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DEVELOPMENT OF CONFIRMATORY METHODS AND DISPOSITION OF SOME VETERINARY DRUGS IN FOOD

ONTWIKKELING VAN BEVESTIGINGSMETHODEN EN UITSCHEIDING VAN BEPAALDE DIERGENEESMIDDELEN IN LEVENSMIDDELEN

door

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Abbreviations

Abbreviations

a	slope
AB	albendazole
AB-SO	albendazole sulphoxide
AB-SO_2	albendazole sulphone
ADI	acceptable daily intake
AL	action limit
AME_n	apparent metabolisable energy, corrected for N-retention = 0
APCI	atmospheric pressure chemical ionisation
b	intercept
BIA	biosensor immunoassay
BW	body weight
BZ	benzimidazole
C	concentration
CCα	decision limit
CCβ	detection capability
CF	concentration factor
CID	collision induced dissociation
CP	crude protein
CRL	community reference laboratory
CRM	certified reference material
CTC	chlortetracycline
CVMP	Commitee for Veterinary Medicinal Products
DAD	diode array detector
DC	doxycycline
DHP	4,6-dimethyl-2-hydroxypyrimidine
DNC	4,4’-dinitrocarbanilide
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EMEA	European veterinary Medicines Evaluation Agency
ESI	electrospray ionisation
FB	fenbendazole
FB-SO_2	fenbendazole sulphone
FE	febantel
FE-SO	febantel sulphoxide
FL	flubendazole
GC	gas chromatography

GC-MS	gas chromatography mass spectrometry
GLP	good laboratory practice
HCl	hydrochloric acid
HFL	hydrolysed metabolite of flubendazole
HME	hydrolysed metabolite of mebendazole
HPLC	high performance liquid chromatography
ICTC	isochlortetracycline
IP	identification point
IPC	ion-pair chromatography
IPL	internal performance limit
IS	internal standard
LC	liquid chromatography = high performance liquid chromatography when used in combination with mass spectrometric detection
LC-MS	liquid chromatography mass spectrometry
LC-MS/MS	liquid chromatography tandem mass spectrometry
LD_{50}	lethal dose
LE	levamisole
LLE	liquid-liquid extraction
LOD	limit of detection
LOQ	limit of quantification
ME	mebendazole
M_r	relative molecular mass
MRL	maximum residue limit
MRM	multiple reaction monitoring
MRPL	minimum required performance limit
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSPD	matrix solid phase dispersion
m/z	mass-to-charge
n	number of experiments
NCB	nicarbazin
NH_2-AB-SO_2	albendazole 2-aminosulphone
NOEL	no-observable-effect-level
NRL	national reference laboratory
OB	oxibendazole
OF	oxfendazole
OTC	oxytetracycline
PTFE	polytetrafluorethylene

rpm	rounds per minute
QA	quality assurance
r	correlation coefficient
RC	regenerated cellulose
RFL	routine or field laboratory
RFL	reduced metabolite of flubendazole
RI	relative intensity of ions
RIA	radioimmunoassay
RME	reduced metabolite of mebendazole
RRT	relative retention time
s	sample
s	standard deviation
s_r	relative standard deviation
SIM	selected ion monitoring
SMT	sulfamethazine
SPE	solid phase extraction
S/N	signal-to-noise
TB	thiabendazole
TC	tetracycline
TC	triclabendazole
TCA	tetracycline antibiotic
TLC	thin layer chromatography
TRL	maximum acceptable total residue level
UV	ultra violet light
WHO	World Health Organisation
4-epi-CTC	4-epimer of chlortetracycline
4-epi-DC	4-epimer of doxycycline
4-epi-ICTC	4-epimer of isochlortetracycline
4-epi-OTC	4-epimer of oxytetracycline
4-epi-TC	4-epimer of tetracycline
6-ICTC	6-isochlortetracycline

CHAPTER 1

General introduction

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Residues of veterinary drugs in food

1.1 The origin of residues in food

1.1.1 Introduction

Antimicrobials were first used in veterinary medicine for the treatment of mastitis in dairy cows shortly after they were developed.[1] Veterinary medicinal agents are generally used in veterinary medicine for three purposes:[2]

1. Therapeutic treatment of active infection or prophylactic treatment in situations that induce high susceptibility using antibiotics and parasiticides
2. Vaccination of prophylactic medication to prevent or to minimise infection
3. Production enhancement, growth promotion and improvement of feed efficiency with antibiotic drugs and hormones

According to these purposes, approximately 42% of all veterinary pharmaceuticals used worldwide are used as feed additives, 19% are used as anti-infectives (e.g. antibacterials, antifungals and antivirals), 13% as parasiticides (e.g. anthelmintics), 11% are used as biologicals and 15% represent other pharmaceuticals.[1]

According to their chemical structure, the most commonly used antimicrobials in food-producing animals can be grouped into five major classes. These include the β-lactams (e.g. penicillins and cephalosporins), tetracyclines (e.g. oxytetracycline, tetracycline and chlortetracycline), aminoglycosides (e.g. streptomycin, neomycin and gentamicin), macrolides (e.g. erythromycin) and sulfonamides (e.g. sulfamethazine).

The most frequently used anthelmintics can be divided into two major classes. These are the benzimidazoles (e.g. oxibendazole, flubendazole and mebendazole) including the tetra-hydro-imidazole levamisole and the macrocyclic lactones (e.g. avermectines as eprinomectine and milbecynes).

1.1.2 Improper or illegal use of veterinary drugs

The administration of any pharmacologically active chemical to a food-producing animal can lead to the occurence of residues in food. Violations can occur as a result of improper use of licensed products or through the illegal use of unlicensed substances.[3] Several factors have contributed to the residue problem such as poor treatment records or failure to identify treated animals, but most violations result from the use of a drug in an inconsistent manner with the product label. This occurs mainly by neglecting withdrawal periods before putting the milk and the eggs on the market or before slaughtering of the animal. Treatments involving any other application than stated on the product label (e.g. different species, increased dosage, different route of administration, different frequency of treatment) are classified as off-label usage, in which case withdrawal times are difficult or impossible to determine.[1]

Providing that a licensed drug is used in accordance with its product licence and providing that the drug withdrawal periods are respected by the farmers, drug residues should not occur in human food in harmful concentration. However, some contamination processes have to be taken into account.

1.1.3 Contamination of feed

Cross-contamination in feed mills is a recognised problem.[3] Residual quantities of medicated feed may be retained at various points along the production line, contaminating subsequent batches of feed as they are processed. The electrostatic properties of some drugs, particularly those in powder form, aggravate the problem, making it more difficult to purge the equipment between batches. Manufacturers have responded to the problem by producing granular preparations with reduced electrostatic properties. It has been reported that the carry-over of sulfamethazine (SMT) in feed was four times higher with powdered water-soluble preparations than with granular forms of the drug. The preparation of nicarbazin-free withdrawal diets can be hindered since nicarbazin (NCB) powder is strongly electrostatic.[4] NCB is a widely used anticoccidial drug, which is licensed in Belgium as a feed additive for broiler chickens, but not for laying hens. There is a problem in many countries world-wide with the occurrence of NCB residues in poultry tissues and eggs. Cannavan *et al.*[4] have reported a proportional relationship between the concentration of 4,4'-dinitrocarbanilide (DNC) and 4,6-dimethyl-2-hydroxypyrimidine (DHP) in eggs, the two marker residues for nicarbazin, and the feed levels. Feed contaminated with NCB at concentrations higher than about 2 mg/kg gave rise to residue concentrations of DNC in eggs higher than 100 µg/kg. Cannavan and Kennedy[5] have reported the occurrence of violative residue concentrations for DNC, as marker residue for nicarbazin, in tissues of broiler chickens due to feed contamination. No maximum residue limit (MRL) has yet been established by the EU for nicarbazin in tissues. Residue concentrations above 200 µg/kg may occur in muscle tissue of broiler chickens not exposed to nicarbazin during rearing, but fed with withdrawal rations accidently contaminated at at least 2.4 mg/kg.

1.1.4 Environmental contamination

Potential environmental contamination may arise e.g. from commercial fish farming where veterinary drugs, given as feed additives, accumulate in sediments and may be ingested by marine species in the vicinity of farms.[3] A large portion of the medicated feed is not eaten or not digested by the farm fish. It is spoiled and falls through the holding cages on the seabed. Relatively large quantities of veterinary drugs can contaminate the surrounding environment and can be absorbed by other marine animals and fauna. The presence of drug residues may pose a risk to health, if these wild species are harvested for human consumption.

1.1.5 Animal-to-animal transfer

Recycling of drugs as a result of ingestion of faeces and/or urine significantly contributes to the concentration and persistence of residues in porcine tissues, poultry tissues and eggs.[3,5,6] Brief exposure of unmedicated animals to the excretions of medicated animals in improperly cleaned housing, perhaps during transport or in the lairage of a slaughter house can result in violative residues. With banned compounds, having zero tolerance, it is obvious that severe problems can arise.

Cannavan and Kennedy[5] have observed a tenfold higher DNC concentration in liver of broiler chickens treated with NCB and housed on litter compared with those housed on wire flooring. Kennedy *et al.*[3] have studied the faecal recycling of NCB in laying hens. DNC concentrations above 2.0 µg/kg in the eggs were obtained up to 16 and even up tot 60 days after withdrawal of NCB medication at feed contamination levels (2.3 mg/kg) of caged hens and of hens kept on a deep litter, respectively. Complete exchange of the litter was the only way to prevent recycling.

1.2 The risks to human health and drawbacks

1.2.1 Toxicity and allergenicity

Quite a large number of antibiotic and anthelmintic drugs administered in therapeutic and subtherapeutic form to domestic animals are also approved for human use. These drugs have been shown to be relatively safe. Acute and chronic toxicities have been evaluated and are well documented.[2] In most cases, the amount ingested by an individual who consumes the drugs as residue in animal foodstuffs will be considerably less than that consumed as a primary drug. The likelihood of acute toxicity from veterinary drugs or their metabolites originating from animal tissues is extremely low. However, the possibility of chronic toxicity expressed in longterm, cumulative allergenic, mutagenic, teratogenic or carcinogenic effects, which are difficult to assess, may occur.

Veterinary drugs with high potential to cause one or more of these toxic effects for human health were banned by the EC for veterinary use. Chloramphenicol produces toxic aplastic anemia that is not related to dosage. Chloramphenicol has been implicated as the causative agent in several cases of fatal aplastic anemia after its use as an ophthalmic drug at an estimated total dose of 82 mg only. Nitrofurans and nitroimidazoles require restrictions as carcinogens, mutagens or inducers of DNA synthesis. But the inherent hazards of their genotoxicity could be overcome by appropriate drug use and by application of a two- or three-fold increased conservative withdrawal time. Sulfonamides have been used widely at subtherapeutic and therapeutic concentrations in food-animal production, but increasing concern over their carcinogenic and mutagenic potential and their thyroid toxicity has led to decreased use, longer withdrawal times and more intensive residue monitoring.

Carry-over of tetracycline residues can reversibly slow down the growth of the skeleton and irreversibly discolor the teeth of children younger than 8 years since tetracyclines are deposited in bones and teeth. The extractable residues of benzimidazole anthelmintics have a defined toxic potential.[7] Some benzimidazoles are mutagenic, but the effect is limited or low in mammals even at high doses. Teratogenicity may be considered as a general property of this drug group. Teratogenic metabolites have been identified and quantified in animal products as milk, eggs and meat. Long and Rupp[8] have reported limb effects associated with teratogenic activity in rats fed albendazole. For levamisole, the most important of the observed adverse effects, which are rare, were agranulocytosis and neutropenia.[9]

To safeguard human health, the EU has established safe maximum residue limits (MRLs) for residues of licensed veterinary drugs in animal tissues entering the human food chain. The term MRL may be defined as the maximum concentration of marker residue (e.g. parent compound, metabolites, etc.) resulting from the use of a veterinary drug, expressed in µg/kg, that is legally permitted or recognised as acceptable in or on food.[1] The MRL is based on the acceptable daily intake (ADI). The ADI is the result of a scientific risk assessment following pharmacological and toxicological studies. The ADI is determined by the no-observable-effect level (NOEL) taking into account a safety factor of usually 100.[10] The maximum acceptable total residue level (TRL) is calculated from the ADI considering the consumption pattern. The MRL is the detectable proportion of the marker residue that corresponds to the TRL value. The MRL value refers to the permissible level for the marker residue.

In addition to conventional toxicological effects, other effects such as the effects of drugs on the immune system and pharmacological effects including specific effects of residues of veterinary antibiotics on the human gut flora, should be taken into account considering safe residue levels.[11]

Besides the toxicological problems, hypersensitive reactions in humans from ingesting antibiotic-contaminated foods of animal origin can occur. Most reactions result from β-lactam (e.g. penicillin) antibiotic residues in milk and meat[1,2,12] in sensitive individuals. Many people that went through prior medical treatment were hypersensitised to such a degree that following oral exposure a response was evoked.

Nevertheless, the risk to human health due to residues of veterinary drugs in foodstuffs have to be relativated. Microbial contamination of food is a major health problem worldwide.[13] Infections of poultry products with *Salmonella* and *Campylobacter* are greater risks for public health than are residues of veterinary drugs in food.[14] Nevertheless, the public health risks of antibiotics and their metabolites in food are difficult to define, and the presence of violative levels of residues in food is illegal and therefore subjected to financial penalties in many countries. The consumer does not like food with veterinary drug residues, unlike the residue concentration is below the published human safety MRL level.

1.2.2 Antibiotic resistance

Antibiotic resistance is a well documented major health threat around the world that has been given high priority by many health agencies.[1,2,12,14] The potential for animal-to-human transfer of resistance is existing. Clearly, the use of antibiotics in lifestock production has been associated with the development of human antibiotic resistance. Animals fed with low (prophylactic) levels of antibiotics may develop bacteria evolving resistance to these and/or other drugs. Humans may be exposed to these bacteria during the preparation or consumption of food. It has been documented that humans developed drug resistant salmonellosis from food of animal origin.[15] Examples of drugs which have been shown to cause the growth of resistant bacteria in food animals are fluoroquinolones and avoparcin.

The resistance of microorganisms arising from subtherapeutic use of penicillin, tetracyclines and sulfa drugs in agriculture is suggested by the WHO to be a high priority issue.[2] Curbing the use of antibiotics in subtherapeutic disease prevention and growth promotion might offer the greatest opportunity to reduce the amount of antibiotics used in food-producing animals. The concept of the integrated control where an optimal animal treatment starts with an optimal housing and hygiene has to be promoted.[12]

1.2.3 Technological problems

The initial concerns with regard to antimicrobial residues in food were not expressed by consumers but by dairy processors who found that contaminated milk was inhibiting the starter cultures used in the production of fermented milk products as well as the influence on the results of the dye reduction tests used for milk quality.[1] The sensitivity of thermophilic and mesophilic starter cultures for manufacturing high-value fermented products as yoghurt, cheese, butter or raw sausages, needs biotechnological safe raw materials.[15]

1.2.4 Effect of processing on residues

Although most food of animal origin is heated before consumption, none of the required studies for licensing drugs include the effect of processing on residues. Information about this influence is required to obtain more accurate estimates of consumer exposure to residues or possible breakdown products. The fate of several drug residues during normal cooking and processing procedures is described by Rose *et al*.[17] Oxytetracycline was not stable but showed no apparent major breakdown products measurable by UV detection. Ellis[18] reported that tetracyclines are relatively labile to mild heating in acid solutions, which destroys the tetracycline ring structure. Residues of levamisole were found to be stable to heating, but a fraction was lost from the meat into the juice.[17] Some evidence of oxfendazole instability in boiling water was found after 3 hours. Heating of samples with incurred residues of oxfendazole nevertheless destroys the drug residues. Moats[19] reported that ordinary heating procedures for meat, even to “well-done”,

can not be relied on to inactivate even the more heat sensitive compounds such as penicillins and tetracyclines. More severe heating as for canning or prolonged cooking with moist heat will inactivate the more heat sensitive compounds. The relevance to food safety is uncertain since the nature of the degradation products is unknown in most cases. The identity of these degradation products should be established and their toxicity assessed.

The use of data generated from surveillance of raw tissue for dietary intake calculations and consumer exposure estimates has to be considered in the light of the effect of cooking on these residues.

1.3 Legislative aspects

1.3.1 Overview of EC Decisions, Council regulations and guidelines

The occurrence of residues of veterinary drugs is a world-wide problem. Food-producing animals and animal products are transported between the different EU countries and between EU countries and third countries. A lot of energy is spent by the EU to harmonise the European legislation to control the residue problems. This European legislation and the reference methods to analyse the animal products are summarised by Heitzman.[20] European legislation aspects are published in the Official Journal of the European Communities. The European Commission had regulated the inspection of animals and of fresh meat for the presence of residues of veterinary drugs and specific contaminants by Council Directive 86/469/EEC.[21] The current monitoring (paragraph 1.7) is governed by national surveillance schemes, established under Council Directive 96/23/EC[22] on measures to monitor certain substances and residues thereof in animal products and under Commission Decision 97/747/EC,[23] laying down levels and frequences of sampling in order to monitoring some substances and residues thereof in certain animal products. This EU Decision establishes numbers of samples to be tested for each compound group.

Quality criteria for the analysis of residues are described in Commission Decision 89/610/EEC[24] laying down the reference methods and the list of national reference laboratories for detecting residues. Quality criteria for residue analyses are also described in Commission Decision 93/256/EEC[25] laying down the methods to be used for detecting residues of substances having a hormonal or a thyreostatic action. Commission Decision 93/257/EEC[26] lays down the reference methods and the list of national reference laboratories for detecting residues. The two Decisions are revised regularly in order to take into account the current scientific knowledge and the latest technical improvements. The revised version was submitted by the Directorate General for Agriculture as draft Commission Decision SANCO/1805/2000[27] laying down performance criteria for the analytical methods to be used for certain substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC.[22] This draft

document was revised and published as Commission Decision 2002/657/EC[28] implementing Council Directive 96/23/EC[22] concerning the performance of analytical methods and the interpretation of results. The validation of analytical methods is discussed in paragraph 1.6.

The establishment of MRLs in the EU is governed by Council Regulation EEC/2377/90,[29] amended by several EC-Regulations. This regulation establishes lists of compounds that have a fixed MRL (Annex I), that need no MRL (Annex II), or that have a provisional MRL (Annex III).[30] Certain compounds, including some antibiotics, are listed in Annex IV of this Regulation, prohibiting their use in livestock production. Annex V provides information and data needed to determine the MRL values. Since 1 January 2000, the administration to food-producing animals of veterinary medicinal products containing pharmacologically active substances which are not listed in Annex I to III is prohibited. Tables 1.1 and 1.2 present the pharmacologically active substances, involved in this thesis, for food-producing animals including the established MRL values. The current status of 25 November 2002 (fixed or provisional) of the MRL values are indicated.

In addition to the protection of consumers from the potential risk of harmful residues, MRLs are also essential to facilitate international trade. However, the introduction of the MRL concept has a severe impact on the availability of veterinary medicinal products. The risks of the occurence of residues in food when food-producing animals are treated with unlicensed products or with products used in an off-label manner, has potentially increased.[31]

1.3.2 Belgian legislation

For the residue control of slaughtered animals, the kidney of the slaughtered animal is used. The Ministerial Decision[32] of 19 June 1995 describes the new Belgian kidney test which is a microbial inhibition test. The quality criteria including limits for residues of anti-infection and antiparasitic substances for consumption milk and milk products are stipulated in the Royal Decision[33] of 14 September 1995. The Ministerial Decision[34] of 6 October 2000 describes the official determination of the quality and the composition of milk delivered to buyers. This Decision refers to Council Regulation EEC/2377/90.[29]

The financial penalties imposed on producers who violate residue regulations are increasing. After successive violations a R-statute (R of residue) can be imposed, excluding the producer from the market for a certain time.

1.4 Pharmacokinetics of veterinary drugs

1.4.1 Introduction

Pharmacokinetics may be defined as the mathematical description of drug concentration changes in the body.[35] Pharmacokinetics in veterinary medicine has been used to describe the

Table 1.1 Anti-infectious agents (antibiotics)

Group	Pharmacologically active substance	Marker residue	Animal species	MRL (µg/kg)	Target tissues	Status
TETRACYCLINES	Tetracycline	Sum of parent drug and its 4-epimer	All food-producing species	100 100 200 300 600	Milk Muscle Eggs Liver Kidney	Fixed
	Oxytetracycline	Sum of parent drug and its 4-epimer	All food-producing species	100 100 200 300 600	Milk Muscle Eggs Liver Kidney	Fixed
	Chlortetracycline	Sum of parent drug and its 4-epimer	All food-producing species	100 100 200 300 600	Milk Muscle Eggs Liver Kidney	Fixed
	Doxycycline	Doxycycline	- Porcine - Poultry - Bovine not allowed for lactating dairy cows and laying hens	100 300 300 600	Muscle Liver Skin + fat Kidney	Fixed

Table 1.2 Antiparasitic agents (anthelmintics)

Group	Pharmacologically active substance(s)	Marker residue	Animal species	MRL (µg/kg)	Target tissues	Status
(PRO-) BENZIMIDAZOLES	Febantel Fenbendazole Oxfendazole	Sum of extractable residues which may be oxidised to oxfendazole sulphone	- Bovine - Ovine - Porcine - Equidae	10 50 50 500	Milk (not for porcine and equidae) Muscle Kidney, fat Liver	Fixed
	Thiabendazole	Sum of thiabendazole and 5-hydroxythiabendazole	Bovine	100	Milk, muscle liver, kidney and fat	Fixed
	Albendazole(oxide)	Sum of albendazole sulphoxide(oxide), albendazole sulphone and albendazole 2-aminosulphone expressed as albendazole	- Bovine - Ovine	100 500 1000	Milk, muscle and fat Kidney Liver	Fixed
	Oxibendazole	Oxibendazole	Porcine	100 200 500	Muscle, kidney Liver Skin + fat	Fixed
	Netobimin	Sum of albendazole oxide, albendazole sulphone and albendazole 2-aminosulphone expressed as albendazole	- Bovine - Ovine	100 500 1000	Milk, muscle and fat Kidney Liver	Fixed

Table 1.2 Antiparasitic agents (anthelmintics) (continuation)

Group	Pharmacologically active substance	Marker residue	Animal species	MRL (µg/kg)	Target tissues	Status
(PRO-) BENZIMIDAZOLES	Triclabendazole	Sum of extractable residues that may be oxidised to ketotriclabendazole	- Ovine - Bovine not allowed for use in animals from which milk is produced for human consumption	100	Muscle, liver and kidney	Fixed
	Flubendazole	Sum of flubendazole and hydrolysed metabolite (2-amino-1*H*-benzimidazol-5-yl)(4 fluorophenyl)-methanone	- Poultry: chicken, turkey - Game birds - Porcine	50 50 300 400 400	Muscle Skin + fat Kidney Liver Eggs	Fixed
	Mebendazole	Sum of mebendazole and its hydrolysed ((2-amino-1*H*-benzimidazol-5-yl)(4 fluorophenyl)-methanone) and reduced ((methyl (5-(1-hydroxy, 1-phenyl) methyl-1*H*-benzimidazol-2-yl)-carbamate) metabolites expressed as mebendazole equivalents	- Ovine - Caprine - Equidae not allowed for use in animals from which milk is produced for human consumption	60 400	Muscle, fat and kidney Liver	Fixed
TETRA-HYDRO-IMIDAZOLES	Levamisole	Levamisole	- Poultry - Ovine - Porcine - Bovine: not allowed for lactating dairy cows	10 100	Muscle, fat and kidney Liver	Fixed

processes of drug absorption, drug distribution and drug elimination (metabolism and excretion) in animals, factors influencing the amount of residue remaining in consumable products. The complex processes that determine duration of drug action and circulation of drugs through the body, are schematically outlined in Figure 1.1. These processes are influenced by many different factors, a number of which arising from the physiology of the subject receiving the drug. In addition, pathologic processes can alter drug disposition by modifying the physiological functions that influence the circulation of drugs in the body. The variety of animal species influences the different processes. The variations in drug response can be attributed to species differences. For the majority of therapeutic agents, the species variations in response are due to differences in disposition kinetics of the drugs. If a drug is administered extravascularly, the accessibility of the drug to the systemic circulation, or systemic availability, will also influence the clinical outcome. This process is especially complex when the extravascular route is oral, as the drug must gain access to the portal circulation from the enteric environment and survive passage through the gut wall and liver with their respective ability to metabolise and inactivate drugs.

Absorption and distribution of a drug influence the concentration obtained in the immediate vicinity of its receptor sites (biophasic concentration), while biotransformation (metabolism) and excretion are responsible for determining the action of the drug. Either directly or indirectly, all processes shown in Figure 1.1 involve passage of drugs across membranes. Both nonpolar lipid-soluble compounds and polar water-soluble substances that possess sufficient lipid solubility can cross the predominantly lipoid plasma membrane by passive diffusion. Most drugs are weak organic acids or bases and exist in solution as both nonionised and ionised forms. The nonionised form is usually lipid-soluble and can readily diffuse across the cell membrane to achieve the same equilibrium concentration on either side. In contrast, the ionised moiety is often virtually excluded from transmembrane diffusion because of its low lipid solubility.

1.4.2 Drug administration

Most veterinary drugs, mainly as prepared form, are administered to animals by intramuscular, intravenous or subcutaneous injection, orally in the feed or the water, topically on the skin or by intramammary or intra-uterine infusions.[1]

Parenteral administration where the gastrointestinal tract is bypassed, is mainly performed as intravenous, intramuscular or subcutaneous injection when systemic effects are desired.[35] Injection of a drug solution directly into the bloodstream gives a predictable concentration of the drug in plasma and, in most cases, produces an immediate pharmacologic response.

Topical application and intramammary and intra-uterine infusions are mostly employed when local effects are desired.

Although some oral administration procedures either aqueous or under the form of elixirs or suspensions are available, other oral dosage forms are solids and include tablets, bolusses for

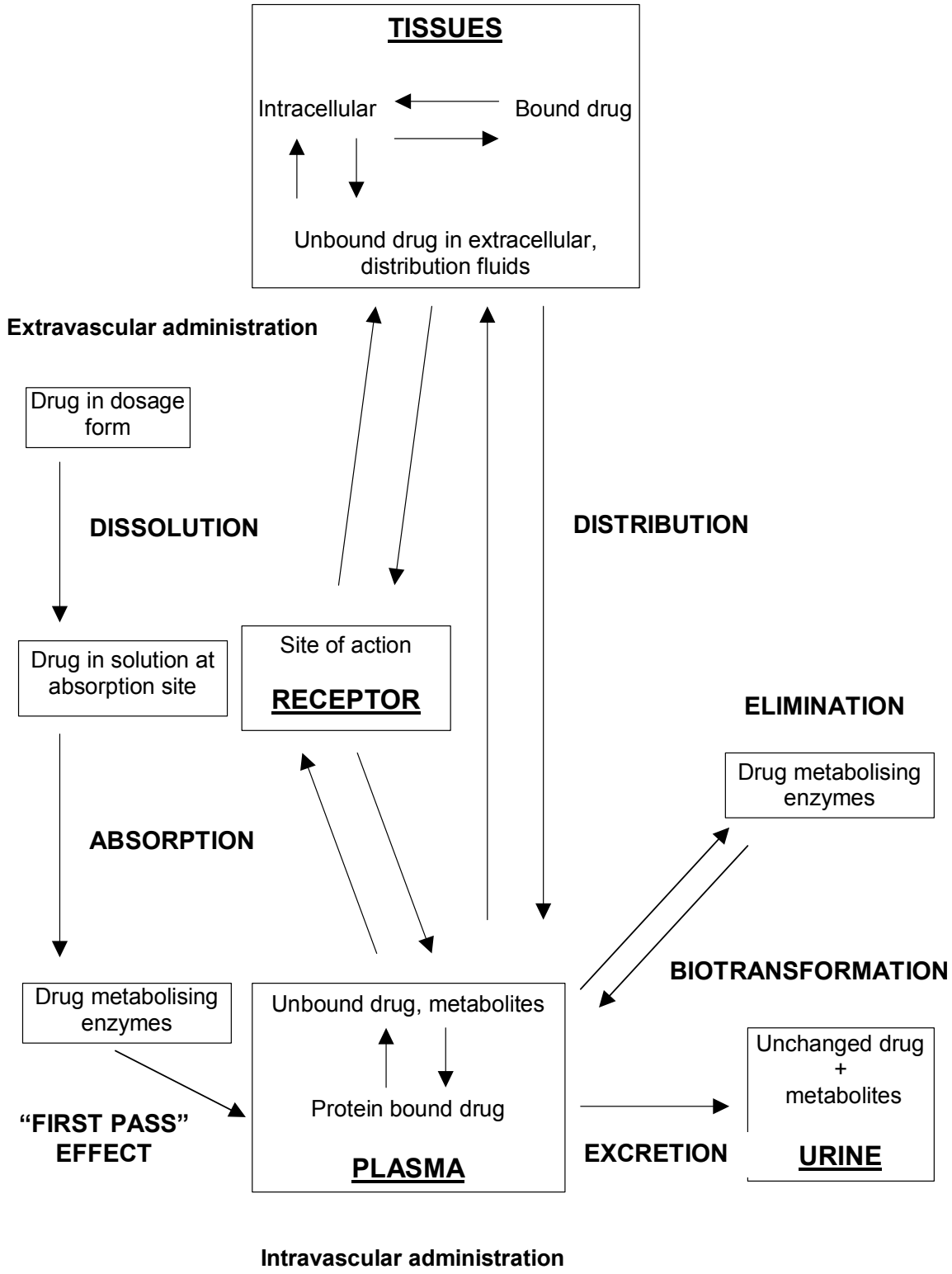

Figure 1.1 Diagram of the interrelationships of the processes that determine the disposition of a drug in the body and the extent and duration of drug action[36]

large animals, pellets, capsules, or a variety of specialised sustained-release products for ruminant animals.

1.4.3 Drug absorption and bioavailability

Drug absorption is the rate at which the drug enters the general circulation and is generally defined as the passage of the drug from its site of administration into the bloodstream. The absorption process is governed by the solubility of the dosage form, the route of administration and certain physicochemical properties of the drug substance.[35] A low degree of ionisation and a high lipid solubility favour absorption.

Absorption of most drugs from intramuscular and subcutaneous injection sites is rapid when it is administered as aqueous solutions.

The ability of a drug, applied topically as a dermatologic preparation, to be absorbed through the skin depends on two consecutive events. It must first dissolve and be released from the vehicle and then penetrate the keratin layer and cells of the epidermis. Since absorption takes place by passive diffusion, lipid solubility is the most important physicochemical property of the drug.

Before entering the systemic circulation, a drug administered as a solid form must undergo three events: release from the dosage form, be transported across the gastrointestinal mucosal barrier and passage through the liver. Dissolution is the rate-limiting step that determines release of the drug from a solid dosage form and can be enhanced by administering the drug in salt form, by micronisation or by decreasing the particle size. A drug that is stable in gastrointestinal fluids and that is not completely ionised and lipid-soluble, would be expected to be well absorbed. The rate of gastric emptying is the most important physiologic factor controlling the drug absorption rate, since the small intestine is the principal site of absorption.

Bioavailability is defined as the rate and extent to which a drug administered as a particular dosage form enters the systemic circulation intact. This pharmacokinetic parameter determines the relationship between drug dosage and intensity of action.

1.4.4 Drug distribution

Drugs are conveyed through-out the body in the circulating blood and reach tissues of each organ in an amount determined by blood flow to the organs. The distribution, i.e. the drug concentration in various tissues at a given time after administration, is dependent on the drug's polarity, molecular size and structure and the amount of protein binding that occurs.[15] Binding of a drug to plasma proteins, mainly albumin, restricts its distribution, thereby limiting its biophasic availability, and can influence elimination of the drug from the body.[35] Protein binding is a reversible interaction, which implies that the drug-protein complex serves as a circulating reservoir of potentially active drugs.

Rapid administration of a high dose of drugs that slowly distributes to body tissues poses a risk of toxic predistribution plasma concentrations.[37]

1.4.5 Drug elimination

Mechanisms of drug elimination, i.e. the processes responsible for removal of the drug from the body, are biotransformation (hepatic metabolism) and renal excretion.[35] The rate of elimination may be influenced by extensive (> 80%) binding to plasma proteins, degree of perfusion of the eliminating organ(s), activity of drug-metabolising enzymes and the efficiency of renal excretion. The rate of elimination of a drug is usually an important determinant of duration of pharmacologic effect. The fate of a drug is largely determined by some of its physicochemical properties, specifically the lipid solubility and the degree of ionisation. Lipid solubility appears to be a prerequisite for biotransformation of drugs by the hepatic microsomal enzyme system.

Drugs undergo metabolic changes in the body that are primarily directed toward formation of metabolites which have physicochemical properties favorable to their excretion. Products of biotransformation are generally less lipid-soluble and are polar in nature. Biotransformation of the parent drug can also occur during metabolism which may serve to activate or deactivate the drug. Apart from the liver, metabolism of drugs takes place in blood plasma and lumen of the gut, where hydrolytic and reductive reactions may occur, as well as in other tissues. The general pattern of drug metabolism is usually biphasic.[15] Common “phase I” biotransformation reactions include oxidation, reduction or hydroxylation. Although metabolic reactions usually yield products with decreased activity, some may give rise to products with similar or even greater activity. “Phase II” reactions involve synthetic reactions as conjugations or combinations with endogenous substances as glucuronic acid, glycine, cysteine, methionine, acetyl and sulphate. The resulting conjugates are water-soluble, often polar and facilitate the elimination of the drug from the body.

Available evidence suggests minimal metabolism for tetracycline, chlortetracycline and doxycycline. Benzimidazole anthelmintics are extensively metabolised by mammals following oral administration.[7] The parent drug is usually short-lived. The free, primary metabolites are usually the products of normal oxidative or hydrolytic processes and are more soluble than the parent drug. A small amount of benzimidazole metabolites becomes bound to endogenous macromolecules in a relatively non-extractable form.

Polar drugs and compounds with low lipid solubility are mainly eliminated by excretion. Although the kidney is by far the most important organ of excretion, the liver, salivary, sweat, mammary glands and lungs constitute nonrenal routes of excretion. Renal excretion is the principal process of elimination for drugs that are predominantly ionised at physiologic pH and for compounds with limited solubility in lipid.

Drugs excreted unchanged (not altered by a metabolic reaction), mainly in urine, include many antibiotics such as penicillin (except nafcillin), cephalosporines, aminoglycosides and oxytetracycline.

Some compounds with a relative molecular mass (M_r) greater than 300 and with polar groups, are excreted mainly by the liver into bile. Conjugation with glucuronic acid, which takes place in the hepatocytes, may be the determining factor for excretion of a drug or metabolites and certain endogenous substances in bile. Compounds excreted in bile enter the small intestine. Depending on their lipid solubility, some drugs (e.g. tetracyclines) are reabsorbed.

1.4.6 Withdrawal times

The time when the residue content of the edible tissues of 99% of the total projected population of animals does not exceed the required MRL is the withdrawal time or the minimal period of time between the last recommended treatment and the time of slaughter or the time of collection of animal products as milk and eggs.[1] This time allows the veterinary drug and its residues to decrease to levels below the established MRL.

Data on the depletion of the marker residues in the target tissue are used to estimate this withdrawal time. Characterisation of the elimination times for drugs from edible tissue or animal products is an important part of the drug approval process so that appropriate withdrawal times can be set to minimise the incidence of drug residues.

One of the obvious limitations of tissue residue studies is the lack of sufficient tissue samples per individual test animal to characterise individual tissue depletion kinetics. Although there is great potential for the population approach to address drug tissue disposition and residue avoidance, adequate strategies for its implementation still have to be explored.[36] Knownledge of the pharmacokinetic characteristics of a drug reduces the number of animals required by conventional residue testing.[38]

The primary pharmacologic determinant of a withdrawal time is the administered dose and the rate of depletion of the drug in the tissue.[39] The kinetics of drug depletion are often reported in the literature when a pharmacokinetic study has been conducted. Withdrawal times after off-label use of a drug increasing the dose for a disease covered by the label or using a normal dose for a disease not covered on the label, can be estimated. When a higher dose is used, the concept of half-life, which is the time required for 50% of a drug to be eliminated from an animal or tissue, can be used. Application of extrapolation of drug disposition parameters across species are not appropriate to determine withdrawal times. The best rule of thumb to follow is that, in general, half-lives are shorter in a smaller species.

The Committee for Veterinary Medicinal Products (CVMP) approved an approach towards harmonisation of withdrawal periods which was published by the European Agency for the Evaluation of Medicinal Products (EMEA) and which is in operation since January 1997.[40] The calculation is based on a regression approach so that it can be assured with a 95% confidence

that in at least 99% of the animals, levels will have declined to below the MRL within the specified time. There is a need for harmonisation of the calculation of withdrawal periods. No specific recommendations exist in the current EU regulations for the setting of withdrawal times for veterinary drugs in tissues.

Several other approaches are described in the literature. Concordet and Toutain[41] have presented a non-parametric method. Fisch[42] has proposed a set of models with varying assumptions.

1.5 Determination of veterinary drug residues

1.5.1 Introduction

Analytical methods can include screening, determinative and confirmatory procedures. Screening methods are designed to be rapid, easy to use and to give a positive or negative respons for a drug at a specified concentration level in a matrix.[15] Traditionally, microbial inhibition tests have been used to screen large numbers of samples for antibiotics and these tests are still widely used today.

In addition, new rapid test kits as microbial receptor, receptor binding, radio- or enzyme immuno assays are being used to screen samples for specific drugs. These screening tests, however, are not always accurate at or below the test threshold, are often drug class but not compound specific and do not give quantitative information. Therefore, additional analytical tests may be needed to determine if a sample is actually violative for an animal drug residue. Some tests as e.g. immuno assays, provide semi-quantitative results.

Determinative methods are designed to separate and to quantify and provide some qualitative information on the analyte of interest. Most gas and liquid chromatographic methods would fall into this category. Qualitative assays classify samples as positive or negative relative to a specific drug concentration.

Confirmatory methods are meant to provide unambiguous identification of the drug residue in question. Because of its sensitivity and specificity, mass spectrometry is the preferred method for confirmation.

1.5.2 Screening assays

The screening assays make a distinction between non-violative and suspected samples. The most important requisite for a screening assay is that the β-error (possibility of false negative result or a risk for the consumer) is lower then 5%.[43]

The earliest methods used for detection of antimicrobial residues in food were based on the detection of growth inhibition of various sensitive bacterial strains.[1] The major disadvantages of these assays are that they are not very specific for antibiotic identification purposes, that they are not quantitative and that they have limited detection levels for many antibiotics. Moreover,

they are time consuming requiring several hours before results are available. However they are inexpensive, easy to perform, adaptable to screen large numbers of samples and they have a reasonable broad antimicrobial detection spectrum.

The CHARM I and II tests are qualitative microbial receptor assays for rapid detection of β-lactams, macrolides, aminoglycosides, tetracyclines, chloramphenicol and sulfonamides in milk and tissues. The tests use two types of bacterial cells containing either the natural receptor sites for antibiotics or an antibody coating and a radiolabelled antibiotic. Any unbound receptor site on the bacterial cell will bind with the radiolabelled antibiotic. Binding is measured with a scintillation counter and compared to control samples.

The Penzyme test is a qualitative enzymatic method for the rapid detection of β-lactam antibiotic in milk. The antibiotics will specifically bind with an enzyme and inactivate it. The end products are measured by use of a redox colour indicator and the final colour is compared with a colour chart.

The SNAP and Delvo-X-Press tests for β-lactam antibiotics in milk are qualitative enzyme linked receptor binding assays in which β-lactams are captured by a penicillin binding protein conjugated to an enzyme. The absence of β-lactams in a sample results in all of the enzyme conjugate remaining unbound and available for binding to the immobilised β-lactam, while the presence of β-lactams results in a portion of the enzyme conjugate being bound and unavailable to bind with the immobilised β-lactam. Samples are declared positive or negative on the basis of a visual comparison of the intensity of colour development.

The specificity of the immune system is demonstrated by its ability to distinguish subtile differences between antigens. Examples of immunoassays are radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). Lactek tests are examples of commercial test kits commonly used for drug residue testing in milk. On the basis of the relationship between colour development and concentration, it is possible to design qualitative, quantitative or semi-quantitative ELISA's.

Jackman[44] described class specific enzyme immunoassays (EIA's) for the detection of tetracyclines and most benzimidazole drugs. For the structural very different thiabendazole, a compound specific EIA had to be developed.

Elliott *et al.*[45] described a biosensor immunoassay (BIA), an automatical variant of RIA. The bulk of the work relied upon the use of a commercially available surface plasmon resonance biosensor instrument. A positive sample can be detected by a reduction in the level of antibody binding to a chip due to binding of drugs of the sample. Results are generated in real time without the need of time consuming sample preparation. These techniques have the possibility to perform high throughput analysis where animals raised for meat are slaughtered. Also the feasibility to perform milk residue analysis for antimicrobial compounds is investigated.

1.5.3 Confirmatory methods

1.5.3.1 Introduction

Suspected samples have to be further analysed by a confirmatory analytical method. Chromatographic techniques play an important role in the confirmatory tests for residues of veterinary drugs. There are several types of chromatographic methods currently in use for residue analysis. These include thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC). TLC has found some use, generally for screening or qualitative analysis only. Most veterinary drugs are to polar, non-volatile (M_r > 1500), heat sensitive and/or difficult to derivatise for GC.[46] Therefore, HPLC is the most commonly used analytical technique for residue analysis. Mass spectrometry (MS) is the preferred detection method for confirmation. Hyphenated techniques as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are the most powerful analytical tools in the analytical laboratory.[43]

1.5.3.2 HPLC

1.5.3.2.1 Extraction techniques

One aspect of residue analysis that has received comparatively little attention is the initial extraction of the analyte from the sample matrix. Some applications of high-speed homogenisation for the solvent extraction of drug residues are used. McCracken *et al*.[47] have compared four different techniques. The use of a high speed mixer/emulsifier, an ultrasonic bath, a Stomacher and an end-over-end mixer were used to extract both incurred and fortified residues from chicken muscle sample. Comparable analytical recoveries from fortified muscle samples were obtained using each of the extraction techniques. However, for each analyte the highest drug concentration detected in incurred samples was obtained using a mixer/emulsifier extraction.

The total drug residue content of a tissue comprises all free residues (parent compound plus all low molecular weight metabolites) plus all residues covalently bound to endogenous macromolecules. A residue is designated as bound if it is not released from the macromolecular fractions after mild but exhaustive extraction procedures. Extraction of bound residues needs hydrolysis by chemical or enzymatic means.[48]

1.5.3.2.2 Clean-up procedures

In the past, liquid-liquid extraction (LLE) was the most used clean-up procedure. Moats and Harik-Khan[49] described this procedure for the determination of tetracycline antibiotics in milk. The tetracyclines were recovered in the water layer formed by liquid-liquid partitioning of the acetonitrile extract with hexane and methylene chloride as purification agents. This clean-up procedure requires more organic solvent. Matrix solid phase dispersion (MSPD) is a technique

where the sample is directly blended with chromatographic material such as bonded silica. Long *et al*.[50] have used this clean-up technique for the determination of tetracyclines in milk. After washing the column material with hexane, the tetracyclines were eluted with a mixture of ethyl acetate and acetonitrile. Solid phase extraction (SPE) is the most frequently used clean-up procedure for the determination of residues of veterinary drugs. Österdahl *et al*.[51] have described a SPE procedure for the determination of levamisole in milk. The sample extract was transferred to the prepacked SPE cartridge. After washing with hexane, levamisole was eluted from the cartridge with methylene chloride.

1.5.3.2.3 Ion-pair chromatography

Ion-pair chromatography (IPC) allows acidic, basic or amphoteric compounds to be separated simultaneously with neutral analytes on column.[52] IPC is most popular in the reversed-phase mode. The mobile phase consists of an aqueous buffer (plus an added organic co-solvent such as methanol or acetonitrile for bonded-phase separations) and an added anionic or cationic counter-ion of opposite charge to the analyte molecule. Alkylsulphonate and tetrabutyl ammonium salts are the most commonly used ion-pair reagents.

The principle of ion-pair chromatography is explained by using the ion-pair model shown in Figure 1.2.

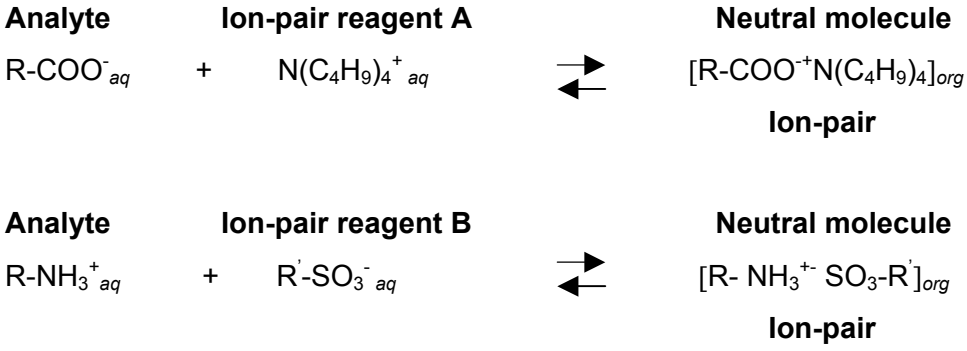

Figure 1.2 The ion-pair model

Ionic analytes, acids or bases, do not easily adsorb into the hydrophobic layer of reversed-phase columns due to the charge on the analyte molecule. The analyte and the ion-pair reagent form a neutral ion pair that will readily partition into the reversed-phase packing. In the simplist case of ion-pair chromatography, it can be assumed that the analyte and counter ions are soluble only in the aqueous mobile phase, and that the ion-pair formed from these ions is soluble only in the organic stationary phase. The subscripts "*aq*" and "*org*" in Figure 1.2 refer to aqueous and organic phases, respectively. Ion-pair reagents of the same charge as the analyte may decrease retention while those of opposite charge may increase retention on reversed-phase columns, presumably by forming a complex which is less polar than the original analyte.[53]

Retentions can be further increased by adding ionic compounds with hydrocarbon tails. Retention is then related to the length of the hydrocarbon tail. An octanesulphonic acid (C_8) ion-pair reagent will cause basic compounds to be retained longer than a hexanesulphonic acid (C_6) ion-pair reagent. The hydrophobic portion of the ion-pair reagent contributes to the overall hydrophobicity. The more hydrophobic the ion-pair reagent, the greather the retention of the ion-pairs. Retention of non-ionic sample components is not influenced. The pH of the mobile phase and the type and concentration of buffer can both be used to control ion-pair separations.

By using IPC, residues can be separated effectively from interferences without further clean-up. Tetracyclines are very polar bases and can be effectively separated from endogenous interferences in milk extracts by adding an alkylsulphonate as ion-pair reagent.[49] Fletournis *et* al.[54] have succesfully applied IPC with octanesulphonate as ion-pair reagent for the determination of fenbendazole in milk. In the absence of the octanesulphonate, fenbendazole, which in highly acidic conditions exists in its protonated form, shows severe peak tailing. This indicates that a relatively strong adsorptive interaction between the protonated fenbendazole and the silica-based reversed-phase packing material had occurred. The negatively charged octanesulphonate ions were used in order to form hydrophobic ion-pairs with the analyte. This addition eliminated peak tailing, increased the retention time slightly and a two-fold higher peak height was obtained. A stable and increased column temperature (30 to 50°C) is important for IPC determinations. The better mass transfer of the analyte between the stationary and mobile phase can improve the peak shape.

IPC in the reversed-phase mode was used for the determination of tetracyclines in eggs and broiler chicken muscle and of anthelmintics in milk described in Chapters 2 and 3, respectively. In the other analytical methods using liquid chromatography-mass spectrometry (Chapters 4, 5 and 6), reversed-phase chromatography was applied with mobile phases containing non-volatile buffers or aqueous solvent mixtures.

1.5.3.2.4 Detection techniques

For screening purposes, universal variable wavelength ultra violet (UV) detectors could be used.[55] However, for the confirmation of suspected samples, more analytical data than just a retention time and a detector response are expected. With a fluorescence detector more specific analyses at lower detection limits could be performed and spectral data for each time point are generated. In most cases some kind of derivatisation of the analyte (pre- or post column) is needed. Analytical evidence for each time point with spectral data and not only signal strength could also be gathered by using a diode array detector (DAD). The DAD is a special UV detector that continuously takes a UV spectrum of the eluent throughout the complete HPLC run. The DAD (Figure 1.3) utilises a deuterium or xenon lamp that emits light over the UV spectrum range.[56] Light from the lamp is focused by means of an achromatic lens through the sample cell and onto a holographic grating. The dispersed light from the grating is arranged to

fall on a linear diode array. The resolution of the detector will depend on the number of diodes in the array and on the range of wavelengths covered. A chromatogram can be reconstructed by monitoring at a specific wavelength depicting only those substances that adsorb UV light at the chosen wavelength and that have unique absorbance characteristics.

The determinations of tetracyclines in eggs and broiler chicken muscle and of anthelmintics in milk described in Chapters 2 and 3, respectively, were performed with a DAD. The mass spectrometric detector, using the principles of mass spectrometry and generating mass spectral data is very important in residue analysis. The principles of a mass spectrometer detector are described in next paragraph.

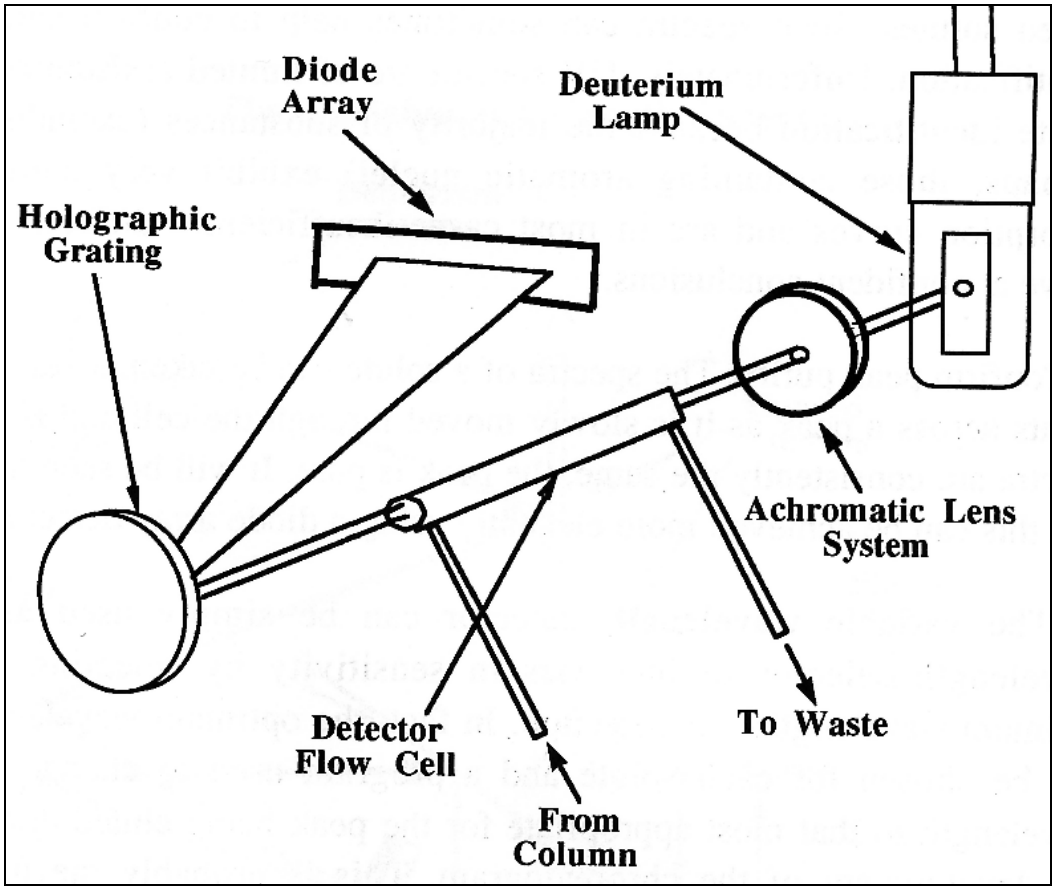

Figure 1.3 Schematic view of a UV photo diode array detector[56]

1.5.3.3 Liquid chromatography-mass spectrometry

1.5.3.3.1 Introduction

The liquid chromatography-mass spectrometry (LC-MS) technique should be regarded as a complementary analytical tool compared with GC-MS that particularly enables the determination of compounds with high molecular masses and polar, non-volatile or thermolabile analytes without derivatisation, which makes this technique particularly interesting for residue analysis of veterinary drugs. [57] Mass spectrometers generate mass spectral data that can provide valuable

information about the identity, the molecular mass and the structure of an analyte. A mass spectrometer is more sensitive and far more specific than all other HPLC detectors.

The three primary processes associated with mass spectrometer analysis are ion production (source), ion separation (analyser) and ion collection (detector). The movement of ions is controlled by applying electric fields and vacuum. Ions are separated and identified according to their mass-to-charge (*m/z*) ratios.

1.5.3.3.2 Ionisation modes

The elimination of the mobile phase needs an interface. The most currently used interface is the atmospheric pressure interface. The ionisation of the analytes takes place at atmospheric pressure and afterwards the analyte ions are mechanically and electrostatically separated from neutral molecules and transferred into the mass spectrometer through a series of chambers with decreasing pressure. The two types of ionisation most common in use are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI).

A schematic view of a mass spectrometer with electrospray interface is presented in Figure 1.4.

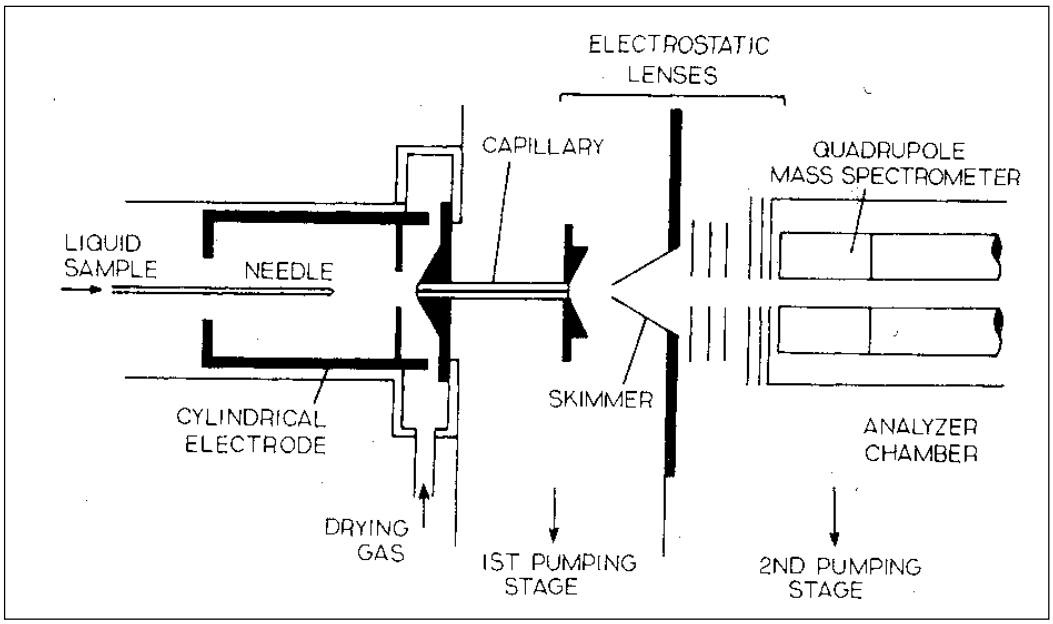

Figure 1.4 Schematic diagram of a mass spectrometer with electrospray interface[58]

The ESI operates by the process of ion evaporation where ions are emitted from a droplet into the gas phase.[57] The eluent from the LC is pumped through a stainless steel capillary which carries a high potential, typically 2 to 5 kV.[50] The strong electric field generated by this potential causes the liquid to be sprayed from the end of the capillary and highly charged droplets are produced (Figure 1.5). As the droplets evaporate, ions are emitted from the droplets into the gas phase. This process is supported by a flow of nitrogen used as drying and as nebuliser gas. Basic compounds can form a protonated molecule ($[M+H]^+$) which can be analysed in the

positive operating mode. Acidic compounds can form a deprotonated molecule ($[M-H]^-$) which can be analysed in the negative mode.

As ESI is a very soft ionisation technique, there is usually little or no fragmentation and the mass spectrum contains only the quasi-molecular ion. Some in-source fragmentation can be induced by adjusting the source parameters.[60] The cone voltage assisted fragmentation delivers fragments which are very useful structural information tools. The energies involved in the collisions are such that usually only weaker bonds such as C-N and C-O bonds are broken.

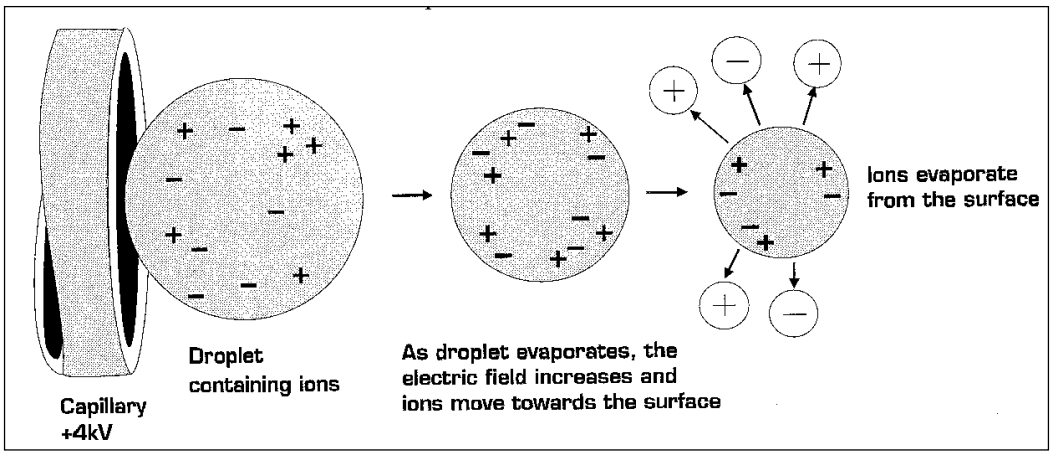

Figure 1.5 Ion evaporation mechanism of the electrospray ionisation process[59]

APCI tends to give better sensitivity for less polar compounds. In APCI operation, the eluent from the LC is carried through a probe heated at 350 to 600°C and elutes into the APCI source which is also heated.[57] A heated probe and coaxially introduced nitrogen cause the liquid to form an aerosol which is evaporated and passes a corona pin held at a voltage of 2 to 3.5 kV. The resulting discharge that is formed ionises solvent molecules.

1.5.3.3.3 Mass spectrometer analysers

Quadrupole and ion trap mass analysers are used most often in mass spectrometers for residue analysis. The quadrupole mass analyser actually is a mass filter.[58] It consists of four hyperbolic or circular rods (poles) equally spaced around a central axis in a square. A mass selector is created by applying precisely controlled voltages to opposing sets of poles. Ions of different *m/z* value are transmitted by the quadrupole filter by ramping the voltages on each set of poles.

An ion trap mass analyser can be imagined as a quadrupole bent on itself which consists of a circular ring electrode as outer rod plus two end caps that together form a chamber.[58] The inner rod is reduced to a point. Ions entering the chamber are trapped there by electromagnetic fields. By raising the potential, the trajectories of ions of successive *m/z* values are made unstable and these ions are ejected out of the trap. Ion traps are able to perform multiple stage mass

spectrometry generating a lot of successive fragments delivering structural information which is very useful in mass spectrum research for qualitative residue analysis.
Most mass spectrometers are equipped with a photomultiplier for the detection of the ions.

1.5.3.3.4 Determination modes

Quadrupole mass analysers can operate in the scan mode or in the selected ion monitoring (SIM) mode, where only a few *m/z* ratios are monitored. This last mode is significantly more sensitive and is used for quantification and monitoring of target compounds. The highest sensitivity and selectivity with the quadrupole technique for target residue analysis is obtained by means of tandem mass spectrometry (MS/MS) using a triple quadrupole LC-MS/MS system operating in the multiple reaction monitoring (MRM) mode. The first quadrupole is used to select the precursor ions.[59] Fragmentation or collision induced dissociation (CID) of the precursor ions takes place in the second stage (octopole or quadrupole), which is called the collision cell. A low argon gas flow enhances these CID. The selected fragment ions are monitored in the third stage or quadrupole. Because of the high selectivity, specificity and resulted sensitivity, thorough sample preparation, clean-up steps and sufficient chromatographic separation for LC-MS/MS methods seem no longer necessary. Although this may hold true to a certain extent for clean samples, complex matrices, such as liver, kidney or muscle which have to be analysed in laboratories involved in residue control, require an appropriate sample preparation combined with effective chromatography in order to avoid quantitative errors associated with matrix effects of the final extract.[57]

1.5.3.3.5 Mobile phase limitations

The transformation of a HPLC method to a LC-MS method may sometimes be hindered because for LC-MS only volatile components for the mobile phase can be used.[55] Non-volatile salts as ion-pair reagents and phosphate crystallise in the source of the mass spectrometer and have to be substituted by e.g. ammonium acetate.

A triple quadrupole LC-MS/MS system operating with electrospray ionisation was used for the multiresidue determinations of anthelmintics in milk (Chapter 4), of flubendazole and its metabolites in eggs and poultry tissues (Chapter 5) and of mebendazole and its metabolites in sheep tissues (Chapter 6).

1.6 Validation of quantitative confirmatory methods

1.6.1 Introduction

Confirmatory methods that provide full or complementary information enable the analyte to be identified unequivocally at the level of interest.[61] These methods are specifically designed to provide unequivocal identification of unauthorised substances and substances with an

established MRL and for quantification of analytes. For quantitative methods, the degree of precision and trueness must be specified. According to Council Directive 96/23/EC,[22] reference methods have to be used for the confirmation of all samples suspected for unauthorised substances. These methods must be based on molecular spectrometry providing direct information on the molecular structure of the analyte under examination. On behalf of the EC, Heitzman[20] published a lot of analytical methods for veterinary drug residues. However, most of these methods are very laborious and time consuming and are not suitable for routine analysis. Therefore, surveillance laboratories are developing their own methods. These have to be validated to demonstrate their reliability. Validation parameters to be evaluated for quantitative confirmatory methods and described in the Draft Commission Decision SANCO/1805/2000[27] are: specificity, linearity, trueness, precision, recovery, analytical limits, stability and applicability/ruggedness. These parameters are the same as those described in the definitive Commission Decision 2002/657/EC.[28] However, the Draft is cited as reference because this Draft was followed during the validation procedures in this thesis.

1.6.2 Validation parameters[27]

1.6.2.1 Specificity

The specificity describes the ability of the method to measure unequivocally the analyte of interest in the presence of all other components as interfering analytes, metabolites, endogenous material and matrix constituents in a complex matrix.[62]

1.6.2.2 Linearity

The mathematical relationship between the measured response and the concentration of the analyte in the matrix has to be established. Whenever possible, a suitable internal standard (IS), preferably deuterated and certified reference material (CRM) should be used for the quality control of the calibration curves. De Wasch *et al*.[63] advised to make a five-point calibration curve based on 10 datapoints grouped as 3 at the lowest and highest concentration, 2 in the middle and 1 in between. Removal of some outliers at the edges of the curve will not have a major impact then on the slope of the curve.

1.6.2.3 Trueness

Trueness is a component of accuracy and is defined as the difference between the mean value measured for an analyte in a CRM and its certified value, expressed as a percentage of this value. If no CRM is available, relevant parameters may be evaluated by analysing fortified sample material.

1.6.2.4 Precision

The precision, another accuracy component, is the closeness of agreement between the results obtained by applying the experimental procedure several times under prescribed conditions and covers repeatability, within-laboratory reproducibility and inter-laboratory reproducibility.

1.6.2.5 Recovery

Matrix effects in biological samples, e.g. lipids in plasma or residual protein, can cause loss of analyte during the analytical procedure and can affect the recovery or extraction efficiency.[64] If there is no CRM available to calculate the trueness, the recovery has to be determined by experiments using fortified blank matrix material. Whenever possible, incurred residue matrix material has to be used to optimise the method to recover as much incurred material as possible.[47]

1.6.2.6 Analytical limits

The analytical limits comprise the limit of detection (LOD), the limit of quantification (LOQ), the detection capability (CCβ) and the decision limit (CCα). The LOD is the lowest concentration of analyte that can be detected in a sample after applying the appropriate identification criteria. The LOQ is the lowest concentration of the identified analyte in a sample that can be quantified with a specified degree of accuracy and within-laboratory reproducibility.

Two new analytical limits are introduced on revision of Commission Decision 93/256/EEC.[25] The definition of these analytical limits are based on CCβ and on CCα.[65] The detection capability is the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error probability of β. The β-error, defined as the probability that the tested sample is truly violative, even though a non-violative measurement has been obtained (false negative decision), should be less than or equal to 5%. In case of banned substances, CCβ is the lowest concentration at which a method is able to detect truly residues in samples with a statistical certainty of 1-β. For substances with an established MRL, CCβ is the concentration at which the method is able to detect MRL concentrations with a statistical certainty of 1-β.

The decision limit is the limit from which on it can be decided that a sample is truly violative with an error probability of α. The α-error, which is the probability that the tested sample is not truly violative, even though a violative measurement has been obtained (false positive decision), shall be 1% or lower and 5% or lower for banned and MRL substances, respectively. In the case of banned substances, CCα is the lowest concentration level at which a method can discriminate with a statistical certainty of 1-α whether the identified analyte is present. For substances with an established MRL, CCα is the concentration above which it can be decided with a statistical certainty of 1-α that the identified analyte content is truly above the MRL.

1.6.2.7 Stability

The validation protocol should always include an investigation of the stability of the analyte or matrix constituents in the sample matrix as well as the stability of the standard analyte in solution.[62] Whenever possible, incurred samples should be used to check the stability in matrix material.

1.6.2.8 Applicability/ruggedness

Pre-investigation studies have to be carried out by selecting factors of the sample pretreatment, clean-up, instrument and analysis, which may influence the measurement results. Such factors may include the operator, the reagents and the laboratory circumstances. The determination of the within-laboratory reproducibility during the real validation is covering some of these factors contributing to the ruggedness/applicability of the method.

1.6.3 Validation criteria

The validation procedures, the quality and the validation criteria for the analytical methods for residues were previously described in Commission Decisions 89/610/EEC[24] and 93/256/EC.[25] The decisions have to be updated systematically in order to take into account the scientific knowledge and the technical improvements. The updated criteria[27] of Commision Decision 93/256/EC have introduced a number of new concepts for the confirmation of veterinary drug residues described in detail by De Brabander *et al*.[65] A system of identification points (IPs) has been introduced to define the number of ions and their corresponding ratios that must be measured for both authorised and unauthorised substances when mass spectrometric analysis is employed. An identifier, e.g. UV absorption or MS ions, is described by Stefany[61] as the part of the total information needed to unambiguously identify the structure of an analyte as being identical with an assumed structure or with the known structure of the compound tested for. For LC-MS/MS analysis, the precursor ion accounts for 1 IP and each fragment ion (transition product) earns 1.5 IPs. According to the criteria, for the positive confirmation of substances listed in group A (banned substances) of Annex I of Council Directive 96/23/EC,[22] at least four IPs must be acquired. This means that at least 2 diagnostic fragment ions from 1 precursor ion has to be measured. For substances listed in group B (authorised substances), a minimum of 3 IPs is required. The main advantage of the IP system is that new techniques are very easily introduced in a system of quality criteria.

In the Draft Commission Decision SANCO/1805/2000,[27] some words have also been changed. A “positive” and “negative” result is replaced by “violative” and “non-violative”, respectively. For unauthorised substances, the result of the analysis is considered as violative if all relevant identification criteria for the analyte are met and if the $CC\alpha$ is exceeded. For MRL substances, the result of the analysis is considered as violative if the determined content of the identified

analyte in the sample is greater than the appropriate CCα. For substances with a zero tolerance, a non-violative result is obtained if the identity of the analyte has not been proven and the criteria are not fulfilled, or if the CCα is not exceeded. For MRL substances, the result is non-violative if the measured content of the identified analyte is below the CCα.

In addition, a new regulatory limit, the minimum required performance limit (MRPL), has been introduced in order to establish more officially the level at which European control has to be made for banned substances.[65]

Recently, the Draft Commission Decision SANCO/1805/2000[27] has been approved and has been published as definitive Commission Decision 2002/657/EC[28] after some minor revisions. The Decision is valid since 1 September 2002. The responsibility of the surveillance laboratories is very high now, because the obtained decision limit is determining the interpretation of results according to this Decision. The result of a residue determination has to be considered non-compliant if the decision limit of the confirmatory method for the analyte is exceeded.

1.6.4 Validation of methods for depletion studies

Producers of veterinary drugs require residue methods for use in depletion studies, which are part of the evaluation dossier of new veterinary medicinal products. Depletion studies are carried out under completely controlled conditions and in compliance with the requirements of good laboratory practise (GLP) and quality assurance. Van Leemput and Clayton[66] described the validation of analytical methods for residue analysis in depletion studies. The validation has to be done for the selected marker residue, which is not necessarily identical to the parent substance. It can be a metabolite or a combination of analytes. The validation must be performed in the edible matrices of concern in depletion experiments. These matrices usually include liver, kidney, muscle and fat, and where appropriate skin with fat in natural proportions, milk or eggs. The method must be specific in the conditions of the depletion experiment. Linearity, accuracy and precision are determined in the working range of the depletion studies by spiking of blank matrices obtained from animals of the same type as used in the experiments. There is no need to explore the LOD, because the MRL is the reference. One and half the MRL can be considered as the LOQ. The repeatability must be determined, but practicability and applicability are no issues.

1.7 Monitoring and surveillance

1.7.1 Introduction

MRLs are only of value if backed up with good residue control programmes. The EU requires from each member state to have in place surveillance and monitoring programmes according to Council Directive 96/23/EC[22] and Commission Decision 97/747/EC.[23] These programmes have to ensure safe food from animal origin controlling that the MRLs are not violated and that no

residues of banned substances are found. Residue analyses are not only related to public health, but international trade or environmental aspects are also involved.

1.7.2 Integrated systems

Residue monitoring or surveillance programmes require the analysis of a large number of samples. Traditionally screening techniques, such as microbiological assays and immunoassays are commonly used in residue laboratories.[67] Multiresidue screening methods are needed. Screening methods reduce the number of samples requiring more detailed analysis, but care must be taken to ensure whether the number of false negatives is kept to a minimum. Screening assays are an important part of the integrated approach to residues monitoring. It is necessary to combine different methods in an integrated system in which a number of different tests are applied consecutively depending on the targets or objectives of the analysis. For regulatory purposes such a strategy should include at least two or more independent methods:[1]

1. Screening with a method optimised to prevent false negative results and with an acceptable number of false positive results at low cost (e.g. microbial growth inhibition tests)
2. Intermediate tests to identify the residue type (e.g. microbial receptor assays, receptor binding assays)
3. Quantitative confirmation with an independent method optimised to prevent false positive results

Heeschen and Suhren[16] have described an integrated system for the detection of antimicrobials in milk ensuring toxicological and technological safety. The dominating residues in milk in most countries are β-lactam antibiotica and sulfa drugs including dapsone. Tetracyclines, aminoglycosides, chloramphenicol and some others may occur. An integrated detection system for antimicrobials and inhibitors in raw and heat-treated milk has to distinguish between aspects of the method applied and the responsibilities of milk producers, treatment/processing plant and food inspection within this system. Positive results with microbiological inhibitor tests or with starter cultures are indicative for a technologically unsafe product. These tests could be used as MRL screening tests to select highly suspicious samples for the presence of antimicrobials exceeding the MRL. Additional tests have to be used, which can detect specific groups of antimicrobials (e.g. tetracyclines, aminoglycosides and chloramphenicol) which are often not sufficiently detected by microbiological inhibitor tests.

A modern strategy to avoid residues of veterinary drugs must involve producers, processors/manufacturers and regulators. The farmer/producer has to respect the withdrawal period which is the most important critical control point in the prevention of unwanted residues.

Products from the farm (e.g. milk or the slaughter animals) should regularly and randomly be tested for inhibitors, using appropriate tests. The food processing plant must check the incoming raw material by using rapid test kits (e.g. receptor tests) as MRL screening tests. Under regulatory aspects, food inspection authorities have to identify residues of antimicrobials qualitatively and quantitatively applying appropriate methods including MRL screening tests and physicochemical tests for final identification.

1.7.3 Drug residue laboratories

The EU has gradually created an extensive network of laboratories entrusted with the surveillance of residues in animal products.[68] A hierarchical system is installed. Routine or field laboratories (RFLs) involved in the annual residue monitoring programmes are coordinated and controlled in each EU member state by at least one national reference laboratory (NRL) designated by the national government. The NRLs are supported, advised and controlled by four EU community reference laboratories (CRLs) designated by the EU in 1991. Besides these official laboratories, many other laboratories are involved in residue analysis.

Good laboratory quality assurance (QA) practises are required for the analytical procedures for detection, identification and/or quantification of drugs in food of animal origin.[69] Quality standard regulations require a formally established and documented QA program to ensure the validity of results from analytical laboratories. Accreditation of the QA programme by an independent organisation will provide customers confidence in the analytical results. Additional residue measures concerning the official control of food require official control laboratories to be accredited according to ISO 17025 from January 2002 on.[28] The quality of results of monitoring tests shall also be ensured by a QA system accredited according to this regulation.

A database, accessible on Internet, provides a carefully judged inventory of analytical methods available for the determination of residues of veterinary drugs.[70] The monitoring programme subdatabase provides useful qualitative and quantitative data obtained from monitoring of Belgian animal food products. The Federal Agency for Food Safety belonging to the Belgian Federal Ministry of Social Affairs, Public Health and Environment coordinates all monitoring programmes and is responsible for surveillance and compliant actions.

1.7.4 Interpretation of results

Sometimes, a different way of interpretation of analytical results exists between inspection services and laboratories.[71] Inspection services are mainly interested in a yes/no answer concerning the violation of a sample. In Belgium, a contradiction between a first and a second analysis automatically starts up a quality assurance procedure. The remnants of both samples are analysed by the NRL. The NRL has to investigate the methods used and has to find out on which quality criteria the laboratory results are based.

Qualitative methods may be used for illegal drugs having a so called zero tolerance. If the analyte is detected, the concentration can be determined or estimated and an action limit (AL) is used to convert the results in yes (violative) or no (non-violative) answers. The AL is an agreement between inspection services and laboratories. The AL is based on the analytical possibilities of the moment taking into account a safety factor to protect the producers of food of animal origin. This limit should be the basis to take compliant actions. Quantitative analysis is necessary especially for drug residues with a MRL. Anyway, qualitative criteria must be fulfilled before quantification.

Today's consumers require the safest possible food in sufficient quantities and at a reasonable price. Therefore, the precautionary principle has also been implemented in a systematic way (already included in the veterinary medicines approval system) as an approach of risk management.[31] This is applied to circumstances of scientific uncertainty, reflecting the need to take action in the face of a potentially serious risk without awaiting the results of scientific research.

Surveillance with analytical methods of independent authorities could increase consumer confidence in safe food, certainly when the obtained results would be published.[66] Setting unreachable objectives and promising those to the consumers can only reduce the consumer confidence. Residue control without publication of the results will not improve consumers confidence. Residue control with publication of violative tests only and with their definitions of residues reduces consumer confidence, as consumers assume that residues have been found accidently.[31] Systematic publication of well-established results of residues is highly desired, as the consumer can be convinced that, despite large efforts to detect residues, only a small part of food contains some residues.

The surveillance and monitoring programmes must be harmonised in all EU member states and the publication of results must be made uniform, so that only those in excess of the MRL and confirmed by validated confirmatory methods, are published.

1.8 Research objectives and relevance of the presented research work

1.8.1 Introduction

The two main objectives of the research work described in two separate parts in this doctoral thesis are:

1. The development of chromatographic confirmatory methods for the quantitative determination of residues of tetracyclines and anthelmintics including metabolites in the following animal products: milk, eggs, poultry and sheep tissues

2. Measuring of disposition, distribution and depletion of residues of tetracyclines and anthelmintics including metabolites in milk, eggs, poultry and sheep tissues

Because of the objective to apply the methods in routine analysis, simple, reliable, accurate, relatively sensitive and flexible methods which are relatively fast have to been developed. Before the analytical methods were applied in depletion studies or were proposed for analysis in monitoring programmes, a reliable and comprehensive validation according to European requirements was performed.

Throughout this thesis, there has been an evolution in analytical technology. In the two first described methods, HPLC with DAD detection was used. Later on, only LC-MS/MS methods were developed.

1.8.2 Monitoring of residues in food

The laboratory involved in screening of samples for veterinary drug residues of the Department of Animal Product Quality and Transformation Technology was looking for analytical tools for confirmation of the violative screened samples of Belgian monitoring programmes. Firstly, a method for tetracyclines in milk had to be developed and had to be optimised later for analysis of eggs and poultry muscle tissue. Most of the available methods were not practical for routine analysis because of their time consuming and laborious sample clean-up. Ion-pair chromatography reduces sample clean-up to a minimum. In Chapter 2, a HPLC method using ion-pair chromatography and DAD detection for the determination of tetracyclines in eggs and broiler chicken muscle is described.

Besides the tetracycline antibiotics, all other research work presented is dealing with anthelmintic veterinary drug residues. Because until then, there was no control on anthelmintic residues, it took a long time before the farmers were convinced that anthelmintic veterinary drugs also could give rise to residues in animal products of treated animals. Anthelmintic residues can not be screened efficiently by conventional screening methods due to the lack of antimicrobial activity of the anthelmintics and due to the lack of commercially available test kits. Therefore, in order to be able to propose the monitoring of Belgian milk, a confirmatory method for anthelmintics in milk had to be developed. The determination of anthelmintic residues in milk was performed with two HPLC methods using ion-pair chromatography and DAD detection (Chapter 3) which were simpler and faster than the existing methods.

More sensitivity, specificity, selectivity and confidence for surveillance purposes is obtained with mass spectrometry, certainly when an LC-MS/MS system operating in the MRM mode is used. The LC-MS/MS multiresidue method for anthelmintics in milk, used actually in the Belgian monitoring programme, is presented in Chapter 4. Compared to available, published methods,

the advantage of the developed method, is the ability to determine simultaneously benzimidazoles and levamisole using electrospray tandem quadrupole mass spectrometry.
The new approach of supplementary measurement of metabolite compounds was introduced. The tissue residue concentrations of metabolites of flubendazole and mebendazole, widely used in anthelmintic treatments of poultry and sheep, respectively, can be very high. Very sensitive LC-MS/MS procedures for the simultaneous determination of flubendazole and its metabolites in eggs and poultry muscle tissue are described in Chapter 5. The methods for mebendazole and its metabolites in some sheep tissues are discussed in Chapter 6.

1.8.3 Disposition of residues in food-producing animals

The determination of some validation parameters has to be performed, if possible, on incurred samples obtained after treatment of animals with veterinary products containing the target drug analyte. The applicability of the developed methods could be intensively tested on the numerous real animal product samples containing residues, which were analysed to study the disposition and distribution of residues in food-producing animals. Potential human health risks were indicated by off-label use of drugs including higher dosage levels and application to related, nonlabeled species.
Because no recent depletion data of tetracyclines in eggs and broiler chickens after oral administration and of levamisole in milk after topical administration were available, depletion studies were performed (Chapters 7 and 8, respectively).
The disposition and distribution of flubendazole and its metabolites in tissues of turkeys and guinea fowls and species differences between both avian species are described in Chapter 9.
The disposition of mebendazole and its metabolites in sheep tissues is presented in Chapter 10.

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PART I

DEVELOPMENT OF CONFIRMATORY METHODS FOR RESIDUES OF SOME VETERINARY DRUGS

CHAPTER 2

High performance liquid chromatographic method for the determination of tetracycline residues in eggs and chicken muscle tissue

Relevant publication:

De Ruyck H., De Ridder H., Van Renterghem R. and Van Wambeke F.
Validation of HPLC method of analysis of tetracycline residues
in eggs and broiler meat and its application to a feeding trial.
Food Additives and Contaminants, 1999, 16, 47-56

Abstract:

HPLC with ion-pair chromatography and UV diode array detection at 355 nm was used to determine residues of tetracycline antibiotics in eggs and chicken muscle tissue.
The samples were deproteinised with hydrochloric acid and the tetracycline antibiotics were extracted with acetonitrile. The extracts were purified by liquid-liquid extraction with methylene chloride and petroleum ether and were concentrated by evaporation using vacuum.
The analytical methods were optimised and validated. The mean recovery values for oxytetracycline in eggs and for tetracycline in breast muscle tissue were 76%. The within-day precision ranged from 8.0 to 11.8% for oxytetracycline in eggs and from 6.1 to 15.5% for tetracycline in breast muscle tissue. The between-day precision was 4.8% and 5.0% respectively for oxytetracycline in eggs and tetracycline in breast meat. The limit of detection and the limit of quantification for oxytetracycline in eggs were 2.2 and 13.0 µg/kg, respectively. These limit values for tetracycline in breast muscle tissue were 10.5 and 20.9 µg/kg, respectively.

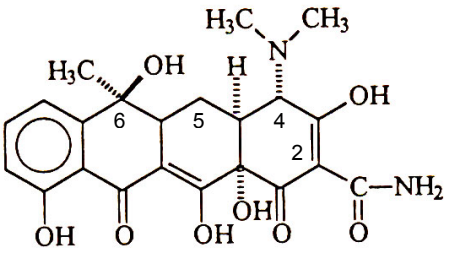
Keywords: tetracycline residues, eggs, chicken muscle tissue, HPLC

2.1 Introduction

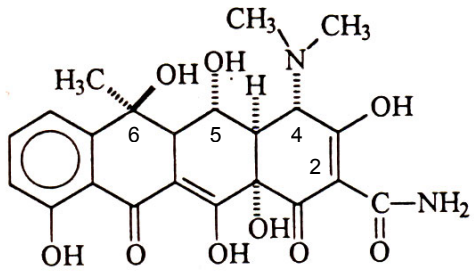
2.1.1 Description of tetracycline antibiotic compounds

Tetracycline antibiotics (TCA's) are broad-spectrum antibacterial drugs active against a number of gram-positive and gram-negative bacteria.[1] These group of substances are widely used as veterinary drugs for food-producing animals because of their broad-spectrum activity and cost effectiveness. The commonly used TCA drugs in veterinary medicine are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC). TC, OTC and CTC are natural TCA's obtained through fermentation with tetracycline-producing *Streptomycetes* bacteria. Doxycycline is a modified, semisynthetic derivative prepared by chemical conversion of OTC via methacycline.

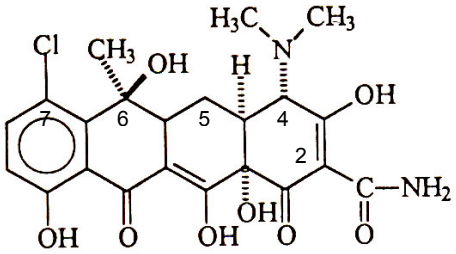
The TCA compounds have the basic "2-naphthacenecarboxamide" structure, which is hydroxy substituted at the 5-position.[2] The chemical structure of the molecules is presented in Figure 2.1.



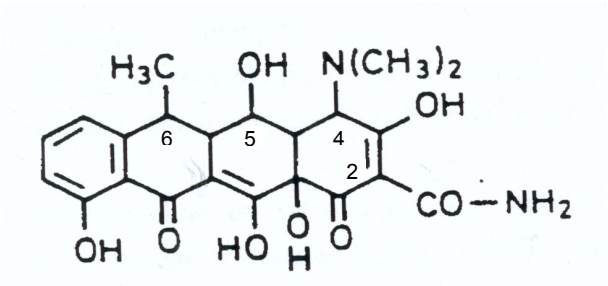
Tetracycline



Oxytetracycline (5-hydroxytetracycline)



Chlortetracycline (7-chlorotetracycline)



Doxycycline
(α-6-deoxy-5-hydroxytetracycline)

Figure 2.1 Molecular structures of tetracycline antibiotics[2]

The various TCA compounds are relatively good soluble in water, but are insoluble in non-polar organic solvents. The TCA's are ionised in the pH range between 3 and 8. The molecules exist as cations at a pH around 3 and lower, as zwitter-ion between a pH of 3.5 and 7.5 and as anions at a pH above 7.5. Tetracyclines form highly fluorescent complexes with metallic ions. The chelate formation is pH dependent. The emitted fluorescence from the chelate complex formed with a zirconium cation (Zr^{4+}), is optimal at a pH of 2.0.[3] Epimerisation and isomerisation

of the tetracycline, the oxytetracycline and the chlortetracycline molecules can occur during the sample pretreatment and during the determination. However, the formation of the C_4-epimer of the TCA's can be minimised using a mobile phase at a pH of 2.0.[4] Zurhelle *et al.*[5] have observed that the formation of 4-epi-chlortetracycline (4-epi-CTC), 6-iso-chlortetracycline (6-iso-CTC) and 4-epi-tetracycline (4-epi-TC) in eggs depends on temperature and time. No epimerisation or isomerisation occurred in spiked egg yolk and egg white samples kept refrigerated at 8°C. Neither could these chemical reactions be observed during the analytical procedure performed at ambient temperature. The epimerisation of OTC probably needs a temperature above 37°C or reaction times longer then 25 h.

2.1.2 Objectives

There are many HPLC methods available for the determination of residues of TCA's in eggs and muscle tissue. However, most of them are impractical for routine applications because of their time consuming and laborious sample clean-up.[6,7,8,9,10] More-over, several analytical methods are using fluorescence detection, which needs a supplementary derivatisation procedure.[7,8,9,11]

The objective of this study was to optimise a fast and quantitative multiresidue HPLC method for the routine determination of residues of TC, OTC, CTC and DC in eggs and chicken muscle tissue. Tissue samples of breast meat obtained from broiler chickens were used for the optimisation and validation of the analytical procedure.

The MRLs, set in the EU according to Council Regulation EEC/2377/90[12] and amendments, are 200 µg/kg for OTC, TC and CTC in eggs. Doxycycline is not licensed for treatment of laying hens. The MRL level for OTC, TC, CTC and DC in chicken muscle tissue was established at a concentration of 100 µg/kg.

The optimised method had to be used in depletion studies of oxytetracycline in laying hens and of tetracycline in broiler chickens, which are described in Chapter 7.

2.2 Materials and methods

2.2.1 Reagents

OTC-dehydrate, TC, CTC-hydrochloride and DC-hydrochloride, used to prepare standard solutions of the drug analytes, were obtained from Sigma (St. Louis, MO, USA). Methanol (Panreac, Barcelona, Spain), acetonitrile (Panreac for sample preparation and column flushing and Merck (Darmstadt, Germany) for mobile phase) and methylene chloride (Sigma) were of HPLC grade. Petroleum ether (b.p. 40-60°C, Panreac), 2-methyl-2-propanol (Aldrich, Milwaukee, WI, USA), phosphoric acid (Aldrich), hydrochloric acid (Panreac), trifluoroacetic acid (Pierce, Rockford, IL, USA) and sodium 1-decanesulphonate (Sigma) were of analytical grade. Water of HPLC grade was obtained by a Maxima LC 113 Ultra-pure water purification system (Elga, Bucks, UK).

2.2.2 Standard solutions

TCA analyte stock solutions (1 mg/ml) were prepared in methanol and stored at -20°C. The solutions were replaced by fresh solutions every month. The working standard solutions, prepared immediately before use, were made by dilution of the stock solution in 0.01 N hydrochloric acid (HCl).

2.2.3 Apparatus

The breast meat samples were minced and grinded with a Knifetec sample mill (Tecator, Höganäs, Sweden). The egg samples and the ground tissue samples were homogenised with an Unimix homogeniser (Haagen and Rinau, Bremen, Germany). The HPLC system (Perkin-Elmer, Norwalk, CT, USA) consisted of a Sec-4 Solvent Environmental Control delivery system with helium degassing, a Series 410 quaternary pump system, an ISS 200 autosampler and a 235 UV diode array detector. A reversed-phase polymeric column type PLRP-S, 100 Å, 5 µm, 150 X 4.6 mm i.d. was used in combination with a PLRP-S guard cartridge of 5 X 3.0 mm (Polymer Laboratories, Church Stretton, UK).

2.2.4 Sample preparation and clean-up

2.2.4.1 Eggs

Two ml of 1 N HCl was added as deproteinisation reagent to 10 g of homogenised whole egg in a beaker. To fortify samples, homogenised egg was spiked before the addition of HCl. After swirling, 50 ml of acetonitrile were added as extraction solvent and the sample liquid was homogenised. After standing for 10 min, the mixture was filtered through a plug of glass wool in the stem of a funnel. The extraction beaker was rinsed with 30 ml of acetonitrile. Fifty ml of filtrate was collected, which was purified in a separatory funnel by liquid-liquid extraction with 45 ml of methylene chloride and 45 ml of petroleum ether. The mixture was shaken vigorously. After standing for 5 min, the lower water layer was collected in a side-arm flask. This fraction was evaporated to 1-2 ml in a water bath at 45°C using vacuum. The remaining phase was transferred into a graduated tube. The flask was rinsed with 1 ml of 0.01 N HCl. The combined liquids were evaporated to about 0.8 ml under a stream of nitrogen. The concentrate was made up to 1 ml with 0.01 N HCl. Finally, the drug residue concentrate was filtered through a 0.2 µm regenerated cellulose (RC) filter cartridge (Alltech, Deerfield, IL, USA) in an autosampler vial.

2.2.4.2 Muscle tissue

A 10 g sample of ground breast muscle tissue was homogenised in 30 ml of water. To prevent foaming, five drops of 2-methyl-2-propanol were added. To fortify samples, muscle tissue was spiked before adding water. After standing for 5 min, 2.5 ml of 1 N HCl and 80 ml of acetonitrile were added to 20 ml of the homogenised mixture, while swirling. After standing for 10 min, the

liquid was filtered through a plug of glass wool in the stem of a funnel. Eighty ml of filtrate was purified in a separatory funnel by liquid-liquid extraction with 80 ml of methylene chloride and 80 ml of petroleum ether. The mixture was shaken vigorously. After standing for 10 min, the concentration phase was performed as described for eggs in paragraph 2.2.4.1.

2.2.5 Liquid chromatography

The separation of the tetracycline analytes while minimising endogenous interferences on the column, was performed by ion-pair chromatography with 1-decanesulphonate as ion-pair reagent. The mobile phase consisted of a buffer solution containing 0.02 M phosphoric acid with 0.01 M sodium 1-decanesulphonate (A), filtered through a 0.45 µm hydrophilised polypropylene filter disc (Millipore, Bedford, MA, USA), and pure acetonitrile (B). Gradient elution was applied and the gradient programme consisted of 75A:25B (0 min), 75A:25B to 63A:37B (1-22 min), 63A:37B (23-26 min), 63A:37B to 100B (27-28 min), 100B (29-43 min), 100B to 75A:25B (44-46 min) and 75A:25B (47-56 min). The flow rate was 1.0 ml/min, the injection volume was 150 µl and the detection wavelength of the diode array detector was set at 355 nm. The column was flushed daily with 120 ml of a solvent mixture consisting of 87.5% acetonitrile and 12.5% water. The column was back-flushed weekly with 960 ml of a solvent mixture consisting of 50% acetonitrile and 50% water containing 0.1% trifluoroacetic acid.

2.2.6 Method validation

2.2.6.1 Calibration curves

The linearity of the HPLC detection was checked by analysing a series of OTC, TC, CTC and DC standard solutions on four different days, in the concentration range of 50 to 5000 µg/l. The calibration curves were obtained using the linear least squares regression procedure of the peak area versus concentration ratios. The accuracy is expressed as the relative standard deviation (s_r) of the slope of the curves.

2.2.6.2 Extraction recoveries

The recovery of the four tetracyclines was determined by analysing blank egg and blank breast meat samples spiked with a standard mixture solution. The samples were fortified on three different days at two concentrations of respectively 100 and 200 µg/kg for eggs and 50 and 100 µg/kg for breast meat. The obtained mean recovery values were used for calculations of the residue concentrations and the precision data.

2.2.6.3 Precision

The precision of the assays was checked by repeatedly analysing blank egg and blank breast meat samples spiked at the respective MRL concentrations. The within-day precision

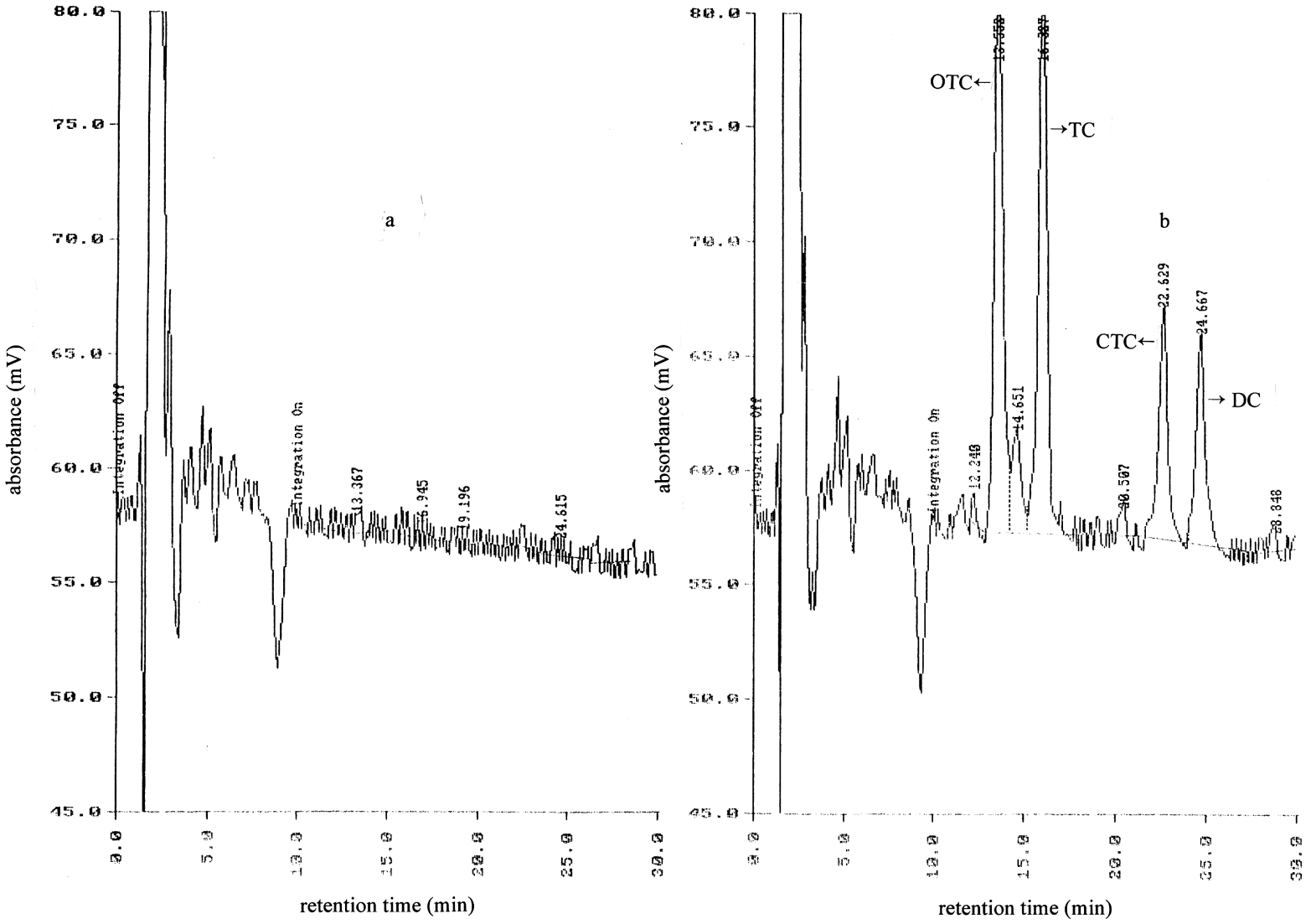


Figure 2.2 Chromatograms of a blank egg sample (a) and an egg sample spiked at a concentration of 200 µg/kg for each compound of a mixture of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) (b)

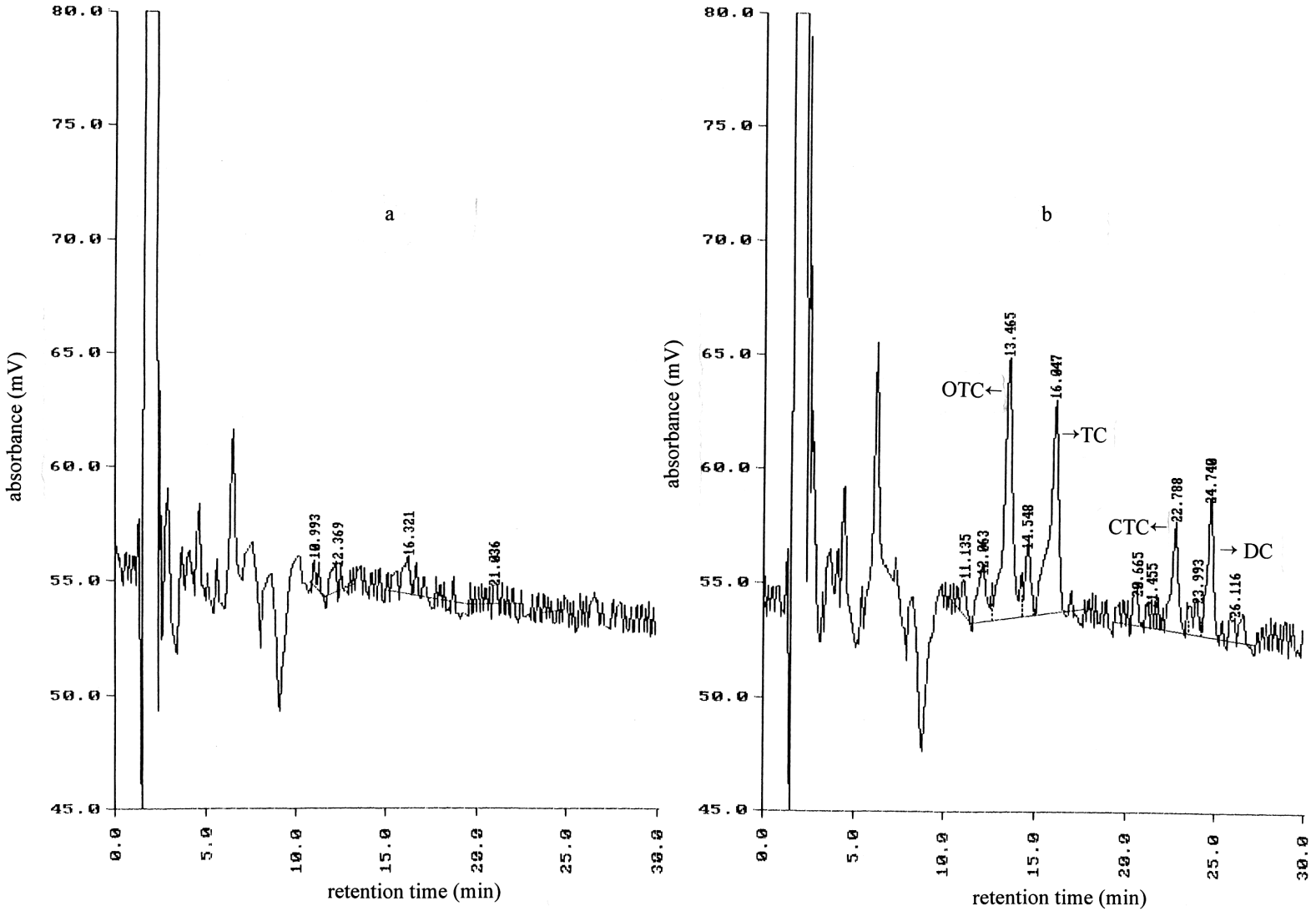


Figure 2.3 Chromatograms of blank breast meat sample (a) and breast meat sample spiked at a concentration of 100 µg/kg for each analyte of a mixture of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) (b)

(repeatability), the between-day precision and the overall precision were determined during three different days and are expressed as s_r value.

2.2.6.4 Limit of detection and limit of quantification

The detection limits (LODs) and quantification limits (LOQs) were estimated as those analyte concentrations which yield a signal-to-noise (S/N) ratio of at least 3/1 and 5/1, respectively. These analytical limits were determined by extrapolating the five-point matrix calibration curves made with blank samples spiked at 0, 100, 200, 300 and 400 µg/kg for eggs and at 0, 50, 100, 150 and 200 µg/kg for breast meat, respectively.

2.3 Results and discussion

2.3.1 Analytical method

In this study, a relatively simple, fast and accurate method, suitable for routine analysis, was developed. The discussed analytical method was an optimisation of available procedures described by Moats,[13] Moats and Harik-Khan,[14] Moats and Harik-Khan[15] and White *et al.*[16] Some previous investigation had indicated a higher stability of TCA analytes in standard solutions and even in spiked sample extracts when using methanol as solvent. The TCA's are slightly unstable in highly acidic (1N HCL) conditions.[6]

Moats and Harik-Khan[15] obtained better results when the tissue samples were first homogenised in water before the tetracycline residues were extracted. TCA's are polar, ionisable compounds which do not generally partition effectively into organic solvents.[13,17] The analytes could be concentrated in the water layer by adding equal volumes of petroleum ether and of methylene chloride to the acetonitrile filtrate.

Careful selection of pH and of ionic strength and the addition of ion-pair reagents improve the partitioning of TCA's into organic solvents.[17] The application of ion-pair chromatography reduces the sample clean-up to a minimum. Moats and Harik-Khan[14] have reported that tetracyclines could be effectively separated from endogenous interferences in milk extracts by ion-pair chromatography without further clean-up.

A polymeric analytical column was chosen, to minimise possible chromatographic problems which can be expected with silica-based bonded columns.[18] With a bonded column, interactions between analytes and the residual silanol groups present in the silica-based support materials may occur. A polymeric column type is widely used in the residue analysis of TCA's to obtain higher recoveries and lower peak tailing.[4,7,9,11]

For multiresidue analysis, gradient elution is advantageous because sharp peak shapes can be maintained throughout the elution and early eluting peaks are not excessively co-eluted.[16] An acceptable analyte separation with relatively sharp peaks was obtained applying a elution programme with linear gradients. Representative chromatograms of a blank egg sample and an

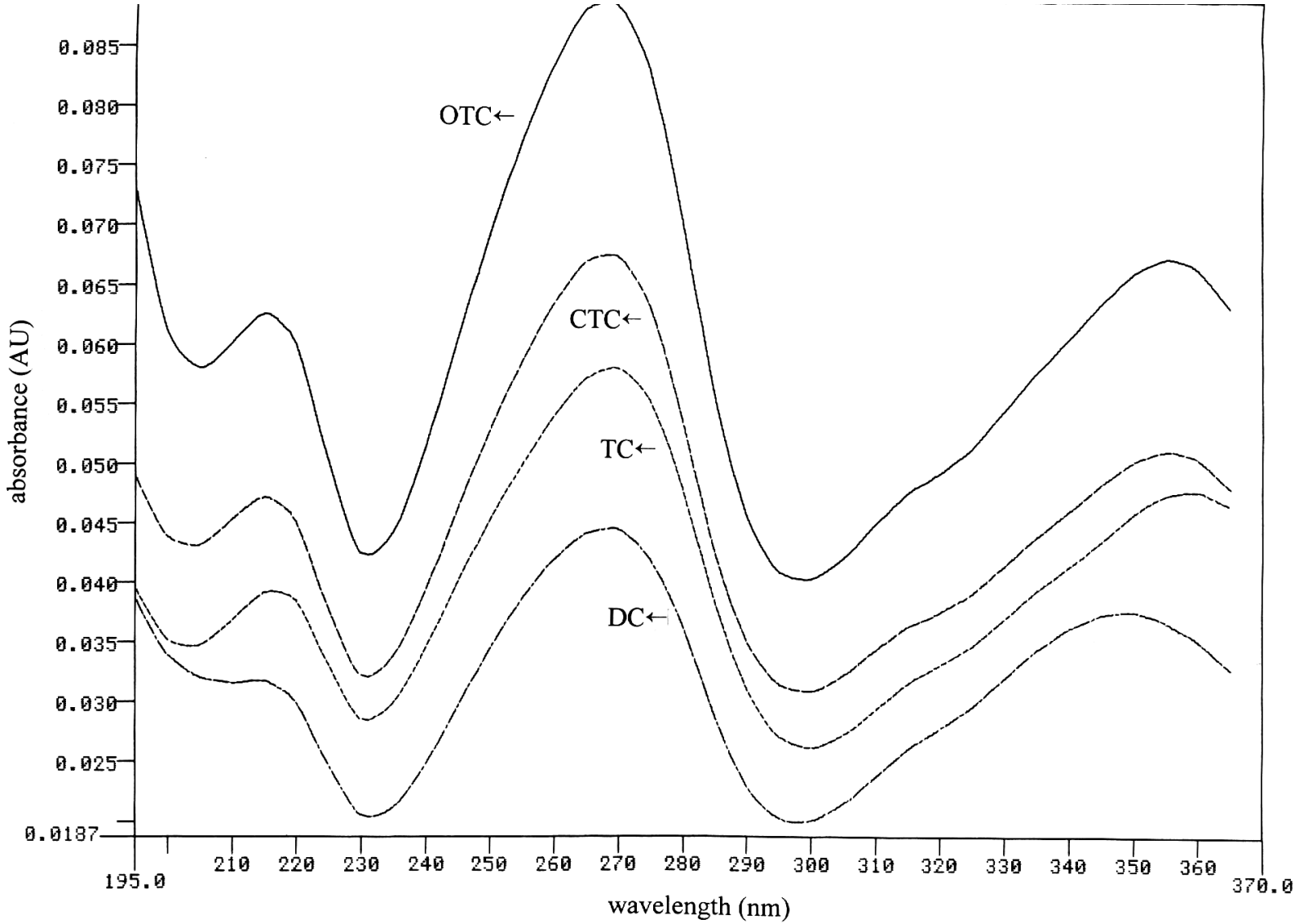


Figure 2.4 UV spectra of tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) at a concentration of 10 g/l

egg sample fortified at MRL concentration are illustrated in Figure 2.2. Figure 2.3 shows chromatograms of blank breast meat sample and a breast meat sample spiked at MRL concentration.

The UV detection by the DAD was carried out at a wavelength of 355 nm. This wavelength was chosen as mean optimum value for the four tetracycline substances. As shown in the representative UV spectra in Figure 2.4, the UV absorption values were much higher at 268 nm. However, in preliminary research more interference and non-specific absorption was obtained at this wavelength. With the specific UV spectra, supplementary analyte confirmation was obtained.

2.3.2 Validation of the analytical method

2.3.2.1 Calibration curves

The regression parameters of the external calibration curves obtained with six calibration points (50, 100, 200, 500, 1000, 5000 µg/l) with standard solutions made with mixtures of the TCA's are summarised in Table 2.1. The curves are described by the equation $Y = aX + b$ ("Y" represents peak area, "*a*" represents slope, "X" represents concentration in µg/l and "*b*" represents intercept). The HPLC respons was linear in the concentration range of 50 to 5000 µg/l. The s_r values on the slope of the calibration curves obtained on four different days were below 9%.

2.3.2.2 Recovery studies

Table 2.2 presents the recoveries obtained for egg and breast meat samples fortified at two concentrations with a TCA analyte mixture. The mean recovery values for OTC, TC, CTC and DC in eggs and in breast meat were 76, 78, 62 and 58% and 78, 76, 76 and 68%, respectively. The s_r values on the mean recovery were 8.4% and 10.4% for OTC in eggs and for TC in breast meat, respectively. These analytes were intensively measured in the depletion studies. The recovery of the analytical procedure can probably be increased by replacing acetonitrile for ethyl acetate as extractant. Cooper *et al.*[17] have observed a highest recovery of 90% for OTC in spiked cattle kidney tissue after extraction with ethyl acetate. They also investigated the effect of spiking procedures (spike contact time, spike solvent and tissue state) on the recovery. The increase of the spike contact time caused a small but statistical significant reduction in recovery due to a slight degradation of OTC.

The obtained recoveries for breast meat were slightly lower than those reported by Moats and Harik-Khan[15] for beef muscle tissue. McCracken *et al.*[8] have obtained comparable results for the residue determination in muscle tissues of pigs, cattle and poultry. Much lower recovery values for pork muscle tissue and eggs were measured by Croubels *et al.*,[9] probably due to the

extensive sample clean-up. The selection of the SPE cartridges, applied for sample clean-up, is critical.[10] Only cartridges providing high and consistent recoveries should be used.

Table 2.1 Regression analysis of calibration curves obtained on four different days

Analyte	Day	Slope (*a*)	Intercept (*b*)	*r*
OTC	1	1166.4	-14104.5	0.9999
	2	1141.6	-17854.0	0.9999
	3	1142.4	-23437.7	0.9999
	4	1183.2	-19024.9	0.9999
		s_r = 1.7%		
TC	1	1133.7	-46188.1	0.9998
	2	1034.9	-3850.0	0.9999
	3	1067.1	-9725.6	0.9999
	4	1111.7	-14337.9	0.9999
		s_r = 4.1%		
CTC	1	567.7	13368.2	0.9999
	2	586.2	-10758.4	0.9999
	3	631.7	-29740.4	0.9996
	4	539.9	-12821.9	0.9999
		s_r = 4.5%		
DC	1	588.1	4176.9	0.9999
	2	606.6	-25925.0	0.9998
	3	702.0	-54513.6	0.9992
	4	607.8	-22152.2	0.9999
		s_r = 8.2%		

OTC: oxytetracycline TC: tetracycline CTC: chlortetracycline DC: doxycycline

Table 2.2 Extraction recoveries for egg and breast meat samples spiked with tetracyclines [mean recovery (%) (s_r (%))]

	Eggs					Breast meat				
	Concentration (µg/kg)	OTC	TC	CTC	DC	Concentration (µg/kg)	OTC	TC	CTC	DC
Day 1	100[a]	80 (5.3)	77 (9.0)	57 (15.4)	54 (14.0)	50[c]	73 (9.6)	70 (14.1)	70 (9.3)	67 (14.6)
	200[b]	72 (9.8)	76 (9.3)	60 (7.6)	57 (8.4)	100[a]	88 (10.5)	83 (7.4)	76 (13.1)	69 (17.3)
Day 2	100[a]	79 (8.1)	79 (7.5)	58 (7.0)	58 (6.6)	50[d]	80 (13.0)	80 (7.0)	75 (12.8)	68 (8.9)
	200[b]	70 (4.4)	75 (5.4)	65 (6.5)	57 (11.3)	100[c]	71 (10.9)	79 (5.3)	76 (14.2)	66 (11.0)
Day 3	100[a]	75 (6.8)	77 (7.6)	61 (14.4)	58 (12.2)	50[c]	74 (8.6)	72 (6.6)	73 (13.8)	71 (2.9)
	200[a]	81 (4.8)	84 (2.8)	69 (10.9)	65 (8.6)	100[c]	79 (8.2)	70 (8.9)	85 (5.2)	70 (8.0)
	Overall	76 (8.4)	78 (7.8)	62 (12.5)	58 (11.1)	Overall	78 (12.5)	76 (10.4)	76 (12.4)	68 (11.2)

[a] $n = 8$ [b] $n = 7$ [c] $n = 6$ [d] $n = 5$ OTC: oxytetracycline TC: tetracycline CTC: chlortetracycline DC: doxycycline

Table 2.3 Precision of the multiresidue tetracycline determinations [s_r (%) (mean value (µg/kg))]

	Eggs					Breast meat			
	OTC	TC	CTC	DC		OTC	TC	CTC	DC
Within-day					Whitin-day				
Day 1[b]	8.0 (210)	6.6 (208)	7.3 (207)	7.9 (194)	Day 1[c]	10.1 (108)	6.1 (95)	10.4 (113)	11.2 (105)
Day 2[a]	11.8 (211)	14.6 (195)	9.0 (208)	10.2 (213)	Day 2[b]	6.0 (93)	15.5 (96)	12.4 (107)	9.1 (102)
Day 3[b]	9.2 (205)	8.0 (192)	6.0 (197)	5.6 (209)	Day 3[b]	8.1 (103)	8.7 (110)	21.9 (96)	16.4 (98)
Between days[d]	4.8 (203)	5.3 (199)	7.8 (201)	6.5 (211)	Between days[d]	4.3 (107)	5.0 (91)	12.2 (107)	12.8 (108)
Overall	9.4 (208)	10.5 (198)	7.7 (204)	8.8 (207)	Overall	9.7 (101)	12.6 (100)	15.6 (105)	12.3 (102)

[a] n = 11 [b] n = 8 [c] n = 6 [d] n = 3 OTC: oxytetracycline TC: tetracycline CTC: chlortetracycline DC: doxycycline

2.3.2.3 Precision of the assay

The mean concentrations and the precision values, expressed as s_r values, obtained for the repetitive analyses are summarised in Table 2.3. The repeatability or within-day precision ranged from 8.0 to 11.8% for OTC in eggs and from 6.1 to 15.5% for TC in breast meat. The between-day precision was 4.8% for OTC in eggs and 5.0% for TC in breast meat, respectively. The overall precision values for these analytes and these matrices were 9.4 and 12.6%, respectively.

2.3.2.4 Limit of detection and limit of quantification

The linearity data of the determination assay and the analytical limit values obtained with spiked egg and breast meat samples are shown in Table 2.4. The LOD and the LOQ values calculated for OTC in eggs were 2.2 and 13.0 µg/kg, respectively. These limit values obtained for TC in breast meat were 10.5 and 20.9 µg/kg, respectively.

Table 2.4 LOD (µg/kg) and LOQ(µg/kg) values for tetracycline determinations

	Eggs			Breast muscle tissue		
	r	LOD	LOQ	*r*	LOD	LOQ
OTC	0.9986	2.2	13.0	0.9999	8.4	17.6
TC	0.9994	4.7	16.1	0.9999	10.5	20.9
CTC	0.9996	28.6	51.6	0.9999	18.2	37.4
DC	0.9977	27.3	47.3	0.9996	18.6	34.5

OTC: oxytetracycline TC: tetracycline CTC: chlortetracycline DC: doxycycline

The calculated values for OTC and TC in breast meat were comparable with those reported by Moats and Harik-Khan.[15] The obtained values for CTC were somewhat lower. McCracken et al.[8] and Blanchflower et al.[7] have obtained comparable results for the analysis of meat samples using more elaborate equipment and fluorescence detection. Much lower detection limits can be obtained when a more intensive or a more selective sample clean-up is performed. Also, low detection limits can be obtained measuring the analytes by more specific fluorescence detection. Agasoster[11] has described an automated method for control of OTC residues in milk, eggs and several animal tissues. Tissue homogenate, skimmed milk or whole egg solution was dialysed and the dialysate was enriched on a small polystyrene column on-line to HPLC. The analytes were eluted by ion-pair chromatography and were measured by a fluorescence

spectrophotometer. Oxytetracycline could be detected (S/N of 3/1) at concentrations between 1 and 8 µg/kg depending on the matrix.

Croubels *et al.*[9] have proposed an appropriate method for trace residue analysis of TCA's in animal tissues and egg. The sample clean-up was performed by metal chelate affinity chromatography based on the chelate complex formation of zirconium with the tetracycline compounds. To enable low detection limits, further concentration on an extraction membrane with cation-exchange properties was carried out. The TCA's were measured by fluorescence detection after post-column derivatisation. The LOD values (S/N of 4/1) ranged from 0.4 to 5.0 µg/kg, depending on tissue and compound. The described procedure was successfully used in other applications. De Wasch *et al.*[19] have reported the detection of residues of TC, OTC, CTC and DC in pork and chicken meat. The detection limits (S/N of 4/1) were estimated at concentrations between 0.4 and 1.4 µg/kg depending on the compound. In a tissue depletion study in turkeys, doxycycline could be detected at an estimated LOD (S/N of 3/1) of 1.0 and 1.2 µg/kg in muscle and liver tissue, respectively.[20]

2.4 Conclusions

The optimised chromatographic method for the simultaneous determination of tetracycline, oxytetracycline, chlortetracycline and doxycycline in eggs and in chicken muscle tissue was proven to be reliable and suitable for routine analysis. The sample preparation could be kept minimal to be able to perform the procedure in a relatively short time. An acceptable separation of the analytes could be obtained using ion-pair chromatography. Supplementary analyte confirmation was obtained with the specific UV spectra of the diode array detector.

The optimised method is used in depletion studies of oxytetracycline in laying hens and of tetracycline in broiler chickens, which are described in Chapter 7.

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CHAPTER 3

Determination of anthelmintic drug residues in milk by high performance liquid chromatography

Relevant publication:

De Ruyck H., Van Renterghem R., De Ridder H. and De Brabander D.
Determination of anthelmintic residues in milk by high performance liquid chromatography.
Food Control, 2000, 11, 165-173

Abstract:

The optimisation of two HPLC methods for the determination of residues of anthelmintic drugs in milk is described. Satisfactory analytical separations and detection limits were obtained with ion-pair liquid chromatography and UV diode array detection at 225 nm for levamisole and at 295 nm for fenbendazole, thiabendazole, albendazole and oxibendazole.

Levamisole was extracted with chloroform after the milk sample was made alkaline. The analyte extract was concentrated by evaporation using vacuum. For the determination of the benzimidazoles, the milk sample was deproteinised with acetonitrile and the analytes were extracted with ethyl acetate. After centrifugation, the supernatant was defatted with iso-octane. The extract was further purified by liquid-liquid extraction with methylene chloride. The remaining organic phase was washed with di-sodium hydrogen phosphate and was concentrated by evaporation using vacuum.

The mean extraction recoveries ranged from 68 to 85%. The limits of detection for fenbendazole, thiabendazole, albendazole, oxibendazole and levamisole were 3.1, 3.4, 3.8, 1.3 and 0.5 µg/l, respectively. The limits of quantification for these anthelmintics were 6.8, 4.3, 6.9, 2.7 and 1.4 µg/l, respectively. The overall precision data, based on within-day and between-day variations during three different days were 11.9, 3.9, 8.2, 7.4 and 12.7%, respectively.

Keywords: anthelmintic drug residues, milk, HPLC-DAD

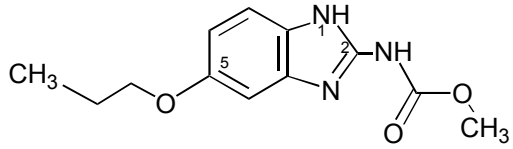
3.1 Introduction

3.1.1 Description of anthelmintic compounds

Anthelmintic veterinary drugs are widely used prophylactically as well as curatively in food-producing animals against endoparasitic worm infections. The anthelmintic drug substances involved in the analytical procedure described in this chapter are fenbendazole (FB), thiabendazole (TB), albendazole (AB), oxibendazole (OB) and levamisole (LE). These synthesised compounds have a broad-spectrum activity and are very suitable for the control of most gastrointestinal nematodes and lungworms in cattle, sheep, pigs and poultry species.[1,2]

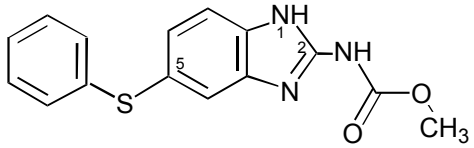
FB, TB, AB and OB belong to the benzimidazole (BZ) group of antiparasitic drug substances. Benzimidazole anthelmintic compounds have as common central chemical structure a "1,2-diaminobenzene" moiety (Figure 3.1) which gives rise to the "benzimidazole" nucleus.[1] Levamisole is defined as the L-isomer of tetramisole, an imiothiazole (imidazothiazole) anthelmintic substance consisting of a racemic mixture of D- and L-isomers.[2,3,4]

The molecular structures of the anthelmintic drug compounds are illustrated in Figure 3.1.



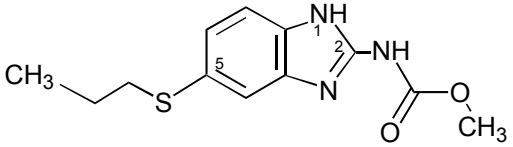
oxibendazole

methyl-[5-(propoxy)-1*H*-benzimidazole-2-yl] carbamate



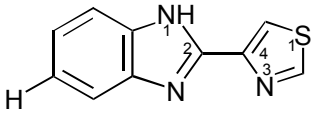
fenbendazole

methyl-[5-(phenylthio)-1*H*-benzimidazole-2-yl] carbamate



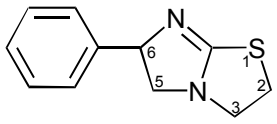
albendazole

methyl-[5-(propylthio)-1*H*-benzimidazole-2-yl] carbamate



thiabendazole

[2-(1,3-thiazol-4-yl)-1*H*-benzimidazole]



levamisole

[L-(-)-2,3,5,6-tetrahydro-6-phenylimidazo-(2,1-b)-thiazole]

Figure 3.1 Chemical structures of the examined anthelmintics[1,2]

In general, benzimidazoles are insoluble or only slightly soluble in water.[1] The BZs are generally soluble in dimethyl sulphoxide and dimethyl formamide. Levamisole is very soluble in water and in polar organic solvents.[2] Benzimidazoles intensively absorb UV light.[1] Thiabendazole shows fluorescence properties. The examined BZs are weakly basic compounds due to their secondary amino group.

The heat stability of levamisole was investigated by Rose *et al*.[5] Levamisole was found to be stable in cooking water, but unstable in cooking oil at 260°C, with a half-life of about 5 min. Levamisole was stable in incurred and fortified samples under heating conditions during microwaving, boiling, grilling or frying. However, some migration of levamisole from the tissue into the meat juices does occur. Roasting was the only heating process where a loss of levamisole was observed. Levamisole intake from contaminated meat can be reduced by discarding the liquids used for cooking or by discarding the meat juices.

3.1.2 Toxicity

Benzimidazole anthelmintic residues are of little human health concern. Toxicological data include studies on metabolism, carcinogenicity, genotoxicity, effects on reproduction and teratogenicity.[1] Short term studies suggest that this group of compounds is efficacious for parasitic control while demonstrating a great margin of safety in mammals, including humans. The most sensitive indicators of developmental toxicity associated with BZs appear to be limb defects associated with teratogenic activity in rats fed with AB. Armour[3] also reported that many BZs including oxibendazole, fenbendazole, albendazole and other analogues, are characterised by a very low toxicity for mammalian hosts, in general. It was virtually impossible to find a median lethal dose (LD_{50}) for fenbendazole. Albendazole, known to be teratogenic, should not be used in early pregnancy at higher than therapeutic dose. Delatour and Parish[6] have reported teratogenicity for albendazole in sheep. The malformations observed were predominantly skeletal.

The mammalian toxicity of levamisole is greater than with the benzimidazoles although, in normal usage, toxic effects are seldom seen and adverse effects are rare.[2,3] Levamisol is also used in human medicine as an anthelmintic, as an immunomodulator and as an adjuvant in the treatment of colorectal cancer.[2]

3.1.3 Objectives

Residue concentrations of anthelmintic drugs in milk can be very high if farmers do not respect the recommended withdrawal times after treatment of dairy cows. Adequate safety margins (MRLs) should be established to prevent consumer health problems since in many cases teratogenic metabolites have been identified in animal products such as milk, eggs and meat.[6] The MRL values in milk are 10 µg/kg for FB, 100 µg/kg for TB and for AB and 50 µg/kg for OB.[7]

The MRL value for OB is provisional and still under consideration. Levamisole is not more allowed for lactating dairy cows.

Several analytical methods are described in the literature. De Baere *et al.*[8] described a HPLC-UV method for the determination of levamisole in animal plasma. A liquid-liquid extraction procedure in alkaline medium, using hexane-isoamyl alcohol as extraction solvent, was performed to clean-up the plasma samples. Chromatographic separation was achieved by gradient elution with a mobile phase containing ammonium acetate buffer and pure acetonitrile. However, the method is not sensitive. A LOD of 77 µg/l was obtained.

Chappell *et al.*[4] presented a method for the determination of levamisole in milk at low concentrations with a hyphenated multidimensional technique consisting of a coupled HPLC-GC system. The HPLC system was used to provide a sample clean-up phase prior to on-line GC analysis. A solid phase extraction (SPE) sample clean-up procedure was applied. Detection limits of 0.4 and 2.2 µg/l could be obtained by flame ionisation and nitrogen-phosphorus GC detection, respectively.

A multiresidue HPLC procedure with diode array detection for the determination of eight benzimidazole anthelmintics in foodstuffs of animal origin was reported by Neri *et al.*[9] The sample was extracted with acetonitrile using an ultrasonic bath. The organic layer was evaporated and the residue was dissolved in hydrochloric acid. After an initial washing with hexane to remove fats, the aqueous layer was purified by SPE. The concentrated and cleaned sample extract was analysed by gradient elution with a mobile phase consisting of phosphate buffer and acetonitrile. Limits of detection lower than 10 µg/kg were reported.

In another procedure, matrix solid phase dispersion (MSPD) was compared with SPE for the determination of fenbendazole and metabolites in milk. The MSPD method showed some advantages such as shorter times of analysis and smaller amounts of reagents. However, lower LODs were obtained with the more laborious and expensive SPE procedure.

For monitoring purposes, an analytical method for anthelmintics in milk had to be developed. This Chapter reports an analytical study dealing with the optimisation of sensitive and quantitative HPLC methods with UV diode array detection for routine analysis of cow's milk on residues of the benzimidazoles fenbendazole, thiabendazole, albendazole, oxibendazole and of the imidazothiazole levamisole. The method had to be suitable for quantification of LE residues in milk during a depletion study with dairy cows. The excretion study of levamisole in milk of treated dairy cows is reported in Chapter 8.

3.2 Materials and methods

3.2.1 Reagents

FB, TB, AB, OB and LE-hydrochloride, obtained from Sigma (St. Louis, MO, USA), were used to prepare the standard solutions. Dimethyl sulphoxide (Panreac, Barcelona, Spain), acetonitrile

(BDH (Poole, UK) for sample preparation and column flushing and Merck (Darmstadt, Germany) for mobile phase), methanol (Merck), methylene chloride (Panreac), iso-octane (Panreac) and chloroform (Panreac) were of HPLC grade. Ethyl acetate (Sigma), di-sodium hydrogen phosphate (Merck), sodium 1-octanesulphonate (Sigma), ortho-phosphoric acid (> 85%, UCB, Ghent, Belgium) and sodium hydroxide (1 N, Panreac) were of analytical grade. Water was of HPLC grade and was obtained from a Maxima LC 113 Ultra-pure water purification system (Elga, Bucks, UK).

3.2.2 Standard solutions

The benzimidazole stock solutions (0.4 mg/ml) were prepared in a mixture of 40% dimethyl sulphoxide and 60% acetonitrile. These stock solutions were stored refrigerated at 5°C and were replaced every three weeks. The working standard solutions were prepared by mixing one part of acetonitrile with one part of 0.01 M sodium 1-octanesulphonate which was adjusted to a pH of 3.5 with ortho-phosphoric acid. These working solutions were prepared immediately before use.

The LE stock solution (0.4 mg/ml) was prepared in a mixture of 40% dimethyl sulphoxide and 60% methanol. This stock solution was also stored refrigerated at 5°C and replaced every three weeks. The working standard solutions were made in a mixture of one part of methanol and one part of 0.01 M sodium 1-octanesulphonate. The solutions were adjusted to a pH of 3.0 with ortho-phosphoric acid. These solutions were also prepared immediately before use.

3.2.3 Apparatus

A RC-5B Sorvall centrifuge (DuPont Instruments, Wilmington, DE, USA) was used during sample preparation. The HPLC system[11] described in Chapter 2, was used. During this analytical study, the reversed-phase column was an Alltima C_{18} column (Alltech, Deerfield, IL, USA) 100 Å, 5 µm, 150 X 3.2 mm i.d. protected with an Alltima C_{18} guard cartridge of 7.5 X 3.2 mm i.d. The column was thermostated at 35°C using a Column Heater Model 7971 (Jones Chromatography, Hengoed, Mid Glamorgan, UK).

3.2.4 Sample preparation and clean-up

3.2.4.1 Procedure for benzimidazoles

Twenty five ml of acetonitrile and 5 ml of ethyl acetate as deproteinisation reagent and as extraction solvent, respectively, were added to 10 ml of milk sample in a centrifuge tube. After shaking vigorously during 30 s, the sample extract was centrifuged for 5 min at 5000 rpm. The clear supernatant was defatted with 30 ml of iso-octane in a separatory funnel. After mixing, the lower aqueous layer was purified by liquid-liquid extraction with 60 ml of methylene chloride in a second separatory funnel and the upper iso-octane layer was discarded. The mixture was

shaken vigorously during 30 s. After standing for 5 min, the lower organic layer was transferred into another separatory funnel and the upper layer was discarded. The remaining organic phase was washed with 20 ml of 0.1 M di-sodium hydrogen phosphate adjusted to a pH of 10.0 with sodium hydroxide. The mixture was shaken for 30 s. After standing for 5 min, the bottom organic layer was transferred into a side-arm flask and was evaporated to 4-5 ml using vacuum in a water bath heated at 50°C. The remaining fraction was transferred into a graduated tube. The flask was rinsed with 1 ml of a mixture of one part acetonitrile and of one part 0.01 M sodium 1-octanesulphonate adjusted to a pH of 3.5 with ortho-phosphoric acid. The combined liquids were evaporated to dryness under a stream of nitrogen. The residue was made up to 1 ml with the above mentioned mobile phase mixture. This cleaned and concentrated sample extract was finally filtered through a 0.2 µm regenerated cellulose (RC) filter cartridge (Alltech, Deerfield, IL, USA) in an autosampler vial.

3.2.4.2 Procedure for levamisole

Sixty ml of chloroform as extraction solvent and 20 ml of 0.1 N sodium hydroxide were added to 10 ml of milk sample in a separatory funnel. This sample mixture was shaken for 1 min. After standing for 5 min, the lower organic layer was collected in a side-arm flask and the concentration of this fraction was performed as previously described in the procedure for BZs (paragraph 3.2.4.1). However, the flask was rinsed with 1 ml of chloroform and the volume correction was performed with a mixture of one part methanol and one part 0.01 M sodium 1-octanesulphonate adjusted to a pH of 3.0 with ortho-phosphoric acid.

3.2.5 Liquid chromatography

The separation of the anthelmintic analytes was performed by ion-pair chromatography with 1-octanesulphonate as ion-pair reagent. The analytes were detected by a diode array detector (DAD). The benzimidazoles and levamisole were determined by separate HPLC methods.

3.2.5.1 Multiresidue method for benzimidazoles

The mobile phase consisted of a buffer solution (A), acetonitrile (B) and water (C). The buffer solution contained 0.01 M sodium 1-octanesulphonate adjusted to a pH of 3.5 with ortho-phosphoric acid, and was filtered through a 0.45 µm hydrophilised polypropylene filter disc (Millipore, Bedford, MA, USA). Gradient elution was applied and the gradient programme consisted of 80A:20B (0 min), 80A:20B to 50A:50B (1-2 min), 50A:50B (3-13 min), 50A:50B to 50C:50B (14-15 min), 50C:50B (16-35 min), 50C:50B to 80A:20B (36-37 min) and 80A:20B (38-57 min). The mobile phase flow rate was 0.5 ml/min and a volume of 50 µl was injected by the autosampler. The analytes were detected by the diode array detector at a wavelength of 295 nm. The column was flushed daily with 150 ml of a solvent mixture consisting of 50% acetonitrile and 50% water.

3.2.5.2 Method for levamisole

The mobile phase consisted of a filtered buffer solution (A), methanol (B) and water (C). The buffer solution contained 0.01 M of the ion-pair reagent and was adjusted to a pH of 3.0 with ortho-phosphoric acid. Isocratic elution was applied with a mobile phase containing 48A:52B. The complete HPLC run consisted of 48A:52B (0-8 min), 48A:52B to 48C:52B (9-10 min), 48C:52B (11-33 min), 48C:52B to 48A:52B (34-35 min) and 48A:52B (36-58 min). The flow rate for the mobile phase was 0.5 ml/min. A sample extract volume of 150 µl was injected and the analytes were measured at a wavelength of 225 nm by the DAD. The same column flushing procedure was applied as described in the method for benzimidazoles (paragraph 3.2.5.1).

3.2.6 Validation of the HPLC assays

3.2.6.1 Standard curves

The linearity of the HPLC respons was studied by analysing a series of mixed benzimidazole and levamisole standard solutions in the concentration range 20-5000 µg/l. The analyses were performed on three different days. The standard curves were obtained using the linear least squares regression procedure of the peak area versus concentration ratios. The between-day variation is expressed as the relative standard deviation (s_r) of the slope of the curves.

3.2.6.2 Recovery

The extraction efficiency for the five examined anthelmintic compounds was determined by analysing spiked blank milk samples. The samples were fortified at two concentration levels, 10 and 100 µg/l. For the benzimidazoles, milk samples were fortified with a mixture of standard solutions. The recoveries were calculated by extrapolating of standard curves taking into account the concentration factor of 10. The accuracy of the recovery determinations is given by the s_r values.

3.2.6.3 Linearity, limit of detection and limit of quantification

The linearity of the complete analytical assay including sample preparation, was checked by analysing blank milk samples fortified separately with a mixture of the examined benzimidazoles and with levamisole. The analyses were performed on three different days on samples spiked at concentrations between 0 and 1000 µg/l (seven calibration points). The matrix calibration curves were calculated using the linear least squares regression analyses of the peak area to concentration ratios. The s_r value of the slope of the curves represents the between-day variation. The limit of detection (LOD) and the limit of quantification (LOQ) were estimated as those concentrations of analyte which yield a signal-to-noise (S/N) ratio of at least 3/1 and 5/1, respectively. These analytical limits were determined by extrapolating of linear matrix calibration

curves, which were obtained by plotting peak height versus concentration with data obtained from the linearity study.

3.2.6.4 Trueness

This accuracy parameter was studied by analysing blank milk samples spiked with anthelmintic standard solutions at a concentration of 100 µg/l. The four benzimidazole substances were spiked with a mixed spiking solution. The trueness was determined by comparing the measured concentration, calculated with matrix calibration curves, to the fortified concentration.

3.2.6.5 Precision

The precision of the assays was studied by repeatedly analysing milk samples, fortified at the respective MRL concentration for the various benzimidazole compounds and at 10 µg/l for levamisole. The drug concentrations were calculated by extrapolating of matrix calibration curves. The within-day precision (repeatability), the between-day precision and the overall precision were determined on three different days and were expressed as s_r value.

3.3 Results and discussion

3.3.1 Analytical methods

In this study, an easy, relatively fast and reliable method for the routine determination of anthelmintic residues in milk had to be developed. Due to the great difference in chemical properties of benzimidazole compounds and levamisole, the optimisation of a multiresidue procedure for the analysis of milk samples on the five anthelmintic analytes, was not successful. The BZ analytes were determined with a optimised multiresidue method based on a procedure for fenbendazole described by Fletournis *et al.*[12]

Preliminary experiments indicated that a slightly higher recovery for the BZs was obtained by addition of ethyl acetate to the acetonitrile deproteinisation and extraction reagent. The BZs are weakly basic hydrophobic compounds with appreciable solubility in polar organic solvents.[12] Ethyl acetate has a high solvating power for weakly basic compounds and is able to form emulsion-free interfaces.[13] Other research workers[14,15] also used ethyl acetate for the extraction of anthelmintic residues in animal product samples.

As co-extracted lipids can interfere in the analysis, especially when sample extracts were concentrated to a small volume, a defatting phase is recommended. Washing of the extracts with iso-octane could efficiently remove lipids.[12] Some clean-up of the milk extract could be affected by addition of methylene chloride. By this addition, most of the water of the extract was separated, which favours removal of highly basic and acidic hydrophilic interfering compounds. Interfering weakly acidic compounds could effectively be removed by further purification by

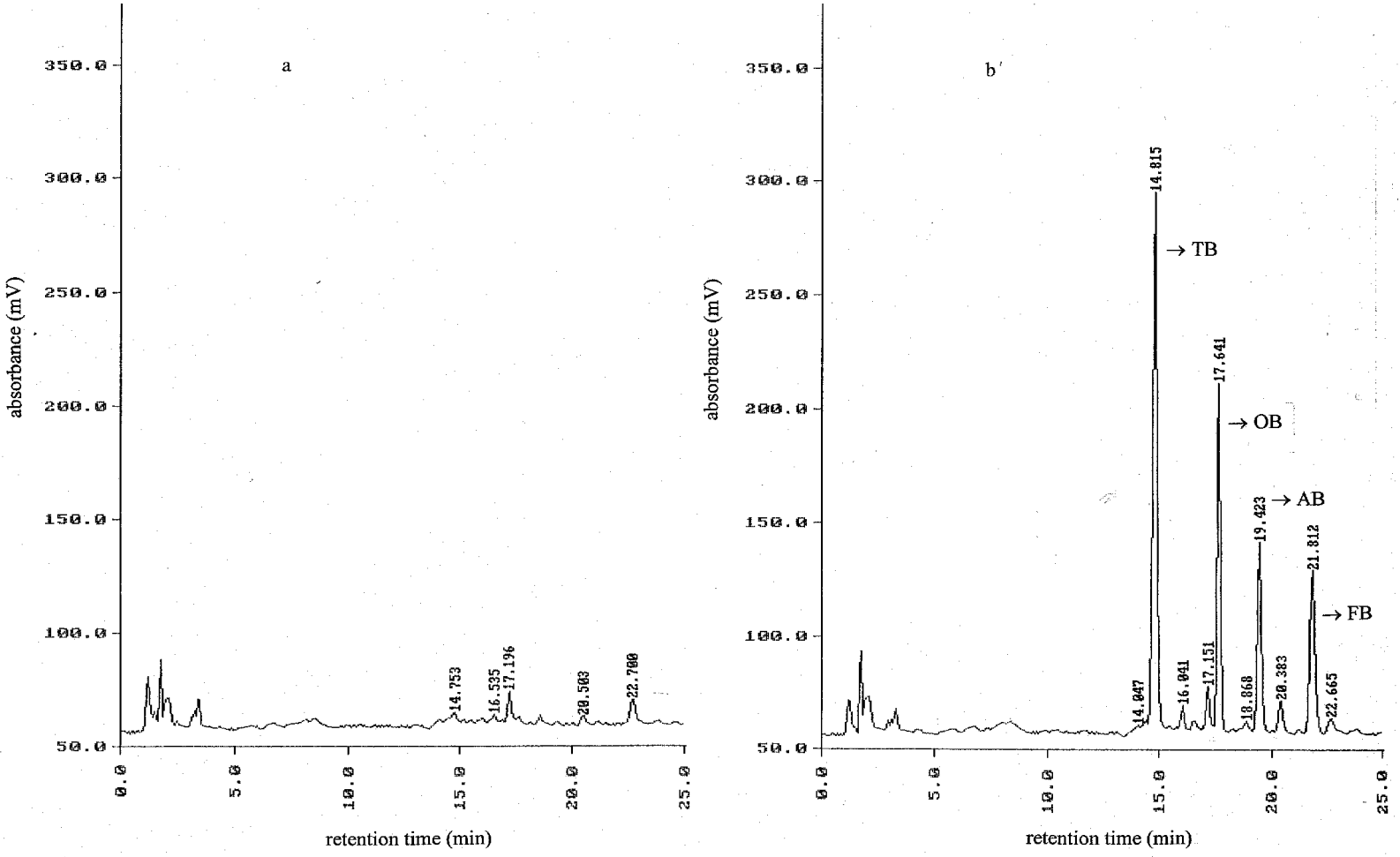


Figure 3.2 Chromatograms of a blank milk sample (a) and a milk sample spiked at a concentration of 100 µg/l for each compound of a mixture of thiabendazole (TB), oxibendazole (OB), albendazole (AB) and fenbendazole (FB) (b)

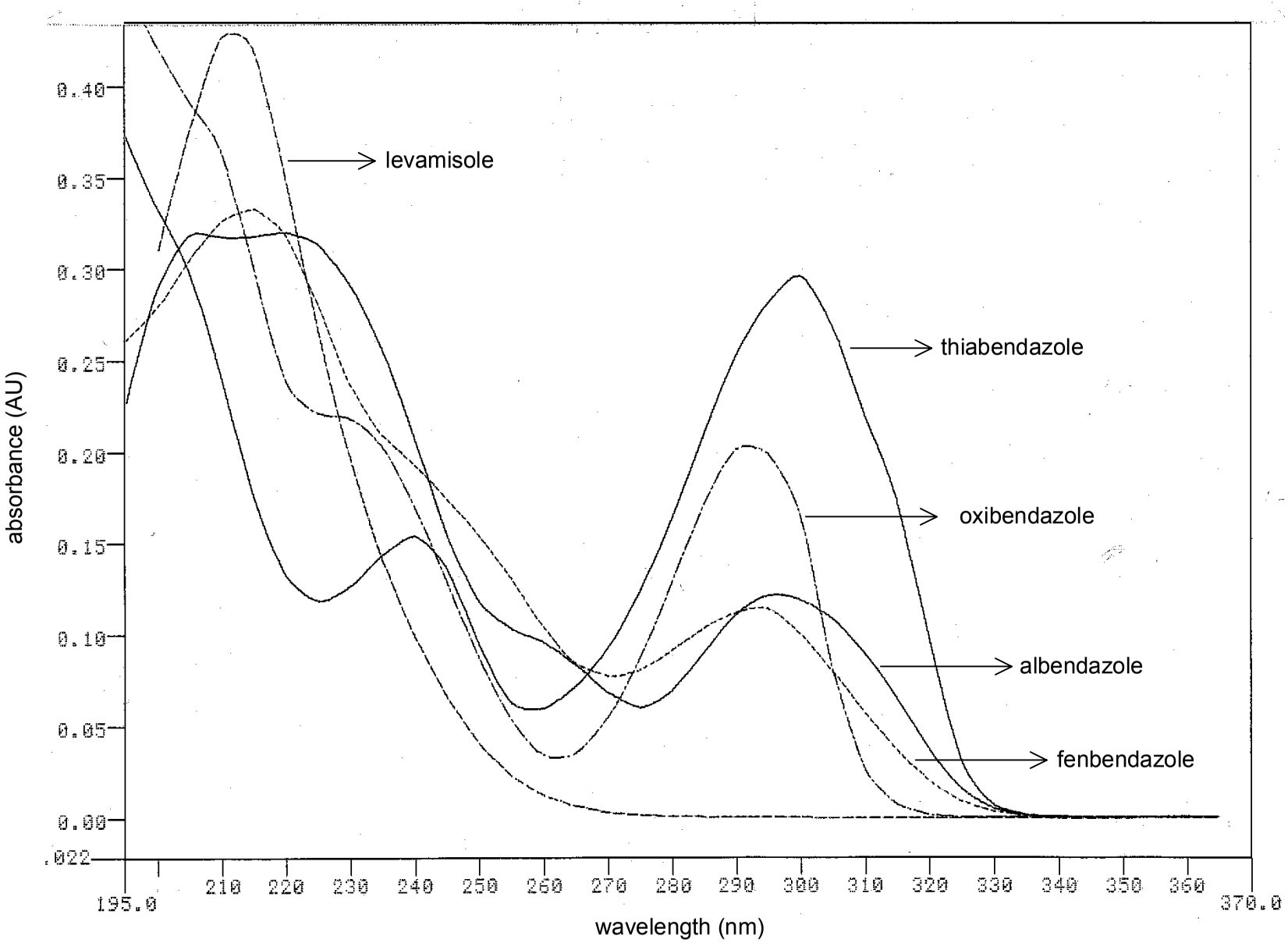


Figure 3.3 UV spectra of thiabendazole, oxibendazole, albendazole, fenbendazole and levamisole at a concentration of 10 g/l

washing the remaining organic extract with phosphate buffer. The formed emulsion-free interface greatly facilitates the quantitative separation of the organic phase. A very satisfactory HPLC separation of the benzimidazole analytes was obtained by ion-pair chromatography and gradient elution. An optimum separation with sodium 1-octanesulphonate as ion-pair reagent was obtained by adjusting the pH of the mobile phase buffer solution at 3.5. A relatively strong adsorptive interaction between the protonated analytes and the silica-based reversed-phase packing material may occur. The negatively charged octanesulphonate ions were used in order to form hydrophobic ion-pairs with the analytes. Peak tailing was avoided, the retention times increased slightly and a considerable increase in peak height was obtained. Representative chromatograms of a blank milk sample and a milk sample spiked at 100 µg/l with the examined BZs are presented in Figure 3.2.

The maximum UV absorption values for FB, TB, AB and OB (Figure 3.3) were obtained with the DAD at wavelengths of 294, 300, 297 and 292 nm, respectively. In the optimised multiresidue method, the eluted analytes were detected with a mean wavelength of 295 nm.

Levamisole was determined with an optimised procedure based on the method applied by Archambault *et al*.[16] The assay is easy and fast and is very suitable for routine analysis. After extraction with chloroform, only a concentration step has to be carried out. A clean-up of the sample extract can be omitted. This method is easier and faster than the method described by Österdahl *et al*.[17] They prefer a more laborious sample pretreatment with a clean-up by SPE on an Extrelut column. The HPLC-DAD method published by De Baere *et al*.[8] is also relatively fast to perform. After liquid-liquid extraction with hexane/iso-amylalcohol, a separation of the extract was performed by centrifugation. A concentration phase evaporating the extract was followed. However, the method was not fully optimised to obtain a high sensitivity (LOD = 77 µg/l). For their HPLC-UV determination method for animal tissues, Dreassi *et al*.[18] also proposed the use of chloroform for the extraction of LE. A SPE sample clean-up was applied. Recovery values up to 89% for muscle samples were obtained. A LOQ value of 4 µg/kg was reported.

Maximum UV absorption for levamisole was obtained at a wavelength of 211 nm (Figure 3.3). However, more endogenous interference due to non-specific absorption at this wavelength was observed in previous research work. The HPLC eluate was analysed by the diode array detector at an optimised wavelength of 225 nm.

A satisfactory separation of the LE analyte from endogenous interfering material was obtained with the described sample preparation procedure. Figure 3.4 shows representative chromatograms of a blank milk sample and a milk sample fortified with levamisole at a concentration of 100 µg/l.

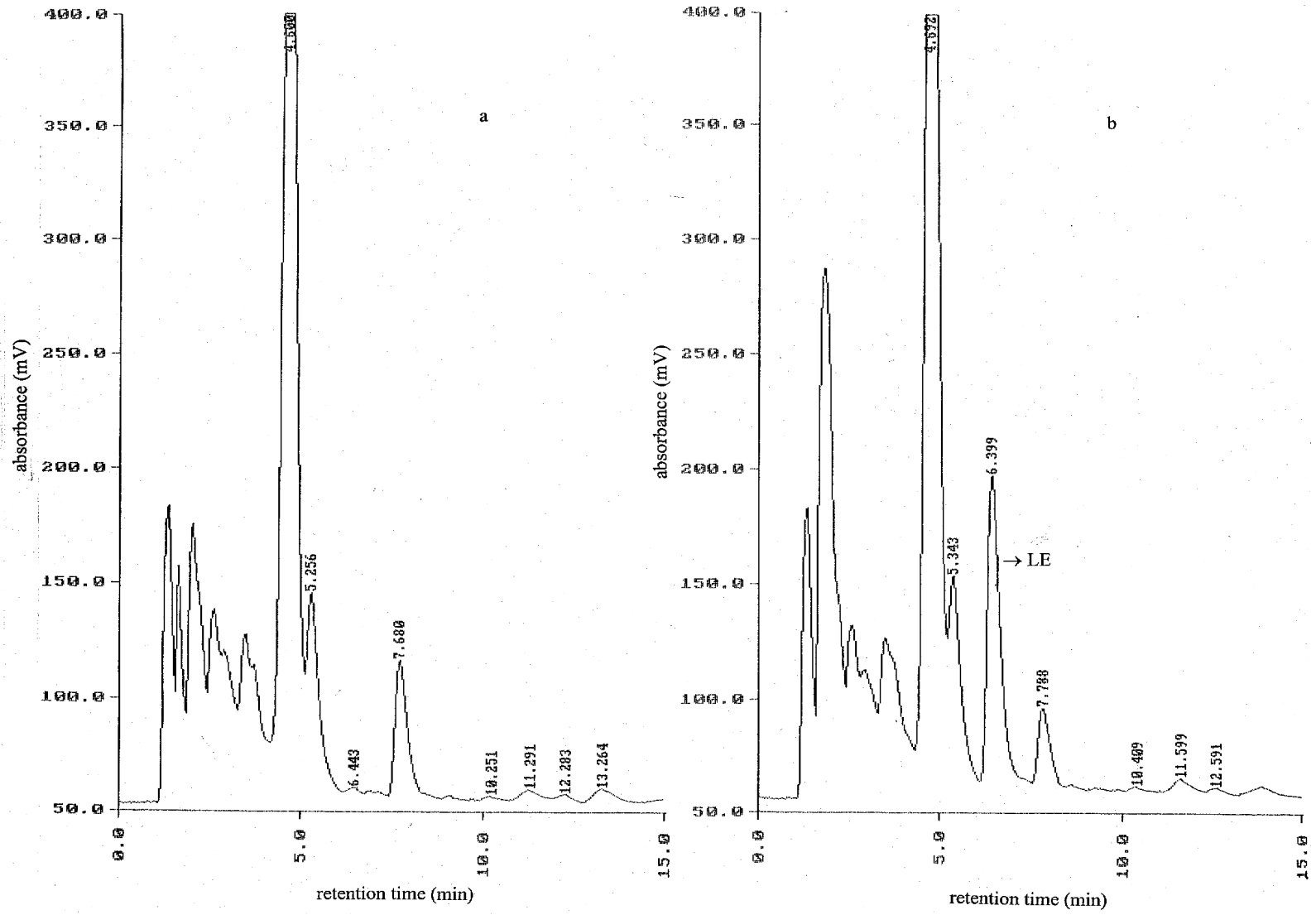


Figure 3.4 Chromatograms of a blank milk sample (a) and a milk sample spiked at a concentration of 100 µg/l with levamisole (LE) (b)

Potential additional analyte characterising data could be obtained by DAD detection. The DAD can assist in the screening of suspected peaks, allowing immediate comparison of the UV spectra of the sample with the specific UV spectra of standard compounds.

Preliminary experiments on the HPLC separation of anthelmintic analytes were performed with a polymeric column with i.d. of 4.6 mm. Changing this type of column for an Alltima C_{18} with an i.d. of 3.2 mm was very advantageous. Significant higher peak heights resulting in more sensitive analysis could be obtained and the mobile phase consumption could be halved.

3.3.2 Validation of the analytical methods

3.3.2.1 Standard curves

The linearity of the HPLC response was verified by running a mixed BZ standard solution and a LE standard solution in a series at seven different concentrations of 20, 100, 200, 500, 1000, 2000 and 5000 µg/l. These analyses were repeated on three successive days. Regression analysis of the analytical data showed a linear response between drug concentration and peak area in the examined range between 20 and 5000 µg/l. The between-day variation, expressed as s_r value of the slope of the curves obtained on the three days, was below 3% for each of the five anthelmintic compounds.

The average standard curves ("Y" represents peak area, "X" represents concentration in µg/l) calculated with the mean regression parameters are given below:

FB: Y = 1253.2 X - 9550.9 r = 0.9999

TB: Y = 3335.0 X + 840.7 r = 0.9999

AB : Y = 1060.5 X - 2610.0 r = 0.9999

OB: Y = 1618.0 X - 4699.0 r = 0.9999

LE: Y = 4752.6 X + 97793.3 r = 0.9998

3.3.2.2 Recovery study

Table 3.1 summarises the mean extraction recovery values obtained for milk samples spiked separately with a mixture of the four benzimidazole substances and levamisole. The recovery values ranged from 68 to 85% and are comparable with those obtained by Long *et al*.[19] These authors have also described a multiresidue HPLC-DAD method for BZs in milk using a more laborious sample pretreatment including matrix solid phase dispersion. In spite of their more intensive sample preparation, Macri *et al*.[20] reported for their multiresidue method also comparable recovery values ranging from 73 to 83% for the same analytes. The extraction of the analytes was performed with ethyl acetate/methylene chloride on milk samples which were previously made alkaline. A liquid-liquid extraction clean-up and a back extraction was performed. Diethylamine was used to form a counter ion in the ion-pair chromatographic separation of the analytes. The extraction recovery for fenbendazole obtained in the presented

Table 3.1 Extraction recoveries obtained for milk samples fortified at concentrations of 10 and 100 µg/l with anthelmintic compounds [mean value (%) (s_r (%))]

Analyte	10 µg/l	100 µg/l
Fenbendazole	71 (9.5)[a]	68 (9.0)[b]
Thiabendazole	80 (4.9)[a]	77 (5.1)[b]
Albendazole	74 (10.5)[a]	73 (7.4)[b]
Oxibendazole	85 (8.6)[a]	73 (6.4)[b]
Levamisole	72 (7.1)[c]	72 (5.9)[d]

[a] n = 10 [b] n = 9 [c] n = 12 [d] n = 11

method was significantly lower than that published by Fletournis *et al*.[12] They reported recovery values near 100%. However, these researchers have optimised their method only for fenbendazole.

The extraction efficiency for LE obtained in the proposed procedure was somewhat lower than that obtained by Archambault *et al*.[16] (80%).

3.3.2.3 Linearity and analytical limits

The results of the regression analysis of the data, obtained by analysing the spiked milk samples, are summarised in Table 3.2. The linearity of the matrix calibration curves, obtained with seven different concentrations (0, 5, 10, 100, 200, 500 and 1000 µg/l), was demonstrated in the concentration range of 0-1000 µg/l. The curves are described by the equation Y = aX + b ("Y" represents peak area, "*a*" represents slope, "X" represents concentration in µg/l and "*b*" represents intercept). The between-day variation expressed as s_r values of the slope of the calibration curves obtained on three different days, was below 10%.

The LODs and LOQs for the anthelmintic residue determinations are summarised in Table 3.3. Acceptable sensitivity was obtained and all LOD values for the BZs were below the respective MRL values. The obtained analytical limit values for the BZ anthelmintics are comparable or somewhat lower than those obtained by Fletournis *et al*.[12,13,21] Macri *et al*.[20] reported a LOQ value of 10 µg/kg for the various BZ compounds.

As wanted, the presented procedure for the unauthorised substance levamisole was very sensitive. The efficiency of the chromatographic procedure and the effectiveness of the sample preparation procedure permitted quantification of LE residue values in milk samples as low as 1.4 µg/l. The calculated LOD was 0.5 µg/l. The detection limit was much lower than those published by Österdahl *et al*.,[17,22] Archambault *et al*.[16] and Simkins *et al*.,[23] which were 20, 40, 50 and 10 µg/l, respectively.

Table 3.2 Regression analysis of calibration curves of anthelmintics obtained on three days

Analyte	Day	Slope (*a*)	Intercept (*b*)	*r*
Fenbendazole	1	8805.6	-9263.8	0.9936
	2	8329.0	85319.4	0.9964
	3	8151.2	100357.3	0.9985
		s_r = 4.0%		
Thiabendazole	1	30284.5	-122121.2	0.9995
	2	30864.6	-82184.6	0.9998
	3	28252.9	24215.7	0.9979
		s_r = 4.6%		
Albendazole	1	8424.0	-101813.0	0.9975
	2	7654.2	54225.2	0.9972
	3	8188.2	48465.9	0.9994
		s_r = 4.9%		
Oxibendazole	1	14768.3	-58279.4	0.9994
	2	13118.0	129299.3	0.9970
	3	15936.2	70596.8	0.9993
		s_r = 9.7%		
Levamisole	1	33916.0	50692.9	0.9999
	2	32985.1	-90191.8	0.9993
	3	33811.6	123028.9	0.9998
		s_r = 1.5%		

Table 3.3 Analytical limit values for the anthelmintic determinations

Analyte	LOD (µg/l)	MRL (µg/kg)	LOQ (µg/l)
Fenbendazole	3.1	10	6.8
Thiabendazole	3.4	100	4.3
Albendazole	3.8	100	6.9
Oxibendazole	1.3	50	2.7
Levamisole	0.5	Not allowed	1.4

3.3.2.4 Trueness

This accuracy parameter was found to be within acceptable limits. The mean calculated concentrations and s_r values (%) in milk samples spiked at 100 µg/l for the BZ compounds (n = 13) FB, TB, AB, OB and LE (n = 9) were 105.4 (9.1), 102.1 (6.4), 106.3 (6.4), 106.0 (5.2) and 96.5 (6.8) µg/l, respectively. The allowed range for trueness recommended by the EU[24] for the analysis of an analyte content > 10 µg/l is between - 20% and + 10%. The mean concentrations of all examined anthelmintic drugs fell within the range of 80 to 110 µg/l.

3.3.2.5 Precision of the assays

The mean measured concentrations and the precision data of the anthelmintic determinations, expressed as s_r values, are given in Table 3.4. The BZ samples were spiked at respective MRL concentrations and the LE samples were fortified at 10 µg/l. These precision data fell within the allowed ranges stipulated by the EU for analysis of drug residues by confirmation methods. The s_r values of the repeatability (within-day precision) of the different anthelmintic compounds are lower than one-half of the values calculated according to the Horwitz equation ($s_r = 2^{(1-0.5\log C)}$, where "C" represents the residue concentration expressed as a power of 10).[24] These maximum allowed values for a residue concentration of 10 and of 100 µg/kg are 32 and 23%, respectively. The overall precision values for the determinations of FB, TB, AB, OB and LE were 11.9, 3.9, 8.2, 7.4 and 12.7%, respectively.

Table 3.4 Precision of anthelmintic determinations at respective MRL concentration for fenbendazole, thiabendazole, albendazole, oxibendazole and at 10 µg/l for levamisole [s_r (%) (mean value (µg/l))]

	Fenbendazole	Thiabendazole	Albendazole	Oxibendazole	Levamisole
Within-day					
Day 1	9.6 (8.9)[a]	2.9 (97.9)[a]	7.1 (94.2)[a]	3.6 (45.2)[a]	13.1 (11.7)[b]
Day 2	11.6 (10.3)[c]	3.4 (102.2)[c]	6.3 (105.7)[c]	4.7 (52.0)[c]	7.6 (10.1)[d]
Day 3	9.0 (9.3)[e]	4.1 (97.9)[e]	7.4 (99.3)[e]	5.8 (48.6)[e]	10.1 (9.9)[f]
Between days[g]	7.3 (9.2)	3.7 (98.5)	8.1 (97.6)	9.6 (48.3)	7.6 (9.9)
Overall	11.9 (9.5)	3.9 (99.3)	8.2 (99.8)	7.4 (48.6)	12.7 (10.5)

[a] n = 10 [b] n = 10 [c] n = 10 [d] n = 12 [e] n = 10 [f] n = 8 [g] n = 3

3.4 Conclusions

Two easy, relatively fast and reliable chromatographic analytical methods for the quantitative determination of the benzimidazole substances fenbendazole, thiabendazole, albendazole, oxibendazole and the imiothiazole levamisole in milk at low residue concentrations, were optimised. The benzimidazole compounds and levamisole have to be determined by two separate HPLC assays. The methods have shown satisfactory validation characteristics with respect to recovery, sensitivity, selectivity, trueness and precision. The detection limits are much lower than the respective MRL concentrations. Additional analyte identification was obtained with the specific UV spectra recorded by the diode array detector.

The methods are suitable as tests assays for routine screening of anthelmintic residues in milk. The HPLC-DAD methods were used several years for the monitoring of Belgian farm milk.

The HPLC assay for levamisole was suitable for quantitative determination of residue values in a depletion study with dairy cows administered with levamisole, which is described in Chapter 8.

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CHAPTER 4

Liquid chromatographic-electrospray tandem mass spectrometric multiresidue method for anthelmintics in milk

Relevant publication:

De Ruyck H., Daeseleire E., De Ridder H. and Van Renterghem R.
Development and validation of a liquid chromatographic-electrospray tandem mass spectrometric multiresidue method for anthelmintics in milk.
Journal of Chromatography A, 2002, 976, 181-194

Abstract:

A liquid chromatographic-tandem mass spectrometric multiresidue method for the simultaneous quantitative determination of levamisole and the benzimidazoles thiabendazole, oxfendazole, oxibendazole, albendazole, fenbendazole, febantel and triclabendazole in milk has been developed. The anthelmintic residues were extracted with ethyl acetate after the milk sample was made alkaline. The extract was centrifuged and the supernatant was concentrated by evaporation using a stream of nitrogen. The liquid chromatographic separation was performed on a reversed-phase C_{18} column with gradient elution. The analytes were detected by tandem quadrupole mass spectrometry after positive electrospray ionisation by multiple reaction monitoring. The confirmatory method has low detection limits and each component can be detected at a residue concentration lower than 1 µg/l. The method is validated according to the revised European Union requirements and all determined parameters were found conform the criteria. The evaluated parameters were linearity, specificity, stability, recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (detection limit, decision limit and detection capability). This analytical assay is applied in the Belgian monitoring programme for residues of classical anthelmintic veterinary drugs in raw farm cow's milk.

Keywords: food analysis, milk, validation, anthelmintics, benzimidazoles, levamisole

4.1 Introduction

4.1.1 Usage of anthelmintic veterinary drugs

Within the group of antiparasitic drugs, anthelmintic products are widely used in veterinary medicine in cattle. Anthelmintics are used prophylactically and curatively to treat acute and chronic infections.[1] The anthelmintic drugs involved in this chapter, have unique wide-spectrum properties against parasitic helminths.[2] Control treatments reduce worm burdens, enhance productivity and substantially reduce the build-up of infective worm larvae on the pasture or eggs in the environment. Gross *et al*.[3] reported a mean increased milk production of 0.63 kg/day due to an effective anthelmintic treatment.

A lot of antiparasitic immunity is already achieved through treatment of young cattle. Nevertheless, in wet periods, the medication of dairy cows with endoparasiticides can also be necessary for protecting or treating the animals mainly against gastrointestinal nematodes, lungworms and liver flukes. Most forms of parasitic gastroenteritis in ruminants tend to occur in the second half of the summer.[1] Most infection pressure of lungworms arises in summer or early autumn.

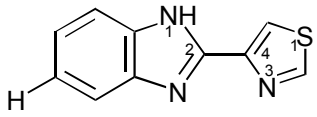
4.1.2 Chemical properties of anthelmintic compounds

The veterinary drug substances involved in this study are the synthetic compounds thiabendazole (TB), oxfendazole (OF), oxibendazole (OB), albendazole (AB), fenbendazole (FB), triclabendazole (TC), flubendazole (FL), mebendazole (ME), levamisole (LE) and febantel (FE). TB, OF, OB, AB, FB, TC, FL and ME belong to the group of benzimidazole (BZ) anthelmintics. FL, a BZ substance very similar to most of the target compounds (TB, OF, OB, AB, FB, TC, LE and FE), was used to verify the specificity of the developed analytical method. ME was used as internal standard (IS). LE is an imidazothiazole substance defined as the L-isomer of the drug preparation tetramisole, a racemic mixture of D- and L-forms.[4] FE is a pro-benzimidazole drug, which is closely related to the benzimidazoles and it converts in the host to both FB and OF (paragraph 4.1.4).

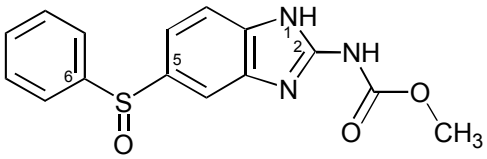
The chemical structures of the examined anthelmintic compounds are shown in Figure 4.1. The benzimidazole compounds have a “benzimidazole” nucleus and most substances contain a “carbamate (-NHCOOCH$_3$) type” functional group.[5]

General chemical properties of the analytes and the heat stability of levamisole are described in paragraph 3.1.1 of Chapter 3.

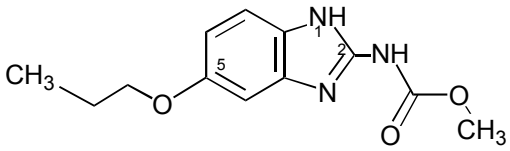
The heat stability of oxfendazole in water, in cooking oil and in incurred residues in cattle liver was investigated by Rose *et al*.[6] Some evidence of instability was found in boiling water after 3 h. This degradation was associated with the formation of an amine product formed by hydrolysis of the functional carbamate group on the OF molecule. In oil heated at 150 and 180°C, the half-live of OF was 15 and 6 min, respectively. In tissue, OF residues were present



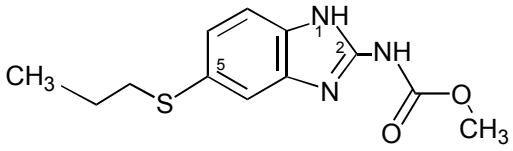
thiabendazole M_r = 201.2
[2-(1,3-thiazol-4-yl)-1*H*-benzimidazole]



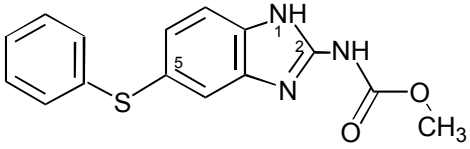
oxfendazole M_r = 315.3
methyl-[5(6)-phenylsulfinyl-1*H*-benzimidazole-2-yl] carbamate



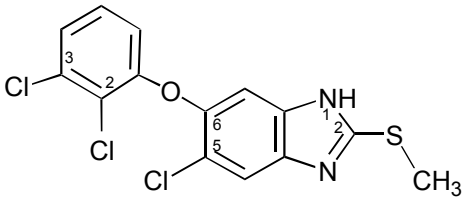
oxibendazole M_r = 249.3
methyl-[5-(propoxy)-1*H*-benzimidazole-2-yl] carbamate



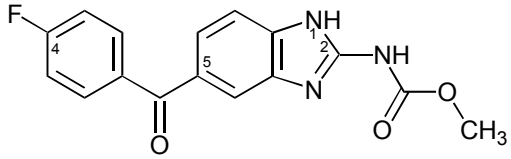
albendazole M_r = 265.3
methyl-[5-(propylthio)-1*H*-benzimidazole-2-yl] carbamate



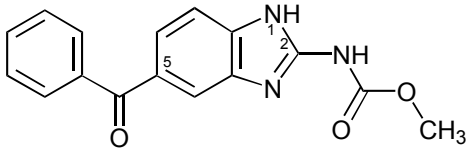
fenbendazole M_r = 299.3
methyl-[5-(phenylthio)-1*H*-benzimidazole-2-yl] carbamate



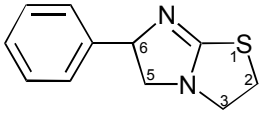
triclabendazole M_r = 359.7
[5-chloro-6-(2,3-dichlorphenoxy)-2-methylthio-1*H*-benzimidazole]



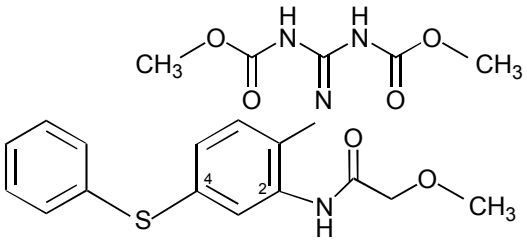
flubendazole M_r = 313.3
methyl-[5-(4-fluorobenzoyl)-1*H*-benzimidazole-2-yl] carbamate



mebendazole (internal standard) M_r = 295.3
methyl-[5-benzoyl)-1*H*-benzimidazole-2-yl] carbamate



levamisole M_r = 204.3
[L-(-)-2,3,5,6-tetrahydro-6-phenylimidazo (2,1-b)-thiazole]



febantel M_r = 446.5
dimethyl-[[2-[(methoxyacetyl)amino]-4-(phenylthio) phenyl]carbonimidoyl] biscarbamate

(M_r = relative molecular mass)

Figure 4.1 Molecular structures of the examined anthelmintics[7]

as an equilibrium between the parent compound and its oxidation (OF sulphone) and reduction products (FB). Heating did not destroy residues in incurred tissue, but it affected the equilibrium point. OF in fortified samples were found stable during six weeks at frozen storage. Frozen storage of incurred tissue resulted in a decrease of residues already after four weeks.

4.1.3 Toxicity

Several toxicological effects of benzimidazoles and levamisole were described in paragraph 3.1.2 in Chapter 3.

BZs appear to be characterised by very low toxicity in mammals.[5] Nevertheless, the extractable residues (parent drug and/or free metabolites) are chemical species with defined toxic potential for which safety margins may be defined.[2] Mutagenic activity and mainly teratogenicity are reported as the most harmful toxicological problems. Oxfendazole and albendazole are known to be teratogenic and an eventually applied dose in early pregnancy should be limited and not greater than therapeutic level.[4] In many cases, teratogenic metabolites have been identified in animal products as milk, eggs and meat.[2]

Mammalian toxicity of levamisole is greater than with the BZs although, in normal usage, toxic effects are seldom observed.[4] However, LE has also found clinical application as human anthelmintic medicine and in the treatment of cancer.[8,9]

4.1.4 Metabolism

Benzimidazole anthelmintics and their primary metabolites appear to deplete from tissues relatively rapidly.[2] No prolonged or unusual drug or metabolite retention in any tissue has been reported among the examined compounds. Biliary secretion of metabolites and conjugates does occur with BZs. BZ anthelmintics are extensively metabolised by mammals following oral administration. The parent drug is usually short-lived. Metabolites, rather than the parent drug, often dominate in blood, bile, tissues and urine. These free, primary metabolites are usually the products of normal oxidative or hydrolytic processes and are more soluble than the parent drug. However, a small amount of BZ metabolites becomes bound in relatively non-extractable form to endogenous macromolecules.

Carbamate hydrolysis is a major metabolic route for several BZ carbamates. The resulting 2-aminobenzimidazoles, which are often additionally metabolised in the 5-substituent as for FB and OF, are readily eliminated in the urine. In the case of AB, the 2-aminosulphone metabolite is the longest residing free metabolite in tissue.The alkyl groups of the 5-substituents of AB are subject to metabolic oxidation, which leads to alcohol metabolites. Oxidation in the benzene ring of the 5-substituent to produce phenols is an important metabolic process for FB and OF.

The metabolism of TB has been studied in several animal species including cattle.[10,11] The major metabolic steps were hydroxylation of the benzimidazole ring at the 5-position to form 5-hydroxythiabendazole and subsequent conjugation to the glucuronide and sulphate.

Metabolism of AB was studied by several research workers.[12,13,14] Primary oxidation of the sulphide group of AB leads rapidly to albendazole sulphoxide (AB-SO). AB-SO is slowly metabolised to albendazole sulphone (AB-SO_2), which is formed as a 2-step oxidation product. Albendazole 2-aminosulphone (NH_2-AB-SO_2) is formed by deacetylation of the carbamate group of AB-SO_2 to an amine. Fletournis *et al*.[13] have performed a depletion study for AB with milk cows treated with the recommended dose. AB could not be detected (LOD = 7 µg/l). The residue concentration of AB-SO is highest at 12 h (658 µg/l) after dosing and declines below 3 µg/l at 36 h. AB-SO_2 attains its highest level more slowly (705 µg/l at 24 h) and also disappears more slowly (< 5 µg/l at 72 h). The residue concentration of NH_2-AB-SO_2 is highest at 36 h after dosing (110 µg/l). It slowly disappears at 108 h after dosing.

Other research workers have described the occurrence of metabolites of FB.[15,16,17] After oral administration, FB is absorbed from the intestine and is rapidly metabolised to fenbendazole sulphoxide, a metabolite also defined as OF, which is an active anthelmintic drug. Afterwards, OF is oxidised to fenbendazole sulphone (FB-SO_2). Fletournis *et al*.[13] have also performed a depletion study for FB with milk cows treated with the recommended dose. FB was measured until the 48 h milking after application of a concentration of 10 µg/l. It seems that FB is readily oxidised to OF, because residue values of 196 µg/l were measured at 12 h after dosing. A maximum value of 248 µg/l was obtained 24 h after dosing which was declines below 3 µg/l at 60 h. FB-SO_2 appears more slowly, reaching a peak at 48 h after dosing and declines below 5 µg/l at 84 h after dosing.

Pro-drugs as FE, lead to active benzimidazoles by hydrolytic or cyclisation processes.[2] They are converted either chemically or metabolically to the biologically active BZ soon after administration. FE is converted either directly to FB or to OF, which is obtained via febantel sulphoxide (FE-SO) as an intermediate. The complex metabolic pathway of FE is described by Rose.[18] The structures and relationship of 11 compounds known to be part of the metabolic pathway of OF are illustrated in Figure 4.2. Several metabolites and breakdown products were identified in tissue. There was also evidence of an unstable equilibrium between OF, oxfendazole sulphone and FB in incurred tissue. Both FE and FE-SO were found to be unstable as they converted to FB during the extraction and clean-up procedure. Because of their mode of action, residues of these compounds are unlikely to be found in animal tissue.

The metabolism of levamisole is discussed in Chapter 8 dealing with the depletion study of levamisole in milk.

4.1.5 Objectives

If the recommended withdrawal times after anthelmintic treatment of dairy cows are not respected or if unauthorised substances are administered, the residue concentration in milk can be very high.[19] Because a number of anthelmintic compounds have been shown to cause

teratogenic and embryotoxic effects in some species, MRL values were laid down by the EC to protect the consumer against potential toxic residues.[20]

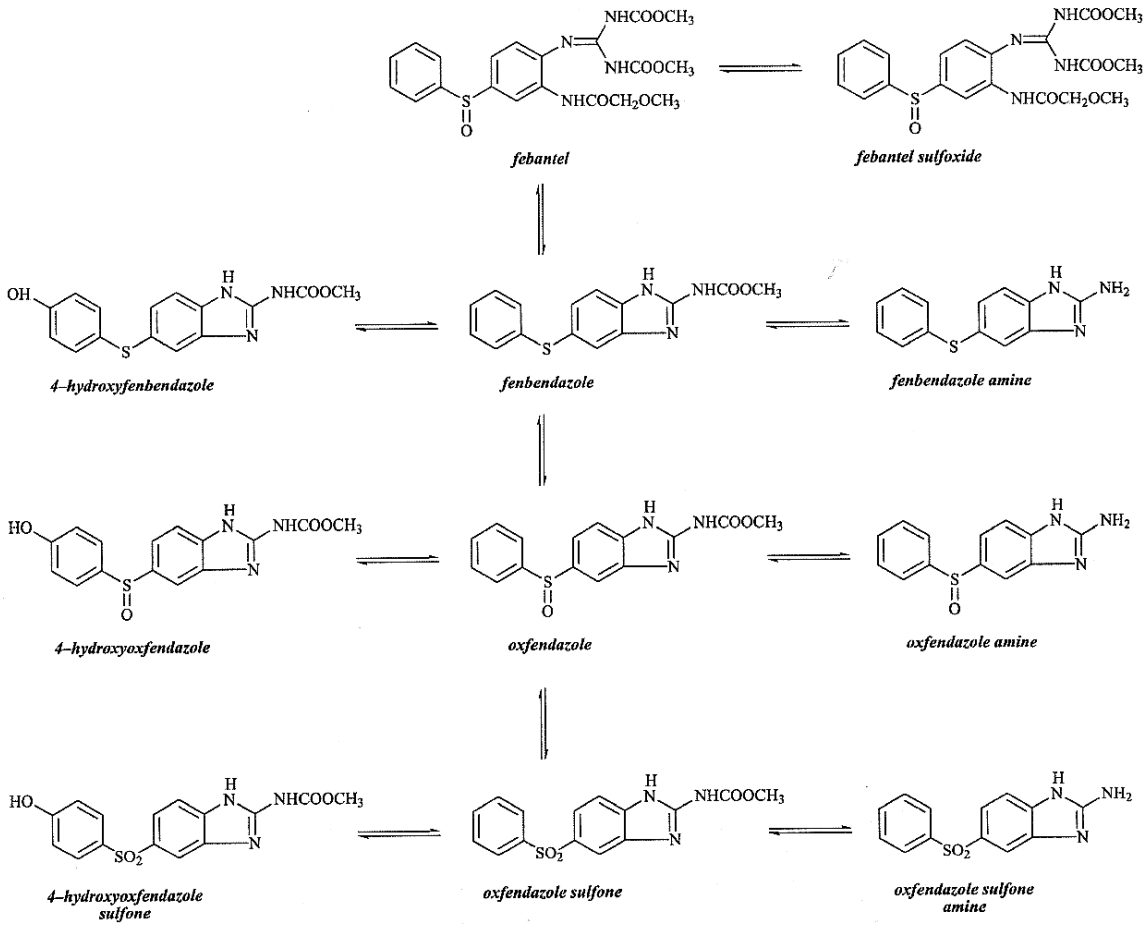


Figure 4.2 Metabolic pathway of oxfendazole[18]

A lot of chromatographic methods exist for the residue analysis of one of more anthelmintics in a variety of animal products. UV, UV diode array or fluorescence detection were the most commonly used detection techniques.[12,13,15,16,17,18,21,22,23] However, detection based on the chemical structure of the compound with mass spectrometry (MS) provides mass-related data which become desirable for confirmation in residue analysis. A lot of mass spectrometric detection methods for anthelmintic residues have already been described.[8,24,25,26,27,28,29,30,31,32] Several methods used a rather old ionisation mechanism or only single MS. Wilson *et al.*[24] have described a multiresidue assay for confirmation of BZ anthelmintics in several animal tissues by gas chromatography with selected-ion monitoring electron impact mass spectrometry. Facino *et al.*[25] have discussed the application of collisionally activated decomposition mass-analysed ion kinetic energy spectrometry in the electron impact mode for BZ residues in sheep milk. A hyphenated technique comprising of an on-line HPLC-GC-tandem ion trap mass spectrometer operating in the chemical ionisation and electron impact mode for the determination of LE in

milk is presented by Chappell *et al*.[8] Blanchflower *et al*.[26] have developed a method for the determination of FB and OF in liver and muscle tissues using single liquid chromatography-mass spectrometry (LC-MS) with thermospray ionisation. The same analytical technique for the determination of LE in animal tissues was proposed by Cannavan *et al*.[27] Balizs[28] described the determination of several BZ residues in eggs and animal tissues using liquid chromatography and tandem mass spectrometry (LC-MS/MS) with electrospray ionisation (ESI). Cherlet *et al*.[29] presented a quantitative LC-MS/MS method with atmospheric pressure chemical ionisation (APCI) for the analysis of porcine tissues on residues of LE. De Baere *et al*.[30] compared a HPLC-UV assay and a LC-MS/MS (ESI) procedure for the quantitative determination of LE in animal plasma. The LC-MS/MS method was considered as the superior method due to the possibility of a high sample throughput, the excellent specificity and the low analytical limits.

To our knowledge, no LC-MS/MS method with ESI for the simultaneous determination of residues of benzimidazoles, febantel and levamisole in milk was published. The objective of this study was to develop such an analytical procedure for the multiresidue detection and quantitative determination of the benzimidazoles TB, OF, OB, AB, FB, TC, the pro-benzimidazole FE and the imidazothiazole LE. Because levamisole and triclabendazole are not licensed for lactating dairy cows, a method with low decision limits was needed. An internal performance limit (IPL) of 1 µg/l was chosen. The MRL values for TB, OF, OB, AB, FB and FE are 100, 10, 50 (provisionally), 100, 10 and 10 µg/kg, respectively. A fast and relatively simple sample preparation procedure was required to obtain a method suitable for routine analysis in a monitoring programme to detect residues of anthelmintic veterinary drugs in raw farm cow's milk.

4.2 Experimental

4.2.1 Reagents and chemicals

Analytical standard material of TB, OB, AB, FB, LE-hydrochloride and the internal standard ME, were purchased from Sigma (St. Louis, MO, USA). Standard material of OF, FE, TC and FL were kindly provided by Merial (Toulouse, France), Bayer (Leverkusen, Germany), Novartis (Munchwilen, Switzerland) and Janssen Animal Health (Beerse, Belgium), respectively. Dimethyl sulphoxide (HPLC grade), ethyl acetate (HPLC grade) and formic acid (analytical grade) were from Panreac (Barcelona, Spain). Acetonitrile and methanol of HPLC gradient grade and sodium hydroxide of analytical grade were obtained from Merck (Darmstadt, Germany). Water was purified with a Maxima LC 113 Ultra-pure water purification system (Elga, Bucks, UK) to HPLC grade. Blank milk samples were received from the Department of Animal Nutrition and Husbandry, Section cattle and pig husbandry (DVV-CLO, Merelbeke, Belgium).

4.2.2 Standard solutions

The analytes were dissolved in 10 ml of dimethyl sulphoxide. Stock solutions of 0.1 and 0.2 mg/ml were prepared by dilution with methanol. The stock solutions were stored refrigerated at 5°C and replaced every two months. The working standard solutions were prepared immediately before use by dilution with mobile phase consisting of water containing 0.1% formic acid and acetonitrile (50/50, v/v). Tuning of the mass spectrometer and acquisition of the analyte identification spectra were performed with standard solutions of 1 µg/ml. During the validation procedure, the IS was spiked using a solution of 1 and 5 µg/ml for fortification to a final milk sample concentration of 10 and 100 µg/l, respectively. The anthelmintic analytes were fortified using a standard solution of 0.1, 1 and 5 µg/ml for matrix concentrations up to 2.5 µg/l, between 2.5 and 50 µg/l and above 50 µg/l, respectively.

4.2.3 Apparatus

A shaker (Bühler, Hechingen, Germany), a RC-5B Sorvall centrifuge (DuPont Instruments, Wilmington, DE, USA) and a vortex mixer (Scientific Industries, Bohemia, NY, USA) were used during the sample preparation. A high-performance liquid chromatograph combined with a mass spectrometer already applied for previous research studies[31,32,33] in our laboratory was used. The LC-MS/MS system consisted of a LC system (Kontron, Biotech Instruments, Milan, Italy) with a type 325 ternary pump system, a vacuum degasser and a type 465 autosampler, coupled with a Quattro LCZ tandem quadrupole mass spectrometer (Micromass, Altrincham, UK) provided with a “z”-spray electrospray ion interface. The mass spectrometer was fully controlled by MASSLYNX software version 3.3. A model 11 syringe pump of Harvard Apparatus (Holliston, MA, USA) connected to the interface, was very useful for tuning purposes.

4.2.4 Sample preparation

Five ml of milk sample was transferred into a centrifuge tube of 50 ml. The sample was spiked with the compounds and/or the IS. After standing for 30 min, the milk sample was made alkaline with 100 µl of a 10 M sodium hydroxide solution. The extraction of the analytes was performed with 15 ml of ethyl acetate on a shaker (amplitude: 30 mm, frequency: 90 per min) for 5 min. The extract was centrifuged (5000 rpm, 10 min) and the supernatant was removed using a pipette and was transferred into a graduated tube. The organic fraction was evaporated to dryness under a stream of nitrogen in a water bath at 50°C. The dry residue was dissolved in 600 µl of mobile phase consisting of 0.1% aqueous formic acid/acetonitrile (50/50, v/v) while vortex mixing. The mixture was heated at 50°C in a waterbath for 5 min. After vortex mixing and after cooling down to room temperature, the mixture was made up to 1 ml with mobile phase and was homogenised by vortex mixing. The cleaned sample extract was filtered through a

0.2 µm polytetrafluorethylene (PTFE) Chromafil filter (Macherey-Nagel, Düren, Germany) into an autosampler vial.

4.2.5 Liquid chromatography

The LC separation of the anthelmintic analytes was performed on a reversed-phase column and was based on a previously developed procedure for the analysis of sheep liver for residues of mebendazole.[32] The LC runs were carried out at room temperature on an Alltima C_{18} column (Alltech, Deerfield, IL, USA), 5 µm, 150 x 2.1 mm i.d. protected by an Alltima C_{18} guard cartridge of 7.5 x 2.1 mm i.d. The mobile phase consisted of water containing 0.1% formic acid (to increase the ionisation intensity) (A) and pure acetonitrile (B). Gradient elution was applied: A-B (65:35) (0 min), A-B (65:35) to (50:50) (0-0.1 min), A-B (50:50) to (25:75) (0.2-3 min), A-B (25:75) (4-5 min), A-B (25:75) to (50:50) (6-7 min), A-B (50:50) to (65:35) (8-15 min) and A-B (65:35) (16-25 min). The mobile phase flow rate and the injection volume were 0.25 ml/min and 20 µl, respectively.

4.2.6 Mass spectrometry

The analytes were detected and identified with a tandem quadrupole mass spectrometer without splitting the LC eluent. Atmospheric pressure electrospray ionisation in the positive mode (ESI^+) was applied. The nitrogen gas flows were set at 80 and at 600 l/h for nebulising the LC eluent and for drying the solvents, respectively. The source block and solvent desolvation temperatures were 130 and 250°C, respectively. The parent molecular ions were fragmented in the collision cell with argon gas.

The analytes were detected by tandem MS using the multiple reaction monitoring (MRM) function of two transitions, each from the molecular precursor ion to two different product ions. The mass spectrometer was tuned while optimising the specific cone voltage and the specific collision energy to maximise the ion current of the three diagnostic ions of each drug compound. The ESI^+ mode leads to addition of a H-atom to the ions so that protonated pseudo-molecular positive ions are formed. The optimisation of the transition of the precursor ion, which was the molecular parent ion, to the two most abundant product ions was performed by infusion of a standard solution of 1 µg/ml using a syringe pump.

Quantification was obtained by internal calibration. The data of the samples generated by MRM of the transition from the precursor ion into the most abundant product ion were evaluated by an internal standard procedure based on matrix calibration curves. The quantitative results were calculated automatically by MASSLYNX software version 3.3 of the mass spectrometer. The calibration curves were always calculated using the best fit of two replicated determinations per concentration level. The calibration curves were obtained using at least five concentration points, including the zero level. The response factors (Y values) (response factor = peak area

ratio of sample and IS multiplied by the concentration of the IS) were plotted against the concentrations (X values).

4.2.7 Validation procedure

4.2.7.1 Introduction

The validation of the proposed LC-MS/MS method was based on the revised requirements for detecting residues of veterinary drug substances in animal products defined by the EC in the Draft Commission Decision SANCO/1805/2000.[34] Several supplementary validation parameters were also evaluated.

The examined validation parameters for the analytical assay were linearity, specificity, stability, recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (limit of detection, decision limit and detection capability). During the validation procedure, blank milk samples were fortified with a standard solution of a mixture of the anthelmintic compounds, unless otherwise stipulated. The IS was added with a separate standard solution.

The method was validated at two residue levels. To evaluate the usefulness of the quantitative method for the unauthorised substances levamisole and triclabendazole at residue concentrations as low as possible, the validation was performed at the IPL (1 µg/l) concentration. Moreover, the method can also be used in depletion studies to quantify low residue concentrations. On the other hand, the method was also validated on MRL level which is very important to make the right decisions for the licensed substances for statutory testing purposes.

4.2.7.2 Liquid chromatographic-mass spectrometric analysis

The specific criteria for LC separation and MS detection were verified during the whole validation procedure. Concerning the chromatographic separation, the difference between the relative retention time (RRT) of the analytes in the validation sample and those of the matrix calibration standards was measured. Concerning the ion recognition, each diagnostic ion was measured when it had a signal-to-noise (S/N) ratio of at least 3/1. Furthermore, the difference of the relative intensities of the detected ions (RI), expressed as a percentage of the peak area of the most intense ion in the validation samples and those of the matrix calibration standards, was evaluated.

4.2.7.3 Linearity

The linearity of the response of the LC-MS/MS system was checked by repeatedly analysing standard solutions of a mixture of the anthelmintics at concentrations of 0, 1, 5, 25, 100 and 500 µg/l.

The linearity of the LC-MS/MS assay was demonstrated using blank milk samples spiked with the analytes at concentrations of 0, 1, 5, 25, 100 and 500 µg/l.

4.2.7.4 Specificity

To evaluate the specificity, potential endogenous interfering material during the LC separation was measured by analysing 20 blank milk samples. Potential hindered identification and quantification was detected by addition of flubendazole, a substance very similar to most of the target compounds. Hindered identification was checked by analysing blank milk samples separately spiked at MRL concentration for the authorised substances and at 10 µg/l for LE, TC and FL. Interferences on the quantification were evaluated by comparing the peak areas of blank samples spiked at 100 µg/l respectively with a mixture of FL+LE+TB+OF+OB+AB+FB+FE+TC and with a mixture of LE+TB+OF+OB+AB+FB+FE+TC.

4.2.7.5 Stability

The stability of fortified samples was tested by repeatedly analysing a spiked milk sample during three months at 2-week time intervals. The samples were fortified at MRL concentration for the authorised compounds and at 10 µg/l for LE and TC and were stored frozen at -18°C until investigation. The regression data and measured residue values were compared with those obtained for a freshly fortified milk sample.

Furthermore, the stability of a frozen (-18°C) incurred milk sample containing LE residues, was measured for 4.5 months.

4.2.7.6 Recovery

Because no certified reference material of the anthelmintic substances exists, the analyte extraction recovery instead of the trueness was determined. The recovery was evaluated at two concentration levels. In a first series of analyses, blank milk samples spiked at concentrations of 1.0 IPL (1.0 µg/l), 1.5 IPL (1.5 µg/l) and 2.0 IPL (2.0 µg/l) were analysed. This validation parameter was also calculated at MRL level with blank milk samples spiked with the authorised compounds at their respective MRL concentration. To obtain the recovery efficiency, the IS was added after the clean-up procedure and six replicates at each level were analysed.

4.2.7.7 Precision

To verify the precision of the method, the repeatability and the within-laboratory reproducibility were determined. As described for the recovery, these validation parameters were also measured at the two concentration levels. The repeatability was calculated by analysing six replicates on three different days. The within-laboratory reproducibility was performed by two operators on five different days, using own standard solutions. To obtain six values, one value of the repeatability data was also taken into account.

4.2.7.8 Analytical limits

The limits of detection (LODs) were calculated as the apparent residue content corresponding to the value of the mean plus three times the standard deviation (*s*) obtained for at least 20 representative blank sample determinations. If only noise was obtained, technical LODs were calculated as those concentrations which yield a S/N ratio of 3/1.

Besides the more commonly used LOD, the new concept of decision limit (CCα) was also studied. The CCα values of the presented method are defined as the mean values of the obtained concentrations by determining blank milk samples spiked at MRL (authorised compounds) or IPL (authorised and unauthorised compounds, 1 µg/l) level plus 1.64 times the corresponding standard deviations (*s)*. This validation parameter was calculated for the examined substances with already obtained values of the calibration curves calculated for the investigation of repeatability, recovery, within-laboratory reproducibility and stability. The analytical limits obtained for the authorised compounds at IPL concentration can also be defined as LOD values calculated according to the criterion for measuring of CCα values.

The detection capability (CCβ) values were calculated as the sum of the respective CCα value plus 1.64 times the *s* value obtained on the measured concentration values by determining 20 blank milk samples spiked at the respective analytical limits obtained at MRL and at IPL level. The analytical limits obtained for the authorised compounds at IPL concentration can also be defined as LOQ values calculated according to the criterion for measuring of CCβ values.

4.3 Results and discussion

4.3.1 Mass spectrometry

Deuterated analytical standards of the anthelmintics involved in this study are not available. Therefore, mebendazole, a BZ compound very similar with most of the examined analytes, was used as IS. ME is authorised for use in food animals, but not for lactating dairy cows. The presented method is therefore only applicable for milk samples. It is practically impossible to find ME residues in milk samples. Furthermore, the tandem MS detection technique has a very high sensitivity and specificity. Any ME residue in unknown samples will immediately be detected and can be compensated for after double analysis.

A summary of the monitored protonated ions and the optimised MS operating parameters obtained for the examined anthelmintics is given in Table 4.1. Identification and confirmation of a violative sample for one of more anthelmintic residues is only proven by detection of their three diagnostic ions above the detection limit.

The specific mass spectra for each analyte are presented in Figure 4.3. The full scan mass spectrum (a) shows the molecular precursor ion. The product ions obtained by fragmentation of the molecular ion are presented in the fragmentation mass scan of the precursor ion (b).

The fragmentation pathway from the compound molecules into the fragment diagnostic ions, can be explaned as follows. The most abundant protonated fragment ion for LE with *m/z* 91.1 is induced by fragmentation of the protonated molecular ion to C_6H_5 CH–. The product ion with *m/z* 123.1 can be explained by splitting off the fragment –$NCSCH_2CH_2N$– from the LE molecule. The consecutive loss of –CN and –CS from the molecular structure of TB gives rise to the fragments with *m/z* 175.2 and *m/z* 131.1. The two product ions with *m/z* 175.2 and *m/z* 159.1 of OF originate from consecutive losses of –SOC_6H_5 and –OCH_3.

Table 4.1 Summary of the protonated diagnostic ions and the MS operating parameters

Analyte	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone voltage (*V*)	Collision energy (*eV*)
Levamisole	205.0^+	123.1^{+a}, 91.1^+	40	29
Thiabendazole	202.0^+	175.2^{+a}, 131.1^+	45	28
Oxfendazole	316.2^+	191.2^{+a}, 159.1^+	40	22
Oxibendazole	250.1^+	176.2^{+a}, 218.2^+	35	23
Albendazole	266.2^+	191.2^{+a}, 234.3^+	35	27
Fenbendazole	300.1^+	268.2^{+a}, 159.0^+	40	28
Febantel	447.4^+	383.4^{+a}, 415.3^+	25	15
Triclabendazole	359.0^+	274.1^{+a}, 344.2^+	40	30
Flubendazole	314.3^+	282.1^{+a}, 123.0^+	29	40
Internal standard (Mebendazole)	296.5^+	264.4^{+a}, 105.0^+	35	25

[a] most abundant product ion

The loss of –OCH_3 from the molecular structures of OB, AB, FB, FE, FL and the IS results in product ions with *m/z* 218.2, 234.3, 268.2, 415.3, 282.2 and 264.4, respectively. The fragment with *m/z* 105.0 from IS originates from fragmentation into –COC_6H_5. The remaining product ion with *m/z* 123.0 for FL originates from fragmentation into –COC_6H_4F. The loss of –$CH_2CH_2CH_3$ from the product ion with *m/z* 218.2 of OB results in a second fragment with *m/z* 176.2. The second fragment of AB with *m/z* 191.2 is obtained by loss of –CONH from the main product ion with *m/z* 234.3. A second loss of –OCH_3 from the product ion of FE with *m/z* 415.3 results in the remaining fragment with *m/z* 383.4. The fragment with *m/z* 159.0 for FB originates from the loss of –SC_6H_5 from the fragment with *m/z* 268.2. The derivatives of TC with *m/z* 344.2 and *m/z* 274.1 can be explained by the consecutive loss of –CH_3 and 2 Cl-atoms, respectively.

For the confirmation of the drug compounds involved in this study and belonging to the drug substances of group B, veterinary drugs and contaminants of Annex I of Council Directive 96/23/EC, at least three identification points (IPs) have to be obtained using MS detection.[34] The detection of the analytes in standard solutions and spiked milk samples in this study was performed with the transitions of the molecular precursor ion $(M+H)^+$ to the two most abundant product ions. These two studied MRM transitions represent 4 IPs because the precursor ion accounts for 1 IP and its transition product earns 1.5 IPs.

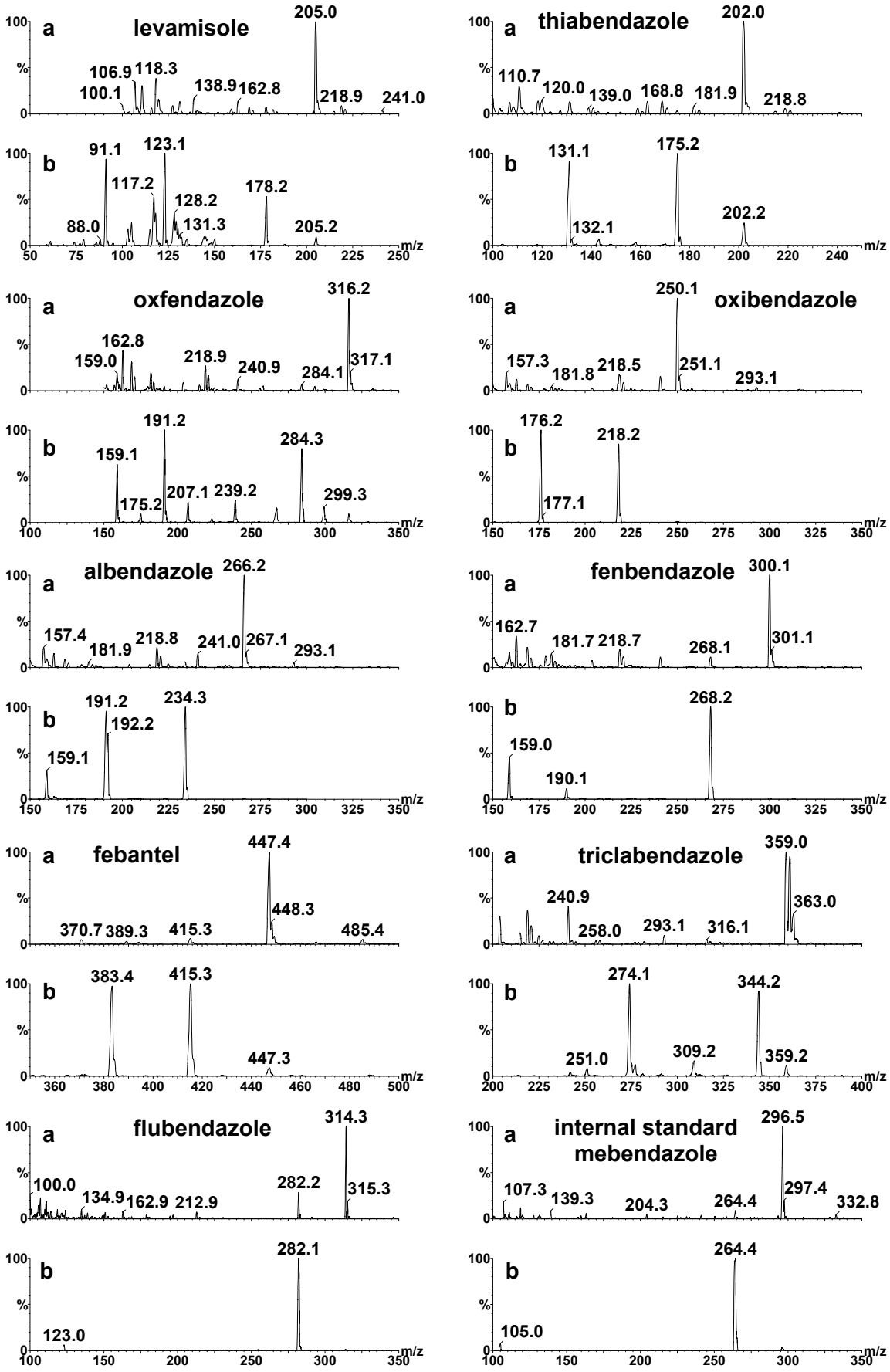


Figure 4.3 MS (a) and MS/MS (b) spectra of the examined anthelmintic compounds

4.3.2 Liquid chromatographic-mass spectrometric analysis

A relatively simple, very fast, very sensitive and reliable quantitative multiresidue method for the simultaneous detection of LE, TB, OF, OB, AB, FB, FE and TC at residue concentrations lower than 1 µg/l was developed. The method was based on the principles of a presented method (Chapter 6) for the determination of mebendazole and its metabolites in sheep liver.[32] Mebendazole belongs to the same anthelmintic benzimidazole group as the examined compounds. The analytical procedure was optimised for analysis of milk samples. Preliminary experiments modifying the analyte extraction and the sample clean-up procedures indicated that defatting of the sample extract with *n*-hexane can be omitted. Furthermore, a slightly higher response for some of the substances was obtained without this supplementary sample preparation step.

Netobimin and albendazole sulphoxide, two other authorised benzimidazole anthelmintics,[20] were also involved in this study. However, the discussed determination procedure, which is very useful for the examined compounds, was not successful for these substances. A new separation method has to be developed. Probably, the sample extraction and/or the MS ionisation mode should have to be changed.

Compared with the described HPLC-DAD method[19] in Chapter 3, this LC-MS/MS method is much faster, the scope of anthelmintic compounds is broader and specific mass spectra are obtained to allow unambigously identification of the presence of an analyte. Because of the high selectivity of tandem MS applied in the MRM mode resulting from the decrease in chemical noise, very high S/N ratios and very low LODs for the analytes of interest are obtained in spite of the very simple sample clean-up.[35]

The mobile phase composition used for the determination of mebendazole and its two major metabolites in sheep liver and the developed gradient programme was successful for the LC separation of the eight analytes and the IS in this study. To increase the sensitivity, 20 µl instead of 10 µl of cleaned sample extract was injected in the LC-MS/MS apparatus. To shorten the run time, the mobile phase flow rate of 0.20 ml/min, which is recommended for a column with an i.d. of 2.1 mm, was increased to 0.25 ml/min.

Identification and quantitative determination of the eight target compounds, flubendazole and the internal standard were carried out by MRM. Two transitions from precursor ion to product ions are measured. An example of a chromatogram of a blank milk sample fortified at 1 µg/l for each compound of a mixture of the anthelmintic analytes and at 10 µg/l for the IS is shown in Figure 4.4. The chromatogram shows two peaks per compound, one peak per transition from precursor ion to product ion. All compounds eluted within 11 min. As can be seen in the chromatogram, levamisole and thiabendazole are not chromatographically separated. However, LC separation of these compounds was not needed because of the powerful separation capacity of tandem MS in the MRM mode. Mosely *et al*.[35] reported that the mass spectrometer

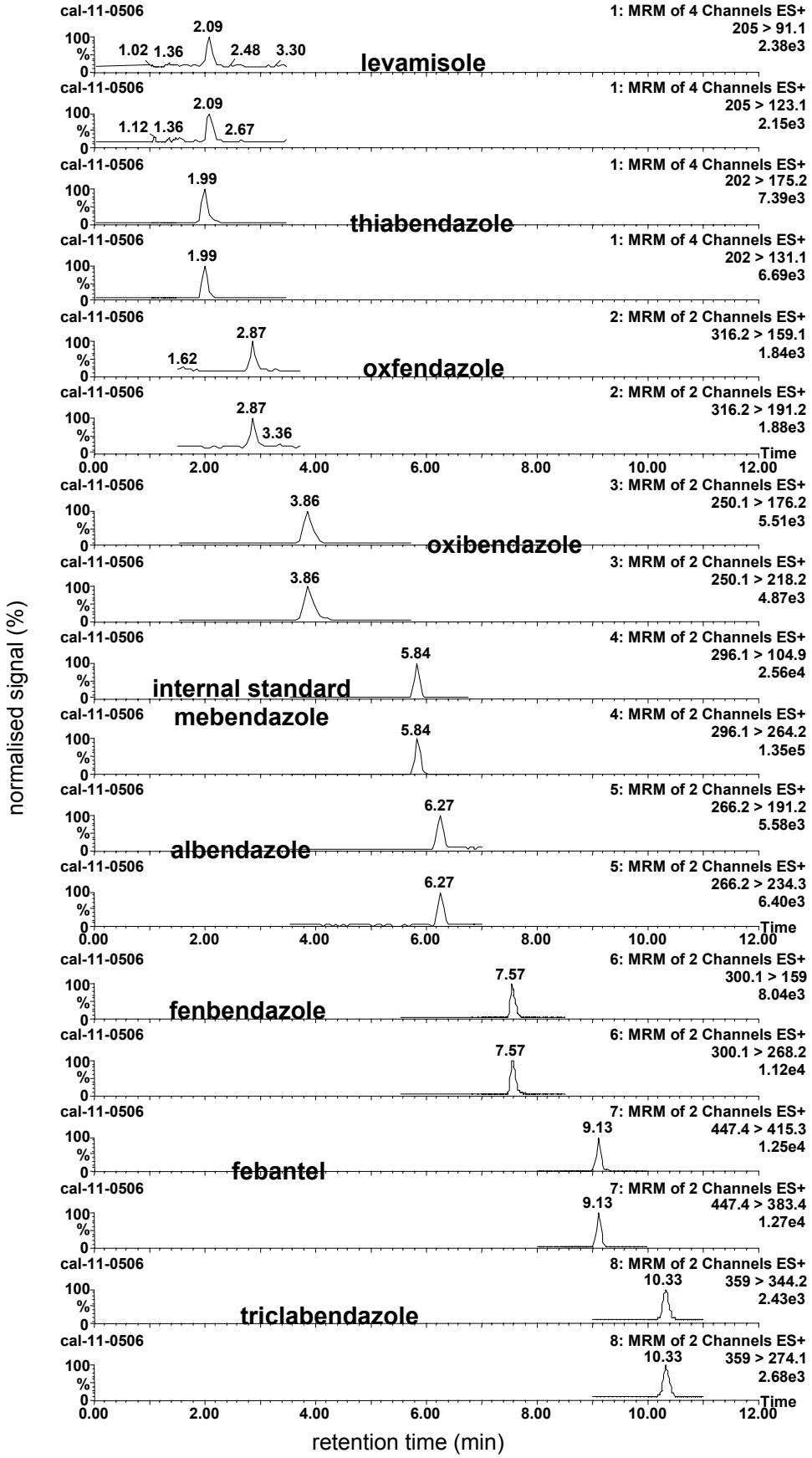


Figure 4.4 Chromatogram of a blank milk sample spiked with a mixture of anthelmintics at 1 µg/l for each compound and with the internal standard mebendazole at 10 µg/l

is used for the bulk of the separation process and that the LC is serving mainly as an automated sample injection technique.

During the LC-MS/MS run in the presented method, the initial mobile phase composition was already obtained after a run time of 15 min. The column had to be equilibrated for a further 10 min. The total run time was 25 min which means that 38 cleaned samples can be determinated per night. The sample preparation was more time consuming and was the time-limiting step. Twenty-four samples could be prepared during a 7-h working day per operator. Because the proven stability for spiked samples, prepared samples could be collected and stored refrigerated or deep frozen until determination during the validation study (see paragraph 4.3.3.4).

4.3.3 Validation study

4.3.3.1 Liquid chromatographic-mass spectrometric analysis

The specific criteria on relative retention time and on ion ratio were examined for all the samples analysed for the validation study. The values of RRT and of RI obtained for all samples measured to evaluate the validation parameters, were in agreement with the EU criteria.[34] As an example, the values of a sample spiked at 1 µg/l and of a matrix calibration sample spiked at 1 µg/l to evaluate the repeatability, are given. The RRT values in the spiked sample were 0.31, 0.31, 0.49, 0.51, 1.00, 1.27, 1.69 and 1.86 for LE, TB, OF, OB, AB, FB, FE and TC, respectively. These values are within the tolerance ranges of ± 2.5% obtained with the values of the calibration samples, which are 0.31±0.01, 0.31±0.01, 0,49±0.01, 0.51±0.01, 1.00±0.02, 1.27±0.03, 1.69±0.04 and 1.86±0.05 for LE, TB, OF, OB, AB, FB, FE and TC, respectively. The values for the RI in the spiked sample were 31, 96, 81, 83, 80, 77, 84, 94 and 22% for LE, TB, OF, OB, AB, FB, FE, TC and the IS, respectively. These values are within the allowed ranges obtained for the calibration sample. These ranges are 27±25, 99±20, 92±20, 87±20, 81±20, 79±20, 81±20, 90±20 and 22±25% for LE, TB, OF, OB, AB, FB, FE, TC and the IS, respectively.

4.3.3.2 Linearity

The instrumental response was linear for the eight anthelmintic analytes in the concentration range 0-500 µg/l obtained by six-point standard calibration. The slope and the intercept values for the standard regression lines of LE, TB, OF, OB, AB, FB, FE, TC were 0.33 and -0.002, 1.44 and 20.71, 0.21 and 0.002, 0.66 and -0.0002, 0.46 and 0.35, 0.82 and 0.20, 0.11 and 0.08, 1.21 and 0.03, respectively. Because of the rather broad concentration range, a better fit for the low concentrations was obtained with a weighting factor of $1/y^2$ calculated automatically by the MASSLYNX software.

The linearity of the whole LC-MS/MS assay was demonstrated by six-point matrix calibration analysis for all compounds in a residue concentration range of 0 to 500 µg/l. The obtained linear

regression parameters slope and intercept for LE, TB, OF, OB, AB, FB, FE, TC were 0.19 and -0.0001, 0.66 and 0.017, 0.14 and 0.00005, 0.83 and 0.014, 0.43 and 0.0069, 0.67 and 0.0043, 0.82 and 0.0041, 0.24 and 0.0026, respectively. Again, to increase the fit for the low residue concentrations, weighted least square regression was applied with a weighting factor of $1/y^2$.

4.3.3.3 Specificity

No interferences of endogenous material above a S/N ratio of 3/1 for all the analytes could be detected by analysing 20 blank milk samples. False positive or false negative results might be generated due to hindered identification. The absence of potential interference due to a similar substance belonging to the same chemical drug class as the examined analytes was demonstrated. No false positive or false negative results were obtained by analysing blank milk samples, separately spiked at MRL concentration with the licensed compounds and at 10 µg/l for LE, TC and FL. The quantification was not influenced by the presence of flubendazole in the samples. The differences on the peak areas between the samples with and without FL were very small and within the normal ranges of repeated analysis. Clearly, tandem mass spectrometry is a very powerful analytical technique showing a very high specificity. The identification and confirmation potentials of the LC-MS/MS technique are superior in comparison with the more traditional HPLC-DAD technique applied in our previous determination methods.[19]

4.3.3.4 Stability

The stability of fortified milk samples, stored at -18°C, was tested by repeatedly determining the spiked milk samples at 2-week time intervals. The regression data and calculated concentration values were comparable with those obtained for freshly spiked milk samples during an observation period of three months. For practical reasons, this observed stability for fortified samples is important. The numerous samples to be analysed can be collected, stored and analysed in the most efficient way. The demonstrated instability of an incurred milk sample indicated that laboratories have to be careful with the conservation of real samples for testing purposes of anthelmintic residues for a long period of time. A decrease of 30% of the residue concentration of levamisole after three months storage at -18°C was observed in a violative milk sample of the Belgian monitoring programme. During subsequent storage under frozen conditions, the levamisole residue concentration decreased from 183.8 to 128.8 µg/l. The residue concentration stabilised the next 1.5 months of measurement.

4.3.3.5 Recovery

The results of the sample extraction efficiency analyses are summarised in Table 4.2. The overall recovery values ranged from 89.6 to 102.0%. The obtained recovery values are in agreement with the requirements[34] which are correlated with the concentration. The variabilities

Table 4.2 Analyte recovery values for the determination of anthelmintics in fortified milk samples (mean (%), s_r (%), n = 6 for each concentration)

Spiked level (µg/l)	LE		TB		OF		OB		AB		FB		FE		TC	
	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
1.0	102.9	6.1	94.8	5.3	102.1	13.1	104.7	7.9	88.8	8.0	85.1	7.4	96.6	6.4	96.8	14.6
1.5	100.0	7.2	98.8	5.1	96.6	8.9	109.3	2.2	99.4	3.1	95.2	4.7	101.7	1.0	91.6	4.6
2.0	89.6	8.3	93.9	4.0	82.7	6.5	101.7	1.3	90.3	3.6	82.7	8.4	98.4	2.6	87.0	9.1
5	-	-	-	-	108.7	9.5	-	-	-	-	103.6	1.9	79.9	8.2	-	-
10	-	-	-	-	100.5	2.2	-	-	-	-	102.2	2.8	79.9	5.3	-	-
15	-	-	-	-	104.3	3.3	-	-	-	-	106.0	1.8	81.3	5.4	-	-
25	-	-	-	-	-	-	99.0	2.1	-	-	-	-	-	-	-	-
50	-	-	85.6	15.8	-	-	96.1	2.1	95.1	4.4	-	-	-	-	-	-
75	-	-	-	-	-	-	101.1	1.4	-	-	-	-	-	-	-	-
100	-	-	90.5	3.9	-	-	-	-	96.2	3.7	-	-	-	-	-	-
150	-	-	98.4	1.8	-	-	-	-	100.5	0.4	-	-	-	-	-	-
Overall recovery	97.3	9.2	93.7	8.2	99.1	11.4	102.0	5.4	95.0	6.1	95.8	10.5	89.6	11.6	91.8	10.8

LE: levamisole TB: thiabendazole OF: oxfendazole OB: oxibendazole AB: albendazole FB: fenbendazole FE: febantel TC: triclabendazole

on the overall recoveries, expressed as s_r values, were acceptable and varied from 5.4 to 11.6%. The obtained variabilities are in agreement with the criteria of the Horwitz equation[34] ($s_r = 2^{(1-0.5\log C)}$, where "C" represents the residue concentration expressed as a power of 10). The presented extraction recoveries are higher than those of our previous HPLC-DAD method.[19] The supplementary clean-up during the sample preparation probably unfavourably influenced the analyte recovery. The extraction efficiency is probably also higher when the milk sample is firstly made alkaline. In spite of the very simple sample preparation, Facino *et al*.[25] reported somewhat lower recoveries (80 to 90%), probably due to the not fully optimised extraction procedure or to the choise of extraction solvents (mixture of diethylether/*n*-hexane). Chappell *et al*.[8] used a more intensive solid phase extraction sample clean-up for the determination of LE in milk. The obtained recovery was acceptable and reached 82.6%.

4.3.3.6 Precision

The precision of the method was determined by means of the repeatability and the within-laboratory reproducibility.

The repeatability was evaluated at IPL and at MRL levels. The results of the repeatability study performed at IPL and at MRL levels, are summarised in Tables 4.3 and 4.4, respectively. The tables present mean values of measured residue concentrations and the variability on these values expressed as s_r values. Almost all these mean values fell within the ranges stipulated by the EU criteria,[34] except for a few very small deviations. Following the validation criteria, the repeatability or within-day precision expressed as s_r values for analyses carried out under repeatability conditions would have to be between half and two thirds of these values according to the Horwitz equation. The repeatability of the presented confirmatory method is acceptable. All calculated s_r values were within the allowed range. Moreover, the overall precision values were lower than half of the Horwitz values. The overall precision ranged from 5.2% for the determination of albendazole at 100 µg/l to 21.7% for the determination of thiabendazole at 1 µg/l.

An overview of the within-laboratory reproducibility results of the determinations at IPL and MRL levels are given in Tables 4.5 and 4.6, respectively. The analyses were performed on 5 different days and by two analysts with their own standard solutions. A sixth value for each concentration was obtained from data of the repeatability study. The method studied showed a good within-laboratory reproducibility. The allowed ranges for the mean concentration values of this validation parameter are equal to those of the repeatability.[34] This criterium was fulfilled for all the calculated mean values at the found concentration levels. The variability for the within-laboratory reproducibility is allowed to be higher than for the repeatability and the s_r values should be lower than the values of the Horwitz equation correlated with the concentration level.[34] The values in Tables 4.5 and 4.6 are much lower than these limits. The within-laboratory reproducibility, expressed as s_r value of the mean determined residue concentrations ranged

Table 4.3 Repeatability results for the determination of anthelmintic residues in milk samples spiked at 1.0, 1.5 and 2.0 IPL concentration (= 1.0, 1.5 and 2.0 µg/l) (mean (µg/l), s_r (%), n = 6 for each concentration)

Spiked level (µg/l)	LE		TB		OF		OB		AB		FB		FE		TC	
	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
Within-day																
Day 1																
1.0	1.1	17.1	1.1	16.4	0.9	11.9	0.9	10.8	0.9	6.2	0.8	12.6	1.0	15.9	0.9	9.8
1.5	1.5	8.6	1.4	7.9	1.5	12.1	1.4	3.5	1.3	10.3	1.3	10.2	1.4	16.3	1.2	14.4
2.0	2.1	2.4	2.1	7.0	1.9	9.0	1.9	5.6	1.9	3.5	1.7	5.9	1.6	6.3	1.4	8.9
Day 2																
1.0	0.9	19.0	0.8	24.7	1.0	9.6	0.9	14.2	0.8	7.5	1.0	5.8	1.1	7.9	1.0	5.3
1.5	1.6	10.1	1.4	12.4	1.5	10.1	1.4	2.8	1.2	8.8	1.4	11.0	1.5	9.9	1.3	6.0
2.0	2.1	10.6	2.0	9.8	1.9	14.7	2.0	6.3	1.8	5.4	1.7	7.4	1.7	13.2	1.4	11.2
Day 3																
1.0	1.0	13.6	0.8	18.5	0.8	28.5	0.9	11.7	1.0	10.7	1.0	12.5	1.1	8.4	1.0	10.0
1.5	1.6	8.7	1.3	11.4	1.1	8.7	1.3	9.3	1.4	5.5	1.4	6.3	1.5	11.8	1.5	10.0
2.0	1.8	6.8	1.6	3.9	1.6	13.8	1.7	4.0	1.9	8.4	1.8	4.7	1.9	3.1	1.8	10.9
Between-day																
Overall (n = 18)																
1.0	1.0	18.2	0.9	21.7	0.9	18.9	0.9	11.9	0.9	11.5	1.0	15.3	1.0	11.0	0.9	9.4
1.5	1.5	9.1	1.4	10.8	1.4	16.0	1.4	6.1	1.3	9.0	1.3	10.3	1.5	12.2	1.4	13.8
2.0	2.0	10.2	1.9	13.9	1.8	14.5	1.9	8.8	1.9	6.1	1.7	7.0	1.7	12.2	1.6	16.0

LE: levamisole TB: thiabendazole OF: oxfendazole OB: oxibendazole AB: albendazole FB: fenbendazole FE: febantel TC: triclabendazole

Table 4.4 Repeatability results for the determination of anthelmintic residues in milk samples spiked at 0.5, 1.0 and 1.5 MRL concentration (mean (µg/l), s_r (%), n = 6 for each concentration)

Analyte	Spiked level (µg/l)	Within-day						Between-day	
		Day 1		Day 2		Day 3		Overall (n = 18)	
		Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
TB	50	41.8	6.9	46.4	9.8	48.9	2.8	45.7	9.4
	100	83.6	3.0	91.8	12.3	97.3	1.0	90.9	9.4
	150	133.4	6.0	145.0	5.0	157.4	6.2	145.3	8.8
OF	5	5.4	7.1	4.6	7.8	4.9	4.0	5.0	9.1
	10	9.0	9.6	9.7	8.5	10.1	7.7	9.6	9.5
	15	15.1	3.5	15.3	8.5	15.2	5.3	15.2	5.8
OB	25	22.0	3.5	24.7	1.6	25.1	2.7	24.0	6.3
	50	42.8	3.1	49.0	5.9	49.9	2.9	47.2	8.0
	75	66.9	3.9	77.2	4.5	78.9	3.8	74.3	8.3
AB	50	44.3	7.1	48.3	4.9	52.0	3.0	48.2	8.2
	100	93.9	2.2	96.4	6.4	102.0	1.3	97.4	5.2
	150	143.8	2.8	151.3	4.4	161.8	4.5	152.3	6.3
FB	5	5.5	5.1	5.4	8.1	5.8	5.0	5.5	6.8
	10	10.7	4.1	10.3	8.2	11.1	2.4	10.7	6.0
	15	16.1	5.1	16.6	4.8	16.8	4.1	16.5	4.7
FE	5	4.8	5.6	4.5	8.0	5.0	5.6	4.8	7.5
	10	9.8	5.9	8.9	10.5	9.9	3.1	9.5	8.3
	15	15.0	5.1	14.7	8.0	16.5	6.9	15.4	8.3

TB: thiabendazole OF: oxfendazole OB: oxibendazole AB: albendazole FB: fenbendazole FE: febantel

Table 4.5 Within-laboratory reproducibility for determination of anthelmintics residues in milk samples spiked at 1.0, 1.5 and 2.0 IPL concentration (mean (µg/l), s_r (%) n = 6 for each concentration)

Spiked level (µg/l)	LE		TB		OF		OB	
	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
1.0	1.0	11.4	0.9	14.7	0.9	15.2	1.0	10.2
1.5	1.5	6.5	1.4	9.0	1.5	21.8	1.5	8.8
2.0	2.0	4.6	1.9	12.6	1.7	7.0	2.0	8.8

Spiked level (µg/l)	AB		FB		FE		TC	
	Mean	s_r	Mean	s_r	Mean	s_r	Mean	s_r
1.0	1.0	1.0	1.1	13.0	1.1	19.2	1.1	13.8
1.5	1.5	6.6	1.5	10.0	1.6	5.5	1.6	7.1
2.0	2.0	5.6	2.0	14.8	1.9	27.2	2.0	25.4

LE: levamisole TB: thiabendazole OF: oxfendazole OB: oxibendazole
AB: albendazole FB: fenbendazole FE: febantel TC: triclabendazole

Table 4.6 Within-laboratory reproducibility for determination of anthelmintic residues in milk samples spiked at 0.5, 1.0 and 1.5 MRL concentration (mean (µg/l), s_r (%) n = 6 for each concentration)

Analyte	Spiked level (µg/l)	Mean	s_r	Analyte	Spiked level (µg/l)	Mean	s_r
	50	49.2	6.5		50	51.3	3.4
Thiabendazole	100	99.7	7.4	Albendazole	100	101.0	1.9
	150	159.3	9.9		150	151.8	3.7
	5	4.8	7.8		5	5.5	12.1
Oxfendazole	10	10.1	4.5	Fenbendazole	10	10.8	10.6
	15	14.5	18.9		15	16.4	7.0
	25	25.0	5.3		5	5.2	9.0
Oxibendazole	50	49.9	8.5	Febantel	10	10.7	11.3
	75	77.8	12.7		15	16.3	3.5

from 1.0% for the determination of albendazole at 1.0 µg/l to 27.2% for the determination of febantel at 2.0 µg/l. As expected, this range was somewhat higher than the range obtained for the repeatability.

4.3.3.7 Analytical limits

The development and the full optimisation of this LC-MS/MS method to obtain low detection limits was one of the objectives of this study. An IPL level of 1 µg/l was chosen to be able to detect low residue concentrations for the monitoring of anthelmintic residues in raw farm milk, especially because two unauthorised substances (levamisole and triclabendazole) are involved. As already expected from the study of the specificity, no endogenous interfering matrix material could be measured for any compound to calculate the LOD values while analysing 20 blank milk samples. Technical LOD values were estimated as those concentrations which yielded a S/N ratio of 3/1 by replicated analysis of blank milk samples spiked at a concentration series of 1.0, 1.5, 3.0 and 5.0 µg/l. The sensitivity of the multiresidue confirmation method was very high and all compounds could be detected at a residue concentration lower than 1 µg/l. The measured LOD values (MRL values within brackets expressed in µg/kg) for LE, TB, OF, OB, AB, FB, FE and TC were 0.5 (0), 0.1 (100), 0.6 (10), 0.1 (50), 0.3 (100), 0.1 (10), 0.1 (10) and 0.6 (0) µg/l, respectively. As expected, these LOD values are much lower than those obtained in the previously presented HPLC-DAD methods.[19] The performance of the discussed LC-MS/MS method is also better than these of existing methods. Macri *et al*.[36] obtained a LOD of 10 µg/kg with their multiresidue HPLC-DAD method. LODs in the range of 0.6-2.8 µg/kg were obtained with the LC-MS method reported by Facino *et al*.[25] Branchflower *et al*.[26] reported a LOD of 50 and 100 µg/kg for LC-MS analysis of liver tissue for FB and OF, respectively. Chapell *et al*.[8] demonstrated a comparable detection limit (0.5 µg/kg) for LE in milk by a procedure with online LC-GC including an ion trap multimass spectrometry detector. However, their method is very complex and can only determine levamisole.

The decision limit values were calculated for the licensed drug substances as well as for the unauthorised compounds levamisole and triclabendazole. The analytical limit values for the MRL compounds were also calculated at IPL level and can also be defined as detection limit values. The analytical limit values at IPL level were calculated with concentration values already obtained for blank milk samples spiked at 1.0 µg/l for calculating the calibrations curves for the evaluation of the recovery, the repeatability and the within-laboratory reproducibility. The $CC\alpha$ values at MRL level for the licensed compounds were calculated with values already obtained for the determination of the recovery, the repeatability, the within-laboratory reproducibility and the stability. The calculated mean values plus 1.64 times the corresponding standard deviation equals the analytical limit ($CC\alpha$ and LOD values). These analytical limit values are summarised in Table 4.7. Obviously, the highest percentile deviation on the target concentration was

obtained for the analyses at IPL level. The highest value of 1.3 µg/l should be taken into account for decisions concerning the residues of febantel and triclabendazole. The highest percentile deviation (12%) at MRL level was also observed for the determination of febantel. The obtained CCα value was 11.2 µg/l. The decision limit should be handled as a very useful tool for the laboratory in order to make the right decision concerning a violative sample. The LOD values obtained with this criterion are somewhat higher (> 1 µg/l) than the detection limits obtained with the S/N ratio criterion. Therefore, LODs of different methods have to be interpreted in view of the used criterion.

The detection capabilities were obtained by analysing 20 blank milk samples spiked at IPL or at MRL level for the unauthorised and authorised substances, respectively. The respective decision limit values plus 1.64 times the corresponding standard deviations equals the detection capabilities. Applying this criterion for the MRL compounds at IPL level leads to analytical limits which can be defined as limits of quantification. The CCβ and LOQ values are also given in Table 4.7.

Table 4.7 Analytical limit values (CCα, CCβ, LOD and LOQ in µg/l) for the determination of anthelmintic residues in milk at IPL and MRL concentrations

Spiked concentration	Levamisole		Thiabendazole		Oxfendazole		Oxibendazole	
	CCα	CCβ	LOD	LOQ	LOD	LOQ	LOD	LOQ
IPL (1.0 µg/l)	1.2	1.4	1.1	1.2	1.1	1.3	1.1	1.2
			CCα	CCβ	CCα	CCβ	CCα	CCβ
MRL	-		105.8	118.2	10.5	12.1	52.6	58.9
Spiked concentration	Albendazole		Fenbendazole		Febantel		Triclabendazole	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	CCα	CCβ
IPL (1.0 µg/l)	1.1	1.2	1.1	1.3	1.3	1.3	1.3	1.4
	CCα	CCβ	CCα	CCβ	CCα	CCβ		
MRL	104.6	110.5	10.9	12.1	11.2	12.9	-	

- Decision limit: CCα (or LOD) = mean value of spiked milk samples + 1. 64 *s*
- Detection capability: CCβ (or LOQ) = CCα + 1.64 *s* (*n* = 20)
- *n* = 9 for IPL level and *n* = 12 for MRL level

4.4 Conclusions

A simple, very fast and specific LC-MS/MS confirmatory multiresidue method has been developed for the simultaneous, quantitative determination of levamisole, thiabendazole,

oxfendazole, oxibendazole, albendazole, fenbendazole, febantel and triclabendazole in milk at residue levels around 1 µg/l. The presented method is also suitable to quantify residues of anthelmintics at MRL level, providing the laboratory with a powerful tool to make decisions in violations for statutory testing purposes. The reliability of the liquid chromatographic-mass spectrometric procedure was proven by fulfilling all validation criteria of the final version of the European Commission Decision concerning analytical methods for detecting substances and residues thereof in live animals and animal products according to Council Directive 96/23/EC.[34]

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4.6 References

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CHAPTER 5

Determination of flubendazole and its metabolites in eggs and poultry muscle tissue by liquid chromatography-tandem mass spectrometry

Relevant publications:

- De Ruyck H., Daeseleire E., Grijspeerdt K., De Ridder H., Van Renterghem R. and Huyghebaert G.
  Determination of flubendazole and its metabolites in eggs and poultry muscle with liquid chromatography-tandem mass spectrometry.
  Journal of Agricultural and Food Chemistry, 2001, 49, 610-617
- De Ruyck H., De Ridder H., Van Renterghem R. and Daeseleire E.
  Determination of flubendazole and metabolites in eggs and poultry meat with LC-MS/MS.
  Proceedings of EuroResidue IV: Conference on residues of veterinary drugs in food, 8-10 May 2000, Veldhoven, Nederland, 962-968
- De Ruyck H., Daeseleire E., De Ridder H., Van Renterghem R. and Huyghebaert G.
  Determination of flubendazole and metabolites in eggs and poultry meat with LC-MS/MS.
  Proceedings of 9th Annual meeting of the Flemish society for veterinary epidemiology and economics: Risk analysis and quality control of animal products, 25 oktober 2001, Melle, 71-73

Abstract:

The optimisation of a quantitative LC-MS/MS method for the determination of residues of flubendazole and of its hydrolysed and reduced metabolites in eggs and poultry muscle is described. The benzimidazole components were extracted twice from the two matrices with ethyl acetate after the sample mixtures had been make alkaline. The collected organic supernatant fractions were concentrated by evaporation using vacuum and afterwards defatted with *n*-hexane. The LC separation was performed on a reversed-phase C_{18} column with gradient elution using a mobile phase containing ammonium acetate and acetonitrile. The analytes were detected after atmospheric pressure electrospray ionisation on a tandem quadrupole mass spectrometer in MS/MS mode. The MS/MS transition of the molecular precursor ion to the most abundant product ion of each analyte was measured. The overall extraction recovery values for flubendazole, the hydrolysed metabolite and the reduced metabolite in eggs (fortification levels 200, 400 and 800 µg/kg) and in muscle tissue (fortification levels 25, 50 and 100 µg/kg) were respectively 77, 78 and 80% and 92, 95 and 90%. The trueness (fortification level of 400 and 50 µg/kg, respectively for eggs and muscle tissue), expressed as a percentage of the added values for these compounds, was respectively 89, 100 and 86% and 110, 110 and 98%. The presented MS detection method operating in the MS/MS mode is very selective and has low detection limits. The limits of detection for flubendazole and its hydrolysed and reduced metabolites in egg and muscle tissue were respectively 0.19, 0.29 and 1.14 µg/kg and 0.14, 0.75 and 0.31 µg/kg. The limits of quantification were respectively 1, 1 and 2 µg/kg and 1, 1 and 1 µg/kg.

Keywords: flubendazole, metabolites, residues, poultry, LC-MS/MS

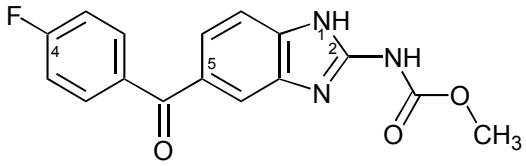
5.1 Introduction

5.1.1 Properties of flubendazole and its metabolites

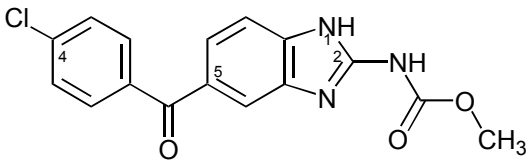
Flubendazole (FL) is a broad-spectrum benzimidazole (BZ) anthelmintic, which is effective against endoparasites such as gastrointestinal roundworms, gapeworms and tapeworms.[1] This antiparasitic drug is widely used in the veterinary medicine of poultry.

Benzimidazole anthelmintics are extensively metabolised following oral administration.[2] The major metabolites arising after treatment of poultry species with flubendazole are the hydrolysed (HFL) and the reduced compound (RFL), which are formed by hydrolytic and reduction reactions, respectively.[3,4]

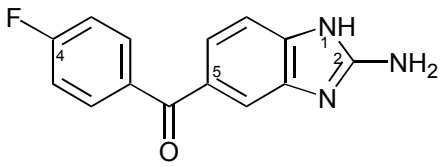
The molecular structures of FL, its two metabolites and the internal standard which will be used for the analytical method, are presented in Figure 5.1.



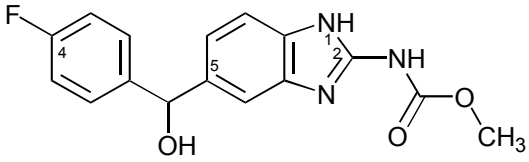
flubendazole M_r = 313.3
methyl-[5-(4-fluorobenzoyl)-
1*H*-benzimidazole-2-yl] carbamate



internal standard M_r = 329.7
methyl-[5-(4-chlorobenzoyl)-
1*H*-benzimidazole-2-yl] carbamate



hydrolysed metabolite of flubendazole M_r = 255.3
(2-amino-1*H*-benzimidazole-5-yl)(4-fluorophenyl)
methanone



reduced metabolite of flubendazole M_r = 315.3
methyl-[5-[(4-fluorophenyl)hydroxymethyl]-
1*H*-benzimidazole-2-yl] carbamate

(M_r = relative molecular mass)

Figure 5.1 Chemical structures of flubendazole, its metabolites and the internal standard

The BZ compounds, shown in Figure 5.1, have a common central “benzimidazole” nucleus and, except for HFL, contain a “carbamate (-$NHCOOCH_3$) type” functional group each.[5] Flubendazole is the *p*-fluor analogue of mebendazole, another anthelmintic drug compound which is widely used for treatment of sheep.

Some general properties of the BZ analytes are described in paragraph 3.1.1 in Chapter 3.

5.1.2 Toxicity

Short term studies suggest that benzimidazoles are efficacious for parasitic control while demonstrating a great margin of safety in mammals, including humans.[2]

Thienpont *et al*.[6] described the biological and pharmacological properties of flubendazole in seven animal species. Toxicity studies were performed in various laboratory animals. The LD_{50} value in acute toxicity tests was above 2560 mg/kg for all animal species. In chronic toxicity studies with rats and dogs, no side-effects were observed, neither clinically nor histopathologically. The teratological results for rat and rabbit were normal, as well as the peri- and postnatal effects and fertility experiments in rats. The teratogenicity of flubendazole in rats was studied in detail by Yoshimura.[7] The highest administered dose (160 mg/kg) was embryocidal and resulted in a significant increase in the fetal resorption rate. There was a dose-dependent decrease in fetal body weight which was significant at a dose of 40 mg/kg or more. The 40 and 160 mg/kg doses induced significant fetal (gross, skeletal and internal) malformations.

Benzimidazole anthelmintic residues are of little human health concern. Toxicological data include studies on metabolism, carcinogenicity, genotoxicity, effects on reproduction and teratogenicity. For consumers of edible muscle tissues, drug residues covalently bound in tissue should not present significantly toxicological risks.[2] However, the extractable residues (parent drug and/or free metabolites) are chemical compounds with defined toxic potential.

5.1.3 Objectives

Administration of flubendazole to poultry species can result in the presence of residues of the parent flubendazole compound and its hydrolysed and reduced metabolites in eggs and in muscle tissues. By Council Regulation EEC/2377/90 and updates,[8] the EC sets the MRLs for the sum of FL+HFL as marker residue in eggs and in poultry muscle tissue at 400 and 50 µg/kg, respectively.

Only a few chromatographic methods for the determination of residues of flubendazole in eggs and/or muscle tissue have been published.[3,4,9,10] Some depletion studies of flubendazole with laying hens and excretion data for residues in eggs are available.[3,4] Balizs[4] reported the determination of residues of different benzimidazoles in several animal tissues and eggs using liquid chromatography and tandem mass spectrometry (LC-MS/MS). A practical application of the assay has been described for incurred egg material of laying hens treated with flubendazole. The residue depletion curves in egg yolk as well as in egg white were drawn. Kan *et al*.[3] studied the residue formation in eggs and the metabolism in laying hens after long-term low-level dose (carry-over dose) and normal dose level exposure of hens treated with

flubendazole via the feed. The residue concentrations of flubendazole and its two major metabolites in egg yolk and in egg white were quantified with a HPLC-UV method. Marti *et al*.[9] described a multiresidue HPLC-UV method for the determination of eight BZ anthelmintics in meat samples. For verification of violative samples, the drug substances were derivatised for detection by GC-MS in the electron-impact or chemical ionisation mode. The simultaneous determination of flubendazole, triclabendazole and metabolites of triclabendazole in milk, muscle tissue and liver samples by HPLC-UV was published by Takeba *et al.*[10]

To our knowledge, no depletion studies with residue formation data in poultry muscle tissue after administration of flubendazole are reported in the open literature.

The objective of this study was to optimise a sensitive and quantitative LC-MS/MS method for the simultaneous determination of residues of FL, of HFL and of RFL in eggs and in muscle tissue of poultry species. The developed method is applied in depletion studies (Chapter 9) with turkeys and with guinea fowls which were orally treated with flubendazole. The residues concentrations of FL, HFL and RFL were monitored in muscle and in liver tissues.

5.2 Materials and methods

5.2.1 Reagents

Analytical standard material of FL, HFL, RFL and IS was kindly provided by the manufacturer Janssen Animal Health (Beerse, Belgium). Acetonitrile (HPLC grade), methanol (HPLC grade) and acetic acid (100%) of analytical grade were from Merck (Darmstadt, Germany). Dimethyl sulphoxide (HPLC grade), ethyl acetate (HPLC grade), ammonium acetate (analytical grade), and 0.1 N sodium hydroxide (analytical grade) were purchased from Panreac (Barcelona, Spain). *n*-Hexane (analytical grade) was from BDH (Poole, UK). All water used was of HPLC grade and was purified by a Maxima LC 113 Ultra-pure water purification system (Elga, Bucks, UK).

5.2.2 Standard solutions

The standard material was dissolved in dimethyl sulphoxide at a concentration of 0.3125 mg/ml. These solutions were further diluted with methanol to stock standard solutions of 0.1 mg/ml. The stock standard solutions were stored refrigerated at 5°C and were replaced every two months. The working standard solutions were diluted with methanol and prepared immediately before use. Standard solutions of 1 µg/ml in ammonium acetate and acetonitrile (30/70, v/v) were used for tuning the mass spectrometer and for acquisition of the analyte identification mass spectra.

5.2.3 Instrumentation

The muscle tissue samples were minced and ground with a Knifetec sample mill (Tecator, Höganäs, Sweden). The ground muscle tissue samples and the egg samples were

homogenised with an Unimix homogeniser (Haagen and Rinau, Bremen, Germany). A shaker (Bühler, Hechingen, Germany), a RC-5B Sorvall centrifuge (DuPont Instruments, Wilmington, DE, USA) and a vortex mixer (Scientific Industries, Bohemia, NY, USA) were used during the sample preparation. The LC-MS/MS system consisted of a LC system (Kontron, Biotech Instruments, Milan, Italy) with a 325 ternary pump system, a vacuum degasser, a 465 autosampler and a 433 UV detector, coupled with a quattro LCZ tandem quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, UK) with "z"-spray electrode and electrospray ion interface. The mass spectrometer was fully controlled by MASSLYNX software version 3.3. A model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA) was connected to the interface for tuning purposes. The LC separations were carried out on a reversed-phase column Alltima C_{18} (Alltech, Deerfield, IL, USA), 5 µm, 150 x 2.1 mm i.d., in combination with an Alltima C_{18} guard cartridge of 7.5 x 2.1 mm i.d.

5.2.4 Sample Preparation

5.2.4.1 Muscle and liver tissue

One gram of ground muscle or liver tissue sample was weighed into a 50 ml beaker. If necessary, FL, HFL, RFL, and/or IS solutions were added. After standing for 30 min, the mixture was homogenised in 10 ml of water for 30 s. The mixture was made alkaline with 5 ml of 0.1 N sodium hydroxide. The analytes were extracted with 20 ml of ethyl acetate in centrifuge tubes of 50 ml on a shaker (amplitude: 30 mm, frequency: 90 per min) for 10 min. After centrifugation (5 min, 5000 rpm), the supernatant was transferred into a sidearm flask. The extraction procedure was repeated once on the lower layer with 10 ml of ethyl acetate. The collected organic fractions were evaporated to 4-5 ml in a water bath at 60°C using vacuum. The residual fraction was transferred into a graduated tube and was evaporated to dryness under a stream of nitrogen. The residue was dissolved with 600 µl of methanol. After mixing, the mixture was heated at 60°C in a water bath during 5 min. The mixture was defatted with 2 ml of *n*-hexane. After standing for 5 min, the upper hexane layer was removed. If necessary, for recovery experiments, the IS was added at this stage. The solution was made up to 1 ml with methanol and was heated at 60°C in a water bath for 2 min. After mixing, the mixture was filtered through a 0.2 µm polytetrafluorethylene (PTFE) Chromafil filter (Macherey-Nagel, Düren, Germany) into an autosampler vial.

5.2.4.2 Egg

One gram of mixed whole egg sample was weighed into a centrifuge tube. If necessary FL, HFL, RFL and/or IS solutions were added. After standing for 30 min, the mixture was made alkaline with 1 ml of 0.1 N sodium hydroxide. The following steps were identical to those applied for the extraction and clean-up of muscle and liver samples described in paragraph 5.2.4.1.

5.2.5 Liquid chromatographic-mass spectrometric method

The LC separation of flubendazole, its metabolites and the IS was carried out on a reversed-phase column with a buffer solution. The mobile phase consisted of pure acetonitrile (B) and a buffer solution (A) containing 0.04 M ammonium acetate adjusted to a pH of 5.2 with a few microlitres of acetic acid (100%). Gradient elution was applied and the elution programme consisted of 50A:50B (0 min), 50A:50B to 25A:75B (1-3 min), 25A:75B (4-5 min), 25A:75B to 50A:50B (6-7 min) and 50A:50B (8-15 min). The mobile phase flow rate was 0.25 ml/min and the injection volume was 10 µl.

The analytes were detected and identified with a tandem quadrupole mass spectrometer. An incorporated split device was set to a split ratio of 1:1. Atmospheric pressure electrospray ionisation in the positive mode (ESI^+) was applied. The molecular precursor ions were fragmented in the collision cell with argon. FL, HFL, RFL and IS were determined with tandem mass spectrometry (MS/MS) by the multiple reaction monitoring (MRM) function. One transition from precursor ion to product ion, was measured. Cone voltage and collision energy were tuned to optimise the transition from the molecular precursor ion to the most abundant product ion.

5.2.6 Method of data calculation

The measured residue data of the samples by MRM of one transition are evaluated by an internal standard procedure based on calibration curves. Quantification is conducted by internal calibration following the formula:

$$C_S\ (\mu g/kg) = [(area_S \times C_{IS}\ (\mu g/kg) / area_{IS} - intercept) / slope] \times 1/CF$$

were C: concentration s: sample IS: internal standard CF: concentration factor

The results were calculated automatically by the MASSLYNX software version 3.3 of the mass spectrometer.

5.2.7 Validation of the analytical method

5.2.7.1 Introduction

The validation of the analytical procedure was based on the EC guidelines for determination methods of veterinary drug residues.[11] The validation procedure was carried out completely for egg and muscle tissue matrices. Breast muscle tissue of broiler chickens was used as validation matrix for muscle tissue.

The IS was spiked or added with a working standard solution of 1 µg/ml. For determination of the linearity, the analytical limits and the repeatability, the IS was spiked to concentrations of

100, 10 and 100 µg/l or µg/kg, respectively. The IS concentrations used for the recovery and the trueness experiments, are summarised in paragraph 5.2.8.

5.2.7.2 Linearity

The linearity of the LC-MS/MS response was checked by analysing three series standard solutions of a mixture of FL, HFL and RFL at concentrations of 0.5, 1, 5, 25, 100, 500, 600 and 800 µg/l.

Matrix calibration curves were obtained by analysing three series of spiked egg and muscle tissue samples with a mixture of FL, HFL and RFL at concentrations of 0.5, 1, 5, 25, 100, 500, 600 and 800 µg/kg. The curves were calculated using the linear least squares regression analyses of the peak area to concentration ratios.

The variation on the linearity is expressed as the relative standard deviation (s_r) of the slope of the curves.

5.2.7.3 Recovery

The extraction efficiency for the BZ analytes was determined for 10 blank samples fortified at three concentration levels, namely, 0.5 MRL, 1.0 MRL and 2.0 MRL. For determination of the recovery, the IS was not extracted but added just before injection on the LC column. The recovery values were calculated with calibration curves especially drawn during the determination of this validation parameter for muscle tissue samples and egg samples. These samples were spiked at concentrations of 10, 25, 50, 100 and 200 µg/kg, and of 100, 200, 400, 800 and 1000 µg/kg, respectively.

5.2.7.4 Analytical limits

The detection limits (LODs) were calculated as the apparent concentration corresponding to the value of the mean plus three times the standard deviation (s) obtained for at least 20 representative blank sample determinations. If only noise was obtained, a technical LOD was calculated as those concentrations that yield a signal-to-noise (S/N) ratio of 3/1.

The quantification limits (LOQs) were defined as the lowest concentration of the analytes for which the method is validated with an accuracy and precision that fall within the ranges recommended by the EC.[11]

5.2.7.5 Trueness

The trueness was calculated as the closeness between the mean measured residue value and the fortified concentration. This validation parameter was determined by analysing 10 blank samples spiked with the analytes at MRL level. The trueness values were calculated using calibration curves obtained with spiked concentrations of 25, 50 and 100 µg/kg for muscle tissue and of 200, 400 and 800 µg/kg for eggs, respectively. The trueness is expressed as the

percentual agreement between the mean measured value and the added concentration. This validation parameter gives the measured concentrations after compensation for recovery which is incorporated in the calibration curves.

5.2.7.6 Repeatability

The accuracy of the LC-MS/MS method was evaluated by determination of the repeatability or the within-day precision of the assay. The repeatability of the assay for eggs was studied by repeatedly analysing a blank egg sample, spiked at 100 µg/kg with a fortification solution containing the three analytes. The repeatability of the assay for muscle tissue was verified by repetitively analysing an incurred turkey breast muscle tissue sample taken during the administration period of the depletion study described in Chapter 9. The repeatability was expressed as s_r value.

5.2.8 Sample fortification procedure for the determination of recovery and trueness

5.2.8.1 Recovery

For the recovery experiments, the standard solutions of the target analytes and of the IS were added after the *n*-hexane removal during the sample preparation of fortified blank egg samples and fortified blank muscle tissue samples.

To fortify egg samples at 200, 400 and 800 µg/kg (IS at 400 µg/kg), 40, 80 and 160 µl of a standard solution of 5000 µg/l of a mixture of the compounds were added, respectively. To determine the calibration curves (concentration levels of 100, 200, 400, 800 and 1000 µg/kg), 100 µl of a standard solution of 1000 µg/l and 40, 80, 160 and 200 µl of a solution of 5000 µg/l, respectively, were used.

To fortify muscle tissue samples at 25, 50 and 100 µg/kg (IS at 100 µg/kg), 25, 50 and 100 µl of a standard solution of 1000 µg/l, respectively, were added. The calibration curves (10, 25, 50, 100 and 200 µg/kg) with this matrix were made with 100 µl of a standard solution of 100 µg/l and 25, 50, 100 and 200 µl of a solution of 1000 µg/l, respectively.

5.2.8.2 Trueness

To determine the trueness, equal volumes and spiking concentrations of standard solutions as described for the evaluation of the recovery were used. The standard solutions were added to the blank samples into the centrifuge tube at the beginning of the extraction step.

The egg samples were spiked at 400 µg/kg with the target analytes and with the IS. Calibration was done with matrix curves made with concentrations of 200, 400 and 800 µg/kg. The muscle tissue samples were fortified at 50 µg/kg with the target analytes and at 100 µg/kg with the IS. The concentrations fortified for the calibration curves were 25, 50 and 100 µg/kg.

5.3 Results and discussion

5.3.1 Analytical method

A relatively simple, fast and reliable method was developed for the quantitative determination of residues of flubendazole and its metabolites in eggs and poultry muscle tissue.

Because of good results in previous research work on benzimidazole drugs in milk, ethyl acetate was also used as extraction reagent in this study.[12] Preliminary experiments as well as the experience of Issar *et al*.[13] have indicated that the alkaline conditions of the sample mixture have a great influence on the extraction recovery of the weak basic benzimidazole compounds. Sodium hydroxide was added to the sample to make the sample mixture alkaline. Interfering residual fat particles were removed with *n*-hexane. The sample preparation and clean-up procedures applied in the presented procedure are comparable with those reported by Kan *et al*.[3] and Balizs[4].

Identification and determination of FL, its metabolites and the IS was carried out by tandem MS operating in the MRM mode. The mass spectrometer was previously tuned and optimised for maximum ion response of each analyte by infusion of a standard solution of 1 µg/ml with a syringe pump. A summary of the diagnostic ions obtained for the examined compounds and the optimised MS operating parameters are given in Table 5.1.

Table 5.1 Summary of the diagnostic ions and MS operating parameters

Analyte	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone voltage (*V*)	Collision energy (*eV*)
FL	314.1^{+a}	282.0^{+a}, 122.7^{+}	40	25
HFL	256.1^{+a}	122.9^{+a}	50	30
RFL	316.2^{+a}	284.1^{+a}, 238.4^{+}, 159.9^{+}	45	25
IS	330.3^{+a}	297.9^{+a}, 138.5^{+}	50	25

[a] ions used for MRM detection FL: flubendazole

HFL and RFL: hydrolysed and reduced metabolites of flubendazole IS: internal standard

The ions indicated in Table 5.1 are protonated. The detection of the analytes in standard solutions and in spiked samples was performed by measuring the transition of the precursor (parent) ion $(M+H)^+$ to the most abundant product ion (indicated in Table 5.1). Preliminary tests indicated that the ratio of the intensity of the different product ions was constant. For unknown samples out of control and monitoring programmes for example, a supplementary transition in the MRM mode has to be measured for the confirmation of a violative sample. The specific

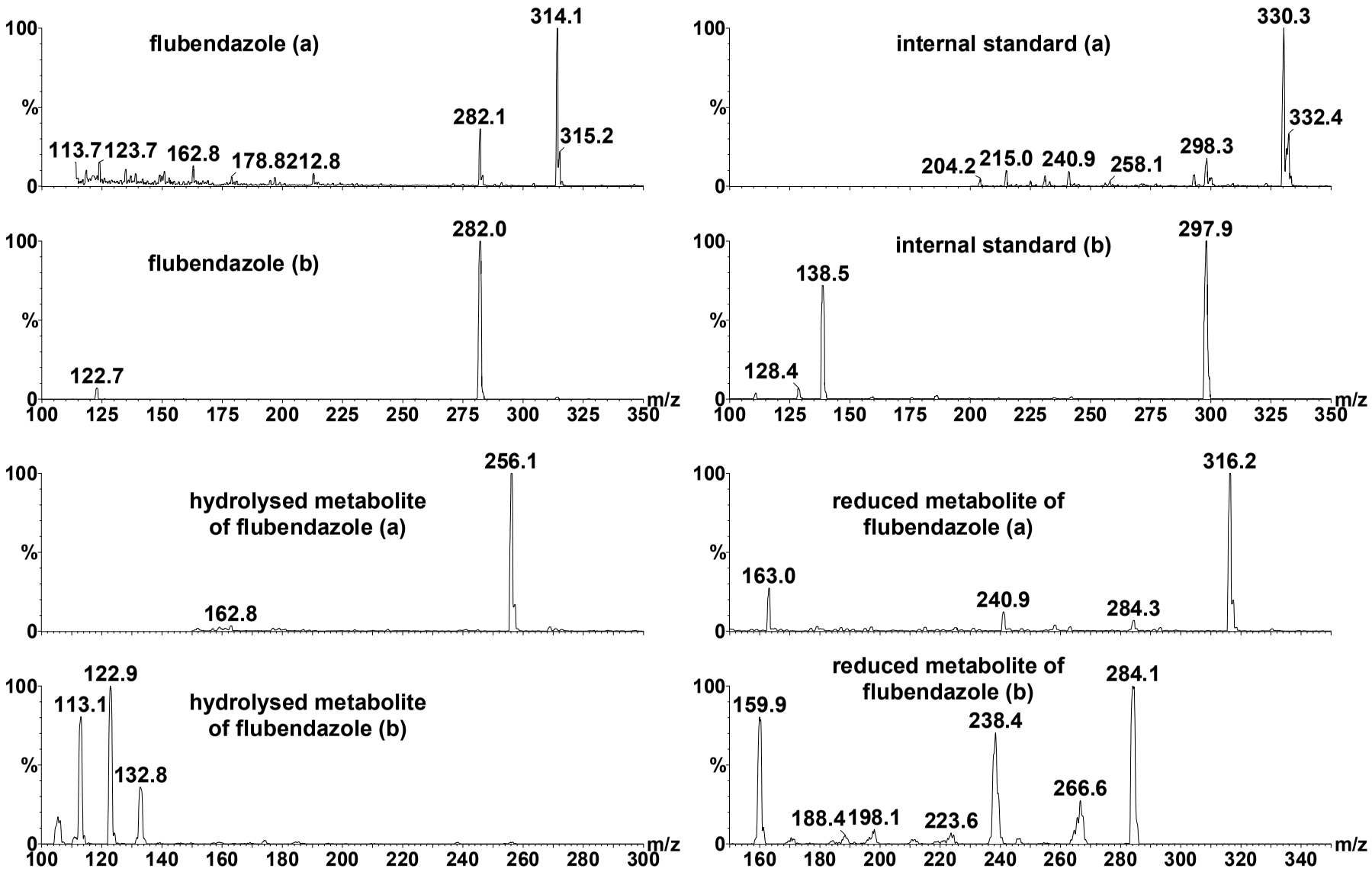


Figure 5.2 MS (a) and MS/MS (b) spectra of the examined anthelmintic compounds

mass spectra for each examined compound are presented in Figure 5.2. The full scan mass spectrum (a) and the fragmentation mass scan (b) of the precursor ion of each analyte are given.

The LC separation of the analytes was obtained by gradient elution with 0.04 M ammonium acetate, adjusted to a pH of 5.2 and acetonitrile. Several LC methods using a mobile phase containing ammonium acetate are published.[3,4,14] Previous work showed that methanol did not sufficiently separate the analytes. First of all, a gradient elution programme was optimised for the separation of FL, HFL and the IS. Increasing the flow rate from 0.20 to 0.25 ml/min decreases the total LC run time to 15 min. The preliminary analyte detection was performed by UV absorption at a wavelength of 254 nm. Afterwards, the reduced metabolite was also involved in this study. RFL could be determined with the same LC procedure. For further experiments, only MS detection was applied. As illustrated in Figure 5.3, the two metabolites are not chromatographically separated. However, this LC separation is not necessary because of the powerful separation capacity of the mass spectrometer operating in the MRM mode.

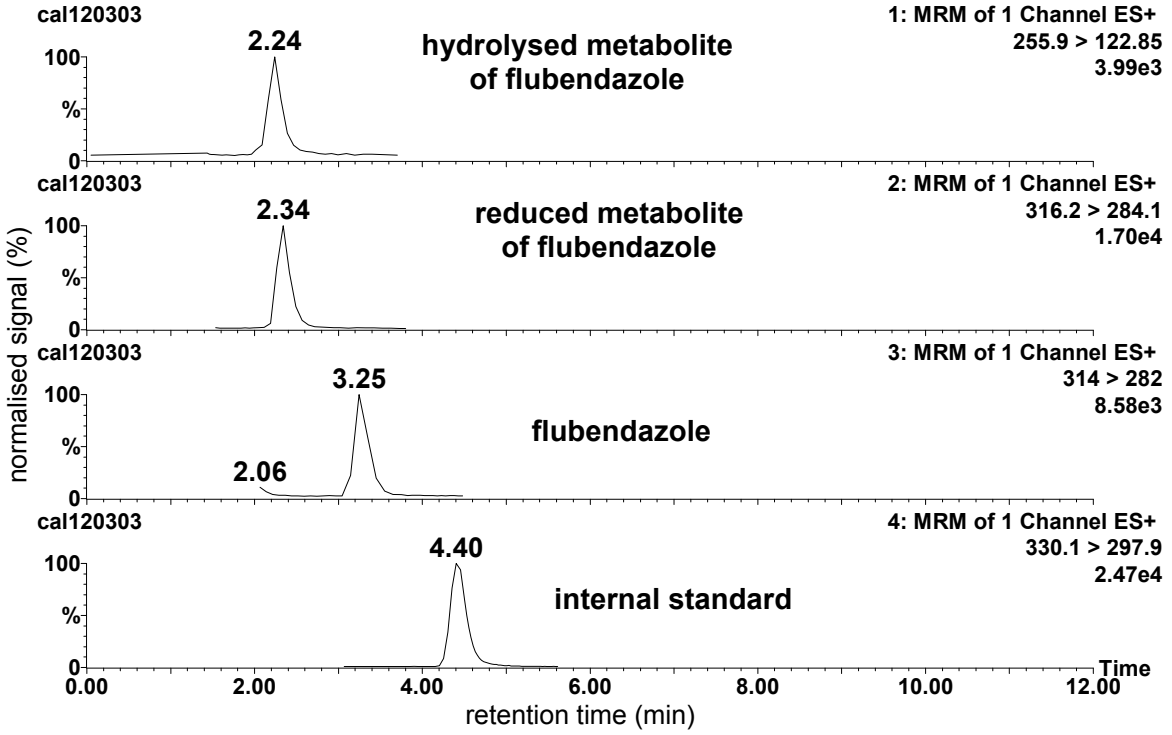


Figure 5.3 Chromatogram of a blank muscle tissue sample spiked with a mixture of flubendazole and its metabolites at 1 µg/kg and with the IS at 10 µg/kg

Figure 5.3 shows a chromatogram of a blank muscle tissue sample spiked with a mixture of the analyte compounds at a concentration of 1 µg/kg and with the IS at a concentration of 10 µg/kg.

Table 5.2 Regression analysis of matrix calibration curves for the determination of flubendazole and its metabolites in eggs and muscle tissue

Analyte	Egg				Muscle tissue			
	Curve	Slope	Intercept	*r*	Curve	Slope	Intercept	*r*
FL	1	2.81	2.37	0.9926	1	2.18	1.80	0.9643
	2	2.95	1.39	0.9977	2	2.35	0.98	0.9829
	3	2.87	0.77	0.9952	3	2.29	9.55	0.9831
		s_r = 2.4%				s_r = 3.8%		
HFL	1	3.64	2.63	0.9820	1	2.71	0.41	0.9215
	2	4.04	1.45	0.9844	2	2.80	0.28	0.9711
	3	3.99	0.97	0.9946	3	2.60	8.12	0.9927
		s_r = 5.6%				s_r = 3.7%		
RFL	1	3.98	8.59	0.9825	1	1.84	3.28	0.9563
	2	4.34	6.22	0.9749	2	2.04	1.35	0.9774
	3	4.02	5.13	0.9754	3	1.99	8.17	0.9984
		s_r = 4.8%				s_r = 5.3%		

FL: flubendazole HFL and RFL: hydrolysed and reduced metabolites of flubendazole

Table 5.3 Recovery values for blank egg and blank muscle tissue samples fortified at 0.5, 1.0 and 2.0 MRL concentration (µg/kg) with flubendazole and its metabolites [mean recovery value (%) (s_r(%)), n = 10]

Analyte	Egg				Muscle tissue			
	200	400	800	Overall	25	50	100	Overall
FL	70.2 (7.7)	76.8 (9.0)	83.3 (7.0)	76.7 (10.4)	92.4 (6.8)	91.0 (6.8)	92.8 (6.1)	92.1 (6.4)
HFL	69.9 (11.8)	91.4 (9.4)	74.0 (7.9)	78.4 (15.4)	104.4 (6.4)	88.9 (9.4)	90.7 (7.4)	94.7 (10.5)
RFL	71.6 (9.3)	88.1 (8.2)	79.8 (5.9)	79.8 (11.5)	90.9 (6.8)	90.6 (8.4)	87.6 (6.5)	89.7 (7.3)

FL: flubendazole HFL and RFL: hydrolysed and reduced metabolites of flubendazole

5.3.2 Validation of the analytical method

5.3.2.1 Linearity

The LC-MS/MS response was linear in the concentration range of 0.5 to 800 µg/l obtained with eight calibration points (0.5, 1, 5, 25, 100, 500, 600 and 800 µg/l). Three series of standard solutions were measured. Very stable linearity relationships were obtained for the three examined analytes. The s_r values of the slope of the standard curves for FL, HFL and RFL were 3.6, 1.8 and 1.0%, respectively.

The results of the regression analysis of the calibration data obtained by analysing spiked egg and muscle tissue samples are summarised in Table 5.2. The linearity of the eight-point (0.5, 1, 5, 25, 100, 500, 600 and 800 µg/l) matrix calibration curves was proven in the concentration range 0.5 to 800 µg/kg. The s_r values of the slope of the three different curves were very low and were below 6% for each of the target compounds.

5.3.2.2 Extraction recovery efficiency

Table 5.3 reports the mean extraction recovery values obtained for egg and muscle tissue samples spiked with a mixture of the analytes. The recovery values ranged from 77 to 95% and were higher for muscle tissue than for eggs. The overall extraction recovery values for FL, HFL and RFL in eggs and muscle were 77, 78 and 80%, and 92, 95 and 90%, respectively. The values for eggs are slightly lower than those obtained by Kan *et al*.[3] These authors reported mean values of respectively 80, 82 and 89% for these compounds. Balizs[4] obtained a mean recovery value of 72% for FL in eggs. The reported recovery value of 92% for FL in muscle tissue with the presented method is somewhat higher than that obtained by Takeba *et al*.[10] (86%). Even lower recoveries of 73 and 50% for FL in muscle tissue were published by Marti *et al*.[9] and Balizs,[4] respectively.

5.3.2.3 Analytical limits

In contrast with the preliminary results reported in our earlier short publication,[15] final detection limit values were determined more in detail by analysing series of 24 blank egg and 23 blank muscle tissue samples. The calculated LOD values are summarised in Table 5.4. For the hydrolysed metabolite in the two matrices, only noise was measured. Therefore, a technical LOD with fortified samples was calculated as that concentration which yields a S/N of 3. These results illustrate the extreme sensitivity of the presented LC-MS/MS method. Except for RFL in egg, all analytes can be detected at a residue level lower than 1 µg/kg. The obtained detection limits are lower than those reported Kan *et al*.[3] These authors obtained LODs of 7, 12 and 28 µg/kg for HFL, FL and RFL in whole egg, respectively. Less sensitivity was demonstrated by the methods of Balizs,[4] Marti *et al*.[9] and Takeba *et al*.[10] These research workers reported

detection limit values for FL of 32 µg/kg in eggs, 50 µg/kg in muscle tissue and also 50 µg/kg in muscle tissue, respectively.

Table 5.4 Detection limits (LOD) (µg/kg) for the determination of flubendazole and its metabolites in eggs and muscle tissue

Compound	Egg	Muscle tissue
FL	0.19	0.14
HFL	0.29[a]	0.75[a]
RFL	1.14	0.31

[a] technical LOD with S/N = 3 FL: flubendazole

HFL and RFL: hydrolysed and reduced metabolites of flubendazole

An overview of the quantification limit values is given in Table 5.5. Very low LOQs of 1.0 to 2.0 µg/kg were obtained with the described LC-MS/MS method. With exception for RFL in eggs (2 µg/kg), the method was validated with an accuracy and precision that fall within the ranges recommended by the EC[11] for samples spiked with the analytes in eggs and muscle tissue samples at a concentration of 1.0 µg/kg.

5.3.2.4 Trueness

The calculated trueness values for muscle tissue samples are expressed as the percentual agreement between the mean measured concentration (n = 10) and the fortification concentration of 50 µg/kg (MRL). These values (s_r (%) in parentheses) for FL, HFL and RFL were 110 (10.1), 110 (4.9) and 98% (7.1), respectively. The corresponding values obtained with blank egg samples (n = 10) fortified at a concentration of 400 µg/kg (MRL) were 89 (5.6), 100 (8.7) and 86% (6.9), respectively.

5.3.2.5 Repeatability

A summary of the within-day precision or repeatability results of the method is given in Table 5.6. For most of the analytes, the repeatability, expressed as s_r value, was not higher than 10%. The repeatability of the assay was least favorable for the HFL compound measured in the incurred turkey breast muscle sample at a residue concentration of 243 µg/kg. A repeatability of 13.4% was obtained.

Table 5.5 Quantification limits (LOQ) for the determination of flubendazole and its metabolites in eggs and muscle tissue

Compound	Egg (n = 6)			Muscle tissue (n = 7)		
	LOQ (µg/kg)	Measured concentration (mean ± s (µg/kg))	s_r (%)	LOQ (µg/kg)	Measured concentration (mean ± s (µg/kg))	s_r (%)
FL	1.0	1.13 ± 0.13	11.5	1.0	1.05 ± 0.09	8.4
HFL	1.0	1.14 ± 0.18	15.6	1.0	1.01 ± 0.14	13.6
RFL	2.0	1.62 ± 0.14	8.5	1.0	1.15 ± 0.11	9.3

FL: flubendazole HFL and RFL: hydrolysed and reduced metabolites of flubendazole

Table 5.6 Repeatability results for the determination of flubendazole and its metabolites in eggs at 100 µg/kg and of incurred turkey breast muscle tissue [(s_r (%)) mean value (µg/kg)]

Analyte	Egg	Muscle tissue
FL	7.0 (83.6)	8.7 (61.6)
HFL	10.5 (86.8)	13.4 (243.1)
RFL	7.2 (95.5)	not detected

n = 10

FL: flubendazole HFL and RFL: hydrolysed and reduced metabolites of flubendazole

5.4 Conclusions

A relatively fast, sensitive and very selective LC-MS/MS method for the determination of flubendazole and its hydrolysed and reduced metabolites in eggs and poultry muscle tissue is described. Due to the relatively intensive sample preparation and the very classic equipment required, it was not possible to determine more than 16 muscle tissue samples or 32 egg samples per day per analyst.

Because of the lack of screening methods for these group of drug substances, the method can be proposed for screening purposes in monitoring programmes for poultry products. Regarding the new EC rules for residues analysis, one supplementary transition in the MRM mode has to be measured to make the presented method suitable for confirmation purposes of violative samples in surveillance programmes.

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CHAPTER 6

Determination of residues of mebendazole and its metabolites in sheep tissues by liquid chromatography-tandem mass spectrometry

Relevant publications:

- De Ruyck H., Daeseleire E. and De Ridder H.
  Development and validation of a liquid chromatography-electrospray tandem mass spectrometry method for mebendazole and its metabolites hydroxymebendazole and aminomebendazole in sheep liver.
  The Analyst, 2001, 126, 2144-2148
- De Ruyck H., Daeseleire E., De Ridder H. and Van Renterghem R.
  Liquid chromatographic-electrospray tandem mass spectrometric method for the determination of mebendazole and its hydrolysed and reduced metabolites in sheep muscle.
  Analytica Chimica Acta, 2003, 483, 111-123

Abstract:

A quantitative liquid chromatographic-electrospray tandem mass spectrometric method for the determination of residues of mebendazole and its hydrolysed and reduced metabolites in sheep muscle and liver tissues has been developed. The benzimidazole substances were extracted twice with ethyl acetate after the sample mixture had been made alkaline. After centrifugation, the collected organic fractions were concentrated by evaporation using a vacuum evaporator and defatted with *n*-hexane. The LC separation was performed on a reversed-phase C_{18} column with gradient elution using a mobile phase consisting of water containing 0.1% formic acid and acetonitrile.

Using a very selective tandem quadrupole mass spectrometer, the presented drug residue analysis method has low detection limits. The analytes were detected in multiple reaction monitoring mode after atmospheric pressure electrospray ionisation. The MS/MS transitions of the molecular precursor ion to the two most abundant product ions of each component were measured. The confirmatory method was validated according to the revised EU requirements. The evaluated validation parameters were stability, specificity, recovery, precision (repeatability and within-laboratory reproducibility) and analytical limits (decision limit, detection limit and quantification limit). Mebendazole-derived residues could be detected in sheep tissues at concentrations lower than 2 µg/kg. The validation limit or the internal performance limit was set at 10 and 50 µg/kg for muscle and liver tissue, respectively. The obtained overall recovery was higher than 90% for the three analytes. The relative standard deviation on the repeatability ranged from 5 to 11%. The range for the within-laboratory reproducibility was between 2 and 17%. The decision limits for mebendazole, the hydrolysed and the reduced metabolites in liver tissue were 445.9, 466.5 and 463.4 µg/kg, respectively. The limits of quantification for these substances were 60.0, 86.1 and 90.9 µg/kg, respectively.

The decision limit values obtained for mebendazole, the hydrolysed and the reduced metabolite in muscle tissue were 72.5, 69.2 and 69.8 µg/kg, respectively. The quantification limits for these substances were 13.1, 15.2 and 15.8 µg/kg, respectively.

To be suitable for analysis in a depletion study, the LC-MS/MS method was also optimised for the analysis of kidney and back fat tissues.

Keywords: drug residue analysis, mebendazole, sheep tissues, LC-MS/MS, method validation

6.1 Introduction

6.1.1 Properties of mebendazole and its metabolites

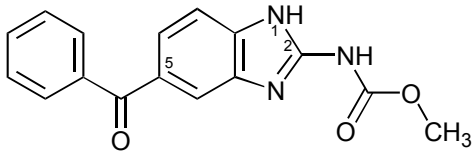
Mebendazole (ME) is a benzimidazole (BZ) antiparasitic substance widely used in veterinary drug products. This pharmaceutical compound is an orally active broad-spectrum anthelmintic which is effective against numerous species of nematodes and cestodes of the gastrointestinal tract of animals.[1] ME is frequently used for sheep and goats against major gastrointestinal nematodes, lung nematodes and tapeworms.[1,2,3,4,5] ME is known to be absorbed from the gastrointestinal tract and to be intensively metabolised in sheep.[1] The major metabolic compound measured in sheep tissues and fluids after administration of mebendazole, is hydroxymebendazole. This reduced or alcoholic metabolite (RME) is formed by carbonyl reduction of the ketone group (Figure 6.1).[1,6,7] The hydrolysed metabolite or aminomebendazole (HME) arises by hydrolysis of the carbamate group of the mebendazole molecule (Figure 6.1).[6]
The chemical structures of the benzimidazole (BZ) compounds involved in this study are presented in Figure 6.1. All shown compounds have a “benzimidazole” nucleus. With exception for the hydrolysed metabolite, the substances have a “carbamate (-$NHCOOCH_3$) type” functional group. Flubendazole (FL), an anthelmintic drug compound developed as the *p*-fluoro-derivative of mebendazole,[8] was used as internal standard in this analytical method. The specificity of the assay was verified with oxfendazole (OF).
Several general chemical properties of the BZ compounds were already given in paragraph 3.1.1 in Chapter 3.

6.1.2 Toxicity

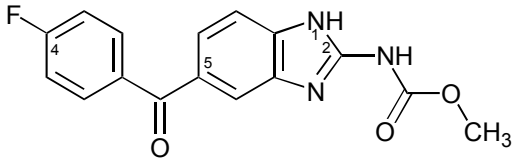
Concerning teratogenic toxicity, a great deal of information is available on embryopathic effects of benzimidazoles in rat.[6] Embryolethal and teratogenic effects have been described for mebendazole. Teratogenicity occurs at fairly low dosages compared with acute toxicity to adult animals. Mebendazole is reported to be nonteratogenic at the therapeutic dosage in sheep. The hydroxymebendazole metabolite is more embryotoxic than mebendazole itself in rat.
From the standpoint of public safety, the toxicological evaluation of benzimidazole residues is of considerable importance. Bound residues do not present a hazard for the consumer. The extractable residues are chemicals with defined toxic potential for which safety margins may be set.

6.1.3 Objectives

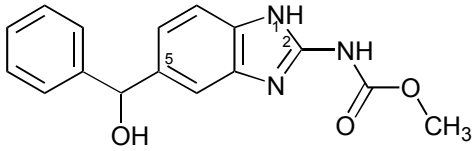
Residues of the parent mebendazole substance and its reduced and hydrolysed metabolites in foodstuffs might occur after treatment of food-producing animals with mebendazole, especially when the recommended withdrawal period is not respected.



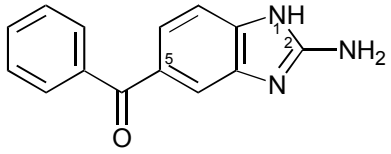
mebendazole M_r = 295.3
methyl-[5-benzoyl)-1*H*-benzimidazole-2-yl] carbamate



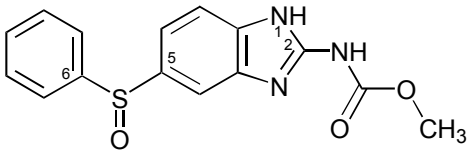
flubendazole (internal standard) M_r = 313.3
methyl-[5-(4-fluorobenzoyl)-1*H*-benzimidazole-2-yl] carbamate



reduced metabolite of mebendazole M_r = 297.3
methyl-[5-[(phenyl)hydroxymethyl]-1*H*-benzimidazole-2-yl] carbamate



hydrolysed metabolite of mebendazole M_r = 237.3
(2-amino-1*H*-benzimidazole-5-yl) (phenyl) methanone



oxfendazole M_r = 315.3
methyl-[5(6)-phenylsulfinyl-1*H*-benzimidazole-2-yl] carbamate

(M_r = relative molecular mass)

Figure 6.1 Molecular structures of the examined benzimidazole compounds

To monitor the safety of food products, validated methods for the determination of mebendazole-derived residues in edible tissues of animals are needed. Several analytical methods for mebendazole-derived residues are developed and described in the literature. Most of them use HPLC.[9,10,11,12] Steenbaar *et al.*[9] developed a HPLC-DAD determination method for residues of ME in eel muscle tissue. The method was extended for the metabolites RME and HME and was published by Hajee *et al.*[10] Al-Kurdi *et al.*[11] presented a HPLC-UV method for the determination of residues of ME and HME in human pharmaceutical dosage formulations. A rapid and selective HPLC-UV assay for the simultaneous quantitative determination of ME and analogous compound in whole blood is described by Ramanathan *et al*.[12]

Only a few methods for residues of mebendazole using liquid chromatography-mass spectrometry (LC-MS) are available.[3,13] A collisional spectroscopy procedure for simultaneous detection of five widely employed BZ anthelmintics including ME in sheep milk was developed

by Facino *et al*.[3] The milk samples were adjusted to an alkaline pH of 10 and the residues were extracted twice with trichloro methane. The combined organic phases were evaporated to dryness and the residues were dissolved in methanol before analysis. The determination involves injection of the extracts into the LC-MS system and selection and collision of the most abundant ion (molecular ion of fragment ion) obtained under electron impact ionisation. Balizs[13] published a more actual multiresidue liquid chromatographic-tandem mass spectrometric (LC-MS/MS) method with electrospray ionisation for benzimidazoles including ME in several animal tissues. The analytes were extracted twice out of the alkaline made tissue samples with ethyl acetate. The collected organic phases were evaporated to dryness. The dried residue was defatted with *n*-hexane and ethanol. The ethanol-acid layer was evaporated and dissolved in a mixture consisting of ammonium acetate and methanol. A solid phase extraction (SPE) was performed. The analytes were eluted from the SPE columns with a mixture consisting of methanol and ethyl acetate (20/80, v/v). The dried residue was dissolved in the LC mobile phase consisting of acetonitrile and 0.01 M ammonium acetate containing 0.5% acetic acid (60/40, v/v). The metabolites of ME were not involved in this method.

To protect the consumer, the EC has established MRLs by Council Regulation EEC/2377/90 and amendments[14] for mebendazole-derived residues in sheep tissues. The MRL value for the sum of residues of ME+RME+HME in liver tissue is set at 400 µg/kg. The MRL value for these residues in muscle, kidney and back fat tissues is established at 60 µg/kg.

The present study deals with the development of confirmatory assays with LC-MS/MS for the quantitative determination of residues of mebendazole, its reduced and its hydrolysed metabolites in muscle, liver, kidney and back fat tissues of sheep. The method is validated for liver and muscle tissues matrices according to the revised EC rules for drug residue analysis.[15]

The analytical study was performed in two parts. Firstly, the method was developed for liver tissues. Afterwards, the analytical procedure was performed for muscle tissue and was optimised for the analysis of kidney and back fat tissues.

The developed methods were used in a depletion study for the quantification of mebendazole-derived residues in tissues of orally treated sheep. This depletion study is reported in Chapter 10.

6.2 Experimental

6.2.1 Reagents and chemicals

Analytical standard material of ME, HME, RME and the internal standard (IS) FL was generously donated by Janssen Animal Health (Beerse, Belgium). OF was kindly supplied by Merial (Toulouse, France). Acetonitrile and methanol were of HPLC grade and from Merck (Darmstadt, Germany). Dimethyl sulphoxide (HPLC grade), ethyl acetate (HPLC grade), 1 M sodium hydroxide (analytical grade), 0.1 M sodium hydroxide (analytical grade) and formic

acid (analytical grade) were purchased from Panreac (Barcelona, Spain). *n*-Hexane (analytical grade) was from BDH (Poole, Dorset, UK). All water used was of HPLC grade and purified by a Maxima LC 113 Ultra-pure water purification system (Elga, Bucks, UK).

6.2.2 Standard solutions

Analytical standard material of the analytes was dissolved in dimethyl sulphoxide at a concentration of 0.3125 mg/ml and further diluted with methanol to stock standard solutions of 0.1 mg/ml. The stock standard solutions were stored refrigerated at 5°C and used for up to two months. The working standard solutions were made immediately before use by dilution with mobile phase consisting of acetonitrile and water, containing 0.1% formic acid (50/50, v/v). Standard solutions of 1 µg/ml were used for tuning the mass spectrometer and for acquisition of identification mass spectra of the analytes.

6.2.3 Apparatus

The slightly thawed muscle, liver, kidney and back fat tissue samples were minced and ground with a Knifetec sample mill (Tecator, Höganäs, Sweden). The ground tissues were homogenised with an Unimix homogeniser (Haagen and Rinau, Bremen, Germany). A shaker (Bühler, Hechingen, Germany), a RC-5B Sorvall centrifuge (DuPont Instruments, Wilmington, DE, USA) and a vacuum evaporator (Büchi, Flawil, Switzerland) were used during the sample pretreatment. The LC-MS/MS instrument already used for previous analytical studies in our laboratory was utilised.[16,17] The LC-MS/MS apparatus consisted of a LC-system (Kontron, Biotech Instruments, Milan, Italy) combined with a Quattro LCZ tandem quadrupole mass spectrometer (Micromass, Altrincham, Cheshire, UK) provided with a “z”-spray electrode and an electrospray ion interface. The LC-system consisted of a 325 ternary pump system, a vacuum degasser and a 465 autosampler. The mass spectrometer and the data acquisition were fully controlled by the MASSLYNX software version 3.3. The tuning experiments were performed with a Model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA) which was connected to the interface. The LC separations were performed on an Alltima C_{18} reversed-phase column (Alltech, Deerfield, IL, USA), 5 µm, 150 x 2.1 mm i.d., protected with an Alltima C_{18} guard cartridge of 7.5 x 2.1 mm i.d.

6.2.4 Sample preparation

6.2.4.1 Liver tissue

One gram of ground sheep liver sample was weighed into a centrifuge tube of 50 ml. If necessary, ME, HME, RME, OF and/or IS solutions were fortified at this stage. The spiked samples were left for 30 min. The sample was made alkaline with 1 ml of 0.1 M sodium hydroxide and was vortex mixed. The analytes were extracted with 20 ml of ethyl acetate on a

shaker (amplitude: 30 mm, frequency: 90 per min) for 10 min. After centrifugation (5 min, 5000 rpm), the supernatant was transferred into a side-arm flask. The extraction procedure was repeated once on the centrifugate with 10 ml of ethyl acetate. The collected organic fractions were evaporated to 4-5 ml in a water bath at 60°C using vacuum. The residual fraction was transferred into a graduated tube. The flask was rinsed with 2 ml of ethyl acetate. The remaining extract was evaporated to dryness under a stream of nitrogen in a water bath at 60°C. The residue was dissolved in 600 µl of methanol and was vortex mixed. The mixture was heated at 60°C in a water bath during 5 min. After vortex mixing, the mixture was defatted with 2 ml of *n*-hexane while swirling. After standing for 5 min, the upper hexane layer was removed and the tube was placed during 2 min in a water bath heated at 60°C. For the recovery experiments, the IS was added after the extraction, at this stage. The solution was vortex mixed and made up to 1 ml with methanol while vortex mixing. The cleaned extract was filtered through a 0.2 µm polytetrafluorethylene (PTFE) Chromafil filter (Macherey-Nagel, Düren, Germany) into an autosampler vial.

6.2.4.2 Muscle and kidney tissue

One gram of ground sheep muscle or kidney tissue sample was weighed into a beaker of 50 ml. If necessary, the analyte and/or IS solutions were spiked at this stage. The fortified samples were left for 30 min. The tissue was homogenised (8000 rpm, 15 s) in 10 ml of water. The sample was made alkaline with 1 ml of 1 M sodium hydroxide. The sample mixture was transferred into a centrifuge tube of 50 ml and the beaker was rinsed with 20 ml of ethyl acetate. The extraction was activated on a shaker (amplitude: 30 mm, frequency: 90 per min) for 10 min. After separation by centrifugation (5000 rpm) during 5 min, the supernatant was transferred into an evaporation tube. The extraction procedure was repeated once on the centrifugate with 10 ml of ethyl acetate. The collected organic fractions were evaporated to 2-4 ml with the vacuum evaporator (260 ± 10 X 10^2 N/m^2, 60°C, 200 rpm). The residual fraction was quantitatively transferred into a graduated tube. The evaporation tube was rinsed with 2 ml of ethyl acetate. The remaining extract was evaporated to dryness under a stream of nitrogen in a water bath at 60°C. The residue was dissolved in 600 µl of mobile phase consisting of acetonitrile and water containing 0.1% formic acid (50/50, v/v), and was vortex mixed. The mixture was heated at 60°C in a water bath during 5 min. After vortex mixing, the mixture was defatted with 2 ml of *n*-hexane while swirling. After standing for 5 min, the upper hexane layer was removed and the tube was placed in a water bath at 60°C during 2 min. For the recovery experiments, the IS was added after the extraction at this stage. The solution was vortex mixed and made up to 1 ml with mobile phase and homogenised by vortex mixing. The cleaned extract was filtered through a 0.2 µm regenerated cellulose (RC) Chromafil filter (Macherey-Nagel, Düren, Germany) into an autosampler vial.

6.2.4.3 Back fat tissue

One gram of ground back fat tissue sample was weighed into a centrifuge tube of 50 ml. If necessary, analyte and/or IS solutions were added at this stage. The fortified samples were left for 30 min. Ten ml of water was added and the sample mixture was made alkaline with 1 ml of 1 M sodium hydroxide while swirling. The tubes were placed in a water bath at 60°C for 10 min while swirling regularly. Twenty ml of ethyl acetate as extraction reagent was added. The extraction was performed on a shaker (amplitude: 30 mm, frequency: 90 per min) for 10 min. After centrifugation (5000 rpm) during 10 min, the supernatant was transferred into an evaporation tube. The extraction procedure was repeated with 10 ml of ethyl acetate on the centrifugate which had been heated before in a water bath at 60°C during 5 min. The next steps were identical to those of the sample preparation procedure of muscle or kidney tissue described in paragraph 6.2.4.2.

6.2.5 Liquid chromatography

The LC separation of mebendazole and its metabolites was performed on a reversed-phase column at room temperature. The mobile phase consisted of pure acetonitrile (B) and water containing 0.1% formic acid (to increase the ionisation intensity) (A). Gradient elution was applied to improve the separation capacity and to decrease the retention times of the analytes. The gradient programme consisted of 65A:35B (0 min), 65A:35B to 50A:50B (0-0.1 min), 50A:50B to 25A:75B (0.2-3 min), 25A:75B (4-5 min), 25A:75B to 50A:50B (6-7 min), 50A:50B to 65A:35B (8-15 min) and 65A:35B (16-25 min). The mobile phase flow rate was 0.25 ml/min. An injection volume of 10 µl of cleaned liver tissue sample extract was injected into the LC system. To increase the sensitivity of the method, the injection volume was increased to 30 µl for the other tissue samples.

6.2.6 Mass spectrometry

The benzimidazole analytes were detected and identified with a tandem quadrupole mass spectrometer. The compounds were ionised by atmospheric pressure electrospray ionisation in the positive ion mode (ESI^+). The LC eluent flow of 0.25 ml/min was sprayed into the mass spectrometer interface without splitting. Nitrogen gas flows of 80 and 600 l/h for nebulising the LC eluent and for drying the solvents respectively, were used. The source block and solvent desolvation temperatures were set at 130 and 250°C, respectively. Fragmentation of the precursor ions in product ions was obtained in the collision cell with argon gas.

The analytes were detected by tandem MS using the multiple reaction monitoring (MRM) function of two transitions. The specific cone voltage and collision energy for each compound were optimised to maximise the ion current of the three induced ions, one precursor (parent) ion and two product (fragment) ions. Protonated positive ions are formed by ESI^+ due to addition of

a H-atom to the ions. The transition of the precursor ion, the molecular parent ion, to the two most abundant fragment ions was optimised by means of infusion of separate standard solutions of 1 µg/ml using a syringe pump. Identification and confirmation of one or more analytes in a violative sample is proven by detection of these three diagnostic ions above the detection limit (signal-to-noise (S/N) ratio of at least 3/1).

6.2.7 Method of quantification

The MRM-generated data of the transition from the precursor ion to the most abundant product ion were evaluated by an internal standard procedure based on matrix calibration curves. Quantification was conducted by internal calibration. The results were calculated automatically by the MASSLYNX software version 3.3 of the mass spectrometer. The calibration curves were always drawn using the best fit of two replicated determinations per concentration point. The calibration curves were obtained using at least five concentration points, including the zero level. The response factors (Y-values) (response factor = peak area ratio of sample and IS multiplied by the concentration of the IS) were plotted against the concentration levels (X-values).

6.2.8 Validation procedure

6.2.8.1 Introduction

The main lines of the revised EC requirements for detecting residues of veterinary drug substances in animal products[15] were applied as guide for the validation of the presented method. The validation was performed completely for liver and muscle tissue matrices.

During the validation procedure for muscle and liver tissues, the IS was added using a solution with concentration of 1 µg/ml. Standard solutions of a mixture of ME, HME and RME were always used to fortify the samples, unless indicated otherwise. Blank muscle tissue samples were spiked using a mixed standard solution of 0.1 and 1 µg/ml for matrix concentrations up to 25 µg/kg and above 25 µg/kg, respectively. Blank liver tissue samples were spiked using a mixed standard solution of 1 and 2 µg/ml for matrix concentrations of 50 to 250 µg/kg and above 250 µg/kg, respectively.

The validation parameters evaluated for the entire analytical assay were the stability, the specificity, the recovery, the precision (repeatability and within-laboratory reproducibility) and the analytical limits (decision limit, detection limit and quantification limit).

During the validation of the analytical methods, the rules for validation of methods applicable for depletion studies by companies which are developing veterinary drugs, were taken into account. The EC requirements prescribe validation at concentration levels of 0.5 MRL, 1.0 MRL and 1.5 MRL for each analyte.[15] For the validation study of sheep tissues, this prescription can not be followed because the MRL for muscle and liver tissue is set at 60 and 400 µg/kg,

respectively, for the sum of residues of ME+HME+RME. The revised European criteria guide shows a hiatus for this kind of veterinary drugs with an established MRL for marker residues consisting of the sum of parent drug and its metabolites or analogous compounds. To estimate useful tolerance levels and validation values in this case, the levels of 60 and 400 µg/kg have to be divided between the examined analytes ME, HME and RME. A validation limit or an internal performance limit (IPL) of 10 and 50 µg/kg for muscle and liver tissue, respectively, was chosen. The two higher concentration levels were chosen at 20 and 60 µg/kg, 100 and 400 µg/kg for muscle and liver tissue, respectively, so that a broad validated concentration range was obtained.

6.2.8.2 Liquid chromatographic-mass spectrometric analysis

The specific criteria for liquid chromatographic separation and mass spectrometric detection were checked during the whole validation procedure. Firstly, there is the retention time criterion for the LC analysis. The difference between the ratio of the retention time of the analytes in the validation samples to that of the IS, i.e. the relative retention time (RRT) of the analytes, and that of the matrix calibration standards was measured. Secondly, two parameters related to the MS detection have to be evaluated. Concerning the ion recognition, each diagnostic ion was measured when it had a S/N of at least 3/1. Furthermore, the difference of the relative intensities of the detected ions (RI), expressed as a percentage of the integrated area of the most intense ion, in the validation samples and these of the matrix calibration standards, was evaluated.

6.2.8.3 Stability

The stability of refrigerated standard solutions was verified by determinations at two week intervals. Standard mixtures of ME+HME+RME at concentration ranges of 0, 50, 100, 250 and 500 µg/l were prepared. The regression data and calculated values were compared with those obtained for freshly made solutions.

6.2.8.4 Specificity

To evaluate the specificity, potential endogenous interferences were checked by analysing 20 blank muscle and 20 blank liver tissue samples from different origin. Hindered identification by the multiresidue method was detected by analysing blank muscle and blank liver tissue samples spiked at 10 and 50 µg/kg, respectively, separately with HME, RME and OF, a benzimidazole substance very similar to the target compounds. Interferences on the quantification were evaluated by comparing the integrated peak areas of muscle and liver tissue samples spiked at 10 and 50 µg/kg, respectively, with a mixture of OF+ME+HME+RME and a mixture of ME+HME+RME.

6.2.8.5 Recovery

Because no certified reference material of the examined compounds exists, the recovery efficiency instead of the trueness was determined. This validation parameter was calculated at the validation concentrations of 10, 20 and 60 µg/kg for muscle tissue, and of 50, 100 and 400 µg/kg for liver tissue. Blank samples were fortified with mixed standard solutions and six replicates at each concentration were analysed. To be able to calculate the recovery, the IS was added after the extraction and the clean-up of the analytes, at the end of the sample preparation procedure.

6.2.8.6 Precision

The precision was evaluated by determining the repeatability and the within-laboratory reproducibility. The repeatability was determined with a comparable procedure as was used for the evaluation of the recovery. Evidently, the IS was now spiked at the beginning of the sample preparation, before the clean-up procedure. To evaluate the within-laboratory reproducibility, for each concentration, the analyses were performed on five different days, with different mixed standard solutions and by two operators. To obtain six values, one value out of the repeatability data was taken. The precision is expressed as the relative standard deviation (s_r value) calculated with the results of the repeatedly analysed samples.

6.2.8.7 Analytical limits

The decision limit (CCα) is the concentration, above which it can be decided with a statistical certainty of 1-α that the identified residue content is truly above 60 and 400 µg/kg, the MRL values for sheep muscle and liver tissue, respectively. The decision limits of the described determination method for mebendazole and its metabolites are defined as the mean values of the calculated concentrations by determining blank tissue samples spiked at the respective MRL values with the analytes plus 1.64 times the corresponding standard deviations. Since this validation parameter was calculated with values already obtained for the determination of the repeatability and the within-laboratory reproducibility, no supplementary analyses had to be performed.

To determine the detection limit (LOD), the criterion for calculation of the decision limit (CCα) was also used. The analyses were performed at the chosen IPL values of 10 and 50 µg/kg for muscle and liver tissue, respectively. The LOD of the presented analytical method for mebendazole and its metabolites was defined as the mean values of the calculated concentrations by determining spiked muscle and liver tissue samples plus 1.64 times the corresponding standard deviations. No supplementary analyses had to be performed. This validation parameter was calculated with already obtained values of calibration curves made for the determination of some other validation parameters.

To calculate the limit of quantification (LOQ), the criterion for determination of the detection capability (CCβ) was used. The LOQ values were calculated as the respective LOD values plus 1.64 times the corresponding standard deviations of the measured concentrations by analysis of 20 blank muscle and tissue samples spiked at the respective LOD values with the analytes.

6.3 Results and discussion

6.3.1 Mass spectrometric detection

Deuterated analytical standards of mebendazole and its metabolites are not available. Flubendazole was used as internal standard. FL is another benzimidazole substance which is very similar to mebendazole and which is only licensed for use in poultry species and in pig, but not for sheep. The described methods are only suitable for determination of residues in sheep tissues, which in practice are very unlikely to contain flubendazole residues. Furthermore, the MS/MS detection technique, based on the structure related principle, is a very powerful tool with a high separation capacity, a high specificity and a high sensitivity. Residues of flubendazole in unknown incurred samples will be detected immediately and can be compensated for.

The identification and the determination of the examined anthelmintic compounds were performed by MRM. Two transitions from the molecular precursor ion $(M+H)^+$ to the two most abundant product ions were measured. For the confirmation of the analytes involved in this study and belonging to substances of group B, veterinary drugs and contaminants of Annex I of Council Directive 96/23/EC, at least three identification points (IPs) have to be obtained when MS detection is applied.[15] With the two involved MRM transitions, four IPs were obtained because the precursor ion accounts for 1 IP and its transition products earns 1.5 IPs.

The mass spectrometer was tuned by infusion of individual standard solutions of each compound at a concentration of 1 µg/ml. The monitored cations and the MS operating parameters optimised for the procedure for liver as well as for muscle tissue, are given in Table 6.1. The indicated ions are protonated. The specific mass spectra of each compound are presented in Figure 6.2. The full scan mass spectrum (a) shows the precursor (parent) ion. The product ions obtained by fragmentation of the precursor ion are presented in the MS/MS scan (b).

An explanation for the fragmentation pathway of the precursor ions into the various product ions is given. The loss of $–OCH_3$ from the analyte molecules gives rise to the fragments with *m/z* 264.4, 266.5 and 282.5 for ME, RME and the IS, respectively. The fragment with *m/z* 105.0 for ME and HME originates from the loss of $C_6H_5CO–$. The fragment with *m/z* 95.1 from HME is obtained by loss of $C_6H_5CO–$ and of $–NHCNH_2$. The fragment with *m/z* 160.3 from RME can be explained by consecutive splitting off the fragments $C_6H_5COH–$ and $–OCH_3$. The fragments with *m/z* 191.3 and 159.2 for OF originates from the consecutive losses of $C_6H_5SO–$ and of both

$C_6H_5SO–$ and $–OCH_3$, respectively. Fragmentation of the IS into $–COC_6H_4F$ results in the remaining fragment with *m/z* 123.1.

Table 6.1 Summary of diagnostic ions and MS operating parameters

Analyte	Precursor ion *(m/z)*	Product ions *(m/z)*	Cone voltage *(V)*	Collision energy *(eV)*
ME	296.4^+	264.4^{+a}, 105.0^+	$35^b/35^c$	$25^b/30^c$
HME	238.3^+	105.0^{+a}, 95.1^+	$45^b/48^c$	$30^b/33^c$
RME	298.5^+	266.5^{+a}, 160.3^+	$40^b/40^c$	$25^b/34^c$
OF	316.5^+	191.3^{+a}, 159.2^+	$35^b/40^c$	$25^b/22^c$
FL (IS)	314.5^+	282.5^{+a}, 123.1^+	$40^b/40^c$	$23^b/29^c$

[a] most abundant fragment ion [b] liver tissue procedure [c] muscle tissue procedure

OF: oxfendazole FL (IS): flubendazole (internal standard)

ME: mebendazole HME and RME: hydrolysed and reduced metabolites of mebendazole

6.3.2 Liquid chromatographic-mass spectrometric analysis

Relatively simple, sensitive and reliable confirmatory assays using LC-MS/MS for the quantitative determination of residues of mebendazole and its metabolites in sheep liver, muscle, kidney and back fat tissues were developed. The presented methods were based on a formerly described method for the residues of flubendazole and relevant metabolites.[16]

During the sample preparation of liver tissue, homogenisation was not necessary because of its relatively soft matrix. The sample preparation procedure for the other sheep tissues was based on the procedure used for the liver tissue. Previous recovery tests indicated that the relatively firm muscle tissue had to be homogenised in water to obtain a better extraction of the analytes. This sample preparation is based on the method described by Balizs.[13] Preliminary laboratory tests indicated that the optimised sample preparation for muscle tissue could also be used for kidney tissue. With respect to the back fat tissue, the efficiency of the analyte extraction was improved when the moistened sample was heated before the first extraction and when the centrifugate was heated before the second extraction. For this tissue, the centrifugation time also had to be increased from 5 to 10 min to reach a sharp separation in the centrifuge tube.

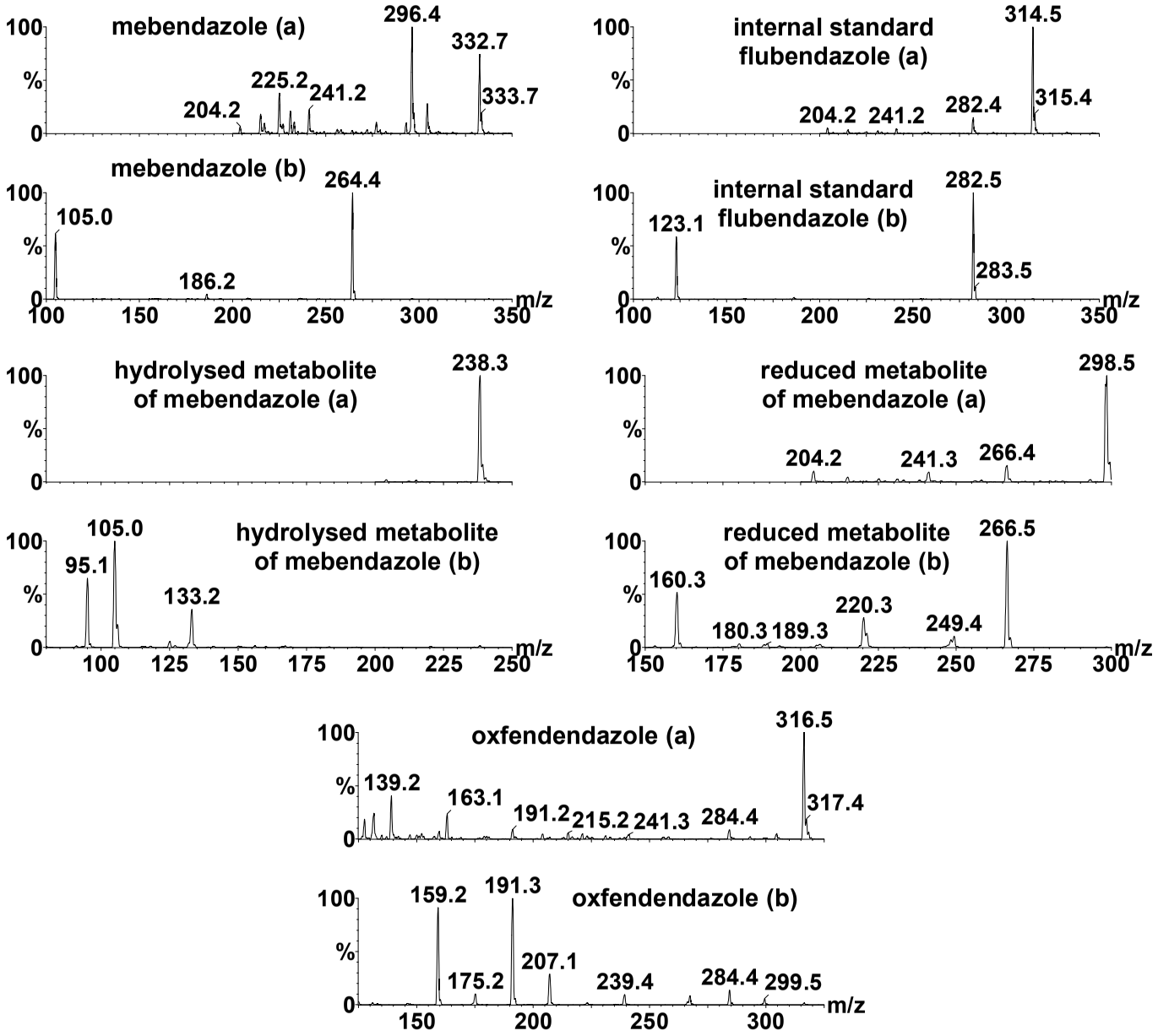


Figure 6.2 Full scan MS (a) and product scan MS/MS (b) spectra of the examined anthelmintic compounds

During sample preparation of muscle, kidney and back fat tissues, a multitube vacuum evaporator was used for the concentration of the sample extracts. This allowed a better reproducibility and increased capacity as compared to a separate evaporation as used in the previous research studies for sheep liver tissue and for flubendazole-derived residues in poultry tissues and eggs.[16] The sample preparation is similar to that reported by Balizs.[13]

Problems with unstability of the ammonium acetate buffer obtained in the previous method[16] were avoided by changing the mobile phase. A new LC separation method was developed. A very good LC-MS/MS separation was obtained with a mobile phase consisting of acetonitrile and water containing 0.1% formic acid to increase the ionisation intensity. A specifically developed gradient was applied. The chromatographic separation procedure developed previously for mebendazole-derived residues in sheep liver tissue was satisfactory and an identical mobile phase and gradiënt programme were used during the study of the other sheep matrices. To increase the detection limit, the cleaned sample volume injected into the LC-MS/MS apparatus was increased to 30 µl.

The described method was validated for liver tissue in the limited concentration range of 50 to 400 µg/kg. However, the presented LC-MS/MS method was able to detect mebendazole and its metabolites at residue concentrations even lower than 1 µg/kg.

The described LC-MS/MS method was validated for muscle tissue at a IPL of 10 µg/kg. However, as can be estimated from the chromatogram in Figure 6.3, the presented method is able to detect mebendazole-derived residues at concentrations even lower than 2 µg/kg. The analyte response intensity is also depending on the cleanliness of the sampling cone and of the baffle plate of the mass spectrometer. Figure 6.3 presents an example of a chromatogram of a blank muscle tissue sample spiked with a mixture of ME, RME and HME at a concentration of 10 µg/kg for each compound and with the IS at 50 µg/kg. Each measured transition from precursor ion to product ion delivers one peak so that two peaks per analyte are shown in the figure. The compounds were already eluted after 6.1 min. The column had to be conditioned further and the total run time was 25 min. HME and RME eluted approximately at the same retention time and were not separated chromatographically. However, because of the powerful separation capacity of the mass spectrometer in MRM mode based on the molecular mass, LC separation is not required and the specificity of LC-MS/MS analysis is very high (Figure 6.4). Thirty eight prepared samples could be determined per night. The sample preparation was more time consuming. Sixteen samples could be prepared during a 7-h working day. Because the stability of the sample extracts, the vials were collected and stored refrigerated until determination.

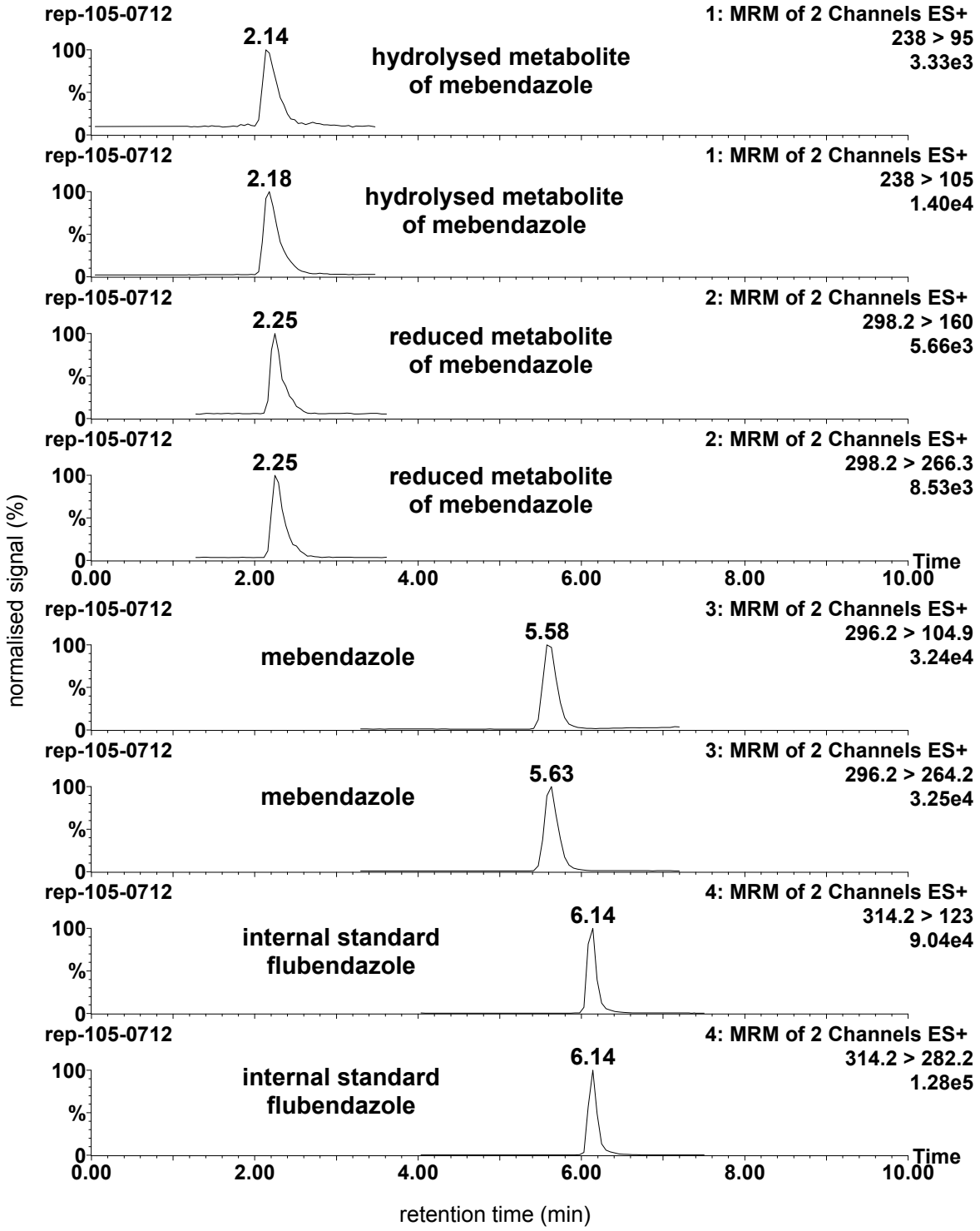


Figure 6.3 Chromatogram of a blank muscle tissue sample spiked with a mixture of mebendazole and its metabolites (10 µg/kg) and with the IS (50 µg/kg)

6.3.3 Validation study

6.3.3.1 Liquid chromatographic-mass spectrometric analysis

The quality of the chromatographic separation and of the mass spectrometric detection was examined by evaluating the specific criteria on RRT and RI for all the samples analysed during the validation study for liver and muscle tissues. For all the samples used to calculate the validation parameters, the values for RRT and for RI were conform with the European requirements.[14] Using liver tissue as an example, the values for a sample spiked at 50 µg/kg and for a matrix calibration sample spiked at 50 µg/kg to evaluate the repeatability, are given. The values for the RRT in the spiked samples were 0.87, 0.41 and 0.40 for ME, HME and RME, respectively. These values were within the tolerance ranges of ±2.5% obtained with the values in the calibration samples namely 0.88±0.02, 0.42±0.01 and 0.41±0.01 for ME, HME and RME, respectively. The values for the RI in the spiked samples were 23, 14, 5 and 11% for ME, HME, RME and IS, respectively. These values fulfilled the allowed ranges obtained with the values of the calibration samples. These ranges were 23±25%, 14±30%, 5±50% and 11±30% for ME, HME, RME and IS, respectively.

As an example for muscle tissue, the values of a sample spiked at 10 µg/kg and for a matrix calibration sample spiked at 5 µg/kg to evaluate the within-laboratory reproducibility, are reported. The values for the RRT in the spiked sample were 0.88, 0.36 and 0.37 for ME, HME and RME, respectively. These values were within the tolerance ranges of ±2.5% obtained with the values in the calibration samples, namely 0.88±0.02, 0.35±0.01 and 0.36±0.01 for ME, HME and RME, respectively. The RI values in the spiked sample were 47, 22, 41 and 44% for MEB, HME, RME and IS, respectively. These values were very close to those obtained for the calibration sample and are between the allowed ranges. These ranges were 47±25%, 22±25%, 42±25% and 44±25% for ME, HME, RME and IS, respectively.

6.3.3.2 Stability

Using standard analyte solutions, the linearity of the LC-MS/MS response was proven with five calibration points in the concentration range of 0 to 500 µg/l. The regression data and measured concentrations for the refrigerated standard solution were compared with those obtained for freshly made solutions at two week intervals. With the results obtained, it can be concluded that the stability of standard solutions of mebendazole and its metabolites was proven during the test period of two months. The values measured in the reference standard solution were very close to those of the freshly made standard solutions.

6.3.3.3 Specificity

The specificity of the described analytical assays for sheep liver and muscle tissues was proven. In none of the 20 blank liver and muscle tissue samples from different origin, endogenous interfering matrix material above the detection limit (S/N ≥ 3/1) could be detected.

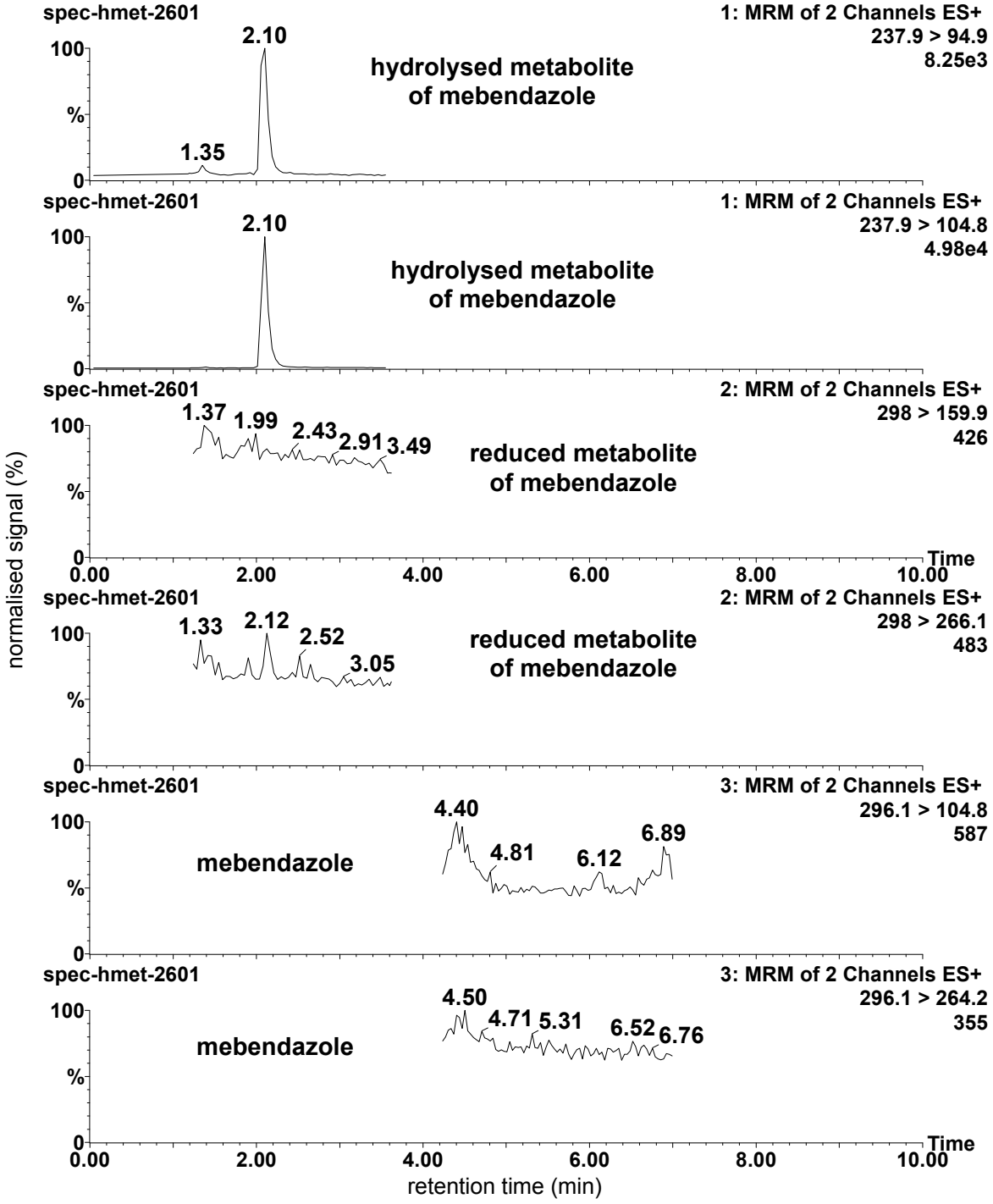


Figure 6.4 Chromatogram of a blank liver tissue sample spiked with the hydrolysed metabolite of mebendazole at a concentration of 50 µg/kg

False positive or false negative results might be generated for mebendazole and its metabolites due to hindered identification. Therefore, oxfendazole, a substance with a benzimidazole structure very similar to the target compounds, was used. Blank samples were spiked separately with oxfendazole, the hydrolysed and the reduced metabolite of mebendazole and were analysed by the multiresidue method. OF eluted at 2.8 min. No false positive or false negative results were obtained. An example of the high specificity of LC-MS/MS analysis operating in MRM mode is demonstrated in Figure 6.4. Figure 6.4 presents a chromatogram of a blank liver tissue sample spiked with HME at 50 µg/kg and analysed with the multiresidue method for ME, RME and HME. Unless the fact that HME eluted at approximately the same retention time (2.1 min) as RME (2.2 min), no interference in the retention window of RME was measured. As expected, only noise could be detected in the retention window of ME. Because of the structure related detection, MS/MS detection is a very powerful tool with a high separation capacity and a high specificity. Also, the quantification of mebendazole and its metabolites was not influenced by the presence of oxfendazole in the samples. The differences in the peak areas between the samples with and without oxfendazole were very small and within the normal ranges for repeated analyses.

6.3.3.4 Recovery

Table 6.2 reports the mean recovery values obtained for blank liver and muscle tissue samples spiked with a mixture of the analytes. The recovery efficiency for the analytes in the sheep tissues was high. The recovery was somewhat higher for muscle tissue. The overall recovery values for ME, HME and RME in liver and muscle tissues were 92, 94 and 91%, and 93, 96 and 96%, respectively. These values were within the permitted range of 80 to 110%. The variabilities on these recovery values were low and the relative standard deviations (s_r values) ranged respectively from 8.3 to 10.9% and from 5.0 to 6.9% for the three substances in liver and muscle tissues at the different fortified concentrations. The variability was in agreement with the criteria on the Horwitz equation ($s_r = 2^{(1-0.5\ \log C)}$, where C is the concentration expressed as a power of 10 (e.g. 1 µg/kg = 10^{-9})). The obtained s_r values for liver tissue were lower than the maximum allowed values of 16.7, 15.1 and 12.2%, respectively for the residue concentrations of 50, 100 and 400 µg/kg. The obtained s_r values for muscle tissue were much lower than the maximum allowed values of 21.3, 19.2 and 16.3%, respectively for residue concentrations of 10, 20 and 60 µg/kg.

The reported recovery values were much higher than those obtained by Balizs.[13] This author obtained a recovery value of only 50% for ME in pig muscle. The recovery was probably negatively influenced by the supplementary sample clean-up. Hajee and Haagsma[10] reported comparable recovery efficiencies for RME (92%), slighty lower values for ME (90%) and much lower values for HME (74%) in eel muscle tissue. Some 20 to 30% (obtained recovery values

Table 6.2 Recovery values for the determination of mebendazole and its hydrolysed and reduced metabolites in spiked sheep liver and muscle tissues [mean recovery value (%) (s_r (%)), n = 6]

Analyte	Liver tissue				Muscle tissue			
	50 (µg/kg)	100 (µg/kg)	400 (µg/kg)	Overall	10 (µg/kg)	20 (µg/kg)	60 (µg/kg)	Overall
ME	99.5 (7.6)	90.8 (11.9)	85.7 (8.2)	92.0 (10.9)	95.5 (5.0)	92.1 (3.8)	92.5 (6.0)	93.4 (5.0)
HME	97.5 (6.8)	97.7 (9.7)	87.0 (5.4)	94.1 (9.0)	94.8 (6.5)	93.0 (5.8)	99.0 (7.8)	95.6 (6.9)
RME	92.7 (6.9)	92.8 (11.2)	88.0 (5.7)	91.2 (8.3)	95.4 (6.9)	95.8 (3.0)	95.2 (6.6)	95.5 (5.5)

ME: mebendazole HME and RME: hydrolysed and reduced metabolites of mebendazole

between 62 and 83%) lower values were reported by Steenbaar *et al*.[9] for ME for the same muscle tissue. However, probably because of the intensive clean-up procedure, the variability was very low and the s_r values were not more than 5%. Excellent analyte extraction efficiency (100 to 101%) was obtained by Al-Kurdi *et al*.[11] for ME and HME in pharmaceutical dosage forms.

6.3.3.5 Precision

The precision of the method was evaluated by means of the repeatability and the within-laboratory reproducibility. The precision of the method is described as the variability on measured mean residue concentrations of spiked samples and is expressed as s_r values. The repeatability results are summarised in Table 6.3. The repeatability or within-day precision for analyses performed under repeatability conditions would have to be between half and two-thirds of the values according to the Horwitz equation.[15] These allowed maximum values for the variability are 16.7, 15.1 and 12.2% for spiked concentrations of 50, 100 and 400 µg/kg in liver tissue, respectively. The repeatability of the determination of mebendazole-derived residues in liver tissue was good and ranged from 5.3 to 10.0%. The obtained mean concentrations were very close to the fortified concentrations. The allowed ranges for these mean values are 40 to 55, 80 to 110 and 320 to 440 µg/kg for spiked concentrations of 50, 100 and 400 µg/kg, respectively. For muscle tissue, the allowed maximum s_r values are 21.3, 19.2 and 16.3% for residue concentrations of 10, 20 and 60 µg/kg, respectively. A small deviation on the criteria for RME was observed. The obtained variability for the determination at a concentration level of 20 µg/kg was 23.0% and somewhat higher than the upper limit value of 19.2%. The calculated mean values were close to the spiked concentrations. The permitted ranges for these mean values were 8 to 11, 16 to 22 and 48 to 66 µg/kg for fortified concentrations of 10, 20 and 60 µg/kg, respectively. The method published by Hajee and Haagsma[10] was more precise, giving a highest within-day repeatability of 4.8% in eel muscle tissue. Al-Kurdi[11] reported a repeatability of 1.4% for the determination of ME in a synthetic matrix used for suspension formulation.

The analyses for the determination of the within-laboratory reproducibility were performed on different days, with different standard solutions and by two analysts. The results are presented in Table 6.4. The method showed an acceptable within-laboratory reproducibility. The variability on the obtained mean values is allowed to be higher than for the repeatability.[15] The values should be lower than the values of the Horwitz equation which are 25.1, 22.6 and 18.3% for the spiked concentrations of 50, 100 and 400 µg/kg in liver tissue, respectively. The variability shown on these mean values was relatively high and the s_r values ranged from 2.9 to 17.8%. The allowed ranges for the mean values obtained for determination of the within-laboratory reproducibility are equal as those for the repeatability and were fulfilled for the method used for

Table 6.3 Repeatability results for the determination of mebendazole and its hydrolysed and reduced metabolites in spiked sheep liver and muscle tissues [s_r (%) (mean measured concentration (µg/kg)), n = 6]

Analyte	Liver tissue			Muscle tissue		
	50 (µg/kg)	100 (µg/kg)	400 (µg/kg)	10 (µg/kg)	20 (µg/kg)	60 (µg/kg)
ME	5.3 (48.5)	6.8 (91.7)	6.5 (406.1)	4.6 (10.4)	16.6 (20.5)	11.4 (63.8)
HME	10.0 (45.5)	5.7 (89.3)	8.6 (394.3)	8.2 (11.3)	8.6 (16.6)	7.0 (52.9)
RME	5.6 (46.4)	5.8 (93.6)	8.8 (413.1)	9.9 (11.8)	23.0 (20.8)	11.3 (59.7)

ME: mebendazole HME and RME: hydrolysed and reduced metabolites of mebendazole

Table 6.4 Within-laboratory reproducibility results for the determination of mebendazole and its hydrolysed and reduced metabolites in fortified sheep liver and muscle tissues [s_r (%) (mean measured concentration (µg/kg)), n = 6]

Analyte	Liver tissue			Muscle tissue		
	50 (µg/kg)	100 (µg/kg)	400 (µg/kg)	10 (µg/kg)	20 (µg/kg)	60 (µg/kg)
ME	7.4 (53.7)	9.0 (99.2)	2.9 (418.4)	9.6 (9.9)	21.1 (21.0)	6.6 (58.7)
HME	16.2 (53.9)	15.7 (105.0)	11.0 (433.3)	6.2 (11.0)	21.5 (21.5)	16.3 (65.1)
RME	16.2 (52.3)	17.6 (105.8)	17.8 (438.0)	9.5 (10.1)	12.7 (21.1)	11.5 (64.5)

ME: mebendazole HME and RME: hydrolysed and reduced metabolites of mebendazole

liver and muscle tissue. The variability values on the measured mean concentrations in the spiked muscle tissue samples have to be lower than 32, 28.8 and 24.4% for concentrations of 10, 20 and 60 µg/kg, respectively. The obtained within-laboratory reproducibility ranged from 6.2 to 21.5% for HME obtained at concentrations of 10 and 20 µg/kg, respectively.

6.3.3.6 Analytical limits

The decision limits were calculated with already measured residue concentrations for blank muscle and liver tissue samples spiked at 60 and 400 µg/kg, respectively, for the determination of the validation parameters repeatability and the within-laboratory reproducibility. The mean values plus 1.64 times the corresponding standard deviations equals the decision limits. These CCα values of the analytical method for mebendazole, the hydrolysed and the reduced metabolites in muscle and liver tissues were 72.5, 69.2 and 69.8 µg/kg, and 445.9, 466.5 and 463.4 µg/kg, respectively.

The detection limits were calculated with the criterion for determination of CCα with data already obtained during the validation study for blank samples spiked at the respective IPL concentration for muscle and liver tissue of 10 and 50 µg/kg. The LOD values calculated as mean measured values plus 1.64 times the corresponding standard deviations for the determination of mebendazole, the hydrolysed and the reduced metabolites in muscle tissue were 11.0, 12.4 and 13.2 µg/kg, respectively. The LODs of the method for determination of ME, HME and RME in sheep liver tissue were 56.6, 61.8 and 64.2 µg/kg.

The limits of quantification were obtained by analysing 20 blank muscle and liver tissue samples spiked at the respective LOD values for the three analytes. The respective LOD value plus 1.64 times the measured corresponding standard deviation of the residue determinations equals the LOQ. The obtained LOQ values for the determination method for ME, HME and RME in muscle and liver tissues were 13.1, 15.2 and 15.8 µg/kg, and 60.0, 86.1 and 90.9 µg/kg, respectively.

6.4 Conclusions

The LC-MS/MS assays described in this chapter provide relatively simple, fast, sensitive, very specific and reliable procedures for the quantitative determination of residues of mebendazole, its hydrolysed and reduced metabolites in sheep muscle, liver, kidney and back fat tissues. The confirmatory methods for muscle and liver tissues are validated in accordance with the main lines of the revised EC requirements for detecting residues of veterinary drug substances in animal products. Validation at 0.5, 1.0 and 1.5 MRL was not applicable because the EC established the MRL values for the sum of ME+HME+RME. The rules for validation of methods needed for depletion studies to be performed by veterinary drugs companies, were taken into account. The lowest possible detection limits were not studied. All examined validation

parameters in liver tissue fulfilled the requirements with respect to the tolerance levels. Except for one small deviation for the repeatabilty of the RME determination, the examined validation parameters fulfilled the requirements for muscle tissue.

The presented determination methods were used to analyse sheep tissues for mebendazole-derived residues in a depletion study. This depletion study is reported in Chapter 10.

6.5 Acknowledgments

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PART II

DISPOSITION OF RESIDUES OF SOME VETERINARY DRUGS IN MILK, EGGS AND EDIBLE TISSUES OF POULTRY SPECIES AND SHEEP

CHAPTER 7

Depletion of tetracyclines in eggs and broiler muscle tissue

Relevant publication:

De Ruyck H., De Ridder H., Van Renterghem R. and Van Wambeke F.
Validation of HPLC method of analysis of tetracycline residues in eggs and broiler meat and its application to a feeding trial.
Food Additives and Contaminants, 1999, 16, 47-56

Abstract:

Residues of tetracycline antibiotics in eggs and broiler muscle tissue were determined during and after oral administration of medicated feed. Medicated feed containing 840 mg of oxytetracycline per kg was provided to laying hens for seven successive days. Two days after the administration was ceased, the mean oxytetracycline residue value in eggs was already lower than the MRL level (200 μg/kg) and reached 118 μg/kg. Broiler chickens were supplied with medicated feed containing 480 mg of tetracycline per kg for seven successive days. Four days after the administration was stopped, the mean tetracycline residue value in breast muscle tissue declined below the MRL value and was 86 μg/kg.

Keywords: tetracycline residues, eggs, chicken muscle tissue, HPLC

7.1 Introduction

7.1.1 Usage of tetracyclines for laying hens and broilers

Tetracycline antibiotics (TCA's) are broad-spectrum antibacterial compounds, widely used in the prevention and treatment of diseases of food-producing animals. The commonly used TCA drugs in veterinary medicine are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC). Among the antibiotics, TCA's are most used in poultry husbandry because of their broad antibacterial spectra.[1] However, since the incidence of resistant strains is very high, these antibiotics are prescribed less than in the past. Administration of tetracycline antibiotics results in healthier and faster-growing animals.[2] TCA's have been used to minimise poultry diseases such as chronic respiratory disease, blue comb and infectious sinusitis. Control of infectious synovitus, infectious coryza and fowl typhoid are also described.[3] TCA's will also act against some pathogenic agents unaffected by other antibiotics e.g. *rickettsiae* and certain large viruses belonging to the psittacosis group in animals.[4] Kan[5] reported that due to there cost-effectiveness and in spite of the increasing resistance, these drugs are still widely used in the poultry industry mostly as prophylactic agents for broiler chickens to prevent general infections and, in particular, respiratory diseases. The infection pressure on laying hens is much lower and for these poultry species, TCA's are only useful for therapeutic purposes.

OTC is the commonest intramuscularly administered TCA.[4] CTC should not be administered intramuscularly because of severe tissue irritation and destruction. DC is more completely absorbed and is more slowly excreted than most other TCA's. It has longer duration of action with attendant advantages of a smaller required dose and a less frequently administered maintenance dose. DC is useful as therapeutic agent in psittacine birds because of the favorable pharmacokinetic parameters to achieve high systemic concentration for longer periods than the more traditional CTC and OTC. Croubels *et al*.[6] described DC as a broad-spectrum antibiotic which is widely used in the treatment of respiratory tract infections in various species. DC is widely used because of its advantageous pharmacokinetic properties.[4,7] These include a better bioavailability from the gastrointestinal tract, a stronger lipophilic character and a higher affinity for tissues. However, the higher lipid solubility of DC may result in higher and more persistent residue amounts in edible tissues.[3,7]

7.1.2 Metabolism of tetracyclines

Rational use of an antimicrobial drug in the treatment of an infectious disease, caused by a bacterial agent, requires knowledge not only of the susceptibility of the pathogen to the drug but also of its concentration in the body fluids of the treated animal.[8] Tetracycline antibiotics are metabolised to various degrees.[4] The most frequently identified substance in urine, faeces and tissues is the parent TCA. As much as 30% will be excreted unchanged in the faeces. TCA's

are reversibly bound to plasma proteins and are widely distributed in the body. They are removed from the blood by the liver and high concentrations can be found in the bile.

Most of the data documented in the literature deal with the epimerisation of TCA's. Several publications describe metabolites obtained after isomerisation of the parent molecule. As example for all TCA's, the chemical structure and full scan MS spectra of the 4-epimer and the 6-isomer of CTC are presented in Figure 7.1.[9] The full scan MS spectra were obtained by

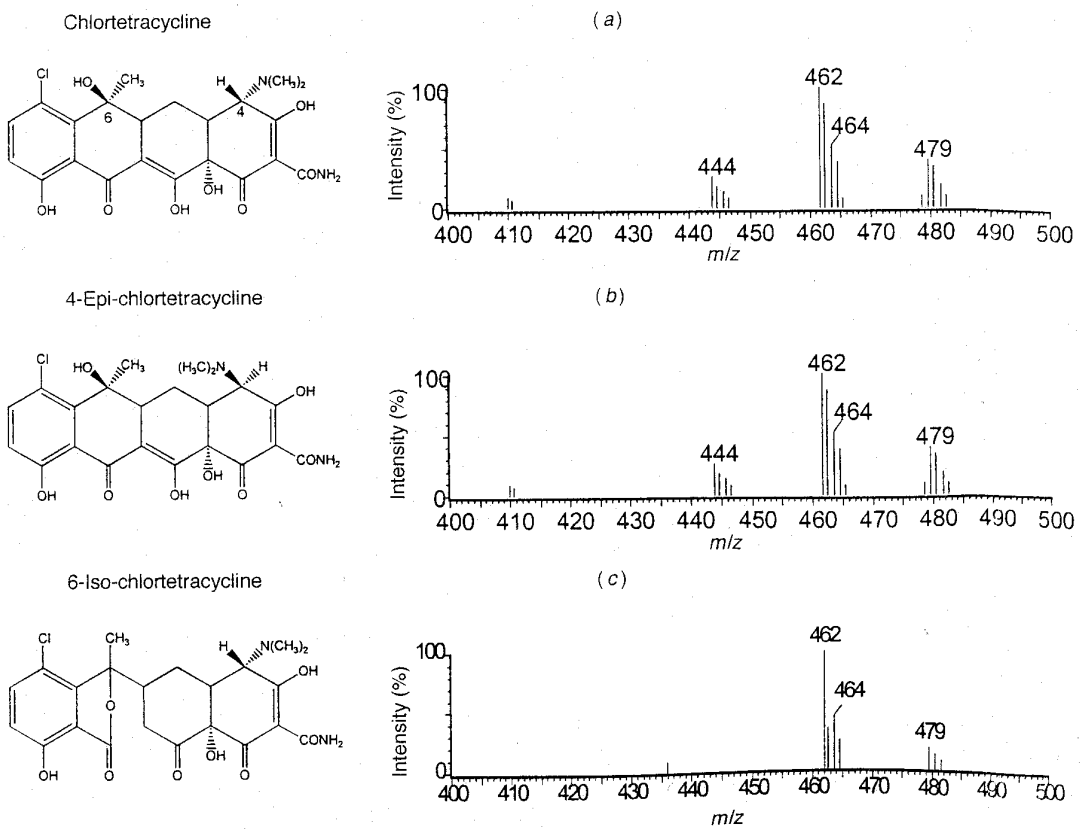


Figure 7.1 Chemical structures and full scan MS spectra of chlortetracycline (*a*) 4-epi-chlortetracycline (*b*) and 6-iso-chlortetracycline (*c*)[9]

atmospheric pressure chemical ionisation. CTC, 4-epi-chlortetracycline (4-epi-CTC) and 6-isochlortetracycline (6-ICTC) show ions with the same *m/z*, but these analytes could be separated chromatographically.

After administration of TC and CTC to animals, these parent substances and their metabolites, 4-epi-tetracycline (4-epi-TC) and 4-epi-chlortetracycline (4-epi-CTC) are excreted.[10] Isochlortetracycline (ICTC) and 4-epi-isochlortetracycline (4-epi-ICTC) are not known as major mammalian CTC metabolites, but CTC is known to be degraded to its isomers under alkaline conditions. This might be the reason for the degradation of CTC to isochlortetracycline and its 4-epimer in slurry. The degradation products of CTC have no antimicrobial activity.

Zurhelle *et al*.[11] described the metabolites of OTC, TC and CTC and their distribution in egg white, egg yolk and hen plasma in feeding studies with either OTC, TC or CTC. These authors concluded that any residue besides the parent compounds in egg and plasma samples must have its origin from the in vivo metabolisation. The epimerisation rates of OTC, TC and CTC were constant during the whole medication period. The residue concentrations for TCA's in egg yolk are higher than in egg white. 4-epi-tetracycline (4-epi-TC) and N-demethyltetracycline were detected as metabolites of TC in egg yolk and plasma. Metabolism of OTC resulted in 4-epi-oxytetracycline and N-demethyloxytetracycline in these matrices. Twenty one to 24% of the OTC was epimerised in egg yolk. The epimerisation rate for TC was 41-50% in egg yolk and 22-26% in egg white. N-demethylation rates for OTC and TC were 7-8 and 18-21%, respectively, in egg yolk. 6-ICTC, 4-epi-CTC, 4-epi-ICTC and N-demethylisochlortetracycline were detected as metabolites of CTC. The obtained ratios of epimerisation and N-demethylation in eggs did not change during the medication period. The epimerisation rates for ICTC (55-57%) and CTC (41-45%) in yolk were constant during the medication period. The epimerisation rate for ICTC in egg white was 22%.

Croubels *et al*.[7] described a liquid chromatographic separation method for DC and 4-epi-doxycycline (4-epi-DC) in a tissue depletion study for DC in turkeys. The metabolite 4-epi-DC is an antibacterially inactive epimer of DC which is spontaneously formed in an acidic medium at a pH between 2 and 6. These authors concluded that probably the degradation compound was partially formed in vivo and to a lesser extent during sample preparation. However, additional studies are necessary to confirm this hypothesis. Moreover, a 4-epi-DC peak was detected in both incurred turkey liver and muscle tissues, though to a lesser extent in the latter.

Kennedy *et al*.[9] reported that the reversible epimerisation of CTC to 4-epi-CTC also can occur under mild acidic conditions. These authors also studied the metabolism of chlortetracycline in eggs of laying hens fed with a therapeutic dose and with a subtherapeutic dose (feed contamination level). They reported that residues of 6-ICTC plus 4-epi-6-isochlortetracycline rapidly accumulate to concentrations equivalent to more than twice the current MRL value of 200 µg/kg. Residues of these metabolites were also detected after treatment with the low-level dose. It was unlikely to obtain mean residue concentrations in excess of 200 µg/kg. These authors could not explane whether these metabolite compounds are formed as a result of either metabolism or of chemical degradation.

Capolongo *et al*.[12] reported a depletion study of turkeys treated with OTC via drinking water. At the first day after the end of treatment, 4-epi-OTC was detected at very low concentrations (< 26 µg/kg) in muscle tissue, in liver tissue after 1 and 3 days of the withdrawal period and in kidney tissue at all sampling times until 5 days of the withdrawal period.

A residue study of DC and 4-epi-DC in pigs medicated via drinking water was reported by Croubels *et al*.[6] Comparable residue concentrations for DC and its metabolite 4-epi-DC were

measured in kidney 3 days after cessation of the medication. However, the detected response in liver tissue was much higher for the metabolite.

The marker residues of the MRLs for TC, OTC and CTC are set as the sum of the parent compound plus its 4-epi derivative.

7.1.3 Distribution of tetracycline residues in eggs

A large number of studies on drug residues in eggs and edible poultry tissues have been published.[13] Since the experimental designs widely varied, it is difficult to compare the obtained results. In general, the results show that the clearance times vary from a few days in edible tissues to 1 or 2 months in eggs. There is a close correlation between the physiological development of eggs and the length of time in which drug residues are found in egg white and yolk. Recent results indicate that the developing egg yolk serves as an important storage depot for chemical residues.

Kan and Petz[14] reported the distribution of residues of veterinary drugs between yolk and white in eggs in detail. When drug substances reach the bloodstream, they are distributed over the whole body. In the laying hen, this includes the ovary with growing follicles and the oviduct, where the egg white is formed and secreted. The amount of residues of drug substances or their metabolites in each tissue depends on their physicochemical characteristics. Due to the physiological processes of egg formation, the pharmacokinetics of drug residues in egg yolk and white show the following common features. Drug residues first occur in egg white, at least if the drug is distributed towards that compartment. Residues in egg white are a reflection of the plasma concentrations and will therefore show a constant concentration over time when plasma levels are constant. The time needed to achieve a constant concentration in egg white is generally 2-3 days. Residues in yolk reflect the plasma concentrations during the 10 days of their rapid growth. Residues of drugs in yolk generally require exposure for 8-10 days to reach a constant concentration. Disappearance of drugs from white and yolk heavily depends on the plasma concentrations of the drug tested. Drugs that rapidly clear from the body also disappear from egg white in 2-3 days after cessation of exposure. Disappearance of drugs from yolk generally takes 10 days.

Traditionally, the rather lipid-soluble drugs are expected to yield residues only in the fat-rich yolk. However, the rather lipid-soluble DC drug showed higher residues in white than in yolk during long-term administration. Yoshimura *et al*.[3] also made this conclusion. In spite of similarities in structure between DC and OTC, DC was deposited in higher concentrations and lasted in eggs for a longer period. This characteristic was considered to be due to its greater lipophilicity, closely correlated with oral absorption and tissue penetration.

Several research workers reported that residue concentrations of TC, OTC or CTC in egg yolk are higher than in egg white.[1,11,15,16]

Donoghue[17] described mechanisms regulating drug and pesticide residue uptake by egg yolk and developed predictive models. This author reviewed the physiology of egg yolk formation and its relation to residue incursion. Models were developed to understand and to quantify residue patterns. Magnetic resonance imaging was used to detect residue incorporation into eggs. The results obtained support the hypothesis that residues are sequestered in developing pre-ovulatory egg yolks and that they can be contained in laid eggs for weeks after the withdrawal period of the drug. The model is very useful for predicting residues in laid eggs following a hen's exposure to chemicals with short half-lives, such as many veterinary or production drugs used by the poultry industry.

Another publication of Donoghue and Myers[18] was conducted to visualise incorporation and potential compartmentalisation of drug residues in developing egg yolks. Following dosing of hens, high-resolution magnetic resonance images of drug residues in eggs indicate that drugs can be incorporated and compartmentalised into ring structures within individual developing egg yolks. The significant human food safety implications are that sequestered drug residues may be stored and released later to contaminate eggs for days to weeks after dosing.

Donoghue and Hairston[19] studied the OTC transfer into egg yolk or albumen (egg white protein) of laying hens fed with the approved doses of OTC (50 and 200 g OTC per 1000 kg) for breeder hens and broilers. Residues of OTC were not detectable during the predosing, dosing or withdrawal period in egg yolks. OTC residues were detectable (100 to 120 µg/kg) however, in egg albumen during the fifth day of treatment and the first day of the withdrawal period. These data indicate that illegal or unintentional dosage of laying hens with feed medicated at doses allowed for breeder hens or broilers should not necessarily cause detectable residue concentrations of OTC in eggs.

7.1.4 Objectives

The widespread use of tetracycline antibiotic veterinary drugs in the poultry industry may also cause residues in eggs, muscle tissue and other foodstuffs of poultry origin. Some drug residues or its metabolites may be deposited in eggs, as well as in muscle tissue.[16] After metabolism, some antibiotics are excreted while others are stored in the tissues. The residue concentrations could be particularly high in foodstuffs if the recommended withdrawal times are not respected. The MRL values set by the EC[20] for TC, OTC and CTC in eggs are 200 µg/kg, measured for the marker residue consisting of the sum of parent drug plus its 4-epimer metabolite compound. DC is not allowed by the EU for laying hens. The MRLs in muscle tissue of broiler chickens for TC, OTC, CTC and DC are 100 µg/kg. The marker residue for TC, OTC, CTC also is the sum of the parent plus its 4-epimer.

Meridith *et al*.[2] studied the depletion of chlortetracycline in eggs after oral treatment of laying hens with medicated feed. Roudaut *et al*.[15] studied the excretion of tetracycline and chlortetracycline in eggs after oral medication of laying hens either through drinking water and

feed. Yoshimura *et al*.[3] described the depletion of doxycycline in eggs after medication of laying hens via drinking water for seven consecutive days. Kennedy *et al*.[9] reported the metabolism of chlortetracycline with drug accumulation and excretion results in eggs after oral treatment of laying hens with medicated feed. Zurhelle *et al*.[11] performed depletion studies of tetracycline and doxycycline after oral treatment of laying hens via medicated feed. The parent drug residues and the metabolites were measured in eggs and plasma.

To our knowledge, available depletion data for oxytetracycline in eggs after oral administration with medicated feed are rather scarce or old.[1,2,3,11,16] Meridith *et al*.[2] administered oxytetracycline to laying hens with feed containing 1000 mg of OTC per kg feed mixture. However, the residue concentrations in the eggs were measured by a microbiogical inhibition method. The effects of egg cooking processes as poaching and scrambling on the drug residues in the eggs were observed. Roudaut *et al*.[1] described the kinetics of oxytetracycline elimination into eggs after oral administration through either drinking water or feed (300 and 600 mg of OTC per kg feed for seven successive days) and after intramuscular injections. However, the residues were also assayed by a microbiological agar diffusion method. Other residue studies of oxytetracycline in eggs after medication of laying hens via drinking water were reported by Yoshimura *et al*.[3] and Omija *et al*.[16] Also, these authors used microbiological inhibitor assays for their drug residue determinations. A more specific approach was applied by Zurhelle *et al*.[11] by using HPLC-UV for investigating the occurence and distribution of residues of oxytetracycline in egg white and egg yolk. These research workers treated laying hens orally with feed medicated with OTC at 750 and 3000 mg per kg. However, the study mainly focused on the metabolism and the occurence of metabolites of OTC in eggs. The depletion data were not reported in detail.

Meridith *et al*.[2] also reported chlortetracycline and oxytetracycline residues in liver, breast and thigh tissues of broiler chickens after oral administration of the drug substances via medicated feed. Black[21] studied the pharmacodynamics of oxytetracycline in broiler chickens. The birds were orally, intramuscularly or subcutaneously treated and the disposition of oxytetracycline in tissues was measured. Croubels *et al*.[7] determined the depletion of doxycycline in liver and muscle tissues of turkeys treated with medicated drinking water. The depletion of oxytetracycline in muscle, liver and kidney tissues of turkeys administered with OTC via medicated drinking water, was described by Capolongo *et al*.[12]

No recent depletion data about tetracycline in chicken muscle tissue could be found in the open literature. Anadon *et al*.[8] described the pharmacokinetics of tetracycline by means of plasma levels and biliary excretion in broiler chickens after intravenous administration.

The objective of this study was to follow the disposition and distribution of residues of oxytetracycline in eggs and of tetracycline in chicken breast muscle tissue during and after oral medication of laying hens and broiler chickens, respectively. The animal treatments were performed at the Department of Animal Nutrition and Husbandry, Section Small Stock Husbandry (DVV-CLO, Merelbeke, Belgium). The residue analyses were carried out at the

Department of Animal Product Quality and Transformation Technology (DVK-CLO, Melle, Belgium).

7.2 Materials and methods

7.2.1 Veterinary drug products

Virginiamycine was supplemented as feed antibiotic in the feed rations of laying hens and of broiler chickens by means of Stafac^R (Smith-Kline, Rixensart, Belgium). The broiler chickens were vaccinated against Newcastle disease with Hitchner spray (Intervet, Boxmeer, The Netherlands) and against infectious bronchitis with H120 spray also purchased from Intervet. The vaccination against Newcastle disease was repeated with La Sota (Clone 30) delivered by Intervet. The oxytetracycline medicated feed for the laying hens contained OTC-hydrochloride obtained from VMD (Arendonk, Belgium). The prescribed withdrawal time after treatment of laying hens with this veterinary drug product is 14 days. The tetracycline medicated feed for the broiler chickens was prepared with TC-hydrochloride received from Wolfs-Kela & Eurovet (Heusden-Zolder, Belgium). The recommended withdrawal time for broiler chickens medicated with this veterinary drug product is seven days.

7.2.2 Animal treatments

7.2.2.1 Laying hens

Twenty 64-week-old laying hens of the Isa Warren strain were used in the trials. Average egg production was about 75%. The hens were fed a mash ration containing 11.71 MJ AME_n/kg (apparent metabolisable energy, corrected for N-retention = 0) and 16.8% crude protein (CP). The diet contained 110 mg of Stafac^R per kg. Feed and drinking water were provided ad libitum. The hens were caged individually one week before the start of the medication. During this observation period, a few eggs were taken as blank control samples. Oxytetracycline was supplemented at a therapeutic level of 840 mg per kg diet. The medicated feed was supplied for seven consecutive days. The individual feed consumption was determined during this administration period. During the experimental period, eggs were collected twice a day at 8 a.m. and 3 p.m. The eggs were refrigerated until determination of the OTC residues. As stipulated by EU legislation concerning antibiotic residue studies, ten eggs per day were analysed.

7.2.2.2 Broiler chickens

Day-old male and female broilers (Ross ‘208’, 75 of each gender) were obtained from a commercial hatchery. They were reared separately under infra-red bulbs. The broiler chickens received a diet containing 12.6 MJ AME_n/kg and 22% CP. Their ration also contained Stafac^R at a concentration of 40 mg/kg. Feed and drinking water were provided at libitum. The broilers were vaccinated the first day of age against Newcastle disease (Hitchner spray) and bronchitis

(H120 spray). At 16 days of age the vaccination against Newcastle disease was repeated with La Sota (Clone 30). At 19 days of age, during the observation period and when a vaccination reaction was no longer expected, some birds were sacrified, after stunning, by cervical dislocation. The breast muscle tissue was removed for investigation as blank control muscle tissue. Tetracycline was supplemented at a therapeutic dose level of 480 mg per kg diet. The broilers were provided with the medicated feed for seven consecutive days. Following EU legislation concerning antibiotic depletion studies, the breast muscle tissue of six broiler chickens was investigated daily. During the experimental period, three males and three females were weighed individually and slaughtered. These birds were taken ad random. The breast muscle tissue samples were refrigerated until investigation. The feed consumption per gender was determined during the observation time and the administration period.

7.2.3 Analysis of medicated feed

The concentrations of oxytetracycline and tetracycline in the medicated feeds were determined in the State Analysis Laboratory at Tervuren (Belgium).

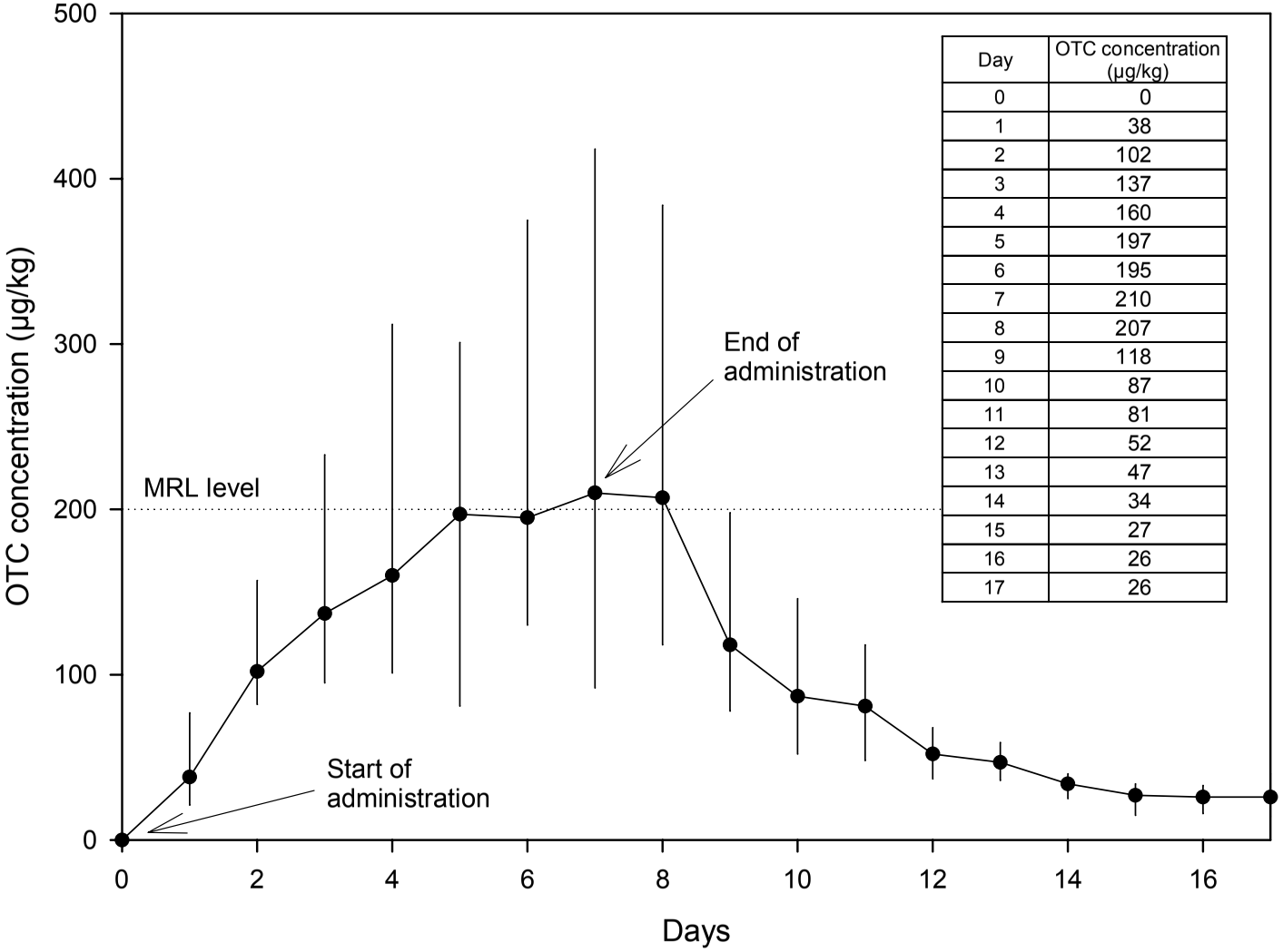
7.2.4 Drug residue analysis

The residue concentrations of oxytetracycline in the egg samples and of tetracycline in the breast muscle tissue samples of the broiler chickens were determined with the quantitative HPLC-DAD method described in Chapter 2.

7.3 Results and discussion

7.3.1 Disposition of oxytetracycline in eggs

The OTC residue values in eggs during (day 0 until day 7) and after (day 8 until day 17) medication of the laying hens are shown in Figure 7.2. Medicated feed was supplemented on day 0 and was changed for a normal feed ration on day 7. Each data point of the excretion curve represents the mean OTC residue concentration of ten eggs of ten different hens. To have ten eggs daily during the whole experimental period, 14 laying hens in total were needed for this investigation. The ranges of the residue concentrations of the individual eggs are indicated in Figure 7.2. The individual variation was high, especially during the medication period. As example, the OTC residue values ranged from 92 to 418 µg/kg on day 7. This fluctuation is mainly caused by physiological differences, but is also influenced by variations in feed intake and in egg weight. The mean residue values of each data point are also summarised in the inset-table in the figure.



Day	OTC concentration (µg/kg)
0	0
1	38
2	102
3	137
4	160
5	197
6	195
7	210
8	207
9	118
10	87
11	81
12	52
13	47
14	34
15	27
16	26
17	26

Figure 7.2 Disposition of residues of oxytetracycline (OTC) in eggs of laying hens during and after oral administration of feed medicated with 840 mg of oxytetracycline per kg (The range of the residue concentrations of the individual eggs per data point is indicated)

The feed intakes observed during the administration period and the mean egg weights are summarised in Table 7.1. The daily feed intake ranged from 97.1 to 132.1 g. The mean egg weights varied from 57.8 to 71.8 g.

Table 7.1 Feed intakes and main egg weights of laying hens medicated orally with 840 mg oxytetracycline per kg feed

Hen	Feed intake (g/day)	Egg weight (g)
1	107.0	62.1
2	108.3	65.3
3	113.9	66.6
4	105.9	71.8
5	132.1	57.8
6	109.3	60.0
7	97.1	58.6
8	121.4	71.7
9	118.6	61.2
10	100.0	64.9
11	121.4	67.0
12	115.4	63.7
13	119.6	63.4
14	122.1	61.6

After one day of administration, an OTC residue concentration of 38 µg/kg was already obtained. This rapid excretion could be expected because tetracyclines are readily absorbed to give peak blood plasma concentrations a few hours already after starting oral administration.[4,21] It took some five days before a plateau residue concentration in the eggs could be observed. On day 5, the OTC concentration reached a value near the MRL level (200 µg/kg). The residue values were fluctuating arround this level during the next two days of the administration period. On day 9, two days after ceasing of the administration, the residue value has already fallen below the MRL level. Afterwards, the residue level decreased slowly. This slow excretion can be explained by the persistence of tetracyclines in blood following absorption in contrast with other antibiotics which are eliminated more readily.[4] Black[2] obtained lower OTC residu concentrations

in tissues and noted a slower depletion after oral administration compared with intramuscular and subcutaneous medication. Also, Anadon *et al*.[8] measured a slower excretion for tetracycline via the bile after oral medication.

On day 17, ten days after ceasing the administration, a mean OTC residue concentration of 26 µg/kg was obtained and the analyses were stopped because some of the ten individual figures approached the limit of quantification of 13 µg/kg.

The presented results, to a certain extent, confirm published studies although they were not always conducted in similar conditions. Meredith *et al*.[2] observed maximum OTC residue concentrations of 580 µg/kg in eggs of hens fed with medicated feed containing 1000 mg of OTC per kg. Roudaut *et al*.[1] used a lower dose of 600 mg per kg of feed and measured a plateau level in OTC residue concentration of 250 µg/kg during day 6 and 7 of the medication. Zurhelle *et al*.[11] reported a lower maximum OTC residue concentration of 120 µg/kg measured at day 14 after administration of medicated feed with 750 mg of OTC per kg feed.

Yoshimura *et al*.[3] compared the depletion of OTC and DC in eggs after medication of laying hens via drinking water at the recommended dosage for OTC of 0.5 g/l. The mean peak residue values were 5 times higher in yolk and more than 10 times higher in albumen for DC than for OTC. This depletion characteristic of DC is considered to be due to its greater lipophilicity, causing more efficient absorption and tissue penetration.

Clearly, oxytetracycline does not accumulate very strongly and hence does not show high residue values in eggs. Huber[4] reported that tetracyclines diffuse throughout the body and are found in highest concentrations in kidney, liver, spleen and lung. Tetracyclines also incorporate in chicken and turkey egg shells. Zurhelle *et al*.[11] observed that only these shells of eggs sampled on the medication period showed a characteristic yellow fluorescence of the calcium-tetracycline complex when irradiated with long-wave UV.

Roudaut *et al*.[1] calculated the total excretion of OTC into eggs. Between 0.015 and 0.020% of OTC supplied was recovered after oral administration and 0.22-0.24% after a intramuscular route. This means that the passage of OTC in egg was about 10 times higher by the latter route. Incomplete intestinal absorption in case of oral administration causes the lower disposition of OTC in eggs. TCA's form complexes with bivalent ions such as Ca^{++} in the digestive tract, which markedly diminishes their absorption. Black[21] reported that birds receiving the OTC drug orally tended to have considerably lower residue concentrations in their tissues. However, there was a tendency for residues to remain for a longer period of time. Oral administration of OTC apparently was a less efficient route. However, because of the savings in labour, the oral medication via feed or drinking water is the preferred route. A significantly greater quantity of drug appears to be required. Administration of antimicrobials to poultry via the drinking water is carried out widely in tropical countries, where the amount of drinking water consumed is considerably larger (350 ml per day per hen) than in moderate zone (200 ml per day per hen).[1,3] Even if the quantity of oxytetracycline ingested daily in the case of oral administration can not

be precisely determined, the holding conditions used in the described experiments offered the advantage of being very close to the practise in large breeding farms of laying hens.

Some research workers studied the distribution of OTC residues between egg yolk and egg white.[1,3,16] In all cases, it was in the egg yolk that OTC residues were present earlier after starting medication, that a higher maximum concentration was reached and that the clearance time was longer. Omija *et al*.[16] confirmed that at higher dosages, OTC was found in the albumen and reached a maximum concentration faster in the albumen than in the yolk. The concentrations persisted for a longer period in the yolk. Clearly, the withdrawal period depends on the dosages of OTC administered.

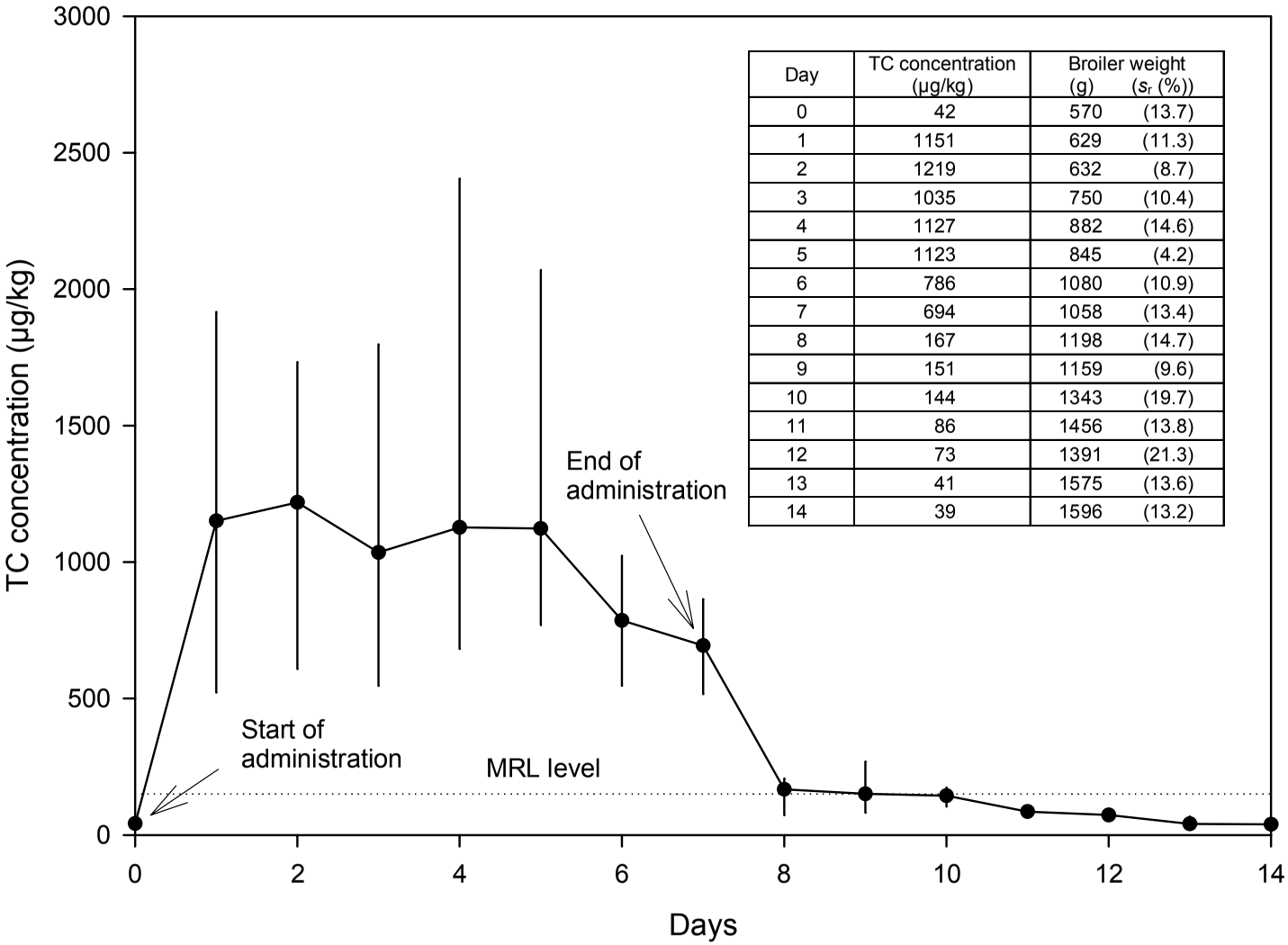
Although oxytetracycline residues are heat labile, the presence of antibiotic residues in eggs might cause some risks for the consumer's health. OTC residues are totally destroyed by a thermal treatment at 100°C for 5 min, through formation of either inactive derivatives as 4-epi-OTC and α- or β-apo-oxytetracycline. They are partially degraded by 60% or 80% whether the eggs are poached or scrambled.[2,22] The temperature during cooking had the largest impact on the loss.

7.3.2 Disposition of tetracycline in breast muscle tissue of broiler chickens

The disposition of TC residues in breast muscle tissue of broilers during (day 0 until day 7) and after (day 8 until day 14) oral administration is presented in Figure 7.3. The shown excretion curve is drawn with mean values calculated with the residue values of six broilers, three males and three females. The mean residue values of each data point of these trials are also summarised in the inset-table in the figure.

The range of the individual residue concentrations is indicated in Figure 7.3. The variability of the residue values during the medication period was high but somewhat lower than those obtained for the depletion of OTC in eggs. These residue fluctuations are mainly caused by physiological differences but also by variations in antibiotic intake and feed conversion. The observed mean daily feed intake during the medication period was 106.8 g for males and 88.1 g for females. The mean value and the relative standard deviation (s_r value) of the broiler weights, obtained just before slaughter, are also presented in the inset-table in the figure.

Following the oral administration, tetracycline is rapidly absorbed and efficiently distributed to breast muscle tissue of the broilers in the trials. On day 0, the birds were slaughtered only a few hours after supplying the medicated feed and the TC concentration already reached 41.7 µg/kg. Huber[4] reported that following oral administration, TCA's are readily absorbed from the stomach and the small intestine to give peak plasma concentrations in 2-4 h. Plasma concentrations of TC and OTC administered intramuscularly are detectable within 15 min, reach a peak within 1 h, maintain significant blood concentrations for about 12 h and decline to trace amounts



Day	TC concentration (µg/kg)	Broiler weight (g)	(s_r (%))
0	42	570	(13.7)
1	1151	629	(11.3)
2	1219	632	(8.7)
3	1035	750	(10.4)
4	1127	882	(14.6)
5	1123	845	(4.2)
6	786	1080	(10.9)
7	694	1058	(13.4)
8	167	1198	(14.7)
9	151	1159	(9.6)
10	144	1343	(19.7)
11	86	1456	(13.8)
12	73	1391	(21.3)
13	41	1575	(13.6)
14	39	1596	(13.2)

Figure 7.3 Disposition of residues of tetracycline (TC) in breast muscle tissue of broiler chickens during and after oral administration of feed medicated with 480 mg of tetracycline per kg (The range of the individual residue concentrations per data point is indicated)

approximately 24 h after injection. Black[21] was able to detect a measurable OTC peak (> 100 µg/l) in the blood serum of day-old chickens already 1.5 h after oral administration of a very low dosage of 5 mg per kg feed. This assessment is also in accordance with other published TCA depletion data. Anadon *et al*.[8] measured a maximum TC plasma concentration of 690 µg/ml already 15 min after intravenous treatment of broiler chickens at a concentration of 65 mg per kg body weight (BW). Capolongo *et al*.[12] reported a mean maximum OTC residue concentration of 126 µg/kg in muscle tissue of turkeys already 1 day after cessation of the oral treatment of the birds via the drinking water with a varied dose of 57 to 103 mg/kg BW during three consecutive days. Croubels *et al*.[7] found a similar rapid absorption for DC in turkey muscle tissue. However, these authors obtained much higher residue concentrations due to the higher lipid solubility of this antibiotic drug substance. A mean DC residue value above 2000 µg/kg was measured 12 h after last dosage of a medicated drinking water at a total daily dose of 25 mg/kg BW during four successive days.

The obtained residue accumulation of TC in breast muscle tissue was very high. The applied therapeutical TC dosage level in the feed in the experiment (480 mg per kg) was also higher than the recommended dose of 50 to 450 mg OTC per kg reported by Black.[21] On day 1, the TC residue concentration was already higher than 1000 µg/kg. A maximum TC concentration in the breast muscle tissue was found on day 2 and reached 1219 µg/kg. Meredith *et al*.[2] reported a maximum OTC residue value of 1440 µg/kg in poultry muscle tissue after oral treatment of broilers with medicated feed containing 1000 mg of OTC per kg feed.

A plateau residue concentration in the depletion curve in Figure 7.3 is already obtained after one day of medication which afterwards decreased until the end of the administration period. On day 7, a mean residue value of 694 µg/kg was obtained. This decreasing tendency is probably mainly caused by an increase of the feed conversion.

The absorption of tetracycline in breast muscle tissue appears to be higher than the absorption of oxytetracycline in eggs. The maximum residue concentration for OTC in eggs was 210 µg/kg compared with a maximum TC residue concentration of 1219 µg/kg in muscle tissue.

After the medication was stopped, the TC residue concentration decreased very rapidly. On day 11, four days after the administration was stopped, the TC concentration was 86 µg/kg and declined below MRL level (100 µg/kg). Afterwards the residue concentration decreased slowly. On day 14, seven days after ceasing administration, a residue concentration of 39 µg/kg was still found. On day 15, the TC residue concentration of two samples was below the limit of quantification of 20.9 µg/kg. Anadon *et al*.[8] and Black[12] also obtained a slow elimination of tetracyclines after oral administration. Anadon *et al*.[8] also observed a deep tissue sequestration of the TC drug and suggested a slow elimination process. Huber[4] reported that TCA's persist in blood following absorption in contrast with other antibiotics that are eliminated more rapidly.

Black[12] compared the depletion of OTC in tissues and serum of day-old and one-week-old broilers after either oral, intramuscular or subcutaneous administration of the birds. The birds

receiving the drug orally tended to have considerably lower concentrations of OTC in their tissues. However, this author observed a tendency for residues to remain for a longer period of time.

Meredith *et al*.[2] studied the difference in OTC depletion in several tissues of laying hens fed during 6 months with feed medicated with 1000 mg of OTC per kg feed. The greatest antibiotic residue concentration was measured in the liver (4050 µg/kg). The highest residue concentration in muscle tissue was generally found in dark meat taken from the thigh (1440 µg/kg) of the bird. The lowest residue concentration was measured in the breast muscle tissue (1200 µg/kg).

As Meridith *et al*.[2] have reported, adequate heat treatment of broiler chicken muscle tissue as well as of eggs before consumption will further minimise the tetracycline residue concentrations. The effect of a range of heating processes including microwaving, boiling, roasting, grilling, braising and frying on OTC residues in incurred animal tissues was also investigated by Rose *et al.*[22] Substantial net reductions in OTC concentration of 35-94% were measured. Migration of residues from the tissue into the surrounding liquid or meat juices was observed during the different heating processes.

7.4 Conclusions

From the described depletion studies for OTC in eggs and for TC in broiler breast muscle tissue can be concluded that when the recommended withdrawal periods of 14 days for use of OTC for laying hens and seven days for use of TC for broiler chickens are respected, there is no risk for the public health. After these periods of time, the obtained residue concentrations were far below the MRL levels. However, the total elimination of tetracycline residues via eggs and via breast muscle tissue after oral administration takes several days.

7.5 Acknowledgements

The authors wish to thank Ir. K. Haustrate of the State Analysis Laboratory (Tervuren, Belgium) for the analysis of the medicated feed samples. The preparation of the figures by Ing. P. Traest and Mr G. Vancalbergh was also greatly appreciated.

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CHAPTER 8

Depletion of levamisole in milk

Relevant publication:

De Ruyck H., Van Renterghem R., De Ridder H. and De Brabander D. Determination of anthelmintic residues in milk by high performance liquid chromatography.
Food Control, 2000, 11, 165-173

Abstract:

The disposition and distribution of levamisole in milk of dairy cows medicated with two doses was investigated. Cows were separately topically treated with a recommended dose of 10 mg for non-lactating cows and a higher dose of 20 mg of levamisole per kilogram body weight. Seven hours after administration of the recommended dose, a maximum residue value of 1896 µg/l was measured in the milk. Seventy nine hours after the treatment, the mean levamisole concentration had declined to 21 and 83 µg/l for the low and the high dose, respectively.

The highest obtained total excretion of levamisole via the milk was 0.55% of the dosed amount, measured after 76 hours of excretion with the cows administrated with the high dose.

Keywords: anthelmintic drug residues, levamisole, milk, depletion

8.1 Introduction

8.1.1 Usage of anthelmintics for dairy cows

Anthelmintic drugs are widely used in the veterinary medicine for protecting or treating animals mainly against gastrointestinal nematodes and lungworms. The wide-spectrum anthelmintics currently available for cattle originate mainly from the chemical groups of benzimidazoles (BZs), tetramisoles (levamisole) and avermectins.[1] The BZs are characterised by a very wide spectrum of activity and low mammalian toxicity. BZ efficiency increases as the exposure time of the nematode to a threshold level of the drug substance increases. Repeated administration of divided doses to prolong exposure and administration by the oral route may therefore enhance drug activity. BZs given orally are particularly effective in ruminants in which prolonged passage through the rumen and hence a reduced rate of absorption leads to a continued exposure of the parasite to the drug compound. For similar reasons, the most effective BZ are the less water-soluble compounds such as fenbendazole, oxfendazole and albendazole, which remain in solid form precipitates within the gut lumen for longer periods of time. When the BZ drug passes directly to the abomasums following oral administration, the time for passage through the gastrointestinal tract is reduced and efficiency is correspondingly decreased. Anthelmintic efficiency against adult and developing larval stages of the common gastrointestinal nematodes is high, but only the less soluble compounds which promote the maintenance of a treshold level of drug for a few days are effective against arrested larval stages and lungworms.

Levamisole (LE) has a long history of use as a broad-spectrum anthelmintic which is effective against lungworms and gastrointestinal nematodes in large food-producing animals (cattle, sheep, pigs), poultry species and occasionally in wild animals.[2] LE is used orally and parenterally mainly against lungworms and gastrointestinal nematodes. It is also effective by topical, dermal application of cattle as “pour-on” technique. In contrast to the BZs, the peak concentration of LE is more important since excretion is very rapid. More than 90% of the LE residues excrete within 24 hours.[1] LE is readily absorbed independently of the route of administration and is rapidly excreted, predominantly via the urine. An increasing fraction of the portion of the dose remaining in the animal is bound to larger molecules forming non-extractable residues.[2] The activity of LE against developing larvae and the adult forms of gut nematodes is high and the drug substance is also effective against arrested larvae of lungworms.[1] Because of its broad and effective working spectrum, its short withdrawal time and its easy form of application, LE still remains the anthelmintic agent of choice for use in lactating dairy cows suspected of harbouring burdens of adult gastrointestinal nematodes. Most forms of parasitic gastroenteritis in ruminants tend to occur in the second half of the summer.[3] Treatment of dairy cows in wet periods at the end of summer can be very useful to protect and to treat the animals against lungworms.

The effective therapeutic dose is best guaranteed when the drug is administered to cattle as a drench, paste or by injection.[1] Administration in feed or drinking water may be labour saving but has some disadvantages. Because the cattle feed or drink individually there is no guarantee that a therapeutic dose will be obtained by each animal and subtherapeutic doses may promote the development of drug tolerance. Moreover, since depression of appetite is a common consequence of internal parasitism, the quantity of anthelmintic consumed may be sub-optimal. Where topical preparations of anthelmintics have been used, the activity, while good, has been generally less than that achieved following oral or parenteral administration. Unless the strong economic or management advantages of in-feed or topical application of cattle anthelmintics, the traditional route is to be preferred, particularly when the agents are being used therapeutically, rather than prophylactically.

Resistance against thiabendazole and the other BZs has been recorded mainly from geographic areas where *Haemonchus* spp. predominate and the numbers of annual treatments are more numerous than in moderate zones such as Western Europe.[1] This resistance or tolerance as it is frequently labeled, is sometimes incomplete and may be overcome by using higher dosage rates. Nematodes have sufficient genetic variation to develop resistance to several different anthelmintics at rates at least equal to that which develops to a single drug. On this basis, a good policy would be to alternate the anthelmintics used on farm provided that the alternation occurs between different generations or populations of worms and that the alternative drugs are from a different chemical group.

The anthelmintic drugs are generally of very low toxicity to mammalian hosts but some BZ compounds such as albendazole and oxfendazole are known to be teratogenic.[1] In general, BZ blood concentrations and excretion half-lives are longer in ruminants than in monogastric animals, but in either case BZs appear to be characterised by very low toxicity in mammals.[4] The mammalian toxicity of LE is greater than with the BZ although, in normal usage, toxic effects are seldom seen.[1]

There is a considerable debate on the influence of gastrointestinal parasitism in limiting milk production in dairy cows. Positive evidence on increased milk yields following therapy with anthelmintics has come from various reports. Block *et al*.[5] obtained an improved milk production of 4.8% and a reduced gastrointestinal worm burden after injecting of dairy cows with levamisole at calving. Spence *et al*.[6] also reported an increase of 4.8% in milk production per lactation by treatment of artificially infected cows with broad-spectrum anthelmintics. Gross *et al*.[7] reviewed the results of more than 80 experiments on gastrointestinal parasitism and the impact of anthelmintic treatment on milk production in dairy cattle. Their summary suggests that treatment with an anthelmintic drug produces an overall benefit of approximately 0.6 kg milk per cow per day.

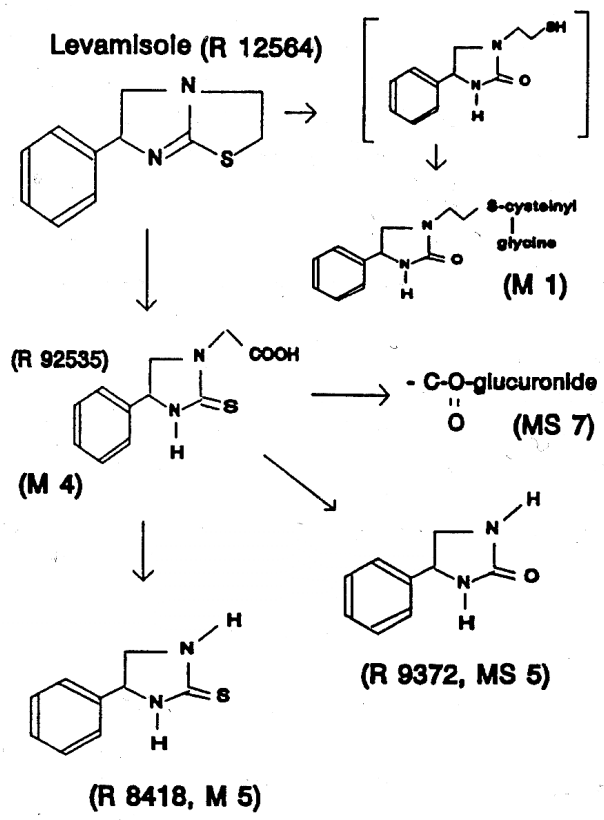
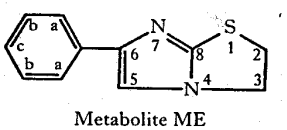


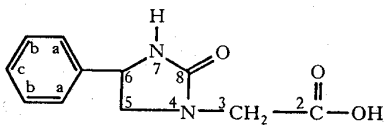
Figure 8.1 Metabolism of levamisole in cattle[2]



Metabolite ME

0.006% of oral dose
0.008% of subcut. dose

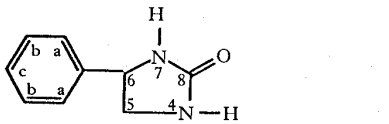
GC-MS (EI-mode) *m/z* 202 (M+), ^{1}H-nmr (CD_3OD)
δ 3·85 – 3·89 (m, H_2);
δ 4·18 – 4·22 (m, H_3);
δ 7·19 – 7·23 (m, H_c);
δ 7·31 – 7·34 (m, H_b);
δ 7·46(s, H_5);
δ 7·62 – 7·64 (m, H_a)



Metabolite MH

0.013% of oral dose
0.019% of subcut. dose

–FAB-MS *m/z* 219 (M–H);
^{1}H-nmr (CD_3OD)
δ 3·35 (t, $J_{5,5'} = J_{5,6} = J_{5',6} = 9{\cdot}0$ Hz, H_5 or 'H_5);
δ 3·64 (d, $J_{3,3'} = 17{\cdot}1$ Hz, H_3 or 'H_3);
δ 3·87 (d, $J_{3,3'} = 17{\cdot}1$ Hz, H_3 or 'H_3);
δ 3·96 (t, $J_{5,5'} = J_{5,6} = J_{5',6} = 9{\cdot}0$ Hz, H_5 or 'H_5);
δ 4·84 (t, $J_{5,6} = J_{5',6} = 9{\cdot}0$ Hz, H_6);
δ 7·28 (t, $J_{b,c} = 7{\cdot}4$ Hz, H_c);
δ 7·35 (t, $J_{b,c} = J_{a,b} = 7{\cdot}4$ Hz, H_b);
δ 7·43 (d, $J_{a,b} = 7{\cdot}4$ Hz, H_a);
GC-MS of monomethylation product, *m/z* 234, 175, 132, 105 and 77;
GC-MS of dimethylation product, *m/z* 248, 132, 105 and 77.



Metabolite MJ

0.005% of oral dose
0.007% of subcut. dose

+FAB-MS *m/z* 163 (M + 1);
GC-MS, *m/z* 162, elemental composition $C_9H_{10}O$;
^{1}H-nmr (acetone-D_6)
δ 3·20 (t, $J_{5,5'} = J_{5,6} = J_{5',6} = 7{\cdot}8$ Hz, H_5 or 'H_5);
δ 3·83 (t, $J_{5,5'} = J_{5,6} = J_{5',6} = 7{\cdot}8$ Hz, H_5 or 'H_5);
δ 4·85(t, $J_{5,6} = J_{5',6} = 7{\cdot}8$ Hz, H_6);
δ 7·30 (t, $J_{b,c} = 7{\cdot}4$ Hz, H_c);
δ 7·38 (t, $J_{b,c} = J_{a,b} = 7{\cdot}4$ Hz, H_b);
δ 7·41 (d, $J_{a,b} = 7{\cdot}4$ Hz, H_a);

Figure 8.2 Metabolites of levamisole in milk[8]

8.1.2 Metabolism of levamisole

Levamisole is rapidly metabolised to a large number of metabolites.[2] Studies based on both in vivo and in vitro in target species and laboratory animals indicate that the routes of metabolism of LE are qualitatively similar in most species. A study using ^{14}C levamisole investigated the metabolism of LE in urine, plasma and liver in cattle. An extensive and rapid metabolism of LE was observed. Unchanged drug accounted for only 0.4-2.3% of the dosed amount in urine or faeces and less than 3% of the total residues in tissues. The main metabolites are shown in Figure 8.1.

Paulson and Feil[8] reported that there are two major routes of metabolism for levamisole. The first route includes the oxidation at the 2-position of the imidazothiazole ring (Figure 8.2) followed by oxidation to a carbonyl and hydrolysis to a thiohydantoic acid. The second route is the hydrolysis of the thiazoline ring to yield a mercapto-ethyl intermediate which forms the polar conjugate S-cysteinyl-glycine. These research workers also investigated and compared the disposition of ^{14}C levamisole in lactating cows after oral and subcutaneous administration. The parent levamisole accounted for 14.1 and 10.5% of the ^{14}C activity in milk collected 0-12 h after oral and subcutaneous dosing, respectively. The percentage of the total ^{14}C activity present as ^{14}C levamisole declined in later collections to 1.7-1.8% of the total ^{14}C residue in milk collected 36-48 h after dosing. The proportion of total ^{14}C residues present as ^{14}C levamisole was similar in milk samples collected from the orally and subcutaneously dosed cows throughout the 48 h collection period. Metabolite ME (Figure 8.2), identified as dehydro-levamisole accounted for 4.4-4.7% of the ^{14}C activity in the 0-12 h milk. Some studies demonstrated that levamisole slowly decomposes under neutral and alkaline conditions to yield dehydro-levamisole.[9] Thus it is possible that metabolite ME was an artifact and not a result of animal metabolism. The urine was the major route of elimination of ^{14}C activity after both oral (82.9%) and subcutaneous (84.1%) dosing.[8] Faecal excretions during the same time period were 10.6% and 9.1% of the administered dose, respectively. The parent drug was not found in the urine. The major ^{14}C labelled fraction isolated from urine was identified as 2-thio-3-(2-carboxymethyl)-5-phenylimidazolidine (metabolite M4 in Figure 8.1).

8.1.3 Objectives

Levamisole is still used for anthelmintic treatment of dairy cows. If farmers do not respect the recommended withdrawal times when using this drug substance for lactating dairy cows, the residue concentrations in the milk can be very high. Since several years, LE is not more approved for usage in lactating dairy cows.[10]

Several depletion studies of benzimidazole anthelmintic compounds have been published recently.[11,12,13,14,15,16] The distribution of albendazole and its major metabolites in ovine milk and milk products after single oral dose is presented by De Liguoro *et al*.[11] Fenbendazole-related drug residues in milk from treated dairy cows were studied by Kappel and Barker.[12] The

depletion of albendazole metabolites from milk of sheep and goat was reported by Cinquina *et al*.[13] The excretion of metabolites of albendazole in goat and sheep milk products and by-products is described by Longo *et al*.[14] Fletournis *et al*.[15] tested a rapid quantitative screening multiresidue HPLC-UV assay for tracing benzimidazoles and metabolites in milk of two cows respectively treated with an albendazole and a fenbendazole formulation. Barker and Kappel[16] investigated the drug residues in milk of cows treated with fenbendazole as a paste, drench or feed top-dressing.

Reports of depletion studies of levamisole in dairy cows are scarce and most of the publications are rather old.[17,18,19] Simkins *et al*.[17] described the excretion of levamisole in milk from cows treated with various formulations. The rate of disappearance of LE in milk from cows given levamisole as drench, feed pellets, boluses and injections was determined. Each formulation was given as a single treatment to each of five cows at a dose equivalent to 8 mg of levamisole-hydrochloride per kg body weight (BW). The LE residues in the milk were measured by a gas chromatographic determination method until 72 h after treatment. Archambault *et al*.[18] performed a study on the plasma bioavailability after a cutaneous application of levamisole in cattle. The experiment was conducted in young animals which received successive doses of 10 and 20 mg per kg BW at one-week interval. To compare the plasma concentrations with those obtained during a more conventional administration, the same animals were also administered an intramuscular injection of LE at a dose of 7.5 mg per kg BW. Dairy cows were cutaneously treated with levamisole at a dose of 10 mg per kg BW. Österdahl *et al*.[19] reported a study of levamisole residues in milk from dairy cows suffering from lungworms. A single intramuscular dose of 7 mg levamisole-hydrochloride per kg BW was administered to a herd of 42 milking cows.

Paulson and Feil[8] investigated the disposition of ^{14}C levamisole in the lactating dairy cow in detail. ^{14}C levamisole was administered orally and subcutaneously to lactating cows at a dose of 8 mg per kg BW. ^{14}C labelled residues of levamisole and metabolites were measured in milk, blood, urine and faeces samples collected from 0 to 48 h after dosing. Tissues were collected and analysed 48 h after dosing. Cannavan *et al*.[20] described the depletion of levamisole from muscle, liver and kidney tissues of sheep orally treated with a commercial drench containing levamisole at the recommended therapeutic dose of 7.5 mg per kg BW.

Levamisole is normally administered to dairy cows in the hydrochloride form and by injection or orally.[19] However, administration of LE to cattle by topical, dermal treatment as “pour-on” technique is another easy and very fast practise. To our knowledge, only Archambault *et al*.[18] reported a depletion study after topical administration in the open literature.

The objective of the presented study was to perform depletion experiments for levamisole with lactating dairy cows treated topically at therapeutic dose levels. The evolution in levamisole residues was measured in the milk of successive milkings. The animal treatments were performed at the Department of Animal Nutrition and Husbandry, Section cattle and pig

husbandry (DVV-CLO, Merelbeke, Belgium). The residue analyses were carried out at the Department of Animal Product Quality and Transformation Technology (DVK-CLO, Melle, Belgium).

8.2 Experimental

8.2.1 Treatment of the dairy cows

Twenty lactating Holstein cows, divided into four groups of 5, were selected for the depletion studies. The cows were treated topically with levamisole (L-Ripercol® pour-on, Janssen-Cilag, Beerse, Belgium) in two trials. In the first trial, the cows were treated with the recommended dose of 10 mg of levamisole per kg BW. The BW averaged 564±40 kg (mean±standard deviation (*s*)). Levamisole was administered to the four groups at different times. To obtain more data points shortly after treatment, group 1, 2, 3 and 4 were already treated, respectively 1, 3, 5 and 7 h before the subsequent milking. The cows were milked twice a day. The first seven milkings after the administration were sampled for determination of LE residues. The samples were frozen and stored at -18 °C until investigation.

Four weeks after the first trial, the same cows were treated with a double dose of 20 mg of levamisole per kg BW in a second trial in the same manner as in the first treatment. The average BW of the cows at the beginning of this second trial was 589±40 kg (mean±*s*). The milk samples were taken in the same way as in the first trial.

A withdrawal time of 48 h for usage of L-Ripercol® pour-on was prescribed for dairy cows.

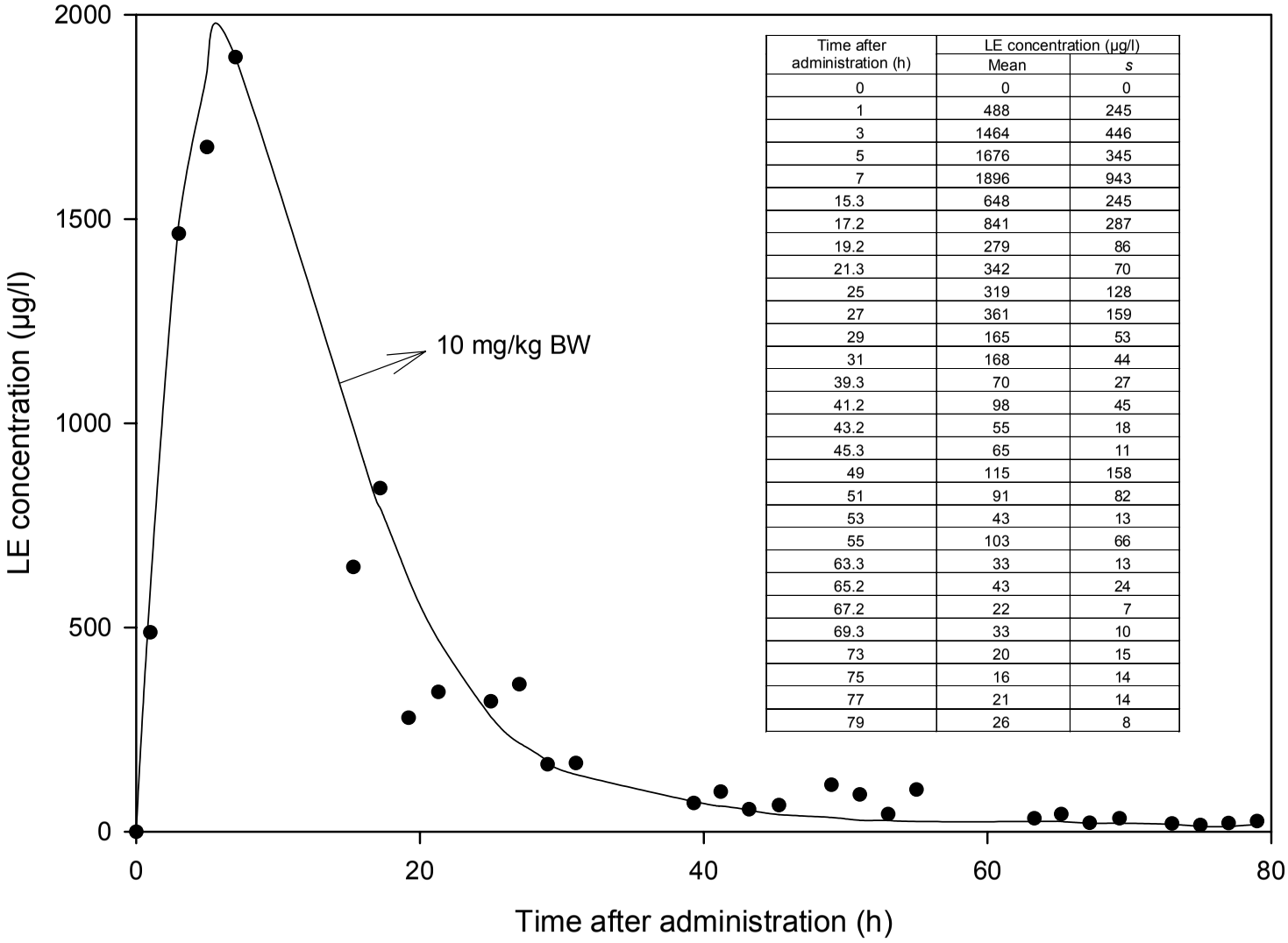
8.2.2 Determination of levamisole residues in milk

The quantitative HPLC-DAD determination method for residues of levamisole, described in Chapter 3, was applied on the milk samples collected during the depletion studies. If necessary, the milk samples were diluted with blank milk to decrease the residue concentration below 1000 µg/l.

8.3 Results and discussion

8.3.1 Depletion of levamisole in milk after treatment with the recommended dose

The depletion of levamisole in the milk after a single administration of the recommended dose of 10 mg per kg BW is shown in Figure 8.3. The figure presents the data points of the mean LE concentrations of the different groups in function of time after administration. A simulated excretion curve is drawn. The mean values and the standard deviation values of the different groups are also indicated in the inset-table in Figure 8.3. In the blank milk samples, no levamisole could be detected. As expected from the literature,[1] the levamisole anthelmintic



Time after administration (h)	LE concentration (µg/l)	
	Mean	*s*
0	0	0
1	488	245
3	1464	446
5	1676	345
7	1896	943
15.3	648	245
17.2	841	287
19.2	279	86
21.3	342	70
25	319	128
27	361	159
29	165	53
31	168	44
39.3	70	27
41.2	98	45
43.2	55	18
45.3	65	11
49	115	158
51	91	82
53	43	13
55	103	66
63.3	33	13
65.2	43	24
67.2	22	7
69.3	33	10
73	20	15
75	16	14
77	21	14
79	26	8

Figure 8.3 Depletion of levamisole (LE) after topical treatment of dairy cows with the recommended dose of 10 mg per kg body weight

substance was very rapidly absorbed and was subsequently excreted in the milk. One h after dosing, a LE residue concentration of 488 µg/l could already be measured. This could be expected from reported residue values in blood samples. Archambault *et al*.[18] measured a mean LE concentration of 334 µg/l in plasma samples of young animals already 1 h after a comparable treatment. Paulson and Feil[8] obtained 1 h after dosing already a total ^{14}C activity in blood samples of 1440 µg/l (oral dose) and 1570 µg/l (subcutaneous dose). Simkins[17] also obtained a rapid absorption and excretion. The LE residue concentration in blood and urine peaked at 2 to 6 h after cattle were treated via a bolus formulation. Österdahl *et al*.[19] obtained a maximum LE concentration of 2200 µg/l in milk after 1 h already. However, the cows in their trials were treated intramuscularly. Archambault *et al*.[18] also found a significantly higher biodisponibility after intramuscular injection. A mean plasma concentration of 1063 µg/l was obtained for the young animals already 1 h after intramuscular treatment with a dose of 7.5 mg per kg BW. The lowest residue concentrations obtained by Österdahl *et al*.[19] were found in the cows treated 7 h before milking probably due to the rapid metabolism of the drug.

In the presented study, a highest LE residue concentration of 1896 µg/l was measured 7 h (group 4) after administration. Archambault *et al*.[18] obtained comparable depletion results. These authors measured a residue concentration of 1100 µg/l at their first data point at 12 h after topical treatment of a dose of 10 mg per kg BW.

The individual fluctuation of the residue concentrations within the groups was high and is probably caused by physiological differences. The most important differences between the individual cows are the biodisponibility and the rates and routes of metabolism and excretion of the parent drug and metabolites. The average daily milk yield of the 20 cows during the four days of the trial was 20.1±7.8 kg (mean±*s*). No relationship could be found between residue concentration and milk production.

As reported by Armour[1] also, the observed depletion of LE is very rapid. The mean residue concentration had decreased to 319 µg/l at 25 h after treatment. After the prescribed withdrawal period of 48 h, a residual LE concentration could still be observed. The mean LE concentration of the 4 groups of this milking was 88 µg/l. This concentration is still higher than the EC zero level, which was established recently. Paulson and Feil[8] obtained approximately similar residue concentrations (60 µg/l) in the milk samples 36-48 h after the oral or subcutaneous administration. These researchers were able to distinguish the parent LE from its metabolites using ^{14}C labelling. They remarked that only 1.7-1.8% of the total residue value was present as parent LE. In the presented depletion study, a HPLC-DAD technique was used and the identity of the LE peak was confirmed with an UV spectrum. In the last sampled milking, at 76 h after treatment, the LE residue concentration had declined to 21 µg/l. Archambault *et al*.[18] was not able to measure a residue concentration above the detection limit (50 µg/l) at this time point. Österdahl *et al*.[19] also reported a rapid residue decrease in the milk. A half-live of about 5 h was calculated.

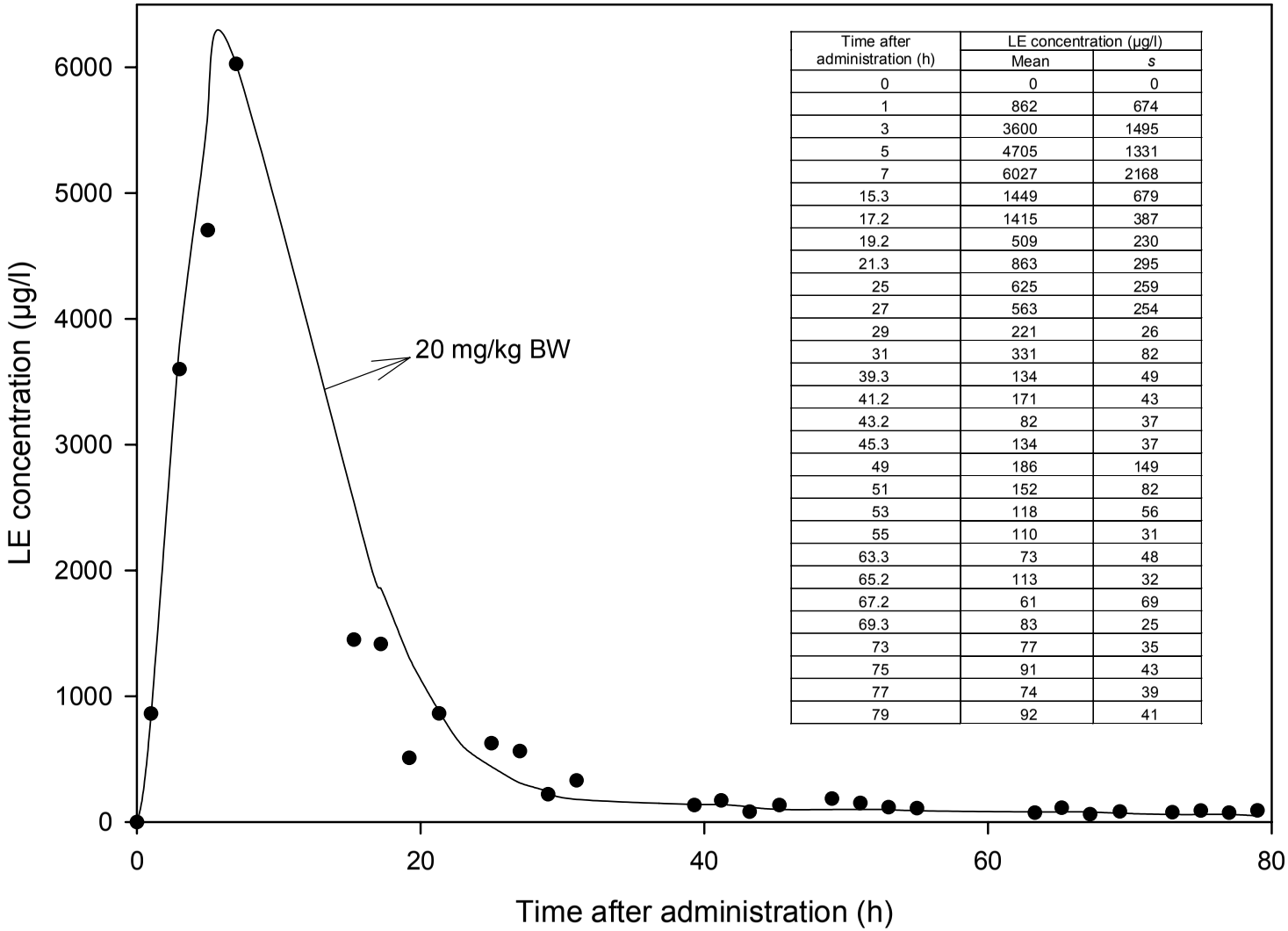
The depletion pattern of levamisole in muscle tissue obtained by Cannavan *et al*.[20] was comparable with that obtained in milk. A LE residue concentration of approximately 10 µg/kg was reported after a withdrawal of 2 days.

The biodisponibility obtained by Archambault *et al*.[18] after intramuscular injection of 7.5 mg per kg BW was significantly higher than that obtained after topical treatment with a dose of 10 mg per kg BW. However, the residue concentrations showed a good dose-reponse relation. With the topical dose of 20 mg, a significantly higher residue response in the plasma samples of young animals was already measured compared with the intramuscular treatment. As expected, the absorption and excretion in blood after topical treatment is somewhat slower, but the peak values were higher (2039 µg/l after 4 h compared with 1274 µg/l after 3 h for the topical and the intramuscular treatment, respectively). Simkins *et al*.[17] reported that the ranges of LE residues in milk at 12 h after treatment are similar, which suggests that LE is rapidly absorbed for all formulations. LE-hydrochloride residues in milk averaged 50, 55, 58 and 32 µg/l at 12 h after the administration of levamisole drench, feed, bolus and injectable formulations. All formulations resulted in LE residues which were less than 10 µg/l after the 48 h milking. The residue concentration in muscle, fat, liver and kidney tissues had also fallen below the detection limit of 10 µg/kg at 48 h after treatment.

8.3.2 Depletion of levamisole in milk after treatment with the higher dose

The depletion of levamisole after the topical treatment of the dairy cows with 20 mg per kg BW is shown in Figure 8.4. This figure presents the mean measured LE residue concentrations at each sampling point of the different groups of cows. A simulated depletion curve is drawn through the data points. The mean residue concentration values and the standard deviations are also given in the inset-table in this figure. No LE residues could be detected in the blank milk samples. The pattern of the depletion curve is comparable with that obtained in the first experiment with the recommended dose of 10 mg per kg BW. The residue values were approximately threefold those of the first trial. A residue concentration of 862 µg/l was already measured at 1 h after treatment. The highest obtained residue concentration averaged 6027 µg/l and was measured as in the first trial, in the sample collected at 7 h after administration. Archambault *et al*.[18] also obtained a threefold higher residue concentration (2039 µg/l at 4 h instead of 661 µg/l at 5 h after treatment) in the plasma samples of the young animals by doubling the topical treatment dose from 10 to 20 mg per kg BW.

Again, the variability of the residue values in the different groups was high. The average daily milk yield during this trial was 20.9±9.0 kg. In this trial also, no relationship was found between residue concentration and milk yield. The residual levamisole concentration of the last milking, at 76 h after administration, had declined to 83 µg/l.



Time after administration (h)	LE concentration (µg/l)	
	Mean	*s*
0	0	0
1	862	674
3	3600	1495
5	4705	1331
7	6027	2168
15.3	1449	679
17.2	1415	387
19.2	509	230
21.3	863	295
25	625	259
27	563	254
29	221	26
31	331	82
39.3	134	49
41.2	171	43
43.2	82	37
45.3	134	37
49	186	149
51	152	82
53	118	56
55	110	31
63.3	73	48
65.2	113	32
67.2	61	69
69.3	83	25
73	77	35
75	91	43
77	74	39
79	92	41

Figure 8.4 Depletion of levamisole (LE) after topical treatment of dairy cows with a dose of 20 mg per kg body weight

8.3.3 Total excretion of levamisole in milk

The total excretion of the administered levamisole amount via the milk was estimated by means of the milk yields and the residue concentrations. The results for the different treated groups and the two applied doses are summarised in Table 8.1. The obtained total excretion of LE in the milk is very low and varied between 0.25 and 0.49% of the administered dose after treatment with the recommended dose. The highest mean excretion (0.49%) was found in group 2, the group that was treated 3 h before the first milking.

The percentual excretion of LE residues via the milk was not influenced by the route of administration. Österdahl *et al.*[19] calculated a comparable total excretion of 0.3 to 0.7% after intramuscular administration. However, as expected, the highest excretion rates were found in the cows treated at 1 h before the first milking. The absorption of LE and the excretion in the milk was faster after intramuscular than after topical treatment.

Paulson and Feil[8] reported that milk collected from 0 to 48 h after dosing, accounted for 0.2% (oral dose) and 0.6% (subcutaneous dose) of the total ^{14}C activity administered as ^{14}C levamisole. These authors have shown that the urinary and the faecal excretion accounted for respectively 84% and 10% of the supplemented LE dose. No parent LE could be detected in the urine.

In the trial with the dose of 20 mg per kg BW, the estimated total excretion after 76 h of depletion was highest and reached 0.55% of the dosed amount. This excretion was found in group 4, the group that was treated 7 h before the first milking.

Table 8.1 Total excretion of levamisole (% of administered amount) in milk after topical administration

Administered dose (mg/kg BW)	Group			
	1	2	3	4
10	0.25	0.49	0.37	0.38
20	0.26	0.52	0.41	0.55

8.4 Conclusions

The absorption of levamisole in the animal body and the excretion of residues in the milk occurred very rapidly. However, the depletion studies indicate that, when a levamisole treatment is performed for lactating cows, a withdrawal period of 48 h is to short to obtain residue free milk. With the recommended dose of 10 mg per kg body weight, a levamisole residue concentration of 88 µg/l was measured after 48 h of withdrawal.

8.5 Acknowledgements

The authors wish to thank Mr A. Opstal of DVV-CLO (Merelbeke, Belgium) for his collaboration during the animal treatments. The authors are grateful to colleague Dr. Ir. K. Grijspeerdt for the preparation of some figures.

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CHAPTER 9

Disposition and distribution of residues of flubendazole and metabolites in edible tissues of avian species

Relevant publications:

- De Ruyck H., Daeseleire E., Grijspeerdt K., De Ridder H., Van Renterghem R. and Huyghebaert G.
  Determination of flubendazole and its metabolites in eggs and poultry muscle with liquid chromatography-tandem mass spectrometry.
  Journal of Agricultural and Food Chemistry, 2001, 49, 610-617
- De Ruyck H., Daeseleire E., Grijspeerdt K., De Ridder H., Van Renterghem R. and Huyghebaert G.
  Distribution and depletion of flubendazole and its metabolites in edible tissues of guinea fowl.
  British Poultry Science, 2002, submitted

Abstract:

Two separate depletion studies of avian species orally treated with medicated feed containing flubendazole were performed. Flubendazole was administered to turkeys and to guinea fowls in two doses. The concentrations of the flubendazole-derived residues in breast muscle, thigh muscle and liver tissues were determined by a liquid chromatographic-mass spectrometric method.

A first pharmacokinetic study was performed with turkeys. Medicated feed containing 19.9 and 29.6 mg per kg feed was provided in two trials for seven consecutive days. In the trial with the recommended dose of 19.9 mg/kg, one day after the end of the treatment, the mean sum of the residues of flubendazole plus hydrolysed metabolite in thigh and breast muscle tissues declined to around the MRL (50 µg/kg) and was 36.6 and 54.1 µg/kg, respectively. The corresponding values with the higher dose of 29.6 mg/kg were 101.7 and 119.7 µg/kg, respectively.

In the second depletion study, guinea fowls were treated at doses of 56.2 and 86.1 mg per kg feed during seven successive days. The highest residue response was obtained for the reduced metabolite. With the therapeutic dose (56.2 mg/kg), the maximum mean residue concentrations obtained for this compound in breast muscle, thigh muscle and liver tissue were 287.6, 311.5 and 1042.5 µg/kg, respectively. After 24 h of depletion, the sum of the residue concentrations of parent and metabolites in muscle tissue still exceeded 50 µg/kg. After eight days of depletion, flubendazole-derived residues at low concentrations could still be measured in both muscle and liver tissues. Generally, the disposition of residues in breast and thigh muscle tissues was comparable.

The European Commission has not established a MRL for flubendazole in edible tissues of guinea fowl. In contrast, the existing MRLs for other bird species are expressed as the sum of parent flubendazole and its hydrolysed metabolites. It could be questionned whether the established MRLs could serve as a reference to evaluate the safety of flubendazole-derived residues in guinea fowl. A withdrawal period of three days will assure residue safety in the edible tissues of guinea fowl treated with flubendazole at therapeutic dose.

Keywords: flubendazole-derived residues, turkey and guinea fowl tissues, residue depletion
LC-MS/MS analysis

9.1 Introduction

9.1.1 Usage of flubendazole for poultry species

Over the years, benzimidazole anthelmintics have provided unique broad-spectrum efficacy against parasitic helminths and have proved to be extraordinarily safe in practical use.[1] In a variety of animal species, flubendazole has a broad-spectrum activity against nematodes, both adult and larval. This veterinary drug substance is widely used in the veterinary medicine of poultry. Flubendazole is very active against gastrointestinal roundworms, gapeworms and tapeworms in poultry (chickens, turkeys, geese) and game birds (pheasants and partridges), and also in minor species as guinea fowls.[2] The tenicidal properties (activity against tapeworms) of flubendazole are limited compared with those of mebendazole.[3] The introduction of a F-atom at the benzyl ring decreases the toxicity of flubendazole for cestodes compared with mebendazole. However, flubendazole differs from mebendazole by its better tolerance especially in those animal species showing higher sensitivity to mebendazole.[4] Helminth infection is less a problem in poultry reared indoors because of their lack of contact with intermediate hosts.[2] Breeding birds are treated before laying. Rearing birds are dosed three weeks after placing on infected ground, maintenance doses being given every six to eight weeks.

Special attention was spent by Froyman and De Keyser[5] to the safety of flubendazole with regard to egg production and reproductive performance. The medication of breeder chickens does not unfavourably affect egg production, fertility or hatchability. Vanparijs[6] obtained a 100% worm elimination, no consistent change in egg-lay performance and a highly significant increase in hatchability after flubendazole medication of naturally infected geese.

In France, the guinea fowl production reached 57000 tons in 1999, representing only 3% of the total poultry meat production; 36% are produced free-range.[7] Also, in Belgium, guinea fowl production is marginally. Guinea fowls are still reared in management systems and housings were the animals are often kept on deep litter and have to be treated for parasitic infections.[5]

9.1.2 Metabolism of flubendazole

Carbamate hydrolysis is a major metabolic route for several benzimidazole carbamates.[1] The hydrolytic reaction of flubendazole (FL) results in its hydrolysed metabolite (HFL) or 2-aminoflubendazole (Figure 5.1 in Chapter 5). This metabolite is often additionally metabolised in the 5-substituent and is readily eliminated in the urine. The anthelmintic benzimidazoles containing 5-keto substituents, such as flubendazole, also undergo metabolic reduction of the carbonyl group to the corresponding alcohol compound. The flubendazole drug substance is reduced in the animal body to the reduced metabolite of flubendazole (RFL) or hydroxyflubendazole (Figure 5.1 in Chapter 5).

Benzimidazole (BZ) anthelmintics have an extremely limited water solubility.[1] Metabolism (biotransformation) of the BZ drug substances decreases the lipid solubility of the compounds. The obtained metabolites are polar in nature and more water-soluble than the parent compound, and are readily excreted.[8] Kan *et al.*[9] found higher residue fractions for the parent flubendazole and its metabolites in the fatty egg yolk than in the egg white. Metabolites, rather than the parent drug, often dominate in blood, bile, tissues and urine. A small amount of benzimidazole metabolites is bound in relatively non-extractable form to endogenous macromolecules. The principles of the pharmacokinetics with disposition and fate of drugs in the animal body were explained in detail by Baggot.[8] Before entering the systemic blood circulation, an orally administered drug must undergo three events: release from the dosage form, transport across the lipoid mucosal barrier and passage through the liver. In general, the kinetics of drug distribution in blood, organs and tissues depend on dose and route of administration, lipid solubility of the drug, extent of binding to plasma proteins and to extravascular tissue constituents and blood flow through organs and tissues. Differences in penetrating the capillary endothelium (influenced mainly by binding to plasma proteins) and in diffusing across biological cell membranes of the two metabolites of flubendazole, can be an explanation for the disposition and depletion of different metabolites in animals.

Attention has to be spent on drug resistance. As microorganisms for antibiotics,[10] the parasites in the enteric fauna may become drug resistant as a result of ingestion of minute amounts of antiparasitics. Armour[11] reported that resistance or tolerance to BZs has been recorded in areas where the numbers of annual treatments are more numerous than in moderate zones such as Western Europe. This resistance is sometimes incomplete and may be overcome by using higher dosage rates. A good policy is to alternate the anthelmintics used on a farm, provided that the alternation occurs between different generations or populations of worms and that the alternative drugs are belonging to a different chemical group.

9.1.3 Objectives

Treatment of poultry species with flubendazole could be accompanied with residues of the parent drug compound, its hydrolysed metabolite and its reduced metabolite in poultry foodstuffs.[9,12]

The extractable residues of benzimidazole anthelmintics, parent drug and/or free metabolites are chemical molecules with defined toxic potential for which safety margins may be defined.[1] The risks from tissue-bound residues are less well defined. However, some theory suggests that they do not present a risk for the consumer. Some BZs are mutagenic, but the effect is marginal or low in mammals, even at high doses. Teratogenicity can be considered as a general property of these pharmacologic substances. In many cases, teratogenic metabolites have been identified and quantified in animal products as milk, eggs and meat. This means that acceptable

daily intake (ADI) values can be defined and that adequate safety margins can be established for the consumer. Research using radiolabeled drugs has shown that several metabolite residues are uncharacterised and persist in tissue much longer than the parent drug.[13] The direct toxic effects for the consumer due to flubendazole-related residues in meat are negligible, because of the fact that the occuring residue concentrations are relatively low. The toxicity of flubendazole and its two metabolites are comparable. The toxicity of flubendazole still exists for its metabolites as long as the "carbamate" structure stays intact during the metabolism. The lethal dose (LD_{50}) in acute toxicological tests for seven tested laboratory animals, namely rat, mouse, chicken, turkey, pig, dog and sheep reported by Thienpont *et al*.[4] was at least 2560 mg/kg. Besides the teratogenic properties, which were obtained at both administration doses of 40 and 160 mg/kg, Yoshimura[14] observed supplementary significant embryocidal effects in rats after treatment with the highest dose of 160 mg/kg.

The EU MRLs set by Council Regulation EEC/2377/90 and amendments for the sum of FL+HFL as marker residue in muscle tissue and liver tissue of the poultry species chicken, turkey, geese and game birds (pheasants and partridges) are 50 and 400 µg/kg, respectively.[15] The prevention of drug residues in animal food products as meat, eggs and milk has to be a top priority in all activities involved in the food production chain.

Depletion data of flubendazole-derived residues in laying hens after treatment are available in the literature.[9,12] Kan *et al*.[9] investigated the excretion of flubendazole and its hydrolysed and reduced metabolites in eggs of orally treated laying hens. Different medication doses were administered to the hens via the layer feeds. A therapeutic dose (27 mg per kg feed), a low dose (feed carry-over concentration) of 2.6 mg and an intermediate dose of 9.4 mg per kg feed were supplemented. Data on residue formation in eggs and metabolism in laying hens after long-term low-level exposure are not available in the literature. Carry-over of feed additives from former dosed batches to blank feed batches occurs during industrial feed manufacturing and transport. These research workers also measured the distribution of flubendazole-derived residues between egg yolk and egg white. Balizs[12] measured the residue concentration of the parent compound flubendazole depleted in egg yolk and in egg white after oral treatment of laying hens via medicated feed containing a therapeutic dose of 30 mg per kg. Michiels *et al*.[16] reported the pharmacokinetics of flubendazole in animals (rats and dogs) and man. Flubendazole was given orally or by subcutaneous injection to the rats at a dose rate of 40 mg per kg body weight (BW). The dogs were treated intramuscularly with flubendazole once or repeatedly for five consecutive days at a dose concentration of either 2.5 or 25 mg per kg BW.

To our knowledge, no depletion data for flubendazole in muscle tissue of poultry animals, game birds or minor species as guinea fowl are available in the open literature. Although not authorised by the EU and because of its very efficacious anthelmintic properties, flubendazole could be useful for treatment of guinea fowls.

The purpose of this study was to evaluate and to measure the disposition and distribution of residues of flubendazole, its hydrolysed and its reduced metabolites in edible tissues of turkeys and of guinea fowls during and after oral treatment of the animals via medicated feed. The thigh muscle, breast muscle and liver tissues of both avian species were analysed for the flubendazole-derived residues. The animal treatments and the collection of the target tissues were performed at the Department of Animal Nutrition and Husbandry, Section small stock husbandry (DVV-CLO, Merelbeke, Belgium). The residue analyses were carried out at the Department of Animal Product Quality and Transformation Technology (DVK-CLO, Melle, Belgium).

9.2 Materials and methods

9.2.1 Veterinary medicinal products, reagents and chemicals

Coccidiosis was prevented by supplementation of the feed with 1 mg of diclazuril (Clinacox®, Janssen Animal Health, Beerse, Belgium) per kg and with 120 mg of amprolium/ethopabaat (25/1.6, w/w) (Amprol® plus, Merial, Lyon, France) per kg for the turkeys and the guinea fowls, respectively. These veterinary medicinal products were supplemented from day 1 onwards with a minimum withdrawal period of 5 days before slaughtering. Nifursol® premix 2.5% (Duphar, Weesp, The Netherlands) at a dose of 75 mg per kg was supplemented to the feed in order to avoid histomoniasis for both animal species. The animals were treated from day 1 onwards with a withdrawal period of 14 days before slaughtering.

The vaccination of the turkeys and the guinea fowls against Newcastle disease was performed with the living vaccine La Sota (ELD-50,® Intervet, Boxmeer, The Netherlands).

The flubendazole medication of the feed was performed with the commercial formulation Flubenol® 5% (Janssen Animal Health, Beerse, Belgium). There is no withdrawal period prescribed before slaughtering and consuming edible tissues of poultry species treated with Flubenol® 5%.

With exception of formic acid, all reagents and chemicals used in the analytical methods for the quantification of the flubendazole-derived residues in the tissue samples are summarised in paragraph 5.2.1 in Chapter 5. Formic acid was of analytical grade and was obtained from Panreac (Barcelona, Spain).

9.2.2 Apparatus

The commercial formulation of flubendazole was blended in the feed in 3 steps: a 2-step premixer (as a 1 and 5% premix in a Varimixer (Dehaeck, Ghent, Belgium) and a RM premixer (Quintijn, Zulte, Belgium), respectively) and the entire batch vertical mixer (Vrieco, Zelhem, The

Netherlands). Crude protein and crude fat were determined with a Kjeltec (Foss Tecator, Höganäs, Sweden) and a Soxhlet system (Gerhardt, Bonn, Germany), respectively.
The apparatus used for the residue analysis of the tissue samples are described in Chapter 5. A supplementary vacuum evaporator of Büchi (Flawil, Switzerland) was used for the sample preparation of feed and tissue samples of the depletion study with the guinea fowls.

9.2.3 Animal treatments

9.2.3.1 Treatment of turkeys

Day-old turkeys of the BUT 9 heavy medium strain (136 of each gender) were obtained from a commercial hatchery. They were reared separately until 8 weeks of age. Afterwards, males and females were allocated to three pens. The feeding program consisted of a phase-feeding system. Apparent metabolisable energy, nitrogen correction = 0 (AME_n), in MJ per kg and crude protein (CP) in percent in the feed for the day-old animals were 11.5 and 28.0, respectively. At 4, 8 and 12 weeks of age, these composition parameters were respectively, 12.0 and 25.0, 12.2 and 22.0, 12.5 and 19.0. Feed (in mash for the first period and in pellets for the subsequent periods) and water were available at libitum. The turkeys were vaccinated against Newcastle disease at days 1, 21 and 72. In two pens, the turkeys were fed medicated feed containing 19.9±1.4 mg (mean±standard deviation (*s*)) and 29.6±1.4 mg of flubendazole per kg during weeks 13 and 15 of age for females and males, respectively. Feed intake and feed conversion rate were calculated for the respective periods. Three male and three female turkeys were weighed and slaughtered at different ages according to the flubendazole feeding schedule, just before the start, daily during the administration, and 2, 4 and 6 h and 1, 2, 5 and 7 days post administration. At each sampling time the same muscle group of breast and thigh and the liver were removed, frozen and stored at -18°C until investigation.

9.2.3.2 Treatment of guinea fowls

One day-old guinea fowl chicks (136 of each gender) were purchased from a local commercial hatchery. They were reared separately until 6 weeks of age. Afterwards, males and females were allocated to three floor pens, needed for two administration doses and one control group. The feeding programme consisted of a phase feeding system. AME_n in MJ/kg and CP in % of the feed were respectively 11.8 and 22.4 for period 1 to 28 days of age and 12.4 and 20.9 for period 29 days to slaughter. Feed in mash form and water were available at libitum. In order to prevent coccidiosis and histomoniasis, the veterinary drugs were supplemented into the diets. The birds were vaccinated against Newcastle disease at day 1 and 21. In two pens, the healthy birds were fed medicated feed containing 56.2±4.7 mg (mean±*s*) or 86.1±1.6 mg of flubendazole per kg feed during week 9 of age for both females and males (a control diet without any flubendazole was fed to the control group). Three male and three female birds,

taken at random, were weighed and sacrificed at different time points according to the flubendazole feeding schedule, just before the start, daily during the administration period and 2, 4 and 6 h and 1, 2, 5 and 8 days post administration. Each time point, the homogeneous muscle group of breast and thigh and the liver were collected separately and stored frozen at -35°C until investigation.

9.2.4 Growth performance and muscle composition of guinea fowls

The mean feed intake per pen was calculated separately for the observation, the administration and the depletion period. Just before slaughter, the body weight of the birds was noted. The crude protein and the crude fat content of some breast and thigh muscle samples were determined according to the technique of Kjeldahl (calculated as % N X 6.25) and Soxhlet (calculated as the petroleumether extract), respectively.

9.2.5 Analysis of medicated feed samples

9.2.5.1 Medicated feed for turkeys

The concentrations of flubendazole in the medicated feed prepared for the depletion study with the turkeys were determined in the State Analysis Laboratory at Tervuren (Belgium).

9.2.5.2 Medicated feed for guinea fowls

The flubendazole content of the medicated feed material was measured on a homogeneous sample taken before supplementation as well as on the remainder after cessation of the administration. To determine the actual flubendazole dose, 1 g of ground medicated feed sample was weighed into a centrifuge tube of 50 ml. The FL and/or internal standard (IS) solutions were added at this stage. Ten ml of methanol was added and the mixture was homogenised by vortex mixing. The analyte extraction was performed on a shaker (amplitude: 30 mm, frequency: 90/min) for 15 min. Afterwards, a clean-up of the sample mixture by centrifugation at 5000 rpm during 5 min was carried out. The organic upper layer was filtered through a 0.2 µm regenerated cellulose (RC) Chromafil filter (Macherey-Nagel, Düren, Germany) into an autosampler vial. Ten µl of filtrate was injected into the LC system and the flubendazole concentration was determined with the LC-MS/MS determination procedure described in the next section.

9.2.6 Flubendazole-derived residue determination method

9.2.6.1 Tissue samples of turkeys

The residue analysis of the edible tissue samples of the depletion study with the turkeys were performed with the LC-MS/MS method for muscle and liver tissue samples described in Chapter 5. For the incurred turkey tissue samples, the IS was spiked to a concentration of 50 µg/kg.

9.2.6.2 Tissue samples of guinea fowls

9.2.6.2.1 Introduction

The residue concentrations of the tissue samples of the depletion study with the guinea fowls were quantified with an optimised LC-MS/MS method.

9.2.6.2.2 Standard solutions

Analytical standard material of the analytes were dissolved in 16 ml of dimethyl sulphoxide. The solutions were further diluted with methanol to stock standard solutions of 0.1 mg/ml which were stored refrigerated at 5°C and which were replaced every two months. A stock standard solution of 0.2 mg/ml flubendazole and the IS were prepared for the analyses of the medicated feed samples. The working standard solutions were made immediately before use by dilution with mobile phase consisting of 50/50 (v/v) acetonitrile and water containing 0.1% formic acid. Solutions of 1 µg/ml were used for tuning the mass spectrometer and for acquisition of the analyte identification spectra. During the preparation of matrix calibration samples, the analytes were spiked using a mixed standard solution of 0.01, 0.1, 1 and 5 µg/ml for concentrations up to 1, 5, 100 and above 100 µg/kg, respectively. The IS was added to the calibration samples and to the incurred samples using a standard solution of 1 µg/ml.

9.2.6.2.3 Sample preparation

9.2.6.2.3.1 Breast and thigh muscle tissue

One gram of ground guinea fowl muscle tissue sample was weighed into a beaker of 50 ml. The analyte and/or IS solutions were added at this stage. The spiked samples were left for 30 min. Ten ml of water was added and the tissue was homogenised for 15 s at 8000 rpm. The sample mixture was transferred quantitatively into a centrifuge tube of 50 ml. The beaker and the homogeniser rotor were rinsed twice with 10 ml of ethyl acetate, the extraction solvent. The rinse solutions were transferred into the tube. The mixture was made alkaline with 5 ml of 0.1 M sodium hydroxide and a pH of approximately 11.3 was obtained. The extraction was activated on a shaker (amplitude: 30 mm, frequency: 90 per min) for 10 min. After separation by centrifugation at 5000 rpm for 5 min, the supernatant was transferred into an evaporation tube. The extraction procedure was repeated once on the centrifugate with 10 ml of ethyl acetate.

The collected organic fractions were evaporated to 2-4 ml with the vacuum evaporator ((260±10) X 10^2 N/m^2, 60°C, 200 rpm). The remainder was transferred quantitatively into a graduated tube. The evaporation tube was rinsed with 2 ml of ethyl acetate and the rinse solution was also transferred into the graduated tube. The remaining extract was evaporated to dryness under a stream of nitrogen in a water bath at 60°C. The residue was dissolved in 600 µl mobile phase consisting of acetonitrile and water containing 0.1% formic acid (50/50, v/v). The mixture was vortex mixed. The concentrated drug residue solution was heated at 60°C in a water bath for 5 min. After vortex mixing, the extract was defatted with 2 ml of *n*-hexane. After standing for 5 min, the hexane upper layer was removed and the tube was placed in a water bath at 60°C for 2 min. After cooling down to room temperature, the solution was made up to 1 ml with mobile phase. The final residue solution was homogenised by vortex mixing and filtered through a 0.2 µm RC Chromafil filter into an autosampler vial.

9.2.6.2.3.2 Liver tissue

One gram of ground guinea fowl liver tissue sample was weighed directly into a centrifuge tube of 50 ml. The analyte and/or IS solutions were added at this stage. The spiked samples were left for 30 min before the extraction of the benzimidazole compounds was performed.

The next steps were identical to those of the sample pretreatment of muscle tissue as described in paragraph 9.2.6.2.3.1.

9.2.6.2.4 Liquid chromatographic–mass spectrometric analysis

The liquid chromatographic separation and mass spectrometric detection (LC-MS/MS) of the flubendazole-derived residues were similar with the presented LC-MS/MS methods for determination of residues of anthelmintics in milk and of mebendazole-derived residues in sheep tissues, described in Chapters 4 and 6, respectively.

The LC runs were performed at room temperature on a reversed-phase column with a mobile phase consisting of acetonitrile (B) and water containing 0.1% formic acid (to increase the ionisation intensity) (A). The elution gradient programme consisted of 65A:35B to 50A:50B (0-0.1 min), 50A:50B to 25A:75B (0.2-3 min), 25A:75B (4-5 min), 25A:75B to 50A:50B (6-7 min), 50A:50B to 65A:35B (8-15 min) and 65A:35B (16-25 min). A volume of 30 µl of the cleaned sample extracts was injected into the LC apparatus.

Atmospheric pressure electrospray ionisation in the positive ion mode (ESI^+) was applied with the tandem quadrupole mass spectrometer. The LC eluent flow of 0.25 ml/min was sprayed directly into the mass spectrometer interface, without splitting. The analytes were detected by tandem mass spectrometry using the multiple reaction monitoring (MRM) function of two transitions. The optimisation of the transition of the molecular precursor ion to the two most abundant product ions was performed.

The generated data of the quantitative determinations by MRM of the transition from the precursor ion to the most abundant product ion were evaluated by an internal standard procedure based on matrix calibration curves and calculated automatically by the MASSLYNX software version 3.3. The calibration curves were obtained using at least five concentration points, including the zero level. They were calculated always using the best fit of two replicated determinations per concentration level.

9.2.6.2.5 Determination of the analytical limits

The limits of detection (LODs) and limits of quantification (LOQs) were determined according to the EU requirements for drug residue analyses recently published by the EC.[17] LOD and LOQ were calculated by performing a validation criterion for determination of the decision limit and the detection capability, respectively.

The LOD values are defined as the mean values of the calculated concentrations by determining at least 20 blank tissue samples spiked at the internal performance limit (IPL) chosen at 1 µg/kg with the analytes plus 1.64 times the corresponding standard deviations. The LOD values for muscle tissue were determined with fortified blank guinea fowl breast muscle tissue samples. The LOD values for liver tissue were determined with spiked blank liver tissue samples.

The LOQ values were calculated as the respective LOD values plus 1.64 times the corresponding standard deviations of the measured concentrations by determining at least 20 blank breast muscle and liver tissue samples spiked at the respective LOD.

9.3 Results

9.3.1 Rearing of the animals

9.3.1.1 Turkeys

The turkeys were reared from 25 November 1999 until 16 March 2000. The experimental time could be divided into three successive periods. During the observation or acclimatisation period, some female and male turkeys were slaughtered and breast and thigh muscle tissue and the liver tissue were sampled for analysing as blank controls. The turkeys were treated with the medicated feed for seven consecutive days during the administration period. The trial schedule was made so that the last group of turkeys was slaughtered when these birds were 13 and 15 weeks old for female and male, respectively, which is the normal practical rearing period for turkeys. After the anthelmintic treatment, the depletion of the residues was observed until seven days after cessation of the administration.

During the animal trials, all turkeys were in good general health as indicated by the absence of any clinical sign. The growth results were in line with the target of BUT. The 12-week body weights were 12.7 and 7.6 kg for males and females, respectively. The corresponding feed

conversion rates were about 1.84 and 1.91 for males and females, respectively. The treatment with flubendazole did not influence daily feed intake and average values of 530 and 290 g per day were obtained for males and females, respectively.

The mean body weights just before slaughter in the trial with the recommended dose of 19.9 mg per kg feed were 8.1 and 12.9 kg for females and males, respectively. The mean BWs in the depletion study with the medicated dose of 29.6 mg per kg feed were 8.1 and 12.5 kg respectively, for females and males.

9.3.1.2 Guinea fowls

The guinea fowls were reared from 21 January 2002 until 2 April 2002. The trial schedule was made so that the last group of eight guinea fowls were 10 weeks old at the time of slaughter, which is the normal practical rearing period for this avian species. Because the slaughter weights for females and males were supposed to be comparable, the trials for both genders were performed parallel.

The animals were observed for one week before the birds were treated with the antiparasitic drug. During this acclimatisation period, two female and two male guinea fowls were slaughtered and breast and thigh muscle tissue and the liver tissue were sampled for analysing as blank controls. The medicated feeds were administered to the guinea fowls during seven

Table 9.1 Mean feed intakes (g per bird per day) obtained per pen

Birds	Period	Control group	Dose 56.2 mg/kg	Dose 86.1 mg/kg	Mean
Females	Acclimatisation	158.4 (*n* = 24)	131.0 (*n* = 52)	135.5 (*n* = 52)	141.6
	Administration	169.0 (*n* = 22)	144.3 (*n* = 52)	169.3 (*n* = 52)	160.9
	Depletion	115.6 (*n* = 15)	128.2 (*n* = 31)	128.2 (*n* = 31)	124.0
Males	Acclimatisation	135.7 (*n* = 24)	135.5 (*n* = 52)	130.0 (*n* = 52)	133.7
	Administration	129.3 (*n* = 22)	160.1 (*n* = 52)	139.9 (*n* = 52)	143.1
	Depletion	138.9 (*n* = 15)	113.8 (*n* = 31)	115.6 (*n* = 31)	122.8

n = maximum number of guinea fowls per pen

Table 9.2 Body weights (mean±*s*, kg) of the guinea fowls per pen just before slaughter during the whole experimental period

Period	Day	Control group (*n* = 1)		Dose 56.2 mg/kg (*n* = 3)		Dose 86.1 mg/kg (*n* = 3)		Female (mean, *n* = 7)	Male (mean, *n* = 7)
		Female	Male	Female	Male	Female	Male		
Acclimatisation	-7	1.165±0.106[a]	1.175±0.021[a]	-	-	-	-	1.165[a]	1.175[a]
Administration	0	1.390	1.600	1.463±0.101	1.410±0.066	1.470±0.026	1.440±0.159	1.456	1.450
	1	1.680	1.610	1.530±0.010	1.593±0.257	1.477±0.116	1.523±0.025	1.529	1.566
	2	1.640	1.578	1.670±0.096	1.630±0.159	1.393±0.198	1.553±0.107	1.547	1.590
	3	1.500	1.500	1.490±0.121	1.497±0.084	1.443±0.095	1.623±0.064	1.471	1.551
	4	1.590	1.700	1.617±0.067	1.587±0.117	1.547±0.140	1.730±0.030	1.583	1.664
	5	1.480	1.350	1.737±0.237	1.493±0.112	1.477±0.149	1.607±0.180	1.589	1.521
	6	1.780	1.700	1.600±0.050	1.547±0.221	1.627±0.146	1.567±0.104	1.637	1.577
Depletion	7	1.540	1.700	1.700±0.236	1.777±0.115	1.800±0.182	1.687±0.087	1.720	1.727
	7.08	1.680	1.750	1.667±0.021	1.617±0.076	1.500±0.176	1.780±0.115	1.597	1.706
	7.17	1.460	1.750	1.770±0.040	1.880±0.157	1.707±0.117	1.813±0.023	1.699	1.833
	7.25	1.610	1.680	1.640±0.266	1.777±0.237	1.710±0.123	1.663±0.050	1.666	1.714
	8	1.670	1.650	1.560±0.165	1.723±0.090	1.533±0.123	1.670±0.062	1.564	1.690
	9	1.670	1.650	1.680±0.121	1.740±0.147	1.763±0.119	1.753±0.253	1.714	1.733
	12	1.690	1.750	1.957±0.268	2.003±0.087	1.930±0.075	1.863±0.055	1.907	1.907
	15	1.610	2.250	2.013±0.116	2.117±0.136	1.970±0.089	2.200±0.035	1.937	2.171

[a] *n* = 2

consecutive days. The excretion of flubendazole-derived residues was measured already during the treatment. The flubendazole contents of the remaining medicated feed materials were 61.3±1.9 (mean±s) and 87.2±4.2 mg of flubendazole per kg feed for the recommended and the higher dose, respectively. After cessation of the administration, the depletion of the residues was observed for eight days.

During the whole experimental period, the housing temperature could be kept relatively constant and ranged from 18.0 to 22.5°C. All guinea fowls were in good general health as indicated by the absence of any clinical sign. The mean feed intakes per pen obtained during the acclimatisation, the administration and the depletion period are summarised in Table 9.1.

The feed intakes per animal varied from 115.6 to 169.3 g/day for females and from 113.8 to 160.1 g/day for males. The increase in feed intake from observation to administration period during the rearing seemed normal for growing guinea fowls. The administration of flubendazole did not influence the daily feed intakes.

Table 9.2 presents the mean body weights of the birds per pen obtained just before slaughter during the whole experimental period.

Five remaining guinea fowls out of the trials of each gender were sampled to measure fat and crude protein composition of breast and thigh muscle tissue. For females and males, a same protein and fat content of 19.9 and 2.9% respectively, was measured in the homogenised and mixed breast muscle sample. The composition of thigh muscle was quite different. The protein and fat contents were 18.7 and 8.5% and 19.1 and 7.1% for females and males, respectively.

9.3.2 Depletion of residues of flubendazole and metabolites in animal tissues

9.3.2.1 Depletion study with turkeys

9.3.2.1.1 Disposition and distribution of residues in tissues of turkeys treated with the recommended dose

The results of the depletion study with the recommended dose of 19.9 mg per kg feed are shown in Figure 9.1, which represents the mean FL and HFL residue values in breast and thigh muscle tissues of turkeys during and after the administration. In some breast and thigh muscle tissue samples, residues of the reduced metabolite of flubendazole could be detected in concentrations around the LOD. These residue values are not shown in Figure 9.1.

In the blank muscle tissue samples, no flubendazole-derived residues could be detected. The shown residue values in Figure 9.1 are mean values obtained from six turkeys, three males and three females. The residue values of HFL were much higher than these of FL in both the thigh and breast muscle. Figure 9.1 shows a clear trend of higher residue concentration in thigh muscle.

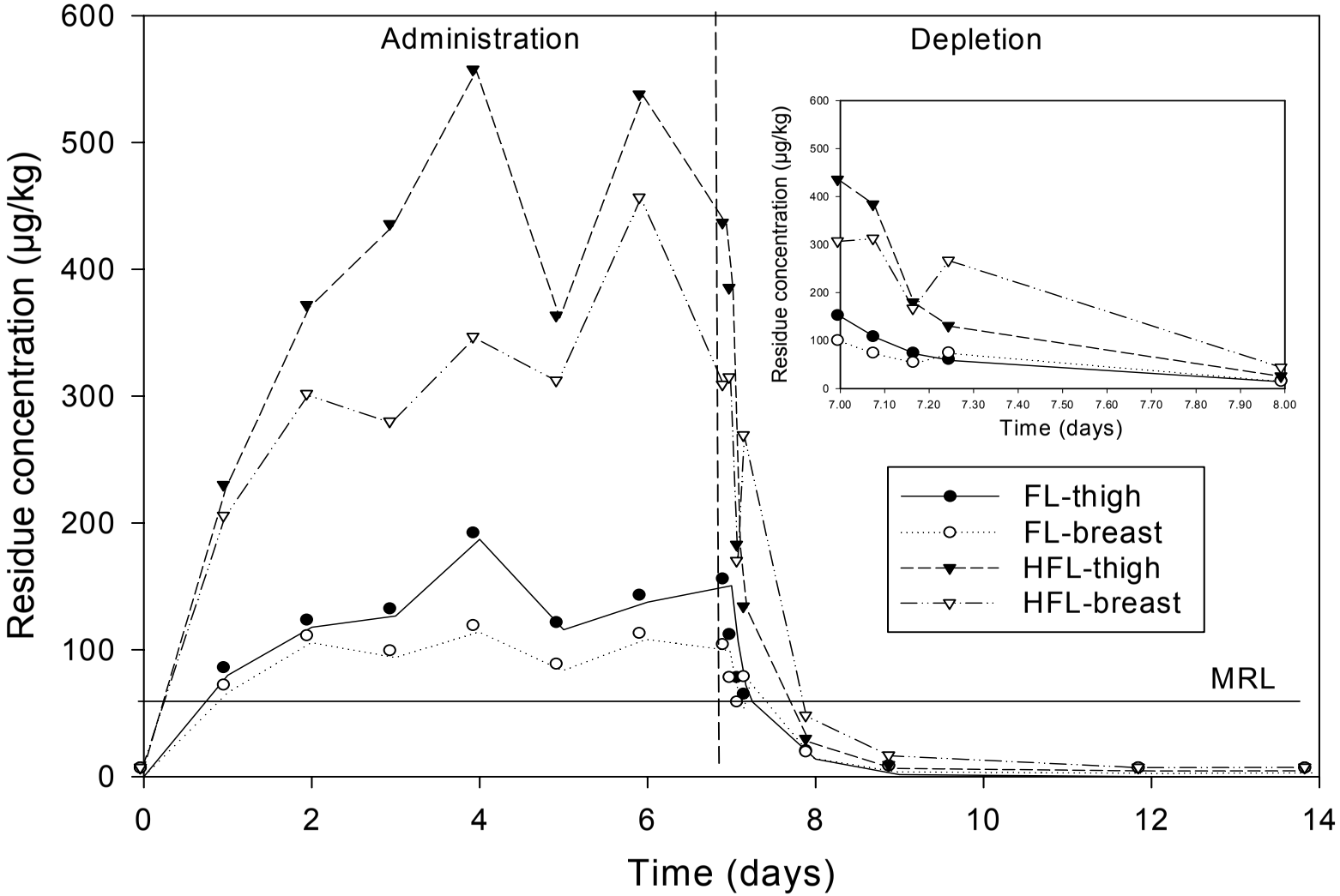

Figure 9.1 Depletion of residues of flubendazole (FL) and its hydrolysed metabolite (HFL) in turkey breast and thigh muscle tissues during and after oral administration of 19.9 mg of flubendazole per kg feed for seven successive days

After one day of treatment, the sum of the residue concentrations of FL plus HFL was already above the MRL value (50 µg/kg). The concentrations in thigh and breast muscle tissue were respectively, 304.8 and 266.6 µg/kg. Some liver tissue samples were also analysed at this time point and the residues were quantified with calibration curves obtained with spiked blank liver tissue samples. The residue response in liver tissue was very high and reached a concentration of 8304.6 µg/kg.

A mainly steady state situation in residue concentration was obtained after four days of administration. The maximum concentration for the sum of residues of FL plus HFL in the thigh and breast muscle tissues were respectively, 742.8 and 561.1 µg/kg and were obtained after respectively, four and six days of treatment. The measured residue value in liver tissue after four days of administration was 10604.2 µg/kg. On day 7, just before the treatment was stopped, this residue value was 10275.7 µg/kg.

The decline in residue concentration after cessation of the flubendazole administration is very rapid. After one day of withdrawal, the residue concentration already decreased to a level around the MRL value. To show clearly the decline of the residue concentration on the first day after the medication, a detailed inset-figure is given inside in Figure 9.1. As already mentioned, the drug residue excretion in breast muscle was somewhat slower. The sum of the residue concentrations of FL plus HFL in thigh and breast muscle tissue was respectively 36.6 and 54.1 µg/kg. A residue concentration of 916.9 µg/kg was found in liver tissue. After five days of withdrawal, the flubendazole-derived residue depletion in muscle tissue was negligible and no residues could be detected anymore in the muscle tissue samples.

The variability of the residue values of the six turkeys taken per sampling time was high and is probably caused by physiological differences. The most important differences between the individual turkeys are the biodisponibility and the rates and routes of metabolism and excretion of the parent drug and the metabolites. The variability of the residues in thigh muscle tissue per sampling time, expressed as relative standard deviation (s_r), for FL and for HFL ranged (mean values in parentheses) from 15.3 to 81.5% (36.5) and from 12.1 to 74.3% (40.4), respectively. These ranges in breast muscle tissue were respectively 20.0 - 50.1% (32.8) and 18.9 - 72.7% (38.7).

9.3.2.1.2 Disposition and distribution of residues in tissues of turkeys treated with the higher dose

The results of the depletion study with turkeys treated with the higher dose of 29.6 mg per kg are shown in Figure 9.2. Again, the depletion curves are drawn with mean residue values for FL and HFL in thigh and breast muscle tissues of six turkeys, three females and three males. No residues could be detected in the blank muscle tissue samples. No relationship could be found between residue concentration and body weight. The same conclusions as for the first trial with

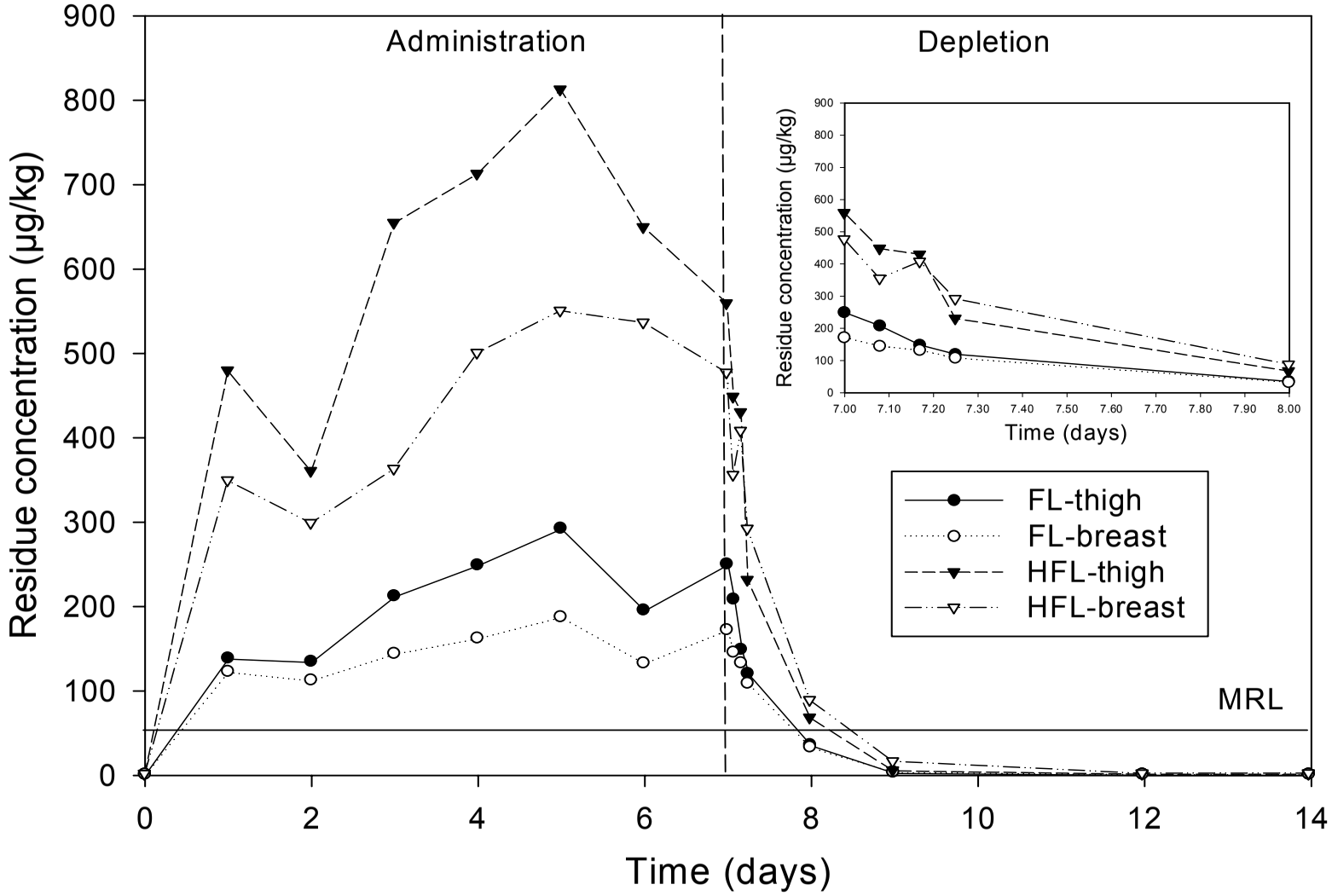

Figure 9.2 Depletion of residues of flubendazole (FL) and its hydrolysed metabolite (HFL) in turkey breast and thigh muscle tissues during and after oral administration of 29.6 mg of flubendazole per kg feed for seven successive days

the recommended dose could be made. The shape of the depletion curves for the two trials were very similar. As expected, the residue concentrations of this trial with the higher dose were higher. The obtained residue concentrations for HFL were much higher than those for FL in both thigh and breast muscle tissues. Again, a clear trend of a higher residue concentration for both FL and HFL in thigh muscle tissue compared to the breast muscle tissue was observed.

After one day of treatment, the sum of the residue concentrations for FL plus HFL in thigh and breast muscle tissue was respectively, 616.6 and 470.2 µg/kg. A residue concentration of 14206.7 µg/kg was already measured in liver. The maximum concentrations for the marker residues of FL plus HFL in thigh and breast muscle tissues were respectively, 1104.3 and 736.5 µg/kg and were obtained after five days of treatment. At this time, this residue value in liver tissue was 18127.0 µg/kg. After one day of withdrawal, the residue concentrations of FL plus HFL in thigh and breast muscle tissues had dropped to respectively, 101.7 and 119.7 µg/kg. The residue value in liver had declined to 2333.9 µg/kg. The detail inset-figure in Figure 9.2 clearly shows the drop of the residue concentration the first withdrawal day. Generally, the depletion of the drug residues in breast muscle tissue was somewhat slower. Seven days after treatment of the turkeys, a low mean HFL residue concentration of 0.6 µg/kg could be detected in breast muscle tissue.

The fluctuation on the individual residue values was high. The variability of the residues in thigh muscle tissue for one sampling time expressed as s_r value respectively for FL and HFL ranged (mean values in parentheses) from 17.9 to 67.6% (39.5) and from 22.9 to 65.8% (40.2). These ranges in breast muscle tissue were, respectively, 11.0 - 56.7% (31.7) and 9.5 - 63.8% (35.9).

9.3.2.2 Depletion study with guinea fowls

9.3.2.2.1 Analytical method

The monitored protonated ions and the optimised MS operating parameters for the determination method of FL, HFL, RFL and the IS are given in Table 9.3.

The residue concentrations in the tissue samples were calculated using matrix calibration curves drawn with calibration points of 0, 1, 5, 25, 100, 250, 500 and 800 µg/kg. For each calibration point, two blank tissue samples were fortified and only the samples giving the best fit were used for the calibration curve. When residue concentrations above this range were expected in the incurred samples, these were diluted twice by weighing 0.5 g of tissue sample instead of 1 g. An example of a LC-MS/MS chromatogram of a breast muscle sample of a female guinea fowl sampled at day 3 of the administration period with the higher dose is shown in Figure 9.3. Chromatographic separation of the two metabolites was not needed. Because of the different *m/z* value, the two co-eluting compounds could be separated in the mass spectrometer. The analytes and the IS were eluted within 8 min.

Table 9.3 Summary of the diagnostic ions and the MS operating parameters

Analyte	Precursor ion (*m/z*)	Product ions (*m/z*)	Cone voltage (*V*)	Collision energy (*eV*)
FL	314.4^{+}	282.3^{+a}, 123.1^{+}	35	30
HFL	256.2^{+}	122.7^{+a}, 112.9^{+}	45	32
RFL	316.4^{+}	160.0^{+a}, 284.4^{+}	35	35
IS	330.3^{+}	138.5^{+a}, 298.3^{+}	35	30

[a] most abundant product ion FL: flubendazole

HFL and RFL: hydrolysed and reduced metabolites of flubendazole

IS: internal standard, methyl [5-(4-chlorobenzoyl)-1*H*-benzimidazole-2-yl] carbamate

Fairly, the LC-MS/MS tissue residue quantification method is very selective, very specific and low detection limits can be obtained. The three benzimidazole compounds could be detected at residue concentrations between 1 and 3 µg/kg. The measured limits of detection and limits of quantification in muscle tissue were 1.9, 1.7 and 2.7 µg/kg and 4.5, 2.3 and 3.6 µg/kg for FL, HFL and RFL, respectively. These analytical limit values in liver tissue were 1.6, 1.1 and 1.7 µg/kg and 2.6, 1.5 and 2.6 µg/kg, respectively. No endogenous interferences with residues of the medication with amprolium-ethopabaat, Nifursol® and ELD-50® vaccine were observed.

9.3.2.2.2 Disposition and distribution of residues in edible tissues

9.3.2.2.2.1 Introduction

The results of the disposition and depletion of residues of flubendazole and metabolites in the guinea fowl tissues during and after the administration of flubendazole are shown in Figures 9.4, 9.5, 9.6 and 9.7. The residue curves were drawn with mean residue values of six guinea fowls, as prescribed by European drug residue legislation. At each time point, three treated females, three treated males, one control untreated female and one control untreated male were sampled. All the control muscle and liver tissue samples were free of flubendazole-derived residues.

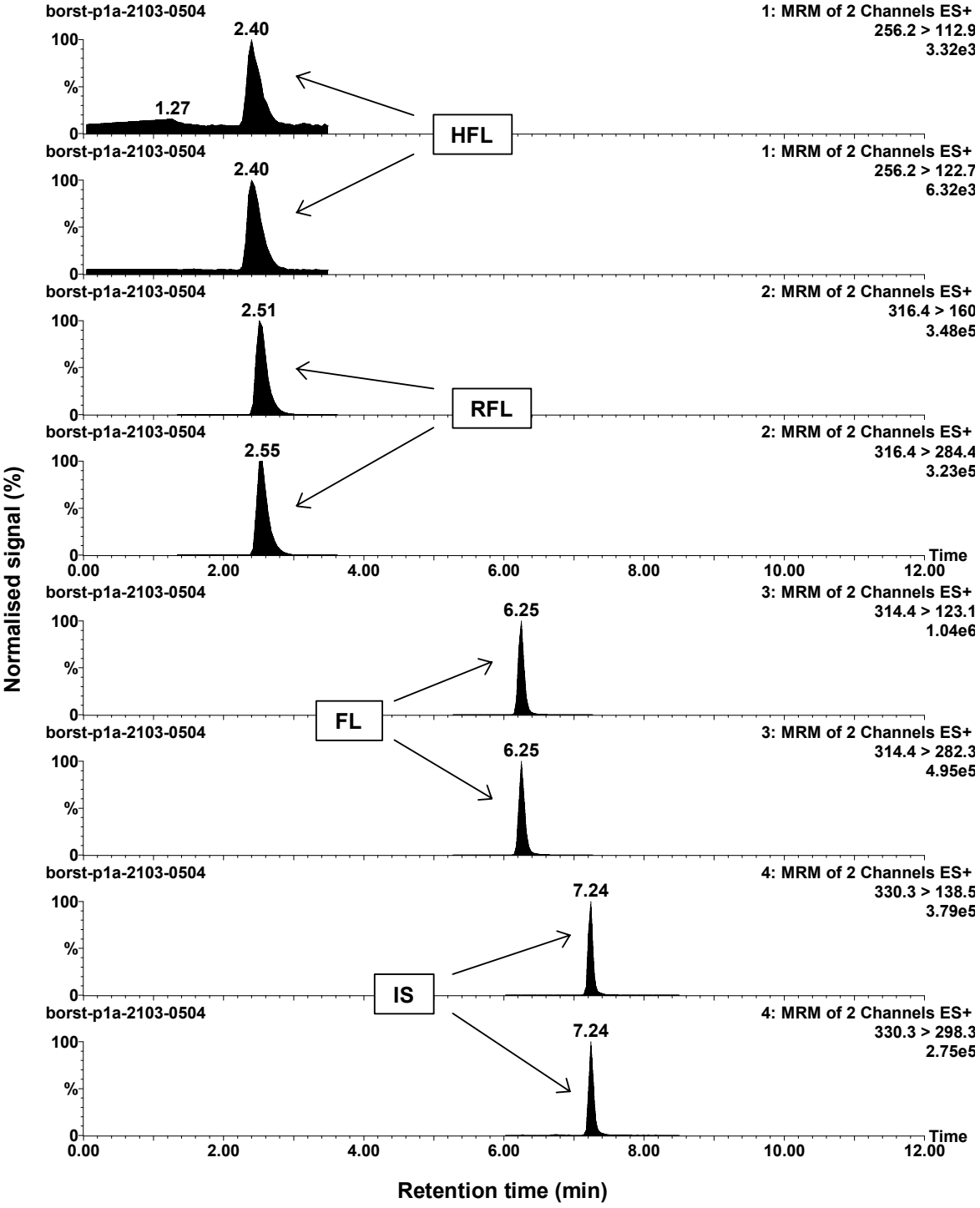

Figure 9.3 LC-MS/MS chromatogram of a breast muscle tissue sample of a female guinea fowl with residue concentrations of flubendazole (FL), the hydrolysed (HFL) and the reduced metabolite (RFL) of 154.3, 2.9 and 381.3 µg/kg, respectively. The internal standard (IS) was fortified at a concentration of 100 µg/kg.

9.3.2.2.2.2 Disposition in muscle tissue

9.3.2.2.2.2.1 Trial with the therapeutic dose

Figure 9.4 illustrates the residue concentration curves in breast and thigh muscle tissues during and after the administration with medicated feed at the therapeutic dose of 56.2 mg/kg. Residue values for FL and for RFL are given in Figure 9.4. The hydrolysed metabolite residues in the muscle tissues are omitted because the response of HFL was very low and varied around the detection limit. The MRL concentration of 50 µg/kg for muscle tissue in target poultry species is indicated in Figure 9.4 as reference. The disposition of flubendazole-derived drug residues in the muscle tissues starts very rapidly after the start of treatment. After oral administration of flubendazole, a large fraction of flubendazole was metabolised to the reduced metabolite. After one day of medication, considerable high residue concentrations for RFL and FL of 287.6 and 97.0 µg/kg, respectively in breast muscle tissue and of 180.6 and 54.7 µg/kg, respectively in thigh tissue were already observed. A plateau residue concentration was then already obtained for both analytes and in both muscle tissues. This steady state concentration remained during the whole administration period. The difference in residue concentrations between the two types of muscle tissues for both FL and RFL was negligible. During the administration period, the residue concentration for RFL varied between 136.8 µg/kg (measured on day 6) and 311.5 µg/kg (measured on day 3). This range for FL was between 28.7 µg/kg (measured on day 4) and 113.7 µg/kg (measured on day 7, just before the administration was stopped). All these values were measured in thigh muscle tissues. The considerable decline in residue concentration of both compounds and in both muscle tissues from experimental days 3 until 5 was surprising.

The decline in residue concentration after cessation of medication was very rapidly. To clearly illustrate the decline of residue concentration on the first day after cessation of the administration, a detailed figure is given inside Figure 9.4. After one day of depletion, on experimental day 8, the marker residue concentration of the sum of FL plus HFL was already lower than 50 µg/kg. However, the highest residue concentration was still 80.6 µg/kg for RFL and 14.6 µg/kg for FL in thigh and breast muscle tissue, respectively. After eight days of depletion, on experimental day 15, the highest residual concentration for RFL was still 13.5 µg/kg, observed in breast muscle tissue. No residues for FL could be detected in the analysed muscle tissues.

During this depletion study, the variability on the residue values of the six individual animals per sampling time was high and is probably caused by physiological differences. Important differences between the individual animals are the biodisponibility and the rates and the routes of metabolism and excretion of the parent drug and its metabolites. The variability on the individual residues in thigh muscle tissue per sampling time expressed as relative standard deviation (s_r) (mean values in parentheses) varied from 29.7 to 87.0% (46.8) and from 13.7 to

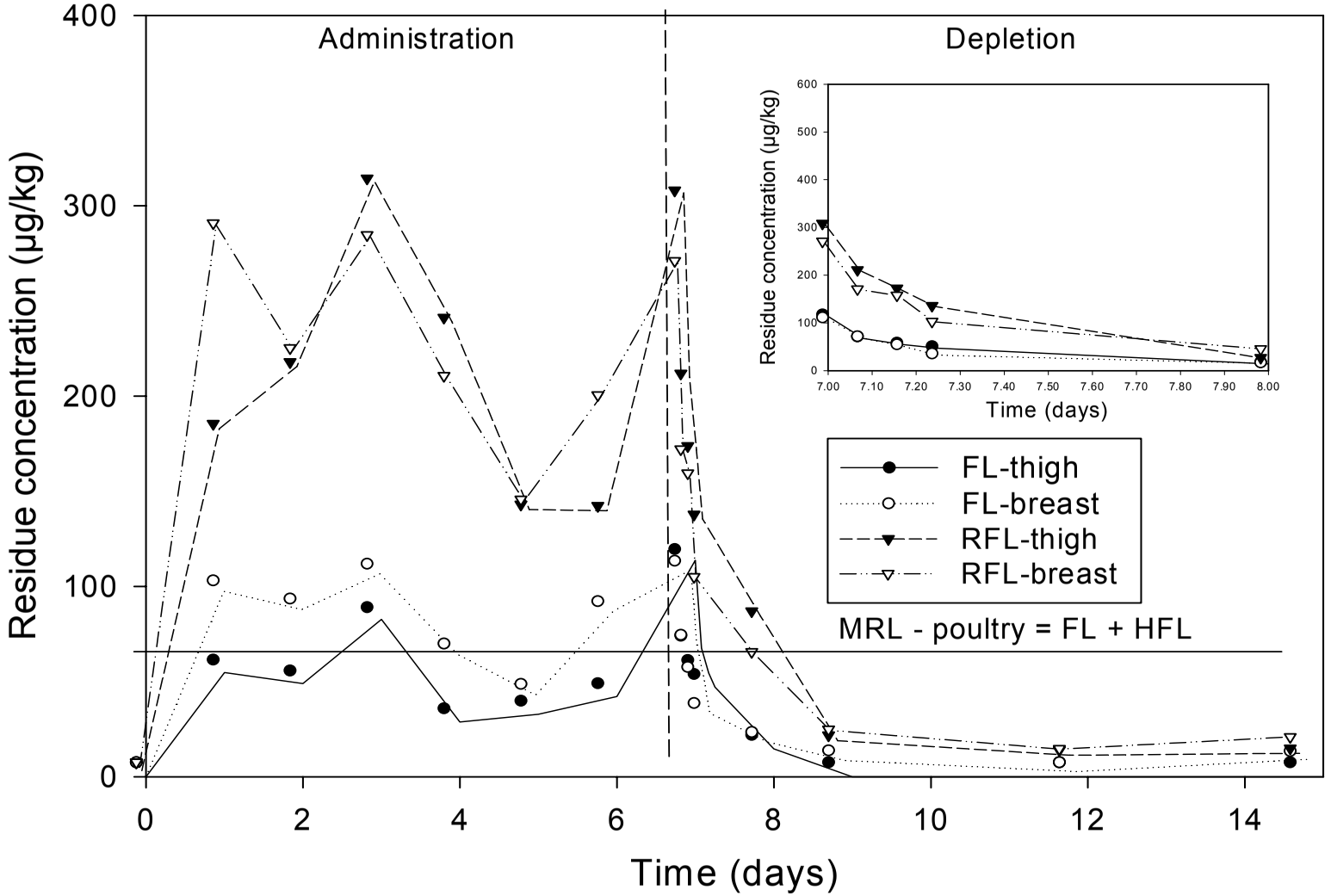

Figure 9.4 Disposition of residues of flubendazole (FL) and its reduced metabolite (RFL) in guinea fowl breast and thigh muscle tissues during and after oral administration of 56.2 mg of flubendazole per kg feed during seven successive days

61.8% (31.8) for FL and RFL, respectively. These s_r ranges for breast muscle tissue were 19.1 - 63.1% (34.4) and 11.9 - 66.5% (35.0), respectively.

9.3.2.2.2.2.2 Trial with the higher dose

The results of the depletion study for FL and RFL in the muscle tissues of the guinea fowls medicated at the higher dose of 86.1 mg per kg feed are presented in Figure 9.5. Nearly the same trends in depletion of residues could be observed as for the experiment with the therapeutic dose. Obviously, the measured residue concentrations were higher. During the administration of the veterinary drug, the steady-state concentration for RFL varied between 263.3 µg/kg (measured on day 5) and 401.2 µg/kg (measured on day 1). Both values were measured in breast muscle tissue. The residue concentration for FL ranged between 58.5 µg/kg (measured in thigh muscle tissue on day 1) and 161.5 µg/kg (measured in breast muscle tissue on day 7, just before the administration was stopped). During this experiment, only a decline in residue concentration of RFL in breast muscle from day 1 until day 5 could be observed. The residue values for both FL and RFL and in both sampled muscle tissues were comparable. The residues of HFL were slightly higher than those obtained with the lower medication dose. The highest concentration reached 4.3 µg/kg and was obtained on day 7. The values for this metabolite were omitted also in Figure 9.5. As reference, the MRL concentration of 50 µg/kg for muscle tissue in target poultry species is indicated in the figure.

Again, the decline of residue concentration after cessation of the administration is very rapidly. A detailed figure is given inside Figure 9.5, to show more clearly the decline tendency of residue concentration the first withdrawal day. On experimental day 8, after 24 h of depletion, the highest residue values measured for RFL and FL were 85.4 and 14.3 µg/kg, respectively. These residue values were obtained in thigh and breast muscle tissue, respectively. After eight days of depletion, a residue concentration of 18.1 µg/kg for RFL was obtained in breast muscle tissue. Residues of FL could be quantified in this kind of muscle tissue only and at a concentration of 8.5 µg/kg.

Again, the variation on the residue values of the individual guinea fowls was high. The variability on the individual residues in thigh muscle tissue per sampling time expressed as s_r value varied (mean values in parentheses) from 21.0 to 63.4% (31.8) and from 10.2 to 51.0% (26.9) for FL and RFL, respectively. These s_r ranges for breast muscle tissue were between 10.9 and 52.0% (29.5) and between 14.2 and 54.5% (34.2), respectively.

9.3.2.2.2.3 Disposition in liver tissue

9.3.2.2.2.3.1 Depletion of the reduced metabolite of flubendazole

The highest residue response for the reduced metabolite of flubendazole was obtained in liver tissue. The disposition of residues of RFL in liver tissue of guinea fowls treated with the two

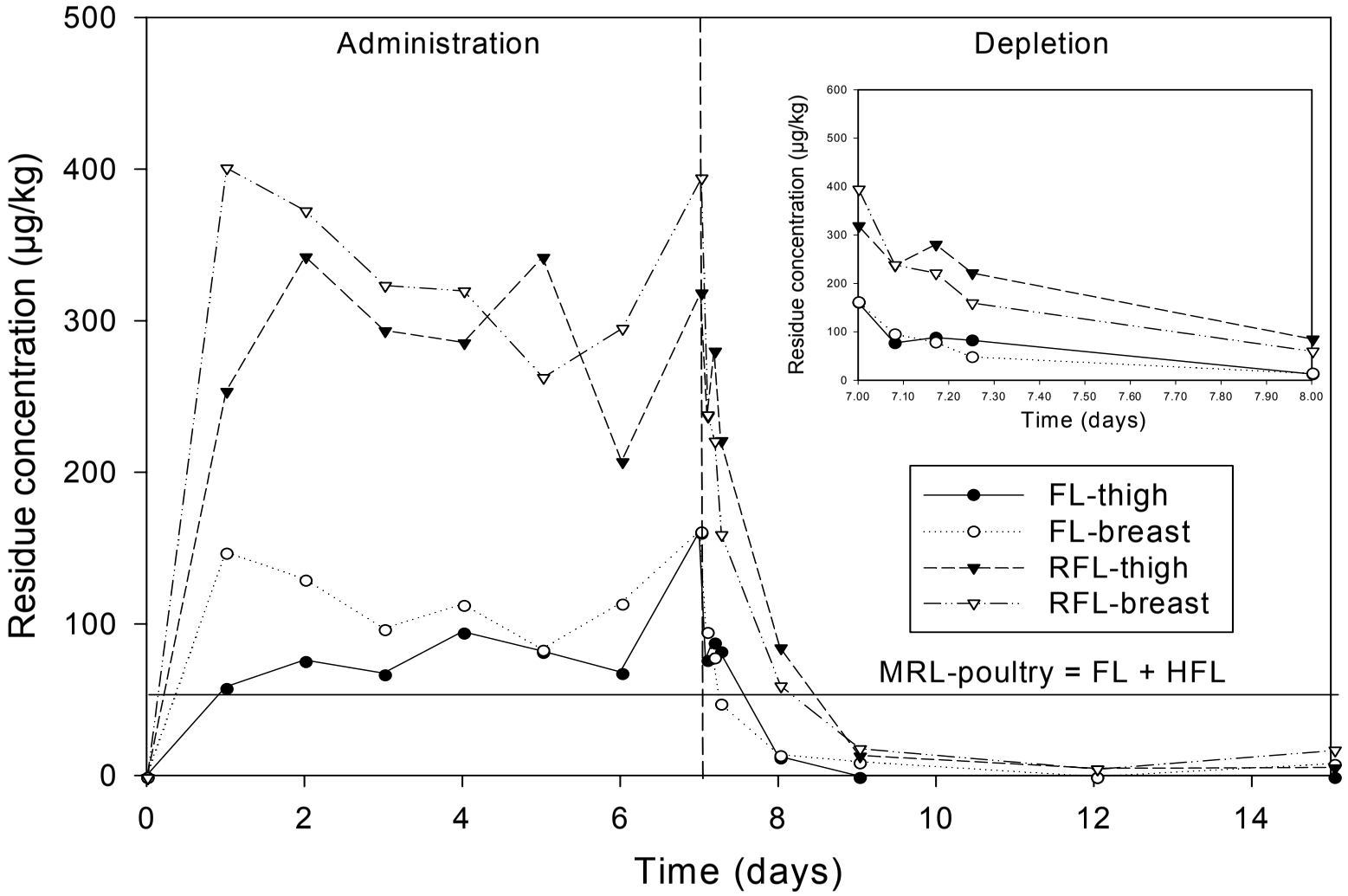

Figure 9.5 Disposition of residues of flubendazole (FL) and its reduced metabolite (RFL) in guinea fowl breast and thigh muscle tissues during and after oral administration of 86.1 mg of flubendazole per kg feed during seven successive days

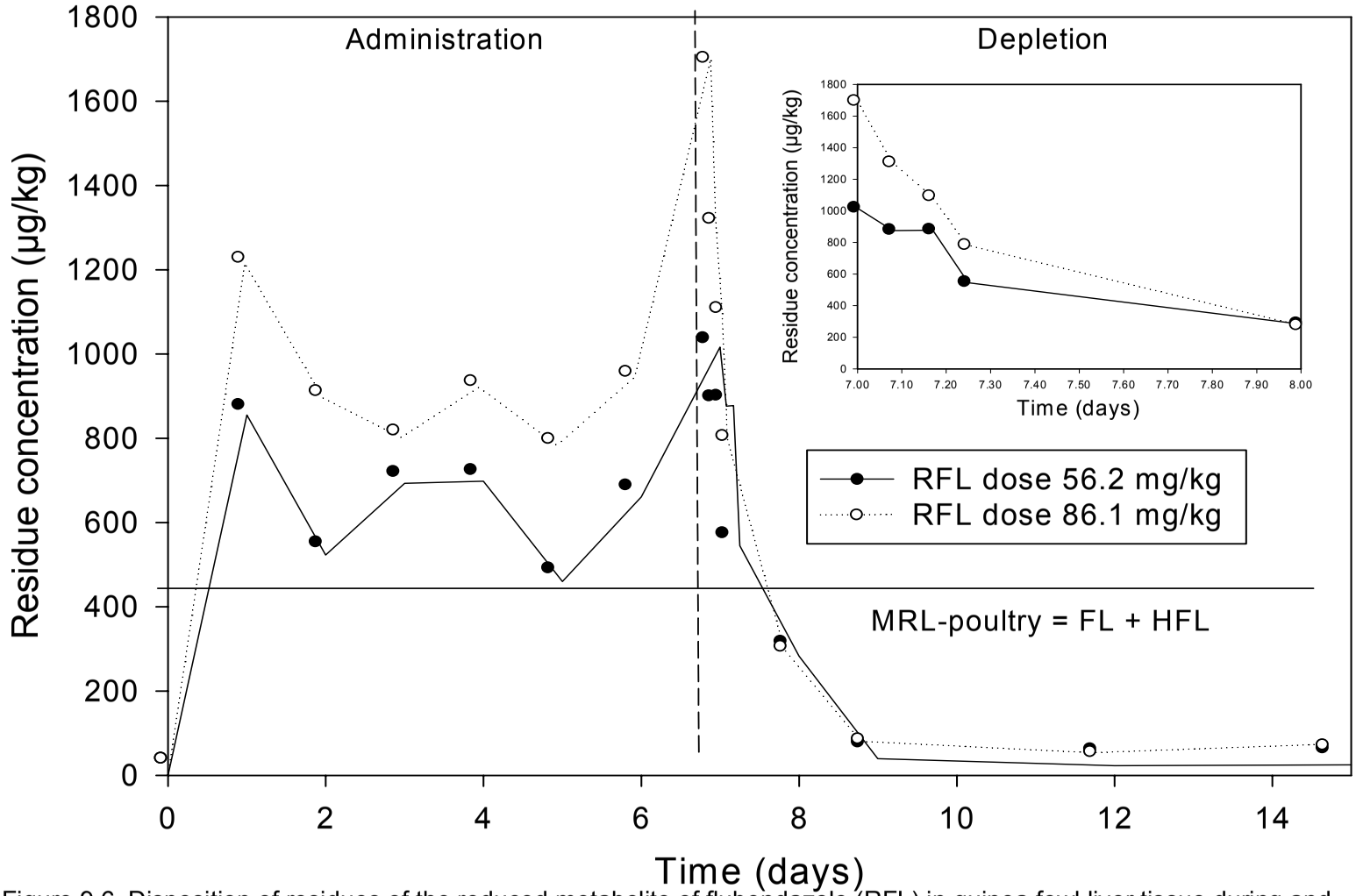

Figure 9.6 Disposition of residues of the reduced metabolite of flubendazole (RFL) in guinea fowl liver tissue during and after oral administration of 56.2 and 82.1 mg of flubendazole per kg feed during seven successive days

administration doses of 56.2 and 86.1 mg/kg is illustrated in Figure 9.6. The shape of the two residue curves are similar and comparable with the residue concentration curve obtained for this compound in the muscle tissues (Figures 9.4 and 9.5). The obtained residue values in liver tissue with the high treatment dose were about 1.5-fold higher of those obtained with the low administration dose. The values were about threefold and fourfold of those obtained in the muscle tissues with the low and the high dose, respectively. After one day of treatment, residue values of 855.2 and 1210.8 µg/kg were obtained for the low and high dose, respectively. During the administration period, the residue value varied between 472.5 and 1042.5 µg/kg and between 772.8 and 1694.5 µg/kg for the low and high dose, respectively. The same trend in decreasing of residues until experimental day 5 as observed in the muscle tissues could be observed, especially with the low dose. After the treatment was stopped, the depletion was very rapidly. As shown in detail in the inset-figure in Figure 9.6, within 24 h of depletion, the residue concentration decreased from 1042.5 to 282.9 µg/kg and from 1694.5 to 270.6 µg/kg for the low and the high dose, respectively. The MRL value of 400 µg/kg for the sum of residues of FL plus HFL in liver tissue in target bird species is indicated in Figure 9.6 as reference. The remaining residue concentrations obtained at the last sampling time after eight days of depletion were 24.8 and 32.1 µg/kg for the low and the high dose, respectively. Also in liver tissue, the differences between the residue values of the individual guinea fowls were high. The variability per sampling time (mean values in parentheses) ranged from 13.7 to 69.5% (41.6) and from 8.9 to 56.7% (27.9) for the low and the high dose, respectively.

9.3.2.2.2.3.2 Depletion of flubendazole and its hydrolysed metabolite

The depletion results for FL and HFL in liver tissue with the two administered doses are shown in Figure 9.7. The residue values were considerably lower than those obtained for RFL. The residue concentrations for FL in liver tissue were slightly lower than those measured in the muscle tissue with both doses. A detectable response for HFL was observed in liver tissue. The values for HFL were lower than those obtained for FL. After one day of administration, the residue concentrations for FL were 45.5 and 59.3 µg/kg for the low and the high dose, respectively. The residue values for HFL were 10.2 and 17.0 µg/kg, respectively. During the whole antiparasitic treatment period, the residue values for FL ranged between 45.1 and 107.5 µg/kg and between 59.3 and 156.1 µg/kg for the low and the high dose, respectively. These ranges for HFL were from 10.2 to 22.9 µg/kg and from 17.0 to 37.5 µg/kg, respectively. The decreasing trend of the residue concentrations until experimental day 5 could be observed again for both kinds of residues and for both doses. The rapid decline of the residue concentrations during the first depletion day, is illustrated in the inset-figure in Figure 9.7. After one day of depletion, the residue concentrations for FL were 16.8 and 23.8 µg/kg for the low and the high dose, respectively. These residue values for HFL were 11.3 and 13.9 µg/kg,

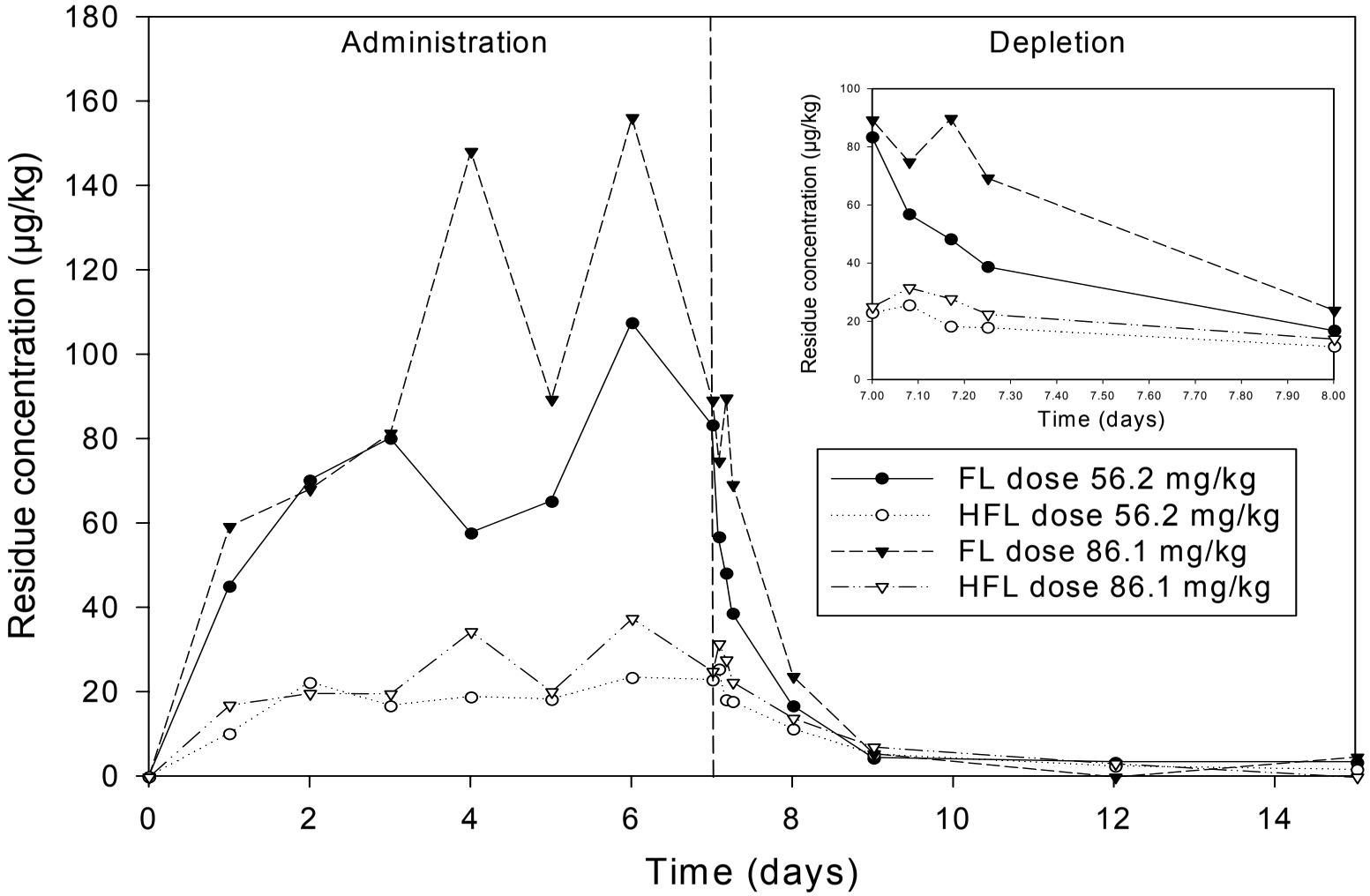

Figure 9.7 Disposition of residues of flubendazole (FL) and its hydrolysed metabolite (HFL) in guinea fowl liver tissue during and after oral administration of 56.2 and 82.1 mg of flubendazole per kg feed during seven successive days

respectively. At the last sampling point, on experimental day 15, the residual drug concentrations for FL and HFL were 3.4 and 1.7 µg/kg and 4.7 µg/kg and below detection limit for the low and the high dose, respectively. The variability per sampling time (mean values in parentheses) for FL ranged from 21.9 to 86.5% (38.2) and from 18.5 to 45.2% (35.6). The s_r values for HFL varied from 21.7 to 57.1% (33.1) and from 20.3 to 42.2% (32.9) for the low and the high dose, respectively.

9.4 Discussion

9.4.1 Depletion study with turkeys

The used LC-MS/MS analytical technique was very specific. No interference of residues of the other supplemented veterinary medicinal products diclazuril and Nifursol® was obtained.

During the administration period, no relationship was found between residue concentration and body weight. The effect of increasing body mass on residue level was probably compensated by the higher feed intake.

To verify whether the differences in residue values between thigh and breast muscle tissue were statistically significant, a four-parameter Weibull function was fitted to both data series of the two trials with different administered dose. Comparing the confidence intervals of the estimated Weibull parameters showed a clear overlap and thus no significant difference for all cases. Nevertheless, when individual points of the breast-thigh series were compared with a t-test assuming homogenous variances, as validated by a Browne-Forsyth test, some measurement points proved to be significantly different. The majority of the individual points were not significantly different, which can be attributed to the substantial variance on the individual residue values of the six animals. Nevertheless, the higher fat content of thigh muscle can be an explanation for the supposed higher residue concentration. In the fat-rich egg yolk, higher flubendazole-derived residue concentrations were measured as compared to egg white.[9,12] Another potential explanation could be the higher blood flow in the thigh, which is necessary for the mobility. Figure 9.1 shows a somewhat more rapid depletion of both analytes in thigh muscle, probably also due to the higher blood flow.

The intensity of the metabolism of flubendazole to its hydrolysed metabolite, as observed with the residues in the tissues, was considerably high. The highest intensity was measured in liver tissue. In the trial with the recommended dose, the mean calculated percentual ratios of HFL in thigh and breast muscle to the total of FL plus HFL were respectively, 73 and 78%. This transformation value in liver tissue was 93%. The intensity of metabolism obtained with the higher dose was comparable. The calculated conversion values were 71, 75 and 95%, respectively in thigh muscle, breast muscle and liver tissue.

The disposition and depletion obtained for flubendazole-derived residues in muscle tissue is higher than these published for eggs. Kan *et al*.[9] obtained a mean value for the sum of residues

of FL plus HFL of 250 µg/kg in whole egg during a treatment of laying hens with feed medicated with a dose near the recommended dose.

9.4.2 Depletion study with guinea fowls

An antiparasitic treatment of poultry species with flubendazole is strongly recommended by the oral route of administration. The applied veterinary medicinal product is prepared as a premix for medication of the feed diet. The presence of feed in the gastrointestinal tract enhanced the absorption of the drug. Michiels *et al*.[16] also have reported higher plasma residue concentrations for FL in man when the medicinal product was supplemented directly after a meal. The increased absorption of flubendazole can be explained by the increased dissolution of the drug in the lipoid components of food and in bile acids. Hence, it is recommended to give systemic localised helmintics, such as flubendazole, during or after a meal. As prescribed for medication of game birds, a standard medicated feed with a dose of 60 mg of flubendazole per kg was supplemented to the guinea fowls. Compared with the used dose for turkeys, 1.5-fold higher flubendazole administration dose of 90 mg/kg as highest dose was applied for this depletion study with the guinea fowls.

Compared with broiler chickens and turkeys, the growth rate of guinea fowls is much lower. Broiler chickens and turkeys show a higher gender related dimorphism than guinea fowls. The normal growth period for guinea fowls until slaughter takes ten weeks for a body weight of 2.0 to 2.25 kg. Broiler chickens of 1.5 kg can be slaughtered already after a growth period of five weeks.[18] Compared with males, females showed a higher feed intake. The mean feed intakes (Table 9.1) obtained for the three pens per gender in the acclimatisation, the administration and the depletion period were 141.6 and 133.7, 160.9 and 143.1, 124.0 and 122.8 g/day for females and males, respectively. This observation is opposite to the results for broiler chickens[18] and certainly to those obtained for turkeys. During the medication period, the mean feed intakes were 88.1 and 106.8 g/day (for broilers) and 290 and 530 g/day (for turkeys) for females and males, respectively.

The feed conversion is much lower for guinea fowls than for broiler chickens and turkeys. Baeza *et al*.[7] and Sales *et al*.[19] obtained higher BWs for the female guinea fowl birds than for the males. In the present study, rather heavy male birds were obtained. The effect of gender on body weight during the experimental period was assessed by an analysis of variance (ANOVA) of the data of Table 9.2. The difference on body weight between the two genders was not significant ($P > 0.05$) for most days, except for day 8 and 15. An almost significant difference ($P = 0.055$) was found at the sampling point day 7.08 (2 h after cessation of the drug administration at day 7). For these three cases, the male birds had a significant higher body weight than the female birds. The antiparasitic treatment of the guinea fowls at the two applied doses had no significant influence on the body weight ($P > 0.05$). As observed by Baeza *et al*.,[7]

males showed a higher feed conversion, compared with the females. Their feed intake was lower and in the presented study, male birds were obtained being at least as heavy as the female birds.

Concerning the chemical composition of the guinea fowl body tissues, thigh muscle tissue had markedly higher fat contents than breast muscle tissue. The analysed fat contents in thigh muscle were 8.5 and 7.1% for females and males, respectively. A value of 2.9% in breast tissue was obtained for both genders. The same conclusions with respect to the influence of gender on protein and fat content of the guinea fowl body at the same age were made by Sales *et al*.,[19] in spite they had analysed the whole guinea fowl body after plucking. A higher fat content and a lower protein content for the female body, compared with the male body, was measured. The contents of protein and fat, which were close to the values obtained in the presented study were respectively, 16.8 and 6.6% and 17.1 and 5.9% for females and for males.

The application of the sample pretreatment procedure, previously optimised for eggs and turkey tissues, confirmed the versatility of these extraction and clean-up procedure. Some improvements were implemented. A distinction in the sample preparation between muscle and liver samples was made. To shorten the sample preparation time, the homogenisation step of liver tissue in a small quantity of water could be omitted, because of the softness of this matrix.[20]

The evaporation of the sample extracts was standardised by an automatic multitube vacuum evaporator. The LC separation was performed with a mobile phase consisting of acetonitrile and water containing 0.1% formic acid, which was successfully applied in analytical LC-MS/MS methods described in Chapters 4 and 6. The gradient applied gave a satisfactory LC-MS/MS separation and detection, avoiding problems with unstability of a mobile phase containing ammonium acetate. To increase the sensitivity of the determination method, the injection volume was increased from 10 to 30 µl. This modified LC-MS/MS method is relatively fast and very selective for the determination of flubendazole-derived residues in guinea fowl tissues. The determination method shows relatively low detection limits. The three drug compounds could be detected at residue concentrations lower than 3 µg/kg in muscle and liver tissues.

The decline in residue concentration observed at the low dose, disappeared by treating the animals with a higher dose, probably due to an excessive drug uptake.

Using TableCurve2D (SYSTAT software, Richmond, USA), it was determined that a four-parameter linear polynomial function could best describe the different excretion curves (Figures 9.4 and 9.5) overall. To verify whether the differences in depletion curves between thigh and breast muscle tissues were statistically significant, this function was fitted to all curves. Comparing the 95% confidence intervals of the estimated parameters showed clear overlap and thus no significant difference for all cases.

Flubendazole is, in principle, not authorised for use in guinea fowl and its use is not supported by the veterinary medicinal product manufacturer. Nevertheless, flubendazole might be

employed by guinea fowl breeders in view of its effectiveness as documented in the literature. It is the objective of surveillance labs to monitor the residue safety of food. In the absence of MRLs for flubendazole in the edible tissues of guinea fowls, and with regard to the observed species difference in residue disposition between the guinea fowl and the other bird species, for which MRLs have been established, residue safety can only be guaranteed by recommending withdrawal periods which are sufficiently long for the elimination of the residues. The data on the depletion curves indicate that the residue concentrations do not longer decrease substantially after three days of withdrawal. A withdrawal period of three days will assure residue safety in the edible tissues of guinea fowls treated with flubendazole at a therapeutic dose during seven consecutive days. Instead of using the prescribed statistic calculation procedure, this withdrawal time was estimated as the time point where the mean concentration of all flubendazole-derived residues in all tissues were low and negligible and not longer decreasing substantially.

9.4.3 Species differences and similarities between turkeys and guinea fowls

Surprisingly, a large fraction of flubendazole was metabolised to its reduced metabolite in the guinea fowl body. Only small amounts were present as hydrolysed metabolite in the analysed tissues. This species difference between guinea fowl and turkey was unexpected. For the depletion study with turkeys, the residue concentrations found for RFL were around the detection limit and were negligibly. Kan *et al*.[9] also reported higher residue concentrations for the hydrolysed than for the reduced metabolite in eggs. They measured whole eggs, egg white and egg yolk of laying hens treated orally with flubendazole. The maximum residue values for FL, HFL and RFL obtained in whole eggs were 140, 120 and 50 µg/kg respectively during the administration of medicated feed with a dose of 27 mg per kg feed.

Delatour and Parish[1] reported very important information about metabolisation and disposition of benzimidazole veterinary medicinal products. Unfortunately, for flubendazole, they reported no data on differences in excretion of the two metabolites in poultry species. Compared with the muscle tissues, RFL is excreted mainly by the liver into the bile of the guinea fowls. A relative molecular mass larger than 300 and the presence of polar groups appear to facilitate its biliary excretion.[8]

The decline in residue concentration during the medication of guinea fowls with the low dose until day 5 (Figure 9.4) was also obtained in the depletion study with the turkeys during the administration of the lowest dose. A clear explanation for this phenomenon can not be given, but a residue dilution in the tissue of a faster growing bird with an increased body weight can be possible. The residue concentrations in muscle and liver tissues showed a good dose-response relation. The ratio of higher to lower dose is comparable with the ratio of the residue

concentrations obtained with these doses. Similar conclusions were made for the residue disposition with turkeys. However, Michiels *et al*.[16] reported that the absorption of flubendazole was completely dose-independent for medication of man with much higher doses. The plasma residue values increased only 1.4-fold for a 20-fold higher dose.

During the experiments with the turkeys as well as with the guinea fowls, complete depletion of all residues in each kind of tissue was difficult and would probably take a long time. The residue values declined very rapidly the first two days of depletion. Afterwards, only a very slow disappearance of the remaining drug residues was observed until the last data point. The avian birds in these trials were reared on the floor and recycling of drugs through faecal, dust or litter contamination could be a potential reason for this generally observed phenomenon.[13]

9.5 Conclusions

The results of the depletion study with turkeys demonstrated that there is no risk for the public health when a withdrawal period of one day is respected after treatment of flubendazole with the recommended dose. One day after cessation of the administration, the drug residue concentration of the sum of flubendazole and its hydrolysed metabolite in muscle tissue declined to or below MRL level.

From the depletion study with the guinea fowls it can be concluded that a longer withdrawal time is needed. Residue safety in the edible tissues after treatment at therapeutic dose will be assured after a withdrawal period of three days.

Species difference in flubendazole residue metabolism between turkey and guinea fowl was observed. In edible tissues of guinea fowl, a high residue concentration for the reduced metabolite of flubendazole could be detected. In tissues of turkey, a high response of the hydrolysed metabolite compound was observed.

9.6 Acknowledgements

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CHAPTER 10

Disposition of residues of mebendazole and metabolites in sheep tissues

Relevant publication:

De Ruyck H., Daeseleire E., De Ridder H. and Van Renterghem R.
Liquid chromatographic-electrospray tandem mass spectrometric method for the determination of mebendazole and its hydrolysed and reduced metabolites in sheep muscle.
Analytica Chimica Acta, 2003, 483, 111-123

Abstract:

The disposition and distribution of mebendazole-derived residues in tissues of sheep was studied after a single oral treatment.

The residues of mebendazole, its hydrolysed and its reduced metabolites were quantified in muscle, liver, kidney as well as back fat tissues by means of a liquid chromatographic-electrospray tandem mass spectrometric method.

After one day of excretion, the residue concentrations ranged from 21 to 7630 µg/kg. The highest residue concentrations were found in liver tissue. With exception of back fat tissue, the highest residue responses were always obtained for the reduced metabolite. The reduced metabolite in liver tissue and the hydrolysed metabolite in kidney tissue were found until 14 days after treatment.

Keywords: mebendazole-derived residues, sheep tissues, residue depletion, LC-MS/MS analysis

10.1 Introduction

10.1.1 Usage of mebendazole for treatment of sheep

Mebendazole is a benzimidazole, orally active broad-spectrum anthelmintic drug effective against numerous nematodes and cestodes of the gastrointestinal tract and against lung nematodes of different animals and humans.[1,2] Mebendazole is the prototype of a series of broad-spectrum anthelmintics widely used in both animal and man.[3]

Sheep is an animal species which is very susceptible to parasitic infections caused by helminths. These animals are often reared in circumstances (wet pastures) posing high parasitic risk. Bishop[4] described mebendazole as a substance which is highly effective against gastrointestinal roundworms and tapeworms in sheep. Although adult tapeworms do not usually cause discernible disease, treatment is often necessary for public health purposes, to prevent disease due to larval stages in farm livestock and to minimise meat losses. Tapeworm infection is mostly seen in lambs during their first summer on pasture but rarely causes ill-effect, except perhaps for a marginal influence on growth. Infections often disappears spontaneously in the summer and are not common in older animals. Control of infection includes treatment in late spring or early summer and again in autumn. Prophylactical programmes in sheep are more complicated and often less satisfactory. The ewe acts as an extra source of infection to the lamb and the pre-lambing treatment is therefore necessary to eliminate the periparturient rise in faecal egg-counts. Additional treatment of lambs in spring may be necessary especially in a high-risk season due to climatic conditions.

The pharmacokinetics of an anthelmintic drug and thus its efficacy may be affected by the level of infection. Bauer and Conraths[5] reported the comparative efficacy of mebendazole and moxidectin (a macrocyclic lactone anthelmintic drug compound) against gastrointestinal nematodes in experimentally infected lambs. Pathological changes were therefore induced in the lambs by the inoculation of large doses of gastrointestinal nematode larvae. The mebendazole formulation was administered at the recommended therapeutic dose of 15 mg per kg body weight (BW). The nematode eggs were counted in individual collected faecal samples. The worm burdens were assessed in the gastrointestinal tracts of lambs euthanased seven days after treatment. The results of the trial demonstrate that mebendazole was highly effective against several adult gastrointestinal nematodes with an activity up to 100%. The failure to achieve a reduction of more than 76% against some species was most probably attributable to benzimidazole-resistance.

Van der Westhuizen *et al*.[6] described the anthelmintic efficacy of a mebendazole suspension against induced helminth infections in sheep and cattle. The mebendazole suspension was administered intraruminally via a stomach tube at a dosage rate of 15 mg of mebendazole per kg BW. Daily inoculations of larvae of various helminth parasites were given to the sheep over a period of several days. All animals were euthanased seven to 31 days after treatment. Several

gastrointestinal worms and lungworms and their stages were identified and the efficacy of the mebendazole treatment against each of the parasite species was calculated. The controlled efficacy of the mebendazole suspension obtained ranged from 79.8 to 100% against a wide variety of investigated larval and adult stages of sheep nematodes.

Cavier and Notteghem[7] performed a pharmacological study on the tenicidal properties (activity against tapeworms) of mebendazole and flubendazole. The tenicidal properties of the two compounds with similar molecular structure (flubendazole is the fluoro-substituted analogue of mebendazole) were comparatively studied on experimentally infested mice. Only mebendazole had sufficient activity warranting its therapeutical use against cestodes.

10.1.2 Metabolism of mebendazole

Mebendazole (ME) is known to be absorbed from the gastrointestinal tract and to be intensively metabolised in sheep.[1] The major metabolic reaction of mebendazole in sheep is the carbonyl reduction of the 5-keto group (Figure 6.1 in Chapter 6) to the corresponding alcohol compound hydroxymebendazole (RME).[1,8] Hydrolysis of the “carbamate” group of ME to the resulting 2-aminomebendazole (HME) (Figure 6.1 in Chapter 6) is the second metabolic pathway in sheep.[9] The benzimidazole ring itself appears to be metabolically stable. The 2-aminobenzimidazoles of other benzimidazoles are often additionally metabolised in the 5-substituent and are readily eliminated in the urine. No reported major metabolic pathway involving breakage of the benzimidazole ring system exists. Mebendazole metabolites appear to be extensively excreted in bile and this route is more important than urine.

Also, HME is described by Al-Kurdi *et al*.[10] as the main degradation impurity product of mebendazole in pharmaceutical dosage formulations such as tablets and suspensions, which are used in human medicine. HME was obtained in the laboratory by alkaline treatment of mebendazole. ME raw material was dissolved in 1 M sodium hydroxide solution, heated to boiling point under reflux cooling for 30 min, cooled down and neutralised with 1 N nitric acid.

Delatour and Parish[9] reported that in contrast with other benzimidazole anthelmintics as oxfendazole, albendazole and febantel, mebendazole appears to be nonteratogenic in sheep. Excellent toxicological correlation exists between rat and sheep with benzimidazoles. Compounds which are teratogenic in sheep are also teratogenic in rat. The reverse also appears to be true with the possible exception of mebendazole, which is reported to be nonteratogenic at the therapeutic dosage. However, embryolethal and teratogenic effects have been described for mebendazole in rats. The hydroxymebendazole metabolite is more embryotoxic than mebendazole itself.

Mebendazole itself appears to be the active anthelmintic drug substance. The metabolites have very little anthelmintic activity.[8,9]

Galtier and Alvinerie[11] investigated the pharmacological basis for hepatic drug metabolism in sheep. The effect of experimental fascioliasis of sheep, proposed as a pathological model, on

the pharmacokinetics of various hepatic tracers and veterinary drugs, such as mebendazole, was investigated. The mean residence time of mebendazole was increased 1.5 to 2.7-fold when the lambs were treated 13-25 weeks post infection with consequences on the elimination of RME. The characteristic decrease in liver cytochrome could be responsible for most of the pharmacokinetic and pharmacodynamic changes observed in fluke-infected ruminants. A decrease of 59% in ME reduction was measured in liver microsomes prepared from 12-week infected sheep. The intermediate and late stages of fascioliasis (by weeks 8-25) were characterised by decreases (26 to 66%) in metabolism of ME to RME.

A residue study of Iosifidou *et al*.[12] clearly demonstrates that mebendazole given to European eel via the water is absorbed rapidly and is metabolised into its reduced and its hydrolysed metabolite. The hydrolysed metabolite proved to be the major metabolite of mebendazole in the eel.

It was demonstrated that ME and its hydroxy metabolite (RME) constitute the residues of toxicological concern, because they were demonstrated to have embryotoxic potential.[12] If the amino metabolite (HME) persists in edible parts of muscle tissues, it should be taken as the compound of interest for proving the animals treatment with mebendazole and estimating the withdrawal time for consumption of muscle tissue of the animal treated with mebendazole.

10.1.3 Objectives

Blanchflower *et al*.[13] measured residues in liver and muscle tissues after treatment of sheep with fenbendazole and oxfendazole. It can be expected that after treatment with mebendazole, a very similar anthelmintic drug compound, also residues remain in these tissues. Mebendazole-derived residues can still occur in food derived from sheep especially when the withdrawal time is not respected. To protect the consumer, the EC has established MRLs for residues of mebendazole and its two major metabolites in sheep tissues with as marker residue the sum of ME plus RME plus HME.[14] The MRL value in liver tissue is set at 400 µg/kg. The MRL value for these residues in muscle, kidney and back fat tissues is established at 60 µg/kg.

Several pharmacokinetic studies described the depletion of mebendazole-derived residues in plasma of sheep and goats.[1,2,8,11] Galtier *et al*.[1] described the pharmacokinetics of mebendazole in infected sheep after oral administration of mebendazole at 25 mg per kg BW. They reported the effect of *Fasciola hepatica* infestation on sheep plasma concentration of mebendazole and its reduced metabolite after administration to sheep before and after 4, 8, 13, 19 and 25 weeks of infection. The parent drug and especially its reduced metabolite were present in plasma of the animals. Behm *et al*.[8] published residue concentrations of mebendazole and metabolites in sheep plasma at intervals up to 48 h after treatment of sheep with an intraruminal injection at dose rates of 12.5, 25, 50 and 100 mg per kg BW. Peak plasma concentrations occurred between 9 and 24 h for all dose rates and declined rapidly. Residues of the hydrolysed and reduced metabolite of mebendazole were detected and their concentrations exceeded that of

mebendazole at all dose rates. Galtier and Alvinerie[11] determined the pharmacokinetics of mebendazole in sheep before and 4-25 weeks following a similar experimental fascioliasis. Age-related changes in hepatic drug metabolising activities of ewes were determined in foetal, neonatal, growing, pregnant and adult animals. After oral administration of mebendazole at 25 mg per kg BW, the parent drug and especially its reduced metabolite were present in the animal plasma. The disposition kinetics of mebendazole in plasma, milk and ruminal fluid of goats were described by Pandey and Roy.[2] Their experiment was designed to study the pharmacokinetics of mebendazole after single dose intravenous (5 mg per kg BW) and oral (40 mg per kg BW) administration in two separate groups of female goats. The plasma half-life, the total body clearance and the residue concentration of the parent drug in the experimental fluida was calculated. The highest residue response was obtained in the ruminal fluid and reached 40.1 µg/ml at 4 h after the single-dose oral administration.

Facino *et al*.[15] applied a liquid chromatographic-mass spectrometric analytical method to investigate the persistence of five benzimidazole anthelmintics, including mebendazole, in sheep milk. The healthy sheep, which were free of helminth parasites, were treated with a single oral therapeutic mebendazole dose of 25 mg per kg BW. Residues of mebendazole could be detected (detection limit of 2 µg/l) in the milk until 60 h after treatment.

To our knowledge, no depletion data of mebendazole-derived residues in sheep tissues are available in the open literature.

Michiels *et al*.[3] investigated the pharmacokinetics of mebendazole and flubendazole in rats. Plasma mebendazole residue concentrations after oral and subcutaneous treatment at a dose rate of 40 mg per kg BW were about tenfold higher than those of flubendazole because of its better solubility. The comparative absorption experiment in rats suggested a marked strain difference with respect to the systemic absorption of oral mebendazole. Subcutaneous mebendazole appeared to be absorbed more gradually but at a similar rate for either strains.

The extensive use of mebendazole in eel culture for anthelmintic treatment may lead to mebendazole-derived residues in eel tissues. Iosifidou *et al*.[12] performed a residue study of mebendazole and its reduced and hydrolysed metabolites in liver, kidney, fat, skin and muscle tissue samples after batch treatment at a dose of 1 mg per l of water for 24 h. Liver and kidney were found to contain the highest concentrations of metabolites and fat contained the highest residue values of the parent compound. Skin contained higher residue concentrations for all three compounds, compared with muscle tissue. The hydrolysed metabolite could be detected (detection limit of 10 µg/kg) in the edible tissues (muscle and skin) sampled 14 days after the end of the treatment.

The objective of this study was to investigate the depletion of the mebendazole-derived residues from tissues of sheep after a single oral treatment at 20 mg per kg body weight. The disposition and distribution of mebendazole and its hydrolysed and reduced metabolites was measured in thigh muscle, rib muscle, liver, kidney and back fat tissues. The incurred tissue

samples were received from Janssen Animal Health (Beerse, Belgium) who performed the sheep treatments. The residue analyses were carried out at the Department of Animal Product Quality and Transformation Technology (DVK-CLO, Melle, Belgium).

10.2 Experimental

10.2.1 Treatment of sheep

Eleven male, mixed Texel/Suffolk sheep of approximately 25 kg body weight were used in the depletion study performed by Janssen Animal Health. On day 0, ten sheep were treated with Ovitelmin® at a single oral dose equivalent to 20 mg mebendazole per kg body weight. The sheep were weighed before the start of the experiment and samples were taken from the lightest and heaviest sheep at every time point. On day 1, two sheep were sacrificed using a captive bolt gun. After exsanguination, the liver, kidneys, 25 g of thigh muscle, 25 g of rib muscle and 25 g of back fat were collected in standard plastic bags, which were sealed, appropriately labeled and deep frozen. This procedure was repeated on days 7, 14, 21 and 28. The remaining, untreated sheep was sacrificed, sampled on day 28 and analysed as blank control. Samples were kept deep-frozen until needed for analysis.

The recommended withdrawal period before slaughtering sheep after treatment with Ovitelmin® is set at one week.

10.2.2 Determination of mebendazole-derived residues

The incurred sheep tissue samples were analysed for residues of mebendazole and its hydrolysed and reduced metabolites with the quantitative liquid chromatographic-electrospray tandem mass spectrometric (LC-MS/MS) procedures described in Chapter 6.

10.3 Results and discussion

10.3.1 Depletion of mebendazole-derived residues in sheep tissues

The applicability of the determination methods for mebendazole and its metabolites in tissues of sheep could be evaluated with the incurred samples of the limited depletion study. Samples of thigh muscle, rib muscle and the liver as well as the kidney and back fat were collected. The different kinds of samples were prepared by the different sample pretreatment procedures described in Chapter 6.

The residue concentrations measured in the different tissue samples are summarised in Table 10.1. The values of the two sheep sampled at the same time point are given separately. The differences in the individual residue values between the sheep were high. It was therefore impractical to calculate mean values because the number of sheep involved in the trials was

Table 10.1 Residue concentrations (µg/kg) of mebendazole, its hydrolysed and its reduced metabolite in sheep tissues after a single oral treatment with Ovitelmin® at an equivalent mebendazole dose of 20 mg per kg body weight

Time (days)	Thigh muscle tissue			Rib muscle tissue			Liver tissue			Kidney tissue			Back fat tissue		
	ME	HME	RME	ME	HME	RME	ME	HME	RME	ME	HME	RME	ME	HME	RME
1	21	119	585	53	110	706	1628	41	1127	302	213	238	215	28	207
	41	51	1467	67	25	1491	1042	47	7630	1160	237	1330	778	8	549
7	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	6	4	< 0.5[a]	< 3[b]	< 0.5[a]	< 1.5[a]	< 5[a]	< 5[a]
	< 0.5[a]	< 7[a]	< 2[a]	5	< 7[a]	< 2[a]	< 0.5[a]	7	5	< 0.5[a]	< 3[b]	< 0.5[a]	< 1.5[a]	< 5[a]	< 5[a]
14	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 3[a]	< 1[a]	< 0.5[a]	< 2[a]	< 0.5[a]	< 1.5[a]	< 5[a]	< 5[a]
	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 3[a]	3	< 0.5[a]	< 3[b]	< 0.5[a]	< 1.5[a]	< 5[a]	< 5[a]
21	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 3[a]	< 1[a]	< 0.5[a]	< 2[a]	< 0.5[a]	< 1.5[a]	< 5[a]	< 5[a]
	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 3[a]	< 1[a]	< 0.5[a]	< 2[a]	< 0.5[a]	< 1.5[a]	< 5[a]	< 5[a]
28	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 3[a]	< 1[a]	< 0.5[a]	< 2[a]	< 0.5[a]	< 1.5[a]	< 5[a]	< 5[a]
	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 7[a]	< 2[a]	< 0.5[a]	< 3[a]	< 1[a]	< 0.5[a]	< 2[a]	< 0.5[a]	< 1.5[a]	< 5[a]	< 5[a]

[a] limit of detection: S/N = 3 [b] limit of quantification: S/N = 5

ME: mebendazole HME and RME: hydrolysed and reduced metabolites of mebendazole

low.

From the residue depletion results, it can be concluded that mebendazole was absorbed from the gastrointestinal tract very rapidly. ME metabolised mainly to the reduced metabolite and depleted in the tissues very rapidly. After one day of depletion, the residue concentrations ranged from 21 to 7630 µg/kg for ME in thigh muscle tissue and for RME in liver tissue, respectively. The highest residue response was found in liver tissue. Except for the back fat samples where the residue values for ME were higher, the highest residue values in the various tissues were measured for RME. No significant differences in residue response between thigh muscle tissue and rib muscle tissue were observed. The residue concentrations of the three compounds were comparable in each muscle tissue. Higher residue values were found for HME than for ME with maximum values of 119 and 67 µg/kg, respectively. The residue values for RME in the tissue samples varied between 585 and 1491 µg/kg. In kidney, liver and especially in back fat tissues, the concentrations for ME were higher than for HME. The maximum ME residue concentrations in kidney, liver and back fat tissue samples were 1160, 1628 and 778 µg/kg, respectively. The maximum measured values for HME in these tissues were 237, 47 and 28 µg/kg, respectively.

Considerable residue concentrations were found only in the tissue samples taken after one day of depletion. From the tissues involved in this study, almost all residues disappeared during the first week of depletion. A rapid decrease of residue values occurs during this first week. Only a few residue values could still be measured at the second time point. Seven days after treatment, the sum of the residue values of ME plus RME plus HME in the various tissues had declined below the respective MRL values. Measurable residues of RME at a concentration of 3 µg/kg were still found in liver samples until 14 days after treatment.

From a practical point of view, detection limits (LODs) and quantification limits (LOQs) were used as analytical limits in order to be able to report more quantitative data for this depletion study with a limited number of samples. Decision limits and detection capabilities were of less practical use because higher minimum values for these analytical limits were obtained. The LOD and the LOQ for the various tissues in this depletion study were calculated as those concentrations that yield a signal-to-noise (S/N) ratio of at least 3/1 and 5/1, respectively. These analytical limit values were estimated as mean values at the calibration points of 1, 5 and 10 µg/kg and are reported for the various tissues in Table 10.1.

10.3.2 Discussion

A comparable residue depletion pattern in sheep liver was obtained by Blanchflower *et al*.[13] one day after oral treatment of fenbendazole and oxfendazole with the recommended therapeutic dose of 5 mg per kg BW. The mean residual concentrations of fenbendazole and oxfendazole were approximately 1050 and 2500 µg/kg, respectively. However, shown elimination rates of these compounds in the liver and muscle tissues were slower than that obtained with

mebendazole in this study. The half-lives of fenbendazole and oxfendazole in liver tissue were 33.2 and 44.6 h, respectively. The residue concentrations of fenbendazole were much lower in muscle tissue than in liver tissue. A maximum concentration of 180 µg/kg was reached on day 2. On day 4, the concentrations had declined below the detection limit of the assay of 50 µg/kg. However, the obtained residue response of fenbendazole was considerably higher than that obtained with mebendazole in the presented study. No oxfendazole was detected (detection limit of 100 µg/kg) in any of the muscle tissue samples tested.

Good correlations between residue amounts in plasma samples found in the literature and the depletion results obtained in this study, especially for muscle and liver tissues, could be observed. The rapid absorption and distribution of mebendazole after oral treatment could be expected from the results of Galtier *et al*.[1] After oral treatment of sheep with a dose of 25 mg per kg BW, ME and RME peaked, respectively, at 12 h (110 µg/l) and 11 h (930 µg/l) in the plasma of the sheep uninfested with fascioliasis. In animals treated 4 to 25 weeks postinfection, the observed maximum plasma concentrations were unchanged, whereas the corresponding times of plasma maxima obtained by week 14 were significantly increased by 256% for ME and 154% for RME. Comparable results are published by Behm *et al*.[8] They concluded in their study that mebendazole is absorbed rapidly into the blood in sheep and that it is quickly metabolised to the hydrolysed and the reduced metabolite. These research workers were able to measure ME residues in sheep plasma already 3 h after treatment with administration doses variing from 12.5 to 100 mg per kg BW. They observed a peak concentration between 10 and 24 h of depletion. These research workers obtained a maximum residue concentration of 78 µg/l at 24 h after treatment by intraruminal injection of 25 mg per kg body weight. The total concentration of the two major metabolites were six- to eightfold higher than mebendazole itself, throughout the 48 h after treatment. They obtained a peak concentration of 1600 µg/l estimated for the sum of the two metabolites between 9 and 24 h of excretion after treatment with a dose of 100 mg per kg BW. A more rapid and higher residue response for ME was obtained in plasma of goats by Pandey and Roy.[2] After a single-dose oral administration of 40 mg per kg BW, ME appeared in plasma after 15 min. The maximum residue concentrations of 5.8 and 6.1 µl/ml were observed after 2 and 12 h of depletion. The mean plasma concentration of ME after intravenous administration of 5 mg per kg BW was 49.6 µg/ml obtained after 5 min. ME plasma concentration time profiles after intravenous and oral treatment, showed a secondary peak during the elimination phase, which led to the determination of additional redistribution (intravenous) and reabsorption (oral) components out of the usual elimination phases. A comparable residue response was obtained in the milk of the goats. The maximum ME residue concentration after the oral administration was 10.1 µg/ml obtained after 12 h of depletion. Residue values in milk could be detected (limit of detection of 0.1 µg/ml) until 120 h of depletion. From the results of Facino *et al*.[15] can be concluded that the clearance of ME residues in milk is more rapid in sheep than in goat. These research workers could measure (detection limit of

2.0 µg/l) ME residues until 60 h of depletion after a single oral administration with a dose of 25 mg per kg BW.

The metabolism of mebendazole in eel, as reported by Iosifidou *et* al.,[12] was quite different compared with that observed in sheep. The hydrolysed metabolite instead of the reduced one was the major metabolite of mebendazole measured in muscle tissue as well as in skin, kidney and fat tissues of eel. HME showed the highest residue concentration in each sample type from one day posttreatment and has the longest half-live. Elimination of ME and hydroxymebendazole from all tissues was comparable with exception for the skin tissue, which shows longer half-lives for ME and RME than the other tissues. The longest persistence of HME was obtained in skin and kidney tissues. The terminal elimination half-lives measured in skin and kidney tissue were 10.2 and 14.5 days. The residual concentrations measured 13 days after treatment were still approximately 1000 and 1300 µg/kg in skin and kidney tissue, respectively.

10.4 Conclusions

From the results of this depletion study can be concluded that the recommended withdrawal time for mebendazole administration to sheep of one week before slaughter is long enough. There is no risk for public health when the withdrawal period of one week before slaughtering of sheep is respected by the farmers and when sheep are not slaughtered during the first week after the mebendazole administration. The residue values in all tissues involved in this study declined below the respective MRL values seven days after the end of treatment. Only negligible mebendazole-derived residue concentrations were observed in rib muscle, kidney and liver tissues. Residues of the hydrolysed and the reduced metabolites of mebendazole could be detected, respectively in kidney and liver tissue, until 14 days of depletion.

10.5 Acknowledgements

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General conclusions

General conclusions

The main part of the experimental work of this doctoral thesis about residues of veterinary drugs considers the development of quantitative confirmatory analytical methods.

The sample pretreatment procedure of the HPLC method with UV diode array detection for several anthelmintic residues in milk, was not fully optimised to obtain optimum analyte recovery (Chapter 3). A moderate extraction efficiency of about 70% can be reached with the described procedure. As proved for the LC-MS/MS method, alkaline conditions of the milk sample mixture seem necessary to improve the recovery substantially (Chapter 4). The extraction efficiency of the weak basic benzimidazole analytes was increased to at least 90% when the compounds were extracted in an alkaline sample matrix.

The introduction of mass spectrometry as analytical tool in the residue analysis was very advantageous. The application of tandem mass spectrometric detection leads to very specific analyte detection. Although the sample clean-up was limited, reliable and accurate residue analysis could be performed and very low detection limits, even lower than 1 µg/kg, were reached. Because of the very easy sample preparation and the fast analyte determination, the LC-MS/MS methods are time and labour effective and very user friendly. Furthermore, in conformity with the recently published EC Decision 2002/657/EC, confirmatory methods for organic residues should provide information on the chemical structure of the analyte. Suitable confirmatory methods for residues of banned pharmacologically active substances for which no MRLs can be fixed need a liquid chromatographic method combined with infra red or mass spectrometric detection. UV diode array detection is acceptable as detection technique in HPLC methods for residues of authorised veterinary drugs. However, some limitations compared with mass spectrometry were discussed. Hence, HPLC methods are rather suitable for screening purposes. The LC-MS/MS analytical methods are very flexible. It is possible to change the parameters for the target substances within two hours.

In the second part of this thesis, the developed analytical methods were applied to investigate the disposition and distribution of residues of veterinary drugs in milk, eggs and animal tissues. From the depletion results obtained for tetracyclines in eggs and broiler muscle tissues and for anthelmintics in milk, turkey, guinea fowl and sheep tissues, it can be expected that residues of these drug substances arise in food. In the past, less attention was paid to the potential occurrence of residues of anthelmintic drugs in food of animal origin. The residue analysis mainly focused on hormones and antibiotics.

Anthelmintic drug substances are very rapidly absorbed, distributed and metabolised in the animal body after administration. Due to metabolisation, the administered compound will not always be the only target substance. Residues of the parent drug substances and metabolites are depleted in milk, eggs and animal tissues and shortly after administration, very high residue

amounts could be found. The anthelmintic veterinary medicinal products administered as topical or oral formulation, show a short efficacy time from one until a few days. The drug compounds do not persist in the animal body. A rapid decline in residue concentration in the animal tissues is observed after treatment. After treatment of healthy animals with the licensed veterinary medicinal products, all recommended withdrawal times were proved to be long enough to obtain safe animal products with residue concentrations below the MRL value. However, the total clearance of residues, as observed for flubendazole with the poultry species, takes several days probably because of recycling.

Anthelmintic treatment of turkeys with flubendazole at the recommended therapeutic dose of 19.9 mg per kg feed leads to residue amounts in muscle tissue up to 13-fold the MRL value during treatment. After 24 h of depletion, the residue concentration of the target residues declined to around the MRL level of 50 µg/kg. Therefore a withdrawal time of 24 h before slaughtering the target poultry species should be recommended.

Species difference between turkey and guinea fowl in flubendazole metabolism was demonstrated. In turkeys, flubendazole was mainly converted to its hydrolysed metabolite while the reduced metabolite was the major metabolite depleted in tissues of guinea fowls. Consequently, it can be questionned whether the established MRLs for turkeys can serve as a reference to evaluate the safety of flubendazole-derived residues in guinea fowl. Flubendazole is not licensed for treatment of guinea fowl. Because of the higher potential toxicity of the reduced metabolite, it is strongly recommended to add this compound to the marker residues for this safety level.

Besides the existing MRL concept, which is excluding as much as possible toxicological risks for public health, there is an arising consumer's tendency for a zero tolerance. However, public health must be the priority in this ethical matter.

Today's consumers require the safest possible food in sufficient quantities at a reasonable price. Therefore, the precautionary principle has also been implemented in a systematic way as an approach to risk management. The ideal of zero risk concerning food safety does not exist. Because of communication problems on food safety issues, the consumer perception of food unsafety arised also. Surveillance and monitoring programmes should be harmonised in all EU member states and the results should be published in an uniform way, so that only those in excess of the MRL value and confirmed by validated confirmatory methods, are published.

With this research study, a modest contribution for increasing the food safety is supplied. Several reliable and accurate analytical methods were developed which are suitable for routine quantitative determination of drug residues in food of animal origin in monitoring programmes or for surveillance analysis. The multiresidue LC-MS/MS method for anthelmintics in milk is currently used under accredited conditions for monitoring of Belgian farm milk.

Summary

Summary

In the general introduction of this doctoral thesis, theoretical aspects and background information about residues of veterinary drugs in food are emphasised (Chapter 1). Residues of veterinary drugs not only originate from improper, illegal or off-label use of veterinary medicinal products. Small residue concentrations below MRL level can arise also after proper use of veterinary drugs. Otherwise, cross-contamination in feed mills is a recognised problem. Also environmental contamination may be a source of unwanted residues. Animal-to-animal transfer by recycling of veterinary drugs should not be neglected and can lead to very long clearance times, especially with eggs. Veterinary drug residue aspects are regulated in the European legislation. Recently, the EC Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results was published. This Decision established that the result of a residue analysis should be considered as non-compliant if the decision limit of the confirmatory method for the drug analyte would be exceeded. The most important disadvantage of existing chromatographic confirmatory methods showing low detection limits is that those are not suitable for routine analysis because of their very laborious and time consuming sample clean-up. Confirmatory methods have to be validated to demonstrate their reliability. The parameters which are thoroughly investigated for the presented quantitative confirmatory methods are: specificity, linearity, trueness, precision, recovery, analytical limits, stability and applicability/ruggedness. The relative retention times and the relative intensities of the measured precursor and product ions of the analytes were also monitored during the liquid chromatographic-mass spectrometric methods.

The experimental part of this study deals with applied research work which recently has been published or which was submitted more briefly for publication in various scientific journals. The presented experimental work originates from the needs of the laboratory of the Department of Animal Product Quality and Transformation Technology (DVK-CLO, Melle, Belgium) which is involved in the screening of animal product samples for veterinary drug residues. There was a need for the ability to confirm samples suspected for antibiotics and to detect anthelmintic residues since no practical screening tests for these compounds were available until now. Most of the experimental work was focused on anthelmintic residues and the animal products under investigation were milk as well as eggs, poultry and sheep tissues.

The experimental work of this doctoral thesis is divided in two separate parts. The development of chromatographic multiresidue confirmatory methods for the quantitative determination of tetracyclines and anthelmintics including metabolites is reported and discussed in the first part (Chapters 2, 3, 4, 5 and 6). A better insight into residue problems and the behaviour of drugs in the animal body is obtained via pharmacokinetic studies, which are described in the second part

(Chapters 7, 8, 9 and 10). The developed methods could be tested thoroughly with numerous incurred samples received from these depletion studies.

Residues of tetracycline, oxytetracycline, chlortetracycline and doxycycline in eggs and poultry muscle tissue were determined by HPLC using UV diode array detection at 355 nm (Chapter 2). An acceptable separation of the analytes was obtained using ion-pair chromatography. Using this ion-pair technique, the sample clean-up could be kept to a minimum. The four tetracycline antibiotics could be detected in eggs and muscle tissue at limit concentrations ranging from 2.2 to 27.3 µg/kg. Additional analyte confirmation was obtained through characteristic UV spectra of the diode array detector.

The risks for human health due to residues of anthelmintics are rather limited. Nevertheless, also for these drug compounds MRL values were established by the European Commission and withdrawal times are recommended also. In the literature, teratogenicity is considered as the major potential risk. In addition to the well documented antibiotic resistance, precautions are also recommended to delay or to prevent the onset of resistance against anthelmintic drugs. Administration of the correct dose, usage of the minimum number of necessary treatments, rotation of the class of anthelmintics used annually and ensurance that animals would not infected with the resistant strains are effective policies for this goal.

The determination of residues of anthelmintics in milk was investigated by two different detection techniques. Two separate HPLC methods were optimised for the determination of levamisole, fenbendazole, thiabendazole, albendazole and oxibendazole (Chapter 3). For these compounds extended with oxfendazole, febantel and triclabendazole, a multiresidue LC-MS/MS method was developed also (Chapter 4). Because of the quite different properties, levamisole had to be treated with a separate sample preparation procedure for the HPLC-DAD method. Satisfactory analyte separations and detection limits were obtained with ion-pair liquid chromatography and UV diode array detection at 225 nm for levamisole and at 295 nm for fenbendazole, thiabendazole, albendazole and oxibendazole. The previously used polymeric column with i.d. of 4.6 mm was changed for a reversed-phase C_{18} column with i.d. of 3.2 mm resulting in less pH-restrictions and higher analyte response values. The obtained detection limits varied from 0.5 to 3.8 µg/l. The introduction of mass spectrometry in the residue analysis was very advantageous. Much more specificity and lower detection limits were obtained using tandem mass spectrometry. The obtained specific mass spectra, showing the characteristic precursor and product ions, are very powerful analyte confirmation tools. Furthermore, compounds of the multiresidue methods do not have to be fully separated chromatographically because of the powerful separation capacity of tandem quadrupole mass spectrometer for compounds showing parent and product ions with different molecular mass. Because of the very specific detection principle, the sample clean-up could be omitted almost completely. The analytes were detected by positive electrospray ionisation. Further miniaturising of the

LC column was carried out. A reversed-phase C_{18} column with i.d. of 2.1 mm was used for all LC-MS/MS methods described in this thesis.

The analytical method for the anthelmintics in milk has very low detection limits. Each compound could be detected at a residue concentration lower than 1 µg/l. The extraction recovery of the anthelmintics could be increased considerably to values of at least 90% by extracting the analytes in an alkaline milk matrix. The LC-MS/MS method was thoroughly elaborated. The validation was performed at two concentration levels. The reliability of the method was proven at MRL level as well as at an internal performance limit which was chosen at 1 µg/l. The decision limit and the detection capability, two analytical limits of the new validation concept, were determined also. For levamisole and triclabendazole, two unauthorised anthelmintic drugs for lactating dairy cows, a decision limit of respectively 1.2 and 1.3 µg/l was obtained. The method is also valid to quantify residues of the authorised anthelmintics at MRL level, providing the laboratory with a powerful tool to make decisions in violations for statutory testing purposes.

A validated LC-MS/MS method is presented for the determination of flubendazole and its hydrolysed and reduced metabolites in eggs and in poultry muscle and liver tissues (Chapter 5). Flubendazole is the most frequently used anthelmintic drug in poultry species. The LC separation of the analytes was performed with gradient elution with a mobile phase consisting of acetonitrile and ammonium acetate buffer. However, this ammonium acetate buffer is not stable, leads to blocking of tubings and hence to less robust analysis. Further optimisation in successive procedures indicated that this buffer could be omitted and changed succesfully for a mobile phase consisting of acetonitrile and water containing 0.1% formic acid to increase the analyte ionisation intensity. The limits of detection obtained for flubendazole, its hydrolysed and its reduced metabolite in egg and muscle tissue are 0.2, 0.3 and 1.1 µg/kg and 0.1, 0.8 and 0.3 µg/kg, respectively.

Sheep are one of the animal species who are most susceptible to parasitic infections caused by helminths. Sample preparation procedures and a LC-MS/MS determination method are developed for residues of mebendazole and its hydrolysed and reduced metabolites in muscle, liver, kidney and back fat tissues (Chapter 6). The method is validated for muscle and liver tissues at residue levels of 10, 20 and 60 µg/kg and 50, 100 and 400 µg/kg, respectively. The decision limits in muscle tissue for mebendazole, its hydrolysed and its reduced metabolite are 72.5, 69.2 and 69.8 µg/kg, respectively. These analytical limit values in liver tissue are 445.9, 466.5 and 463.4 µg/kg, respectively. However, mebendazole-derived residues can be detected in sheep tissues at concentrations lower than 2 µg/kg with the described method.

The studies of dispositon, distribution and depletion of residues of tetracyclines and anthelmintics in eggs, milk and edible tissues of poultry species and sheep are described in the second part of this thesis. The amount of residues remaining in food are influenced by

pharmacokinetic processes as drug absorption, drug distribution and drug elimination (metabolism and excretion) out the animal body. The recommended withdrawal times have to be respected to ensure that the residue concentration in the animal product would be declined below the safety MRL level.

The depletion of oxytetracycline in eggs is investigated during and after oral treatment of laying hens with a therapeutic dose of 840 mg per kg feed during seven successive days (Chapter 7). The absorption and disposition of oxytetracycline residues in eggs arised slowly. Five consecutive days of administration of medicated feed were needed to obtain a mean plateau residue concentration which was measured around the MRL level of 200 µg/kg. Two days after ceasing the administration, the residue concentration has fallen below the MRL level and was slowly declining during the successive withdrawal days. The disposition of tetracycline in muscle tissue is studied during and after oral administration of feed to broiler chickens medicated with tetracycline at a therapeutic dose of 480 mg per kg during seven successive days (Chapter 7). Tetracycline is very rapidly absorbed, efficiently distributed in the body of the broiler chicken and depleted in the muscle tissue. The residue amount of tetracycline accumulated in the muscle tissue was much higher than that of oxytetracycline in eggs. The plateau residue concentration of approximately 1200 µg/kg in muscle tissue was already obtained after one day of treatment. The residue concentration decreased very rapidly after the medication period and declined below MRL level the fourth day of the withdrawal period.

Levamisole is a classic, imidazothiazole drug substance still frequently used for anthelmintic treatment of dairy cows. This anthelmintic drug is no more approved for usage in lactating dairy cows since several years. The depletion of levamisole in milk is investigated in dairy cows which were separately treated topically with the recommended dose of 10 mg for non-lactating cows and with a double dose of 20 mg per kg body weight (Chapter 8). Levamisole was very rapidly absorbed and subsequently excreted in the milk. Seven hours after dosing of the recommended amount, a maximum residue concentration of 1896 µg/l could already be measured. A withdrawal time of 48 h is to short to obtain residue free milk. A levamisole residue concentration of 88 µg/l was still measured.

The pharmacokinetics of flubendazole in avian species are investigated in two separate depletion studies with turkeys and guinea fowls treated orally with two doses (Chapter 9). Flubendazole is not licensed for usage of treatment of guinea fowls. Flubendazole was administered to the turkeys and guinea fowls with medicated feed at respective doses of 19.9 and 29.6 mg and of 56.2 and 86.1 mg per kg feed during seven successive days. The disposition and distribution of flubendazole and its hydrolysed and reduced metabolites was measured in breast and thigh muscle tissues and in liver tissue. During the medication of the turkeys with the recommended dose, the residues accumulated in the tissues exceeded the MRL level which is established at 50 µg/kg for the sum of residues of flubendazole plus its hydrolysed metabolite in muscle tissues. A maximum mean residue concentration of

742.8 µg/kg was obtained in thigh muscle tissue after four days of treatment. Flubendazole was intensively metabolised to its hydrolysed metabolite and the residue concentration for the metabolite was approximately three- to fourfold that of the parent compound. The residue values for the reduced metabolite were negligible. The disposition of residues in thigh and breast muscle tissues was comparable. One day after treatment, the residue concentration already decreased to a level around the MRL. Nevertheless, since during treatment high residue concentrations are obtained it can be questionned if it would not be advisable to recommend a withdrawal time of 24 h after treatment and before slaughtering the animals.

Species differences between turkeys and guinea fowls were observed. In contrast with the results obtained for turkeys, high residue concentrations for the reduced metabolite of flubendazole could be detected in edible tissues of guinea fowl. The disposition of residues in the muscle tissues of guinea fowl started even more rapidly than in the tissues of turkeys. After one day of treatment, a plateau residue level of approximately 400 µg/kg for the sum of residues of flubendazole plus the reduced metabolite was already obtained. After cessation of the administration, the decline of residue amount in the tissues was slower compared with the turkeys. Residue safety in the edible tissues after treatment with the therapeutic dose will be assured after a withdrawal period of three days.

Despite the rapid decline in residue concentration just after cessation of the treatment, the total clearance of residues in tissues of turkeys as well as guinea fowls takes several days. Recycling of flubendazole-derived residues can be a potential cause for this observed phenomenon.

The depletion of mebendazole-derived residues in tissues of sheep is investigated with a limited pharmacokinetic study with sheep orally treated with a single recommended dose equivalent to 20 mg mebendazole per kg body weight (Chapter 10). The residues of mebendazole, its hydrolysed and its reduced metabolite were quantified in rib and thigh muscle, liver, kidney as well as back fat tissues at 1, 7, 14, 21 and 28 days after treatment. After one day of excretion, the residue concentrations ranged from 21 to 7630 µg/kg. With exception for back fat tissue samples, the highest residue response was always obtained for the reduced metabolite. There is no risk for public health when the withdrawal period of one week before slaughtering of sheep is respected. Only negligible amounts of mebendazole-derived residues were observed in rib muscle, kidney and liver tissues at this time point. The reduced metabolite in liver tissue and the hydrolysed metabolite in kidney tissue could be detected until 14 days of depletion.

With this doctoral study, an additional contribution to the pool of analytical methods suitable for monitoring programmes or for confirmation purposes in residue surveillance analysis was made. The presented LC-MS/MS method for residues of anthelmintic drugs in milk is currently used under accredited conditions for monitoring of Belgian farm milk.

Samenvatting

Samenvatting

In de algemene inleiding van dit doctoraal proefschrift worden de theorische aspecten en achtergrondinformatie over residuen van diergeneesmiddelen in levensmiddelen benadrukt (hoofdstuk 1). Residuen van diergeneesmiddelen zijn niet alleen afkomstig van onoordeelkundig, illegaal of buiten label gebruik van diergeneeskundige formuleringen. Ook door oordeelkundig gebruik van diergeneesmiddelen kunnen kleine restconcentraties beneden MRL waarde voorkomen. Kruiscontaminatie in mengvoederbedrijven is een ander gekend probleem. Ook contaminatie uit het milieu kan een bron zijn van ongewilde residuen. Het overbrengen van residuen van dier tot dier door recirculatie mag niet verwaarloosd worden en kan aanleiding geven tot heel lange uitscheidingstijden, en dit speciaal in eieren. De aspecten van residuen van diergeneesmiddelen zijn gereguleerd in de Europese wetgeving. Recentelijk werd de Beschikking 2002/657/EC van 12 augustus 2002 van de Europese Commissie gepubliceerd. Deze Beschikking implementeert Richtlijn 96/23/EG betreffende de prestaties van analysemethoden en de interpretatie van resultaten. Deze Beschikking bepaalt dat het resultaat van een residuanalyse voortaan als niet-conform wordt beschouwd als de beslissingsgrens van de bevestigingsmethode voor het analyt wordt overschreden. Het grootste nadeel van bestaande chromatografische bevestigingsmethoden met lage detectiegrenzen is dat deze niet geschikt zijn voor routinebepalingen omwille van de zeer arbeidsintensieve en tijdrovende staalopzuivering. Bevestigingsmethoden dienen gevalideerd te worden om hun betrouwbaarheid aan te tonen. De parameters die uitvoerig onderzocht werden voor de gepresenteerde kwantitatieve bevestigingsmethoden zijn: specificiteit, lineariteit, juistheid, precisie, recovery, analytische limieten, stabiliteit en toepasbaarheid/robuustheid. De relatieve retentietijden en de relatieve intensiteiten van de gemeten precursor- en productionen van de analyten werden opgevolgd bij de vloeistof chromatografische-massaspectrometrische methoden.

Het experimenteel deel van deze studie handelt over toegepast onderzoekswerk dat recentelijk onder meer beknopte vorm werd gepubliceerd of werd ingediend voor publicatie in wetenschappelijke tijdschriften. Het gepresenteerde experimenteel werk kende zijn ontstaan in behoeften van het laboratorium van het Departement voor de Kwaliteit van dierlijke producten en transformatietechnologieën (CLO-DVK, Melle, België), betrokken bij de screening van dierlijke producten voor residuen van diergeneesmiddelen. Er was noodzaak aan bevestiging van verdachte stalen voor antibiotica en aan detectiemogelijkheden voor residuen van ontwormingsmiddelen wegens het ontbreken van praktische screeningstesten hiervoor. Het grootste deel van het onderzoekswerk was toegespitst op residuen van ontwormingsmiddelen en de bestudeerde levensmiddelen waren zowel melk als eieren, gevogelte- en schapenweefsels.

Het experimenteel werk van dit doctoraal proefschrift is onderverdeeld in twee afzonderlijke delen. De ontwikkeling van chromatografische multiresidu bevestigingsmethoden voor de kwantitatieve bepaling van tetracyclines en ontwormingsmiddelen met inbegrip van metabolieten wordt gerapporteerd en beschreven in het eerste deel (hoofdstuk 2, 3, 4, 5 en 6). Een beter inzicht in de residuproblematiek en het gedrag van geneesmiddelen in het dierlijk lichaam werd bekomen via farmacokinetische studies die beschreven zijn in het tweede deel (hoofdstuk 7, 8, 9 en 10). De ontwikkelde methoden konden uitvoerig worden getest met de veelvuldige praktijkstalen bekomen van de uitscheidingsstudies.

Residuen van tetracycline, oxytetracycline, chloortetracycline en doxycycline in eieren en spierweefsel van gevogelte werden bepaald via HPLC met UV diode array detectie bij 355 nm (hoofdstuk 2). Een aanvaardbare scheiding van de analyten werd bekomen via ion-paar chromatografie. Door gebruik te maken van deze ion-paar techniek kon de staalopzuivering minimaal gehouden worden. De vier tetracycline antibiotica kunnen in eieren en spierweefsel gedetecteerd worden tot een limietconcentratie die varieert van 2.2 tot 27.3 µg/kg. Supplementaire analytbevestiging werd bekomen aan de hand van het karakteristieke UV spectrum via de diode array detector.

De risico's voor de volksgezondheid door residuen van ontwormingsmiddelen zijn eerder beperkt. Niettemin zijn door de Europese Commissie ook voor deze geneesmiddelen MRL waarden vastgelegd en worden hiervoor ook wachttijden aanbevolen. Teratogeniciteit wordt als het grootste potentiële risico aangehaald in de literatuur. Aanvullend aan de welomschreven antibioticaresistentie, worden ook voorzorgsmaatregelen aanbevolen voor het vertragen of voorkomen van het ontstaan van resistentie tegen ontwormingsmiddelen. Effectief zijn het toedienen van de correcte dosis, het strikt toepassen van het aantal noodzakelijke behandelingen, het jaarlijks veranderen van de gebruikte groep ontwormingsmiddelen en het verzekeren dat de kiemvrije dieren niet geïnfecteerd worden met resistente stammen.

De bepaling van residuen van ontwormingsmiddelen in melk werd onderzocht via twee verschillende detectietechnieken. Twee separate HPLC methoden werden geoptimaliseerd voor de bepaling van levamisole, fenbendazole, thiabendazole, albendazole en oxibendazole (hoofdstuk 3). Voor deze componenten aangevuld met oxfendazole, febantel en triclabendazole werd ook een multiresidu LC-MS/MS methode ontwikkeld (hoofdstuk 4). Omwille van zijn afwijkende eigenschappen diende levamisole met een andere staalvoorbereidingsprocedure behandeld te worden in de HPLC-DAD methode. Voldoende analyt scheiding en aanvaardbare detectielimieten werden bekomen met ion-paar vloeistofchromatografie en UV diode array detectie bij 225 nm voor levamisole en bij 295 nm voor fenbendazole, thiabendazole, albendazole en oxibendazole. De voorheen gebruikte polymere kolom met i.d. van 4.6 mm werd vervangen door een omgekeerde fase C_{18} kolom met i.d. van 3.2 mm, wat resulteerde in minder pH beperkingen en hogere analyt responswaarden. De bekomen detectielimieten varieerden

van 0.5 tot 3.8 µg/l. De beschikbaarheid van massaspectrometrie voor de residuanalysen was zeer voordelig. Meer specificiteit en lagere detectiegrenzen werden bekomen via tandem massaspectrometrie. De bekomen specifieke massaspectra met de karakteristieke precursor- en productionen bieden een zeer krachtig analyt bevestigingsmedium. Bovendien dienen de componenten in de multiresidu methoden chromatografisch niet volledig gescheiden te zijn omwille van de krachtige scheidingscapaciteit van de tandem quadrupool massaspectrometer voor componenten met precursor- en productionen met verschillende moleculaire massa. Omwille van het zeer specifieke detectieprincipe kan de staalopzuivering bijna compleet achterwege gelaten worden. De analyten werden gedetecteerd via electrospray ionisatie in de positieve mode. Verdere miniaturisatie van de LC kolom werd doorgevoerd. Een omgekeerde fase C_{18} kolom met i.d. van 2.1 mm werd gebruikt voor iedere LC-MS/MS methode beschreven in deze thesis.

De analysemethode voor ontwormingsmiddelen in melk is heel gevoelig en elke component kon gedetecteerd worden tot een residuconcentratie lager dan 1 µg/l. De terugvinding voor de ontwormingsmiddelen kon verhoogd worden tot 90% door de analyten te extraheren in een alkalische melk matrix. The LC-MS/MS methode werd grondig uitgewerkt. De validatie werd uitgevoerd op twee concentratie niveaus. De betrouwbaarheid van de methode was bewezen zowel op MRL niveau als op het niveau van een interne prestatielimiet welke op 1 µg/l werd vastgelegd. De beslissingsgrens en het detectievermogen, twee analytische limieten van het nieuwe validatieconcept, werden eveneens bepaald. Voor levamisole en triclabendazole, twee verboden ontwormingsmiddelen voor lacterende melkkoeien, werd een beslissingsgrens van respectievelijk 1.2 en 1.3 µg/l bekomen. De methode is ook geschikt voor het kwantificeren van residuen van toegelaten ontwormingsmiddelen op MRL niveau, waardoor het laboratorium voorzien wordt van een krachtig medium om beslissingen te nemen bij overtredingen vastgesteld bij officiële analysen.

Een gevalideerde LC-MS/MS methode wordt voorgesteld voor de bepaling van flubendazole en de gehydrolyseerde en gereduceerde metabolieten in eieren en in spier- en leverweefsel van gevogelte (hoofdstuk 5). Flubendazole is het meest frekwent gebruikte ontwormingsmiddel bij pluimvee. De chromatografische scheiding van de analyten werd uitgevoerd met gradiëntelutie met een mobiele fase bestaande uit acetonitrile en ammoniumacetaat buffer. Deze ammoniumacetaat buffer is echter niet stabiel en geeft aanleiding tot blokkering van de tubings en tot een minder robuuste analysemethode. Verdere optimalisatie in de volgende procedures gaf aan dat het gebruik van deze buffer kon worden vermeden en dat deze met succes kon worden vervangen door een mobiele fase bestaande uit acetonitrile en water dat 0.1% mierenzuur bevat om de ionisatie-intensiteit te verhogen. De bekomen detectielimieten voor flubendazole, de gehydrolyseerde en de gereduceerde metabolieten in eieren en spierweefsel zijn respectievelijk 0.2, 0.3 en 1.1 µg/kg en 0.1, 0.8 en 0.3 µg/kg.

Schapen zijn één van de gevoeligste diersoorten voor parasietinfecties veroorzaakt door ingewandswormen. Staalvoorbereidingsprocedures en een LC-MS/MS bepalingsmethode zijn ontwikkeld voor residuen van mebendazole en de gehydrolyseerde en de gereduceerde metabolieten in spier-, lever-, nier- en vetweefsel (hoofdstuk 6). De methode is gevalideerd voor spier- en leverweefsel op respectievelijke residuniveaus van 10, 20 en 60 µg/kg en 50, 100 en 400 µg/kg. De beslissingsgrenzen in spierweefsel voor mebendazole, de gehydrolyseerde en de gereduceerde metabolieten bedragen respectievelijk 72.5, 69.2 en 69.8 µg/kg. Deze analytische grenswaarden in leverweefsel zijn respectievelijk 445.9, 466.5 en 463.4 µg/kg. Residuen van mebendazole en metabolieten kunnen met de beschreven methode echter gedetecteerd worden tot concentraties lager dan 2 µg/kg.

De distributie- en uitscheidingsstudies van residuen van tetracyclines en ontwormingsmiddelen in eieren, melk en eetbare weefsels van pluimvee en schapen zijn beschreven in het tweede deel van dit proefschrift. De hoeveelheid residuen in levensmiddelen wordt beïnvloed door farmacokinetische processen zoals absorptie, distributie van het geneesmiddel in en verwijdering uit (metabolisatie en uitscheiding) het dierlijk lichaam. De aanbevolen wachttijden dienen gerespecteerd te worden om te verzekeren dat de residuconcentratie in het dierlijk product is afgenomen tot beneden de veilige MRL waarde.
De uitscheiding van oxytetracycline in eieren is onderzocht gedurende en na de orale behandeling van leghennen met een therapeutische dosis van 840 mg per kg voeder gedurende zeven opeenvolgende dagen (hoofdstuk 7). De absorptie en uitscheiding van oxytetracycline residuen in eieren geschiedde traag. Pas na vijf opeenvolgende behandelingsdagen werd een plateauwaarde in residuconcentratie bereikt welke ongeveer overeenkwam met het MRL niveau van 200 µg/kg. Twee dagen na stopzetting van de toediening daalde de residuconcentratie terug beneden het MRL niveau en daalde traag verder gedurende de volgende dagen van de wachtperiode. De uitscheiding van tetracycline in spierweefsel van braadkippen werd bestudeerd gedurende en na orale behandeling via voeder dat gemedicineerd werd met een therapeutische dosis van 480 mg per kg gedurende zeven opeenvolgende dagen (hoofdstuk 7). Tetracycline wordt zeer vlug geabsorbeerd, efficiënt verdeeld in het lichaam van de braadkip en vlug uitgescheiden in het spierweefsel. De geaccumuleerde tetracycline residuhoeveelheid in het spierweefsel is veel hoger dan dat van oxytetracycline in eieren. De plateau residuconcentratie van ongeveer 1200 µg/kg in het spierweefsel werd reeds bereikt na één dag behandeling. De residuconcentratie daalde zeer snel na de medicatieperiode en daalde onder het MRL niveau na de vierde dag in de wachtperiode.
Levamisole is een klassiek imidazothiazole geneesmiddel bestanddeel dat nog frekwent wordt gebruikt voor ontworming van melkkoeien. Het ontwormingsmiddel is sedert enkel jaren niet meer toegelaten voor gebruik bij lacterend melkvee. De uitscheiding van levamisole in melk is

onderzocht met melkkoeien die behandeld werden via de “pour-on” techniek in twee verschillende periodes met de aanbevolen dosis van 10 mg voor niet-lacterende dieren en met een dubbele dosis van 20 mg per kg levend gewicht (hoofdstuk 8). Levamisole wordt zeer vlug opgenomen en snel uitgescheiden in de melk. Een maximale residuconcentratie van 1896 µg/l kon reeds worden gemeten zeven uur na toediening van de aanbevolen dosis. Een wachtperiode van 48 uur is te kort om residuvrije melk te bekomen. Er werd nog steeds een residuconcentratie van 88 µg/l gemeten.

De farmacokinetische eigenschappen van flubendazole bij vogelachtigen zijn onderzocht in twee onderscheiden uitscheidingsstudies met kalkoenen en parelhoenders die oraal behandeld werden met de aanbevolen en met een hogere dosis (hoofdstuk 9). Flubendazole is niet geregistreerd voor behandeling van parelhoenders. Flubendazole werd toegediend aan de kalkoenen en aan de parelhoenders via gemedicineerd voeder met respectievelijke dosissen van 19.9 en 29.6 mg en van 56.2 en 86.1 mg per kg voeder en dit gedurende zeven opeenvolgende dagen. De verdeling en uitscheiding van flubendazole en van de gehydrolyseerde en gereduceerde metabolieten werd gemeten in borstspier-, in bilspier- en in leverweefsel. Gedurende de medicatie van de kalkoenen met de aanbevolen dosis overstegen de geaccumuleerde residuwaarden in de weefsels het MRL niveau dat vastgelegd is op 50 µg/kg voor de som van de residuen van flubendazole plus de gehydrolyseerde metaboliet in spierweefsel. Een maximale gemiddelde residuconcentratie van 742.8 µg/kg werd bereikt in bilspierweefsel na vier dagen behandeling. Flubendazole metaboliseerde intensief en werd getransformeerd in de gehydrolyseerde metaboliet. De residuconcentratie voor de metaboliet was ongeveer 3 tot 4 maal deze van de ouder component. De residuwaarden voor de gereduceerde metaboliet waren verwaarloosbaar. De uitscheiding van residuen in bil- en borstspierweefsel zijn vergelijkbaar. Eén dag na de behandeling daalde de residuhoeveelheid tot het concentratieniveau van de MRL waarde. Niettegenstaande dit en omdat gedurende de behandeling hoge residuwaarden werden bekomen kan overwogen worden om een aanbevolen wachtperiode van 24 uur na behandeling te adviseren alvorens de kalkoenen te slachten.

Soortverschillen tussen kalkoenen en parelhoenders werden vastgesteld. In tegenstelling tot de resultaten bekomen met de kalkoenen konden in de eetbare weefsels van de parelhoenders hoge residuconcentraties voor de gereduceerde metaboliet van flubendazole gedetecteerd worden. De uitscheiding van residuen in spierweefsels van parelhoenders startte nog sneller dan in de weefsels van de kalkoenen. Na één dag behandeling werd reeds een plateau residuwaarde van ongeveer 400 µg/kg voor de som van residuen van flubendazole en de gereduceerde metaboliet bekomen. De afname in residuhoeveelheid in de weefsels na stopzetting van de behandeling was trager dan bij de kalkoenen. Residuveiligheid in de eetbare weefsels na behandeling met de therapeutische dosis kan worden gegarandeerd na een wachtperiode van drie dagen.

Ondanks een snelle daling in residuconcentratie juist na stopzetting van de behandeling neemt de totale uitscheiding van residuen in weefsels van zowel kalkoenen als parelhoenders toch meerdere dagen in beslag. Recirculatie van residuen gestimuleerd door de grondhuisvesting, kan een mogelijke oorzaak zijn voor dit vastgesteld fenomeen.
De uitscheiding van residuen afgeleid van mebendazole in schapenweefsels is onderzocht met een beperkte farmacokinetische studie met schapen die oraal behandeld werden met een enkelvoudige dosis equivalent met 20 mg mebendazole per kg levend gewicht (hoofdstuk 10). De residuen van mebendazole, de gehydrolyseerde en de gereduceerde metabolieten werden gekwantificeerd in ribspier-, bilspier-, lever-, nier- en vetweefsel en dit 1, 7, 14, 21 en 28 dagen na behandeling. Na één dag uitscheiding varieerden de residuconcentraties van 21 tot 7630 µg/kg. Met uitzondering voor het vetweefsel werd steeds de hoogste residurespons bekomen voor de gereduceerde metaboliet. Er is geen gevaar voor de volksgezondheid indien de wachtperiode van één week na behandeling wordt gerespecteerd, alvorens de schapen worden geslacht. Enkel verwaarloosbare hoeveelheden residuen werden waargenomen in ribspier-, nier- en leverweefsel na één week uitscheiding. De gereduceerde metaboliet in leverweefsel en de gehydrolyseerde metaboliet in nierweefsel kon tot 14 dagen na behandeling nog worden gedetecteerd.

Met deze doctoraatsstudie werd een aanvullende bijdrage geleverd aan de verzameling van geschikte analysemethoden voor monitoringsprogramma's en voor bevestigingsdoeleinden in residucontrole analysen. De voorgestelde LC-MS/MS methode voor residuen van ontwormingsmiddelen in melk wordt momenteel onder geaccrediteerde omstandigheden gebruikt voor de monitoring van Belgische hoevemelk.

Annex: Curriculum vitae

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1 Personalia

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geboren	:	op 01/02/63 te Oudenaarde
burgerlijke staat	:	gehuwd, twee kinderen
nationaliteit	:	Belg

2 Loopbaan

2.1 Vanaf 16/01/89

Wetenschappelijke Instelling van de Vlaamse Gemeenschap (voorheen Ministerie van Middenstand en Landbouw)
Centrum voor Landbouwkundig Onderzoek - Gent (CLO)
Departement Kwaliteit van dierlijke producten en transformatietechnologie (DVK)
Brusselsesteenweg 370, 9090 MELLE
tel. 0032 (0)9 272 30 31, fax. 0032 (0)9 272 30 01, e-mail: H.DeRuyck@clo.fgov.be

- functie: assistent
- hoofdtaken:
  - leiden van opeenvolgende onderzoeksprojecten:
    - kwaliteitsproblemen bij de bereiding van melkpoeder
    - ontwikkeling van speciale melkpoeders
    - toepassingsmogelijkheden van membraanfiltratietechnologie
    - toepassingsmogelijkheden van extrusietechnologie
  - huidige onderzoeksprojecten:
    - ontwikkeling van chemische bevestigingsmethoden voor het bepalen van residuen van diergeneesmiddelen in dierlijke producten
    - uitscheidingsstudies van residuen van diergeneesmiddelen in dierlijke producten
  - dienstverlening in opeenvolgende taken:
    - pilootfabriekverantwoordelijke voor technologisch proefwerk betreffende membraanfiltratie, indampen, drogen en extrusie van zuivelproducten, algemene levensmiddelen en diervoeder
    - laboratoriumverantwoordelijke voor het fysico-chemisch laboratorium, geaccrediteerd volgens EN 45001

- huidige dienstverlening:
  - laboratoriumverantwoordelijke voor het laboratorium voor specifieke analysen van gedroogde zuivelproducten
  - laboratoriumverantwoordelijke voor bepaling van residuen van klassieke antiparasitaire diergeneesmiddelen in melk in chromatografisch laboratorium geaccrediteerd volgens ISO/IEC 17025, monitoring van Belgische melk voor residuen van klassieke antiparasitaire diergeneesmiddelen in kader van het Fonds voor de Voedselveiligheid, bescherming van de Volksgezondheid en Leefmilieu in opdracht van het Federaal Agentschap voor de Veiligheid van de voedselketen (FAVV)
  - adjunct-milieucoördinator DVK
- neventaken:
  - begeleiding van thesisstudenten (zie paragraaf 7)
  - referee voor volgende wetenschappelijke tijdschriften: Food Additives and Contaminants, Analytica Chimica Acta, Journal of Pharmaceutical and Biomedical Analysis, Journal of Food Engineering en World Rabbit Science
  - geven van lessen in cursussen en presentatie van posters en lezingen op studiedagen en congressen
  - officiële melkpoederkeurder voor het FAVV
  - behandelen van aanbestedingen

2.2 Van 01/03/88 tot 31/12/88

- assistent aan het Laboratorium voor levensmiddelentechnologie, -microbiologie en -chemie (diensthoofd: Prof. dr. ir. A. Huyghebaert) van de Faculteit Landbouwkundige Wetenschappen aan de Rijksuniversiteit Gent
- IWONL-project "Studie van membraantechnieken bij de isolatie van melkeiwitpreparaten en van hun aanwending in gefermenteerde zuivelproducten"
- assisteren bij laboratoriapractica
- begeleiden van thesisstudent

2.3 Vanaf 01/08/87 tot 31/01/88

- laboratoriumverantwoordelijke bij NV Clovis Matton, Avelgem
- kwaliteitscontrole van land- en tuinbouwzaden en in vitro-bepaling van de voederwaarde van geënsileerde ruwvoeders

3 Studies

- Universitaire studies van 1982 tot 1987 aan de Rijksuniversiteit Gent:

  Ingenieur voor de Scheikunde en de Landbouwindustrieën

  - specialisatie: technologie en bedrijfsleiding van landbouw- en voedingsnijverheden
  - thesis: valorisatie van immunoglobulinen uit wei
  - afgestudeerd met onderscheiding

- Voorbereidend jaar in 1981 - 1982:
  - St. Amandusinstituut Gent
  - Bijzonder Wetenschappelijke klas

- Middelbaar onderwijs van 1975 tot 1981:
  - Vrij Landelijk Instituut Oudenaarde
  - Technisch onderwijs, A_2 - Landbouw

4 Publicaties

- **De Ruyck H.**
  Mogelijkheden van extrusie voor de zuivelindustrie.
  De melk en wij, 1991, (1), 21-26

- **De Ruyck H.**
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- **De Ruyck H.**, Daeseleire E., Grijspeerdt K., De Ridder H., Van Renterghem R. and Huyghebaert G.
  Distribution and depletion of flubendazole and its metabolites in edible tissues of guinea fowl.
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5 Presentaties

- The origin of white flecks of instant whole milk powder.
  IDF-meeting workgroup E2, 15 maart 1990, Universiteit Gent, Gent

- Bereiding van verstuivingsmelkpoeder voor de chocolade-industrie.
  Comilac (Comelco), 21 februari 1991, Aalter

- Toepassingsmogelijkheden van extrusie in de zuivel.
  28e CTL zuivelstudiedag, 22 november 1991, Gent

- Extrusion technology for the production of fish feed.
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- Residuen van antiparasitaire middelen in melk en zuivelproducten.
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- Uitscheiding van residuen van diergeneesmiddelen in eieren en gevogeltevlees. Studienamiddag van “the World’s Poultry Science Association” (WPSA): residuproblematiek in de pluimveevoeding, 30 april 2002, Melle

6 Posterpresentaties

- **De Ruyck H.,** Daeseleire E., De Ridder H., Van Renterghem R. and Huyghebaert G.
  Determination of flubendazole and metabolites in eggs and poultry meat with LC-MS/MS.
  Euroresidue IV: Residues of veterinary drugs in food, 8-10 mei 2000, Veldhoven, Nederland

- Daeseleire E., **De Ruyck H.** and Van Renterghem R.
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  Rapid confirmatory assay for the simultaneous detection of ronidazole, metronidazole and dimetridazole in eggs using LC-MS/MS.
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  Determination of flubendazole and metabolites in eggs and poultry meat with LC-MS/MS.
  9th Annual meeting of the Flemish society for veterinary epidemiology and economics (VEE): Risk analysis and quality control of animal products, 25 oktober 2001, Melle

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- **De Ruyck H.**, Daeseleire E., De Ridder H. and Van Renterghem R.
  Development and validation of a liquid chromatography-electrospray tandem mass spectrometry multiresidue method for anthelmintics in milk.
  7th International symposium on Hyphenated Techniques in Chromatography and hyphenated chromatographic analysers (HTC-7), 6-8 februari 2002, Brugge

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  Congrilait 2002: 26th IDF World Dairy Congress, Food Safety, Residues and Chemical Contaminants, 24-27 september 2002, Paris, Frankrijk

7 Begeleiding van thesissen en eindwerken als promotor, copromotor of begeleider

- Ir. Ketels P.
  Verwijdering van micro-organismen door microfiltratie.
  Ingenieur voor de Scheikunde en de Landbouwindustrieën, Levensmiddelenchemie, -microbiologie en -technologie, Rijksuniversiteit Gent, 1987 - 1988

- Ing. De Moor K.
  Karakterisatie van white flecks bij instant volle melkpoeder.
  Industrieel Ingenieur Landbouw, optie Landbouw- en voedingsindustrieën, specialisatie Zuiveltechnologie, CTL Gent, 1989 - 1990

- Ing. De Kinne L.
  Bepaling van white flecks bij instant volle melkpoeder.
  Industrieel Ingenieur Landbouw, optie Landbouw- en voedingsindustrieën, specialisatie Zuiveltechnologie, CTL Gent, 1990 - 1991

- Ir. Van Waeyenberge S.
  Texturisatie van wei-eiwitten door verhittingsextrusie.
  Ingenieur voor de Scheikunde en de Landbouwindustrieën, Conserveringstechnologie, Katholieke Universiteit Leuven, 1992 - 1993

- Rosseel C.
  HPLC analyse van antibioticaresiduen in melk.
  Gegradueerde in Chemie, optie Chemie, Campus Hoger Technisch Instituut, Brugge, 1996 - 1997

- Verbeke K.
  Bepaling van tetracyclineresiduen in eieren en kippenvlees via HPLC.
  Gegradueerde in Chemie, optie Chemie, Campus Hoger Technisch Instituut, Brugge, 1997 - 1998

- Vermeire P.
  Bepaling van residuen van anthelmintica in melk via HPLC.
  Gegradueerde in Chemie, optie Chemie, Departement Technologie BME-CTL, Gent, 1998 - 1999

- Ing. Van Heghe A.
  Bepaling van residuen van anthelmintica in melk met HPLC.
  Industrieel Ingenieur in Landbouw en Biotechnologie, optie Landbouw- en voedingsindustrieën, specialisatie Zuiveltechnologie, CTL Gent, 1998 - 1999

- De Roover K.
  Uitscheiding van residuen van flubendazole en zijn metabolieten in parelhoenvlees.
  Gegradueerde in Chemie, optie Chemie, Departement Technologie BME-CTL, Gent, 2001 - 2002

- Coudijzer V.
  Bepaling van albendazolsulfoxide en netobimin in melk met vloeistofchromatografie met massaspectrometrische detectie.
  Gegradueerde in Chemie, optie Chemie, Departement Technologie BME-CTL, Gent, 2002 - 2003

8 Professionele lidmaatschappen

- International Dairy Federation (IDF):
  - Standing Committee on Analytical Methods for Additives and Contaminants, Joint Action Team on Antimicrobials and other veterinary medicinal residues
  - Standing Committee on Dairy technology
- Belgian Society for Mass Spectrometry (BSMS)
- KVIV