0499 Correlation coefficient = 0.999

day 3. Y = 0.0885x - 4.4712 Correlation coefficient = 0.999

day 4. Y = 0.0922x - 3.4906 Correlation coefficient = 0.999

Table 3-46 Inter-assay variability of sheep muscle fortified with enrofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
250.00	251.68 ± 52.93	21.03	99.33
500.00	460.15 ± 21.72	4.72	92.03
1000.00	1039.15 ± 36.88	3.55	96.09
1500.00	1548.23 ± 68.19	4.40	96.78
2000.00	1937.95 ± 20.92	1.08	96.90
2500.00	2512.85 ± 51.64	2.05	99.49

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 6.14%

Mean accuracy = 96.77%

day 1. $Y = 0.072x + 5.9403$ Correlation coefficient = 0.998

day 2. $Y = 0.0698x + 7.1957$ Correlation coefficient = 0.999

day 3. $Y = 0.0687x + 6.1350$ Correlation coefficient = 0.999

day 4. $Y = 0.0666x + 9.5047$ Correlation coefficient = 0.992

Table 3-47 Inter-assay variability of sheep muscle fortified with ciprofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
250.00	191.09 ± 102.23	53.50	76.43
500.00	448.65 ± 8.54	1.90	89.73
1000.00	1096.58 ± 67.32	6.14	90.34
1500.00	1627.48 ± 165.22	10.15	91.50
2000.00	1925.82 ± 85.85	4.46	96.29
2500.00	2460.39 ± 80.97	3.29	98.42

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 13.24%

Mean accuracy = 90.45%

day 1. Y = 0.0814x - 0.43506 Correlation coefficient = 0.998

day 2. Y = 0.0782x - 0.33843 Correlation coefficient = 0.997

day 3. Y = 0.0789x - 1.10578 Correlation coefficient = 0.998

day 4. Y = 0.0660x + 21.9446 Correlation coefficient = 0.953

Table 3-48 Inter-assay variability of pig muscle fortified with enrofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
250.00	289.20 ± 17.58	6.08	84.32
500.00	492.15 ± 18.44	3.75	98.43
1000.00	945.41 ± 22.89	2.42	94.54
1500.00	1497.86 ± 60.72	4.05	99.86
2000.00	2023.07 ± 38.16	1.89	98.15
2500.00	2503.31 ± 24.92	1.00	99.87

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 3.2%

Mean accuracy = 95.86%

day 1. Y = 0.0417x - 6.5376 Correlation coefficient = 0.997

day 2. Y = 0.0457x - 3.79898 Correlation coefficient = 0.998

day 3. Y = 0.0561x - 6.75253 Correlation coefficient = 1.000

day 4. Y = 0.0572x - 6.4287 Correlation coefficient = 0.998

Table 3-49 Inter-assay variability of pig muscle fortified with ciprofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
250.00	260.29 ± 19.21	7.38	95.88
500.00	531.80 ± 38.04	7.15	93.64
1000.00	949.89 ± 48.31	5.09	94.99
1500.00	1509.55 ± 40.47	2.68	99.36
2000.00	1957.72 ± 57.59	2.94	97.87
2500.00	2540.75 ± 69.12	2.72	98.37

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 4.66%

Mean accuracy = 96.69%

day 1. Y = 0.0409x - 1.44982 Correlation coefficient = 0.999

day 2. Y = 0.0425x + 1.76232 Correlation coefficient = 0.998

day 3. Y = 0.0519x - 5.26611 Correlation coefficient = 0.991

day 4. Y = 0.0566x - 4.028 Correlation coefficient = 0.999

3.3.2.4 Sensitivity

The sensitivity of this method, in terms of the validated LOQ of enrofloxacin is 210, 370 and 450 ng/g in beef, mutton and pork respectively. The LOD ranges from 65 to 135 ng/g in these species. The LOQ of ciprofloxacin is 315, 110 and 315 ng/g in beef, mutton and pork respectively while the LOD ranges from 35 - 95 ng/g in these species. The data are given in Table 3-50 to 3–55 hereunder.

Table 3-50 LOQ and LOD of assay method for cattle muscle fortified with enrofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
250.00	22.554 ± 0.558	2.47	18.56	61.85
500.00	45.949 ± 0.503	1.95	16.42	54.73
1000.00	92.995 ± 1.810	1.95	58.39	194.63
1500.00	132.751 ± 2.230	1.68	75.59	251.97
2000.00	179.615 ± 0.295	0.16	9.85	32.85
2500.00	213.224 ± 5.839	2.73	194.83	649.43

SD = standard deviation n = 3

CV = coefficient of variation

Mean limit of detection (LOD) = 62.27ng/g

Mean limit of quantification (LOQ) = 207.58ng/g

Table 3-51 LOQ and LOD of assay method for sheep muscle fortified with enrofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
250.00	28.087 ± 9.139	32.54	244.04	813.45
500.00	36.635 ± 3.899	10.65	159.64	532.14
1000.00	79.143 ± 0.517	0.65	19.59	65.32
1500.00	112.735 ± 2.976	2.64	118.79	395.97
2000.00	145.171 ± 2.303	1.59	95.18	317.28
2500.00	182.450 ± 0.314	0.17	12.91	43.03

SD = standard deviation n = 3

CV = coefficient of variation

Mean limit of detection (LOD) = 108.36ng/g

Mean limit of quantification (LOQ) = 361.20ng/g

Table 3-52 LOQ and LOD of assay method for pig muscle fortified with enrofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
250.00	10.931 ± 1.547	14.16	106.14	353.81
500.00	22.771 ± 0.675	2.96	44.46	148.21
1000.00	45.383 ± 2.185	4.82	144.44	481.46
1500.00	78.833 ± 2.412	3.06	137.68	458.94
2000.00	109.238 ± 3.938	3.60	216.30	720.99
2500.00	137.706 ± 2.805	2.04	152.77	509.24

SD = standard deviation n = 4

CV = coefficient of variation

Mean limit of detection (LOD) = 133.63ng/g

Mean limit of quantification (LOQ) = 445.44ng/g

Table 3-53 LOQ and LOD of assay method for cattle muscle fortified with ciprofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
250.00	17.035 ± 1.704	10.00	75.02	250.07
500.00	34.116 ± 0.936	2.74	41.15	137.18
1000.00	81.637 ± 4.357	5.33	160.11	533.70
1500.00	121.740 ± 2.794	2.29	103.27	344.26
2000.00	172.790 ± 4.663	2.69	161.91	539.73
2500.00	210.732 ± 0.702	0.33	24.98	83.28

SD = standard deviation n = 3

CV = coefficient of variation

Mean limit of detection (LOD) = 94.41ng/g

Mean limit of quantification (LOQ) = 314.70ng/g

Table 3-54 LOQ and LOD of assay method for sheep muscle fortified with ciprofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
250.00	19.205 ± 0.376	1.96	14.68	48.95
500.00	36.044 ± 0.086	0.24	3.58	11.93
1000.00	83.057 ± 2.050	2.47	74.05	246.82
1500.00	119.155 ± 1.219	1.02	46.04	153.45
2000.00	147.526 ± 0.828	0.56	33.68	112.25
2500.00	199.407 ± 0.656	0.33	24.67	82.24

SD = standard deviation n = 3

CV = coefficient of variation

Mean limit of detection (LOD) = 32.78ng/g

Mean limit of quantification (LOQ) = 109.27ng/g

Table 3-55 LOQ and LOD of assay method for pig muscle fortified with ciprofloxacin (250-2500ng/g)

Conc. Added (ng/g)	Mean Peak Area ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
250.00	11.821 ± 0.316	2.68	20.05	66.83
500.00	25.237 ± 1.355	5.37	80.54	268.46
1000.00	48.407 ± 3.496	7.22	216.66	722.21
1500.00	82.038 ± 1.465	1.79	80.36	267.86
2000.00	108.263 ± 2.568	2.37	142.32	474.40
2500.00	139.018 ± 4.212	3.03	25.24	84.16

SD = standard deviation n = 4

CV = coefficient of variation

Mean limit of detection (LOD) = 94.20ng/g

Mean limit of quantification (LOQ) = 313.98ng/g

3.3.2.5 Stability

Enrofloxacin and ciprofloxacin are stable for at least 10 days as can be seen from Tables 3–56 and 3-57 hereunder.

Table 3-56 Stability of enrofloxacin in fortified cattle, sheep and pig muscle over time

Theoretical conc. (ng/g)	day			
	Chromatographic response			
	0	% of initial conc.	10	% of initial conc.
Beef				
500	29.972	100	28.874	96
2000	122.606	100	110.970	91
Lamb				
500	28.044	100	22.353	80
2000	123.495	100	111.909	91
Pork				
500	21.970	100	26.368	120
2000	118.651	100	94.025	80

Table 3-57 Stability of ciprofloxacin in fortified cattle, sheep and pig muscle over time

Theoretical conc. (ng/g)	day 0 Chromatographic response	% of initial conc.	day 10 Chromatographic response	% of initial conc.
Beef				
500	14.746	100	16.136	109
2000	97.780	100	92.415	95
Lamb				
500	16.759	100	14.139	84
2000	94.742	100	92.699	98
Pork				
500	24.061	100	28.181	117
2000	106.386	100	102.668	97

3.3.3 Tiamulin

3.3.3.1 Specificity

The bioassay employed in this method was not necessarily specific, as the test organism might inhibit more than a single substance. Negative control tissue samples did not show the presence of inhibitory substances.

3.3.3.2 Linearity and accuracy

A linear response over the entire range was obtained in respect of beef as shown in Tables 3-58 to 3-61 hereunder.

Table 3-58 Linearity and accuracy of cattle muscle fortified with tiamulin (500-3000ng/g), day 1

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	10.250 ± 0.210	2.05	409.00	81.80
1000.00	10.950 ± 0.050	0.65	1082.00	91.80
2000.00	11.850 ± 0.050	0.60	2065.00	96.70
3000.00	12.700 ± 0.058	0.79	2945.00	98.17

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 1.02%

Mean accuracy = 92.12%

Correlation coefficient = 0.997

Table 3-59 Linearity and accuracy of cattle muscle fortified with tiamulin (500-3000ng/g), day 2

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	9.100 ± 0.153	2.90	174.00	34.80
1000.00	11.100 ± 0.058	0.90	1367.00	63.30
2000.00	12.367 ± 0.133	1.87	2082.00	95.90
3000.00	13.767 ± 0.067	0.84	2877.00	95.90

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 1.63%

Mean accuracy = 72.48%

Correlation coefficient = 0.966

Table 3-60 Linearity and accuracy of cattle muscle fortified with tiamulin (500-3000ng/g), day 3

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	9.033 ± 0.120	2.30	389.00	77.80
1000.00	10.000 ± 0.058	1.00	1160.00	84.00
2000.00	11.000 ± 0.289	2.63	1957.00	97.85
3000.00	12.300 ± 0.058	0.81	2994.00	99.80

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 1.69%

Mean accuracy = 89.86%

Correlation coefficient = 0.995

Table 3-61 Linearity and accuracy of cattle muscle fortified with tiamulin (500-3000ng/g), day 4

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	9.033 ± 0.115	1.30	446.00	89.20
1000.00	10.033 ± 0.186	3.21	1086.00	91.40
2000.00	11.400 ± 0.100	1.73	1961.00	98.05
3000.00	13.033 ± 0.120	2.08	3006.00	99.80

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 2.08%

Mean accuracy = 94.61%

Correlation coefficient = 0.998

The data show that the method is linear in respect of cattle muscle. Accuracy values are, with the exception of one value at 500ng/g concentration, within the EU

guideline limits. The mean accuracy for each of the 4 days ranged from 73 - 95%. The correlation coefficients were above 0.966.

A linear response over the entire range was obtained in respect of mutton as shown in Tables 3-62 to 3-65.

Table 3-62 Linearity and accuracy of sheep muscle fortified with tiamulin (500-3000ng/g), day 1

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	9.200 ± 0.231	4.35	391.00	78.20
1000.00	10.267 ± 0.088	1.49	1100.00	90.00
2000.00	11.733 ± 0.088	1.30	2173.00	91.35
3000.00	13.033 ± 0.088	1.17	2936.00	97.87

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 2.08%

Mean accuracy = 89.36%

Correlation coefficient = 0.996

Table 3-63 Linearity and accuracy of sheep muscle fortified with tiamulin (500-3000ng/g), day 2

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	9.800 ± 0.115	2.04	222.00	44.40
1000.00	11.300 ± 0.113	1.77	1240.00	76.00
2000.00	12.733 ± 0.113	1.81	2213.00	89.35
3000.00	13.633 ± 0.145	1.85	2824.00	94.13

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 1.87%

Mean accuracy = 75.97%

Correlation coefficient = 0.973

Table 3-64 Linearity and accuracy of sheep muscle fortified with tiamulin (500-3000ng/g), day 3

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	10.433 ± 0.067	1.11	303.00	60.60
1000.00	11.633 ± 0.033	1.79	1218.00	78.20
2000.00	12.733 ± 0.033	0.21	2057.00	97.15
3000.00	13.867 ± 0.233	0.29	2922.00	97.40

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 1.57%

Mean accuracy = 83.34%

Correlation coefficient = 0.987

Table 3-65 Linearity and accuracy of sheep muscle fortified with tiamulin (500-3000ng/g), day 4

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	10.967 ± 0.033	0.53	330.00	66.00
1000.00	11.867 ± 0.088	1.29	1130.00	87.00
2000.00	13.033 ± 0.033	0.44	2165.00	91.75
3000.00	13.833 ± 0.088	1.10	2875.00	95.83

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 0.59%

Mean accuracy = 85.15% Correlation coefficient = 0.988

The data show that the method is linear. The mean accuracy for each of the 4 days ranged from 76 – 89%. The correlation coefficients were above 0.973.

A linear response over the entire range was obtained in respect of pork as shown in Tables 3-66 to 3-69.

Table 3-66 Linearity and accuracy of pig muscle fortified with tiamulin (500-3000ng/g), day 1

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	13.567 ± 0.321	2.36	423.00	84.60
1000.00	15.867 ± 0.252	1.57	1162.00	83.80
2000.00	18.067 ± 0.351	1.94	1869.00	93.45
3000.00	21.730 ± 0.723	3.31	3046.00	98.47

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 2.30%

Mean accuracy = 90.08%

Correlation coefficient = 0.993

Table 3-67 Linearity and accuracy of pig muscle fortified with tiamulin (500-3000ng/g), day 2

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	13.800 ± 0.173	1.25	246.00	49.20
1000.00	16.800 ± 0.608	3.62	1251.00	74.90
2000.00	19.430 ± 0.100	0.51	2133.00	93.35
3000.00	21.630 ± 0.458	2.12	2870.00	95.67

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 1.88%

Mean accuracy = 78.28%

Correlation coefficient = 0.979

Table 3-68 Linearity and accuracy of pig muscle fortified with tiamulin (500-3000ng/g), day 3

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	13.900 ± 0.200	1.44	346.00	69.20
1000.00	16.270 ± 0.058	0.35	1180.00	82.00
2000.00	18.670 ± 0.058	0.31	2024.00	98.80
3000.00	21.300 ± 0.361	1.69	2949.00	98.30

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 0.95%

Mean accuracy = 87.08%

Correlation coefficient = 0.992

Table 3-69 Linearity and accuracy of pig muscle fortified with tiamulin (500-3000ng/g), day 4

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	Predicted Value (ng/g)	Accuracy (%)
500.00	13.733 ± 0.320	2.33	267.00	53.40
1000.00	16.430 ± 0.757	4.61	1409.00	59.10
2000.00	17.267 ± 0.252	1.46	1765.00	88.25
3000.00	20.333 ± 0.404	1.99	3059.00	98.03

SD = standard deviation n = 3

Mean coefficient of variation (CV) = 2.60%

Mean accuracy = 74.70%

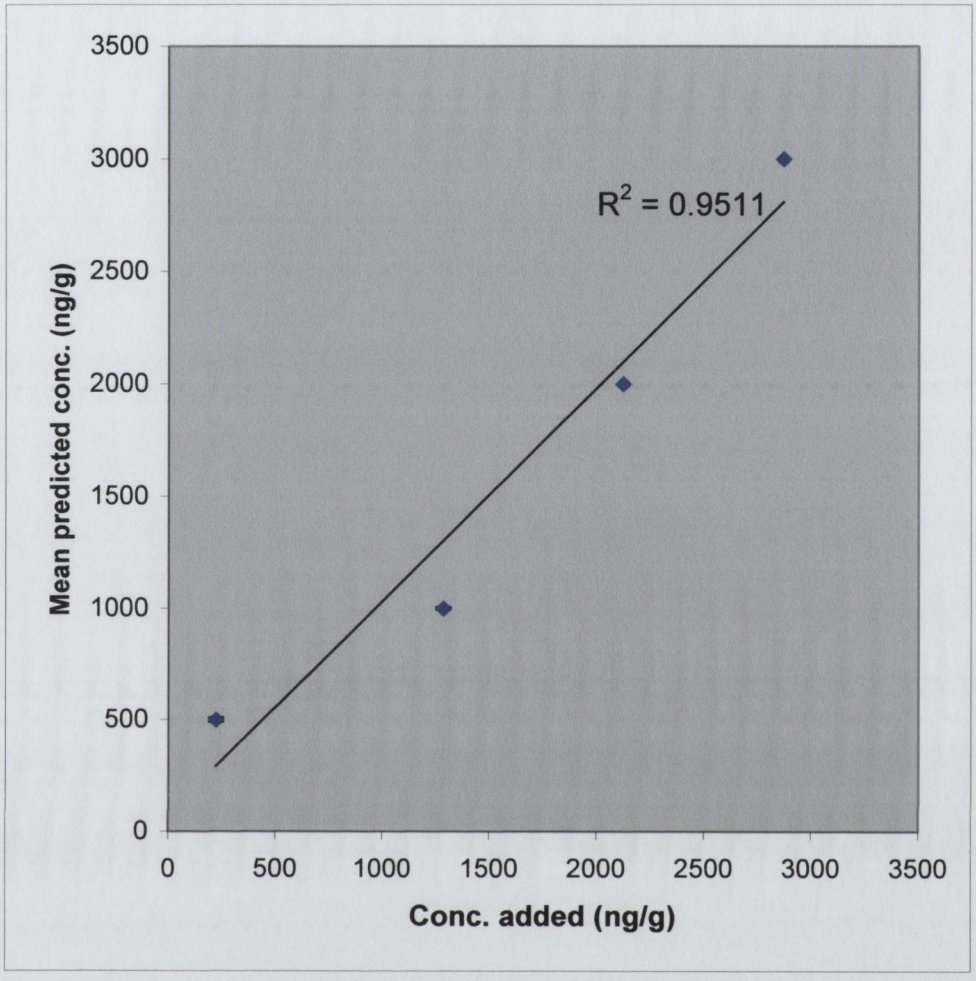
Correlation coefficient = 0.964

The data show that the method is linear. The mean accuracy for each of the 4 days ranged from 75 – 90%. The correlation coefficients were above 0.964.

3.3.3.3 Precision and reproducibility

In respect of precision, the co-efficients of variation (CV) for each of the assays was high at the lowest fortification level as shown in Figures 3-16 to 3–18 hereunder. However, the mean intra-assay CV for tiamulin in respect of all 3 species ranged from 8 - 12%, which is within the EU recommended limit of 15%.

Figure 3-16 Intra-assay variability for cattle muscle fortified with tiamulin (500 – 3000ng/g)



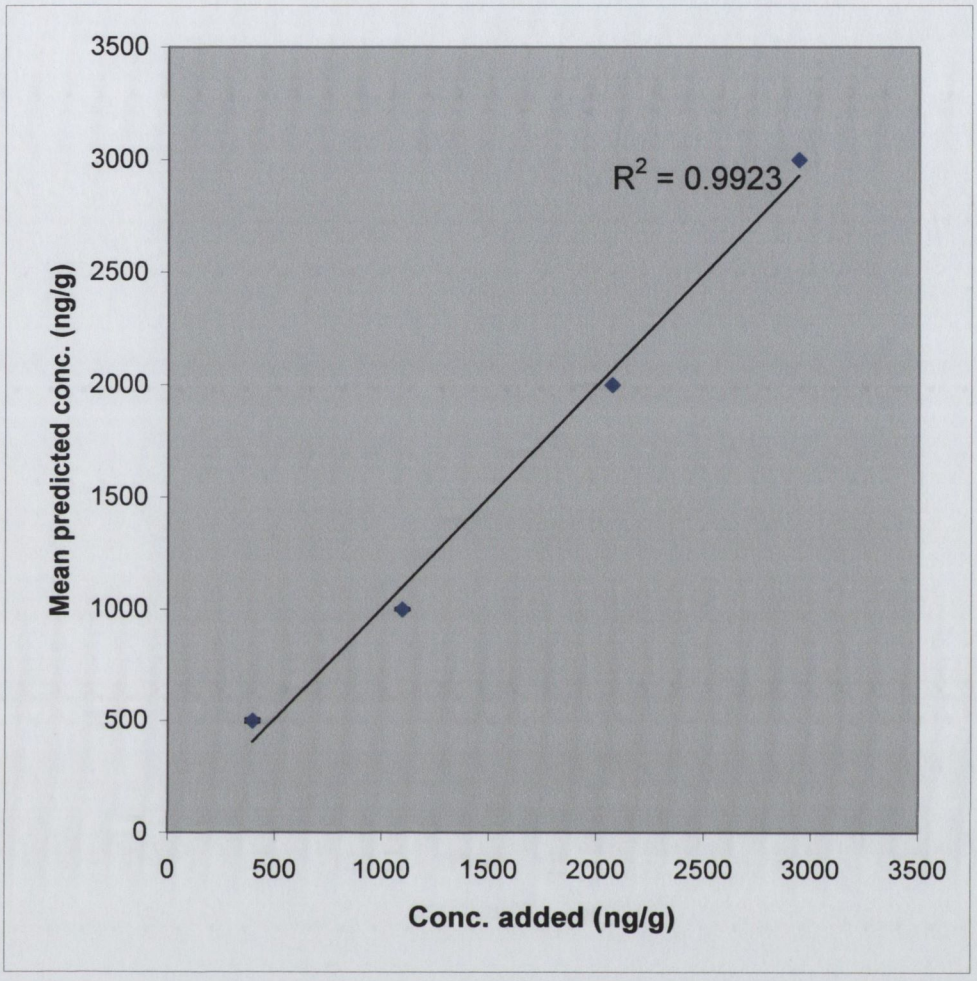
n = 4

Mean accuracy = 76.42%

Mean coefficient of variation (CV) = 12.01%

Error bars indicate standard deviations

Figure 3-17 Intra-assay variability for sheep muscle fortified with tiamulin (500 - 3000ng/g)



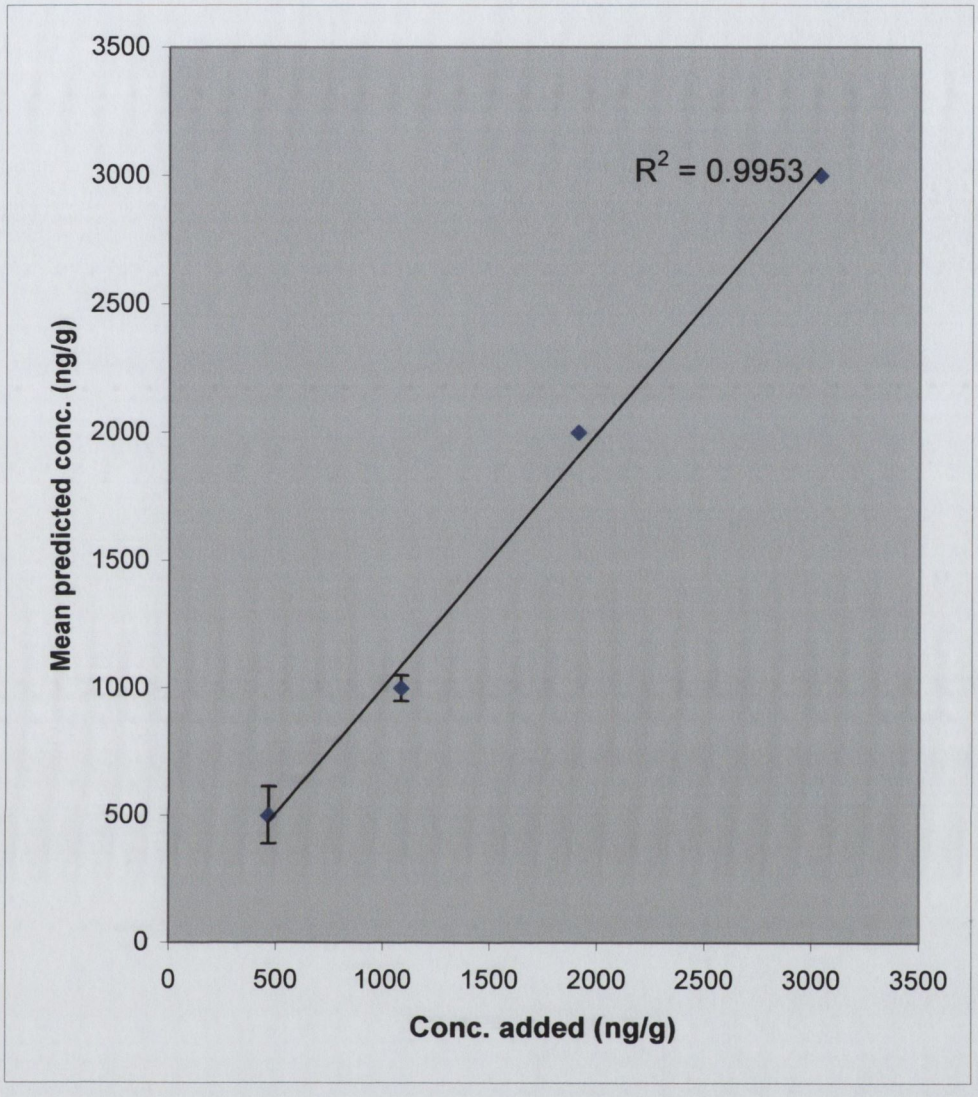
n = 4

Mean accuracy = 91.02%

Mean coefficient of variation (CV) = 7.69%

Error bars indicate standard deviations

Figure 3-18 Intra-assay variability for pig muscle fortified with tiamulin (500 - 3000ng/g)



n = 4

Mean accuracy = 94.88%

Mean coefficient of variation (CV) = 9.64%

Error bars indicate standard deviations

Concerning reproducibility, the methods displayed reasonable performance for a bioassay. The mean accuracy varied from 84 – 89% and the mean CVs were 8 - 12% approximately as can be seen from Tables 3–70 to 3-73 hereunder.

Table 3-70 Inter-assay variability of cattle muscle fortified with tiamulin (500-3000ng/g)

Conc. Added (ng/g)	Mean predicted ± SD conc. (ng/g)	% CV	Accuracy (%)
500.00	354.50 ± 122.63	31.78	71.80
1000.00	1173.75 ± 133.73	10.64	85.54
2000.00	2016.25 ± 66.49	3.19	99.25
3000.00	2955.50 ± 68.61	1.86	98.56

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 11.87%

Mean accuracy = 88.79%

day 1. Y = 9.6610x + 9.8551 Correlation coefficient = 0.997

day 2. Y = 17.6068x + 8.6939 Correlation coefficient = 0.966

day 3. Y = 12.5434x + 8.5449 Correlation coefficient = 0.995

day 4. Y = 15.6275x + 8.3353 Correlation coefficient = 0.998

Table 3-71 Inter-assay variability of sheep muscle fortified with tiamulin (500-3000ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
500.00	312.00 ± 70.10	22.48	62.40
1000.00	1172.00 ± 67.55	5.80	85.32
2000.00	2127.00 ± 74.51	3.50	94.03
3000.00	2889.25 ± 50.72	1.76	96.30

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 8.38%

Mean accuracy = 84.51%

day 1. Y = 15.06x + 8.611 Correlation coefficient = 0.996

day 2. Y = 14.7329x + 9.47241 Correlation coefficient = 0.973

day 3. Y = 13.1098x + 10.0362 Correlation coefficient = 0.987

day 4. Y = 11.2624x + 10.5949 Correlation coefficient = 0.988

Table 3-72 Inter-assay variability of pig muscle fortified with tiamulin (500-3000ng/g)

Conc. Added (ng/g)	Mean predicted conc. (ng/g) ± SD	% CV	Accuracy (%)
500.00	320.25 ± 80.66	25.19	64.05
1000.00	1250.50 ± 112.44	8.99	75.00
2000.00	1947.75 ± 163.02	8.37	97.39
3000.00	2981.00 ± 88.80	2.98	99.37

SD = standard deviation n = 4

Mean coefficient of variation (CV) = 11.38%

Mean accuracy = 83.95%

day 1. Y = 31.4051x + 12.2554 Correlation coefficient = 0.993

day 2. Y = 29.3373x + 13.0654 Correlation coefficient = 0.979

day 3. Y = 28.4271x + 12.9156 Correlation coefficient = 0.992

day 4. Y = 23.6339x - 13. 4996 Correlation coefficient = 0.964

3.3.3.4 Sensitivity

The sensitivity of this method, in terms of the validated LOQ, is 300ng/g approximately in respect of cattle and sheep and 430ng/g in respect of pigs. The LOD is approximately 90ng/g in respect of cattle and sheep and 130ng/g in respect of pigs. The data are given in Tables 3-73 to 3–75 hereunder.

Table 3-73 LOQ and LOD of assay method for cattle muscle fortified with tiamulin (500-3000ng/g)

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
500.00	9.033 ± 0.115	1.28	19.09	63.66
1000.00	10.333 ± 0.321	3.20	93.19	310.66
2000.00	11.400 ± 0.173	1.52	91.05	303.51
3000.00	13.033 ± 0.208	1.60	143.64	478.78

SD = standard deviation n = 3

CV = coefficient of variation

Mean limit of detection (LOD) = 86.74ng/g

Mean limit of quantification (LOQ) = 289.14ng/g

Table 3-74 LOQ and LOD of assay method for sheep muscle fortified with tiamulin (500-3000ng/g)

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
500.00	9.800 ±0.200	2.04	30.61	102.04
1000.00	11.300 ±0.200	1.77	53.09	176.99
2000.00	12.733 ±0.231	1.81	108.85	362.84
3000.00	13.633 ±0.252	1.85	166.36	554.54

SD = standard deviation n = 3

CV = coefficient of variation

Mean limit of detection (LOD) = 89.73ng/g

Mean limit of quantification (LOQ) = 299.10ng/g

Table 3-75 LOQ and LOD of assay method for pig muscle fortified with tiamulin (500-3000ng/g)

Conc. Added (ng/g)	Mean Zone Diameter (mm) ± SD	% CV	LOD (ng/g)	LOQ (ng/g)
500.00	13.567 ± 0.321	2.36	40.00	130.00
1000.00	15.867 ± 0.252	1.57	50.00	170.00
2000.00	18.067 ± 0.351	1.94	120.00	400.00
3000.00	21.730 ± 0.723	3.31	300.00	1000.00

SD = standard deviation n = 4

CV = coefficient of variation

Mean limit of detection (LOD) = 127.50ng/g

Mean limit of quantification (LOQ) = 425.00ng/g

3.3.3.5 Stability

Tiamulin is stable for at least 7 days as can be seen from Tables 3–76 to 3-78 hereunder.

Table 3-76 Stability of tiamulin in fortified cattle muscle over time

Conc. Added (ng/g)	Mean initial zone diameter (mm)	Mean zone diameter after 7 days (mm)	% change in zone diameter
500.00	9.033	9.150	+ 1.29
1000.00	10.033	10.200	+ 1.66
2000.00	11.400	11.390	- 0.087
3000.00	13.033	12.600	- 3.32
	n = 4	n = 4	

Table 3-77 Stability of tiamulin in fortified sheep muscle over time

Conc. Added (ng/g)	Mean initial zone diameter (mm)	Mean zone diameter after 7 days (mm)	% change in zone diameter
500.00	9.167	10.433	+ 13.81
1000.00	10.300	11.633	+ 12.94
2000.00	11.700	12.733	+ 8.83
3000.00	13.033	13.867	+ 6.40
	n = 3	n = 3	

Table 3-78 Stability of tiamulin in fortified pig muscle over time

Conc. Added (ng/g)	Mean initial zone diameter (mm)	Mean zone diameter after 8 days (mm)	% change in zone diameter
500.00	13.57	13.43	- 1.03
1000.00	15.87	15.90	+ 0.19
2000.00	18.07	18.00	- 0.39
3000.00	21.73	22.10	+ 1.70
	n = 4	n = 4	

3.4 Methods performance in different species

The performance characteristics were evaluation in 2 ways: a) a comparison of the pivotal parameters of linearity, LOD and LOQ generated in the validation studies and b) a comparison between the performance of each method in the analysis of fortified tissue of each species prepared and run together in the same assay which was repeated on 3 separate occasions.

3.4.1 Tilmicosin

The method displayed similar performance characteristics in each of the species tested even when run during different weeks or months. The clean-up and analytical conditions were identical and the chromatograms displayed the tilmicosin signal at

the same retention time interval between 5.9 and 7.1 minutes. There was no interference in the blank control muscle tissue samples in any species at this time. Each of the species tested demonstrated similar levels of accuracy, while linearity was also comparable as can be seen from Table 3–79 hereunder.

Table 3-79 Comparison of accuracy and linearity of the methods in muscle fortified with tilmicosin in cattle, sheep and pigs over the concentration range 300 - 3000ng/g

Parameter	Cattle	Sheep	Pigs
Mean CV (%) ± SD	1.76 ± 2.49	2.28 ± 2.74	5.06 ± 5.00
Mean accuracy (%) ± SD	96.01 ± 4.64	92.97 ± 8.43	95.55 ± 5.79
Mean correlation coefficient ± SD	0.9983 ± 0.001	0.9972 ± 0.002	0.9984 ± 0.001

The limits of quantification and detection were also roughly similar as can be seen from Table 3-80 hereunder.

Table 3-80 Comparison of sensitivity of the methods in muscle fortified with tilmicosin in cattle, sheep and pigs over the concentration range 300 - 3000ng/g

Parameter	Cattle	Sheep	Pigs
Mean LOD (ng/g)	38.96	38.05	63.11
Mean LOQ (ng/g)	129.87	126.84	210.37

While the LOD and LOQ are greater for pork than for mutton and beef, the studies were run 1 year apart and the laboratory instruments and equipment were used for other purposes during this period.

In respect of fortified muscle of each species, prepared and analysed on the same day, with repeated samples taken and analysed on 2 other days, the analytical response was very similar as evidenced from Table 3-81 hereunder.

Table 3-81 Comparison of analytical response of the methods in muscle fortified with tilmicosin in cattle, sheep and pigs over the concentration range 300 - 3000ng/g prepared and analysed on the same day and repeated on 3 different days

Conc. Added (ng/g)	Cattle Mean Peak Area ± SD	Sheep Mean Peak Area ± SD	Pigs Mean Peak Area ± SD
300.00	29.841 ± 2.979	35.085 ± 1.859	35.618 ± 5.223
600.00	64.999 ± 4.009	68.114 ± 1.359	71.554 ± 7.802
1200.00	135.628 ± 3.605	141.303 ± 4.818	150.348 ± 11.394
1800.00	218.604 ± 42.239	213.465 ± 16.628	210.957 ± 5.465
2400.00	285.311 ± 31.972	300.973 ± 18.356	316.497 ± 8.811
3000.00	353.856 ± 29.499	379.454 ± 32.807	389.795 ± 11.053

The within animal variation in the analysis of fortified muscle is as large as the variation between the species studied. Therefore it is concluded that the method performs equally in respect of muscle from cattle, sheep or pigs.

3.4.2 Enrofloxacin

The method displayed similar performance characteristics in each of the species tested even when run during different weeks or months. The clean-up and analytical conditions were identical and the chromatograms displayed the enrofloxacin and ciprofloxacin signals at approximately the same times. There was no interference in the blank control muscle tissue samples in any species at this time. Each of the species tested demonstrated similar levels of accuracy, while linearity was also comparable as can be seen from the Tables 3-82 and 3-83 hereunder.

Table 3-82 Comparison of accuracy and linearity of the methods in muscle fortified with enrofloxacin in cattle, sheep and pigs over the concentration range 250 - 2500ng/g

Parameter	Cattle	Sheep	Pigs
Mean CV (%) ± SD	3.80 ± 3.35	7.81 ± 11.51	9.36 ± 7.39
Mean accuracy (%) ± SD	97.01 ± 2.93	93.27 ± 8.71	94.82 ± 6.33
Mean correlation coefficient ± SD	0.9985 ± 0.0009	0.9969 ± 0.003	0.9973 ± 0.001

Table 3-83 Comparison of accuracy and linearity of the methods in muscle fortified with ciprofloxacin in cattle, sheep and pigs over the concentration range 250-2500ng/g

Parameter	Cattle	Sheep	Pigs
Mean CV (%) ± SD	3.80 ± 3.24	2.93 ± 4.21	6.96 ± 6.80
Mean accuracy (%) ± SD	96.56 ± 2.67	88.75 ± 19.86	94.95 ± 4.96
Mean correlation coefficient ± SD	0.9990 ± 0.0004	0.9902 ± 0.015	0.9968 ± 0.004

The limits of quantification and detection were also roughly similar as can be seen from Tables 3–84 and 3–85 hereunder.

Table 3-84 Comparison of sensitivity of the methods in muscle fortified with enrofloxacin in cattle, sheep and pigs over the concentration range 250-2500ng/g

Parameter	Cattle	Sheep	Pigs
Mean LOD (ng/g)	62.27	108.36	133.63
Mean LOQ (ng/g)	207.58	361.20	445.44

Table 3-85 Comparison of sensitivity of the methods in muscle fortified with ciprofloxacin in cattle, sheep and pigs over the concentration range 2500-2500ng/g

Parameter	Cattle	Sheep	Pigs
Mean LOD (ng/g)	94.41	32.78	94.20
Mean LOQ (ng/g)	314.70	109.27	313.98

In respect of fortified muscle of each species, prepared and analysed on the same day, with repeated samples taken and analysed on 2 other days, the analytical response was very similar as evidenced from Tables 3–86 and 3–87 hereunder.

Table 3-86 Comparison of analytical response of the methods in muscle fortified with enrofloxacin in cattle, sheep and pigs over the concentration range 250-2500ng/g, prepared and analysed on the same day and repeated on 3 different days

Conc. Added (ng/g)	Cattle Mean ± SD Peak Area	Sheep Mean ± SD Peak Area	Pigs Mean ± SD Peak Area
250.00	12.672 ± 1.675	11.344 ± 1.661	6.868 ± 0.867
500.00	29.887 ± 4.198	22.506 ± 2.406	16.164 ± 2.211
1000.00	60.175 ± 4.027	62.119 ± 4.844	46.626 ± 6.261
1500.00	87.932 ± 11.339	84.016 ± 5.393	68.467 ± 19.542
2000.00	111.361 ± 1.126	116.500 ± 12.129	96.685 ± 14.650
2500.00	139.812 ± 8.701	138.992 ± 7.641	127.471 ± 13.416

Table 3-87 Comparison of analytical response of the methods in muscle fortified with ciprofloxacin in cattle, sheep and pigs over the concentration range 250-2500ng/g, prepared and analysed on the same day and repeated on 3 different days

	Cattle	Sheep	Pigs
Conc. Added (ng/g)	Mean Peak Area ± SD	Mean Peak Area ± SD	Mean Peak Area ± SD
250.00	15.748 ± 1.157	18.529 ± 3.109	15.077 ± 3.128
500.00	29.340 ± 1.156	31.438 ± 2.192	25.591 ± 2.197
1000.00	59.173 ± 3.056	61.659 ± 6.019	52.745 ± 8.161
1500.00	84.216 ± 1.639	91.883 ± 2.900	79.735 ± 4.234
2000.00	117.740 ± 7.625	122.927 ± 2.243	108.712 ± 6.258
2500.00	140.408 ± 0.595	142.843 ± 3.143	134.476 ± 8.797

Variability in the response was greatest in pork samples as is evidenced by the large standard deviation values seen with enrofloxacin on the comparison table and the higher limits of detection and quantification seen. By contrast, the performance of ciprofloxacin in the pork matrix was similar to that in beef. The reason for this finding is not known.

3.4.3 Tiamulin

The clean-up and analytical conditions were performed under the same conditions throughout. In studies on fortified muscle samples of each species run on the same wells at the same time and repeated on different days, there was no marked difference between the responses obtained in any of the species. There was no interference in the blank control muscle tissue samples in any species at any time. Each of the species tested demonstrated similar levels of accuracy, while linearity was also comparable as can be seen from Table 3–88 hereunder.

Table 3-88 Comparison of accuracy and linearity of the methods in muscle fortified with tiamulin in cattle, sheep and pigs over the concentration range 500-3000ng/g

Parameter	Cattle	Sheep	Pigs
Mean CV (%)± SD	2.08 ± 0.86	1.41 ± 0.98	1.93 ± 1.17
Mean accuracy (%)± SD	87.27 ± 17.12	83.45 ± 15.37	82.53 ± 16.73
Mean correlation coefficient ± SD	0.9890 ± 0.015	0.9860 ± 0.009	0.9605 ± 0.034

The limits of quantification and detection were also roughly similar as can be seen from Table 3-89 hereunder.

Table 3-89 Comparison of sensitivity of the methods in muscle fortified with tiamulin in cattle, sheep and pigs over the concentration range 500-3000ng/g

Parameter	Cattle	Sheep	Pigs
Mean LOD (ng/g)	86.74	89.73	127.50
Mean LOQ (ng/g)	289.14	299.10	425.00

While the LOD and LOQ are significantly greater for pork than for mutton and beef, it must be remembered that the studies were run at different periods and the test micro-organism was from a different batch date. Inhibition zones in the pig assays were larger than for sheep and cattle. This could be a result of a combination of factors including differences in the activity of the test organism *Micrococcus luteus* (although the same batch numbers were recorded for 10/1997 and 10/1998), differences in the ambient temperature in the laboratory (not measured) and other variables (the pig studies were conducted approximately a year after the studies in cattle and sheep).

In respect of fortified muscle of each species, prepared and run on the same day, and conducted on 4 separate days, the analytical response was very similar as evidenced from Table 3-90 hereunder.

Table 3-90 Comparison of analytical response of the methods in muscle fortified with tiamulin in cattle, sheep and pigs over the concentration range 500-3000ng/g prepared and analysed on the same day and repeated on 4 different days

Conc. Added (ng/g)	Cattle Mean ± SD zone diameter (mm)	Sheep Mean ± SD zone diameter (mm)	Pigs Mean ± SD zone diameter (mm)
500.00	12.15 ± 1.40	11.82 ± 1.09	12.05 ± 1.33
1000.00	14.44 ± 0.75	14.32 ± 0.79	14.80 ± 1.34
2000.00	17.33 ± 0.97	17.51 ± 0.16	17.84 ± 1.17
3000.00	20.37 ± 0.36	20.09 ± 0.47	20.67 ± 0.76

3.5 Discussion

In the context of the studies undertaken, the validation data demonstrated that the methods exhibited the performance characteristics required to accurately detect and quantify the analytes at the concentrations of interest. These characteristics included such variables as matrix (various muscle groups), species (cattle, sheep and pigs), gender, age, nutritional status, sampling conditions, storage conditions, sample preparation and other environmental conditions. Regarding the performance characteristics of the individual methods, it is noted that the bioassay method had lower regression coefficients than either of the chemically-based assays. Nevertheless, all of the methods met the objectives set for linearity, accuracy, limits of quantification and detection and were therefore adequate for their purpose.

Regarding the dynamic range of the methods, O'Rangers and van Ginkel (2000) recommended that for linear relationships to be determined, the standard curve should be defined by 5 to 8 concentrations. In the studies conducted, both HPLC assays used 6 concentrations to fortify the muscle tissues. While only 4 concentrations were used in the bioassay the slope of the regression line response curve meant that additional

concentrations within the range tested would not have yielded an improved performance. During test development, lower concentrations were used but did not yield consistent responses. O'Rangers and van Ginkel (2000) also reported that bioassays are often naturally 'non-linear' in response but that this does not mean that the methods are not useful, provided they can provide reproducible and accurate results. While the bioassay employed in the studies reported above had a reduced level of accuracy at the lowest concentration tested, the overall accuracy and precision were acceptable.

Specificity can seldom be fulfilled for microbiological methods that rarely differentiate between individual anti-bacterial substances. However, Korsrud *et al* (1998) reported that the pH and composition of the test medium, as well as other parameters influence the performance of bioassays. Under the particular test conditions that were established to detect tiamulin, including the test organism used, it was believed unlikely that the test would be as sensitive to other classes of anti-bacterials. Moreover, as the test animals were maintained free of xenobiotics for at least 3 weeks prior to the conduct of the studies and did not receive any other drug apart from tiamulin, the most likely interferences in the response of the bioassay would be some matrix effects such as those outlined by Okerman *et al* (1998) or a metabolic product of tiamulin arising from the rapid and extensive metabolism of the drug in ruminants reported by Ziv *et al* (1983).

The analytical methods for tilmicosin, enrofloxacin and tiamulin displayed essentially similar performance in muscle in respect of each species tested. Minor differences in limits of detection, quantification and assay variability between species were less likely to be due to a species effect but rather due to the precision of the individual assays. Comparing the results obtained in the 'species comparison experiments' with those obtained in the 'validation experiments', it is also noted that the responses seen were similar. Thus it is concluded that, under the conditions of the study, the analysis of the marker residues in muscle of cattle, sheep and pigs is essentially similar and that the performance characteristics of the methods used are not species dependant. While HPLC methods may give better accuracy and selectivity by reason of their specific chromatographic operational conditions, these same conditions serve to limit

their potential use in different species tissue matrices. By contrast, bioassays have been demonstrated to have cross-species application. Okerman *et al* (2000) reported on the use of an inhibition test that can be used for the examination of muscle tissue from mammals, birds as well as from fish.

CHAPTER 4: DISTRIBUTION OF RESIDUES IN MUSCLE

4.1 Introduction

The objective of this segment of the thesis was to establish whether residues of the 3 veterinary anti-bacterials tilmicosin, enrofloxacin and tiamulin were distributed homogeneously in the musculature of animals. This knowledge is important for both residue monitoring of meat and for the conduct of residue depletion studies on veterinary drugs to establish product withdrawal periods.

4.2 Materials and methods

Groups containing 3 cattle, 3 sheep and 4 pigs were administered tilmicosin, enrofloxacin and tiamulin parenterally, 7 - 72 hours prior to slaughter, at a time when quantifiable residues were expected to be present in the tissues. Details of the animals, assays and methodology are given in Chapter 2. Muscle tissue (50 – 200g samples) from the external masseter, tongue, shoulder (*supraspinatus*), heart (whole organ), diaphragm, loin (*longissimus*) and gluteal muscle (*gluteobiceps*) were harvested within 6 hours of slaughter into prelabelled, polythene sample bags and stored at – 20°C prior to analysis. None of the samples taken for this study included injection-site residues. Analyses of tilmicosin and enrofloxacin were conducted using HPLC methods while tiamulin was analysed using a bioassay. Standard calibration curves were constructed from control samples fortified with each drug over the expected concentration range. All unknown and fortified samples for chemical assay were analysed in duplicate while the samples for bioassay were analysed in triplicate with the mean result used for calculation of drug concentrations. In order to estimate the homogeneity of residue concentrations within each sample and to increase the reliability of the data generated, a minimum of 3 subsamples of each muscle sample were taken on separate occasions for analysis, while on occasions, up to 6 subsamples from particular muscle samples were taken and analysed. Each subsample was recorded as a separate data point in the results section. The variations in the concentrations found within a muscle sample (i.e. concentrations in the subsamples)

were expressed as the intra-site CV percentage. The variation in residue concentrations between individual muscles in the same animal (the inter-site %CV) was calculated on the basis of the intra-site mean residue concentrations. Residue concentrations between the limits of quantification and detection were recorded as being half the LOQ for the purposes of calculating mean values in the calculations of the percentage coefficients of variation. Residue concentrations below the limit of detection were taken as zero in the calculations to determine the inter-site %CV. Calculations of inter-site %CV was repeated in respect of each particular animal.

4.3 Results

4.3.1 Distribution of test drugs within and between individual muscles

In respect of cattle, sheep and pigs the data show wide variability in residue concentrations in the samples taken both from different muscles and between subsamples taken from the same muscle group. The data are analysed in respect of each drug separately hereunder.

4.3.1.1 Tilmicosin

In respect of cattle muscle, compared to the inter-assay CVs of 8.01% and 5.31% at concentrations of 300ng/g and 600ng/g, it is clear that most of the variation in the responses obtained was outside this range and reflected actual differences in the tissue residue distribution as outlined in Table 4-1 below. The intra-site CV ranged from 5.5 – 59.3 %, while the inter-site CV was 42.5%, 53.1% and 56.5% for animals 1, 2 and 3 respectively. Tilmicosin concentrations were below the LOQ in heart muscle.

In respect of sheep muscle, compared to the inter-assay CVs of 4.25% and 2.99% at concentrations of 300ng/g and 600ng/g, it is clear that most of the variation in the responses obtained was outside this range and reflected actual differences in the tissue residue distribution as outlined in Table 4-2 below. The intra-site CV ranged from 2.6 – 100.6%, while the inter-site CV was 29.4%, 87.2% and 60.1% for animals 1, 2 and 3 respectively.

In respect of pig muscle, compared to the inter-assay CV of 7.9% at a concentration of 300ng/g and an overall fortification range CV of 2.99%, it is clear that most of the variation in the responses obtained was outside this range and reflected actual differences in the tissue residue distribution as outlined in Table 4-3 below. The intra-site CV ranged from 11.9 – 115.0%, while the inter-site CV was 71.8%, 95.3%, 150.9% and 143.3% for animals 1, 2, 3 and 4 respectively.

The variation in concentrations of tilmicosin residues in subsamples within each muscle sample was therefore generally as great as between samples taken from different muscle masses in each of the species examined.

Table 4-1 Distribution of tilmicosin within the musculature of 3 cattle

Muscle tissue samples	No. of subsamples	Drug concentration in muscle (ng/g)					
		Animal 1		Animal 2		Animal 3	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
Masseter	3	510 ± 217	42.5	632 ± 188	29.7	325 ± 62	19.1
Tongue	3	466 ± 85	18.2	406 ± 169	41.6	288 ± 62	21.5
Shoulder	3	213 ± 36	16.9	692 ± 152	22.0	363 ± 97	26.7
Heart	3	nq		nq		nq	
Diaphragm	5	382 ± 96	25.1	425 ± 105	24.7	442 ± 73	16.5
Loin	3	464 ± 275	59.3	419 ± 23	5.5	365 ± 43	11.8
Gluteal	6	678 ± 206	30.0	1030 ± 203	19.7	344 ± 26	7.6

nq = not quantifiable (less than 130ng/g)

SD = standard deviation

CV = coefficient of variation

Table 4-2 Distribution of tilmicosin within the musculature of 3 sheep

Muscle tissue samples	No. of subsamples	Drug concentration in muscle (ng/g)					
		Animal 1		Animal 2		Animal 3	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
Masseter	3	354 ± 32	9.0	192 ± 102	53.1	538 ± 14	2.6
Tongue	3	269 ± 88	32.7	272 ± 105	38.6	415 ± 111	26.7
Shoulder	3	276 ± 69	25.0	274 ± 50	18.3	425 ± 17	4.0
Heart	3	340 ± 42	12.4	nq		358 ± 84	23.5
Diaphragm	3	454 ± 247	54.4	816 ± 821	100.6	683 ± 142	20.8
Loin	3	156 ± 26	16.7	nq		789 ± 43	5.5
Gluteal	6	242 ± 51	21.1	215 ± 20	9.3	756 ± 33	4.4

nq = not quantifiable (less than 130ng/g)

SD = standard deviation

CV = coefficient of variation

Table 4-3 Distribution of tilmicosin within the musculature of 4 pigs

Muscle tissue samples	No. of subsamples	Drug concentration in muscle (ng/g)							
		Animal 1		Animal 2		Animal 3		Animal 4	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
Masseter	4	906 ± 658	72.6	1396 ± 1006	72.1	4257 ± 4896	115.0	1171 ± 832*	71.0
Tongue	3	1208 ± 693	57.7	803 ± 204	25.4	1093 ± 285	26.1	1132 ± 250	22.1
Shoulder	3	283 ± 86*	30.4	218 ± 106*	48.6	201 ± 24*	11.9	329 ± 164	49.8
Heart	3	484 ± 154	31.8	357 ± 44	12.3	508 ± 220	43.3	433 ± 158	36.5
Diaphragm	6	nq		nq		nq		197 ± 77*	39.1
Loin	3	294 ± 113*	38.4	nq		nq		nq	
Gluteal	6	310 ± 113*	36.5	252 ± 69	27.4	215 ± 88*	40.9	379 ± 96	25.3

nq = not quantifiable (less than 210ng/g)

SD = standard deviation

CV = coefficient of variation

* includes values below limit of quantification, taken as 105ng/g for calculation purposes.

4.3.1.2 Enrofloxacin

Given the fact that enrofloxacin is metabolized to ciprofloxacin, and the sum of both compounds is taken as the marker residue, the results are combined in the evaluation conducted hereunder.

In respect of cattle muscle, compared to the overall inter-assay variation for enrofloxacin (CV = 1.8%) and for ciprofloxacin (2.5%), it is clear that most of the variation in the responses obtained was outside this range and reflected actual differences in the tissue residue distribution as outlined in Table 4-4 below. The intra-site CV ranged from 5.6 – 48.6%, while the inter-site CV was 136.6%, 43.3% and 65.5% for animals 1, 2 and 3 respectively. Thus, variability between different muscle samples is somewhat greater than it is within the sample.

In respect of sheep muscle, compared to the overall inter-assay variation for enrofloxacin (CV = 6.1%) and for ciprofloxacin (13.2%), it is clear that most of the variation in the responses obtained was outside this range and reflected actual differences in the tissue residue distribution as outlined in Table 4-5 below. The intra-site CV ranged from 2.7 – 86.5%, while the inter-site CV was 11.6%, 29.3% and 28.4% for animals 1, 2 and 3 respectively. Variability between different muscle samples is less than or similar to that found within the sample.

In respect of pig muscle, compared to the overall inter-assay variation for enrofloxacin (CV = 3.2%) and for ciprofloxacin (CV = 4.66%), it is clear that most of the variation in the responses obtained was outside this range and reflected actual differences in the tissue residue distribution as outlined in Table 4-6 below. The intra-site CV ranged from 8.7 – 48.4%, while the inter-site CV was 8.8%, 12.7%, 13.0% and 8.6% for animals 1, 2, 3 and 4 respectively. These data indicate that the variations within individual muscle samples are as large or larger than those between different muscles.

Table 4-4 Distribution of enrofloxacin plus ciprofloxacin within the musculature of 3 cattle

Muscle tissue samples	No. of subsamples	Drug concentration in muscle (ng/g)					
		Animal 1		Animal 2		Animal 3	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
Masseter	3	467 ± 227	48.6	398 ± 54	13.6	490 ± 235	47.9
Tongue	3	467 ± 168	35.9	446 ± 31	6.9	669 ± 217	32.4
Shoulder	3	316*		616**		nd	
Heart	3	1243 ± 410	32.9	1183 ± 361	30.5	1062 ± 59	5.6
Diaphragm	3	370 ± 43	11.6	485 ± 198	40.8	550 ± 263	47.8
Loin	3	nd		401**		1223**	
Gluteal	6	526**		590**		249*	

SD = standard deviation

CV = coefficient of variation

nd = below limit of detection (135ng/g)

* mean of 2 values (others below limit of detection)

** single response only (others below limit of detection)

Table 4-5 Distribution of enrofloxacin plus ciprofloxacin within the musculature of 3 sheep

Muscle tissue samples	No. of subsamples	Drug concentration in muscle (ng/g)					
		Animal 1		Animal 2		Animal 3	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
Masseter	3	787 ± 143	18.2	329 ± 144	43.8	470 ± 23	4.9
Tongue	3	663 ± 88	13.3	236*		408 ± 36	8.8
Shoulder	3	564 ± 60	10.6	252 ± 218	86.5	263*	
Heart	3	702 ± 201	28.6	508 ± 67	13.2	476 ± 81	17.0
Diaphragm	3	571 ± 202	35.4	278 ± 178	64.0	187*	
Loin	3	593 ± 55	9.3	254*		301*	
Gluteal	6	626 ± 83	13.3	257 ± 7**	2.7	379 ± 25	6.6

SD = standard deviation

CV = coefficient of variation

* mean of 2 values only (others below limit of detection, 110ng/g)

** mean of 4 values only (others below limit of detection, 110ng/g)

Table 4-6 Distribution of enrofloxacin plus ciprofloxacin within the musculature of 4 pigs

Muscle tissue samples	No. of subsamples	Drug concentration in muscle (ng/g)							
		Animal 1		Animal 2		Animal 3		Animal 4	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
Masseter	6	2117 ± 731	34.5	1817 ± 576	31.7	1977 ± 956	48.4	1596 ± 569	35.7
Tongue	3	2248 ± 765	34.0	1841 ± 533	29.0	2199 ± 909	41.3	1641 ± 143	8.7
Shoulder	3	2335 ± 903	38.7	1781 ± 607	34.1	1938 ± 751	38.8	1534 ± 643	41.9
Heart	3	2367 ± 740	31.3	1769 ± 440	24.9	2128 ± 747	35.1	1623 ± 654	40.3
Diaphragm	3	2097 ± 647	30.8	1518 ± 558	36.8	2056 ± 848	41.2	1590 ± 703	44.2
Loin	3	2759 ± 1017	36.9	2354 ± 1032	43.8	2846 ± 1301	45.7	1990 ± 877	44.1
Gluteal	6	2401 ± 847	35.3	1769 ± 654	37.0	2328 ± 1016	43.6	1773 ± 717	40.4

SD = standard deviation

CV = coefficient of variation

4.3.1.3 Tiamulin

In respect of the cattle studied, tiamulin residues in muscle were below the limit of quantification in all animals at all times. In respect of mutton, compared to the overall inter-assay variation for tiamulin (CV = 8.4%), it is clear that most of the variation in the responses obtained were outside this range and reflected actual differences in the tissue residue distribution as is outlined in Table 4-7 below. The intra-site CV ranged from 10.5 – 65.7% although many samples had residue concentrations below the limit of detection. The inter-site CV was 47.0%, 72.3% and 93.1% for animals 1, 2 and 3 respectively. Variability between different muscle samples is as large or larger than it is within each sample.

In respect of pork, compared to the overall inter-assay variation for tiamulin (CV=11.4%), it is apparent that most of the variation in the responses obtained were outside this range and reflected actual differences in the tissue residue distribution as outlined in Table 4-8 below. The intra-site CV ranged from 8.8 – 100.6%. The inter-site CV was 42.7%, 71.2%, 50.8% and 38.7% for animals 1, 2, 3 and 4 respectively. Variability within muscle subsamples is as large or larger than it is within individual muscle samples.

Table 4-7 Distribution of tiamulin within the musculature of 3 sheep

Muscle tissue samples	No. of subsamples	Drug concentration in muscle (ng/g)					
		Animal 1		Animal 2		Animal 3	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
Masseter	3	1963*		943 ± 344	36.5	nd	
Tongue	3	1478**		1055 ± 202	19.1	633**	
Shoulder	3	1903*		1277 ± 375	29.4	457**	
Heart	3	1075 ± 557	51.8	1590 ± 168	10.5	926 ± 316	34.1
Diaphragm	3	752*		619 ± 407†	65.7	613**	
Loin	3	367*		nd		nd	
Gluteal	3	823*		nd ‡		nd	

SD = standard deviation
CV = coefficient of variation

nd = below limit of detection, 90ng/g
*one value only (others below limit of detection,)

** mean of 2 values only (others below limit of detection)
† = includes one sample below limit of quantification, taken as ½ LOQ
‡ includes one sample below limit of quantification, taken as ½ LOQ.

Table 4-8 Distribution of tiamulin within the musculature of 4 pigs

Muscle tissue samples	No. of subsamples	Drug concentration in muscle (ng/g)							
		Animal 1		Animal 2		Animal 3		Animal 4	
		Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV	Mean ± SD	%CV
Masseter	3	2926 ± 1605	54.9	811 ± 816	100.6	1490 ± 452	30.3	1938 ± 585	30.2
Tongue	3	2245 ± 515	22.9	790 ± 261	33.0	1734 ± 427	24.6	1775 ± 367	20.7
Shoulder	3	1730 ± 200	11.6	1250 ± 1070	85.6	1219 ± 640	52.5	907 ± 250	27.6
Heart	3	1821 ± 387	21.3	1446 ± 415	28.7	1882 ± 217	11.5	1997 ± 313	15.7
Diaphragm	6	1941 ± 470	24.2	1586 ± 673	42.4	1850 ± 485	26.2	1680 ± 382	22.7
Loin	3	647 ± 260	40.2	nd		nd**		479 ± 260†	54.3
Gluteal	6	784 ± 503	64.2	nd*		705 ± 194	27.5	1059 ± 93	8.8

SD = standard deviation

CV = coefficient of variation

nd = not detected (less than 130ng/g)

* includes 1 subsample with concentration below the limit of quantification, 425ng/g

** includes 1 subsample with concentration of 927ng/g

† one sample had a residue concentration below the LOQ, taken as 210ng/g for calculation purposes.

4.3.2 Observed distribution of test drugs in musculature of cattle, sheep and pigs.

While it is appreciated that there is little scientific rationale for comparing the distribution of residue of different drugs in each of the target species, (each species was not slaughtered at the same interval post treatment and some received the drugs by a different route of administration) it is interesting to group the results to establish whether there are any trends evident. In respect of tilmicosin, there appears to be some species differences in the residue concentrations recorded at different sites. In cattle, residues were non-quantifiable in heart muscle while gluteal muscle had the highest concentrations found; in sheep all tissues had residue concentrations in the same range, apart from diaphragm which had residue concentrations approximately twice as high as the other muscles; in pigs, residues were non-quantifiable in diaphragm and loin but were most concentrated in the cheek and tongue. These data are outlined in Table 4-9 hereunder.

Table 4-9 Overall species mean concentrations of residues of tilmicosin in different muscles of 3 cattle, 3 sheep and 4 pigs

Muscle location	Species mean residue concentration (ng/g)		
	Cattle	Sheep	Pigs
Masseter	489	361	1933
Tongue	387	319	1059
Shoulder	423	325	258
Heart	nq†	254*	446
Diaphragm	416	651	nq**
Loin	416	337*	nq‡
Gluteal	685	404	287

nq = not quantifiable

† includes 2 subsample values below the limit of quantification

* includes 3 subsample values below the limit of quantification, taken for calculation purposes as ½ the LOQ

**includes 4 subsamples with residue concentrations in the range 220 – 300ng/g

‡ includes 3 subsamples with residue concentrations in the range 250 – 390ng/g

In respect of enrofloxacin, the mean levels of parent compound plus the metabolite ciprofloxacin are quite evenly distributed in sheep and pigs, with all tissues having a similar residue concentration. In respect of cattle, the residue concentration of heart muscle was more than twice that of tongue and masseter muscle, as outlined in Table 4-10 hereunder.

Table 4-10 Overall species mean concentrations of residues of enrofloxacin in different muscles of 3 cattle, 3 sheep and 4 pigs

Muscle location	Species mean residue concentration (ng/g)		
	Cattle	Sheep	Pigs
Masseter	452	529	1877
Tongue	527	436†	1982
Shoulder	310*	360	1897
Heart	1163	562	1972
Diaphragm	468	345†	1815
Loin	nq	383†	2488
Gluteal	nq	421	2068

nq = not quantifiable, residue concentrations below limit of quantitification or detection

* mean of 2 animals only; third animal had residues below limit of detection

† includes data from animals where quantifiable residues were available for only 2/3 of subsamples.

In respect of tiamulin, there are too few data points to give a reliable estimate of species differences. Nonetheless, it appears from Table 4-11 hereunder that in pigs and sheep, residue levels in the loin and gluteal regions were lower than for other muscle tissues.

Table 4-11 Overall species mean concentrations of residues of tiamulin in different muscles of 3 cattle, 3 sheep and 4 pigs

Muscle location	Species mean residue concentration (ng/g)		
	Cattle	Sheep	Pigs
Masseter	nq	958‡	1791
Tongue	nq	1055	1636
Shoulder	nq	1195	1277
Heart	nq	1197	1787
Diaphragm	nq	661	1764
Loin	nq	nq	282*
Gluteal	nq	nq	637†

nq = not quantifiable

‡ includes value from 1 animal with residues below the limit of detection, taken as 0ng/g

* includes values from 2/4 animals below the limit of detection, taken as 0ng/g

† includes one animal with residue concentrations below the limit of detection

4.4 Discussion

There are several possible sources of variation in the results obtained in the distribution studies, including inherent analytical variability, tissue homogeneity variability, distributional variability.

The magnitude of the variation in muscle concentrations of drug residues has been shown to be outside that associated with the conduct of the analysis as evidenced by the relatively higher CV values for the inter-site and intra-site analysis compared to the CVs from the precision and reproducibility of the assay. The data provided are quite comprehensive in so far as they included 3 drugs and involved repeated analysis (including duplicate response measurement and subsampling of individual muscle samples) on several muscles in 3 different species. It is apparent from the data that the drug residues were not homogenously distributed either within or between muscles. These results compare to similar findings on residue concentrations in the published literature including studies on veterinary drug residues in animal muscle by

Rose *et al* (1995b, 1996b and 1996c), Takahashi *et al* (1989), Ekström and Slanina (1982), Jansen *et al* (1989) and in respect of drugs found in human cadavers and reported by Williams and Pounder (1997), Pounder and Jones (1990) and Langford *et al* (1998).

While the data generated indicate a high degree in the concentration of residues present within and between individual muscles, it must be noted that the animals were slaughtered at a time when quantifiable residues were present. At later time points residue limits will have declined further and the variation between samples is expected to change over time. It is not possible to conclude on the reasons for the differences in distribution of residues seen; all drugs had a relatively high volume of distribution. Moreover, all the test drugs are known to be extensively metabolised [Donoho *et al* (1991), Kaartinen *et al* (1997) and Ziv *et al* (1983)] but only the parent compound or marker metabolites were quantified as is usual in respect of residue studies. Nevertheless, it appears that certain tissues e.g. masseter, tongue, heart and diaphragm are more likely to have higher residues than shoulder, loin and gluteal muscle, although the near absence of tilmicosin residues in heart muscle is the exception to this. The theory expounded by Langford *et al* (1998) and Klöppel *et al* (1980) that higher concentrations of drugs in the diaphragm was likely to be the result of greater blood flow and metabolic activity in the active respiratory muscles compared to other skeletal muscles is a possible explanation for the overall trends in residue concentrations found. However, for the same reasons it might be expected that these tissues would be among the first to eliminate extracellular and intracellular tissue residues.

In relation to the comparison of distribution of residue concentrations between the musculature of each species, it would be premature to reach any conclusions on the basis of the results presented. Baggot (1978) reported that superimposed on the numerous factors influencing the half-life of a drug in an individual animal are intra- and interspecies variations. He also reported that significant differences in the half-lives of several drugs, in particular those extensively metabolised, exist among species of domestic animals. While all 3 test drugs are known to be extensively metabolised, the study design did not facilitate an interspecies comparison of

pharmacokinetics and residue distribution. The study design did not require all species to be slaughtered after the same time period post treatment; in respect of tilmicosin cattle and sheep received subcutaneous injections of 10mg/kg bodyweight while pigs received injections at a dosage of 8mg/kg bodyweight by the intramuscular route. Moreover, the huge differences found between concentrations of residues both within the same muscle and between different muscles render comparison impossible.

The results of these studies have important implications for residue surveillance and residue depletion studies used for establishing withdrawal periods for veterinary medicines including the following:

a) surveillance of drug residues should not, on the basis of the findings reported, be targeted at a specific muscle,
b) applicant companies conducting residue studies for regulatory purposes should harvest muscle from several locations and homogenise them prior to analysis. The locations chosen should be identified in the trial protocol and test report,
c) guidance notes for the conduct and interpretation of residue studies should be amended to make reference to the need for sampling in several muscle locations and homogenisation of samples,
d) in the case of a possible dispute over the results of a residue analysis, the sample should be homogenised prior to analysis. The variability shown by results of individual muscle analysis make it highly unlikely that split sampling of incurred muscle tissue, such as occurs where blood or urine sampling for drugs are taken from the live animal, will yield the same result even if the analysis is conducted under identical conditions and at the same time. This finding could pose legal problems for farmers and analytical laboratories where the result of the tissue assay is challenged.

CHAPTER 5: HEAT STABILITY OF TILMICOSIN, ENROFLOXACIN AND TIAMULIN

5.1 Introduction

The objective of this segment of the thesis was to establish whether heating or cooking had any effect on the concentrations of incurred residues of tilmicosin, enrofloxacin and tiamulin in muscle. Since residue studies are conducted on raw tissue whilst most meat is consumed after cooking, information on the effect of cooking is important in order to improve the understanding of the potential hazard of tissue residues and provide a more accurate estimate of consumer exposure to these drugs. In this chapter, both the effects of heating and cooking were studied in order to investigate both the effect of heat on the drug itself as well as the matrix effect on the marker residue. While cooking processes vary widely, assessing the effect of heat on the drug substance itself (*in vitro* heat stability studies) allows for better reproducibility in the conduct of the experiments. Heat stability studies also facilitate direct comparisons between the test drugs as well as with published data on other veterinary drugs.

5.2 Materials and methods

Heat stability studies in aqueous and in vegetable oil solutions of each of the test drugs were conducted as well as cooking studies of incurred residues in muscle under both wet and dry heating conditions.

5.2.1 In vitro heat stability studies

The test drugs were subjected to heating in aqueous solutions of pH 5.5 (being the approximate pH of muscle) and pH 7.0 (being the neutral pH point) at a temperature of 98^0C and in vegetable oil at a temperature of 160^0C for a period of 90 minutes. These conditions were the same as those followed by Rose and his co-workers in their several experiments on various drugs (Rose *et al* 1995a, 1995b, 1995c, 1996b, 1997a, 1997b, 1997c, 1998 and 1999). The experiments were repeated on 3 or 4 occasions in order to give increased reliability to the data generated.

5.2.1.1 Studies on test drugs in aqueous solutions

A 1mg/ml stock solution of each of the test compounds was made and working solutions prepared in various solutions according to Table 5-1 below.

The concentrations of test drugs in the working solutions were chosen to allow for accurate measurement of the concentrations over the validated test range. Working solutions (1.5ml) of the test drugs were taken and placed in 2ml HPLC glass vials. The vials were corked with screw caps and placed in a plastic holder prior to transfer to a water bath that had been preheated to 100^0C. This bath was thermostatically controlled and temperature was maintained within the range 97 - 100^0C. Whole vials were taken from the water bath at the following times for analysis of drug concentration: 0, 5, 10, 20, 40, 60 and 90 minutes. Following removal of sample vials from the water bath, they were immersed in crushed ice. Particular care was taken to shield the vials from light by covering the collection rack with aluminium foil. In the case of experiments in which methanol was part of the heated solution, the samples were weighed before and after heating and if necessary they were refilled to the original weight to compensate for losses caused by evaporation.

Table 5-1 Preparation of working solutions of test compounds for aqueous heating experiments

Test compound	Stock solution made up in	Working solution made up in	pH of working solution
Tilmicosin	Methanol	Methanol	Not applicable
		0.05M phosphate buffer	5.5
		0.05M phosphate buffer	7.0
Enrofloxacin	Methanol	Methanol/ 0.1 M.HCl	Not applicable
		0.05M phosphate buffer	5.5
		0.05M phosphate buffer	7.0
Ciprofloxacin	Methanol/ 0.1 M.HCl	Methanol/ 0.1 M.HCl	Not applicable
	(90:10)	0.05M phosphate buffer	5.5
		0.05M phosphate buffer	7.0
Tiamulin	Methanol/H_2O (50:50)	50:50 Methanol/H_2O	Not applicable
		0.05M phosphate buffer	5.5
		0.05M phosphate buffer	7.0

M = Molar

HCl = Hydrochloric acid

H_2O = Water

5.2.1.2 Studies on test drugs in vegetable oil solutions

The heat stability of tilmicosin, enrofloxacin and ciprofloxacin in cooking oil was evaluated by making up 10mg/ml stock solutions as for the stock solutions prepared for the aqueous experiments. From these stock solutions, authentic standards were run on the HPLC over the concentration range previously established and validated for the test compounds. All the studies followed the same protocol described hereunder. Domestic vegetable oil (Kelkin Pure Sunflower Oil) was obtained and approximately 80ml was poured into a 150ml Pyrex beaker. A volume of 10ml of the oil was taken in a 20ml glass septum vial. A volume of 250µl of tilmicosin stock solution or 200µl/ml stock solutions in the case of enrofloxacin and ciprofloxacin were added to the oil in the septum vial. Each the test drug, including ciprofloxacin, was investigated separately. After adding the drug-containing stock to the oil in the septum vial, the solution was vortexed for 2 minutes. The open-topped septum vial containing the oil-fortified drug was placed in the beaker of oil that was subsequently heated to 160^0C on a hotplate. The aqueous solution boiled off within minutes as the temperature increased to the target, after which the first sample (time zero) was taken from the septum vial and the stop clock started.

The heat stability of tiamulin was evaluated by making up a 1mg/ml stock solution in methanol:water 50:50. Authentic standards were run over the validated concentration range 0.05 - 3.0µg/ml. A volume of 0.25ml of the stock solution was taken and made up to 10ml with the vegetable oil. The emulsion was vortexed prior to heating to a temperature of 160^0C in an open glass septum vial, which was placed in vegetable oil contained within a Pyrex beaker and heated over a hot-plate.

The temperature of the oil in the beaker was monitored with a thermometer throughout the experiments and was not allowed to fall below 140^0C or rise above 180^0C. Samples (100µl) of the heated oil from the septum vial were taken by pipette and placed in pre-weighed glass 10ml volumetric flasks at the following times: 0, 5, 10, 20, 40, 60 and 90 minutes. These volumetrics were again weighed following the taking of the sample to establish the exact weight of the sample taken. The sample

was made up to the 10ml mark using methanol in the case of tilmicosin, methanolic acid in the case of enrofloxacin and ciprofloxacin and methanol:water 50:50 in the case of tiamulin. The sample solutions were shaken vigorously for approximately 30 seconds. For the analysis of tilmicosin, enrofloxacin and ciprofloxacin the solutions were then taken into 2ml plastic millifuge tubes and centrifuged at 10000g for 15 seconds after which time the 1.5ml samples were taken into 2ml glass HPLC vial inserts and analysed by the HPLC methods previously described. For the analysis of tiamulin, 150µl samples were placed in the agar wells for bioassay.

5.2.2 Incurred tissue studies

Incurred residues of tilmicosin, enrofloxacin and tiamulin were obtained from cattle, sheep and pigs. The animals of each species were divided into 3 treatment groups comprising 3 or 4 animals per group. Each group received one of the drugs with the dose divided and administered in both sides of the neck of each animal. Full details of the administration of the test drugs are given in Chapter 2.

Only injection-sites were subjected to the cooking studies, as residues in other muscles were known not to be homogenously distributed (Chapter 4) and, in any event, might not yield sufficient residue concentrations following cooking to allow for accurate quantification. Due to the possible non-homogenous distribution of xenobiotics in muscle, all samples were thoroughly homogenised prior to cooking. This was achieved by thinly slicing approximately 40g samples with a scalpel, followed by mincing using a hand-held domestic meat mincer. Each injection-site was minced twice with the blender being washed and rinsed in methanol between samples. The minced samples were then collected into pre-marked 100ml plastic Starstedt containers and further mixed by hand using a spatula. The containers were identified using the animals' identification number and injection-site location (left or right neck).

Two cooking methods were used: a) microwaving to simulate the effects of dry heat and b) boiling to simulate the effects of moist heat. Minced meat samples containing one of the 3 test compounds were sub-sampled. From each sample, 2 – 3 sub-samples were taken for each cooking procedure. Control samples from an untreated pig or, in

the case of mutton and beef from a local butcher shop were also minced and handled in the same way. Raw (uncooked) sub-samples from each injection-site or control tissue were analysed in the manner previously described in Chapter 2. One 5g sub-sample from each injection-site was transferred into a 100ml Starstedt container for microwaving, with a second 5g sub-sample placed into a pouch, which was made from nylon netting, for boiling. The pouches were closed by means of strings prior to boiling in preheated 1litre Pyrex flasks containing approximately 400ml boiling deionised water that were maintained at 100^0C for 7 minutes on a hotplate. The lids of the Starstedt containers used in the microwave studies were loosened prior to cooking for 14 seconds at 3/5 power (390W). All samples were weighed before and after the cooking procedure in order to adjust the concentration of residue found for weight loss in the muscle. Following cooking, the samples were allowed to cool for 30 minutes. Sub-samples of an appropriate weight (as described in Chapter 2) were taken by means of a scalpel into pre-marked 25ml graduated plastic Sterilins for analysis.

5.2.3 Analyses

Analysis of tilmicosin and enrofloxacin was by HPLC, while tiamulin was analysed using a bioassay. Clean-up and analysis of samples followed the standard protocols described in Chapter 2. Fortified control samples were always run alongside test samples and the predicted concentrations were calculated from these standard curves. All samples for chemical analysis were run in duplicate and the mean response was used for determining the predicted value while the samples taken for the bioassay were run in triplicate with the mean result used for the calculations. Each experiment was run at least 3 times (some were run on 4 occasions) and the trendline used in the graphics to report the results of the heating studies was constructed using the mean values. Concerning the cooking studies, the results were corrected for loss of tissue weight due to heating. Statistical calculations were conducted using the *t* test to compare the residue concentrations in the raw tissue with those following each cooking procedure.

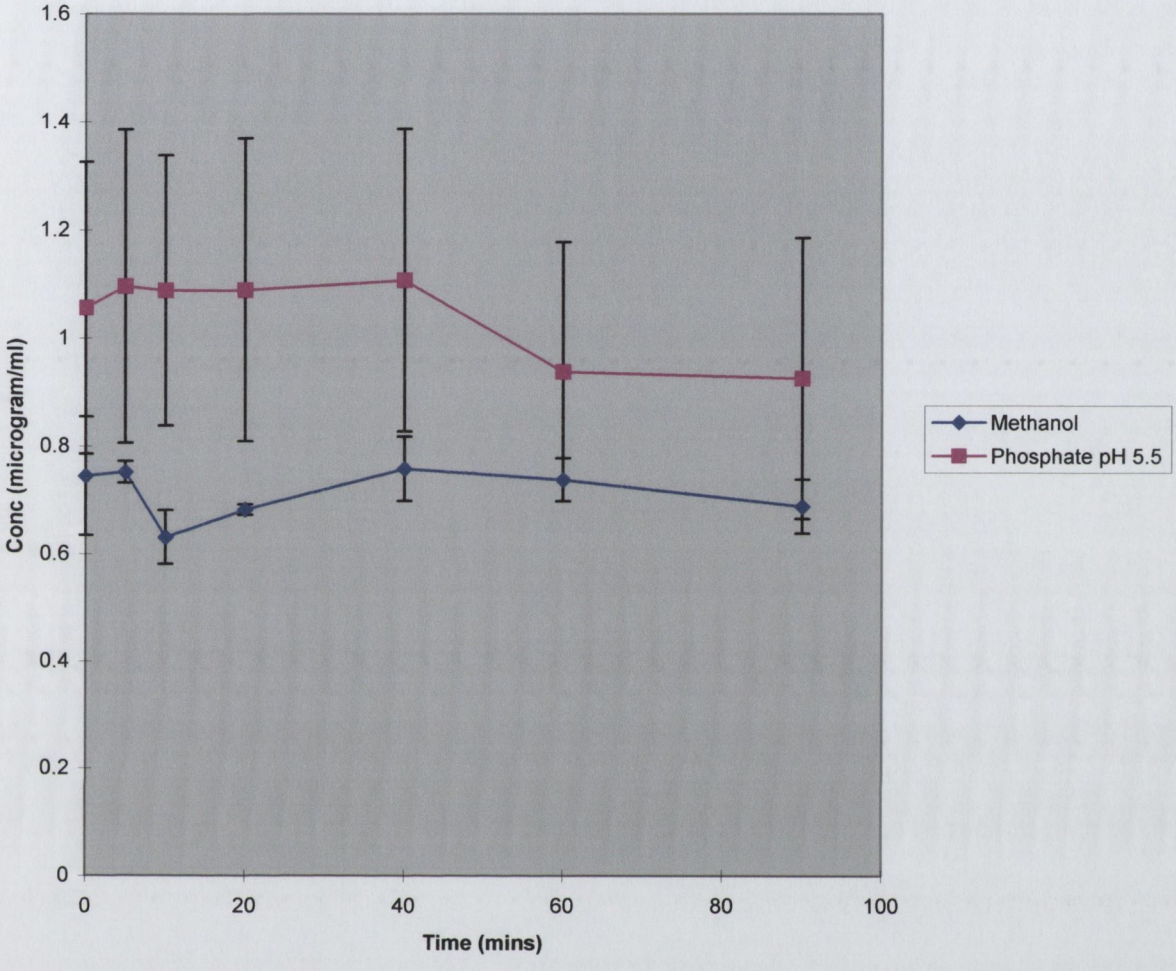
5.3 Results

5.3.1 Results of *in vitro* studies in aqueous solutions

5.3.1.1 Tilmicosin

The effect of heat on the *in vitro* stability of tilmicosin in both aqueous solutions is plotted in Figure 5-1 hereunder.

Figure 5-1 Stability of tilmicosin in methanol and phosphate buffer at 100°C



n = 3,

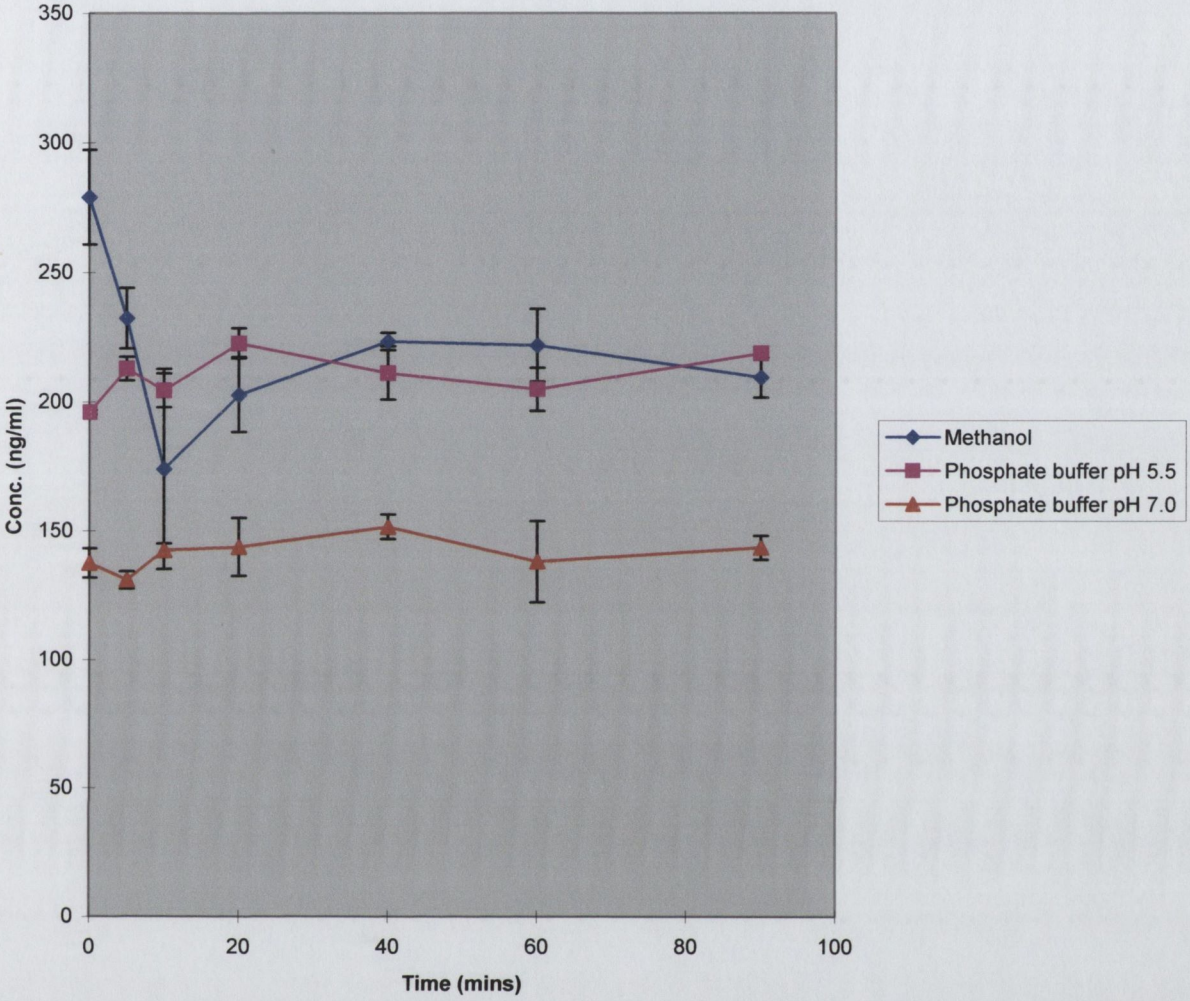
Error bars demonstrate values of the standard error of the mean.

Tilmicosin was not soluble in phosphate buffer at pH 7.0 with no drug being detected on any of the 3 experiments conducted. It was concluded that tilmicosin precipitates at this pH in the phosphate buffer. Tilmicosin was stable when heated in either methanol or buffer pH 5.5 for 90 minutes being approximately 90% of initial concentrations at the end of the heating period.

5.3.1.2 Enrofloxacin

The effect of heat on the *in vitro* stability of enrofloxacin and ciprofloxacin in aqueous solutions is plotted in Figure 5-2 hereunder.

Figure 5-2 Stability of enrofloxacin in methanol and phosphate buffer at 100°C

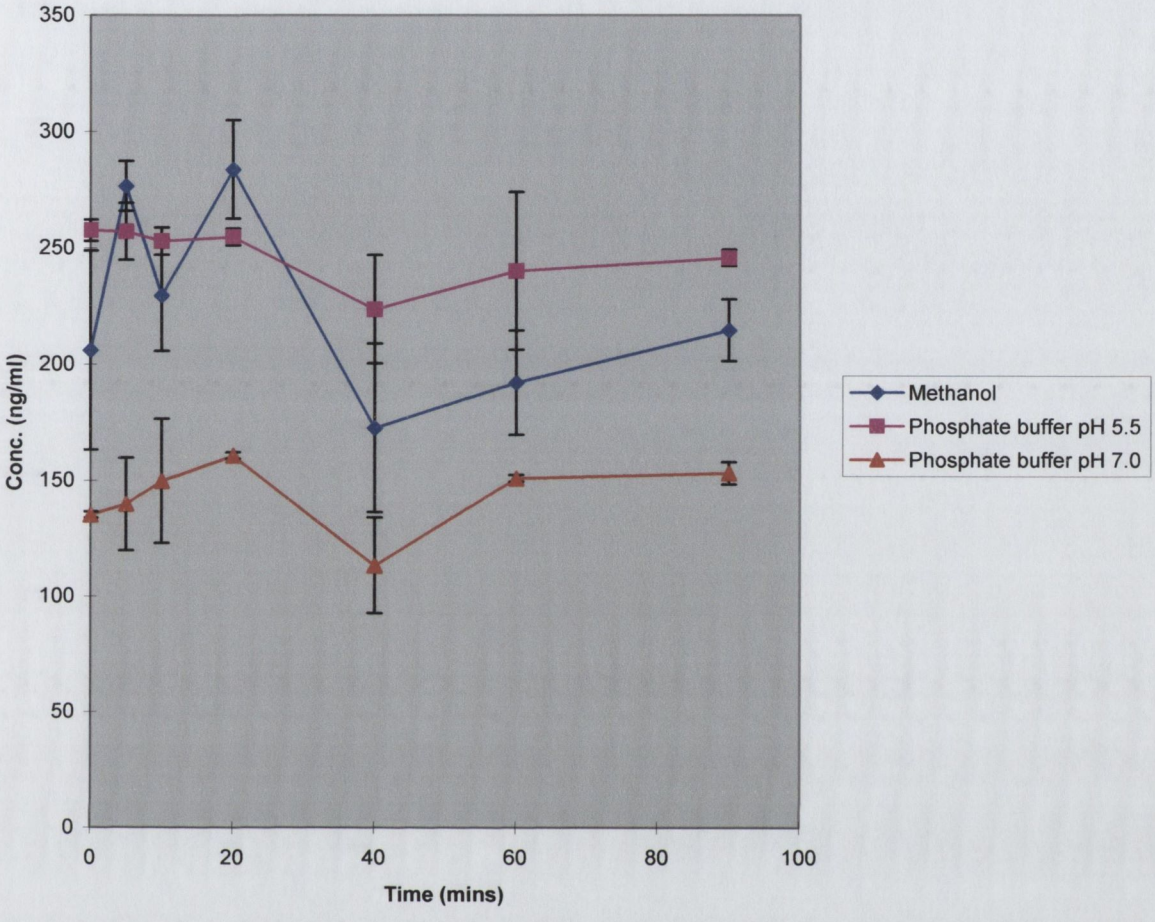


n = 3

Error bars demonstrate values of the standard error of the mean.

Enrofloxacin was stable when heated in phosphate buffer at pH 5.5 and 7.0. In methanol, there was a 25% loss in initial concentration of enrofloxacin over the 90-minute test period. Ciprofloxacin was also stable when heated in methanol or phosphate buffer at pH 5.5 and 7.0 as demonstrated in Figure 5-3 hereunder.

Figure 5-3 Stability of ciprofloxacin in methanol and phosphate buffer at 100°C



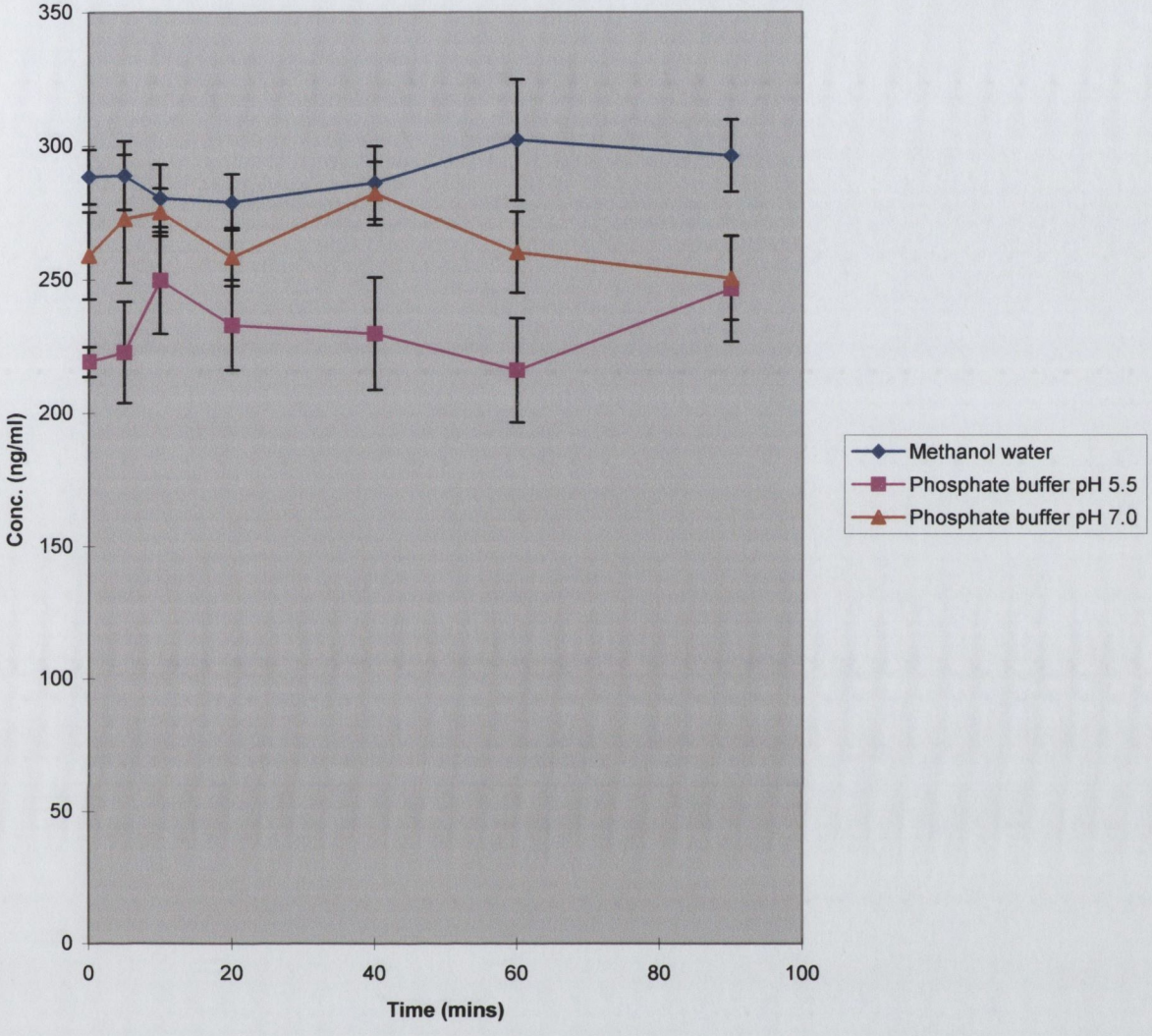
n = 3,

Error bars demonstrate values of the standard error of the mean.

5.3.1.3 Tiamulin

The effect of heat on the *in vitro* stability of tiamulin in aqueous solutions is plotted in Figure 5-4. Tiamulin was stable when heated in methanol water or phosphate buffer at pH 5.5 and 7.0, being 100% of initial concentrations at the end of the heating period.

Figure 5-4 Heat stability of tiamulin in methanol water and phosphate buffer at 100°C



n = 4,

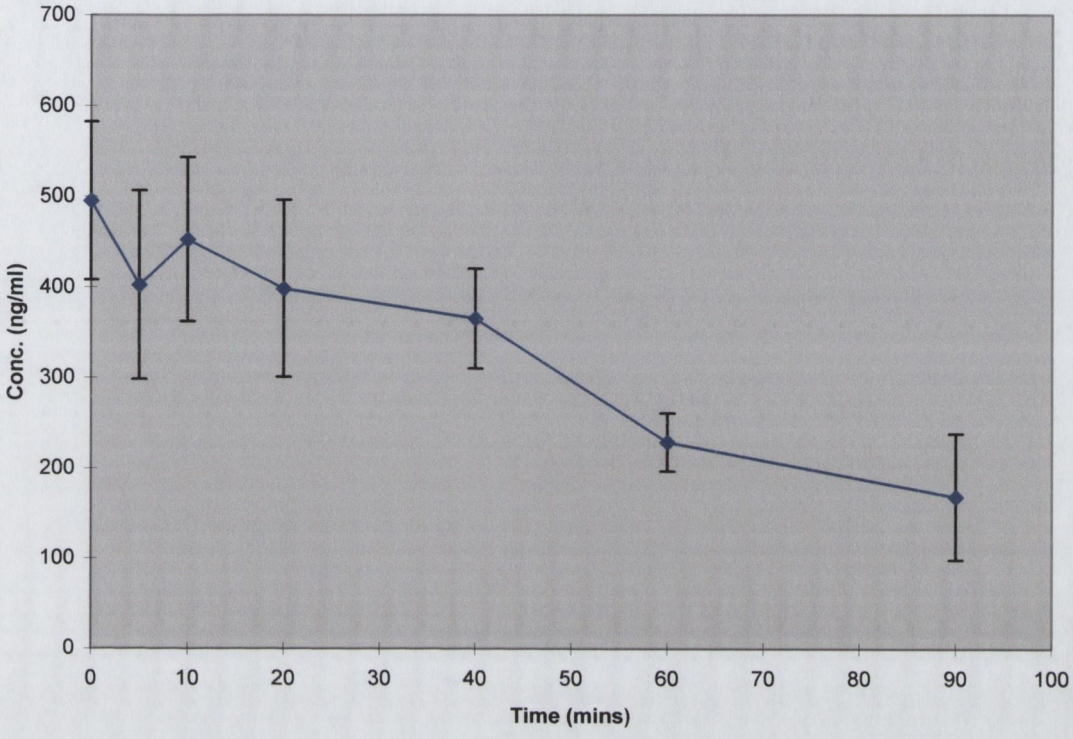
Error bars demonstrate values of the standard error of the mean.

5.3.2 Results of *in vitro* studies in vegetable oil solutions

5.3.2.1 Tilmicosin

There was a clear reduction in the concentration of tilmicosin over the 90 minute period in vegetable oil heated to 160°C, with concentrations being only 34 % of their initial values at the end of the period as can be seen from Figure 5-5 hereunder.

Figure 5-5 Heat stability of tilmicosin in hot vegetable oil at 160°C



n = 4,

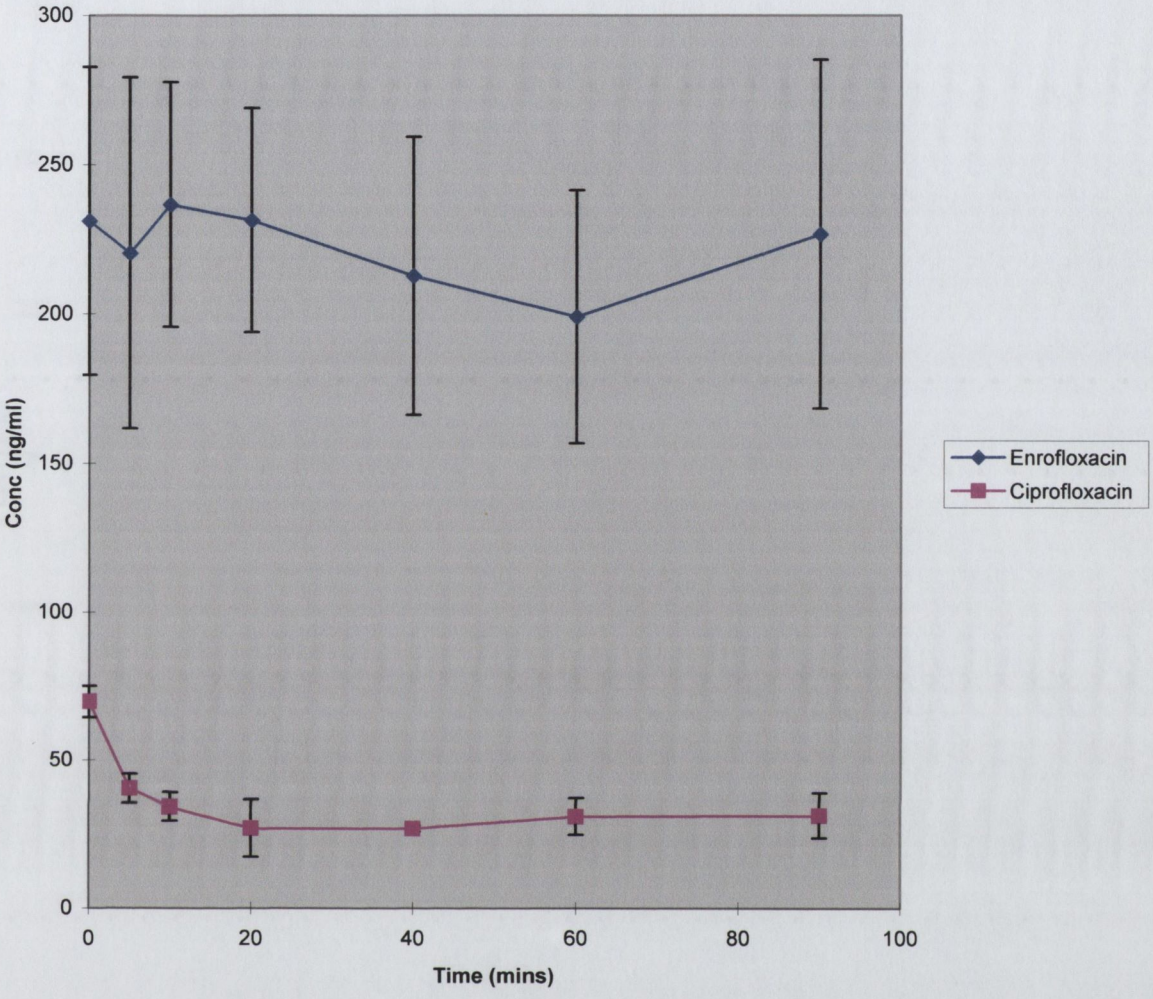
Error bars demonstrate values of the standard error of the mean.

A large unidentified peak was observed in the chromatogram at a retention time of 3.56 minutes, beginning within 5 minutes of starting the experiment and building in a linear manner throughout the 90 minutes. On investigation, this peak was also found in unfortified oil heated for the same period and was not therefore a breakdown product of tilmicosin.

5.3.2.2 Enrofloxacin

The effect of heat on the *in vitro* stability of enrofloxacin and ciprofloxacin in vegetable oil solution at 160°C is plotted in Figure 5-6 hereunder.

Figure 5-6 Heat stability of enrofloxacin and ciprofloxacin in hot vegetable oil at 160°C



n = 3 for both enrofloxacin and ciprofloxacin.

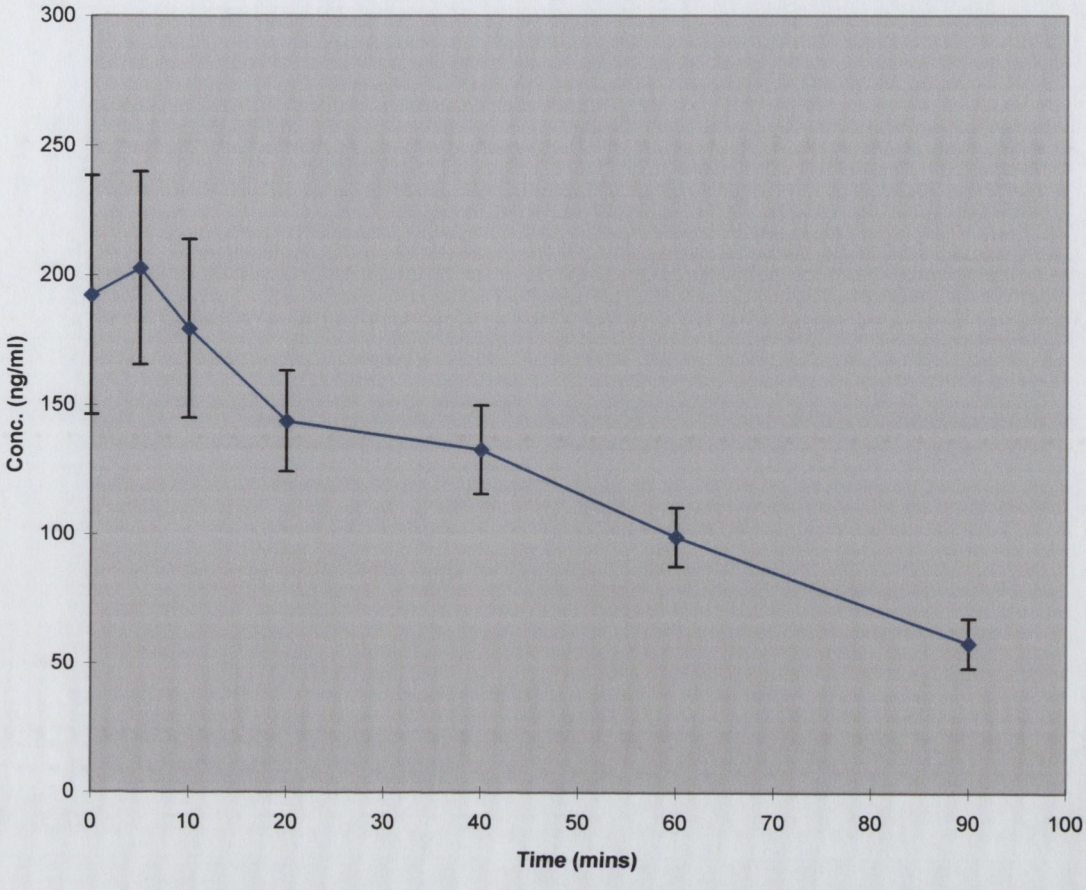
Error bars demonstrate values of the standard error of the mean.

Enrofloxacin was stable in hot vegetable oil over 90 minutes while ciprofloxacin concentrations declined by 50% within 10 minutes but remained stable thereafter.

5.3.2.3 Tiamulin

A plot of tiamulin concentration against time in hot oil at 160°C is shown in Figure 5-7 hereunder. Tiamulin concentrations reduced significantly within 20 minutes of the experiment commencing, being only 30% of the initial concentration at the end of the 90 minutes heating period.

Figure 5-7 Heat stability of tiamulin in hot vegetable oil at 160°C



n = 3,

Error bars demonstrate values of the standard error of the mean.

5.3.3 Results of incurred residue studies

In none of the studies conducted did control samples have residues of any test substance or marker residue in any of the raw or heat-treated samples. Data in respect of individual test substances are presented hereunder.

5.3.3.1 Tilmicosin

The overall effect of microwaving beef at 60% power for 12 seconds was a notional increase in the initial concentration of approximately 2%, while the overall effect of boiling beef for 7 minutes was a reduction of approximately 44% as shown in Table 5-2 hereunder.

Table 5-2 The mean concentration of tilmicosin residues in beef when exposed to different cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (μg/g), n = 3 Animal number								
	1			2			3		
	Mean	SEM	% of initial value	Mean	SEM	% of initial value	Mean	SEM	% of initial value
Raw (uncooked)	18.46	0.75	100	10.11	0.96	100	10.19	0.57	100
Microwaved	17.33	0.56	93	10.37	0.74	103	11.73	0.39	115
Boiled	10.55	0.30	57	5.72	0.39	57	5.42	0.68	53

SEM = standard error of the mean

The low values for the standard errors in respect of each animal sample (raw, microwaved or boiled) show that the subsamples of each tissue sample were homogenous for residue concentrations of tilmicosin. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2 and 3 was 0.092, 0.639 and 0.234 respectively, while those for boiled residues were 0.006, 0.023 and 0.010 respectively. Thus, the effect of microwaving was not significant, while boiling had a significant effect on residues of tilmicosin.

As can be seen from Table 5-3 below, the overall effect of microwaving mutton at 60% power for 12 seconds was a notional increase in the initial concentration of approximately 9%, while the overall effect of boiling mutton for 7 minutes was a reduction of approximately 12%. The data show that the samples taken for analysis were homogenous as evidenced by the low standard error values observed. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2 and 3 was 0.059, 0.003 and 0.071 respectively, while those for boiled residues were 0.006, 0.011 and 0.104 respectively. Thus, the effect of microwaving was not significant overall, while boiling has a significant effect on residues of tilmicosin in animals 1 and 2 but not in animal 3.

In relation to pork, the overall effect of microwaving pork at 60% power for 12 seconds was a notional increase in the initial concentration of approximately 9%, while the overall effect of boiling pork for 7 minutes was a reduction of approximately 20% as outlined in Table 5-4 below. The data show that the samples taken for analysis were homogenous as evidenced by the low standard error values observed. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2, 3 and 4 was 0.08, 0.32, 0.17 and 0.19 respectively, while those for boiled residues were 0.06, 0.018, 0.04 and 0.0008 respectively. Thus, the effect of microwaving was not significant overall, while boiling has a significant effect on residues of tilmicosin in animals 2, 3 and 4 but not in animal 1.

Table 5-3 The mean concentration of tilmicosin residues in mutton when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (µg/g) n = 3 Animal number								
	1			2			3		
	Mean	SEM	% of initial value	Mean	SEM	% of initial value	Mean	SEM	% of initial value
Raw (uncooked)	2.10	0.04	100	2.48	0.01	100	1.62	0.08	100
Microwaved	2.29	0.04	105	2.76	0.01	112	1.76	0.09	108
Boiled	1.83	0.02	87	2.18	0.04	88	1.41	0.01	87

SEM = standard error of the mean

Table 5-4 The mean concentration of tilmicosin residues in pork when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (μg/g) n = 3 Animal number											
	1			2			3			4		
	Mean	SEM	% of initial value	Mean	SEM	% of initial value	Mean	SEM	% of initial value	Mean	SEM	% of initial value
Raw (uncooked)	0.75	0.68	100	2.30	0.02	100	2.32	0.08	100	1.61	0.05	100
Microwaved	0.92	0.01	127	2.52	0.16	110	2.50	0.09	108	1.62	0.04	101
Boiled	0.58	0.02	79	1.77	0.06	77	1.95	0.08	84	1.26	0.05	78

SEM = standard error of the mean

Thus the effects of cooking were similar in respect of all 3 species, being a nominal increase of initial concentration (2 - 9%) on microwaving and a pronounced reduction (and in most cases, this was significant) in respect of boiling (12 - 44%). Nevertheless, as the results have been corrected for weight loss, from a consumer point of view the residues have not been reduced overall, and in the case of microwaved tissues the residue concentrations on a per gram of tissue basis have actually increased by up to 45%.

5.3.3.2 Enrofloxacin

In respect of beef, all the residues present in raw and cooked tissue were ciprofloxacin. The overall effect of microwaving beef at 60% power for 12 seconds was a nominal reduction in the initial concentration of approximately 3%, while the overall effect of boiling beef for 7 minutes was a reduction of approximately 27% as can be seen from Table 5-5 hereunder. The low values for the standard errors in respect of each animal sample (raw, microwaved or boiled) show that the subsamples of each tissue sample were homogenous for residue concentrations of enrofloxacin. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2 and 3 was 0.843, 0.869 and 0.164 respectively, while those for boiled residues were 0.706, 0.103 and 0.054 respectively. Thus, the effect of microwaving and boiling did not have a significant effect overall on residues of enrofloxacin, albeit that the power of the statistical test was low. If no correction for weight loss during cooking is made, residues of ciprofloxacin actually increased over initial concentrations by 18% and 22% for microwaving and boiling respectively, due to moisture loss from the sample, although these increases were not statistically significant.

By contrast to beef, the residues seen in mutton were present as enrofloxacin only. As can be seen from Table 5-6 below, the overall effect of microwaving mutton at 60% power for 12 seconds was a reduction in the initial concentration of approximately 8%, while the overall effect of boiling mutton for 7 minutes was a reduction of approximately 46%. The low values for the standard errors in respect of each animal sample (raw, microwaved or boiled) show that the subsamples of each tissue sample were homogenous for residue concentrations. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2 and 3 was 0.362, 0.792 and 0.073

respectively, while those for boiled residues were 0.084, 0.084 and 0.092 respectively. While these results are not statistically significant, the statistical power of the study was low. If no correction for weight loss during cooking is made, residues of enrofloxacin showed a slight increase over initial concentrations on microwaving and no overall change when boiled, although these increases were not statistically significant.

Table 5-5 The mean concentration of ciprofloxacin residues in beef when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (ng/g) n = 2 Animal number 1			2			3		
	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value
Raw (uncooked)	696.94	64.89	100	556.81	37.89	100	305.28	20.56	100
Microwaved	721.36	72.35	104	542.68	37.89	97	248.27	41.87	81
Boiled	626.29	136.20	90	350.11	10.00	63	163.59	37.71	54

SD = standard deviation

Table 5-6 The mean concentration of enrofloxacin residues in mutton when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (µg/g) n = 2 Animal number								
	1			2			3		
	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value
Raw (uncooked)	0.95	0.06	100	1.02	0.10	100	1.30	0.10	100
Microwaved	0.88	0.003	93	1.01	0.15	99	1.14	0.08	87
Boiled	0.58	0.01	61	0.51	0.0003	50	0.67	0.03	52

SD = standard deviation

In respect of pork, enrofloxacin residues were present primarily as parent compound (in the μg/g concentration range), while ciprofloxacin was also present (in the ng/g concentration range). As can be seen from Table 5-7 below, the overall effect of microwaving pork at 60% power for 12 seconds was no reduction in the initial concentration of enrofloxacin, while the overall effect of boiling pork for 7 minutes was a reduction of approximately 36%. The low values for the standard errors in respect of each animal sample (raw, microwaved or boiled) show that the subsamples of each tissue sample were homogenous for residue concentrations. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2, 3 and 4 was 0.359, 0.742, 0.228 and 0.025 respectively, while those for boiled residues were 0.051, 0.029, 0.017 and 0.049 respectively. Thus, the effect of microwaving did not have a significant effect on residues of enrofloxacin while the reduction in residue concentration following boiling was statistically significant. If no correction for weight loss during cooking is taken, residues of ciprofloxacin actually increased over initial concentrations, although these increases were not statistically significant.

Interestingly, as can be seen from Table 5-8 below, cooking of tissues containing enrofloxacin appears not to lead to the production of ciprofloxacin as there was no increase in the concentrations of ciprofloxacin found. The overall effect of microwaving pork at 60% power for 12 seconds was a reduction in the initial concentration of ciprofloxacin of approximately 12%, while the overall effect of boiling pork for 7 minutes was a reduction of approximately 42%. The low values for the standard errors in respect of each animal sample (raw, microwaved or boiled) show that the subsamples of each tissue sample were homogenous for residue concentrations. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2, 3 and 4 was 0.336, 0.604, 0.087 and 0.446 respectively, while those for boiled residues were 0.227, 0.009, 0.069 and 0.175 respectively. The effect of microwaving and boiling did not have a significant effect overall on residues of ciprofloxacin, albeit that the power of the study was low and boiling tended towards significance.

Table 5-7 The mean concentration of enrofloxacin residues in pork when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (μg/g) n = 2 Animal number											
	1			2			3			4		
	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value
Raw (uncooked)	5.38	0.17	100	1.76	0.02	100	2.37	0.01	100	4.84	0.39	100
Microwaved	5.83	0.23	108	1.66	0.28	95	2.48	0.05	105	4.28	0.43	88
Boiled	3.97	0.01	74	1.06	0.16	60	1.28	0.03	54	2.86	0.18	59

SD = standard deviation

Table 5-8 The mean concentration of ciprofloxacin residues in pork when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (ng/g) n = 2 Animal number											
	1			2			3			4		
	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value
Raw (uncooked)	232.09	40.71	100	185.90	11.24	100	207.66	12.11	100	180.41	16.05	100
Microwaved	212.09	27.21	91	176.71	33.35	95	183.74	7.47	88	133.26	40.18	74
Boiled	142.78	5.50	62	99.95	9.28	54	98.98	29.03	48	122.34	39.19	68

SD = standard deviation

Thus the effects of cooking were similar in respect of all species, being a slight reduction in the initial concentration (3 - 12%) on microwaving and a greater reduction in respect of boiling (23 - 46%) when corrected for weight loss. A variable effect was seen if no allowance is taken for moisture loss in the cooked sample, ranging from a reduction of 26% to a 22% relative increase in concentration.

5.3.3.3 Tiamulin

The overall effect of microwaving beef at 60% power for 12 seconds was a reduction in the initial concentration of tiamulin of approximately 17%, while the overall effect of boiling beef for 7 minutes was a reduction of approximately 25% as shown in Table 5-9 below. The data show that, with the exception of samples from animal 3, the low values for the standard errors in respect of each animal sample (raw, microwaved or boiled) show that the subsamples of each tissue sample were homogenous for residue concentrations of tiamulin. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2 and 3 was 0.056, 0.660 and 0.342 respectively, while those for boiled residues were 0.683, 0.286 and 0.197 respectively. Thus, neither microwaving nor boiling had a statistically significant effect on residues of tiamulin in beef although the statistical power of the test was low. If no correction for weight loss during cooking is taken, residues of tiamulin declined by 8% and 19% from initial concentrations due to microwaving and boiling respectively.

As can be seen from Table 5-10 below, the overall effect of microwaving mutton at 60% power for 12 seconds was a reduction in the initial concentration of approximately 10%, while the overall effect of boiling mutton for 7 minutes was a reduction of approximately 45%.

Table 5-9 The mean concentration of tiamulin residues in beef when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (µg/g) n = 2 Animal number								
	1			2			3		
	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value
Raw (uncooked)	38.90	8.77	100	217.75	19.30	100	417.50	202.52	100
Microwaved	27.57	7.35	71	229.90	9.78	106	290.18	95.30	70
Boiled	34.97	1.44	90	174.08	10.44	80	220.36	113.21	53

SD = standard deviation

Table 5-10 The mean concentration of tiamulin residues in mutton when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (µg/g) n = 2 Animal number								
	1			2			3		
	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value
Raw (uncooked)	267.55	16.19	100	302.80	38.61	100	636.85	36.84	100
Microwaved	234.24	2.06	88	318.63	89.55	105	452.95	0.25	77
Boiled	185.49	3.77	69	181.57	2.13	60	218.39	46.78	35

SD = standard deviation

Using the Student's *t* test, the P value for microwaved residues in mutton samples 1, 2 and 3 was 0.235, 0.736 and 0.090 respectively, while those for boiled residues were 0.068, 0.133 and 0.011 respectively. The low values for the standard errors in respect of each animal sample (raw, microwaved or boiled) show that the subsamples of each tissue sample were homogenous for residue concentrations. If no correction for weight loss during cooking is taken, residues of tiamulin declined by 4% and 26% from initial concentrations due to microwaving and boiling respectively.

The overall effect of microwaving pork at 60% power for 12 seconds was an increase in the initial concentration of tiamulin of approximately 17% (interpreted as no reduction), while the overall effect of boiling pork for 7 minutes was a reduction of approximately 37% as outlined in Table 5-11 below. The data show that the subsamples were quite homogenous, with the exception of raw tissue in animal 1, as evidenced by the low standard deviations observed. Using the Student's *t* test, the P value for microwaved residues in animals 1, 2, 3 and 4 was 0.167, 0.992, 0.323 and 0.151 respectively, while those for boiled residues were 0.589, 0.089, 0.402 and 0.045 respectively. If no correction for weight loss during cooking is made, residues of tiamulin increased by approximately 36% following microwaving and remained constant following boiling (showing a 2% decline over initial concentrations).

Table 5-11 The mean concentration of tiamulin residues in pork when exposed to cooking procedures

Tissue state / cooking procedure	Tissue residue concentration (µg/g) n = 2 Animal number											
	1			2			3			4		
	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value	Mean	SD	% of initial value
Raw (uncooked)	99.80	38.47	100	183.50	30.41	100	3.70	2.40	100	23.52	2.95	100
Microwaved	160.65	7.12	160	183.04	19.01	100	1.30	0.52	35	19.72	1.65	84
Boiled	49.95	4.99	50	133.50	20.47	73	1.40	0.28	38	12.99	1.89	55

SD = standard deviation

Thus the effects of cooking were similar in respect of all species, being a slight reduction of initial concentration (0 – 17%) on microwaving and a greater reduction in respect of boiling (25 – 45%). However, if no allowance is made for moisture loss during cooking, residue concentrations increase by up to 36% following microwaving and decline by up to 26% following boiling.

5.4 Discussion

All test drugs were relatively stable in aqueous solutions during the 90-minute period studied. This period of time represents a period similar to that encountered during normal cooking conditions. In vegetable oil, heating for 90 minutes produced varied results; no effect on the concentration of enrofloxacin, but a 50%, 66% and a 70% reduction in the concentration of ciprofloxacin, tilmicosin and tiamulin respectively. It is probable that the relatively poorer stability of these marker residues in vegetable oil represents the effect of the much higher temperatures reached in this medium. According to Rose *et al* (1995a) most types of cooking using vegetable oil rarely exceed 180°C, and the internal temperatures of the food would be considerably lower than this. The upper limit of the temperatures reached in the experiments conducted was 180°C. The results obtained from the test drugs are in general agreement with the data produced on several veterinary drugs by Rose *et al* (1995a, 1995b, 1995c, 1996b, 1997a, 1997b, 1997c, 1998 and 1999) which show that all the compounds tested were unstable in vegetable oil but had variable stability in aqueous solutions. However, the *in vitro* heating studies in aqueous and vegetable oil solutions were of limited value in predicting the behaviour of the drug in incurred muscle tissue subjected to the cooking processes.

Concerning the cooking studies, the data indicate a degree of variability in the results of particular cooking procedures, even in respect of the same drug and species combination. The reasons for this are not known precisely, but are probably multifactorial and possibly include some experimental variability in the weighing and manipulation of test samples as well as a minor contribution from analytical imprecision. The variability seen in the responses to the cooking experiments is clearly greater than the analytical variability found and documented in Chapter 3 with pronounced effects on the concentration of residues found especially in relation to the

boiling experiments. Regarding homogeneity, every effort was taken to ensure samples were thoroughly minced and blended prior to subsampling. Indeed, this objective was reached as is evident from the low standard deviations and standard error values found in the subsamples analysed. From a statistical viewpoint, the fact that only 2 or 3 subsample replicates were taken is an acknowledged weakness in the study design; nevertheless, the fact that there were minimally 3 animals per cooking process tested and that there were 3 species tested compensates for this.

The overall trends in the data demonstrate that, under the conditions of the studies conducted, cooking generally led to a reduction in the residue concentration of meat from animals treated with tilmicosin, enrofloxacin and tiamulin. In each treatment group and in each animal within each group, boiling lead to a reduction in residue concentrations, which when corrected for weight loss in the tissue sample, were 35 – 90% of the precooking concentrations in individual samples. However, the effect of microwaving was less and in individual cases no reduction in precooking concentrations was demonstrated. From a consumer point of view, although the effect of cooking of incurred muscle containing residues of the 3 test drugs is an overall reduction in residue concentrations found, the moisture loss in the cooked tissue has a compensating effect which means that residue concentrations were not substantially reduced when expressed on a per gram of tissue basis. Indeed residue concentration might actually increase (they reached up to 145% of the precooking concentration in the case of microwaving of meat with incurred residues of tilmicosin).

It is possible that a quantity of the test drugs may have leached out in meat juices (gravy) during the microvaving experiments or into the water in the experiments using boiling. The juices and water were not captured separately for analysis. Meat juices were occasionally seen following microwaving (samples were homogenised before cooking in order to minimise the amount of juice coming from the meat sample).

In relation to the species of origin of the meat used in the experiments, the trends in the data due to the effects of cooking were very similar and do not appear to be species specific. While it would be expected that younger animals would have a

lower metabolic activity compared to older animals [Kaartinen et al (1997)], the finding of residues only of ciprofloxacin in the calves and only of enrofloxacin in the sheep is interesting and contrary to expectations. Further studies are necessary to investigate these findings, especially considering that both marker residues were present in muscle tissues of calves that were harvested from sites remote from the injection-site. However, both marker residues were present in injection-sites in pigs. This could be due to the fact that pigs were slaughtered within 7 hours of treatment while calves and sheep were slaughtered 24 hours following treatment resulting in greater metabolism having taking place in the cattle and sheep than in the pigs.

CHAPTER 6: THE EPIDEMIOLOGY OF INJECTION SITE BLEMISHES

6.1 Introduction

Intramuscular and subcutaneous administration of medicines is frequently carried out in veterinary practice. Parenteral administration of drugs may give rise to the presence of blemishes due to tissue damage (lesions) or abscesses at the site of injection. George *et al* (1997) reported that need breakage was another major concern associated with the intramuscular administration of biologicals and pharmaceuticals in cattle. The objective of this segment of the research was to establish the prevalence and significance of injection-site blemishes in muscle (meat) of Irish cattle, sheep and pigs.

6.2 Materials and methods

Blemishes were harvested from primal and subprimal portions of cattle, sheep and pigs from boning-halls throughout Ireland, as well as from meat cuts prepared by a butcher as described in Chapter 2. There were a total of 2 beef factories involved (Factories *A* and *B*), 1 pork factory and 2 sheep factories (Factories *C* and *D*) as well as a single butcher's outlet. The writer harvested all pork samples, and more than 200 of the cattle blemishes; the quality supervisors on the boning-hall production lines harvested all other samples. Weight loss due to trimming of the blemishes was recorded in the pork factory as well as in one of the beef factories (Factory *B*). The value of the tissue trim was estimated by personnel from the factories based on the commercial value of the unadulterated meat cuts. The throughput of the production lines was recorded using the commercial records of the factories involved. The official government veterinarians recorded details of blemishes and throughput in the sheep abattoirs. The butcher recorded details of the numbers of beef and lamb carcasses processed by him throughout the study period. The blemishes were categorised by the writer as cystic, scar or abscess and the weight of the condemned tissue was recorded. Cystic and scar tissue was analysed for the presence of residues of inhibitory substances as were some meat samples adjacent to abscesses.

6.3 Results

The data are presented separately for each species in Sections 6.3.1 – 6.3.3 hereunder.

6.3.1 Results of the beef survey

From a total of 16821 hindquarters processed from Factory *A*, 219 blemishes were recorded (1.30%). All of the blemishes were located in the cut known as 'silverside'. They were all classified as scars and none contained residues of inhibitory substances as outlined in Table 6-1 hereunder.

Table 6-1 Results of the survey of blemishes in beef primals and subprimals from Factory *A*

	Monthly data			
	June	**September**	**November**	**December**
No. of quarters processed	3925	4482	3984	4430
No. of scars found	66	50	50	53
No. positive to 4-plate-method	0	0	0	0

From a total of 11541 forequarters and 16714 hindquarters processed from Factory *B*, 2160 blemishes were recorded. This represents a prevalence rate of blemishes of 7.64% of quarters processed. Most (98%) of the blemishes were scars (n = 2124) and 9 of the total contained residues of inhibitory substances as outlined in Table 6-2 below. No abscesses were recorded. While quantitative data are not available for all the samples taken, most of the lesions were located in the hindquarters (187/226 or 83%). A staff employee recovered a piece of a broken syringe needle, about the 15mm in length, from the superficial tissue of a primal cut during one of the day's production runs. The average weight of each trim loss due to the presence of a blemish was 289.61g.

Table 6-2 Results of the survey of blemishes in beef primals and subprimals from Factory *B*

	Monthly data								
	February 1999	**June 1999**	**July 1999**	**September 1999**	**October 1999**	**November 1999**	**December 1999**	**January 2000**	**February 2000**
No. of forequarters	639	152	60	624	2023	2112	2568	998	2369
No. of hindquarters	668	250	268	516	3482	4363	2425	1649	3094
Total no. of quarters	1303	402	328	1140	5505	6475	4993	2647	5463
No. of scars	62	54	35	112	590	518	223	164	366
No. of cysts	0	7	1	3	2	4	5	2	12
Total no. of blemishes	62	61	36	115	592	522	228	166	378
No. of scars positive to 4-plate-test	0	1	0	3	2	0	2	0	0
No. of cysts positive to 4-plate-test	0	0	0	0	0	0	1	0	0
Total no. positive to 4-plate-test	0	1	0	3	2	0	3	0	0

Sample of the types of lesions recorded in Factory *B* were photographed. A selection of these photographs are provided as Figures 6-1 to 6-5 hereunder.

Figure 6-1 Typical lesion in beef muscle

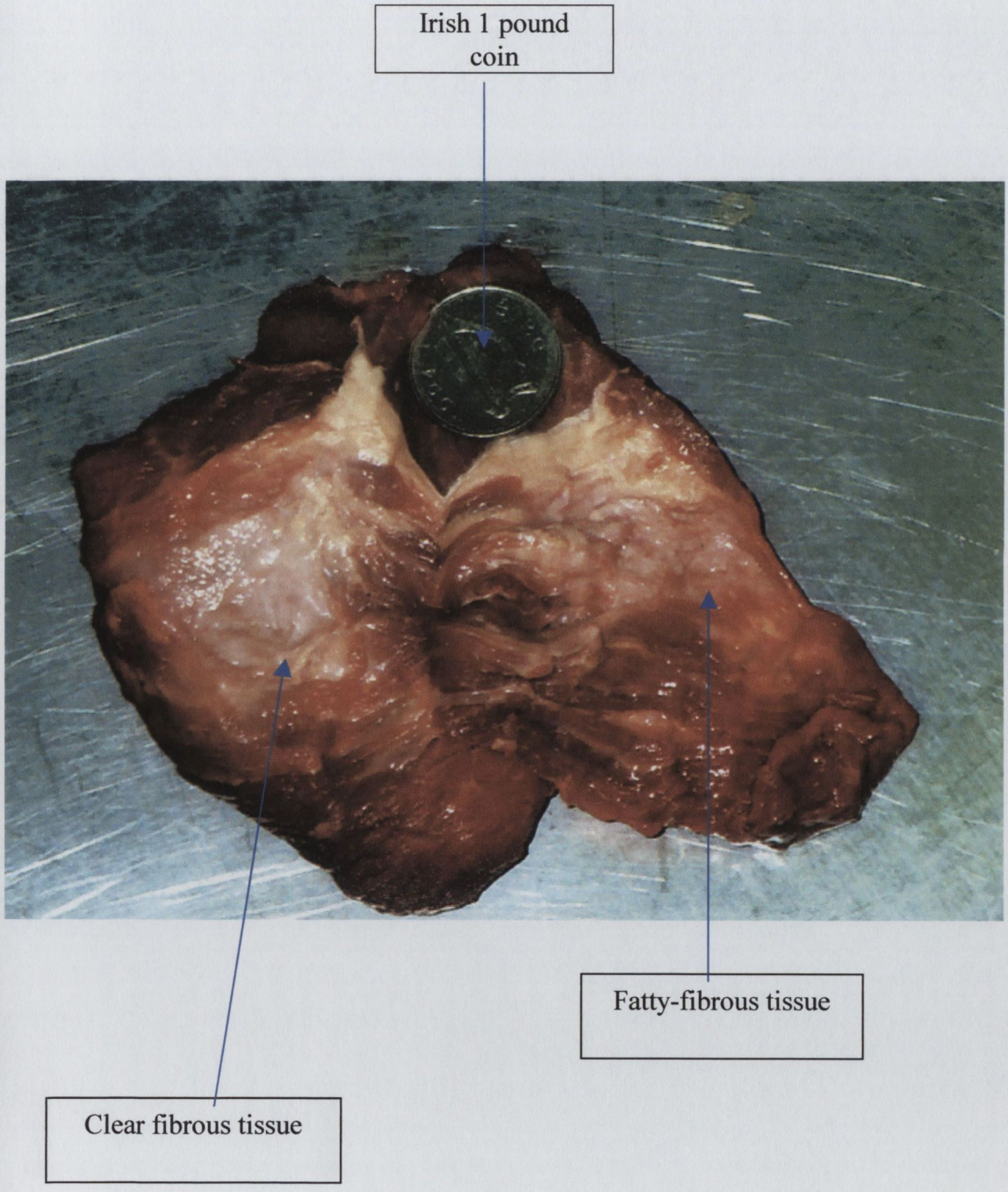


Figure 6-2 Diffuse but well demarcated scar lesions in beef muscle

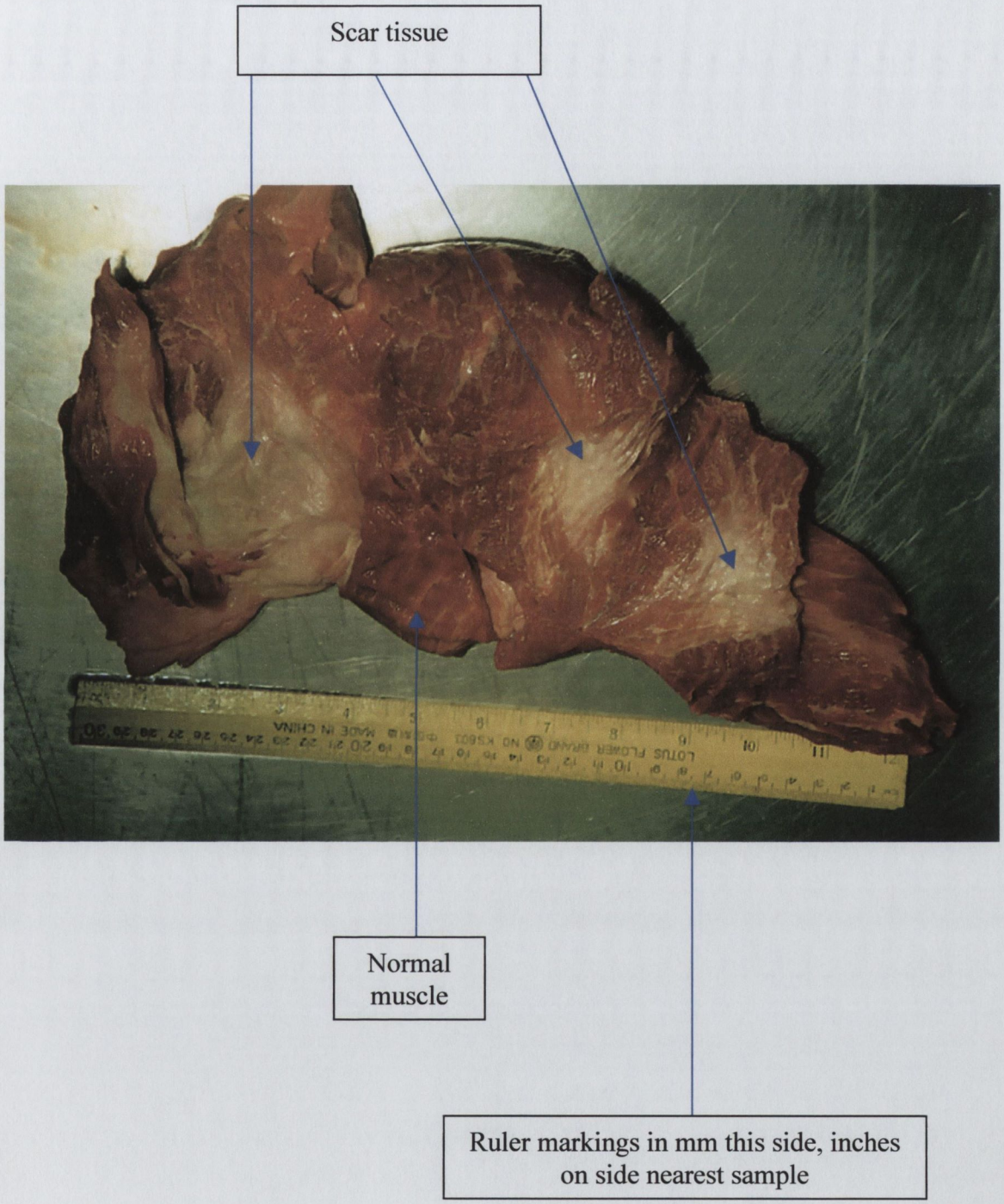


Figure 6-3 Diffuse scar lesion in beef muscle

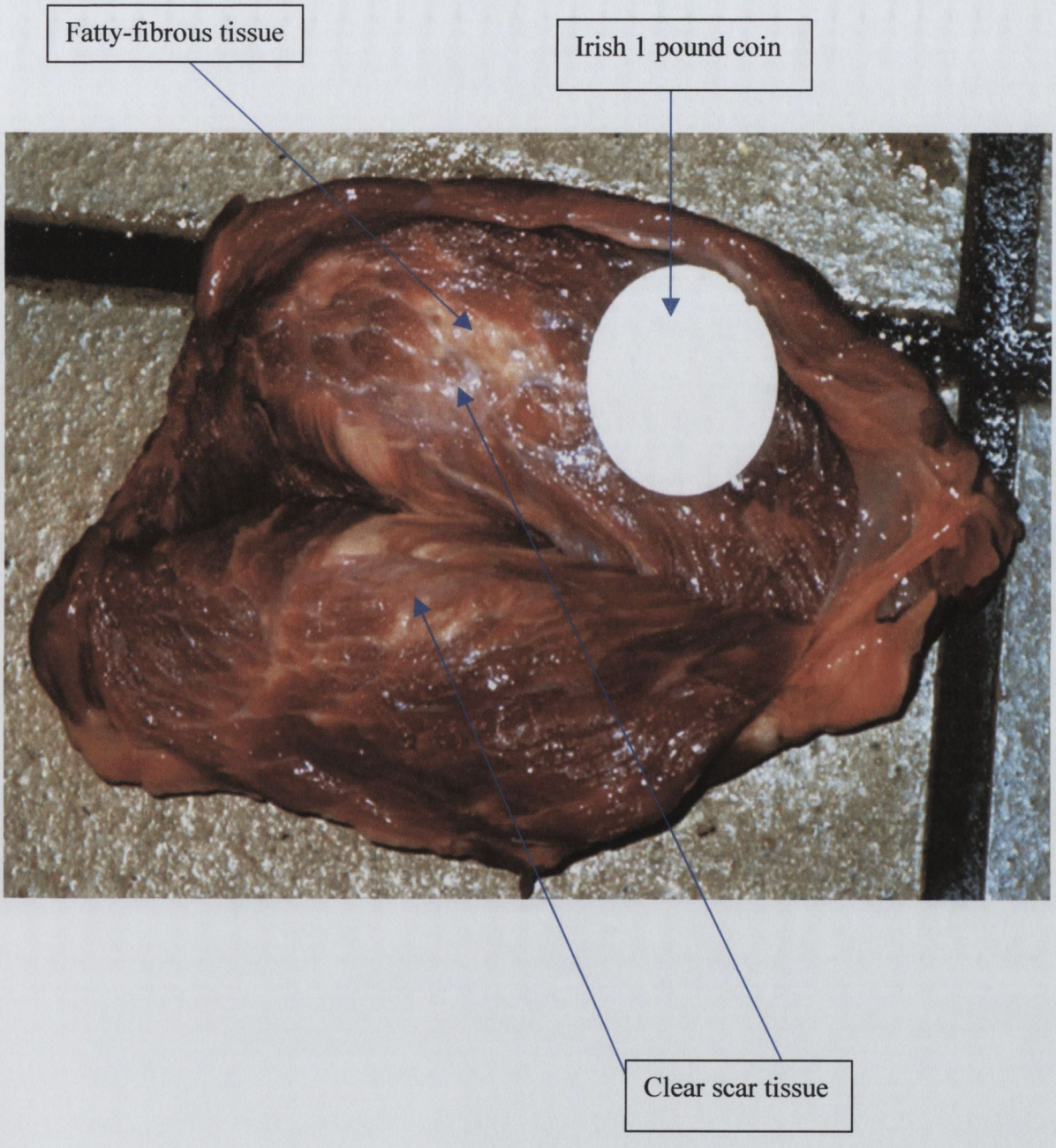


Figure 6-4 Cystic lesion in beef muscle

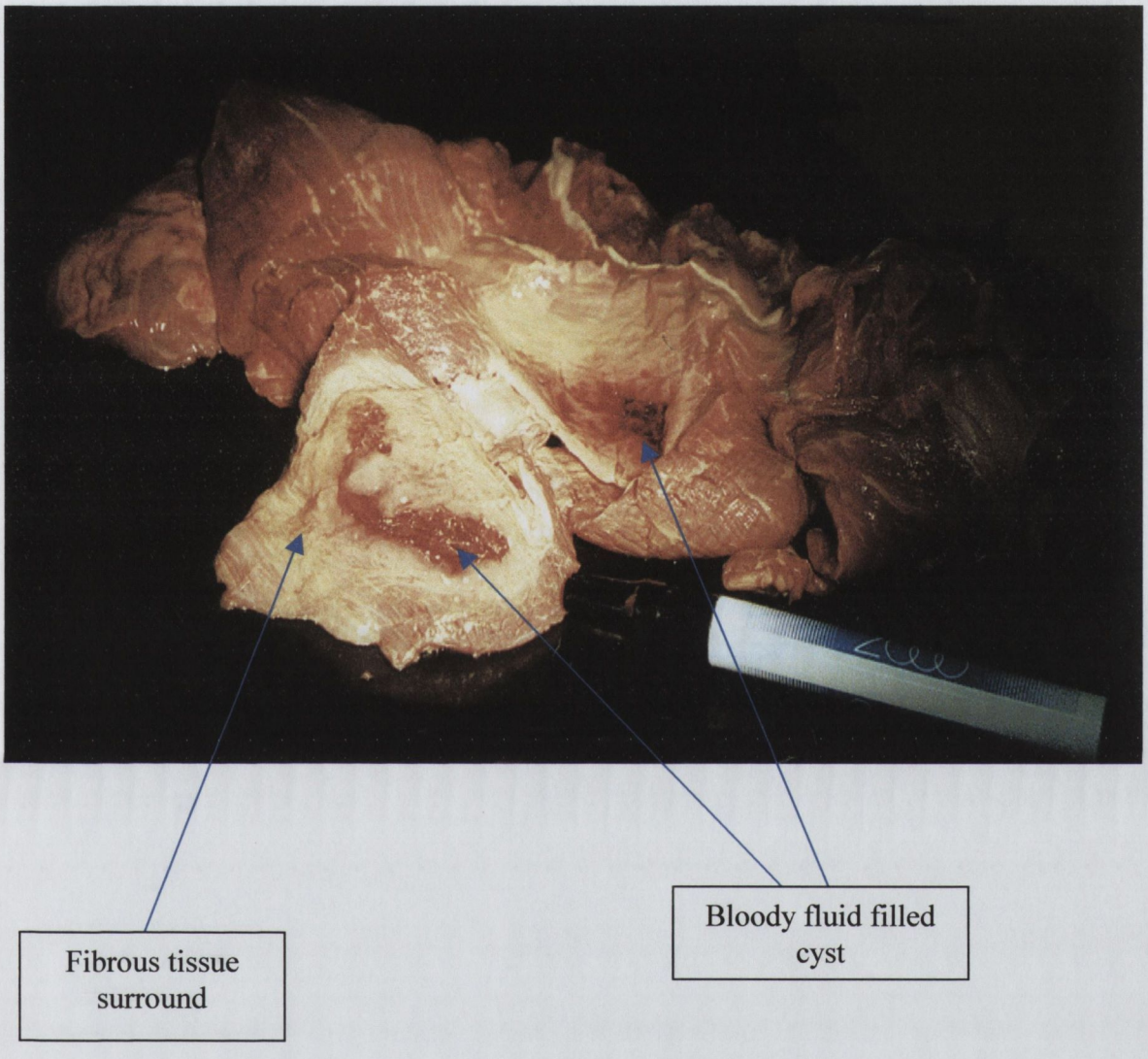
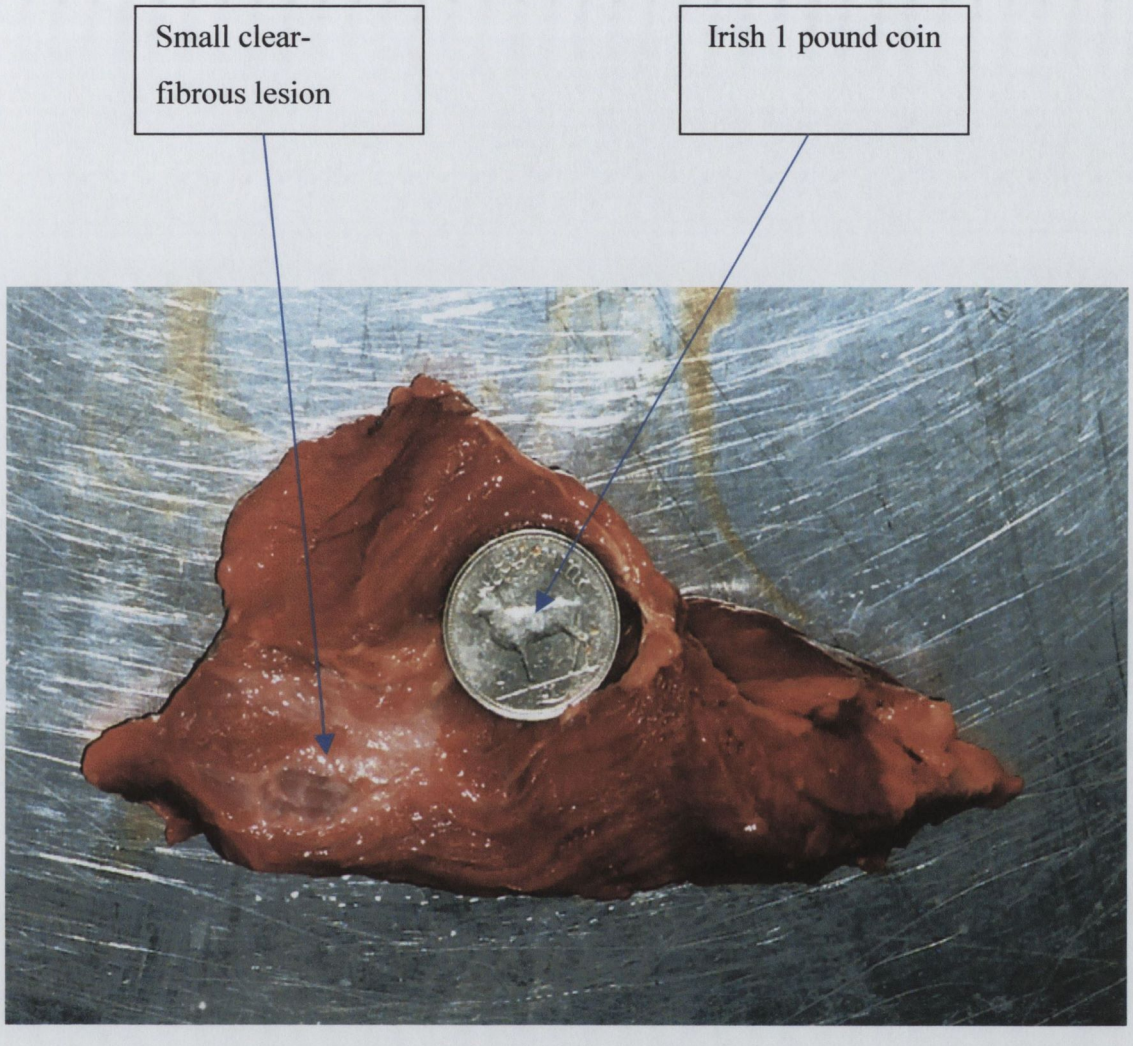


Figure 6-5 Smaller scar lesion in beef muscle



Of the 154 heifers processed by the butcher for domestic consumption, none was reported to contain any lesions. The writer visited this butcher on 6 occasions during 1999 to ensure that he was fully conversant with the study and co-operative. It is expected therefore that these data are accurate and reliable.

The total number of blemishes in beef from all sources is quite high at 21.11% of carcasses (5.28% of quarters) overall.

6.3.2 Results of mutton/lamb survey

No injection-site blemishes were recorded in either of the 2 boning halls or in the butcher's shop where 1522 sheep carcasses were processed into saleable cuts. In the 6-month period from March to September 1999 a total of 25680 carcasses were boned out at Factory *C*. The quality control supervisors and official veterinarians at both factories reported that most injection-site blemishes in lamb carcasses were removed at slaughter and prior to boning out, because they are easily visible in the thin carcasses. A government Veterinary Inspector in Factory *C* therefore monitored the condemnations on the killing line for specific periods during February, July and October 1999. The results of this are presented in Table 6-3 hereunder.

Table 6-3 Sheep trim condemnations recorded in Factory *C*

	February	July	October
Approximate no. of animals slaughtered during monitoring period	4000	9200	10000
Total no. of abscesses	38	36	61
Subcutaneous abscesses	27	27	45
No. and location of condemnations (not including arthritis, emaciation, pleurisy or septic carcasses)	1 forequarter 10 hindquarters	6 forequarters 9 hindquarters	15 forequarters 5 hind quarters

It should be noted it is possible that some of the abscesses recorded may not have been due to injections with veterinary drugs.

In respect of Factory *D*, no blemishes were recorded in any of the 125 carcasses boned out in the presence of the official veterinarian. This was a relatively small facility with 20, 25, 20, 22, 18 and 20 carcasses examined during processing in the months March to September 1999.

6.3.3 Results of pork survey

Concerning pork, the data show that the prevalence of blemishes in Irish pork cuts is 2.2% overall, with 78% of the blemishes being abscesses. No fibrous scars were found. Only a single sample taken from a scar was positive for the presence of an anti-bacterial substance. This scar was haemorrhagic in nature. Many of the abscesses found were in the neck and jowel areas and were not deeply situated within muscle tissue. None of the muscle samples taken from the immediate vicinity of the abscesses tested positive for anti-bacterial residues. Not all muscle samples close to abscesses were taken for analysis for residues as some were well encysted in fibrous tissue surrounded by fascial tissue and fat. The results are tabulated in Table 6-4 hereunder. The total weight of trim loss in pork was 156.60kgs or 665g/lesion.

Table 6-4 Prevalence of blemishes and weight of trim loss in pork cuts

	Monthly data										
	February 1999	**March 1999**	**April 1999**	**May 1999**	**June 1999**	**July 1999**	**September 1999**	**October 1999**	**November 1999**	**December 1999**	**January 2000**
No.of carcasses processed during visit	1000	1200	900	960	1000	1050	1050	1000	900	500	1000
No.of scars found	0	1	1	0	3	3	0	1	2	2	3
No.of cysts found	2	1	4	2	2	10	3	4	3	1	3
No. of abscesses found	14	18	32	7	21	28	15	12	20	6	10
Weight trim (kg)	38.5	18.3	29.1	0.7	16.2	13.6	5.0	11.3	15.1	3.4	4.4
No. of samples analysed for the presence of inhibitory substances	2	2	6	3	6	13	3	5	12	9	15

6.3.4 Results of anti-bacterial screening

From a total of 2379 beef muscle samples and 76 pork muscle samples analysed for the presence of inhibitory substances using the EU 4-plate-method, 10 samples yielded positive results. The responses of the positive samples in each of the 4 test plates are given in Table 6-5 below. According to Bogaerts and Wolf (1980), the results of the pH 6 assay are suggestive of the presence of a compound from the tetracycline family, pH 7.2 is suggestive of a sulphonamide or aminoglycoside while pH 8.0 and 8* are suggestive of β-lactams, macrolides or lincosamines.

Table 6-5 Positive results of samples for presence of inhibitory substances detected by EU 4-plate-method

Species	Laboratory reference no.	Inhibition zone (mm) in test agars			
		pH 6	pH 7.2	pH 8.0	pH 8*
Pork	356/21/02.07.99	5.71	nz	nz	nz
Beef	300/32/09.06.99	nz	nz	9.25	5.68
Beef	300/54/08.09.99	15.36	nz	nz	nz
Beef	300/8/08.09.99	nz	8.32	nz	nz
Beef	300/21/29.09.99	nz	13.52	nz	nz
Beef	300/14/04.10.99	4.65	nz	nz	nz
Beef	300/20/11.10.99	4.11	nz	nz	nz
Beef	300/13/23.12.99	2.38	nz	nz	nz
Beef	300/15/23.12.99	2.00	nz	nz	nz
Beef	300/9/22.12.99	4.22	nz	nz	nz

* Test agar at pH 8.0 based on *Micrococcus luteus*

nz = no zone of inhibition

6.3.5 Monthly incidence of blemishes

A review of the seasonal incidence of blemishes in beef and pork is given in Table 6-6 hereunder. Note that the number of beef primal and subprimals examined for blemishes was very low in June and July and therefore the percentage incidence figure recorded for June may represent the outside. It can be seen that the monthly incidence in pigs is relatively constant.

Table 6-6 Incidence of blemishes in beef and pork 1999/2000

Month/year	Incidence in beef		Incidence in pork	
	No. of carcasses	(%)	No. of carcasses	(%)
February 1999	326	19.03	1000	1.6
March 1999	na		1200	1.68
April 1999	na		900	4.11
May 1999	na		960	0.94
June 1999	101	61	1000	2.5
July 1999	82	43.9	1050	3.9
September 1999	285	40.35	1050	1.71
October 1999	1376	43.02	1000	1.7
November 1999	1619	32.25	900	2.78
December 1999	1248	18.27	500	1.8
January 2000	662	25.09	1000	1.6
February 2000	1366	27.68	na	

na = not analysed

6.4 Discussion

The fact that only 9 samples from more than 2379 blemishes from a total of 11,423 cattle de-boned and packed were positive for antibacterial residues gives reasonable assurance that injection-site blemishes are more a quality problem than a consumer hazard. However, Okerman *et al* (1998) reported that while in routine residue testing the great majority of samples are evaluated on the basis of a screening test, the EU 4-plate-method could not detect fortified meat samples containing ceftiofur,

sulphonamides, streptomycin and some macrolide anti-bacterials at the MRLs set for muscle. However, the same authors reported that the test was capable of detecting tetracylines and β-lactam anti-bacterials at their MRLs established and other substances at levels above their established muscle MRLs. As reported by FEDESA (1999) these 2 drug classes account for 75% of the total therapeutic anti-bacterial used in animal health. It is concluded that the negative responses of the blemish samples are unlikely to be false positives because of the use pattern of the drugs and because residues at the injection-site were likely to be many times above the MRL for normal muscle. In this regard it should be noted that the samples used for residue analysis were taken from the central areas of the scars; a lower residue concentration would be expected from the peripheral areas that do not contain as much fibrous tissue and would therefore be more likely to be consumed. More than 98% of the blemishes were scars that were highly organised into fibrous or fatty-fibrous tissue. According to George *et al* (1996) and Dexter *et al* (1994) the presence of organised scar tissue is indicative of a more long-standing lesion, originating from injections given during preweaning, stocking and early fattening periods. The absence of abscesses may be as a result of their removal immediately following slaughter prior to chilling. The finding of a piece of an injection needle in meat during deboning is a concern from a consumer safety viewpoint although there are metal detectors in operation at each processing facility. George *et al* (1997) reported that in an audit of top sirloin in US beef in 1995, 4 broken needles were recovered from steaks during cutting and that this was a major quality assurance concern.

While the assignment of causality between the blemishes observed in meat and the use of injections cannot be definitely confirmed from this study, the gross nature of the lesions, their location, together with the information from published literature (Dexter and others 1994, George and others 1996 and 1997, Van Donkersgoed and others 1998) makes an alternative causality unlikely.

The prevalence of blemishes in Irish beef is comparable with that reported by George *et al* (1995a), George *et al* (1997) and Van Donkersgoed (1998) for North America at 21.11% of animals slaughtered (5.28% of quarters) overall, although it is appreciated that some carcasses may have had more than one blemish. At a current meat value of

IR£3.30/kg this amounts to a minimum of 95 pence per lesion in trim loss alone. Not infrequently, significant additional losses are also incurred due to downgrading of the affected cut which failed to meet the customer specifications. Further losses are also incurred in the labour required to screen and remove the blemishes. As noted by George *et al* (1997), the problem of injection-sites is primarily a quality control problem for farmers and the meat industry, rather than a consumer risk. George *et al* (1997) demonstrated that meat samples taken from steaks at a point 7.62cm from the centre of the injection-site lesion had average shear values of 5.80 and were therefore not sufficiently tender for the retail trade. These additional losses have not been quantified in this study. It is possible that some blemishes occurred within the primal and subprimal cuts that could not be detected by superficial examination and would only be detected when the meat was further cut into finer meat pieces. It is difficult to find an explanation for the difference in the percentage of blemishes observed between the two factories. The reasons for this are not known but could include the possibility that the factory supervisor in Factory *A* may not have recognised all the lesions present or some of the blemishes may have been discarded by boning hall operatives before reaching the quality control point or that the specifications for the primal cuts were different between the two factories leading to non-detection of blemishes located deeper in the meat cut.

One beef factory processor who boned out culled cows refused the writer access because he was afraid that information on the prevalence of blemishes might harm his business. Almost certainly the prevalence and extent of injection-site blemishes in cull cows is higher than that observed in this study as cows are more likely to have been affected by production diseases e.g. infertility and mastitis and therefore to have received medication or to have received more vaccinations during their lives compared to younger animals slaughtered as prime beef. Guest and Paige (1991) reported that cull cows were the biggest contributors of residues in slaughtered animals in 1985 and 1988 in the USA.

The failure of the butcher to record any blemishes in the 154 beef carcasses processed by him is encouraging. The reasons for this are several and include the following:

a) animals were from young stock (mainly heifers less than 22 months of age),

b) animals were either produced by the butcher himself or bought by him as stores from neighbours and fattened by him (a comprehensive prophylactic health and nutrition regimen was in place that minimised the need for veterinary therapy),
c) the butcher used pour-on anthelmintics in preference to injectables,
d) any superficial lesions observed at slaughter would have been removed before the carcasses were shipped to the butcher.

It is also possible that the butcher deliberately did not disclose the presence of blemishes in case of damage to his reputation; this is felt unlikely as the identity of the individual processors and butcher were not disclosed and full co-operation was given to the writer throughout the survey.

The failure to record a single injection-site blemish in sheep is unexpected as more than 27,000 animals were boned out during the study. That said, approximately 0.58% of carcasses had abscesses. It seems probable, based on personal experience in meat inspection and those of veterinarians involved in meat inspection (Griffin, personal communication, 2000; Matthews, personal communication, 2000) that some of these blemishes, by virtue of their location, arose from veterinary drugs or vaccine administration. The overall significance of injection-site lesions in sheep would appear to be very low.

It is also not possible to establish how many of the blemishes seen in the pork survey were due to injections of drugs or vaccines and how many were due to other sources of contamination e.g. injury or puncture wounds. The finding of the single positive anti-bacterial residue in a haemorrhagic lesion is suggestive of recent use of a veterinary medicine. The absence of fibrous scars in pork meat may indicate that farmers are not routinely using intramuscular therapies in the care of their fattening pigs. While outside the remit of this study, the reasons for this might include the high level of awareness of the residues amongst pig farmers; the fact that pigs are produced under intensive conditions where labour is scarce and therefore oral medication is more convenient; and that pig production is highly integrated in Ireland (most pigs are born and reared on the same farm and shipped directly to the factory for slaughter).

The prevalence of blemishes in pork is much lower than that seen in beef cuts in Ireland or from North America. While the weight of trim condemned as unfit for human consumption was higher for pork than for beef, it is noted that some abscesses required condemnation of large cuts e.g. hams. The average trim loss per pig was 14.7g with much of this occurring in the low value jowel trimmings. The direct economic losses in pork are therefore relatively small.

CHAPTER 7: RISK ASSESSMENT

7.1 Introduction

The objective of this chapter was to estimate the risk of human consumption of injection-site residues of anti-bacterials in Irish beef, mutton and pork. As stated by Wooldridge (1999) a risk assessment is the estimated of a risk: a risk is the probability of an unwanted outcome. Risk assessment may be either qualitative (defining the outcome in words) or quantitative (estimating the risk as a numerical outcome). It is implicit in a quantitative risk assessment that the potential risk pathways have been considered first in order to know which pieces of information are required for the quantitative assessment.

7.2 Qualitative risk assessment

7.2.1 Background

The data from the studies on Irish beef, mutton and pork reported in Chapter 6 clearly point to a low incidence of injection-sites containing anti-bacterial residues and follow the trends found in the routine government surveillance programmes of carcass tissue at slaughter in Ireland as outlined in Table 7-1 hereunder.

Table 7-1 Comparison of survey residue data and residue data from government monitoring for anti-bacterial substances

Species	Government sampling			Survey samples		
	Number of samples	Number positive	% above MRL	Number of samples	Number positive	% positive
Bovine	1488	23	1.55	2379	9	0.38
Ovine	722	0	0.00	0	n.app	n.app
Porcine	41070	349	0.85	76	1	1.3
All species	43280	372	0.85	2455	10	0.41

n. app = not applicable

The data indicate that the overall incidence in primal and subprimal portions in the survey conducted and outlined in Table 7-1 above was less than 50% of that found at slaughter in the government surveillance programme. It should be noted that the blemish muscle samples taken in the survey reported above were intended for discard as unfit for human or animal consumption. Dayan (1993) stated that if some medicinal anti-bacterials are found in food from time to time, it was reasonable to expect that other residues will be present in food, at a frequency related to their use. He also stated that it was likely that the overall frequency of residues in food was low, because of the regulation of farming and veterinary practices.

It has already been demonstrated in the published literature e.g. Rose *et al* (1995a, 1995b, 1995c, 1996b, 1997a, 1997b, 1997c, 1998, 1999) and in Chapter 5 that cooking can have an impact on the concentration of residues in muscle. This can vary from between drug substances, but may reduce significantly the residue concentration present and thereby the risk to human health. On the other hand, as seen in Chapter 5, cooking procedures generally result in a loss of tissue moisture and thereby may actually concentrate any non-heat labile residues present. However, considering that maximum residue limits (MRLs) are generally set on the basis of chronic (life-time) exposure to a particular substance, the occasional presence of residue concentrations above the MRL is of little importance for human health for all but a few substances (those which have acute pharmacological effects or allergenic potential).

7.2.2 Risk pathway

An outline of the risk pathway and the potential risk assessment questions following the approach by Wooldridge (1999) is given in Table 7-2 hereunder. This approach allows each of the risk steps to be characterised.

Table 7-2 An outline of the risk pathway and risk assessment steps for evaluating the risk of adverse of adverse human health effects consequent upon the ingestion of anti-bacterial residues in injection-sites in farm livestock.

Potential risk pathway	Potential risk assessment questions
Disease occurs in farm livestock Anti-bacterials given to farm livestock, a proportion of which are given parenterally. Result: Proportion of animals has injection-site lesions.	What is the probability that farm livestock require preventative or therapeutic anti-bacterial therapy? What is the probability that farm livestock are given parenteral anti-bacterials? What is the probability of farm livestock having injection-site lesions?
Injection-site lesions contain anti-bacterial residues. Lesions remain present at slaughter. Lesions remain in primal cuts at boning-out. Result: Residues of anti-bacterials remain in injection-sites in primal and sub-primal cuts.	What is the probability that injection-site lesions contain anti-bacterial residues? What is the probability that lesions in treated animals will persist through to slaughter? What is the probability that lesions will not be detected at boning out? What is the probability that residues of anti-bacterials remain in injection-sites in primal and sub-primal cuts of livestock origin?
Anti-bacterial residues remain present at all stages in the food chain to final product for eating. Result: Anti-bacterial residues ingested by humans.	What is the probability of anti-bacterial residues being degraded during processing, storage, preparation and cooking of meat? What is the probability that a human will ingest an injection-site containing residues of anti-bacterials?
Humans ingest anti-bacterial residues from an injection-site. Result: A proportion of the human population develop adverse health effects.	What is the probable proportion of the total anti-bacterial residues ingested by humans which comes from the injection-site residues? What is the probability that a human will suffer adverse health effects due to the ingestion of meat containing anti-bacterial residues at the injection-site?

For human consumers to suffer an adverse effect due to the presence of anti-bacterial residues in injection-sites in food-producing animals, it is necessary that anti-bacterials have been administered to those animals by means of injection, for animals to have been slaughtered before the residues had been eliminated, for the residues to remain in meat during chilling, storage, and preparation of the food and for those residues to have the pharmacological or toxicological potential to cause adverse effects in humans. The risk steps are considered hereunder.

7.2.2.1 What is the probability that farm livestock require preventative or therapeutic anti-bacterial therapy?

It is not unusual for animals to fall ill through some infectious or parasitic disease and to require prophylactic, metaphylactic or therapeutic anti-bacterial administration. Nevertheless, in modern agricultural practice, advances that have taken place in the husbandry of animals mean that many diseases are prevented through environmental management, nutrition and the use of vaccines. Anti-bacterials are not routinely administered to every animal. Moreover, in respect of fattening pigs, the requirements for mass medication are usually best achieved through in-feed or in-water medication with parenteral therapy restricted to one or a few individual animals. Sheep and cattle in Ireland are reared for the greater part of their lives outdoors on grass with the result that infectious disease pressures are less than for intensively produced animals such as pigs and poultry. Furthermore, fattened animals used for meat production are generally slaughtered in the prime of their lives; diseases of the older animal, production diseases and mastitis that might necessitate anti-bacterial use do not usually occur in these animals. While accurate information on the amounts of anti-bacterial use in veterinary medicine in respect of each animal species is not available, Boatman (1998) estimated that 50% of the anti-bacterials used for animal health purposes within the EU arose from prescriptions issued for therapeutic purposes while 25% arose from feed additive (oral) usage for growth promotion and 25% for ionophore feed additives (oral) in poultry. The 50% figure estimated for therapeutic use included use in companion animals, horses, fish and other species.

Overall these data indicate that, in the opinion of the writer, the probability that farm livestock require treatment with anti-bacterials is low, but occasionally such use is necessary and does occur.

7.2.2.2 What is the probability that farm livestock are given parenteral anti-bacterials?

According to Boatman (1998), all dosage forms including oral (tablets, boli, in-feed, in-water and drench) formulations, other types of formulations (intramammaries, intrauterine) as well as injectable formulations were included in the 50% figure for anti-bacterials used for therapeutic purposes. While adult cattle are generally treated individually, antibiotics for herd and flock treatment are generally given orally. For the purpose of this exercise, the writer's assumes that about 33% of the therapeutic usage relates to injectables but there are uncertainty limits on this estimate as precise data are not available.

7.2.2.3 What is the probability of farm livestock having injection-site lesions?

Not all formulations of veterinary medicinal products produce lesions at the site of injection. Nouws (1990) reported that irritant formulations and long-acting products are most likely to cause injection-site blemishes. However, George *et al* (1995a) have reported that even vitamins and vaccines given intramuscularly as well as subcutaneously may cause injection-site blemishes. Given the ever-increasing labour shortage on Irish livestock farms, it is likely that the use of long-acting formulations is relatively high. Moreover, transient reactions at the injection-site are known to occur with many drugs. Thus, in the opinion of the writer, the probability of injection-site lesions in farm animals that receive injectable anti-bacterials is relatively high.

7.2.2.4 What is the probability that injection-site lesions contain anti-bacterial residues?

Little information is available from the published literature to assess the probability of this occurrence. Information from the recently conducted survey in Irish beef and pork, described in Chapter 6, demonstrates that this does occur but at a very low frequency (1/240). These data are used in the qualitative risk assessment Section 7-3

below. The fact that residues were detected in injection-site muscle does not necessarily mean that it came from the specific injection given at that site; it may have arisen from an anti-bacterial given at another site, or by the intravenous or oral routes.

7.2.2.5 What is the probability that lesions in treated animals will persist through to slaughter?

George *et al* (1995a) conducted a study on cattle that were treated at branding or weaning and showed that some had lesions at slaughter 7.5 to 12 months later. However, for many veterinary medicines, injection-site damage reduces over time and, in the experience of the writer in evaluating the safety of veterinary medicines, for majority of drugs the injection-site lesion has disappeared by the end of the withdrawal period. However, there are exceptions to this and it is known that vaccines that contain liquid paraffin as an adjuvant as well as certain proprietary veterinary medicines, including some copper injections and anthelmintics, are irritant and cause transient injection-site reactions. Van Dresser and Wilcke (1989) reporting on an investigation on drug residues in food-producing animals conducted in the USA in 1985, stated that failure to follow the withdrawal period was cited as a cause for occurrence of the residue in 70% of cases. They further stated that the withdrawal period was known but disregarded in 50% of cases and that poor or mistaken records, incorrect animal identification, or simple inattention will also lead to residue violations. Presuming farmer compliance with the withdrawal periods and that the withdrawal periods set for the products administered is adequate, the probability that lesions in treated animals will persist through to slaughter is, in the writer's opinion, low with a wide degree of uncertainty surrounding the products used and the lack of published data on this issue.

7.2.2.6 What is the probability that lesions will not be detected at boning out?

Little reliable information is available on this issue. Korsrud *et al* (1994) reported that visual inspection of muscle surfaces did not reliably reveal all injection-site blemishes in yearling beef steers that had received intramusular or subcutaneous injections. Hoogland (2000, personal communication) stated that in a survey in the

Netherlands in 1989 in 8,500 slaughter pigs, the incidence of injection-sites rose depending on the intensity of the inspection conducted; during inspection on the slaughter line the incidence was 0.2% of the animals; in the deboning/cutting plant, injection-sites were found in 5.4% of necks (2.7% of the animals) and in the butchery, injection sites were found in 11% of the necks (5.5% of the animals). It is probable that small lesions which may be present deep within a primal or sub-primal cut will not be detected by quality control inspectors on the production line because to divide the portion into smaller pieces would reduce the value of the piece of meat which must meet the required market specifications. It is also possible that occasionally, larger lesions may go undetected. However, from the single butcher surveyed in the course of the survey reported in Chapter 6, no injection-site blemish was found. It is concluded by the writer, that there is a reasonable probability that some lesions will not be detected at boning out.

7.2.2.7 What is the probability that residues of anti-bacterials remain in injection-sites in primal and sub-primal cuts of livestock origin?

From the information obtained above, in the writer's opinion, it is unlikely that residues of anti-bacterials remain in injection-sites found in primal and sub-primal cuts of livestock origin, but it is possible that injection-site blemishes are present and that some remain undetected.

7.2.2.8 What is the probability of anti-bacterial residues being degraded during processing, storage, preparation and cooking of meat?

From the results of studies published by Rose *et al* (1995a, 1995b, 1995c, 1996b, 1997a, 1997b, 1998, 1999); Ramos *et al* (1993), Hsu and Epstein (1993), O'Brien *et al* (1981), Epstein *et al* (1988), Costa *et al* (1993, 1994), Slanina *et al* (1989), Honikel *et al* (1978), Steffenak *et al* (1993, 1994), Katz and Levine (1978), Inglis and Katz (1978), DePaolis *et al* (1977), Katz *et al* (1978), McCracken and Kennedy, (1997), Askew *et al* (1968), Ekström and Slanina (1982) as well as those conducted during this project and reported on in Chapter 5, it is concluded that storage and preparation of meat has little effect on most veterinary drugs but that cooking of meat has a demonstrable reduction effect in some cases, which may be dependent on the type of cooking method employed. It is however possible that cooking may give rise

to the formation of more toxic degradation products of the residue but data on this are lacking. Thus, in the writer's opinion, the probability of anti-bacterial residues being degraded as a result of the routine processes in the preparation of meat for human consumption is best taken as zero and should not be used in the quantitative risk assessment.

7.2.2.9 What is the probability that a human will ingest an injection-site containing residues of anti-bacterials?

Reliable data on 'table' exposure to injection-sites containing residues of anti-bacterials has not been provided and is difficult, if not impossible, to obtain. The probability of ingestion depends on the dietary habits of the individual, personal taste, age, religious beliefs, and other factors. As reported by Dexter *et al* (1994) injection-sites are generally fibrous in their composition. They are likely to be 'tough' to eat, and according to George *et al* (1997) not very palatable and thus are expected to be rejected on the plate. It is acknowledged however that injection-sites may not be apparent in minced meat or in canned or otherwise mixed meats. Taking the results of the previous steps on risk assessment, it is concluded by the writer that while it is possible that injection-sites containing residues of anti-bacterials may go undetected, they are unlikely to be present in meat dishes at the dining table. However, if present, most are unlikely to have been significantly reduced by meat processing, storage and cooking.

7.2.2.10 What is the probable proportion of the total anti-bacterial residues ingested by humans which comes from the injection-site residues?

It is difficult to separate adverse human health effects associated with veterinary drug residues in foodstuffs from those associated with other uses. Dayan (1993) reported that the general population is now widely and repeatedly exposed to anti-bacterials for medicinal purposes from the earliest infections in childhood. FEDESA (1999) estimated that human use of anti-bacterials in the EU in 1997 accounted for 52% of the total annual use, with animal therapeutic use and feed-additive use accounting for 33 and 15% respectively. They stated that on a comparative 'per kg' weight basis, the

average number of anti-bacterial treatments received by animals is much fewer than the number of treatments administered to the human population, which they estimate conservatively at a ratio of at least 5 to 1. The Scientific Steering Committee [SSC] (1999) reported that although anti-bacterials have also been used as pesticides in some fruits and vegetables and genetic markers in certain crops, significant human dietary exposure was not expected. Dayan (1993) also stated that meat, eggs, fish, milk and products derived from them might, potentially, contain residues of anti-bacterial substances either following treatment of animals or from natural sources. Indeed, so too might honey for which MRLs have been established. Dayan (1993) noted that very low levels of penicillin residues are common in dairy products (estimated by Dayan at 0.4% in the UK) and other foodstuffs (meat, estimated by Dayan at 1 – 2 %) and may be capable of exciting an allergic or anaphylactic reaction.

CVMP (2000b) stated that the individual MRLs in each edible tissue should reflect the kinetics of the depletion of the residues leading to MRL figures proportional to the marker residue concentrations. If this concept is followed diligently, CVMP advised that the MRLs set for the edible tissues should lead to similar withdrawal periods for each edible tissue. However, this concept does not extend to the distribution of residues at the site of injection. Nevertheless, given the fact that injection-sites are small relative to the total edible muscle in a carcass, that other foodstuffs are as likely to contain residues above the MRL if the withdrawal periods are not followed, that anti-bacterial residues may occur in foodstuffs from natural or vegetable sources and that the human population already consumes a large quantity of anti-bacterials for medicinal purposes, in the opinion of the writer, it is probable that the proportion of the total anti-bacterial residues which comes from injection-site is minuscule, albeit with wide confidence intervals.

7.2.2.11 What is the probability that a human will suffer an adverse health effects due to the ingestion of meat containing anti-bacterial residues at the injection-site?

Humans receive oral anti-bacterials routinely for the treatment and prevention of many infectious diseases. While side-effects to treatment have been reported with several anti-bacterials, most are quickly resolved on cessation of therapy. The dosages

employed in human medicines greatly exceed that likely to be present in edible tissues of treated animals, even at the site of injection. The type and volume of anti-bacterials used in animals in the EU and Switzerland are estimated by Boatman (1998) in Table 7-3 hereunder.

Table 7-3 Estimated volumes of anti-bacterials in the EU and Switzerland in 1997 by therapeutic group, reported by Boatman, 1998

Therapeutic group	Volume sold (tonnes of active substance)	Estimated total (% of total)
Penicillins	161	9
Tetracyclines	483	66
Macrolides	319	12
Aminoglycosides	92	4
Fluoroquinolones	32	1
Trimethoprim/sulphonamides	50	2
Others	91	5

The most prescribed group of drug substances are the tetracyclines. This group, while known to cause discolouration of tooth enamel in growing children, is not known for causing generally severe, acute toxicological or pharmacological effects in humans. Dewdney *et al* (1991), in a risk assessment of antibiotic residues of β-lactams and macrolides in food products stated that side-effects caused by macrolides are uncommon and hypersensitivity reactions are of no concern with regard to residues in animal produce. While drugs are foreign molecules, they are generally low molecular weight substances that are not inherently immunogenic. In relation to the β-lactams, the authors considered the risks of penicillin residues in food products to be twofold:

a) propensity for primary or '*de novo*' sensitisation,

b) adverse reactions in pre-sensitised individuals.

With regard to the immunogenic potential of β-lactams residues, the Dewdney *et al* (1991) concluded that there is a significant quantitative gap between the actual residue levels present in food products and the therapeutic and possibly sensitising dose levels resulting from clinical use. They stated that the oral route of exposure has been shown to be much less sensitising than the parenteral route. With regard to the allergenic potential the authors stated that it cannot be totally excluded that residues will evoke allergic reactions in some exceptionally highly sensitive individuals. Borrie and Barrett (1961) reported that penicillin may challenge highly sensitive individuals at doses as low as 3μg but clinical signs remain largely limited to transient skin rashes and urticaria. Nevertheless, Dewdney *et al* (1991) reported that although there have been a few well-documented cases of humans being sensitised or exhibiting allergic reactions that definitely can be traced to an animal drug residue, these cases are very rare (less than 10 cases reported in the last 25 years). They stated that because of the ubiquitous nature of penicillin-producing moulds in nature and the extensive use of β-lactam antibiotics in human medicine, it was unlikely that epidemiological studies could be undertaken that could allow quantification of the minimal risk of allergenic effects. Dayan (1993) supported these findings noting that

a) many antimicrobial substances can only act as effective immunogens by becoming haptens bound to a larger carrier and that these haptens are unlikely to persist in treated animals for more than a few days post treatment,
b) the oral route for immunisation in humans was relatively inefficient,
c) most examples of food-related reactions were of a transient and unrepeatable nature,
d) allergy to antimicrobial residues in the diet was exceeding rare, even though up to 7 - 10% of the human population had true allergenic sensitivity to 'penicillin' due to prior medical treatment.

Dayan (1993) concluded that, on quantitative grounds, exposure via food to free or bound antimicrobial residues was not expected to immunise naïve subjects or to excite an allergic reaction in those already sensitised.

The presence of an anti-bacterial in an injection-site will only produce an adverse effect in the consumer if it is present above a minimum concentration in the food product that is consumed. Current regulatory controls on the marketing authorisation of veterinary medicines require an evaluation of the adequacy of the withdrawal period as well as legal controls on the method of supply of each product. The assessment of the withdrawal period takes into consideration the presence of residues at the injection-site, which according to CVMP (1995) requires that where an MRL is set for muscle, that this MRL will also apply to residues at the injection-site. Where no MRL is set for muscle, the residue concentration in the specified 500g injection-site muscle must be below the acceptable daily intake for residue of the relevant drug. All injectable veterinary anti-bacterials in Ireland are licensed as prescription-only medicines, which ought to mean that the use of these products, including compliance with the stated withdrawal periods, is subject to the direction of a veterinary surgeon. Van Dresser and Wilcke (1989) in a survey of the causes of residue violations in the USA in 1985 cited the failure of the farmer to follow the withdrawal period in 69.1% of the investigations. Nouws (1996), reporting on the situation in the Netherlands, stated that 2 main sources of antimicrobial residues [in foodstuffs] could be distinguished:

a) contamination of animal feed with medicated feed,
b) failure to observe 'sufficient' withdrawal periods following therapeutic and/or prophylactic use of antimicrobials.

Given the increased consumer interest in residues in foodstuffs, together with governmental drug residue surveillance programmes, the licensing system for veterinary medicines and other regulatory controls on the sale, administration and recording of the use of veterinary anti-bacterials, the presentation of treated animals for slaughter before the expiry of the withdrawal period is, in the writer's opinion, unlikely.

The probability of adverse health effects in humans caused by the ingestion of meat containing anti-bacterial residues at the injection-site is, in the opinion of the writer, likely to be extremely low, albeit theoretically possible.

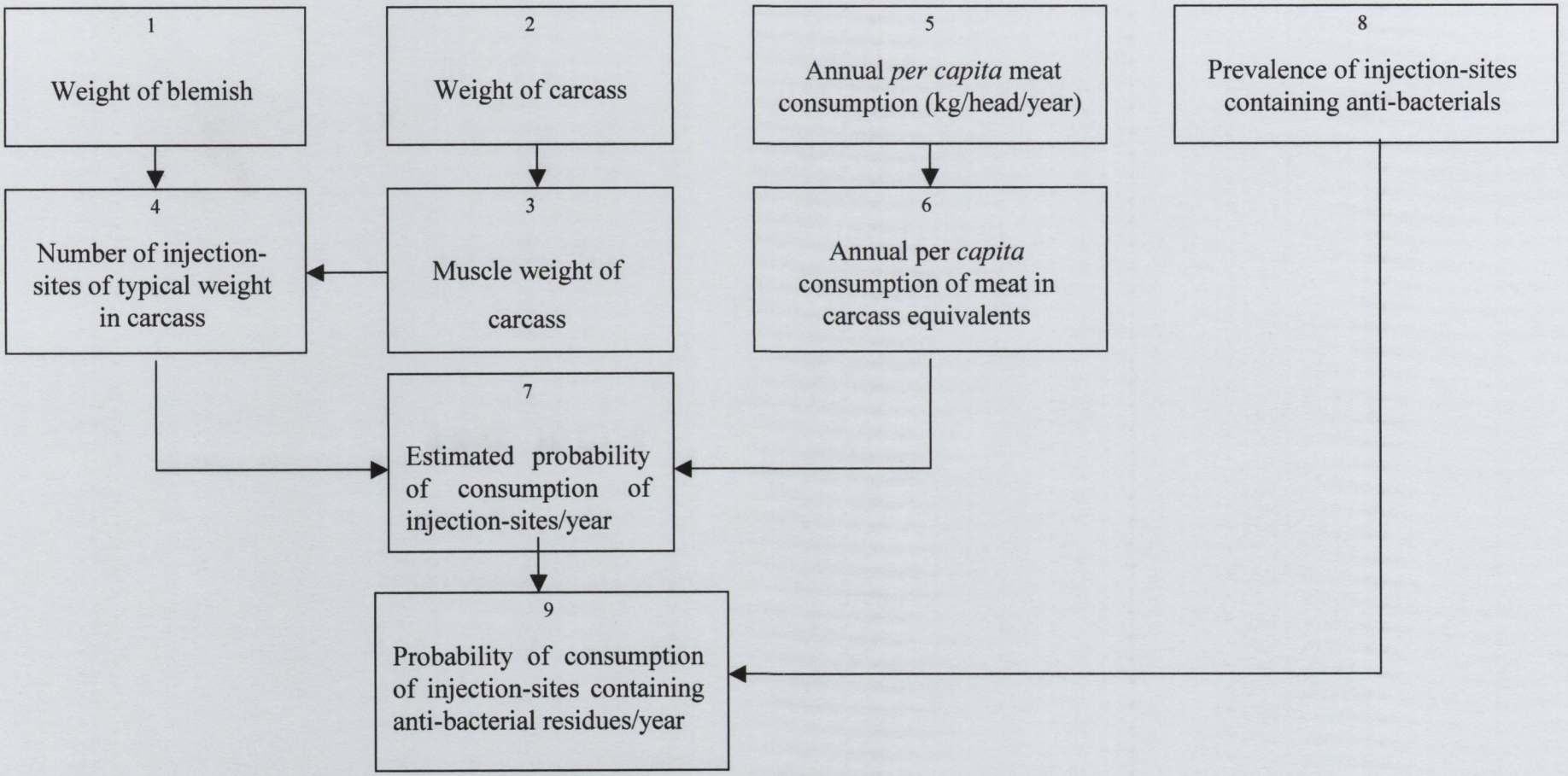
7.3 Quantitative risk assessment

At each step of the qualitative risk assessment the information available suggests only a low probability of the occurrence of the necessary step in the chain of events leading to the unwanted outcome. For several stages there are some data available to quantify the level, albeit with wide uncertainty limits. For others, only an estimate can be given due to lack of precise information. The model for the conduct of the assessment is outlined hereunder.

7.3.1 Risk model

The estimated risk of consumption of injection-site residues containing anti-bacterials is a function of the prevalence of injection-sites containing anti-bacterial residues in the species to be consumed, the size (weight) of the injection-site, and the quantity of meat from that species eaten. It is the outlined in Figure 7-1 hereunder.

Figure 7-1 Model for estimation of risk of consumption of injection-site residues containing anti-bacterials



Superscript numbers indicate data used for model input and are explained hereunder

[1] Weight of blemish: the input for this was from the average weight of meat discarded as unfit for human consumption as taken from the survey data described in Chapter 6. These weights were 290g for beef blemishes and 670g in respect of pork blemishes. As no blemishes were recorded in mutton primals or subprimals, the figure of 500g was taken as being intermediate between the values for beef and mutton. While definitive causality assessment between the presence of blemishes and drug induced damage has not been established, tissue damage caused by the administration of a veterinary medicine is the most plausible explanation for the blemishes recorded.

[2] The carcass weight was estimated based on expert opinion on typical Irish livestock (Matthews, 2000, personal communication; Griffin, 2000, personal communication) to be 320kgs, 26kgs and 72kgs in respect of beef, lamb and pork respectively.

[3] Muscle weight of carcass was estimated to be 60% of the total carcass weight (the remainder being largely made up of bone and fat). The total edible muscle per carcass was calculated to be 192kgs, 16kgs and 43kgs for beef, mutton and pork respectively.

[4] Number of injection-sites of typical weight in a carcass: the input for these values was calculated from the quantity of blemishes of average weight of 290g, 500g or 670g for beef, mutton and pork respectively in the total edible carcass muscle for each species. Theoretically there are 662 blemish sized muscle portions per beef carcass (192 ÷ 0.29); 32 per lamb carcass (16 ÷ 0.50) and 64 per pork carcass (43 ÷ 0.67). Therefore, assuming that the blemishes are not removed during processing the primal and subprimal meat cuts, the probability of the presence of a single blemish from a beef carcass is 1 in 662 = 0.0015 (0.15%). The probability of a single blemish from a lamb carcass being present in a carcass is 1 in 32 = 0.0313 (3.13%) and that for a pork carcass is 1 in 64 = 0.0156 (1.56%).

[5] Annual *per capita* meat consumption: the input for these values came from official government data (Bord Bia, 2000, personal communication) outlined in Table 7-4 hereunder.

Table 7-4 Reported *per capita* annual consumption of beef, mutton/lamb and pork

Meat type	Ireland*			EU*		
	1997	1998	1999	1997	1998	1999
Beef/veal	17.9 kg	18.5 kg	18.8 kg	18.9 kg	19.4 kg	19.6 kg
Mutton/lamb	7.2 kg	7.2 kg	7.2 kg	3.6 kg	3.7 kg	3.7 kg
Pork	38.6 kg	40.8 kg	43.0 kg	40.5 kg	42.8 kg	43.4 kg
Total	63.7 kg	66.5 kg	69.0 kg	63.0 kg	65.7 kg	66.7 kg

*Source GIRA Meat Club December 1999, An Bord Bia, Dublin (based on Eurostat)

[6] Annual *per capita* consumption of meat in carcass equivalents: the input for these values was calculated by dividing the value of the reported *per capita* consumption of meat by the estimated edible muscle weight of the carcass. Using the 1999 Irish and EU average consumption figures the results are given in Table 7-5 hereunder.

Table 7-5 Calculation of *per capita* consumption of carcasses

Meat type	*Per capita* consumption (kg/annum)		Calculation function	Weight of edible muscle per carcass (kg)	Calculated *per capita* carcass consumption *per annum*	
	Ireland	EU			Ireland	EU
Beef	18.8	19.6	÷	192	0.098	0.102
Mutton	7.2	3.7	÷	16	0.450	0.231
Pork	43.0	43.4	÷	43	1.000	1.009

[7] Estimated probability of consumption of injection-sites/annum: the input for this value was calculated by multiplying the calculated *per capita* carcass consumption value by number of injection-sites per carcass. The calculations are given in Table 7-6 hereunder.

Table 7-6 Calculation of risk of consumption of injection-sites/annum

Meat type	Calculated *per capita* carcass consumption *per annum*		Calculation function	Probability of presence of injection-site per carcass	Calculated probability of consumption of injection-sites *per annum*	
	Ireland	EU			Ireland	EU
Beef	0.098	0.102	X	0.0015 (1 in 662)	1.47×10^{-4}	1.53×10^{-4}
Mutton	0.450	0.231	X	0.0313 (1 in 32)	0.0141	7.23×10^{-3}
Pork	1.000	1.009	X	0.0156 (1 in 64)	0.0156	0.0157

EU = European Union

[8] Prevalence of injection-sites containing anti-bacterials: The input for these values came from the analysis for the presence of inhibitory substances of 2367 blemishes originating in Irish beef carcasses and 76 blemishes originating from Irish pork carcasses during 1999 and early 2000. A survey of more than 27000 sheep, conducted during 1999 on carcasses that were boned out did not reveal the presence of any blemishes. These data have been reviewed in Chapter 6. Positive results to the EU 4-plate-test were recorded for 9 blemishes from beef samples (prevalence rate of 1 in 263 or 0.38%) and 1 from pork samples (prevalence rate of 1 in 76 or 1.32%). Okerman *et* al (1998) have reported that the 4-plate test may not detect residues of ceftiofur, sulfonamides, streptomycin and some macrolide anti-bacterials. While this test is used as the routine surveillance test for anti-bacterial residues in Ireland and is expected to indicate the presence of both sulphadimidine (being a positive control disc on plate 2 of the 4 plates) and streptomycin (being a positive control disc on plates 3 and 4 of the 4 plates), for the purposes of the model, a safety factor of 10 is used i.e. the actual prevalence is assumed to be 10 times that found. Therefore, the prevalence figures used in the model are 1 in 26 for beef blemishes and 1 in 8 for pork. Also for the purposes of the model, a prevalence rate of 1 in 15 for mutton was taken, being an intermediate value between that of beef and pork.

[9] Annual probability of consumption of injection-sites containing anti-bacterial residues: This was derived by multiplying the figure for the prevalence of injection-sites containing anti-bacterials by the figure for the probability of consumption of injection-sites/year as outlined in Table 7-7 hereunder. These figures are, in the opinion of the writer, conservative values as the blemishes were destined for rejection as unfit for human consumption on aesthetic grounds.

Table 7-7 Calculated probability of consumption on injection-sites containing anti-bacterial residues

Meat type	Prevalence of injection-sites containing anti-bacterials ‡	Calculation function	Calculated probability of consumption of injection-sites *per annum*		Calculated probability of consumption of injection-sites containing anti-bacterial residues *per annum* ‡	
			Ireland	EU	Ireland	EU
Beef	0.038 (1 in 26)	X	1.47×10^{-4}	1.53×10^{-4}	5.59×10^{-6}	5.81×10^{-6}
Mutton	0.067 (1 in 15)†	X	0.0141	7.23×10^{-3}	9.45×10^{-4}	4.84×10^{-4}
Pork	0.125 (1 in 8)	X	0.0156	0.0157	1.95×10^{-3}	1.96×10^{-3}

EU = European Union

‡ includes a safety factor of 10, in the event that the routine monitoring analytical method underestimated actual prevalence

† this is not a value based on actual data in mutton but is an estimate, being an intermediate value between that for beef and pork

7.4 Discussion

The probability of consumers consuming injection-sites containing anti-bacterial residues ranges between 1.95×10^{-3} and 5.59×10^{-6} per year, (once per year in 1 in 500 to 1 in 200,000 of the population) depending on the species eaten and the dietary habits of the individual. These probability calculations have been established conservatively by the use of safety factor of 10 in their derivation. This factor was chosen on the basis that the standard analytical test for anti-bacterial residues applied to meat samples in Ireland and other EU Member States might not be sufficiently sensitive to all veterinary anti-bacterials. This factor is probably unduly conservative, given the fact that tetracyclines, the most widely used veterinary therapeutic anti-bacterials in the EU (FEDESA, 1999) being 64% of the total administered to animals, were detected at concentrations of 100µg/kg (being the MRL for tetracylines in muscle in the EU) or lower in the review of the efficiency to the EU 4-plate-method conducted by Okerman *et al* (1998). However, the macrolide anti-bacterials, which account for 12% of the total used for therapeutic purposes were not all readily detected in the studies conducted by Okerman *et al* (1998).

While the prevalence of blemishes used in the calculated predictions has been based on the prevalence of blemishes intended for discard and not for human consumption, Korsrud *et al* (1994) found that visual inspection of muscle surfaces did not reliably reveal all injection-site lesions in yearling beef steers which had received intramuscular or subcutaneous injections of penicillin G. It is therefore, in the opinion of the writer, most probable that some injection-site blemishes are not screened out during routine quality control checks in busy production lines in meat factories. Even the subsequent butchering procedure may not detect the presence of deep localised lesions. Nevertheless, the actual prevalence in portions presented for sale is, in the opinion of the writer, probably much less than that used in the calculations.

The weight of the blemishes sampled (290g for beef, 670g for pork) were the weights of total trim loss; in fact the blemishes were often localised and well demarcated and more careful dissection, which was not possible in a busy production environment, would have resulted in a lower trim weight. From a risk assessment viewpoint, the

lower the weight taken for calculation purposes, the lower the overall probability of consumers ingesting an injection-site. In the samples analysed, care was taken to use tissue from the central core to maximise the probability of finding a residue; it is highly probable in the writer's opinion, that if the total sample had been homogenised prior to analysis the concentration of residue would have been reduced, possibly even to undetectable levels.

Other calculations that would reduce the probability of exposure are possible such as the proportion of portions with 0% to 100% residue and the proportion of injection-sites degraded by cooking (although some drugs are not degraded by cooking as is found in Chapter 5). By contrast, should the consumer selectively eat rump or neck muscles (the areas into which injections are mainly given to treated animals in Ireland), the probability of exposure to an injection-site blemish would increase by, in the writers opinion, a factor of 3 (these tissues being estimated to be one third of the total edible carcass muscle.

The factor of 60% used to estimate the weight of muscle in the carcass may be an underestimate but comes from expert opinion (Matthews, 2000, personal communication). A 68 - 70% target of salable meat is the accepted beef industry norm, but this value includes the fat covering the meat portions, as well as gristle and associated tissue. In respect of mutton and pork, it is possible that this value may be an underestimate. The 60% figure chosen was reasonable, if conservative, in the writer's opinion; a few percentage points around the value would not have a marked effect on the probability calculations given.

Consumers are unlikely to choose to eat meat from a single species only and therefore the actual probability of ingestion of injection-site residues is the summation of probabilities of each species. Consumers now enjoy greater varieties of meat products in their diet including game, farmed and wild fish, as well as exotic meat including venison, rabbit and bison. The EU wide per capita consumption of poultry meat for 1999 was 20.99kg (Bord Bia, 2000, personal communication). Poultry, fish and wild game are unlikely to receive injectable anti-bacterials and therefore the estimated risk (i.e. the probability of eating residues of consequence) is low albeit with wide

confidence intervals. However, injection-sites are a significant quality control issue, especially for the beef industry.

While not directly comparable insofar as the calculations reported above relate to the risk assessment for consumers of injection-sites containing anti-bacterial residues, Galer and Monro (1996) reported that there was only a remote chance of a consumer ingesting an injection-site. They estimated that the frequency of human exposure to injection-sites was approximately 0.01 - 0.1% or, for a daily meat eater, 1 day every 3 - 30 years. Stephany (1988) estimated the probability of a consumer ingesting an injection-site from beef during a lifetime at 1 in 21 persons. These data support the conclusion that the probability of a consumer ingesting an injection-site containing an anti-bacterial residue is remote.

CHAPTER 8: OVERALL CONCLUSIONS

8.1 Introduction

In this chapter the results of the previous chapters are reviewed in the context of their overall significance for public health and in relation to their contribution to the primary objective of the research i.e. the risks of ingestion, by consumers, of injection-sites containing anti-bacterial drugs. Furthermore, strategies for blemish reduction and residue avoidance are explored and proposals for further research made.

8.2 Conclusions on analytical method development, validation, and cross-species performance

The studies on analytical method development demonstrated the complexities of achieving analytical methods for determining the residues of tilmicosin, enrofloxacin and tiamulin in muscle that had acceptable performance characteristics over the desired concentration ranges. The importance of method validation has been clearly demonstrated in the application of these residue methods to:

a) the comparison of individual method performance in cattle, sheep and pigs,
b) the mapping of residues in the animal musculature,
c) the effect of cooking on residues at the site of injection.

It is important that all research studies involving analysis of drug residues are adequately characterised by appropriate validation studies in order that the conclusions of the studies are reproducible and can be properly evaluated. This is not to say that all methods should meet the demanding regulatory validation requirements such as those of VICH that may be beyond the capabilities of many universities and other research departments and may not be necessary. The purpose of the method should be the foremost consideration in method development. In the studies conducted, the validation data demonstrated that the methods exhibited the performance characteristics required to accurately detect and quantify the analytes at the concentrations of interest. Regarding the performance characteristics of the individual methods, it is noted that the bioassay method had lower regression

coefficients than either of the chemically-based assays and that specificity was not fully demonstrated. However, as reported by O'Ranger's and van Ginkel (2000) this does not mean that bioassay methods are not useful and indeed as noted by Okerman *et al* (2000) they may have a more useful application in their use across the species barrier. While HPLC methods may give better accuracy and selectivity by reason of their specific chromatographic operational conditions, these same conditions serve to limit their potential use in different species tissue matrices. It is concluded that, under the conditions of the studies conducted, the analysis of the marker residues in muscle of cattle, sheep and pigs is essentially similar and that the performance characteristics of the methods used are not species dependant. As far as the HPLC assays are concerned, it appears to be important to demonstrate that there is no matrix interference at the retention time of the analyte of interest; however, any interference is likely to be easily removed by altering the chromotographical conditions used for the analysis. While muscle is a tissue not known to extensively metabolise substrates, further research is necessary in respect of other target tissues e.g. liver and kidney in order to determine to the extent to which cross-species validation is scientifically justifiable. Nevertheless, as the major edible tissue, the studies on muscle are important especially in the context of lack of availability of veterinary medicines for the so-called 'minor' species of food animals e.g. goats, deer, rabbits and horses. These studies demonstrate that provided some limited knowledge on the conduct of an analytical method is available for muscle in one species, it is likely to be easily adaptable to another species and to have comparable performance in that species. Bio-assays are likely to be even more useful in this regard as the limitations associated with the specific clean-up and chromatographical conditions do not apply.

8.3 Conclusions on distribution of residues in muscle

The distribution of residues in muscle has been demonstrated to be subject to marked variation, both between individual muscles and within the same muscle. The public health significance of these findings, which occur with all 3 drugs studied and accord with similar findings on veterinary drugs by Rose *et al* (1995b, 1996), Takahashi *et al* (1989), Ekström and Slanina (1982), Jansen *et al* (1989) and in respect of drugs found in human cadavers and reported by Williams and Pounder (1997), Pounder and

Jones (1990) and Langford *et al* (1998), is highly important as it challenges the assumption that residues are homogenously distributed in the musculature of animals and that sampling for residues in one muscle is representative of the residue concentration in the whole carcass. While further studies are necessary to investigate these findings in relation to other veterinary drugs, it would appear prudent for investigators conducting residues studies, on the basis of current information, to include samples from several separate muscle sites and to homogenise them prior to analysis. Given the fact that in the studies conducted animals were slaughtered at an interval chosen to yield high residues and that this does not represent the likely farm situation where animals are not slaughtered until after the withdrawal period for the particular product, it is suggested that further residue depletion studies be undertaken at time points closer to the designated withdrawal periods to ascertain whether the variances identified persist or change.

8.4 Conclusions on cooking studies

The data demonstrates that boiling of meat containing incurred residues of tilmicosin, enrofloxacin and tiamulin leads to a reduction in the concentration of drug residues present in the raw meat of 12 – 46%. The effect was more pronounced when meat was microwaved being a reduction of 0 – 17%. Cooking methods and processes are quite diverse and while the studies conducted reflect the 2 primary elements of dry and moist cooking, other cooking processes such as frying, roasting or cooking with alcohol or spices might produce different results. In the studies conducted, the meat samples were homogenised to avoid the formation of meat juices and to facilitate the interpretation of the effect of the cooking process on residue concentrations present. In practice, with the exception of mince and burgers, meat is rarely homogenised prior to cooking and therefore the possibility of the residue being present in the gravy merits further investigation. From a public health point of view, cooking should not be relied upon to reduce the risk of residues; the cooking process itself will result in a loss of moisture in the meat and this effect is likely to reduce or negate any reduction in the residue concentrations in the tissue. Indeed, the residue concentration may even increase relative to the tissue weight, as has been demonstrated with residues of tilmicosin in beef which reached 145% of that present in the precooked samples.

Moreover, the cooking process may result in the degradation of the residue into metabolites, which have not been toxicologically investigated.

It is therefore concluded that current international policy to express MRLs on a fresh meat basis should not be changed.

8.5 Conclusions on study on prevalence of blemishes

The survey of blemishes in primal and sub-primal meat portions from Irish livestock was quite comprehensive in that it comprised data from each of the major livestock species over a one year period and represents the produce of farms throughout the Republic of Ireland. The data demonstrate that while blemishes are not prevalent to a high degree in respect of pork and mutton, they are present in beef primals and subprimals at a rate comparable to that reported by George *et al* (1997) and Van Donkersgoed *et al* (1998). The following conclusions are reached:

a) blemishes are a significant problem in beef cattle affecting 1 in 5 carcasses or 5% of quarters,
b) blemishes are mainly confined to the hind quarters in beef,
c) blemishes are mainly fibrous or fatty fibrous in nature in beef and suggestive of historic administration of veterinary medicinal products,
d) blemishes in pork carcasses are present only to a minor degree (2.2% of carcasses) and mainly involve small abscesses (78% of lesions detected),
e) no blemishes in lamb carcasses were found,
f) the number of blemishes which contain anti-bacterial residues that show a positive result on the industry-wide standard test for inhibitory substances is reassuringly low at a prevalence of 0.0002% (1 positive result per 5000 head slaughtered).

The data generated from the survey of injection-site blemishes in beef are a cause for concern as it is probable that some blemishes may go undetected in primal beef cuts released by the processor to the butcher. The average weight of each beef and pork trim loss associated with the presence of blemishes was 289.61g and 664.96g respectively. However, indirect losses due to the adulteration caused to the primal or sub-primal cut by the removal of the blemish in many cases causes the meat to be

downgraded to lower value portions due to knife and pockmarks. Further losses are also incurred in the labour required to screen and remove the blemishes. As noted by George *et al* (1997), the problem of injection-sites is primarily a quality control problem for farmers and the meat industry, rather than a consumer risk. However, the same authors report that steaks with injection-site lesions may have an undesirable taste and that the full cost of these blemishes must also include customer dissatisfaction. From the public health point of view, while blemishes are most unlikely to adversely affect public health, the presence of an injection-site is unacceptable in any prepared meal and may lead to consumers turning away from meat altogether. Likewise, the presence of a broken needle, albeit detected in a carcass at boning-out is a concern. The needle piece could have survived through further processing, it being unlikely that the metal detector would detect such a small piece of metal.

8.6 Conclusions on risk assessment of injection-site containing anti-bacterials

The actual risk of consumers suffering adverse effects due to the presence of anti-bacterials following their consumption in meat in practically negligible as evidenced by the probability of each of the several events necessary to allow for this to happen. The normal animal husbandry practices, record keeping of drug usage on farms and residue surveillance make it unlikely that animals are presented for slaughter with residues at concentrations above permitted maxima although some farmers may knowingly or unknowingly present animals for slaughter before the expiry of the withdrawal period as was previously reported in the USA by Van Dresser and Wilcke (1989). The quality control systems operated at meat production and packaging facilities, where any lesions are discarded as being unfit for human or animal consumption, also serve to screen out potentially harmful residues that may be present, although it is likely, in the opinion of the writer, that some deep lesions go undetected at this point in the supply chain. The probability that the consumer would suffer harmful effects as a result of consuming residues at the site of injection appears remote, given the fact that the occasional ingestion of a residue above the MRL is likely to be well below the dosage for xenobiotics in humans and is not likely to be repeated within a short time. As MRLs are established on the presumption on lifetime

exposure to the residue at the permitted maxim, only for a minority of drug substances (those which have an acute pharmacological or toxicological potential), will residues at the injection-site present a risk to the consumer. In this context, it is interesting to note the report of the EU's Scientific Steering Committee (1999) quoting the UK Prescription Pricing Authority, wherein it is stated that in 1996, in England and Wales sufficient oral anti-bacterials were prescribed annually in the community to treat every member of the population for 5 days a year. This contrasts to the result of the quantitative risk assessment wherein the conservatively estimated exposure to anti-bacterials present in injection-sites was estimated as once per year in 1 in 500 to 1 in 200,000 of the population. Concerning substances with allergenic potential, the oral route of exposure in humans, which is reported by Dewdney *et al* (1991) to be unlikely to have any immunogenic effect, together with the non-recurring nature of any exposure, make any risk of adverse health effects improbable for consumers.

8.7 Strategies for blemish and residue avoidance

In the USA and in Canada, industry led quality control and educational programmes, such as that outlined by George *et al* (1997), have been undertaken in order to reduce the losses associated with trimming and customer dissatisfaction. There are several stakeholders in the campaign to eliminate or substantially reduce the prevalence of blemishes and the risks of injection-sites for the consumer. These include:

a) the animal producers (including all stages of the animals life from neonate to finished animal),
b) the veterinary and pharmacy professions and those licensed to sell injectable veterinary drugs and vaccines,
c) the meat industry,
d) farm advisory bodies and educators,
e) residues surveillance bodies,
f) consumers and buyers of meat,
g) the pharmaceutical animal health industry.

While not the purpose of this dissertation and therefore not intended to be exhaustive, the essential roles of each stakeholder are considered briefly in Sections 8.8.1 – 8.8.7 hereunder.

8.7.1 The role of the animal producer

Producers of food-producing animals have the dominant responsibility in ensuring that veterinary medicines and vaccines are used under proper professional guidance, only when necessary and are administered correctly. George *et al* (1997) and Van Donkersgoed *et al* (submitted for publication), recommended that cattle producers should avoid all intramuscular injections. It is also suggested by Van Donkersgoed *et al* that when intramuscular administration is necessary, products that cause minimal tissue damage should be used, no more than 10ml be administered per injection-site, and products be administered in the neck rather than in the hind quarters.

In the Irish context, the intramuscular administration of vitamins and mineral preparations would appear to be unnecessary given the fact that several equally efficacious products are available for use by the oral route. When parenteral treatment is deemed essential, the intravenous route of administration, if authorised, is preferred. The use of long-acting formulations and irritant vehicles would appear to increase the likelihood of injection-site blemishes and consideration should be given to their reduction and replacement by alternative therapies in the care of livestock. The need for adequate record keeping and identification of animals is fundamental in any control strategy. Parenteral drugs and vaccines should always be given using a sterile needle and a new needle used for each animal. Animals should be properly restrained during the injection; where a part of a broken needle remains in the animal, the identity of the animal should be recorded and the location of the injection made known to the meat abattoir. Better integration and education of the calf and weaner producers into the finished animal production chain is necessary to ensure that those who cause damage to muscle tissue in young animals are not ignorant of the blemishes seen in the primals and subprimals of the finished animal.

8.7.2 The role of the veterinary and pharmacy professions and those licensed to sell injectable veterinary drugs and vaccines

Veterinarians play a key role in the provision of diagnoses, husbandry and veterinary advice, in farmer education on the correct storage, use and administration of medicines and vaccines, and in the administration of products themselves. Others, including pharmacists and outlets licensed to sell veterinary medicines also play a role in ensuring that medicines and vaccines are supplied with the necessary syringes and appropriate needles and the advice on aseptic technique in administering parenteral injections is given. A code of good practice in the use of veterinary medicines is recommended and consideration should be given to developing a list of medicines that do not cause persistent lesions. George *et al* (1997) noted the critical role of veterinarians in quality assurance in the beef industry in the USA but stated that they must also participate in quality assurance workshops to fully understand the damage that can be caused by intramuscular injections.

8.7.3 The role of the meat industry

It is suggested that the industry should conduct periodic audits on the prevalence of blemishes in meat, including all classes of animals, so that the market classes that lead to the majority of blemishes can be identified and to monitor the effectiveness of existing quality control programmes. The industry should liaise with farm advisors and veterinarians to ensure that current quality assurance programmes are being followed and that the pivotal steps to avoid blemishes are being taken by farmers. The mandatory site for intramuscular injections should be the neck muscles and penalties should be introduced to reduce the occurrence of blemishes in rump muscles which are less easily detected on meat inspection.

8.7.4 The role of farm advisory bodies and educators

An educational campaign to increase awareness among farmers and veterinarians on Good Veterinary Practice in the use of veterinary medicines is needed and an integrated quality control system from birth though to processing put in place. This campaign should focus on the use of lowly irritant products, aseptic injection technique, on the administration of veterinary drugs away from the rump and high value meat cuts and on the importance of record keeping.

8.7.5 The role of residues surveillance bodies

Residue surveillance bodies need to ensure that the surveillance studies are properly conducted according to Good Laboratory Practice and the results are widely published. The need for updating the EU 4-plate-method test in accordance with the suggestions of Okerman *et al* (1998) would appear necessary or, alternatively, the use of additional assay batteries considered. The use of these assays should be based on the sales volumes of the drugs used and their risk for human health.

8.7.6 The role of consumers and buyers of meat

Customers have to set strict requirements to ensure that imperfection and mediocre standards among certain individuals in the use of veterinary drugs and vaccines are not tolerated. They should ensure that properly audited quality monitoring systems are in place to achieve this and proportionate blemish and residue surveillance programmes are instigated.

8.7.7 The role of the pharmaceutical animal health industry

Pharmaceutical companies and vaccine manufacturers must identify excipients and solvents that are cause minimal or no tissue damage and develop 'tissue friendly' products. Irritant formulations should be identified and removed from sale. Alternative delivery systems or routes of administration that can replace the more irritant products should be investigated. In this regard, the application of aerosol therapy, such as that proposed by Lekeux (2000) and which would remove the problem of injection-site blemishes is worthy of further research.

8.8 Governmental regulatory policy on injection-sites in the establishment of withdrawal periods

Based on the results of this dissertation, it is suggested that established scientific policy such as that of CVMP (1995), which advocates the application of the MRL for muscle tissue to injection-site residues, does not represent the true tissue distribution of the drug and is, from a risk assessment viewpoint, counter-productive as the long withdrawal periods are unnecessary for the protection of public health and might not be followed. The designation of an acute reference dose for those products with an acute pharmacological or toxicological effect would appear to be the preferred approach based on risk assessment. In respect of products that do not have an acute

pharmacological or toxicological effect, an alternative approach appears necessary, possibly based on a comparison of injection-site residue concentration to the ADI. However, it also appears important to regulate against products causing persistent lesions at the site of injection.

8.9 Proposals for further studies

A study on the prevalence of injection-site lesions in beef derived from cows would be a useful addition to the knowledge gained, especially as the carcass weight of such animals is much less that that for prime beef and therefore the risk to consumers will be somewhat greater. Further studies on the prevalence of injection-sites in other European countries would also be useful for comparative purposes.

The residue mapping studies on muscle distribution should be extended to other veterinary drugs and using animals slaughtered at a time nearer the recommended withdrawal period to establish whether it is feasible to develop a policy for targeting residue surveillance programmes at particular carcass muscles.

Additional studies on the development, application and validation of residue analytical methods should be conducted to assist in the developing of science based policies to counteract the crisis in the availability of veterinary medicines for minor food-producing species in Europe.

A survey of farmers and veterinarians on their use of veterinary drugs and vaccines should be conducted so that more information is available on the possible products and practices which cause the greatest tissue damage.

Further research into the application of existing human delivery systems to animals is necessary as well as research into delivery systems in animals that do not cause persistent residues at the site of injection. In particular, the use of aerosol delivery systems merits serious consideration.

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