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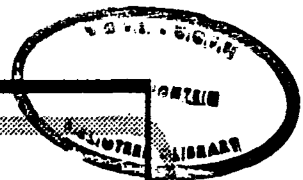

IDENTIFICATION AND CONFIRMATION OF THE PRESENCE OF SOME STEROID-LIKE GROWTH PROMOTERS IN THE URINE OF CATTLE AND SWINE

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IDENTIFICATION AND CONFIRMATION OF THE PRESENCE OF SOME STEROID-LIKE GROWTH PROMOTERS IN THE URINE OF CATTLE AND SWINE

JACOBUS WILHELMUS PIETERSE

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FOREWORD

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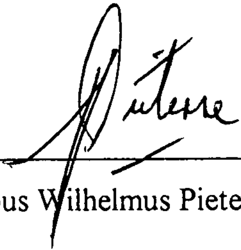
"Our Lord and God! You are worthy to receive glory, honour and power.
For you created all things, and by your will they were given existence and life." ... Revelation 4:11

Dedicated to:

My wife Marietjie, and our three sons Zjak, Hanré and Hansen.

DECLARATION

It is herewith declared that this dissertation for the degree Master of Medical Science at the University of the Orange Free State is the independant work of the undersigned and has not previously been submitted by him at any other University or Faculty for a degree. In addition, copyright of this dissertation is hereby ceded in favour of the University of the Orange Free State.



Jacobus Wilhelmus Pieterse

98-11-25

Date

ABBREVIATIONS AND SYNONYMS

CAS	Chemical Abstracts Service
CI	Chemical Ionization
DES	Diethylstilbestrol
EC	European Community
E-DES	"Entgegen" in German (configurational descriptor : opposite side)
EEC	European Economic Community
EI	Electron Impact / (Ionization)
Epi-nandrolone	17α-19-nortestosterone
Epi-norandrosterone	5α-estran-3β-ol-17-one
Epi-trenbolone	17α-trenbolone
FDA	Federal Drug Administration
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
HPLC	High-Performance / (Pressure) Liquid Chromatography
LC	Liquid Chromatography
LOD	Limit of Detection
MIKES	Mass-analyzed Ion Kinetic Energy Spectrometry
MRL	Maximum Residue Limit / (Level)
MS	Mass Spectrometry
MSD	Mass Selective Detector
MSTFA	N-methyl-N-trimethylsilyl-trifluoroacetamide
Nandrolone	17β-19-nortestosterone
PFTBA	Perfluorotri-n-butylamine
ppb	Parts Per Billion
ppm	Parts Per Million
ppt	Parts Per Trillion
PRE	Pigmented Retinal Epithelum
RIVM	Rijksinstituut voor Volksgezondheid en Milieuhygiene, Netherlands
SIM	Selected Ion Monitoring
Taleranol	7β-zearalanol
Trenbolone	17β-trenbolone
v	volume
Z-DES	"Zusammen" in German (configurational descriptor : same side)
Zeranol	7α-zearalanol
Zeranol	Zearalanol

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1 GENERAL

Over the past several decades, management schemes of livestock production have changed to reflect the changing demands of the consumer. In our consumer-driven economy, these livestock producers need to be using a variety of techniques to produce sufficient meat products of a good quality that meet current human dietary guidelines.

Growth rate and feed efficiency are important traits in livestock production. Anything that can be used to improve liveweight gain on any given feeding system, has a corresponding financial benefit to both the consumer and producer of the meat products. One of the early attempts to chemically manipulate growth was by Stotsenburg (1913) who studied the effects of gonadal hormones on growth [Buttery, *et al*, 1978].

Anabolic agents or "muscle-building" growth promotants are used throughout most of the world to improve growth rates and feed conversion efficiency of livestock. Anabolic agents are compounds that stimulate protein synthesis and thus increase muscle size and strength, both in humans and in animals [Lagana and Marino, 1991].

The mode of action of synthetic anabolic compounds is not fully understood. However, judging by their over-all effects upon metabolism, they may be divided into two classes:

- those which have estrogenic properties, e.g. diethylstilbestrol
- those which have androgenic properties, e.g. trenbolone acetate [Buttery, *et al*, 1978]

Those compounds effective in humans and horses are invariably steroids structurally related to the natural androgen testosterone, whereas in food-producing animals, androgenic- and estrogenic substances, or combinations of both may be used. The estrogens may be derived from the natural steroid 17β-estradiol or may have a non-steroidal structure, e.g. diethylstilbestrol or zeranol [Metzler, 1989].

A minimum concentration of anabolic agent in the blood and tissues is necessary to obtain the maximum growth response in farm animals. An ideal formulation would therefore supply the agent at a constant rate sufficient to maintain this minimum concentration for as long as possible [Harrison, *et al*, 1983].

Compressed pellets of anabolic agents are widely used as subcutaneous implants, although they do not achieve this ideal but tend to release the drug very rapidly at first and then more slowly, so that after a few months the concentrations in the blood and tissues are too low to be effective [Harrison, *et al*, 1983].

As a result much of the drug is wasted and the high concentrations found in the tissues during the early part of the treatment could present a risk to the consumer. To avoid this risk a withholding time or "withdrawal time" between treatment and slaughter of 60 to 90 days is recommended [Harrison, *et al*, 1983].

In food-producing animals, the risk posed by residues of the anabolic agents to the health of the consumer is the major concern. Residues found in carcasses can be classified into three groups:

- the non-altered parent compounds and/or their free metabolites which are extractable by specific organic solvents
- water soluble conjugates (e.g. sulphates, glucuronides)
- non-extractable covalently-bound compounds [Evrard and Maghuin-Rogister, 1987]

The residues in food of animal origin, the liver usually being the main site of disposition, may have pharmacological activity in humans (antiasthmatic, tocolytic, etc.) due to greater or lesser oral bioavailability of these compounds. At present, certain anabolics can be given legally to farm animals in some countries, but are banned in most others because of their proved or alleged toxic and/or carcinogenic properties. The use of these substances is completely forbidden within the European Economic Community (EEC). One of the motives of the total ban, is the protection of the consumer's health [Lagana and Marino, 1991].

In South Africa, an international testing programme is followed whereby residue levels are monitored in fulfilment of the requirements of the European Economic Community (EEC) in order to export meat products to the EEC and earn foreign exchange. However, no national testing program exists for growth stimulants, but only for antibiotics and pesticides.

Because very little is known about the mechanisms underlying the toxic effects of anabolics, the assessment of the risks associated with these agents is a very difficult task at present. For the analysis of biological samples and for an understanding of the toxicity of a compound, pharmacokinetic- and metabolic considerations are of the utmost importance. The pharmacokinetics of these drugs include their absorption, metabolism, detoxification or inactivation and elimination [Chichila, et al, 1988].

The necessity to test for illegal use or to determine residue levels after legal use, has led to a strong interest in developing analytical methods for the identification and confirmation of anabolic agents in biological samples.

Only the following growth-promoting veterinary drugs were studied in cattle and/or swine:

- Clenbuterol
- Diethylstilbestrol
- Nandrolone
- Trenbolone
- Zeranol

1.2 OBJECTIVES

- To develop suitable analytical methods with a view to identify residues of some growth-promoting veterinary drugs and/or their metabolites in the urine of cattle and/or swine
- To confirm the presence of these veterinary drugs unequivocally in the urine
- To establish stability parameters for some of these veterinary drugs in urine under different environmental storing conditions from sampling to analysis

CHAPTER 2

LITERATURE SURVEY

2. LITERATURE SURVEY

2.1 IDENTIFICATION AND CONFIRMATION

Traditional analytical forensic strategies are based on a two step approach:

- screening with a method optimized for a high sample throughput and the prevention of false negative results

- a confirmation method optimized for reliable identification and the prevention of false positive results

According to the guidelines set by the European Community, the methods to be used in forensic analysis have to fulfil a number of criteria. The main objective of these criteria is to prevent the occurrence of false positive results [van Ginkel, et al, 1991]. Apart from general analytical quality criteria, the following criteria are layed down for purposes of identification:

2.1.1 CRITERIA FOR THE IDENTIFICATION OF ANALYTES

In a commission decision 93/256/EEC (1993) the following criteria for the identification of analytes were layed down for the Member States of the European Community:

2.1.1.1 Gas Chromatography

An internal standard should be used if a material suitable for this purpose is available. It should preferably be a stable isotope labelled form of the analyte or, if this is not available, then a related standard with a retention time close to that of the analyte.

The ratio of the retention time of the analyte to that of the internal standard, i.e. the relative retention time of the analyte, should be the same as that of the standard analyte in the appropriate matrix, within a margin of ± 0.5%.

2.1.1.2 Mass Spectrometry

For screening only:

The intensity of at least the most abundant diagnostic ion must be measured.

For confirmation only:

The intensities of preferably at least four diagnostic ions should be measured. If the compound does not yield four diagnostic ions with the method used, then identification of the analyte should be based on the results of at least two independant GC-MS methods with different derivatives and/or ionization techniques, each producing two or three diagnostic ions. The molecular ion should preferably be one of the four diagnostic ions selected.

The relative intensities of the diagnostic ions detected, expressed as a percentage of the intensity of the base peak, must be the same as those for the standard analyte within a margin of ± 10% (EI mode) or 20% (CI mode). GC-MS is the method of choise for confirmatory analysis as direct information concerning the molecular structure of the substance under examination is provided.

2.1.2 METHODS FOR THE IDENTIFICATION OF GROWTH-PROMOTING SUBSTANCES

In an overview by Covey, *et al*, 1988, it was found that a variety of chemical methods exist for the determination of steroid-like growth promoters. Methods have been reported using thin-layer chromatography with a variety of detection methods such as sulfuric acid-induced fluoresence, fluoresence enhanced by derivatization and fluoresence induced by photochemical reaction.

Methods have been reported which use gas chromatography with electron capture detection, as well as high-performance liquid chromatography with electrochemical, fluoresence, ultraviolet, diode array and immunochemical detection.

Several methods using gas chromatography-mass spectrometry (GC-MS) have been reported. A method using mass-analyzed ion kinetic energy spectrometry (MIKES) has also been reported.

General methods for the extraction of estrogens and other steroids using lipophilic gel chromatography for analysis by GC-MS have been extensively developed. Methods employing high-performance liquid chromatography/mass spectrometry (HPLC-MS) were reported, some using the direct liquid introduction technique and others using thermospray LC-MS. Tandem mass spectrometric methods have also been reported.

2.2 CLENBUTEROL

2.2.1 PHYSICAL AND CHEMICAL PROPERTIES

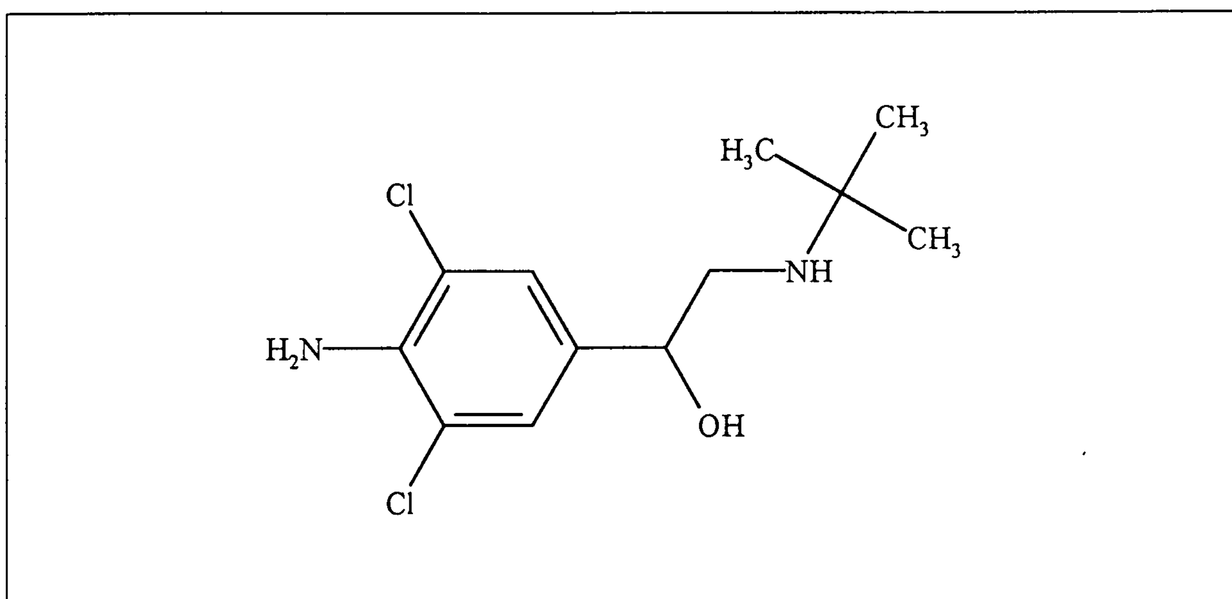

Figure 1 Molecular structure of clenbuterol

Chemical Name

4-amino-3,5-dichloro-α-[[(1,1-dimethylethyl)amino]methyl]benzenemethanol

Molecular Formula $C_{12}H_{18}Cl_2N_2O$

Molecular Weight 277.18

CAS Registration 37148-27-9

Therap Cat. Anti-asthmatic

Therap Cat (vet). Bronchodilator; tocolytic

2.2.2 USES AND ADMINISTRATION

Clenbuterol hydrochloride is a direct-acting sympathomimetic agent with predominantly beta-adrenergic activity and a selective action on beta receptors (beta$_2$-agonists). It is used as a bronchodilator in the management of reversible airways obstruction as in asthma and in certain patients with chronic obstructive pulmonary disease.

A usual dose is 20 µg two or three times daily by mouth; doses of up to 40 µg twice daily have occasionally been employed. Clenbuterol hydrochloride is also given by inhalation in usual doses of 20 µg three times daily.

{**Source** : Budavari, et al, 1989 and Reynolds, et al, 1996}.

The biologically active β-ethanolamino group together with the N-t-butyl substituent is the common feature in the clenbuterol structure.

Clenbuterol is a synthetic orally active β_2-adrenergic agonist which was originally developed only for therapeutic use as a tocolytic agent and for the treatment of bronchial disease. Its positive effect on the growth of slaughter animals was described for the first time in 1984. It causes a reduction of body fat and promotes muscle growth; thus, it is called a "repartitioning" agent [Meyer and Rinke, 1991].

Carcass composition is altered by reducing net fat accretion and enhancing net lean deposition in a variety of species [Geesink, et al, 1993]. For this purpose, clenbuterol needs to be given in dosages 5 to 10 times higher than those required for therapeutic treatments [Meyer and Rinke, 1991]. Such a use of clenbuterol in veal calves led to residue accumulation in all tissues great enough to have potential pharmacological effects on consumers if no withdrawal period was observed [Stoffel and Meyer, 1993].

In veterinary medicine, the recommended therapeutic dosage of clenbuterol is 0.8 µg/kg body weight twice daily [Wilson, et al, 1994].

A significant advantage of β_2-agonists for growth promotion is that they are orally active which allows them to be mixed with animal feed [Collins, et al, 1994]. Clenbuterol has been shown to have a prolonged duration of action and good oral bioavailability [Tsai and Kondo, 1994].

When β_2-agonists are used as growth promoters in animal production, incorporation at 2 to 4 ppm in the diet of cattle increases the growth rate by about 20% over a 3-4 month treatment period. The effects of β_2-agonists in sheep (at about 2 ppm) are similar to those in cattle. In pigs, the effective dose appears to be about 1 ppm over the final 2 months of the finishing period [Tsai and Kondo, 1994].

For these reasons some β_2-agonists (clenbuterol being the most prominent representative) are used on a large scale as growth promoters. The possible adverse effects on the health of consumers of meat originating from treated animals has led in most countries to a total ban of clenbuterol and other β_2-agonists for fattening purposes [Courtheyn, 1991].

In 1990, a number of cases of food poisoning were reported in Spain following consumption of bovine liver which was subsequently found to contain levels of clenbuterol of 160-291 ng/g [Blanchflower, et al, 1993].

2.2.3 METABOLISM AND PHARMACOKINETICS

Confidence in establishing compliance with withdrawal periods or illegal use of clenbuterol as a repartitioning agent, through the determination of residues in edible tissues and body fluids, requires a knowledge of the pharmacokinetic parameters relating to such treatment [Sauer, et al, 1995].

Clenbuterol repartitions energy intake resulting in increased lean muscle deposition and depletion of bodyfat reserves. Reports on the required duration of clenbuterol medication to bring about optimum repartitioning vary.

It is suggested that a period of approximately one month achieves the desired changes in body composition. During prolonged treatment, the compound accumulates in various animal tissues at varying concentrations.. This has been associated with several instances of food poisoning in man following consumption of contaminated bovine tissue [Elliot, et al, 1993b].

β_2-agonists are orally active and effective at around 0.25 to 4 mg/kg in food, depending on the animal species [de Groodt, et al, 1989]. Because the active dose is very low and the apparent distribution volume relatively high, the resulting plasma and tissue concentrations of clenbuterol following oral administration of the drug, are in the parts per trillion (ppt) range [Girault and Fourtillan, 1990].

Clenbuterol residues are detectable in urine and bile for about 5 days after withdrawal of growth-promoting doses, whereas residues have been shown to persist in liver for 25 to 30 days. More recently analysis of retinal extracts has been shown to extend the detection of administration after withdrawal to at least 50 days. The positive repartitioning effects gained by administering a β_2-agonist are gradually lost following removal from the diet. The term "β_2-agonist reverse effect" (muscle depletion and fat accretion) has been used to describe the reversal of the repartitioning properties of β_2-agonists following withdrawal [Elliot, et al, 1993a].

Meyer and Rinke (1991) conducted a trial to examine the pharmacokinetics of clenbuterol in veal calves treated with 5 μg/kg of body weight twice daily for three weeks, representing a dose for growth promotion, in order to obtain basic information about the tissue residues of clenbuterol that can be expected after such treatment. The results show that a rise of clenbuterol concentrations in plasma could be detected within 20 to 60 minutes after treatment. A comparison of clenbuterol concentrations in plasma shows that the absorption of clenbuterol is rapid. The range of urinary concentrations found was 6 to 193 ng/ml. The concentrations in urine were approximately 40 times higher than in plasma during withdrawal.

To determine the elimination rate constant ($t_{½}$) of clenbuterol via urine, two linear regressions were calculated. The first regression represents the course of the elimination until day 3 of withdrawal, and the second regression from day 3.35 onward.

According to these data, the half-life of clenbuterol in urine amounts to 10 hours for the first phase of elimination and to approximately 2.7 days for the second phase. The biphasic elimination agrees with earlier observations in dogs, humans and rats [Meyer and Rinke, 1991].

Meyer and Rinke (1991) found the highest residue concentration in the eye. The release of clenbuterol from the eye is very slow. They also reported that in different animals almost identical levels were found after the same withdrawal, indicating a very consistent mode of distribution into and elimination from the eye.

Because of the strong accumulation and slow elimination, the eye may be the most useful tissue for residue screening. Negative results from analysis of the eye would give a virtual guarantee that no edible tissues contain residues. Residue screening is most practical by analyzing urine. Levels in urine are mostly < 1 ppb after 4 days of withdrawal, at which time levels in edible tissues are reduced and strong pharmacologic effects to the consumer can not be expected [Meyer and Rinke, 1991].

Sauer and co-workers (1995) also investigated the pharmacokinetics, distribution and disposition of clenbuterol residues in male calves (n = 30) treated orally with clenbuterol at a growth-enhancing dose. They found no significant difference between clenbuterol concentrations in kidney and liver for the first 2 days after withdrawal. From day 4 of withdrawal onwards, however, concentrations were consistently higher in liver than in other tissues and fluids. Concentrations in choroid/pigmented retinal epithelum (choroid/PRE) were at least 10 times higher than in liver at all periods following cessation of treatment and 52 times higher 16 days after treatment.

These data suggest that analysis of choroid taken at the abattoir would provide reliable surveillance information on the use or abuse of clenbuterol within the slaughter population. It is proposed that liver samples taken concurrently could be used in the event of a positive result from the choroid/PRE analysis to indicate whether the maximum residue limit (MRL) for edible tissues has been exeeded [Sauer, et al, 1995].

Van Ginkel and co-workers (1991) investigated the conjugation of clenbuterol by treating samples from treated animals with a preparation containing enzymes with β-glucuronidase and -sulphatase activity. They concluded that there is no evidence for conjugation of clenbuterol, nor for instability during prolonged incubation.

Biotransformation, disposition and excretion processes serve to terminate the pharmacologic activity of administered drugs in virtually all living organisms. Drugs and associated metabolites can usually be found in a variety of fluids and tissues, including hair, after drug exposure. The unusually long residence time for drugs in hair has sparked interest in the use of this tissue for the detection of drugs of abuse. The ability to detect drugs in hair for months after use, makes hair analysis highly attractive for drug detection [Cone, 1996].

Hair growth is a highly active metabolic process, yielding immediate information of the active circulating drug concentrations in the not yet keratinized zones of the root. Later this information is stored in the keratinized hair follicles [Moeller, 1996].

Drugs of abuse can appear in hair following active use and from passive exposure. The route of drug entry into hair is not presently known, but it may take place via many complex pathways including entry from blood, sebum, sweat, skin and the environment. Specific binding of basic drugs to hair components is likely to involve both electrostatic attraction and weaker forces such as van der Waals attraction [Cone, 1996].

Hair is easily collected from the living animal and stored until analysis owing to its biological stability and to its physical state. In anti-doping control, the analysis of hair for β_2-agonists could provide complementary information to urine analysis, allowing the theoretical possibility of discriminating acute administration to achieve stimulatory effects from chronic use necessary to obtain the "anabolic" effect [Polettini, et al, 1996].

In their study, Adam and co-workers (1994) showed that, in contrast to tissues, clenbuterol could be detected in hair at least 20 days after the last dose in rats. This accumulation of clenbuterol in hair after chronic administration of the drug is similar to that previously reported for other illicit drugs such as cocaine.

Polettini and co-workers (1996) tested their proposed method on real hair samples obtained from guinea pigs treated with a growth-promoting dose. They concluded that hair is a suitable matrix for the detection of β_2-agonists after intraperitoneal dosing. Accordingly potential applications of hair analysis to prevent the misuse of β_2-agonists both in sports for doping purposes and in zootechnics for growth-promoting purposes can be developed.

However, hair is not a uniform fiber. It consists of very different morphological structures, e.g., cuticle, cortex, medulla, melanin granules and cell membrane complex, each distinct in structure and chemical composition, with different morphological and biochemical properties [Moeller, 1996].

For hair testing to be useful, an understanding must be developed of the fundamental chemical and pharmacologic principles governing the appearance and disappearance of drugs and/or their metabolites in this matrix. Obviously, the mechanism(s) by which drugs are deposited and the site of deposition are key elements [Cone, 1996].

2.3 DIETHYLSTILBESTROL

2.3.1 PHYSICAL AND CHEMICAL PROPERTIES

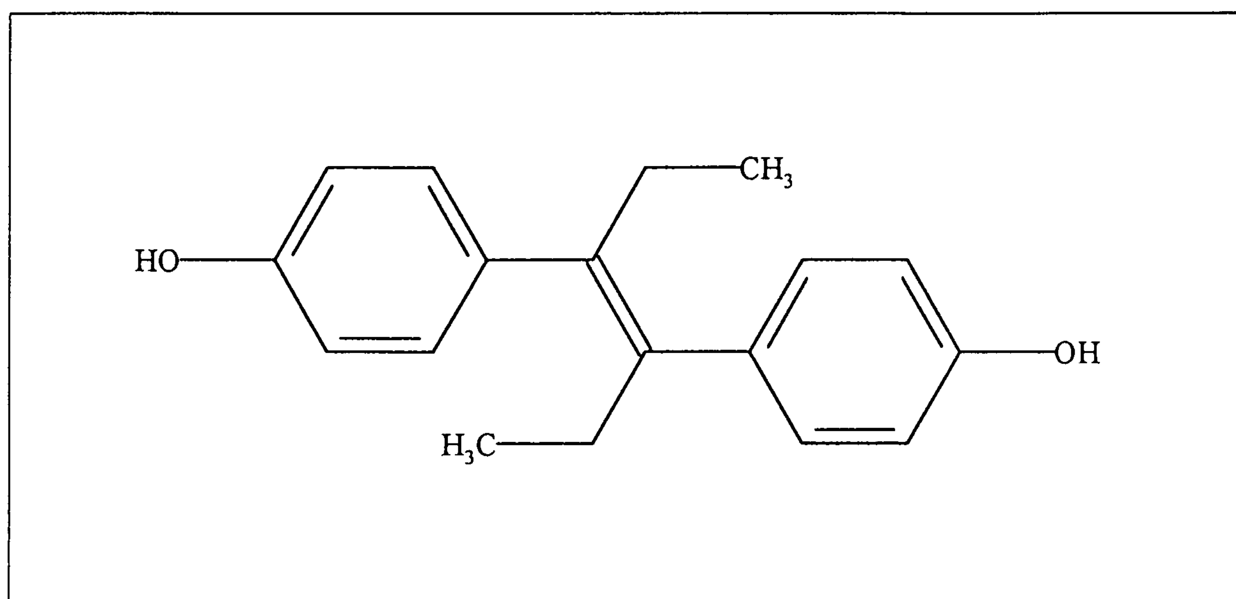

Figure 2 Molecular structure of trans-diethylstilbestrol

Chemical Name

4,4'-(1,2-diethyl-1,2-ethenediyl)bisphenol

Molecular Formula	$C_{18}H_{20}O_2$
Molecular Weight	268.34
CAS Registration	00056-53-1

Therap Cat.	Estrogen
Therap Cat (vet).	Formerly in estrogenic hormone therapy

2.3.2 USES AND ADMINISTRATION

Daily doses of 10 to 20 mg may be used by mouth in the palliative treatment of malignent neoplasms of the breast in postmenopausal women or in men but other agents are usually preferred.

The usual dose in carcinoma of the prostate is 1 to 3 mg daily by mouth; higher doses were formerly given and again other agents have come to be preferred. Diethylstilbestrol has also been used in the treatment of prostatic carcinoma in the form of its diphosphate and diphosphate sodium salts.

{**Source** : Budavari, *et al*, 1989 and Reynolds, *et al*, 1996}.

Diethylstilbestrol (DES) is a non-steroidal estrogen first synthesized and described by Dodds in 1938 [Korach, *et al*, 1978]. Estrogens are defined as those substances that mimic the action of the natural female sex hormone, estradiol [Covey, *et al*, 1988].

Although DES is also known as stilboestrol in some countries, stilboestrol itself, with no diethyl groups, is devoid of oestrogenic activity [Page, 1991].

It is known that diethylstilbestrol can exist in two stereoisomeric forms. E-DES possesses estrogenic activity comparable to estradiol whereas Z-DES is only weakly active, based on its interaction with the estrogen receptor *in vitro*. Non-enzymatic isomerization of E-DES to Z-DES has also been reported previously [Degen and McLachlan, 1983]. Partial isomerization occurs when DES and its metabolites are exposed to daylight [Metzler, 1989].

DES is insoluble in water but soluble in organic solvents and vegetable oils, is active by both oral and parenteral routes and is available as the base or as various esters and ethers including the diphosphate and dipropionate [Page, 1991].

DES is a powerful synthetic estrogen that has been used since the 1950's in human medicine [Bagnati, *et al*, 1990], for a diverse array of indications in small animals [Page, 1991] and as growth promoter in cattle [Bagnati, *et al*, 1990].

As a result of the findings of Burroughs and co-workers (1954), DES has been extensively used in livestock breeding to promote liveweight gain and feed conversion efficiency [Reuvers, *et al*, 1991]. Approximately 10-20% improvement in gain and feed efficiency have been attributed to implanting with 24-36 mg DES per animal during the feedlot period, with some reports indicating that response to implants is maximum during the first half of the feeding period [Rumsey, *et al*, 1974].

The medicinal and veterinary uses of DES had to be re-evaluated several times, due to great concern about the toxicity and possible carcinogenicity of the drug in humans. The carcinogenic potential of DES has now been established by many epidemiological studies, and it has formally proven transplacental carcinogenic action in humans [Marselos and Tomatis, 1993].

The carcinogenicity of synthetic DES was known as early as 1938 [Epstein, 1990]. These results of Lacassagne, perhaps because they involved only a few animals and were published in French, did not attract much attention outside a limited circle of oncologists [Marselos and Tomatis, 1993].

Used as a growth promoter, DES produces the same effect when used as an ear implant as when added to cattle feeds [Short Notes in Nature Vol. 243 May 1973 - DES banned again]. The esters of DES exhibit a sustained action, with the dipropionate derivative having the longest activity [Marselos and Tomatis, 1993].

2.3.3 METABOLISM AND PHARMACOKINETICS

Metzler (1989) published the following data regarding the major pathways in the metabolism of diethylstilbestrol:

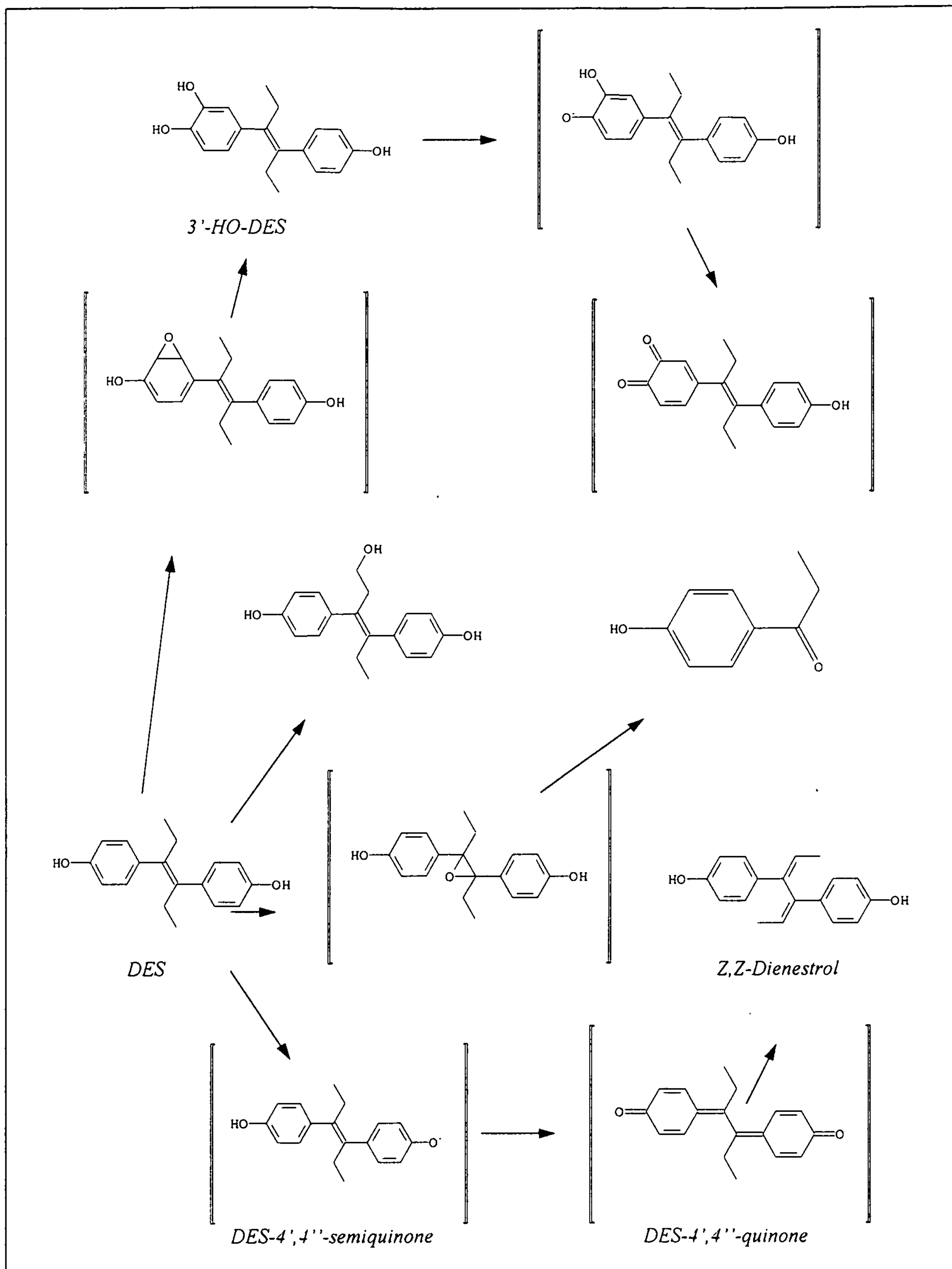

Figure 3 Major pathways in the metabolism of diethylstilbestrol. Only the trans-isomers are shown. Compounds in brackets denote putative reactive intermediates [Metzler, 1989]

Metabolic studies of DES *in vivo* and *in vitro* have shown that DES can be hydroxylated at the aromatic ring and at the methyl group. In addition, a second double bond can be introduced, leading to β-dienestrol and its aromatic and aliphatic hydroxy derivatives. Cleavage of the DES molecule is also observed, giving rise to 4'-hydroxy-propiophenone. Most of these pathways involve potentially reactive intermediates. Aromatic hydroxylation, for example, possibly proceeds through an arene oxide, and the resulting catechol might be further oxidized to the respective ortho-semiquinone and quinone [Metzler and McLachlan, 1979].

The formation of 4'-hydroxy-propiophenone most likely involves an epoxide of the olefinic double bond of DES. The ω-hydroxy-β-dienestrol, which is a major metabolite in most species, is not reactive itself, but could gain reactivity after metabolic esterification, e.g. with sulphuric- and glucuronic acid. Reactive semiquinone- and quinone-like intermediates must be assumed for the metabolic formation of β-dienestrol from DES [Metzler and McLachlan, 1979].

The putative intermediates in DES metabolism imply electrophilic reactivity which is illustrated by the irreversable binding of radiolabelled DES to cellular macromolecules. This has been frequently observed *in vivo* as well as *in vitro* in the presence of suitable metabolic systems. Because there are several potentially reactive intermediates in DES metabolism, the question arises as to which pathway is critical for the genotoxic and possibly also for the carcinogenic effect of DES. Several lines of evidence imply that the pathway leading to Z,Z-dienestrol may be of particular relevance [Metzler, 1984]. The other most likely candidate is aromatic hydroxylation to the catechol 3'-hydroxy-DES [Hey, *et al*, 1986].

DES is readily absorbed after oral administration, although the exact percentage of its intestinal absorption is difficult to calculate due to extensive enterohepatic circulation. DES is a lipid-soluble substance which is readily distributed in the whole organism. In cattle given a single oral dose of radioactive DES (10 mg), the kinetics followed a biphasic depletion curve attributed to hepatic clearance. An initial steeper slope represented a biological half-life of 17 hours, while the half-life for the later phase was 5.5 days [Marselos and Tomatis, 1993].

Pellets of 24-36 mg DES implanted subcutaneously in cattle or steers liberated about 56-74 μg of DES per day into the circulation; the half-life was 80-90 days [Marselos and Tomatis, 1993]. Hale and co-workers (1959) reported that the absorption rate of DES from 12 mg ear implants in beef cattle followed a first order function and that implants were effective for 150-175 days.

2.4 NANDROLONE

2.4.1 PHYSICAL AND CHEMICAL PROPERTIES

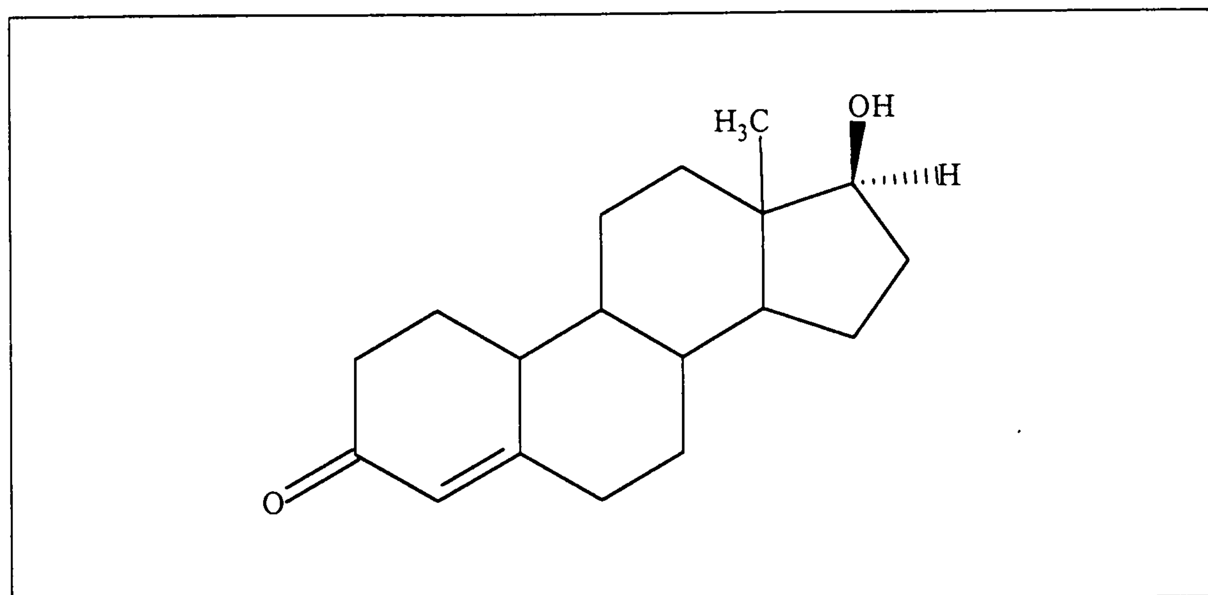

Figure 4 Molecular structure of nandrolone

Chemical Name

17β-hydroxyestr-4-en-3-one

Molecular Formula $C_{18}H_{26}O_2$

Molecular Weight 274.39

CAS Registration 00434-22-0

Therap Cat. Anabolic

2.4.2 USES AND ADMINISTRATION

Nandrolone has anabolic and androgenic properties. It is administered usually as the decanoate or phenylpropionate esters in the form of oily intramuscular injections. Suggested doses of nandrolone decanoate and phenylpropionate are generally the same, but the decanoate ester has a longer duration of action being given generally every 3 or 4 weeks whilst the phenylpropionate is usually given each week.

Doses of 25 to 100 mg have been used as an anabolic after debilitating illness. Doses of 50 mg have been suggested for use in postmenopausal osteoporosis and doses of 25 to 100 mg for postmenopausal metastatic breast carcinoma but other agents are usually preferred in these conditions. The undecanoate has been used similarly in doses of about 80 mg. Doses of between 50 and 200 mg weekly have been suggested for nandrolone decanoate in the treatment of anaemias.

Nandrolone sodium sulphate has been used topically in the treatment of corneal damage. Nandrolone cyclohexylpropionate and nandrolone laurate have been used in veterinary medicine. Nandrolone hexyloxyphenylpropionate and nandrolone propionate have also been used.

{**Source** : Budavari, et al, 1989 and Reynolds, et al, 1996}.

Nandrolone (17β-19-nortestosterone) and its esters are synthetic anabolic steroids which are widely used as therapeutic agents. Since nandrolone promotes an increased formation of tissue protein, while being less androgenic than the natural steroid testosterone, but with similar efficiency [Benoît, et al, 1989], [Meyer, et al, 1989], it has also been used for non-therapeutic purposes; in humans to improve athletic performance, or in the veterinary field as a growth-promoting agent to accelerate weight gain and improve feeding efficiency in cattle [Benoît, et al, 1989].

2.4.3 METABOLISM AND PHARMACOKINETICS

Oruindi et al (1995) published the following data regarding the major metabolic pathway of nandrolone in the bovine:

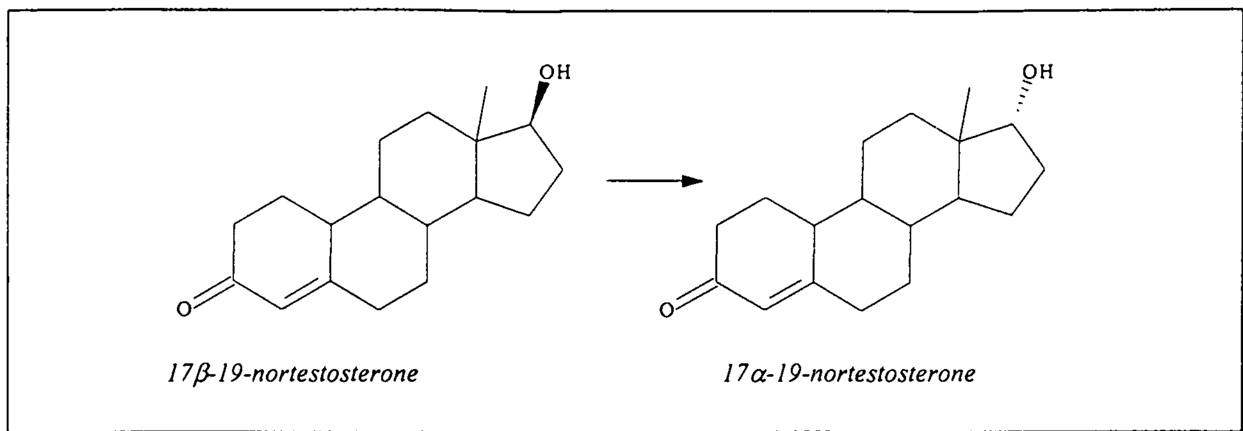

Figure 5 Major metabolic pathway of nandrolone in the bovine [Oruindi, et al, 1995]

Owing to extensive metabolism, the nortestosterone content of bovine urine from treated animals is very low [van Ginkel, et al, 1989]. Several studies demonstrated that the major part of 17β-19-nortestosterone is metabolized and excreted in bovine urine as 17α-19-nortestosterone [Oruindi, et al, 1995].

The results of Meyer and co-workers (1992) indicate that nandrolone may be produced by the bovine placenta. The production of nortestosterone as an intermediate during estrogen synthesis via the C-19 decarboxylation pathway has been proposed earlier. However, it is still unclear whether nandrolone is originally synthesized in the bioactive 17β-form, with a following epimerization, or whether most of it is already produced in the inactive 17α-form.

These new studies of Meyer and co-workers (1992) provide evidence that nandrolone can be produced in cattle and that new threshold values in accordance with the animals' endocrine status are necessary. They suggested that 5 ng/ml could be a useful discriminatory level for treated male and non-pregnant female cattle.

Recent studies have also shown the existence of endogenous 19-nortestosterone in stallions and boars, as well as in mares and in pregnant women. Moreover, the natural presence of the steroid has also been suspected in calves [Vandenbroeck, et al, 1991].

From an excretion study done by Haasnoot and co-workers (1989) in veal calves, half-lives of 2 days for 17β-19-nortestosterone and 15 days for 17α-19-nortestosterone were calculated in urine.

In bovine species, C-17 epimerization is a major pathway of metabolism and 17α-19-nortestosterone is encountered in bovine urine after the administration of 17β-19-nortestosterone-containing veterinary preparations. In contrast to what is observed in bovine species, C-17 epimerization is not a major pathway of metabolism in miniature pigs [Debruyckere and van Peteghem, 1991]. The following metabolites were present in boar, barrow and sow urine after injection of Laurabolin® (100 mg).

- 5β-estran-3α-ol-17-one
- 5α-estran-3β-ol-17-one
- 5β-estran-3α,17β-diol (presumed stereochemistry) [Debruyckere and van Peteghem, 1991]

Hoogenboom and co-workers (1990) used porcine hepatocytes to examine the biotransformation of 17β-19-nortestosterone. Primary cultures of hepatocytes, isolated from livers of food-producing animals, have been shown to be a useful model for studying the biotransformation of growth-promoting agents and veterinary drugs. Initially, the major metabolite of nortestosterone was norandrostenedione, which upon prolonged incubation was further transformed, primarily to the glucuronide of 15α-hydroxy-norandrostenedione.

Hoogenboom *et al* (1990) published the following data regarding the major biotransformation pathway of nortestosterone in primary cultures of pig hepatocytes:

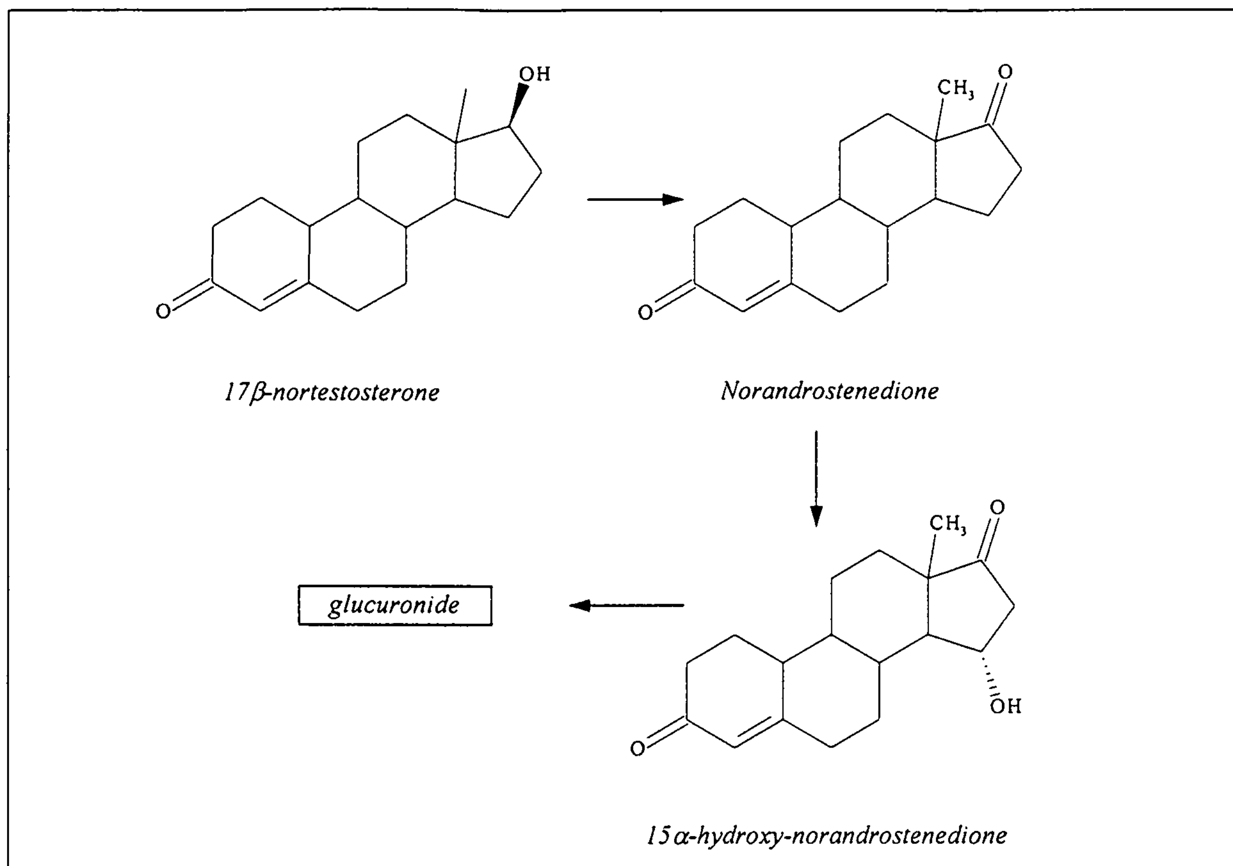

Figure 6 Major biotransformation pathway of nortestosterone in primary cultures of pig hepatocytes [Hoogenboom, et al, 1990]

For validation of the *in vitro* results, castrated male pigs were injected with nortestosterone. 15α-hydroxy-norandrostenedione, primarily as its glucuronide, was identified in the urine of this pig. In addition, norandrostenedione and the glucuronide of the parent compound were present in much smaller amounts [Hoogenboom, *et al*, 1990].

Meyer and co-workers (1989) tested their hypothesis that nortestosterone given orally or parenterally should be metabolized in a different manner and also the residue formation should be different. The results obtained for fat and urine from the same veal calves gave a different pattern of residues depending on prior treatment. Even after a long waiting period of 73 days, residues were still detectable in fat whereas in urine the nortestosterone levels were below 1 ng/ml.

Elevated doses and shorter waiting times resulted in an almost proportional increase in the residue levels in both fat and urine. In contrast, orally given nortestosterone did not cause residue formation in fat, but nortestosterone was present in urine. The results may be explained by the different metabolism depending on the route by which nortestosterone reaches the blood circulation. According to these results, discrimination between orally and parenterally administration seems possible, which may be of future forensic importance [Meyer, et al, 1989].

2.5 TRENBOLONE

2.5.1 PHYSICAL AND CHEMICAL PROPERTIES

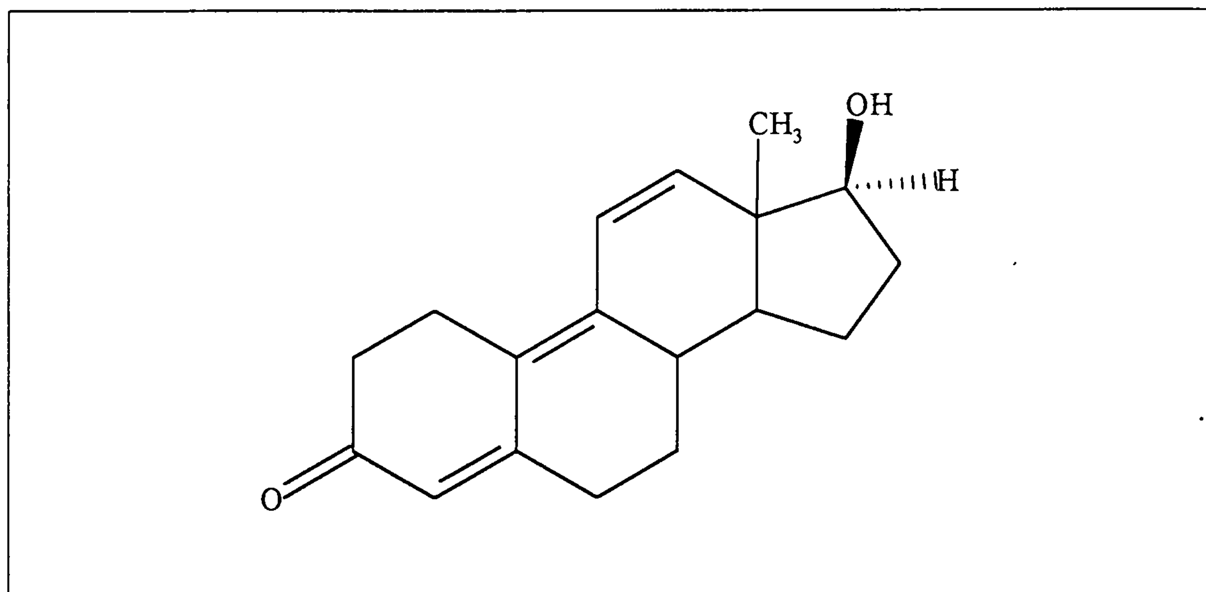

Figure 7 Molecular structure of trenbolone

Chemical Name

17β-hydroxyestra-4,9,11-trien-3-one

Molecular Formula $C_{18}H_{22}O_2$

Molecular Weight 270.38

CAS Registration 10161-33-8

Therap Cat. Anabolic

Therap Cat (vet). Anabolic

2.5.2 USES AND ADMINISTRATION

Trenbolone acetate is used as an anabolic agent in veterinary practice. The hexahydrobenzylcarbonate has also been used for its anabolic properties.

{**Source** : Budavari, et al, 1989 and Reynolds, et al, 1996}.

Trenbolone is a potent synthetic androgen with a trienic steroid structure. In the form of trenbolone acetate, this anabolic steroid has been used as a solid implant to promote growth, usually in combination with 17β-estradiol, in steers, heifers and veal calves [Hewitt, et al, 1993]. It has been exploited for improving the feed conversion rate and carcass characteristics of cattle intended for human consumption [Hsu, et al, 1988].

Free trenbolone can bind to both testosterone and estrogen receptors, altering the rates of protein synthesis and degradation. The net result of these interactions is an increase in skeletal muscle mass [Hewitt, et al, 1993]. Trenbolone acetate is administered as a subcutaneous implant in cattle at levels from 140 to 300 mg per animal [Ryan and Hoffmann, 1978].

2.5.3 METABOLISM AND PHARMACOKINETICS

Metzler (1989) published the following data regarding the metabolism of 17β-trenbolone:

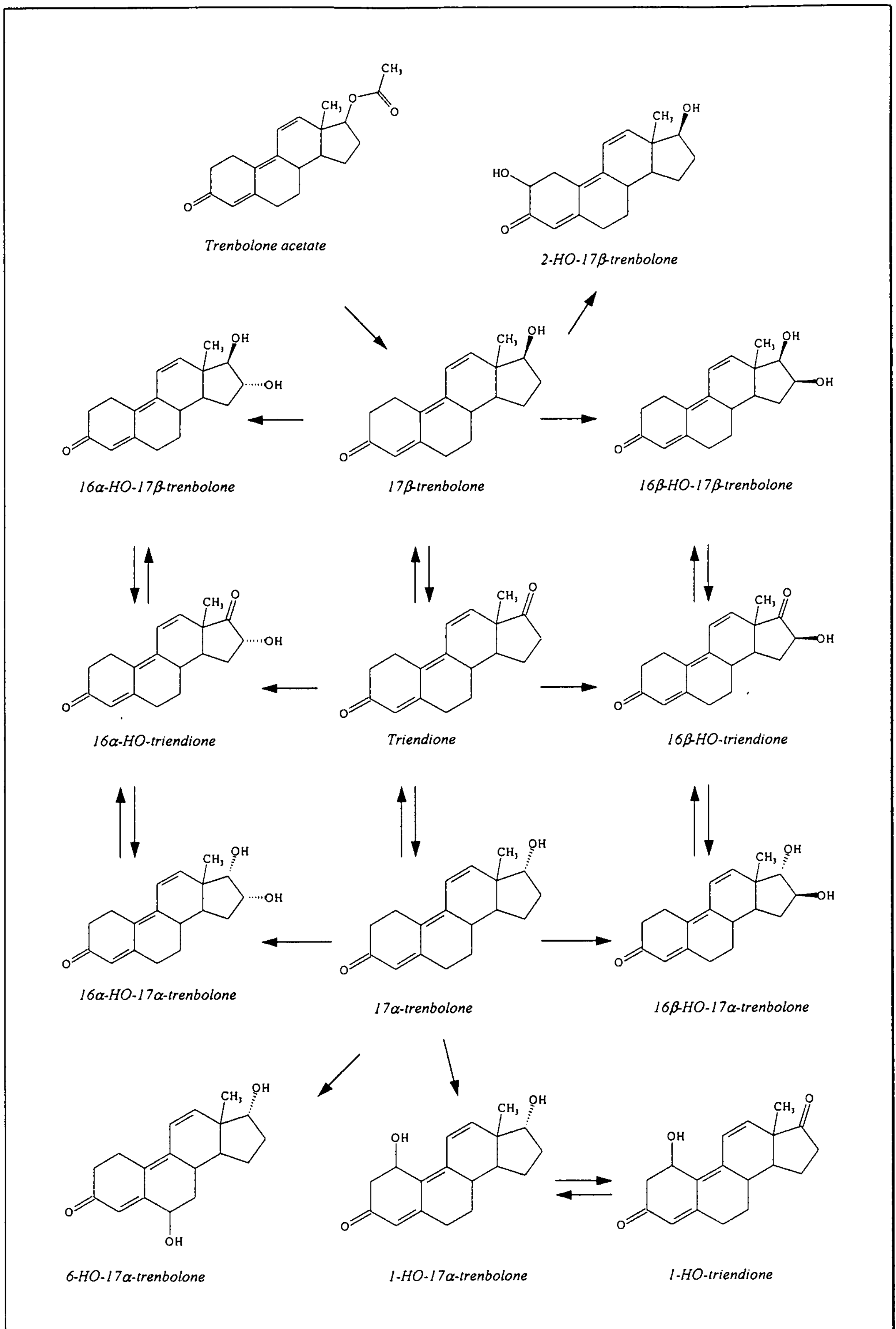

Figure 8 Metabolism of 17β-trenbolone [Metzler, 1989]

Trenbolone is the active anabolic metabolite of trenbolone acetate, formed by the rapid hydrolysis of the acetate group following absorption from the site of implantation [Heitzman and Harwood, 1977].

Previous studies on the metabolism of trenbolone in the cow have shown that after the administration of trenbolone acetate, 17β-trenbolone is the major metabolite in muscle and fat, while the epimer, 17α-trenbolone is the major metabolite in liver and kidney [Hsu, *et al*, 1988]. The 17β-trenbolone undergoes oxidation to trendione, leading to reduction to epi-trenbolone (17α-trenbolone) which, in the form of a glucuronide or sulphate conjugate, is the major biliary metabolite of trenbolone [Hewitt, *et al*, 1993].

Epi-trenbolone is the inactivated form of the original steroid, its remaining androgenic and anabolic activities being 5 and 2% respectively. After conjugation with sulphuric- or glucuronic acid, this metabolite is excreted into bile or urine [Evrard and Maghuin-Rogister, 1987].

In the rat, two major metabolic pathways occur:

- oxidation of the 17β-hydroxyl into the 17-oxo group (trendione)
- hydroxylation at the 16-position

Three major metabolites have been detected in bile:

- 17β-trenbolone
- 16α-hydroxy-trenbolone
- 16α-hydroxy-trendione [Evrard and Maghuin-Rogister, 1987]

Trendione and other hydroxylated derivatives of trenbolone and trendione have also been detected but in small quantities [Evrard and Maghuin-Rogister, 1987].

Trenbolone acetate is widely used as growth promoter, particularly in ruminant species. 17β-Trenbolone must be considered to be the major residue of trenbolone acetate in meat. Urine is the major route of excretion for 17β-trenbolone and its metabolites in humans. This is in contrast to the biliary excretion that predominates in rats and cows [Spranger and Metzler, 1991].

In all three species, the major fraction of the metabolites is excreted as glucuronides. Whereas in rats and cows, the unconjugated fraction accounts only for a minor part, in human urine free metabolites and sulphates are found in equal amounts [Spranger and Metzler, 1991].

The metabolic conversion of β-trenbolone into its α-epimer is known to decrease the androgenic and anabolic potency significantly. This means that metabolism of β-trenbolone should lead to inactivation. The observation of β-trenbolone residues covalently bound to proteins in cattle tissue, indicates that reactive intermediates are indeed formed in β-trenbolone metabolism [Spranger and Metzler, 1991].

2.6 ZERANOL

2.6.1 PHYSICAL AND CHEMICAL PROPERTIES

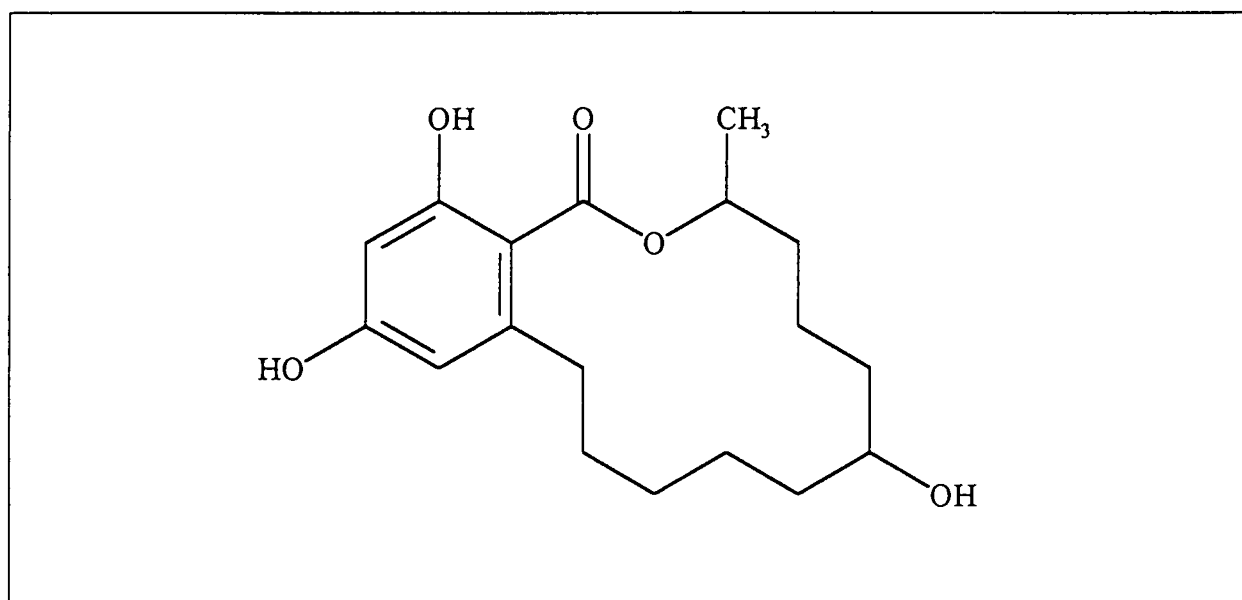

Figure 9 Molecular structure of zeranol

Chemical Name

3,4,5,6,7,8,9,10,11,12-decahydro-7α,14,16-trihydroxy-3-methyl-1H-2-benzoxacyclotetradecin-1-one

Molecular Formula $C_{18}H_{26}O_5$

Molecular Weight 322.41

CAS Registration 26538-44-3

Therap Cat (vet). Anabolic

2.6.2 USES AND ADMINISTRATION

Zeranol is a non-steroidal oestrogenic agent used for the management of menopausal and menstrual disorders and also promoted for the suppression of lactation; doses of 75 to 300 mg daily have been given. It has also been used as a growth promoter in veterinary practice.

{**Source** : Budavari, et al, 1989 and Reynolds, et al, 1996}.

During the early 1950's, researchers at Purdue University observed signs of estrogenic activity in sows fed moldy corn. The mold producing estrogenic activity was identified as *Gibberella zeae*, the perfect form of a common parasitic mold of corn, *Fusarium graminearum*. The active product was isolated, identified, and called zearalenone. More than 150 derivatives have been synthesized, the most promising being the dehydrogenated one at first called zearalanol. Later the name zeranol was adopted [Willemart and Bouffault, 1983]. Zeranol was discovered by chance and isolated in 1962 by Stob and co-workers. [Fumagalli, et al, 1989].

Zeranol/zearalenone and their isomers/metabolites are derivatives of benzoxacyclotetradecin, having the common chemical structure shown in Figure 10 (page 31) [Roybal, et al, 1988]. Zeranol (α-zearalanol), a resorcyclic acid lactone, is a weak synthetic estrogen obtainable, by chemical reduction, from the mycotoxin zearalenone (a compound produced by *Fusarium* moulds and present in contaminated stocks of cereals) [Bagnati, et al, 1991], [Chichila, et al, 1988].

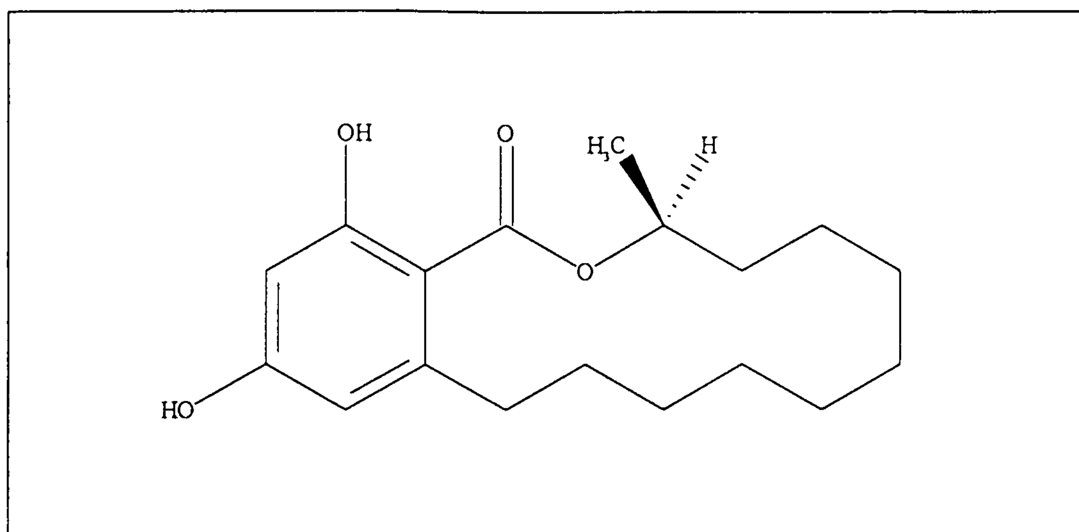

Figure 10 (3S)-3,4,5,6,7,8,9,10,11,12-decahydro-14,16-dihydroxy-3-methyl-1H-2-bonzoxacyclotetradecin-1-one [Roybal, et al, 1988]

Toxicological studies of zeranol have demonstrated that it is nonmutagenic, noncarcinogenic and nonteratogenic. Despite the apparent safety of zeranol, the debate over acceptable levels in animal food products continues [Chichila, et al, 1988].

The possible adverse effects of zeranol can be attributed to its hormonal properties, so acceptable residue levels must be below that which could have a hormonal effect on humans. The FDA has established the recommended dose of zeranol in beef cattle as a 36-mg implant with a 65-day withdrawal time [Chichila, et al, 1988]. The compound is generally given as an ear implant which has been reported to be effective for 84 to 112 days in cattle [Riesen, et al, 1977].

Zeranol is a nonsteroidal anabolic veterinary drug used commercially in cattle and sheep for increasing the rate of weight gain and improving feed efficiency [Roybal, et al, 1988]. The three-dimentional structure of zeranol exhibits relatively close spatial similarity to 17β-estradiol [Bories, et al, 1990]. From an environmental point of view, zeranol is a safe compound, being completely destroyed in cattle faeces at room temperature [Willemart and Bouffault, 1983].

2.6.3 METABOLISM AND PHARMACOKINETICS

Bories et al (1992) published the following data regarding the structures of zeranol and known metabolites:

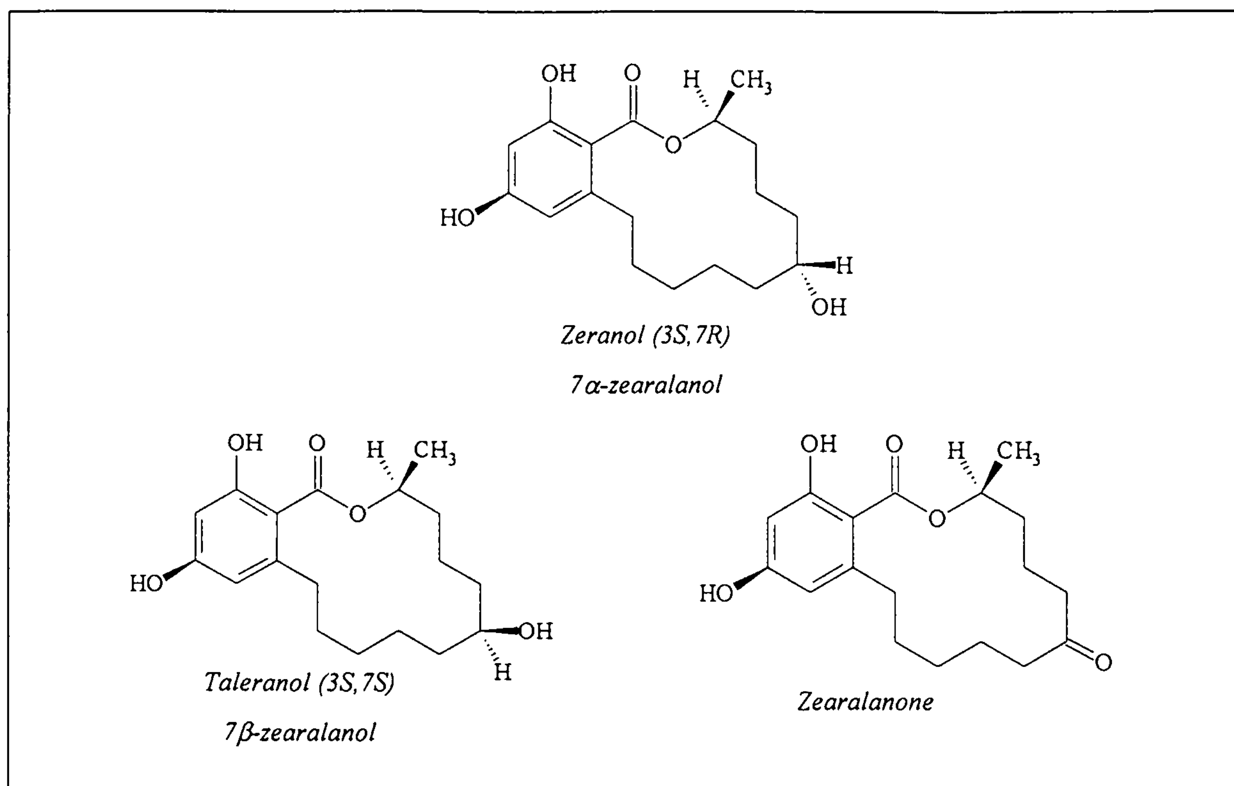

Figure 11 Structures of zeranol and known metabolites [Bories, et al, 1992]

The disposition and metabolism of zeranol has been studied in the rat, rabbit, dog, monkey and human following oral administration, and in the pig after implantation. In all species studied, the major phase-I biotransformation consists of the oxidation of the C-7 secondary alcohol to the corresponding ketone, zearalanone. Furthermore, the diastereoisomer β-zearalanol (taleranol), resulting from the reduction of zearalanone through aldo-keto-reductase action, has been identified in rabbit urine and pig plasma [Bories, et al, 1990]. In all these species, zeranol is absorbed and excreted readily after oral doses, but the relative amounts of zeranol and its metabolites are reported to vary greatly [Bagnati, et al, 1991].

There is some conflicting information regarding the main metabolite of zeranol in the bovine, although all sources agree that there are great differences in the metabolism of zeranol between species. From a study done by Chichila and co-workers (1988), it was concluded that the liver is the primary site of disposition, taleranol is the main metabolite, bile may be the main excretory route and clearance of zeranol from plasma is rapid.

It has been shown that zeranol and its metabolites undergo extensive conjugation as glucuro- and/or sulpho conjugates. Zeranol is metabolized in the pig following similar pathways to those in other species tested, i.e. oxido-reduction to zearalanone and taleranol, then glucuro- and sulpho conjugation as the major metabolic routes [Bories and Suarez, 1989].

Only monoconjugates are produced in the pig. It is suggested that conjugation occurs on the aromatic ring. Whether it is the C-14 or C-16 hydroxyl group that is conjugated, remains to be demonstrated [Bories, *et al*, 1990].

Pharmacokinetic studies using tritiated zeranol have shown that 65 days after implantation, 96.3% of the total dose is absorbed, but less than 0.1% of this absorbed dose remains in edible tissues [Chichila, *et al*, 1988].

2.7 MAXIMUM RESIDUE LEVEL (MRL)

The use of β_2-adrenergic agonists and xenobiotic anabolic agents as repartitioning agent or growth promotant is prohibited in food-producing animals and no residues of these anabolics should be present in animal products imported into or produced within the European Community (Directive 96/22/EC).

CHAPTER 3

EXPERIMENTAL

3. EXPERIMENTAL

3.1 CONSENT

Permission was granted by the ethical committee for animal studies of the University of the Orange Free State to conduct the animal trials in accordance with the protocols submitted.

3.2 ANIMAL TRIALS

3.2.1 CATTLE

Eight (8) steers were used for these experiments. They were kept in a metabolic building, each in a separate shed. Administration of the drugs took place in the second week, the first week being an acclimatisation period. Enough feed and fresh water were available for the duration of the trial.

3.2.1.1 Administration of Drugs

The various drugs were administered to the animals as indicated in Table 1 (page 36). Each animal received a single dose via the appropriate administration route.

Table 1 Drugs and their dosages administered to steers

ID	PROPRIETARY NAME	ACTIVE INGREDIENT	DOSE ADMINISTERED
C-1	TRIBOLIN '75'®	Nandrolone Decanoate	105 mg [a]
C-2	TRIBOLIN '75'®	Nandrolone Decanoate	105 mg [a]
C-3	Ralgro Super®	Zeranol	72 mg [b]
C-4	Ralgro Super®	Zeranol	72 mg [b]
C-5	Coopers Revalor®	Trenbolone Acetate	140 mg [b]
C-6	Coopers Revalor®	Trenbolone Acetate	140 mg [b]
C-7	Stilboestrol®	Diethylstilbestrol	20 mg [c]
C-8	Stilboestrol®	Diethylstilbestrol	20 mg [c]

3.2.1.2 Collection of Samples

Urine samples were collected on days 1, 2, 5, 7, 9, 11 ,14 ,16 and 19 after administration of the drugs. Blank urine samples were also collected before treatment (day 0). All the collected urine samples were stored immediately at -20°C until time of analysis.

3.2.2 SWINE

Eight (8) boars (trial 1 and 2) and six (6) gilts (trial 3) were used for these experiments. They were kept in a metabolic building, each in a separate shed. Administration of the drugs to the boars took place in the second week, the first week being an acclimatisation period. Enough feed and fresh water were available for the duration of the trials.

---

[a] intramuscular

[b] implant in the base of the ear

[c] orally

3.2.2.1 Administration of Drugs

The various drugs were administered to the boars as indicated in Table 2 below. Each of the boars received a single dose via the appropriate administration route.

Table 2 Drugs and their dosages administered to boars

ID	PROPRIETARY NAME	ACTIVE INGREDIENT	DOSE ADMINISTERED
S-1	TRIBOLIN '75'®	Nandrolone Decanoate	35 mg ✍
S-2	TRIBOLIN '75'®	Nandrolone Decanoate	35 mg ✍
S-3	Ralgro Super®	Zeranol	72 mg ☝
S-4	Ralgro Super®	Zeranol	72 mg 🖳
S-5	Coopers Revalor®	Trenbolone Acetate	140 mg ☝
S-6	Coopers Revalor®	Trenbolone Acetate	140 mg 🖳
S-7	Stilboestrol®	Diethylstilbestrol	20 mg 🖳
S-8	Stilboestrol®	Diethylstilbestrol	20 mg 🖳

The six gilts were part of another research study (trial 3) in which the effectiveness of three different growth stimulants were compared in terms of level of response, growth rate, feed conversion rate, carcass characteristics etc. Each of the gilts received a daily dose of 0.5 mg clenbuterol hydrochloride for a period of nine (9) weeks. The clenbuterol hydrochloride was dissolved in water and then administered orally.

✍ intramuscular

☝ implant in the base of the ear

🖳 orally

3.2.2.2 Collection of Samples

Urine samples were collected from the boars that were treated with nandrolone decanoate on days 1, 2, 3, 4, 5, 7, 9, 12, 14, 16 and 19 after administration of the drug (trial 1). Blank urine samples were collected before treatment (day 0).

In a separate trial urine samples were collected from the boars that were treated with zeranol, trenbolone acetate or diethylstilbestrol on days 1, 2, 3, 4, 7, 9, 11, 14, 16, 18 and 21 after administration of the drugs (trial 2). Blank urine samples were also collected before treatment (day 0).

All the collected urine samples were stored immediately at -20°C until time of analysis.

After the gilts (from the other research study previously mentioned - trial 3) received their final treatment of clenbuterol, urine samples were collected on days 0, 2, 4, 6, 8, 10, 12, 15, 17, 19, 22, 24 and 26, and stored immediately at -20°C until time of analysis. Day 0 represents the day on which the final treatment was given.

3.3 MATERIALS

3.3.1 CHEMICALS

All listed chemicals were of Pro Analyse quality or better, unless stated otherwise. Internal standard and derivatization solutions were stored at approximately 4°C.

Table 3 List of chemicals used

NAME	SUPPLIER / REFERENCE
Potassium Carbonate	Merck 4928
Sodium Hydrogen Carbonate	Merck 6329
Ammonium Iodide	Merck 1173
Sodium Acetate	UnivAR 582 098
Acetic Acid	Merck 63
Diethylether [†]	UnivAR 1863040
Methanol	Merck 6009
Toluene	Merck 8325
N-methyl-N-trimethylsilyl-trifluoroacetamide	Sigma M7891
1,4-dithiothreitol	Merck 11474
β-Glucuronidase/Arylsulfatase [Helix pomatia]	Boehringer 127698

[†] pre-distilled over sodium hydroxide

Table 4 List of standards used

NAME	SUPPLIER / REFERENCE
Clenbuterol.HCl	Sigma C-5423
Diethylstilbestrol	Sigma D-4628
Dienestrol	Sigma D-3253
17α-19-nortestosterone (Epi-nandrolone)	Rijksinstituut voor Volksgezondheid en Milieuhygiene, (RIVM) 92 M 2787
5α-estran-3β-ol-17-one (Epi-norandrosterone)	Steraloids, Inc. E 214
Zeranol	Pharmacology departmental store
Taleranol	Rijksinstituut voor Volksgezondheid en Milieuhygiene, (RIVM) 92 M 3532
17α-methyltestosterone	Sigma M-7252

The following chemicals were used as preservatives during the nandrolone stability studies:

- sodium azide
- thimerosal
- 1,4-dithiothreitol

3.3.2 BUFFER SOLUTIONS

3.3.2.1 Acetate buffer pH 5.2 (2 M)

- (a) 25.2 g of acetic acid was dissolved in 500 ml of distilled water.
- (b) 129.5 g of sodium acetate was dissolved in 500 ml of distilled water.

- (b) was added to (a) until a pH of 5.2 was reached.

3.3.2.2 Acetate buffer pH 5.2 (0.25 M)

12.5 ml of acetate buffer (2 M) was diluted with 87.5 ml of distilled water.

3.3.2.3 Potassium carbonate buffer pH 9.6

20 g of potassium carbonate and 20 g of sodium hydrogen carbonate was dissolved in 160 ml of distilled water.

3.3.3 INTERNAL STANDARD SOLUTION

2 mg 17α-methyltestosterone was dissolved in 100 ml of toluene.

3.3.4 DERIVATIZATION SOLUTION

20 mg of ammonium iodide and 40 mg of 1,4-dithiothreitol was dissolved in 10 ml of N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA).

3.4 APPARATUS

Standard laboratory glassware and equipment were used, with in addition:

Aluminium caps

Balances:

- Sartorius (Type 2254)
- Mettler (M3)
- Mettler (PM 400)

Bench-top centrifuge (Hettich Universal II)

Desiccator with vacuum facility

Disposable glass pipettes (Pasteur ISO 7712)

Electric heating block with adjustable thermostat (Thermolyne Type 17600)

Glass injector vials with glass inserts

Glass syringes (Hamilton)

Heating module thermostat adjustable with nitrogen facility

5 ml open amber ampoules (Anchor Rand)

pH-meter (Mettler Toledo M90)

Sep-Pack C18 cartridges (Waters, Millipore)

Semi-automatic pipettes (Adjustable Sample Systems)

3.5 ANALYTICAL INSTRUMENTS

3.5.1 GC-MSD (5970)

Gas Chromatography-Mass Spectrometric analysis was done on a Hewlett Packard gas chromatograph (model HP 5890) which was directly interfaced to a Hewlett Packard mass selective detector (model HP 5970B). An Ultra-1 (Crosslinked Methyl Silicone Gum) column, 17 m x 0.20 mm x 0.33 µm film thickness, was used for chromatographic seperation of the analytes, with Helium as the carrier gas. Injections were made in the splitless mode with a Hewlett Packard autosampler (model HP 7673A), and time to purge of 0.5 minutes.

3.5.2 GCQ (GC-MS-MS)

Gas chromatography with tandem mass spectrometry analysis was done on a Finnigan MAT ion trap instrument. An Ultra-1 (Crosslinked Methyl Silicone Gum) column, 16 m x 0.20 mm x 0.11 µm film thickness, was used for chromatographic seperation of the analytes, with Helium as the carrier gas. Injections were made manually.

3.6 ENZYME IMMUNOASSAY

A competitive enzyme immunoassay (Ridascreen®) from R-Biopharm GmbH, Darmstadt, Germany was used for the quantitative analysis of trenbolone. All reagents required for the enzyme immunoassay, including the standards, are contained in the test kit. The absorbance was measured with a microtiter plate spectrophotometer. The manufacturer claims a recovery rate in urine of 85%.

3.6.1 TEST PRINCIPLE

The basis of the test is the antigen-antibody reaction. The microtiter wells are coated with sheep antibodies directed against anti-trenbolone rabbit IgG. Anti-trenbolone antibodies, trenbolone enzyme conjugate and trenbolone standards or sample solution are added. Free trenbolone and trenbolone enzyme conjugate compete for the trenbolone antibody binding sites (competitive enzyme immunoassay). At the same time, the trenbolone antibodies are also bound by the immobilised rabbit antibodies. Any unbound enzyme conjugate is then removed in a washing step.

Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colourless chromogen into a blue product. The addition of the stop reagent leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm (optional reference wave length ≥ 600 nm). The absorption is inversely proportional to the trenbolone concentration in the sample.

3.6.2 PREPARATION OF SAMPLES

- Evaporate 60 µl of the etheral extract in section 3.8.3 (page 47) to dryness.
- Dissolve the residue in 200 µl of methanol:water (1:1).
- Vortex to homogenize.

3.6.3 TEST PROCEDURE

- Insert a sufficient number of microtiter strips into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions.
- Add 50 µl of diluted enzyme conjugate.
- Add 20 µl of standard or prepared sample to separate duplicate wells.
- Add 50 µl of the diluted trenbolone antibody to each well. Mix thoroughly and incubate for 2 hours at room temperature.
- Pour the liquid out of the wells and tap the microwell holder upside down vigorously against absorbent paper to ensure complete removal of liquid from the wells.
- Fill all the wells with 200 µl of distilled water and pour out the liquid as in the previous step. Repeat 2 more times.
- Add 50 µl of substrate and 50 µl of chromogen to each well. Mix thoroughly and incubate for 30 minutes at room temperature, in the dark.
- Add 100 µl of stop solution to each well. Mix thoroughly and measure the absorbance at 450 nm against an air blank.

3.6.4 TEST RESULTS

The mean values of the absorbance values obtained for the standards and the samples are divided by the absorbance value for the zero standard and multiplied by 100. The zero standard is thus made equal to 100% and the absorbance values are quoted in percentages.

A semilogarithmic calibration curve of % absorbance against concentration is constructed. In order to obtain the trenbolone concentration actually contained in a sample, the concentration read from the calibration curve must be further multiplied by the corresponding dilution factor.

3.7 IDENTIFICATION OF ANALYTES

Gas chromatography-mass spectrometry, which is a combination of two analytical techniques, was used for identification. In order to identify the analytes, it is necessary to derivatize the functional groups present in the molecules, mainly to overcome problems associated with low volatility, high polarity and thermal instability, as described under the analytical procedures section 3.8.4 (page 48), before obtaining the following for each of the analytes:

- the absolute retention time
- the retention time relative to an internal standard
- the characteristic ions resulting from ionization

The instrument, which was calibrated using perfluorotri-n-butylamine (PFTBA), consisted of the components shown in Table 5 below.

Table 5 Instrument components used to record the full-scan mass spectra

COMPONENT	MODEL /DESCRIPTION
Gas Chromatograph	HP 5890
Mass Selective Detector	HP 5970B
GC capillary column	Ultra-1(Crosslinked Methyl Silicone Gum)
Column dimensions	17 m x 0.2 mm x 0.33 μm film thickness
Autosampler	HP 7673A
Computer	HP 216S
Chemstation	HP 59970C
Winchester Disc Drive	HP 9133XV
Plotter	HP 7475A

A full-scan mass spectrum was recorded for each of the analytes under the conditions shown in Table 6 below.

Table 6 Conditions under which full-scan mass spectra were recorded

DESCRIPTION	VALUE / MODE
Ionization mode	Electron Impact
Injection Port Temperature	280 °C
Ion Source Temperature	Factory preset to 200 °C
Transfer Line Temperature	280 °C
Injection mode	Splitless
Injection Volume	2 μl
Scan Range	50 - 550 amu
Electron Energy	70 eV
Carrier Gas	Helium

The oven temperature program of the gas chromatograph is shown in Table 7 below.

Table 7 Oven temperature program of the gas chromatograph (HP 5890)

LEVEL	INITIAL TEMP.	INITIAL TIME	RAMP RATE	FINAL TEMP.	FINAL TIME	TOTAL TIME
1	100 °C	0 min.	10 °C/min.	180 °C	0.00 min.	08.00 min.
2	-	-	04 °C/min.	250 °C	0.00 min.	25.50 min.
3	-	-	14 °C/min.	280 °C	2.40 min.	30.04 min.

3.8 ANALYTICAL PROCEDURES

The drugs given to animals, are excreted in the urine, either in the free or conjugated form. The analytes have to be extracted from the urine, before they can be subjected to gas chromatography-mass spectrometry for identification purposes. To accomplish this, the following techniques were employed.

3.8.1 SOLID-PHASE EXTRACTION

A Sep-Pak® C_{18} cartridge, with reversed phase sorbent, was conditioned by wetting the stationary phase with 5 ml of methanol. Conditioning was followed by an equilibration step where the cartridge was washed with 5 ml of distilled water. 5 ml of the urine sample was loaded onto the cartridge, and washed with 5 ml of methanol:distilled water (40:60 v/v) to remove interferences while retaining the analytes. The analytes were eluted from the cartridge with 3 ml of methanol. The methanolic solution was evaporated to dryness at 50°C under a stream of high purity nitrogen.

3.8.2 DECONJUGATION

The residue obtained in section 3.8.1 was dissolved in 1 ml of sodium acetate buffer (0.25 M, pH = 5.2). Conjugated compounds were hydrolysed enzymatically by adding 25 µl of β-glucuronidase/arylsulfatase and incubating for 2 hours at 50°C.

3.8.3 LIQUID-LIQUID EXTRACTION

The buffered solution (section 3.8.2) was alkalinized with 200 µl potassium carbonate buffer (pH = 9.6) and the analytes of interest were extracted with 4 ml of diethylether by vortexing for 30 seconds. After centrifugation the organic layer was transferred to a 5 ml ampoule containing 50 µl of the internal standard solution, and evaporated to dryness at 50°C under a stream of high purity nitrogen. The residue was dried in an evacuated desiccator for at least one hour.

3.8.4 DERIVATIZATION

40 μl of the derivatization solution was added to the dry residue, the ampoule was sealed off and 15 minutes reaction time was allowed at 60°C. After the ampoule cooled to room temperature, 50 μl toluene was added and the solution transferred to a glass injector vial.

3.8.5 GAS CHROMATOGRAPHY-MASS SPECTROMETRY

2 μl of this solution in the injector vial (section 3.8.4) was injected into the injection port of the instrument with an autosampler, and the run was automatically controlled by the chemstation software.

3.9 RECOVERY OF ANALYTES

The recovery of the analytes of interest is a measure of the efficiency of the extraction procedure where test samples are taken through the whole analytical procedure, and compared with reference samples not taken through the procedure. For the results to be statistically meaningful, 5 replicates were assayed as described below.

3.9.1 REFERENCE SAMPLES

Standard methanolic solutions were prepared for each analyte.. An exact amount of each analyte was spiked into separate 5 ml ampoules, each containing an exact amount of internal standard solution (17α-methyltestosterone). The methanolic solution was evaporated to dryness at 50°C under a stream of high purity nitrogen. The residue was dried in an evacuated desiccator for at least one hour.

3.9.2 Test Samples

The same amount of each analyte taken for the reference sample, was spiked into 5 ml of blank urine, and taken through the whole analytical procedure. At the liquid-liquid extraction step, the organic layer was transferred to a 5 ml ampoule containing the same amount of internal standard solution (17α-methyltestosterone) as the reference sample, and evaporated to dryness at 50°C under a stream of high purity nitrogen. The residue was dried in an evacuated desiccator for at least one hour.

The reference- and test samples were derivatized as described previously (section 3.8.4, page 48) and 2 µl of each solution was injected into the injection port of the instrument with an autosampler, and the run was automatically controlled by the chemstation software.

3.9.3 "Free Fraction" Method

The following extraction method (the so-called "free fraction" method) was used to increase the recovery of clenbuterol.

The test samples were alkalinized with 200 µl potassium carbonate buffer (pH = 9.6) and clenbuterol was extracted with 4 ml of diethylether by vortexing for 30 seconds. After centrifugation the organic layer was transferred to a 5 ml ampoule containing the same amount of internal standard solution (17α-methyltestosterone) as the reference sample, and evaporated to dryness at 50°C under a stream of high purity nitrogen. The residue was dried in an evacuated desiccator for at least one hour.

The reference- and test samples were derivatized as described previously (section 3.8.4, page 48) and 2 µl of each solution was injected into the injection port of the instrument with an autosampler, and the run was automatically controlled by the chemstation software.

3.10 LIMIT OF DETECTION

A representative blank urine sample of the appropriate matrix was prepared by pooling ten randomly selected blank urine samples previously analysed for the analytes in question. An internal standard was used in all the samples to confirm retention time stability by calculating the relative retention times. Because less-than-optimal peaks are expected at the analytical lower limit of a procedure, acceptable chromatography criteria (peak shape, -symmetry, peak and baseline resolution etc.) were not applied too stringently.

A signal-to-noise ratio of 3 of a characteristic ion of the analyte was accepted as significant difference between actual signal and normal background.

3.10.1 PRELIMINARY STUDIES

In preliminary, range-finding experiments, the apparent limits of detection (LOD's) were determined by assaying decreasing concentrations of the analytes to the point where the signal-to-noise ratio of the characteristic ion of the specific analyte in the appropriate matrix was just less than 3. The concentration prior to this dilution was selected as the apparent LOD.

3.10.2 LOD STUDIES

Five replicates of these apparent LOD concentrations were prepared and analysed (as described in section 3.8, page 47) under routine conditions, with injection in duplicate. The signal-to-noise ratio was determined for each injection.

The LOD was calculated from the following equation:

$$\boxed{\text{LOD} = (3 \times \text{apparent LOD})/(\text{average signal-to-noise ratio})} \quad (n = 10)$$

3.11 ANALYSIS OF URINE FROM EXCRETION STUDIES

The frozen urine samples collected previously (sections 3.2.1.2 and 3.2.2.2) were thawed, homogenized and analysed for the various analytes of interest as described in section 3.8 (page 47). Trenbolone was analysed using a competitive immunoassay as described in section 3.6 (page 43).

3.12 CONFIRMATION OF ANALYTES

In order to confirm the presence of a drug unequivocally in a urine sample, an excretion study sample of the analyte in question was extracted according to the analytical procedure described in section 3.8 (page 47) together with a reagent blank, urine blank and reference standard.

The reference standard was prepared by spiking the relevant pure substance into the appropriate normal blank urine matrix at a concentration that corresponds to that of the excretion study sample.

Normally these extracts are then injected into a GC-MSD in the full-scan mode. Recently however, a more sensitive and specific technique became available where more structural evidence about a molecule can be obtained with the GCQ system that can eject all ions exept for one selected parent ion (also called the precursor ion) that remains in the ion trap. This parent ion is then collided with background gas that is present in the mass analyzer.

After many collisions with the background gas, the parent ion gains enough internal energy to cause it to fragment into one or more product ions (also called "daughter"or fragment ions). In addition, because the parent ion gains internal energy slowly, the fragmentation process is very specific; the parent ion usually fragments to the product ion with the lowest energy.

The full scan MS-MS scan mode provides invaluable information on the structure of one particular ion, and therefore on the analyte itself. With this newly developed technology, it is very unlikely for one to get a false positive result.

1 151 55250

To get a false positive result, the interfering compound has to form a parent ion with the same mass-to-charge ratio as the selected parent ion from the target compound, and produce the same full scan mass spectrum of the product ions resulting from the dissociation of the selected parent ion.

Sequence of injection into the GCQ system:

- reagent blank
- excretion study
- urine blank
- reference standard

The instrument, which was calibrated using perfluorotri-n-butylamine (PFTBA), consisted of the components shown in Table 8 below.

Table 8 Instrument components used to record the full scan MS-MS mass spectra

COMPONENT	MODEL / DESCRIPTION
Gas Chromatograph	Finnigan MAT
GC capillary column	Ultra-1 (Crosslinked Methyl Silicone Gum)
Column dimensions	16 m x 0.20 mm x 0.11 μm film thickness
Software	GCQ Version 2.1
Computer	Gateway 2000 (Pentium 120 MHz)

A full scan mass spectrum as well as a full scan MS-MS mass spectrum were recorded for each of the samples and/or analytes under the conditions shown in Table 9 (page 53).

Table 9 Conditions under which full scan MS-MS mass spectra were recorded

DESCRIPTION	VALUE / MODE
Ionization mode	Electron Impact
Injection Port Temperature	250 °C
Ion Source Temperature	180 °C
Transfer Line Temperature	275 °C
Injection mode	Splitless
Injection Volume	1 µl
Constant gas velocity	41 cm/sec
Electron Energy	70 eV
Carrier Gas	Helium

The oven temperature program of the gas chromatograph is shown in Table 10 below.

Table 10 Oven temperature program of the gas chromatograph (Finnigan)

LEVEL	INITIAL TEMP.	INITIAL TIME	RAMP RATE	FINAL TEMP.	FINAL TIME	TOTAL TIME
1	100 °C	0 min.	30 °C/min.	180 °C	0.00 min.	02.67 min.
2	-	-	04 °C/min.	250 °C	0.00 min.	20.17 min.
3	-	-	30 °C/min.	300 °C	0.50 min.	22.33 min.

The GCQ-MS method used to record the full scan MS-MS mass spectra is shown in Table 11 (page 54)

Table 11 GCQ-MS method used to record the full scan MS-MS mass spectra

SEGMENT	START TIME	PARENT MASS	COLLISION ENERGY	PRODUCT MASS	NOTCH	μS
1	4.80 min.	335.0.	0.70 V	50 to 435	1.0	7
2	7.92 min.	412.0	0.80 V	50 to 512	1.0	7
3	9.96 min.	405.0	0.80 V	50 to 505	1.0	7
4	11.02 min.	418.0	1.00 V	50 to 518	1.0	7
5	13.82 min.	446.0	0.80 V	50 to 546	1.0	6
6	15.52 min.	433.0	1.00 V	50 to 533	1.0	6

This is a comprehensive method for clenbuterol (segment 1), diethylstilbestrol (segment 2), epi-norandrosterone (segment 3), epi-nandrolone (segment 4), zeranol and taleranol (segment 6) and 17α-methyltestosterone (internal standard) in segment 5.

3.13 STABILITY OF ANALYTES

The aim of this study was to investigate the stability of some of these analytes in urine from cattle and/or swine under various temperature storing conditions.

3.13.1 URINE SAMPLES AND STORING CONDITIONS

Frozen urine samples from the previous excretion studies were used. One urine sample obtained from the excretion study of each analyte of interest was quantified to obtain a baseline concentration, after which these same samples were divided into fifteen aliquots and stored as follows:

- frozen at -20 °C
- at ambient temperature
- in direct sunlight

3.13.2 PREPARATION OF STANDARDS

Five standards were prepared for each analyte by spiking an appropriate blank urine with a fixed amount of 17α-methyltestosterone (as internal standard) and various amounts of the analyte of interest to bracket the concentrations of the test samples. The blank urine and the stock solutions of the standard analytes were stored at -18°C until the day of analysis. The standards for trenbolone were provided with the kit.

3.13.3 QUANTIFICATION

The samples and standards were analysed at regular intervals (Table 12 and Table 13 below) as described in section 3.8 (page 47), and calibration curves constructed for each. Trenbolone was quantified by using an immunoassay method described in section 3.6 (page 43).

Table 12 Times at which cattle samples were analysed in stability studies

Analyte	Time (hours)				
Diethylstilbestrol	0	24	96	168	240
Epi-nandrolone	0	48	96	168	240
Trenbolone	0	48	96	168	240
Zeranol	0	48	96	168	240
Taleranol	0	48	96	168	240

Table 13 Times at which swine samples were analysed in stability studies

Analyte	Time (hours)				
Clenbuterol	0	24	48	120	192
Diethylstilbestrol	0	48	96	168	240
Zeranol	0	24	96	168	240
Taleranol	0	24	96	168	240

3.13.4 PRESERVATION

Although it was not the aim of this study, preservation of collected urine samples, where freezing facilities are inadequate, was also investigated on a limited scale as an alternative to freezing the samples after collection.

A frozen urine sample from a previous excretion study of nandrolone in the bovine was used. The sample was thawed, homogenized and analysed as described in section 3.8 (page 47), and the remainder divided into twenty four aliquots of 6 ml each. The aliquots were divided into four groups of six each. Six (6) mg of sodium azide was added to each aliquote in the first group, six (6) mg of thimerosal to each aliquote in the second group and six (6) mg of 1,4-dithiothreitol to each aliquote in the third group. No preservatives were added to the aliquotes in the fourth group. All four groups of aliquotes were stored at ambient temperature.

The aliquots were analysed in duplicate on days 2, 4 and 7 as described in section 3.8 (page 47).

The area ratio of the characteristic ions of 17α-19-nortestosterone (nandrolone metabolite in cattle) and 17α-methyltestosterone was used as an indication of the 17α-19-nortestosterone (nandrolone metabolite) content of each sample.

CHAPTER 4

RESULTS AND DISCUSSION

4. Results and Discussion

4.1 Identification of Analytes

4.1.1 Internal Standard (17α-methyltestosterone)

Table 14 Analytical data for the determination of the trimethylsilyl derivative of 17α-methyltestosterone (internal standard)

Absolute Retention Time (min.)	Relative Retention Time	Molecular Mass	Characteristic Ions
26.400	1.000	446	301 ; 446

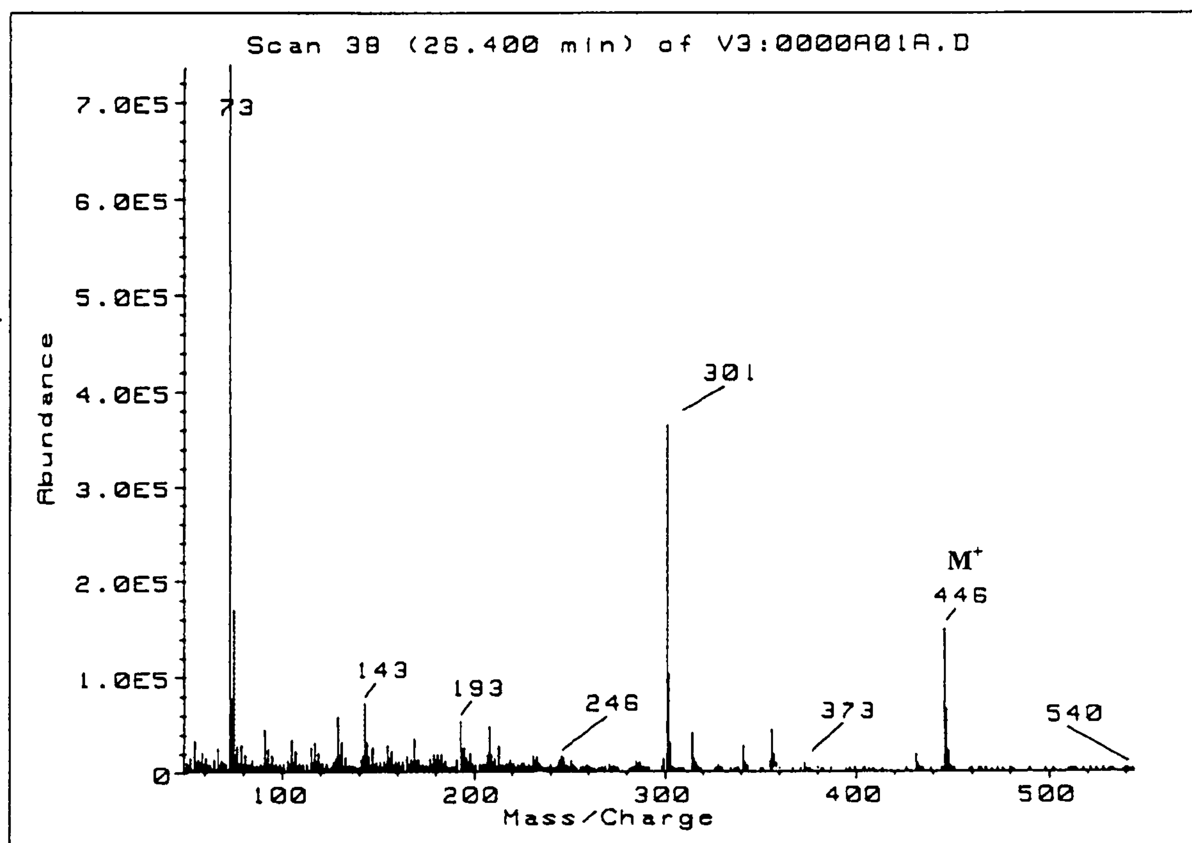

Figure 12 Full-scan electron impact (70 eV) ionization low-resolution mass spectrum of the trimethylsilyl derivative of 17α-methyltestosterone (internal standard)

Table 15 Mass spectral data of the trimethylsilyl derivative of 17α-methyltestosterone (internal standard)

M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE
55.10	4	59.10	3	67.10	3
73.00	100	74.10	10	75.10	23
77.00	3	79.10	4	91.10	6
93.10	3	105.10	4	107.10	3
115.10	3	117.10	4	129.10	8
131.10	4	143.10	10	144.10	4
147.10	3	155.00	3	157.10	3
169.10	4	193.10	7	194.00	3
195.10	3	208.15	6	211.15	2
213.15	3	231.05	2	233.15	2
246.15	2	247.15	2	299.25	2
301.25	49	302.25	14	303.15	4
314.25	5	315.25	2	341.25	3
356.25	6	357.25	2	431.25	2
446.35	20	447.25	9	448.35	3

4.1.2 CLENBUTEROL

Table 16 Analytical data for the determination of the trimethylsilyl derivative of clenbuterol

ABSOLUTE RETENTION TIME (MIN.)	RELATIVE RETENTION TIME	MOLECULAR MASS	CHARACTERISTIC IONS
14.259	0.540	421	86 ; 335

The relative retention time was calculated with respect to the retention time of the internal standard (17α-methyltestosterone).

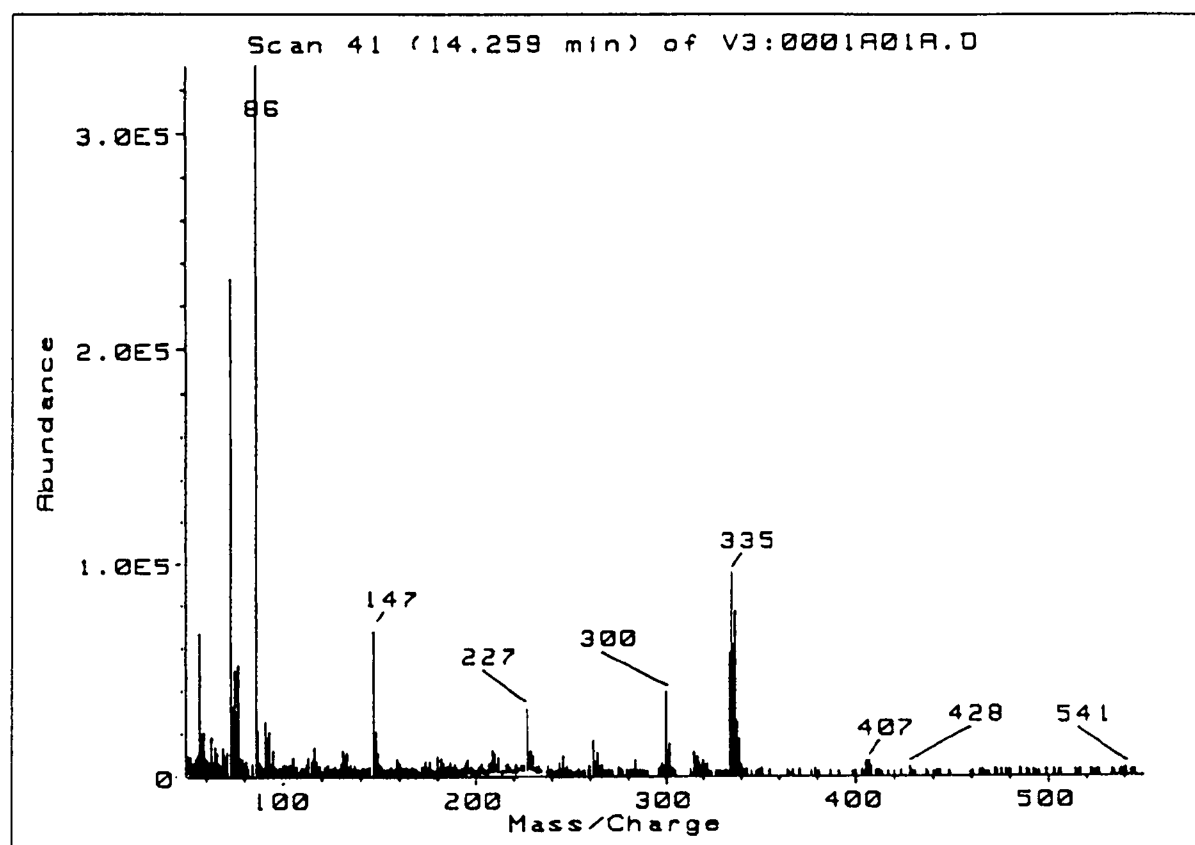

Figure 13 Full-scan electron impact (70 eV) ionization low-resolution mass spectrum of the trimethylsilyl derivative of clenbuterol

Table 17 Mass spectral data of the trimethylsilyl derivative of clenbuterol

M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE
57.10	20	58.10	6	59.10	6
62.90	5	65.00	4	69.00	4
71.10	3	73.00	70	74.10	10
75.00	15	77.00	16	86.10	100
87.10	6	91.10	7	92.10	5
93.00	6	94.90	3	116.10	4
131.00	3	132.10	3	133.00	3
147.00	20	148.10	6	149.10	3
180.10	3	209.05	3	210.05	3
227.15	9	228.05	3	229.05	3
230.05	3	245.95	3	262.05	5
264.05	3	300.05	12	301.05	3
302.15	4	315.15	3	317.05	3
334.05	17	335.05	29	336.05	18
337.05	23	337.95	7	339.05	5

4.1.3 DIETHYLSTILBESTROL

Table 18 Analytical data for the determination of the trimethylsilyl derivative of diethylstilbestrol

ABSOLUTE RETENTION TIME (MIN.)	RELATIVE RETENTION TIME	MOLECULAR MASS	CHARACTERISTIC IONS
19.143	0.725	412	412 ; 397

The relative retention time was calculated with respect to the retention time of the internal standard (17α-methyltestosterone).

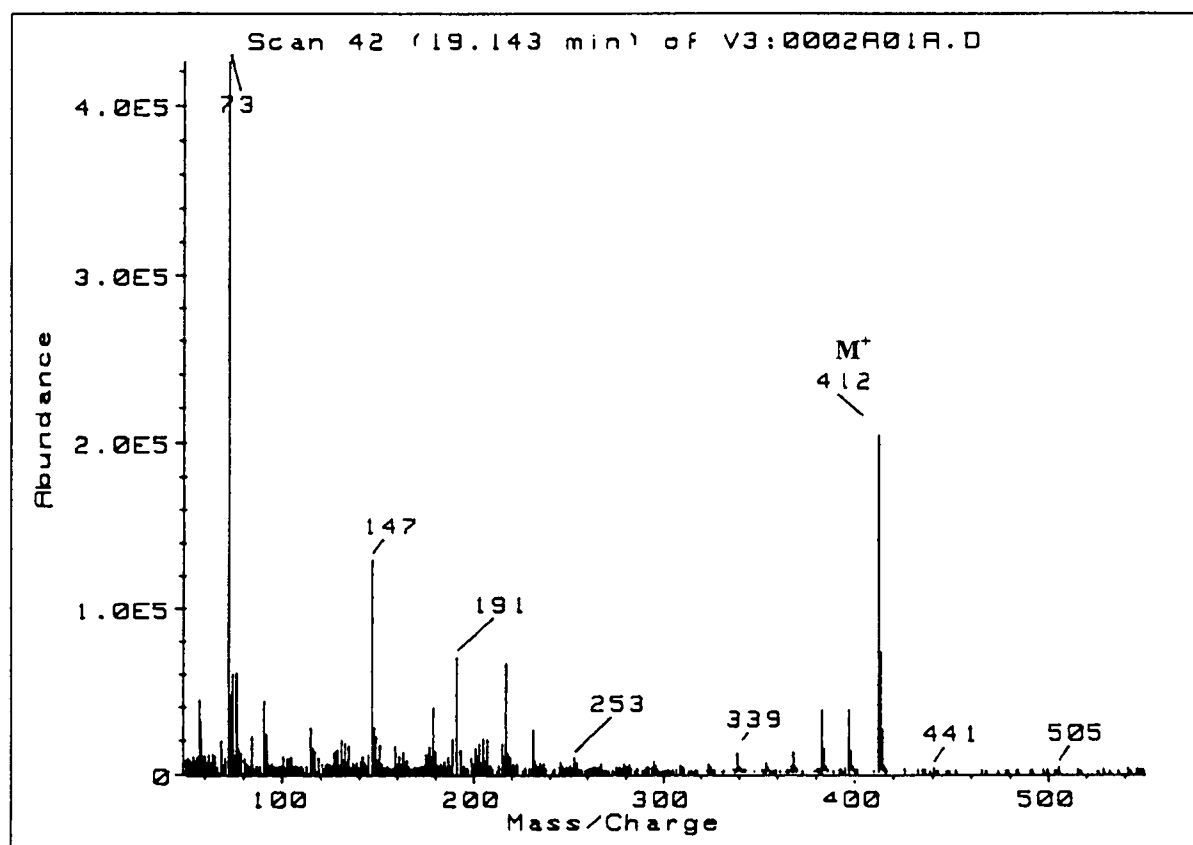

Figure 14 Full-scan electron impact (70 eV) ionization low-resolution mass spectrum of the trimethylsilyl derivative of diethylstilbestrol

Table 19 Mass spectral data of the trimethylsilyl derivative of diethylstilbestrol

M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE
58.10	10	59.00	7	69.00	5
73.10	100	74.10	11	75.00	14
77.00	14	85.00	5	91.10	10
92.10	5	115.00	6	116.10	4
131.00	5	133.00	4	135.00	4
147.10	30	148.00	6	149.00	5
151.10	4	159.00	4	175.10	3
176.10	3	177.00	4	179.10	9
179.90	3	189.00	5	191.10	16
193.10	3	201.10	3	203.00	4
205.10	5	207.15	5	215.05	4
217.05	15	218.05	3	231.15	6
339.25	3	368.15	3	383.15	9
384.15	3	397.25	9	398.15	3
412.25	48	413.25	17	414.25	6

4.1.4 NANDROLONE

4.1.4.1 17β-19-nortestosterone

Table 20 Analytical data for the determination of the trimethylsilyl derivative of 17β-19-nortestosterone

ABSOLUTE RETENTION TIME (MIN.)	RELATIVE RETENTION TIME	MOLECULAR MASS	CHARACTERISTIC IONS
24.074	0.910164	418	418 ; 403

The relative retention time was calculated with respect to the retention time of the internal standard (17α-methyltestosterone).

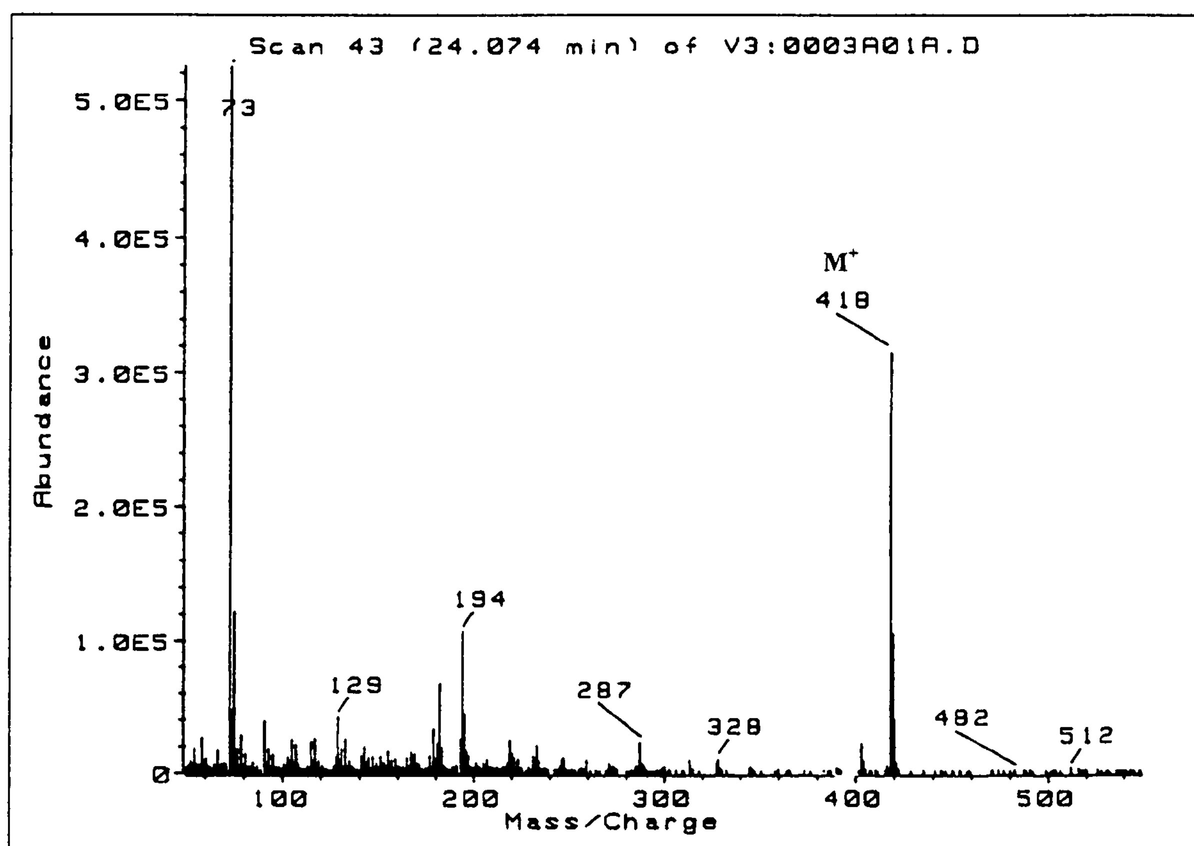

Figure 15 Full-scan electron impact (70 eV) ionization low-resolution mass spectrum of the trimethylsilyl derivative of 17β-19-nortestosterone

Table 21 Mass spectral data of the trimethylsilyl derivative of 17β-19-nortestosterone

M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE
55.00	3	59.00	5	67.10	3
73.10	100	74.10	9	75.10	23
77.00	3	79.00	5	81.10	3
91.10	7	93.10	3	95.10	3
105.10	5	107.10	4	115.00	4
117.00	5	129.10	8	131.10	3
133.10	5	143.00	4	155.10	3
167.10	3	169.10	3	179.10	6
181.10	4	182.10	13	183.10	4
193.20	5	194.10	20	195.10	8
196.10	3	219.15	5	220.15	3
233.15	4	259.25	2	287.15	4
313.25	2	327.25	2	328.25	2
403.15	4	404.15	2	418.25	60
419.25	20	420.25	8	421.25	2

4.1.4.2 17α-19-nortestosterone

Table 22 Analytical data for the determination of the trimethylsilyl derivative of 17α-19-nortestosterone

ABSOLUTE RETENTION TIME (MIN.)	RELATIVE RETENTION TIME	MOLECULAR MASS	CHARACTERISTIC IONS
23.308	0.882	418	418 ; 403

The relative retention time was calculated with respect to the retention time of the internal standard (17α-methyltestosterone).

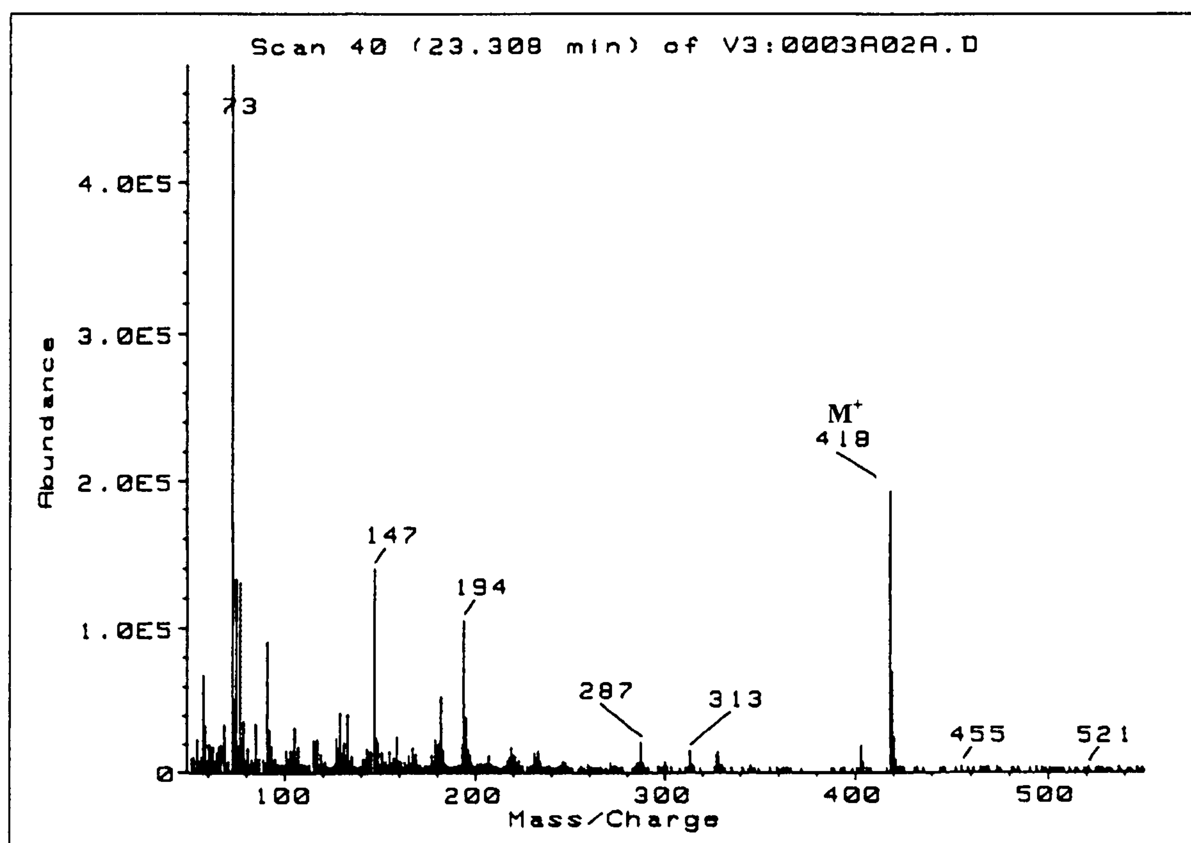

Figure 16 Full-scan electron impact (70 eV) ionization low-resolution mass spectrum of the trimethylsilyl derivative of 17α-19-nortestosterone

Table 23 Mass spectral data of the trimethylsilyl derivative of 17α-19-nortestosterone

M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE
58.10	14	59.00	7	61.10	4
62.90	4	66.10	4	67.00	4
69.00	7	73.10	100	74.10	10
75.00	28	76.10	4	77.00	27
78.10	5	79.00	7	85.00	7
91.10	19	92.10	6	93.10	4
105.10	6	107.10	4	115.00	5
117.10	5	127.00	5	129.10	9
131.10	4	133.00	8	147.00	29
148.10	5	149.00	4	159.00	5
167.00	4	179.10	5	180.00	4
181.10	4	182.10	11	193.20	4
194.10	22	195.10	8	219.05	4
287.15	4	313.25	3	403.25	4
418.25	40	419.25	14	420.25	5

4.1.4.3 Epi-norandrosterone

Table 24 Analytical data for the determination of the trimethylsilyl derivative of epi-norandrosterone

ABSOLUTE RETENTION TIME (MIN.)	RELATIVE RETENTION TIME	MOLECULAR MASS	CHARACTERISTIC IONS
22.003	0.832	420	405 ; 420

The relative retention time was calculated with respect to the retention time of the internal standard (17α-methyltestosterone).

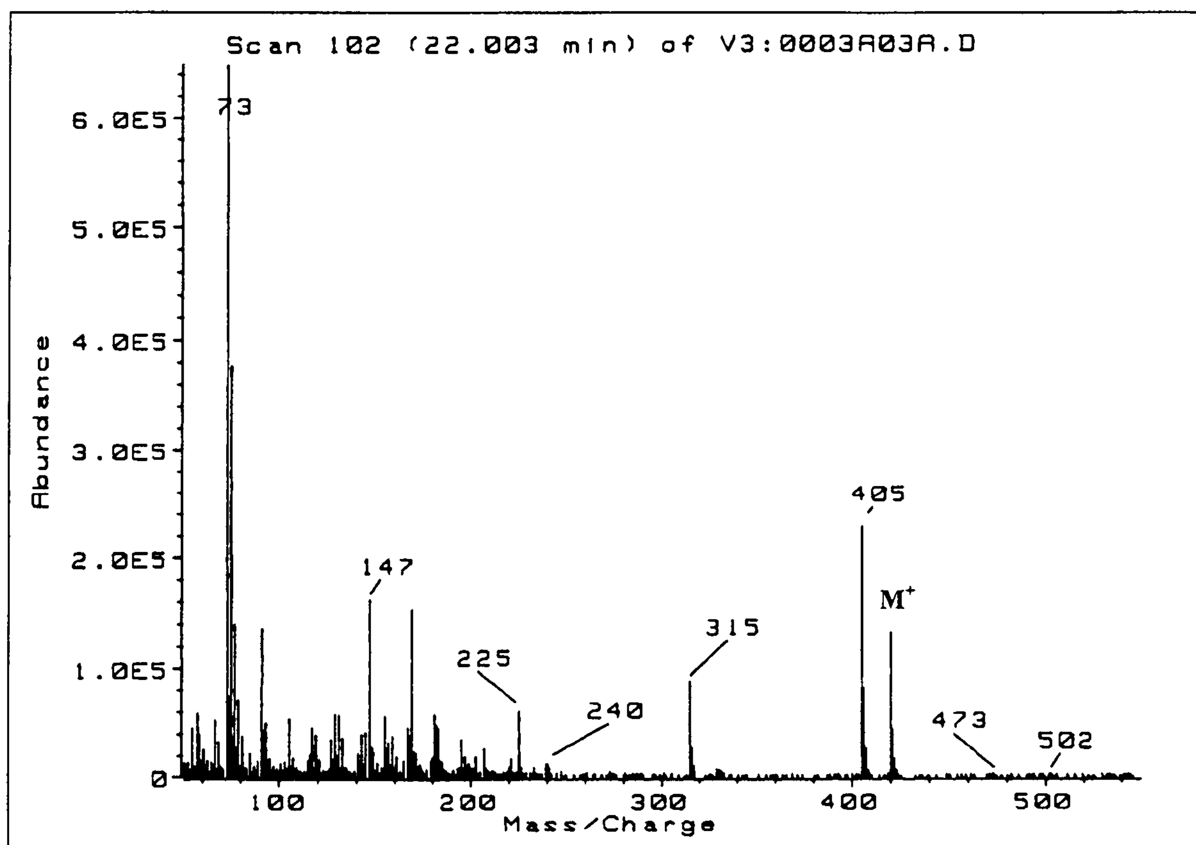

Figure 17 Full-scan electron impact (70 eV) ionization low-resolution mass spectrum of the trimethylsilyl derivative of epi-norandrosterone

Table 25 Mass spectral data of the trimethylsilyl derivative of epi-norandrosterone

M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE
55.10	7	58.10	9	59.00	6
67.00	8	69.00	5	73.10	100
74.10	11	75.10	58	76.00	5
77.00	22	79.10	11	81.10	6
91.10	21	92.10	7	93.10	8
105.10	8	117.10	7	119.00	6
127.00	5	129.00	9	131.10	9
133.00	6	143.00	6	145.10	6
147.10	25	155.10	9	157.00	5
159.00	6	167.10	7	168.10	6
169.10	24	170.10	4	181.10	9
182.10	7	183.10	7	195.10	5
207.15	4	225.15	9	315.25	14
316.25	4	405.25	35	406.25	13
407.25	4	420.25	21	421.25	7

4.1.5 ZERANOL

4.1.5.1 Zeranol

Table 26 Analytical data for the determination of the trimethylsilyl derivative of zeranol

ABSOLUTE RETENTION TIME (MIN.)	RELATIVE RETENTION TIME	MOLECULAR MASS	CHARACTERISTIC IONS
27.311	1.035	538	433 ; 538

The relative retention time was calculated with respect to the retention time of the internal standard (17α-methyltestosterone).

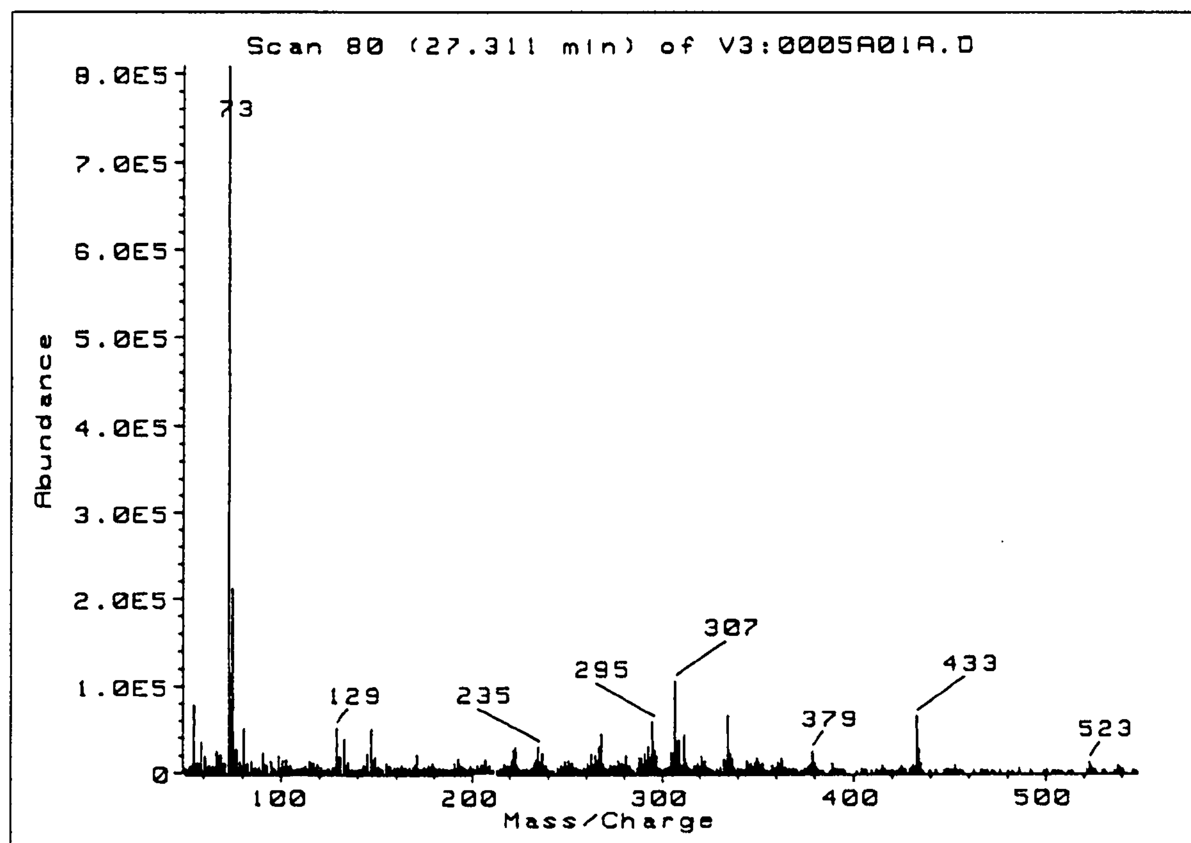

Figure 18 Full-scan electron impact (70 eV) ionization low-resolution mass spectrum of the trimethylsilyl derivative of zeranol

Table 27 Mass spectral data of the trimethylsilyl derivative of zeranol

M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE
55.10	10	59.00	4	67.10	3
69.10	3	73.10	100	74.10	9
75.00	26	77.00	3	81.10	6
91.10	3	129.00	6	133.00	5
145.00	3	147.00	6	171.10	3
222.05	3	223.05	4	235.05	4
237.05	3	263.05	3	267.05	4
268.15	5	291.05	3	293.05	4
295.05	7	296.05	3	305.15	3
307.05	13	308.15	4	309.15	5
312.15	5	323.05	2	333.15	2
335.05	8	336.15	3	337.15	2
345.15	2	349.15	2	350.15	2
351.15	2	361.15	2	363.15	2
379.15	3	433.15	8	434.15	3

4.1.5.2 Taleranol

Table 28 Analytical data for the determination of the trimethylsilyl derivative of taleranol

ABSOLUTE RETENTION TIME (MIN.)	RELATIVE RETENTION TIME	MOLECULAR MASS	CHARACTERISTIC IONS
27.469	1.041	538	433 ; 538

The relative retention time was calculated with respect to the retention time of the internal standard (17α-methyltestosterone).

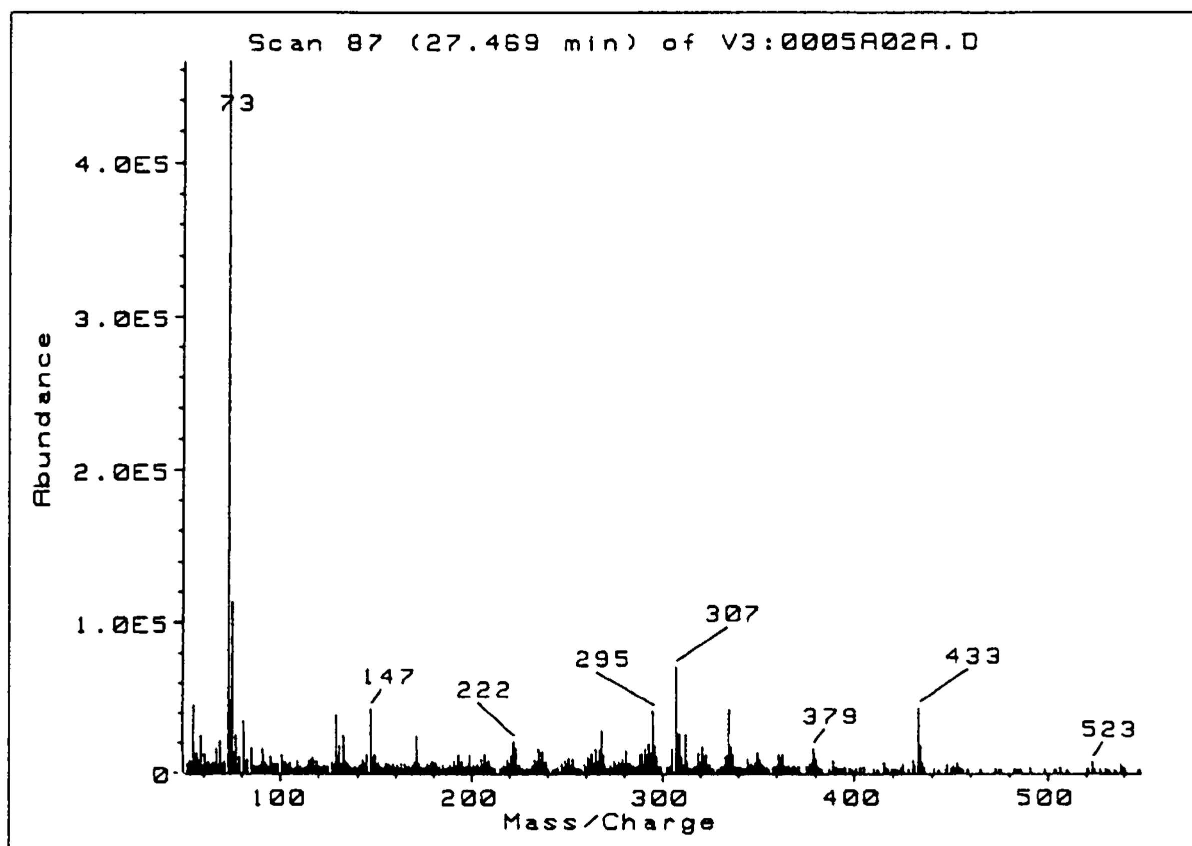

Figure 19 Full-scan electron impact (70 eV) ionization low-resolution mass spectrum of the trimethylsilyl derivative of taleranol

Table 29 Mass spectral data of the trimethylsilyl derivative of taleranol

M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE	M/Z	NORMALIZED ABUNDANCE
55.10	10	59.10	5	69.10	4
73.10	100	74.10	10	75.00	24
77.10	5	81.10	7	91.00	3
129.10	8	130.90	4	133.00	5
147.10	9	149.10	3	171.10	5
207.05	3	222.05	4	223.15	3
235.15	3	237.05	3	263.05	3
265.05	3	267.15	3	268.05	6
281.05	3	289.05	3	291.05	3
293.15	4	294.05	3	295.05	9
296.05	4	305.05	3	307.15	15
308.05	5	309.05	5	312.05	5
319.05	3	321.05	4	335.15	9
336.15	4	337.15	3	350.15	3
379.05	3	433.15	9	434.15	4

The analytes identified in the urine of cattle and/or swine with GC-MSD after administration of some growth-promoting drugs, and their analytical data are summarized below.

Table 30 Analytes identified in the urine of cattle and/or swine after administration of some growth-promoting drugs

DRUG ADMINISTERED	ANALYTE IDENTIFIED	PARENT COMPOUND / METABOLITE	URINE MATRIX	
			CATTLE	SWINE
Clenbuterol	Clenbuterol	Parent Compound	-	✓
Diethylstilbestrol	Diethylstilbestrol	Parent Compound	✓	✓
Diethylstilbestrol	Dienestrol [◊]	Metabolite	✓	✓
Nandrolone	Epi-nandrolone	Metabolite	✓	-
Nandrolone	Epi-norandrosterone	Metabolite	-	✓
Zeranol	Zeranol	Parent Compound	✓	✓
Zeranol	Taleranol	Metabolite	✓	✓

Table 31 Summary of the analytical data for the determination of various analytes as their trimethylsilyl derivatives in the urine of cattle and/or swine, with 17α-methyltestosterone as internal standard

ANALYTE	ABSOLUTE RETENTION TIME (MIN.)	RELATIVE RETENTION TIME	MOLECULAR MASS	CHARACTERISTIC IONS
17α-Methyltestosterone	26.400	1.000	446	301 ; 446
Clenbuterol	14.259	0.540	421	86 ; 335
Diethylstilbestrol	19.143	0.725	412	412 ; 397
Epi-nandrolone	23.308	0.882	418	418 ; 403
Epi-norandrosterone	22.003	0.832	420	405 ; 420
Zeranol	27.311	1.035	538	433 ; 538
Taleranol	27.469	1.041	538	433 ; 538

[◊] minor metabolite

4.2 RECOVERY OF ANALYTES

The data on the recovery of the analytes throughout the whole analytical procedure, are presented in Table 32 below. As this is a broad screening method, some compromise has to be made with respect to the recoveries of the analytes of interest. Although the recovery of clenbuterol is low, the "free fraction" method can be employed to increase the recovery to 99%.

Table 32 Recovery of analytes from test samples in comparison with reference samples

ANALYTE	RECOVERY
Clenbuterol	22%
Diethylstilbestrol	61%
17α-19-nortestosterone	88%
Epi-norandrosterone	81%
Zeranol	93%

The peak area of one of the characteristic ions of each analyte with a mass-to-charge ratio as indicated in Table 33 (page 76) was measured by integrating the extracted ion chromatogram. The peak area of one of the characteristic ions of the internal standard (17α-methyltestosterone) with m/z=446 was also measured by integrating the extracted ion chromatogram. The resulting area ratio (analyte:internal standard) of the test samples was expressed as a percentage of the reference samples.

Table 33 Characteristic ions of the analytes used to determine peak areas

ANALYTE	MASS-TO-CHARGE RATIO (M/Z)
Clenbuterol	86
Diethylstilbestrol	412
17α-19-nortestosterone	418
Epi-norandrosterone	405
Zeranol	433
Taleranol	433

4.3 LIMIT OF DETECTION

Table 34 Limits of detection for the various analytes in the appropriate urine matrix

ANALYTE	CATTLE (ng/ml)	SWINE (ng/ml)
Clenbuterol	0.9	1.2
Diethylstilbestrol	1.1	1.6
17α-19-nortestosterone	1.6	-
Epi-norandrosterone	-	1.4
Zeranol	1.9	2.1

The ability to quantify a trace element or molecule in chemical and biological matrices using specific analytical methods is often viewed in terms of the limit of detection (LOD). This limit of detection is a number, expressed in units of concentration (or amount), that describes the lowest concentration level (or amount) of the element that an analyst can determine to be statistically different from an analytical blank.

Although this definition seems rather straightforward, significant problems have been encountered in expressing these values because of the various approaches to the term "statistically different" [Long and Wineforder, 1983].

The literature suggests two different approaches, statistical and empirical, for determining an LOD [Underwood, et al, 1997]. In the statistical approach, a series of blanks without the analyte of interest is analysed to determine mean background response and variation of the response. The LOD is calculated from the blank mean by adding two to four standard deviation units. This approach establishes an LOD that should be detectable from zero with a defined statistical level of confidence. Needleman and Romberg (1990) criticized the statistical method because, in effect, it measures the average noise level of the procedure and "... defines only the ability to measure *nothing*" instead of a very low concentration of the analyte.

The empirical (experimental) method consists of analyzing a series of samples containing increasingly lower concentrations of analyte. The LOD is the lowest concentration at which the results still satisfy some predetermined acceptance criteria [Armbruster, et al, 1994]. It measures that point beyond which the apparent analytical value no longer has a consistent relationship to the actual concentration present [Needleman and Romberg, 1990].

According to a suggestion by Armbruster, et al, (1994) that the choice of method for LOD determination be tailored to suit the characteristics of the assay and the purpose for which results in the region of the LOD will be used, it was decided to use the empirical method in this study.

The LOD for a particular electron impact GC-MS procedure is also dependent upon several factors including among others:

- electron multiplier voltage
- volume of specimen analysed
- detector threshold
- type of chromatographic column
- extraction efficiency

A practical approach to the determination of LOD values should be realistically achievable and challenge assay and instrument capabilities under routine operating conditions [Underwood, *et al*, 1997].

4.4 EXCRETION PROFILE OF ANALYTES

The methods that were developed for the excretion studies, were only semi-quantitative methods, except for trenbolone that was analyzed quantitatively. This implies that no calibration graphs were constructed. A very good indication of the concentration of the analyte can however be obtained by measuring the peak area of one of the characteristic ions of the analyte by integrating the extracted ion chromatogram. The peak area of one of the characteristic ions of the internal standard (17α-methyltestosterone) can also be measured by integrating the extracted ion chromatogram. The resulting area ratio (analyte:internal standard) is indicative of the concentration for a series of samples under a given set of instrumental conditions.

A competitive enzyme immunoassay method was used for the quantitative analysis of trenbolone. The concentration in the samples were measured using a calibration curve obtained with pure standards. The concentration read from the calibration curve is only semi-quantitative as the extraction recovery is unknown.

The common approach, by reference to urinary creatinine concentrations, was not applied in these excretion studies, since some of the analytes are known to affect protein turnover and therefore creatinine excretion. The total volume of urine excreted could also not be measured because of practical difficulties.

The peak area of one of the characteristic ions of each analyte with a mass-to-charge ratio as indicated in Table 33 (page 76) was measured (by integrating the extracted ion chromatogram) relative to the peak area of one of the characteristic ions of the internal standard (17α-methyltestosterone) with m/z=446.

The aim of these experiments were not to determine an elimination profile of each analyte in urine, but it was only to get an indication of the excretion profiles of the analytes with time in the urine from cattle and/or swine. The results must also be interpreted with the following in mind:

Large differences in concentration levels of the analytes were observed with time in the same animal. Large differences were also observed even when animals received the same treatment. The number of animals was limited and did not allow a statistical analysis of the results (in most cases only two animals were used).

4.4.1 CLENBUTEROL

4.4.1.1 Swine

The data on the excretion of clenbuterol from swine, are presented in Figure 20 and Figure 21 on page 81. These gilts received a daily oral dose of 0.5 mg clenbuterol in a water solution for a period of 9 weeks. After the last treatment, clenbuterol could be detected in the urine above the control level of 1 ng/ml for at least 10 days.

These results contrast with the results published in the literature where it was found that clenbuterol residues were detected in urine for about 5 days after withdrawal of clenbuterol treatment. This difference can be explained as follows:

Different species were used in the two studies (veal calves in the literature study versus gilts in this study). The veal calves received a lower dosage of clenbuterol than the gilts and the calves were treated for only 25 days before treatment was stopped whereas the gilts were treated for 63 days before treatment was stopped.

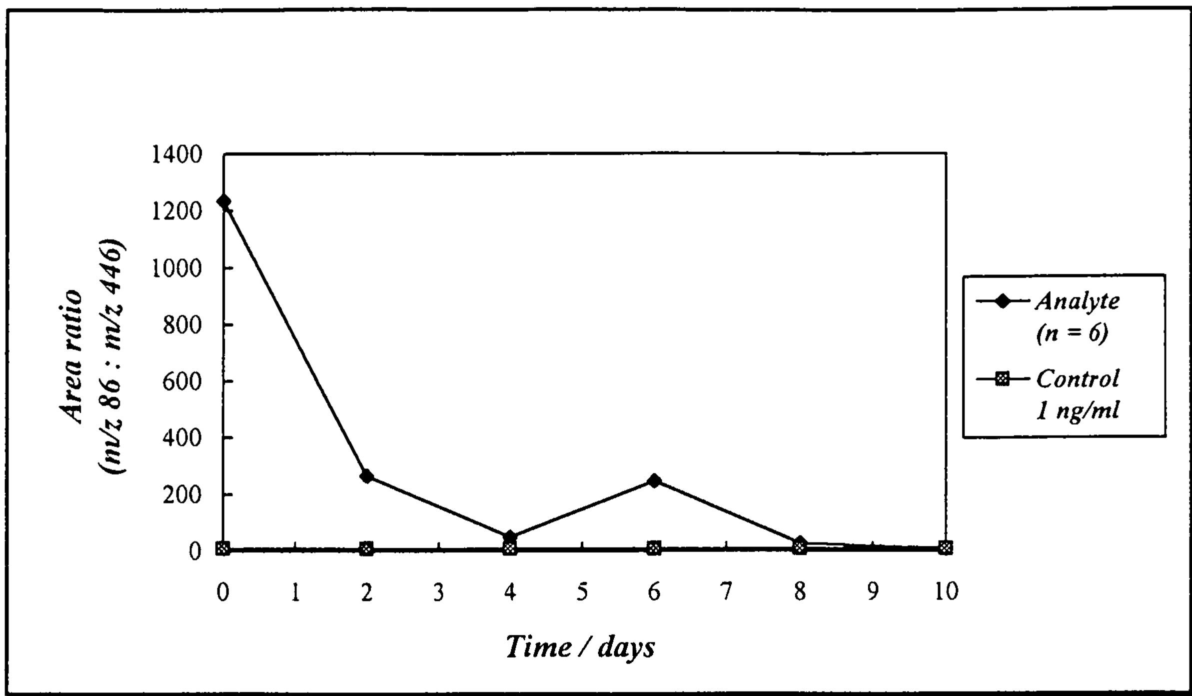

Figure 20 Area ratio of clenbuterol in urine of swine *versus* time after animals received the last dosage

The same results are displayed in Figure 21 below, but in a more visual way.

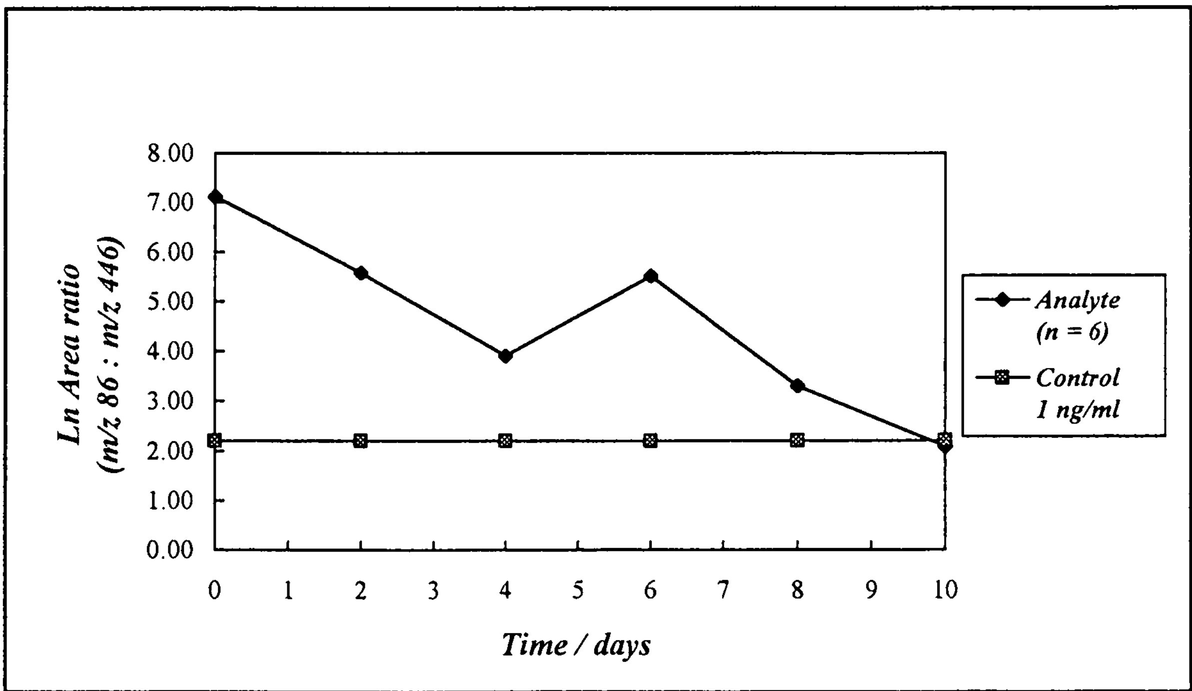

Figure 21 Area ratio of clenbuterol in urine of swine *versus* time after animals received the last dosage (ln transformed)

4.4.2 DIETHYLSTILBESTROL

4.4.2.1 Cattle

The data on the excretion of diethylstilbestrol from cattle, are presented in Figure 22 and Figure 23 on page 83. The parent compound could be detected in the urine above the control level of 2 ng/ml for about 5 days after a single oral dosage of 20 mg diethylstilbestrol.

Dienestrol (minor metabolite of diethylstilbestrol in cattle) could not be detected in the urine above the control level of 2 ng/ml after a single oral dosage of 20 mg diethylstilbestrol.

4.4.2.2 Swine

The data on the excretion of diethylstilbestrol from swine, are presented in Figure 24 and Figure 25 on page 84. The parent compound could be detected in the urine above the control level of 2 ng/ml for about 4 days after a single oral dosage of 20 mg diethylstilbestrol.

Dienestrol (minor metabolite of diethylstilbestrol in swine) could only be detected in the urine above the control level of 2 ng/ml for about 2 days after a single oral dosage of 20 mg diethylstilbestrol.

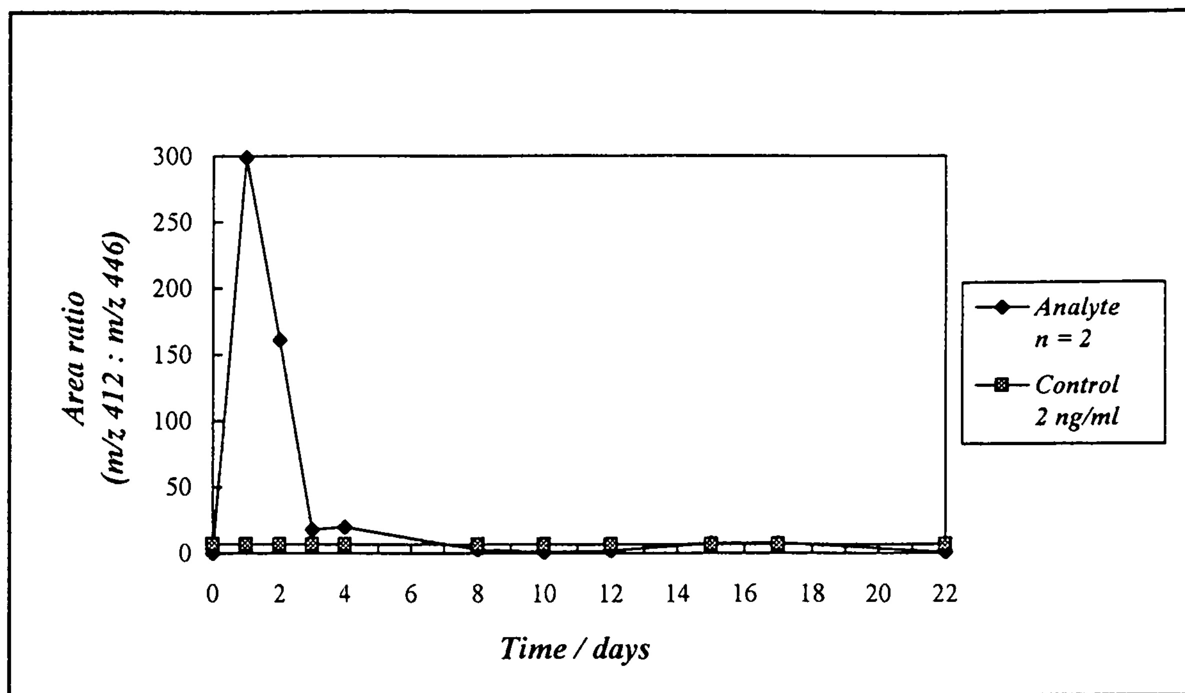

Figure 22 Area ratio of diethylstilbestrol in urine of cattle *versus* time after animals received a single dosage

The same results are displayed in Figure 23 below, but in a more visual way.

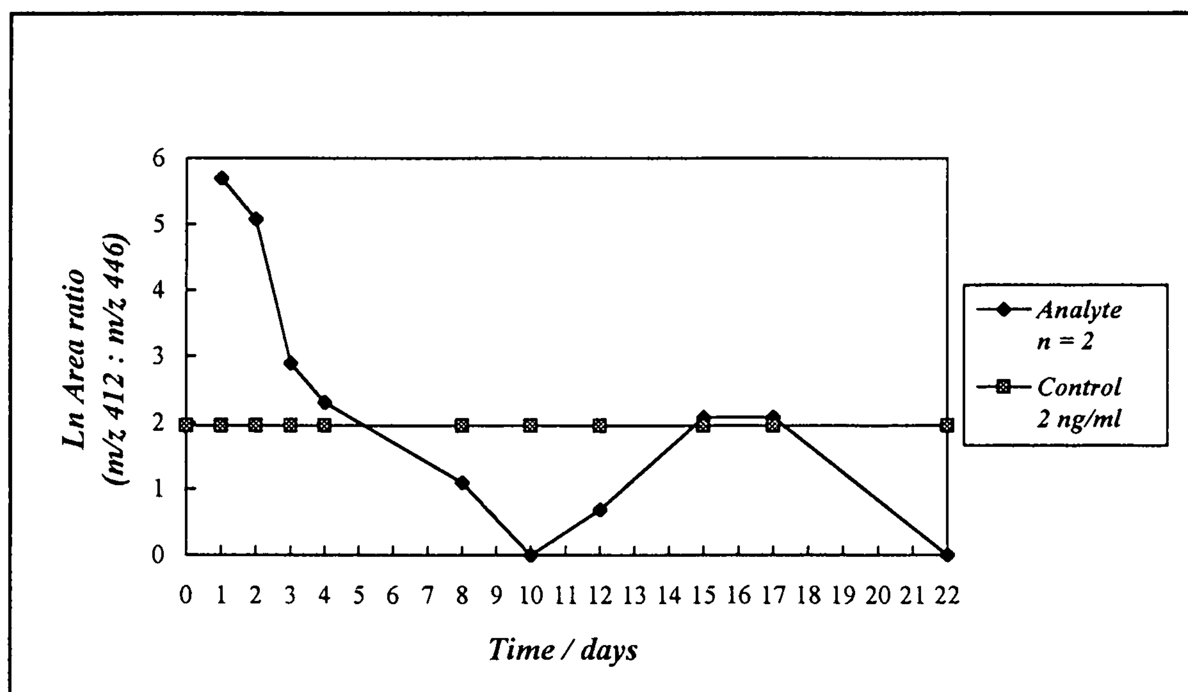

Figure 23 Area ratio of diethylstilbestrol in urine of cattle *versus* time after animals received a single dosage (ln transformed)

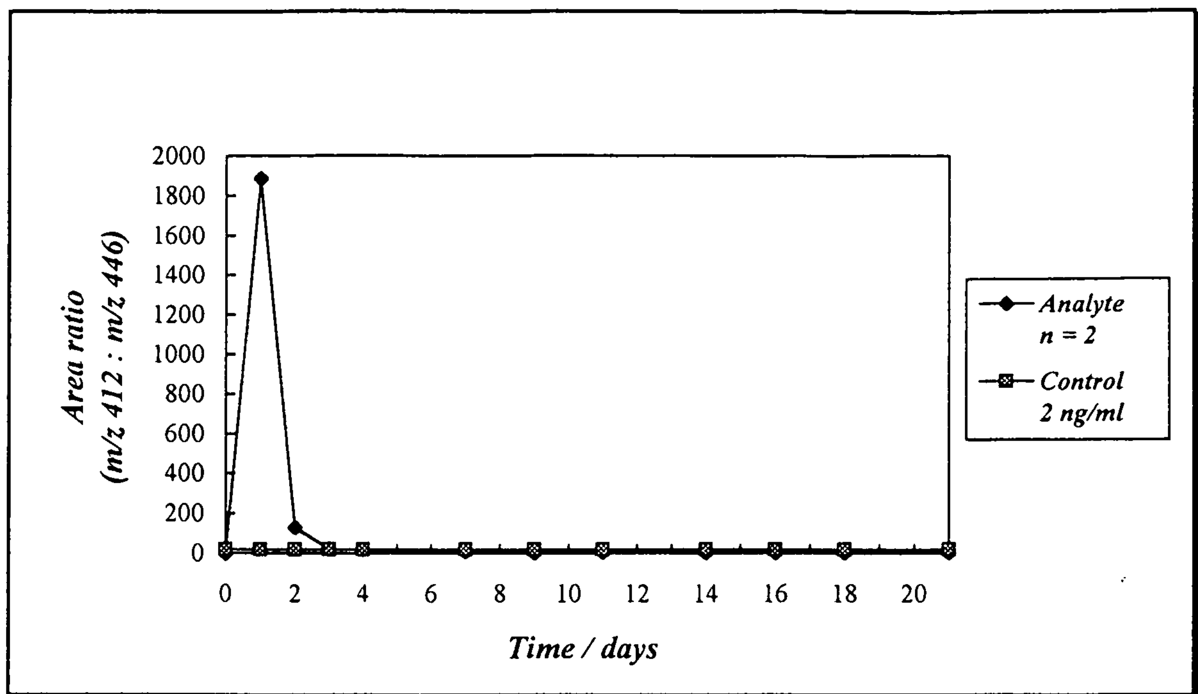

Figure 24 Area ratio of diethylstilbestrol in urine of swine *versus* time after animals received a single dosage

The same results are displayed in Figure 25 below, but in a more visual way.

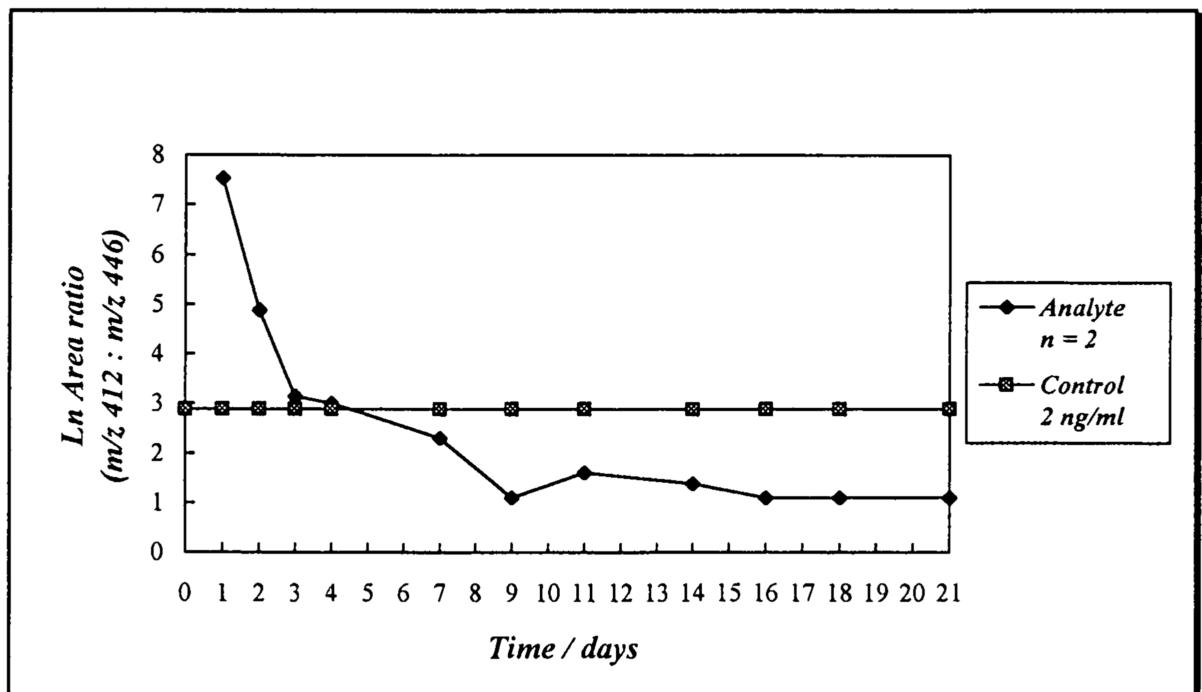

Figure 25 Area ratio of diethylstilbestrol in urine of swine *versus* time after animals received a single dosage (ln transformed)

4.4.3 NANDROLONE

4.4.3.1 Cattle

The data on the excretion of nandrolone from cattle, are presented in Figure 26 and Figure 27 on page 86. The metabolite (17α-19-nortestosterone) could be detected in the urine above the control level of 2 ng/ml for at least 17 days after a single intramuscular injection of 105 mg nandrolone.

4.4.3.2 Swine

The data on the excretion of nandrolone from swine, are presented in Figure 28 and Figure 29 on page 87. The metabolite (epi-norandrosterone) could be detected in the urine above the control level of 2 ng/ml for at least 19 days after a single intramuscular injection of 35 mg nandrolone.

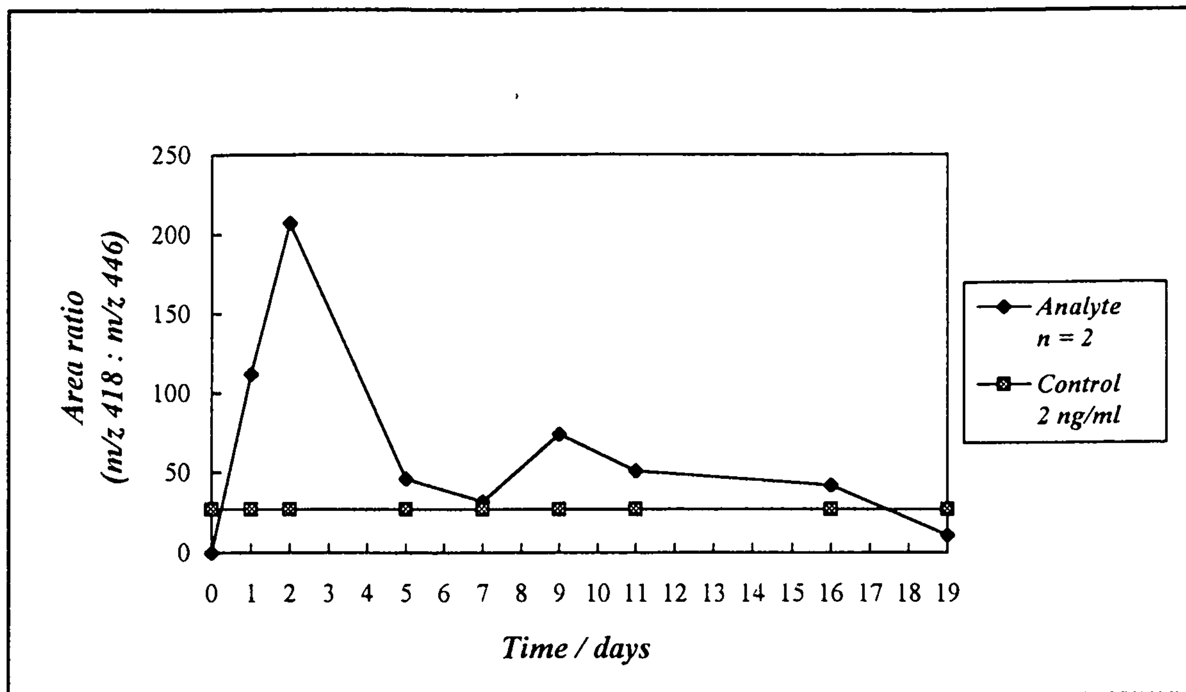

Figure 26 Area ratio of 17α-19-nortestosterone in urine of cattle *versus* time after animals received a single dosage

The same results are displayed in Figure 27 below, but in a more visual way.

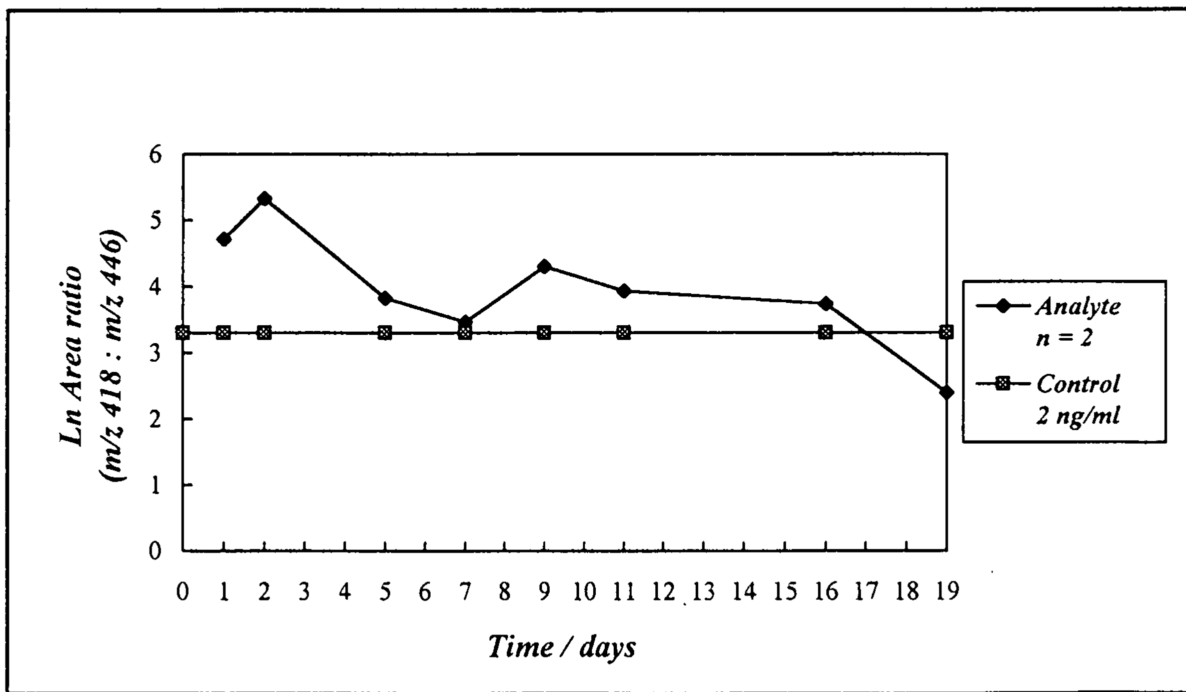

Figure 27 Area ratio of 17α-19-nortestosterone in urine of cattle *versus* time after animals received a single dosage (ln transformed)

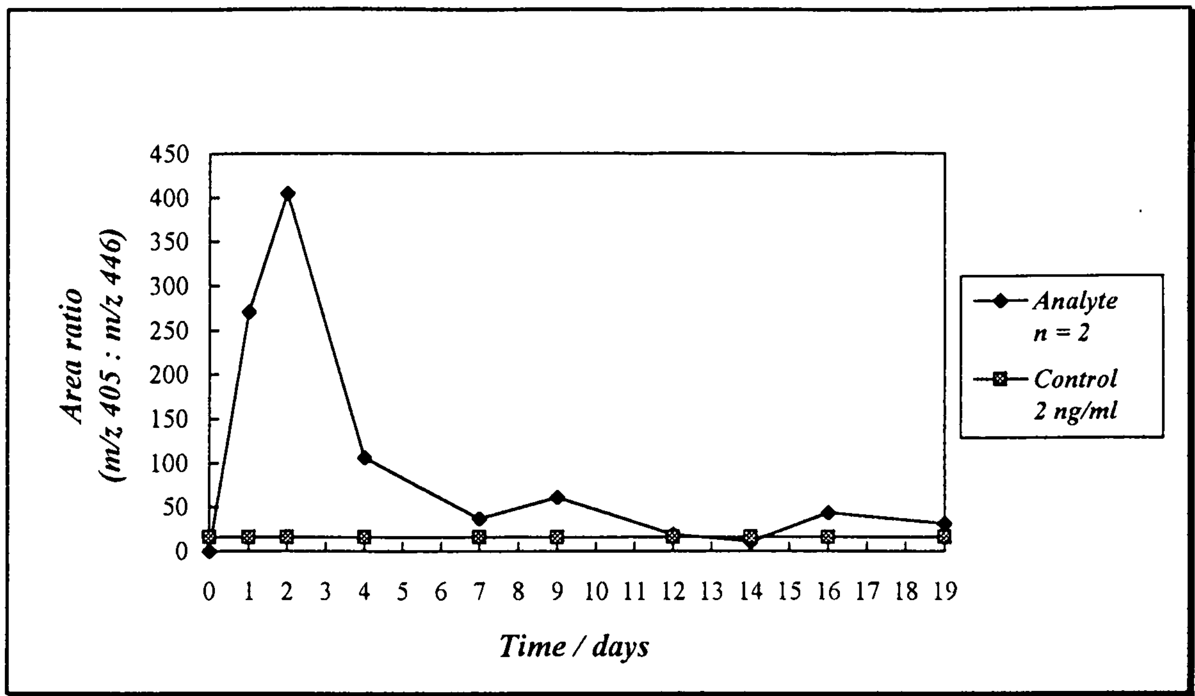

Figure 28 Area ratio of epi-norandrosterone in urine of swine *versus* time after animals received a single dosage

The same results are displayed in Figure 29 below, but in a more visual way.

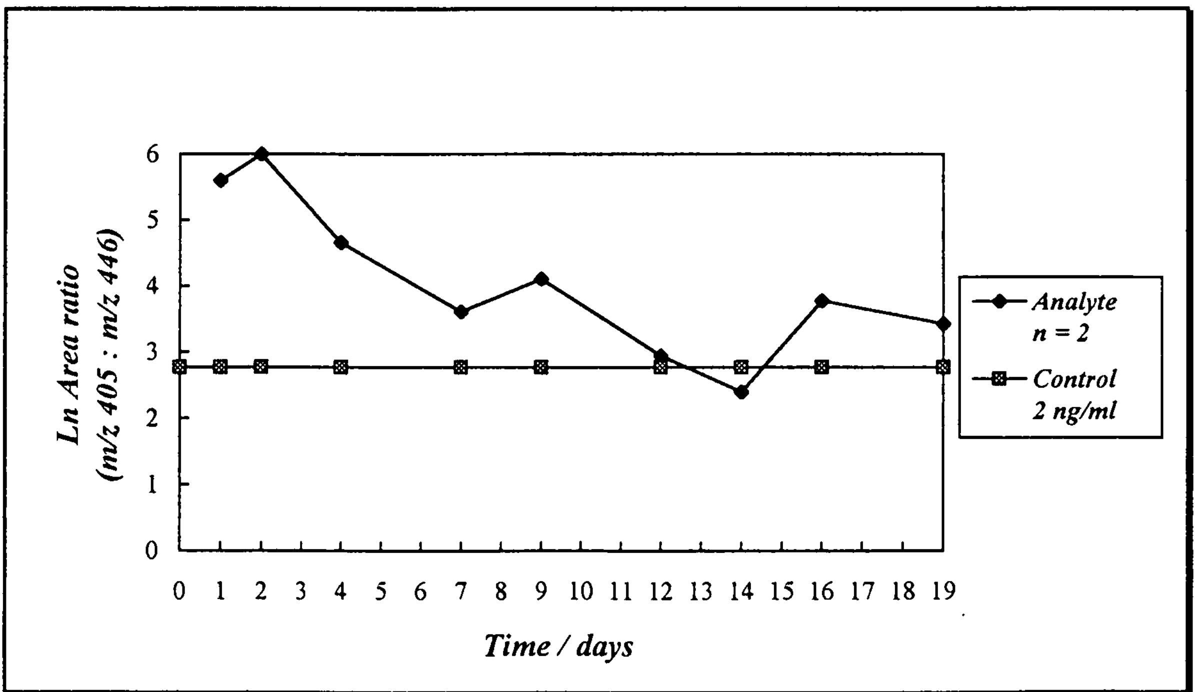

Figure 29 Area ratio of epi-norandrosterone in urine of swine *versus* time after animals received a single dosage (ln transformed)

4.4.4 TRENBOLONE

4.4.4.1 Cattle

The data on the excretion of trenbolone from cattle, are presented in Figure 30 and Figure 31 on page 89. The concentration of 17α-trenbolone (metabolite of trenbolone) was determined with a competitive enzyme immunoassay method.

The metabolite of trenbolone (17α-trenbolone) could be detected in the urine above the control level of 2 ng/ml for about 4 days after a single implant in the base of the ear of 140 mg trenbolone.

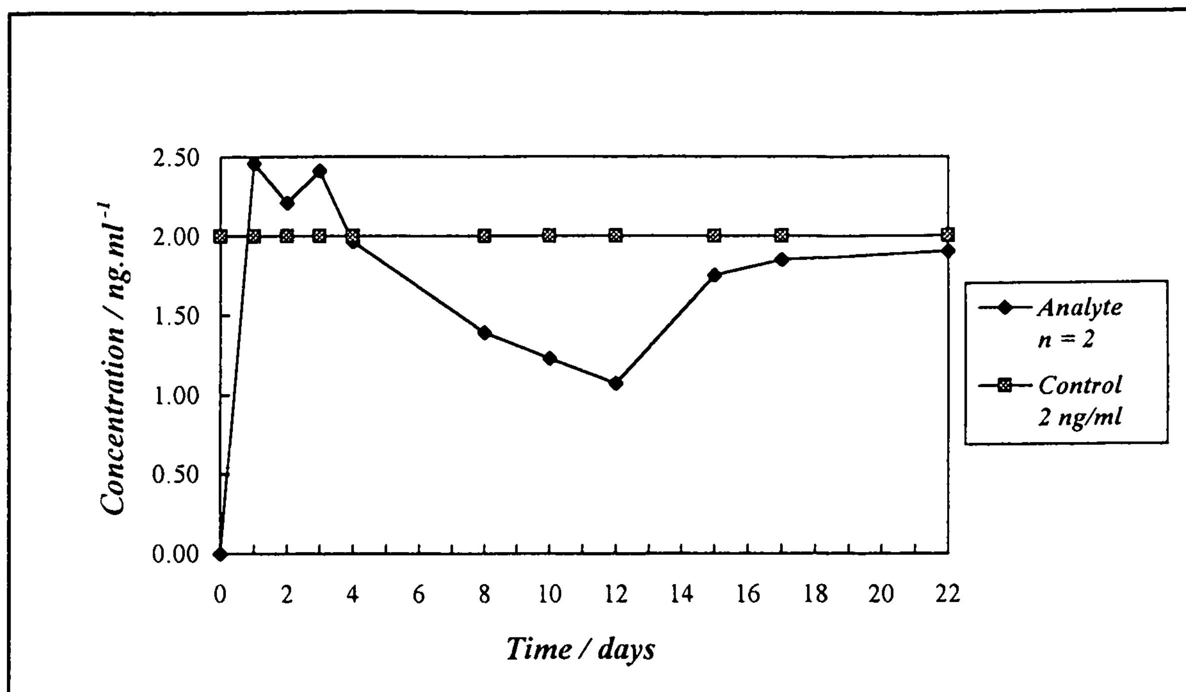

Figure 30 Concentration of 17α-trenbolone in urine of cattle *versus* time after animals received a single dosage

The same results are displayed in Figure 31 below, but in a more visual way.

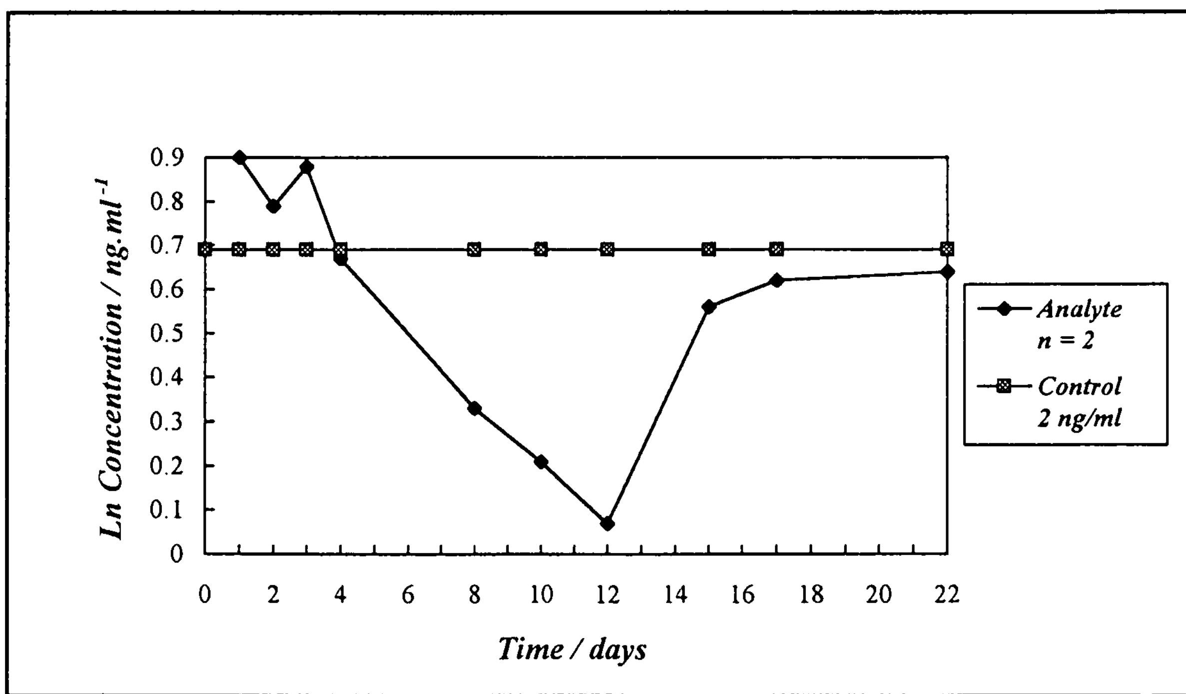

Figure 31 Concentration of 17α-trenbolone in urine of cattle *versus* time after animals received a single dosage (ln transformed)

4.4.5 ZERANOL

4.4.5.1 Cattle

The data on the excretion of zeranol from cattle, are presented in Figure 32 and Figure 33 on page 91. Zeranol could be detected in the urine above the control level of 2 ng/ml for at least 19 days after an implant in the base of the ear of 72 mg zeranol.

The data on the excretion of taleranol (a metabolite of zeranol) from cattle, are presented in Figure 34 and Figure 35 on page 92. Taleranol could also be detected in the urine above the control level of 2 ng/ml for at least 19 days after an implant in the base of the ear of 72 mg zeranol.

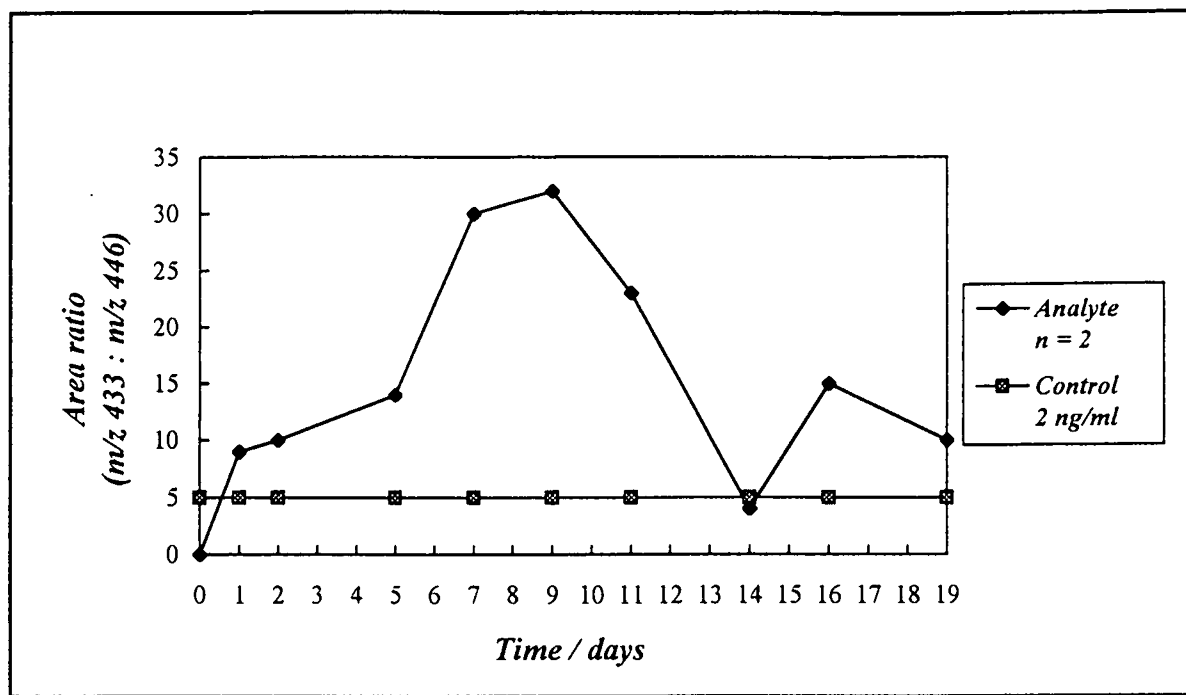

Figure 32 Area ratio of zeranol in urine of cattle *versus* time after animals received a single dosage

The same results are displayed in Figure 33 below, but in a more visual way.

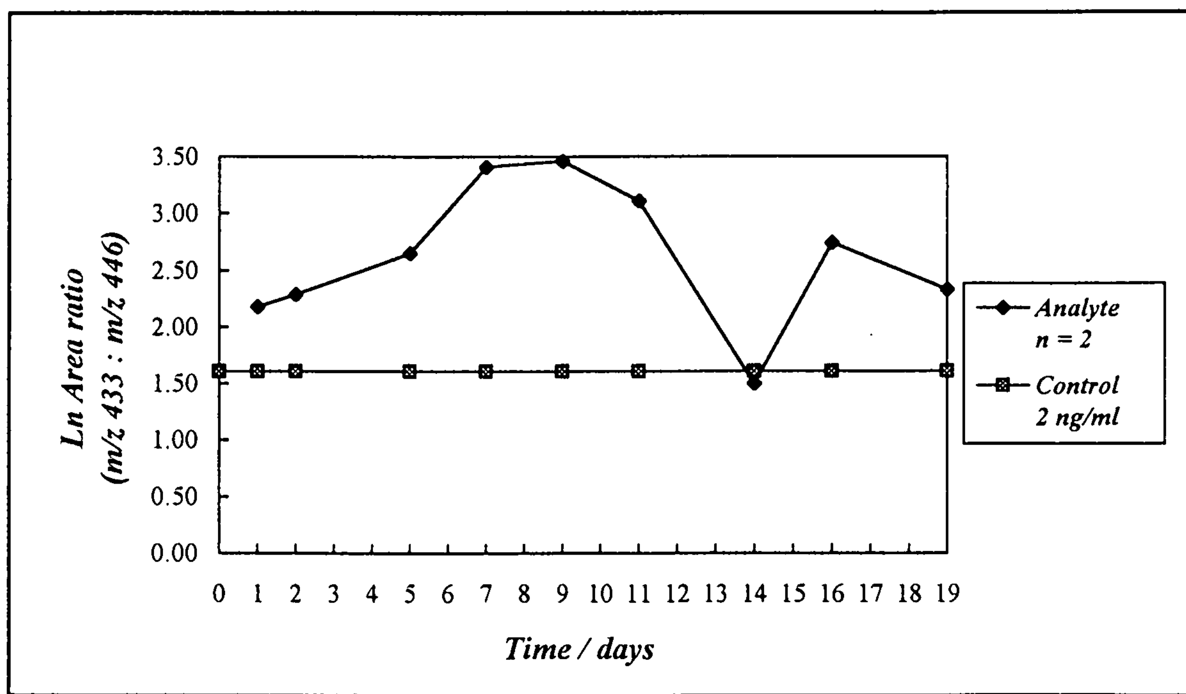

Figure 33 Area ratio of zeranol in urine of cattle *versus* time after animals received a single dosage (ln transformed)

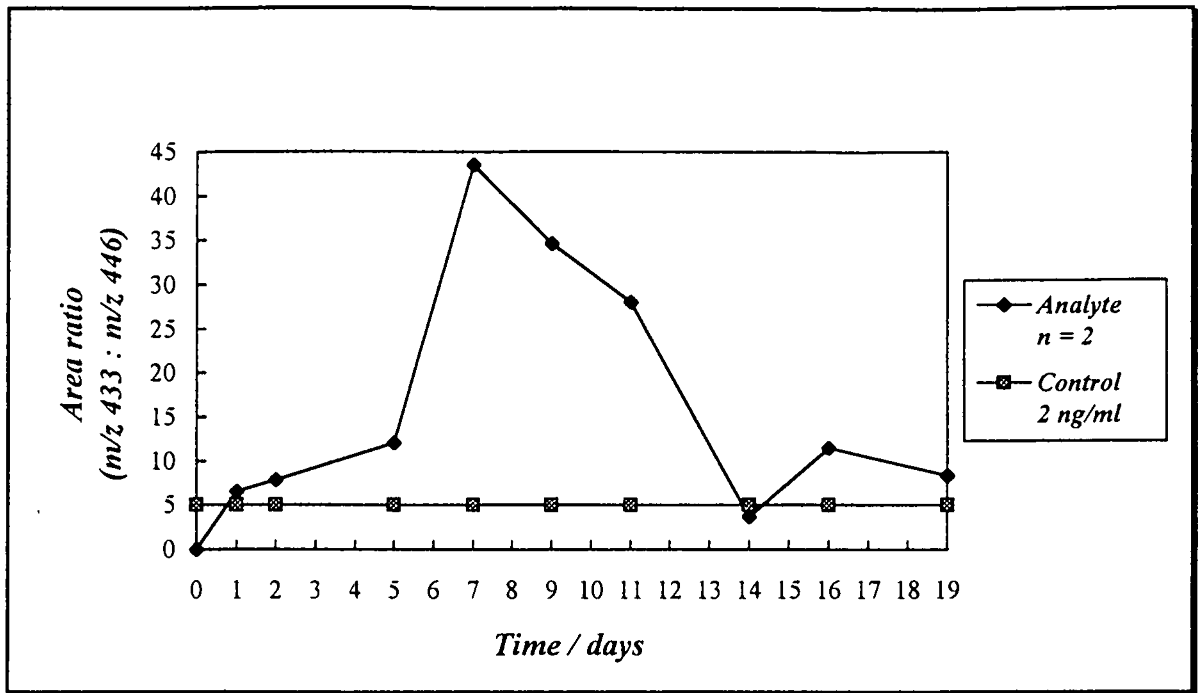

Figure 34 Area ratio of taleranol in urine of cattle *versus* time after animals received a single dosage

The same results are displayed in Figure 35 below, but in a more visual way.

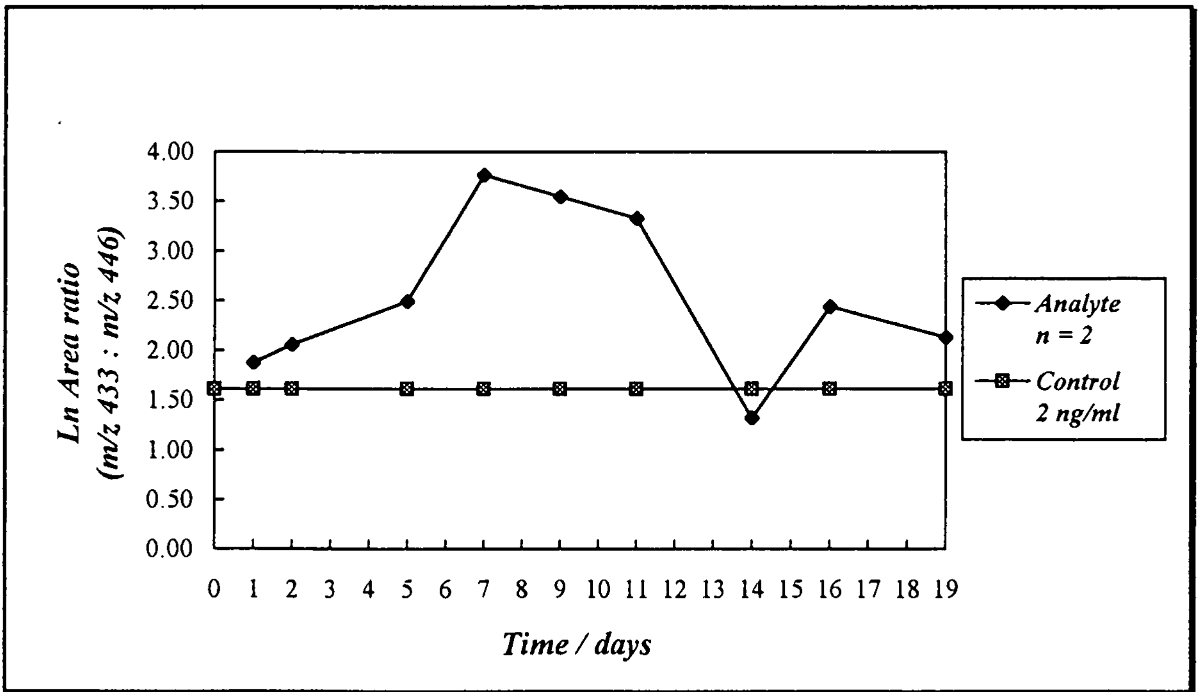

Figure 35 Area ratio of taleranol in urine of cattle *versus* time after animals received a single dosage (ln transformed)

4.4.5.2 Swine

The data on the excretion of zeranol from swine, are presented in Figure 36 and Figure 37 on page 94. Zeranol could be detected in the urine above the control level of 2 ng/ml for at least 21 days after an implant in the base of the ear of 72 mg zeranol.

The data on the excretion of taleranol (a metabolite of zeranol) from swine, are presented in Figure 38 and Figure 39 on page 95. Taleranol could also be detected in the urine above the control level of 2 ng/ml for at least 21 days after an implant in the base of the ear of 72 mg zeranol.

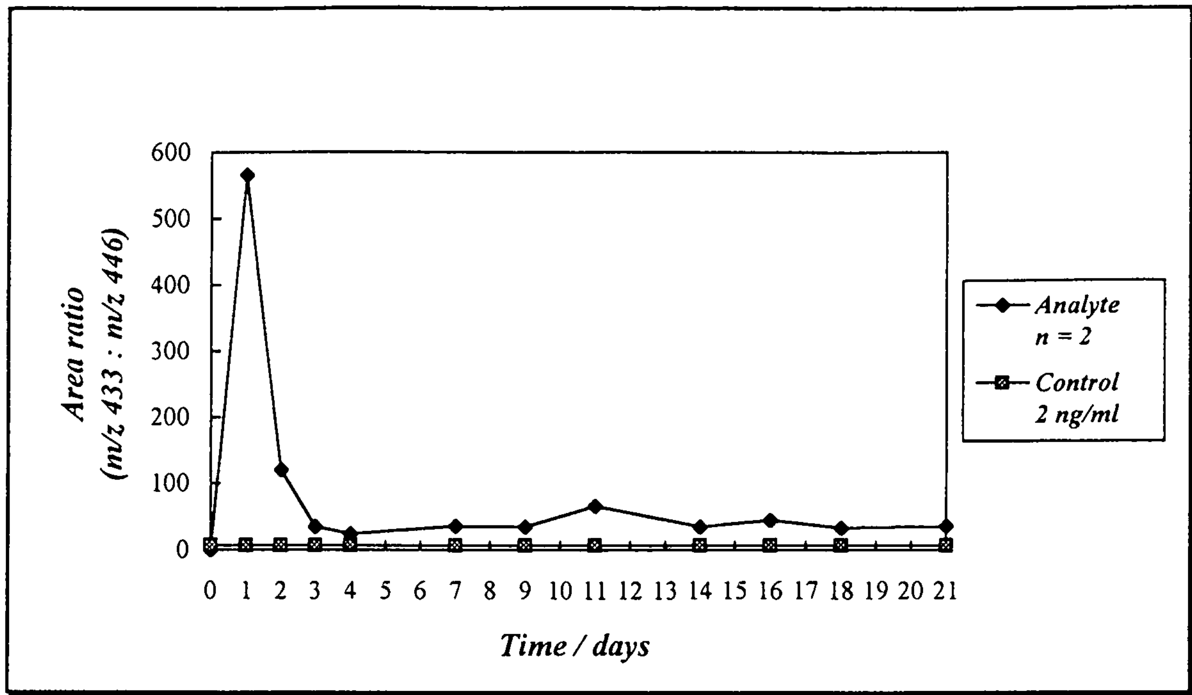

Figure 36 Area ratio of zeranol in urine of swine *versus* time after animals received a single dosage

The same results are displayed in Figure 37 below, but in a more visual way.

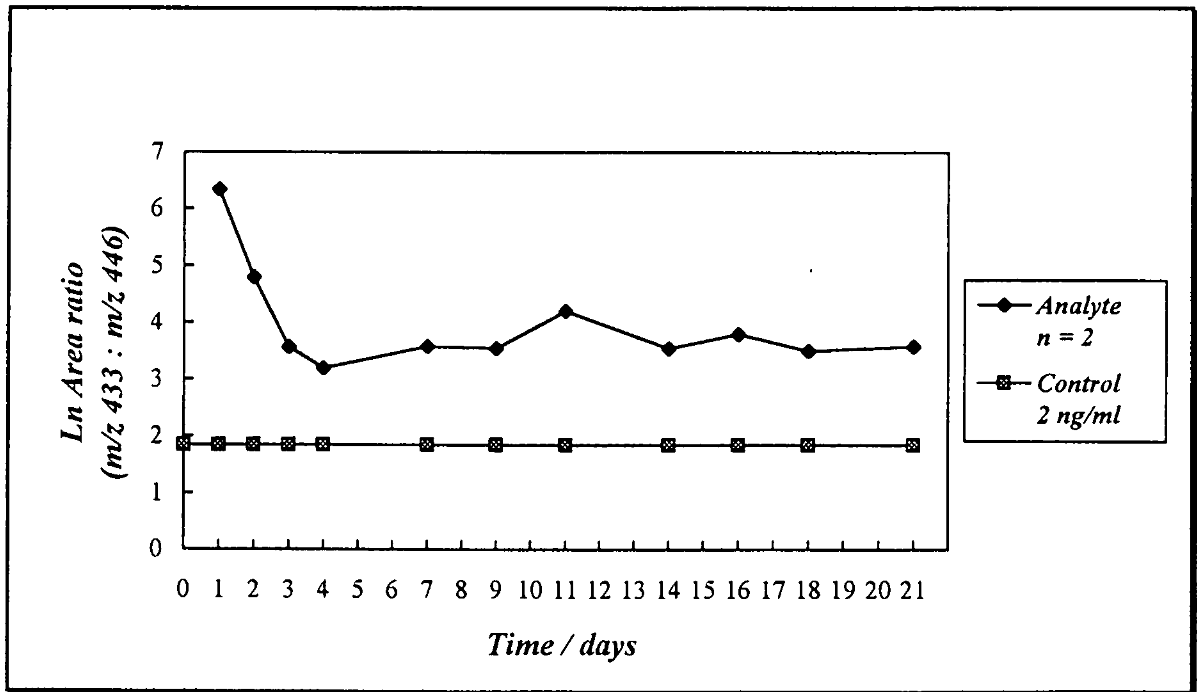

Figure 37 Area ratio of zeranol in urine of swine *versus* time after animals received a single dosage (ln transformed)

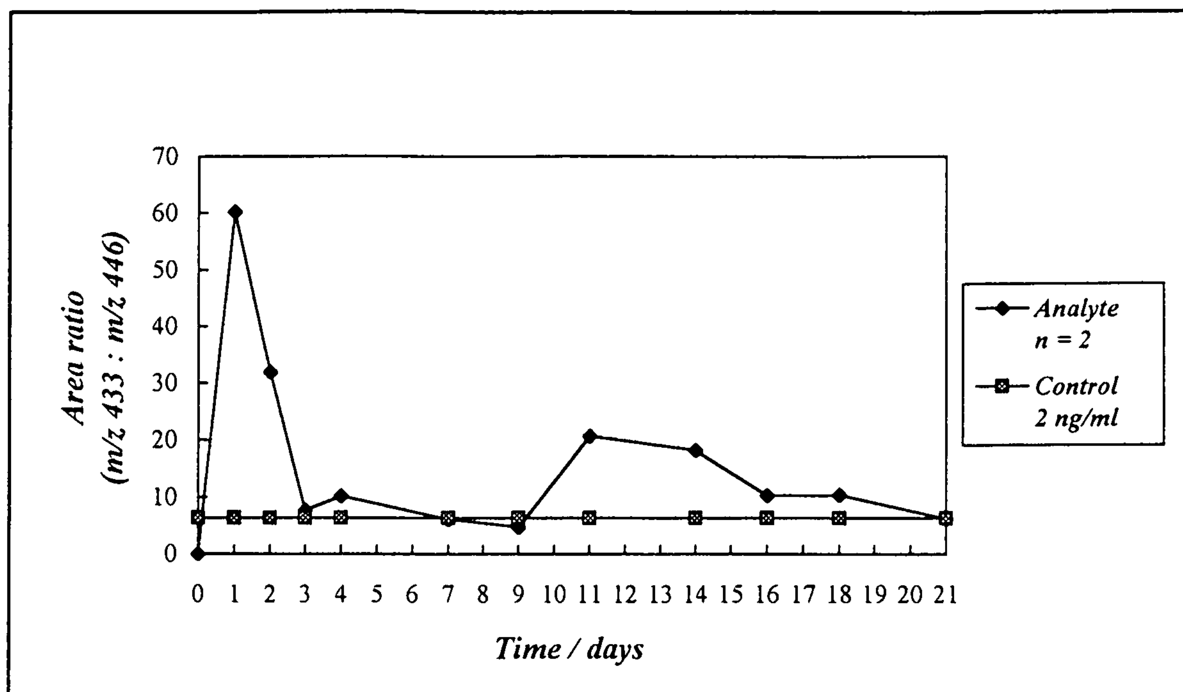

Figure 38 Area ratio of taleranol in urine of swine *versus* time after animals received a single dosage

The same results are displayed in Figure 39 below, but in a more visual way.

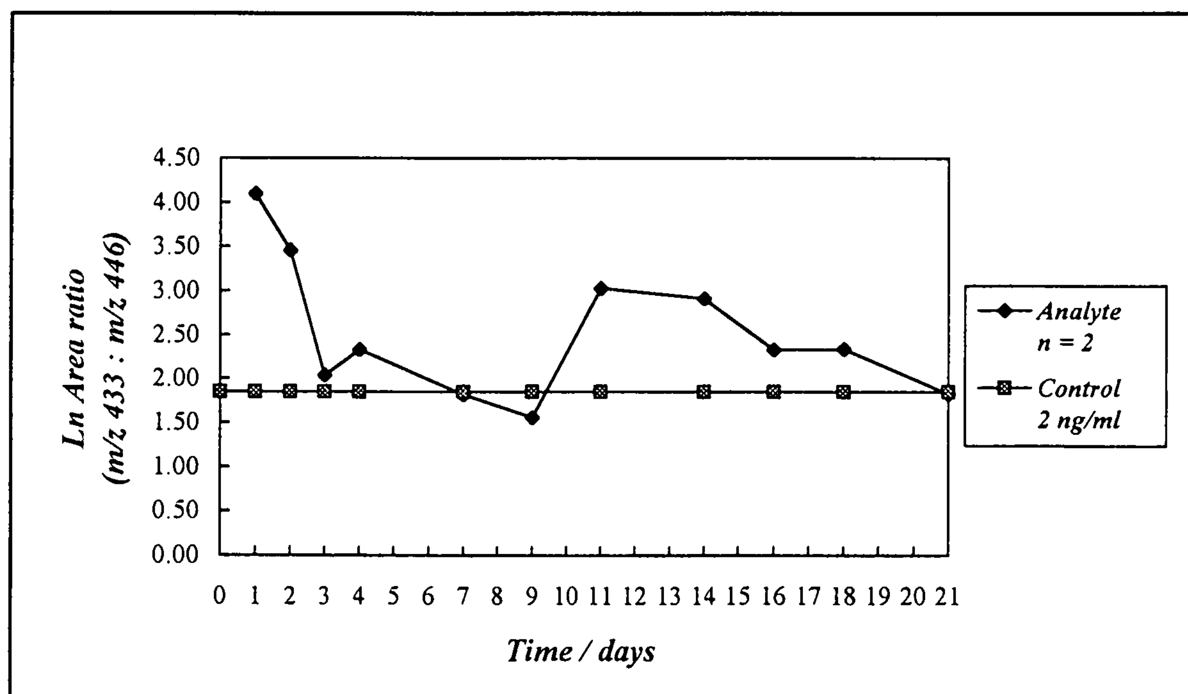

Figure 39 Area ratio of taleranol in urine of swine *versus* time after animals received a single dosage (ln transformed)

4.5 CONFIRMATION OF ANALYTES

The normalized abundances of the GC-MS-MS characteristic ions of the trimethylsilyl derivatives of the analytes are presented in Table 35 below:

Table 35 Normalized abundances of the GC-MS-MS characteristic ions of the trimethylsilyl derivatives of the analytes

ANALYTE (MATRIX)	NORMALIZED ABUNDANCES		
Clenbuterol (Swine)	**m/z = 301**	**m/z = 262**	**m/z = 227**
Standard (Figure 42)	100	25	21
Sample (Figure 41)	100	23	20
Diethylstilbestrol (Cattle)	**m/z = 384**	**m/z = 398**	**m/z = 412**
Standard (Figure 47)	100	31	24
Sample (Figure 46)	100	25	19
Diethylstilbestrol (Swine)	**m/z = 384**	**m/z = 398**	**m/z = 412**
Standard (Figure 51)	100	34	25
Sample (Figure 50)	100	33	23
Epi-nandrolone (Cattle)	**m/z = 313**	**m/z = 328**	**m/z = 404**
Standard (Figure 57)	100	68	33
Sample (Figure 56)	100	87	29
Epi-norandrosterone (Swine)	**m/z = 315**	**m/z = 225**	**m/z = 405**
Standard (Figure 61)	100	80	21
Sample (Figure 60)	100	79	16
Zeranol (Cattle)	**m/z = 389**	**m/z = 415**	**m/z = 433**
Standard (Figure 67)	100	68	26
Sample (Figure 66)	100	66	40
Taleranol (Cattle)	**m/z = 389**	**m/z = 415**	**m/z = 433**
Standard (Figure 71)	100	83	24
Sample (Figure 70)	100	51	50
Zeranol (Swine)	**m/z = 390**	**m/z = 415**	**m/z = 433**
Standard (Figure 75)	100	65	26
Sample (Figure 74)	100	77	34
Taleranol (Swine)	**m/z = 389**	**m/z = 415**	**m/z = 433**
Standard (Figure 79)	100	67	20
Sample (Figure 78)	100	70	18

4.5.1 CLENBUTEROL

A full-scan mass spectrum of a clenbuterol standard, using the ion-trap detection, is shown in Figure 40 (page 98). The ion with m/z=335, used as parent ion in the full-scan MS-MS spectrum, is indicated.

4.5.1.1 Swine

The data on the confirmation of clenbuterol in the urine of swine are presented as follows:

- Figure 41 (page 99) Excretion study of clenbuterol in swine [subject X-3, urine collected on day 2 after treatment was stopped]
- Figure 42 (page 99) Clenbuterol standard
- Figure 43 (page 100) Urine blank
- Figure 44 (page 100) Reagent blank

The retention time of clenbuterol was determined as 5.30 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 0.369.

The background subtracted mass spectra are shown at 5.30 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is unequivocal proof that the excretion study is positive for clenbuterol.

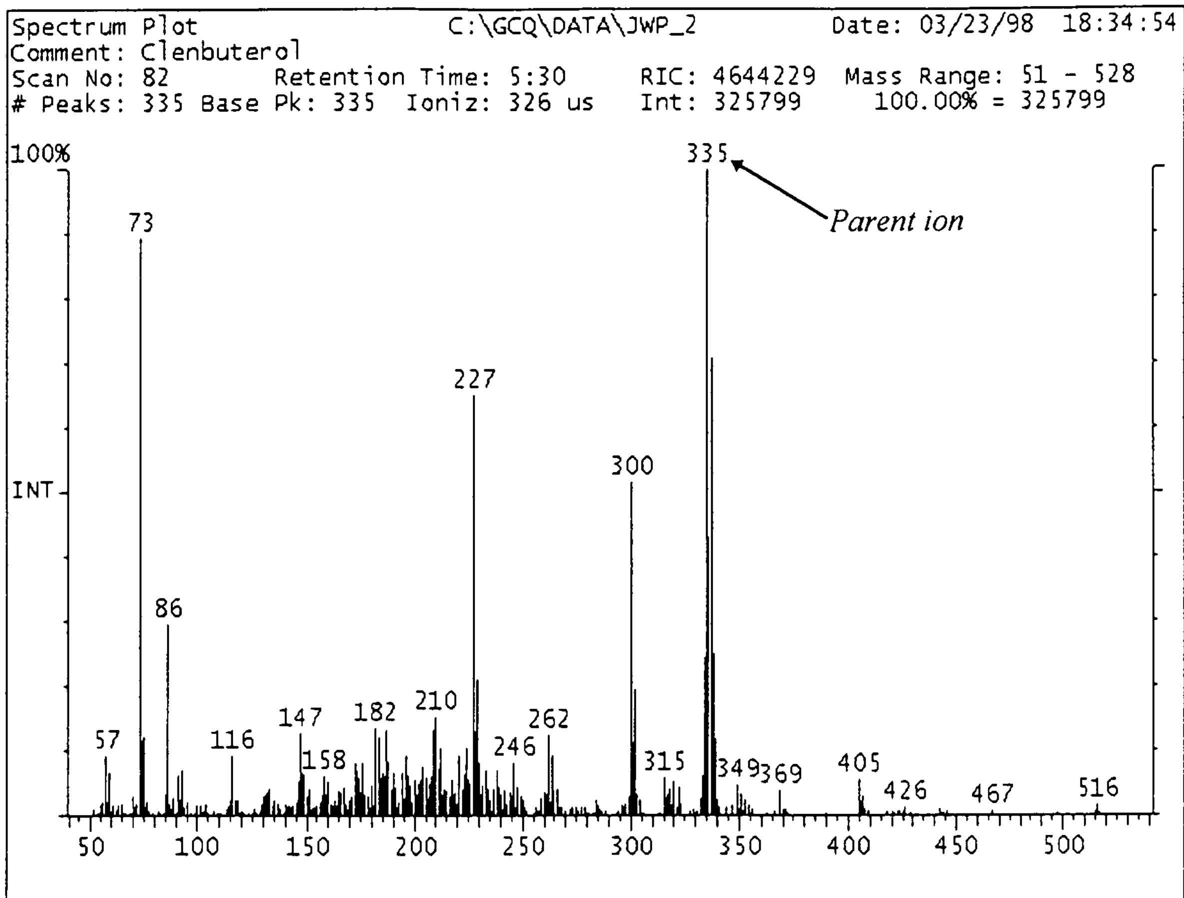

Figure 40 EI-GC/MS mass spectrum of the trimethylsilyl derivative of a clenbuterol standard using the ion-trap detector

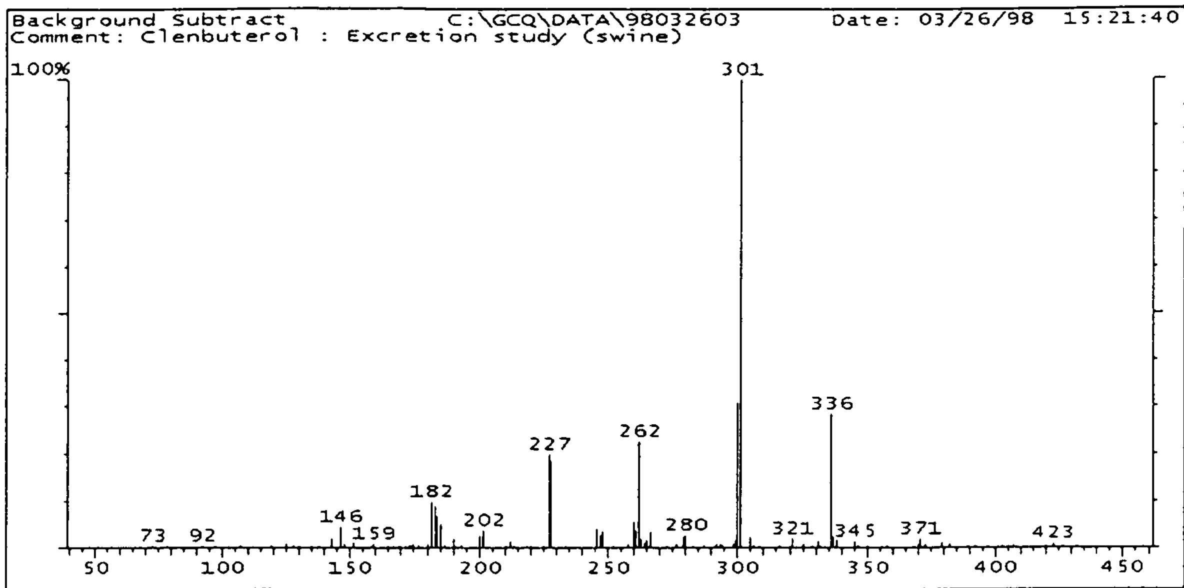

Figure 41 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of clenbuterol in swine

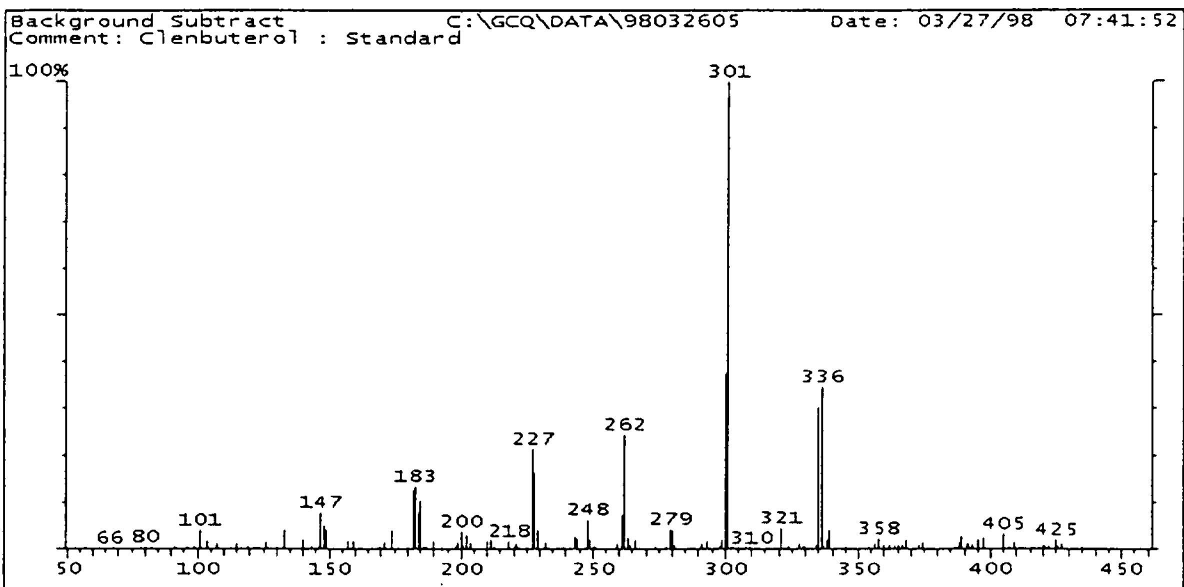

Figure 42 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of clenbuterol

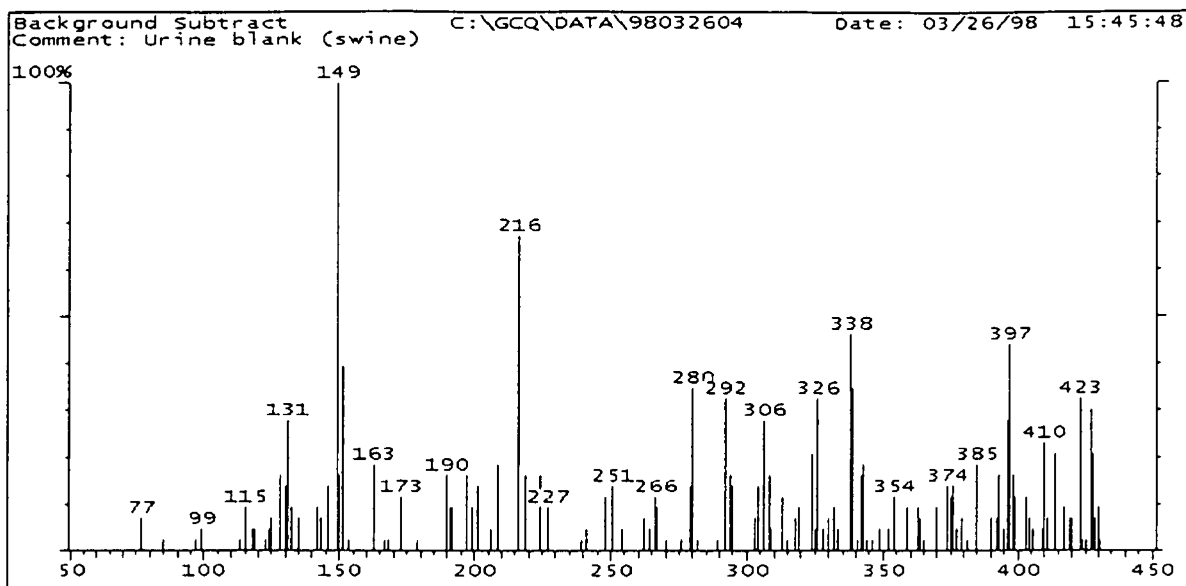

Figure 43 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

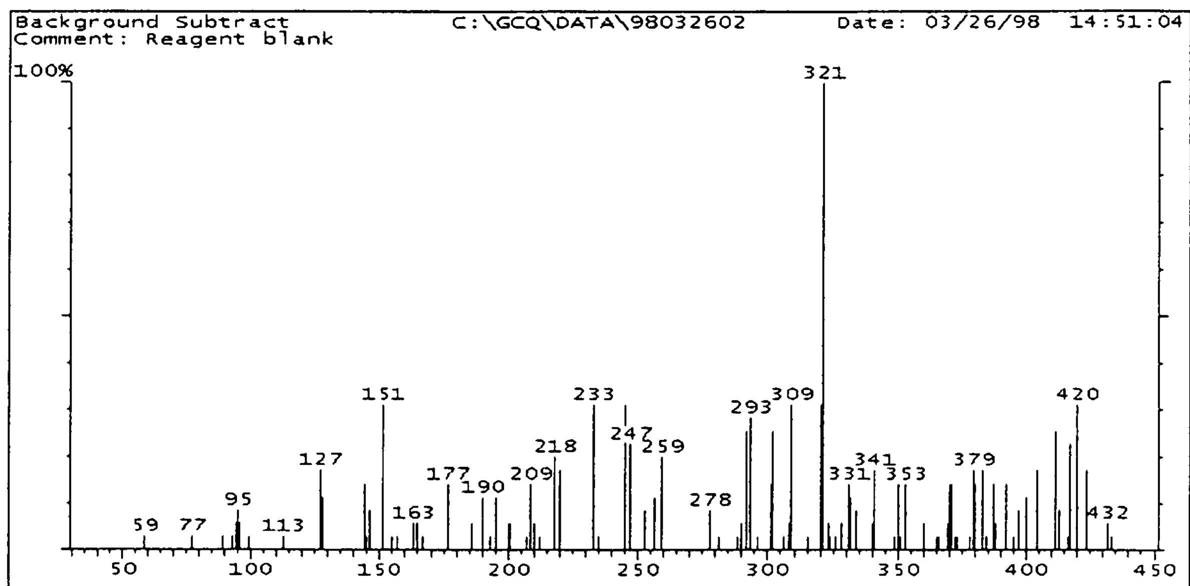

Figure 44 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

4.5.2 DIETHYLSTILBESTROL

A full-scan mass spectrum of a diethylstilbestrol standard, using the ion-trap detection, is shown in Figure 45 (page 102). The ion with m/z=412, used as parent ion in the full-scan MS-MS spectrum, is indicated.

4.5.2.1 Cattle

The data on the confirmation of diethylstilbestrol in the urine of cattle are presented as follows:

- Figure 46 (page 103) Excretion study of diethylstilbestrol in cattle
- [subject C-7, urine collected on day 2 after treatment was stopped]
- Figure 47 (page 103) Diethylstilbestrol standard
- Figure 48 (page 104) Urine blank
- Figure 49 (page 104) Reagent blank

The retention time of diethylstilbestrol was determined as 8.42 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 0.586.

The background subtracted mass spectra are shown at 8.42 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is unequivocal proof that the excretion study is positive for diethylstilbestrol.

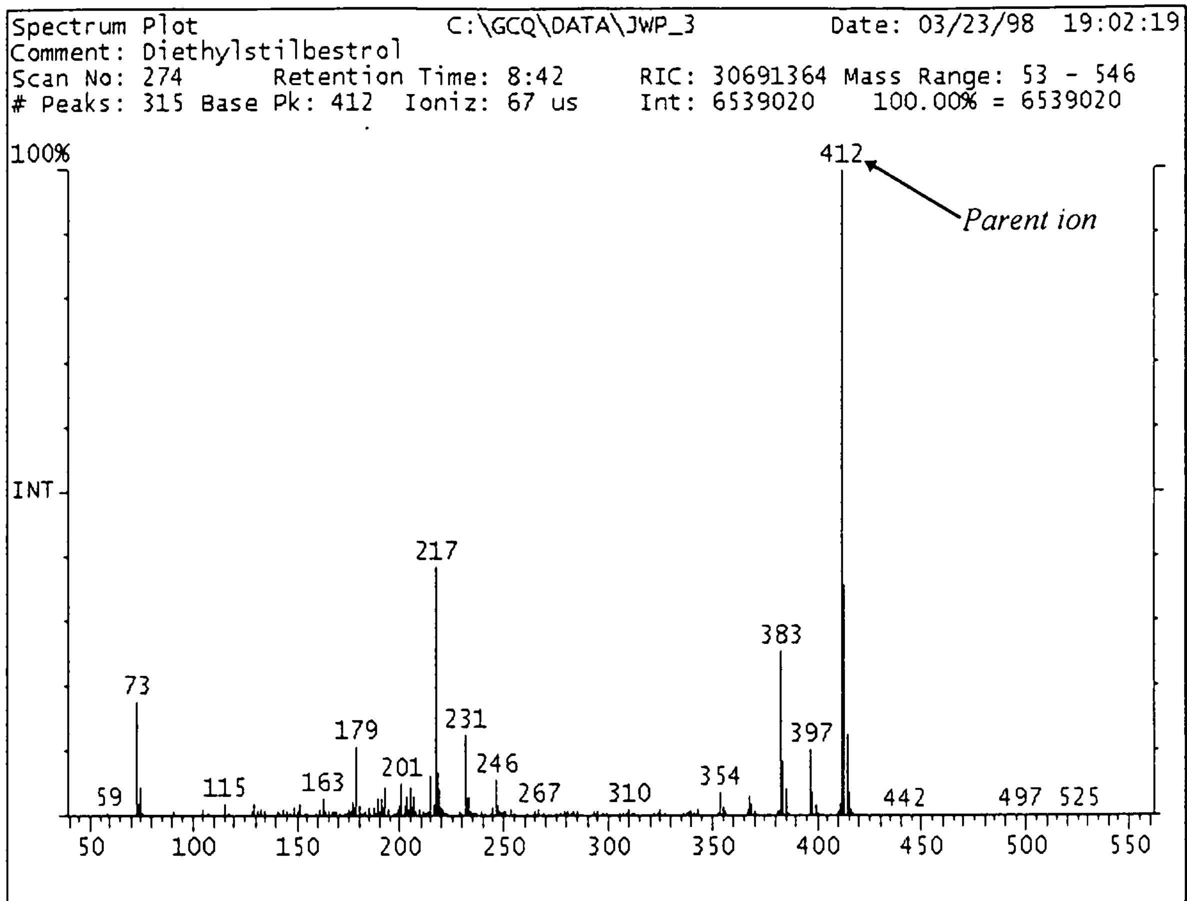

Figure 45 EI-GC/MS mass spectrum of the trimethylsilyl derivative of a diethylstilbestrol standard using the ion-trap detector

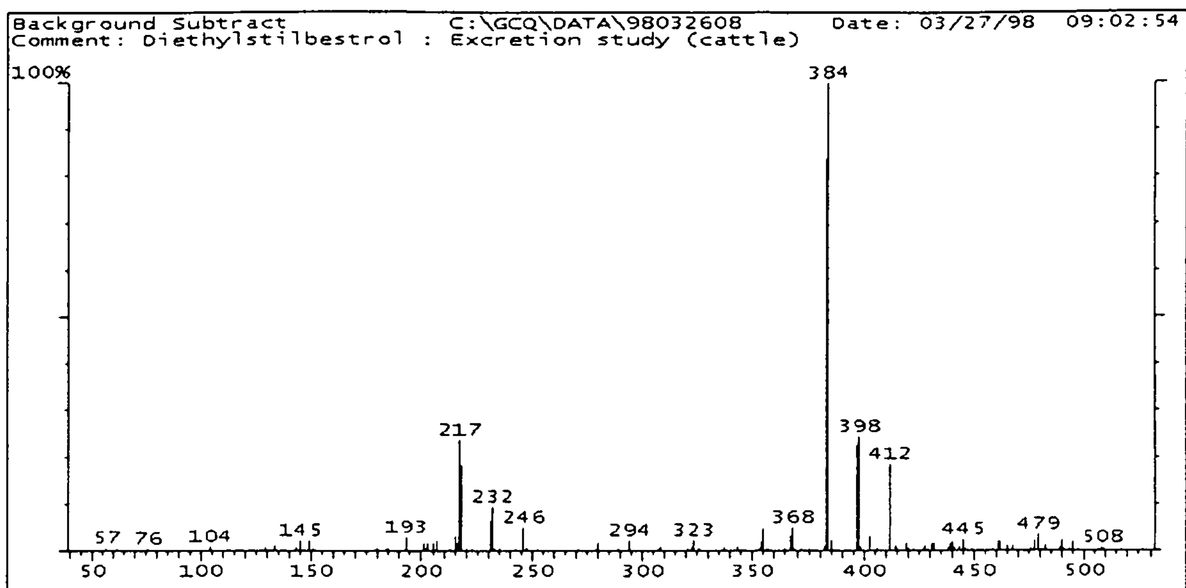

Figure 46 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of diethylstilbestrol in cattle

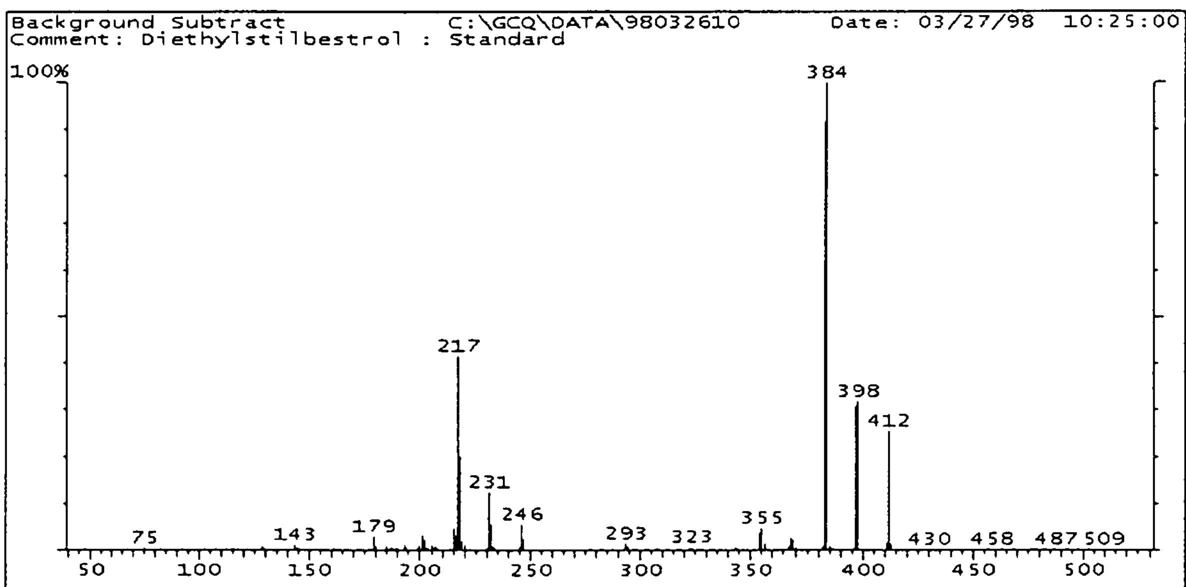

Figure 47 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of diethylstilbestrol

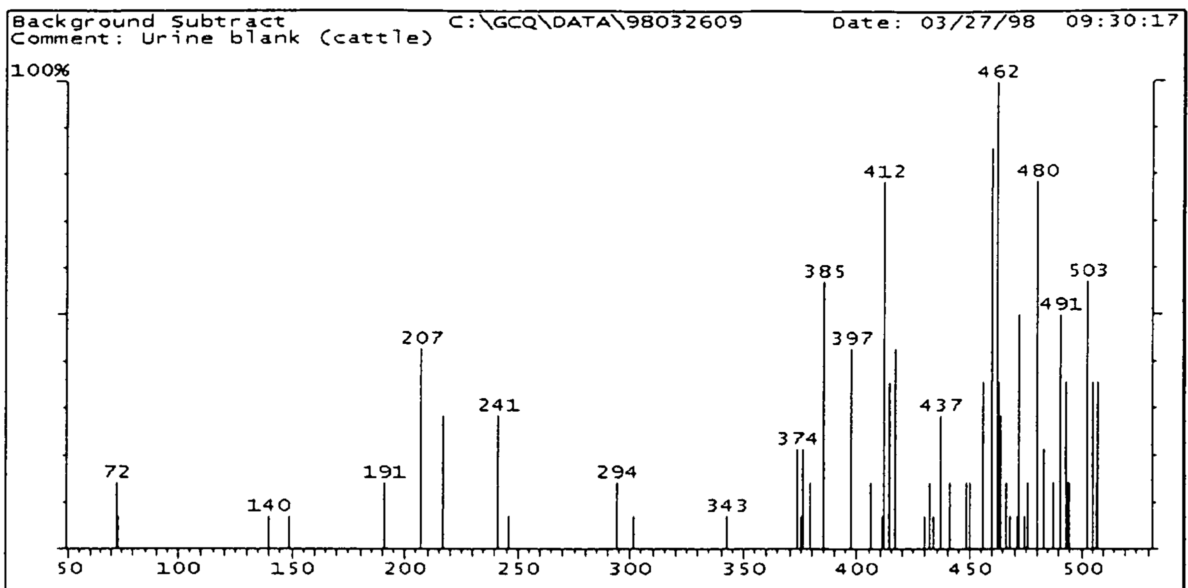

Figure 48 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

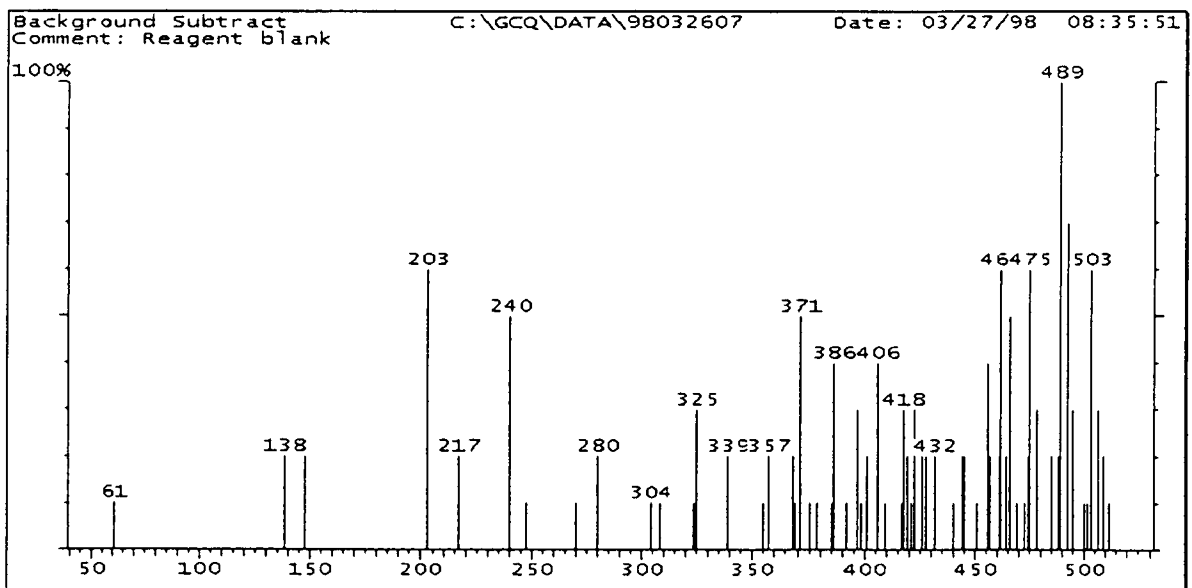

Figure 49 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

4.5.2.2 Swine

The data on the confirmation of diethylstilbestrol in the urine of swine are presented as follows:

- Figure 50 (page 106) Excretion study of diethylstilbestrol in swine [subject S-7, urine collected on day 2 after treatment was stopped]
- Figure 51 (page 106) Diethylstilbestrol standard
- Figure 52 (page 107) Urine blank
- Figure 53 (page 107) Reagent blank

The retention time of diethylstilbestrol was determined as 8.42 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 0.586.

The background subtracted mass spectra are shown at 8.42 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is unequivocal proof that the excretion study is positive for diethylstilbestrol.

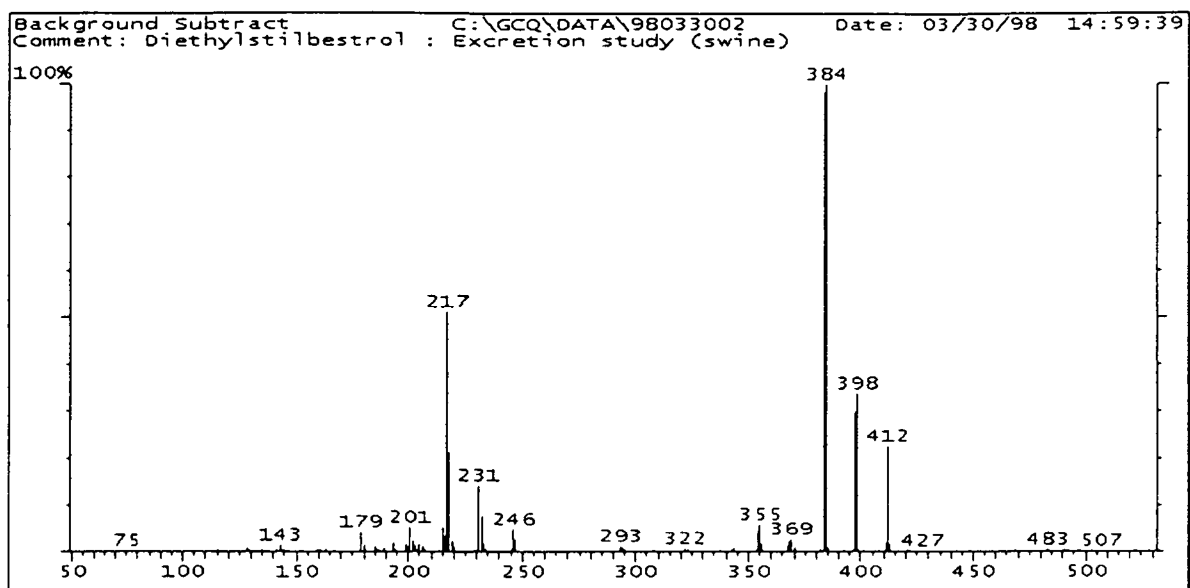

Figure 50 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of diethylstilbestrol in swine

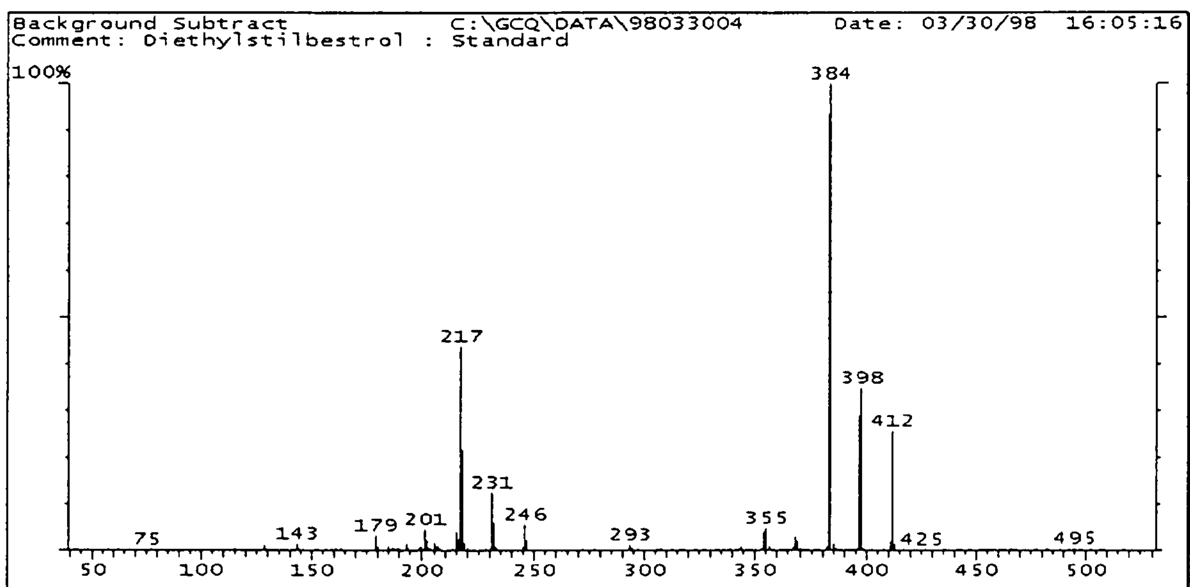

Figure 51 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of diethylstilbestrol

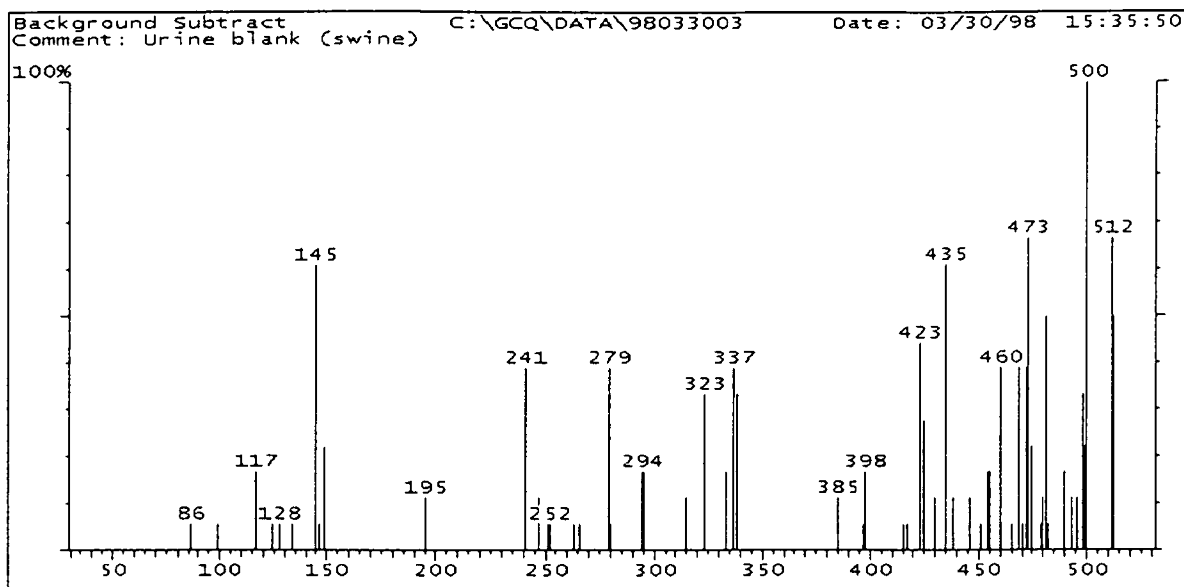

Figure 52 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

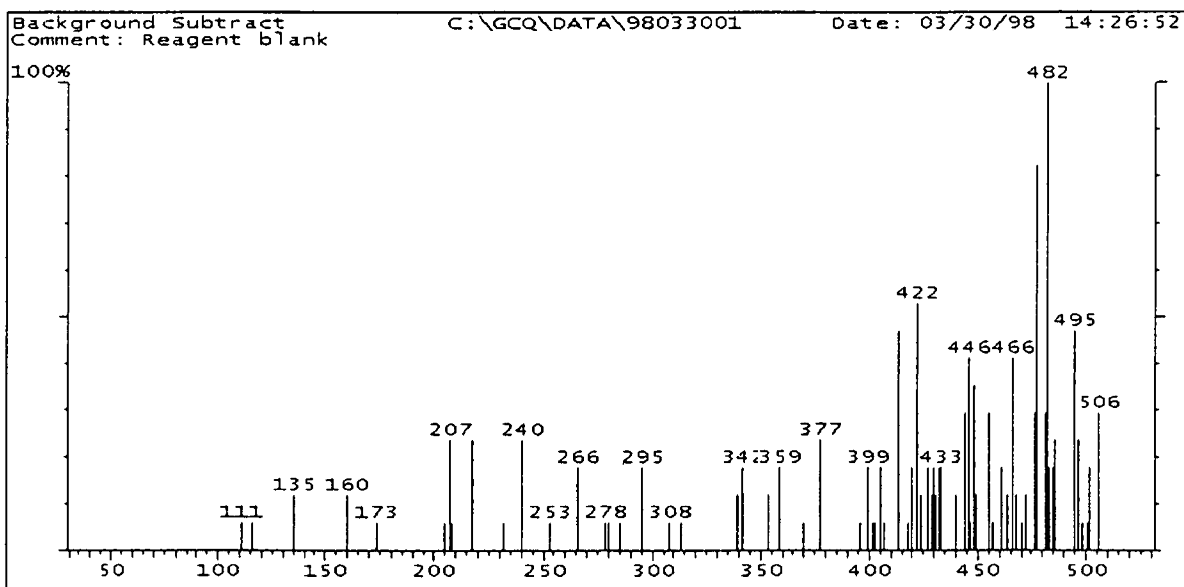

Figure 53 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

4.5.3 NANDROLONE

A full-scan mass spectrum of a epi-nandrolone (nandrolone metabolite in cattle) standard, using the ion-trap detection, is shown in Figure 54 (page 109). The ion with m/z=418, used as parent ion in the full-scan MS-MS spectrum, is indicated. A full-scan mass spectrum of a epi-norandrosterone (nandrolone metabolite in swine) standard, using the ion-trap detection, is shown in Figure 55 (page 110). The ion with m/z=405, used as parent ion in the full-scan MS-MS spectrum, is indicated.

4.5.3.1 Cattle

The data on the confirmation of epi-nandrolone (a metabolite of nandrolone in cattle) in the urine of cattle are presented as follows:

- Figure 56 (page 111) Excretion study of nandrolone in cattle [subject C-1, urine collected on day 2 after treatment was stopped]
- Figure 57 (page 111) Epi-nandrolone standard
- Figure 58 (page 112) Urine blank
- Figure 59 (page 112) Reagent blank

The retention time of epi-nandrolone was determined as 11.52 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 0.802.

The background subtracted mass spectra are shown at 11.52 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is unequivocal proof that the excretion study is positive for nandrolone.

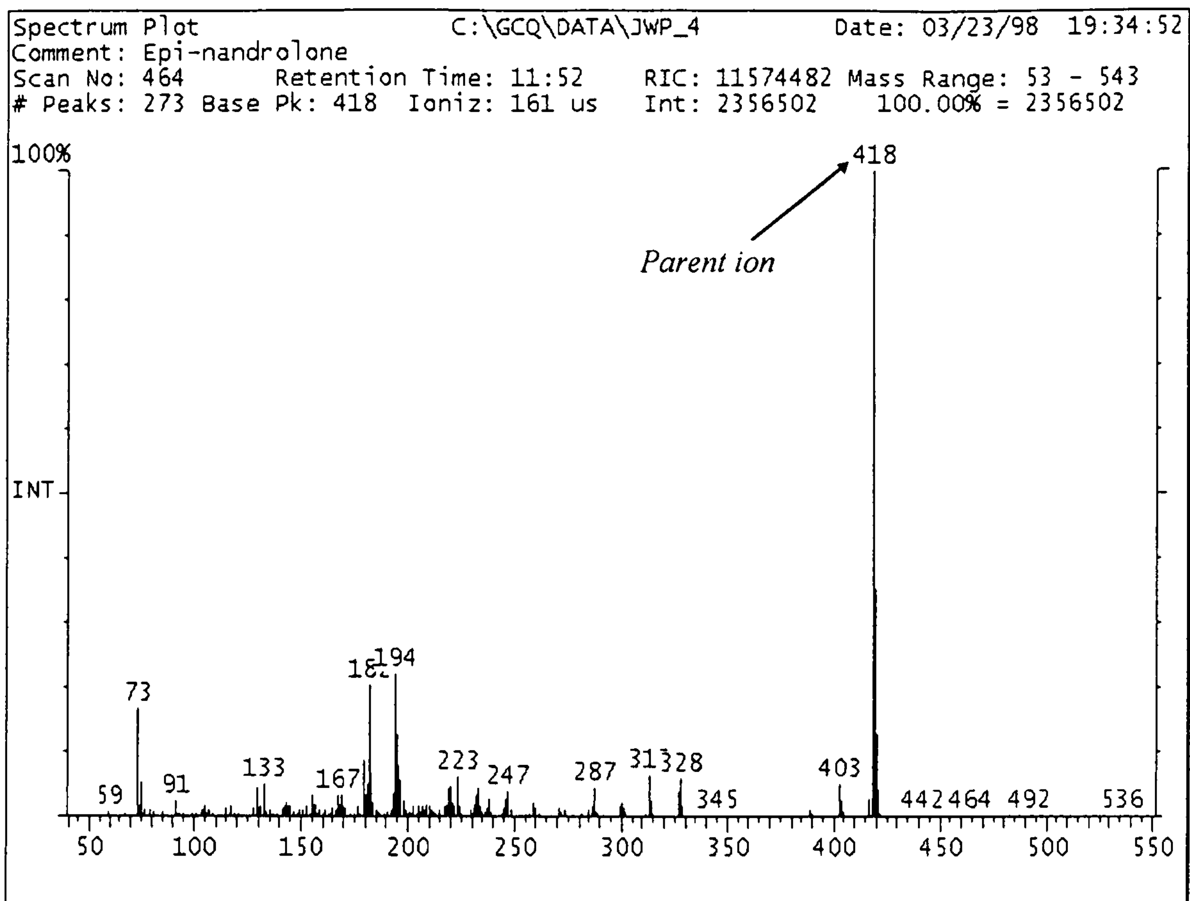

Figure 54 EI-GC/MS mass spectrum of the trimethylsilyl derivative of a epi-nandrolone standard using the ion-trap detector

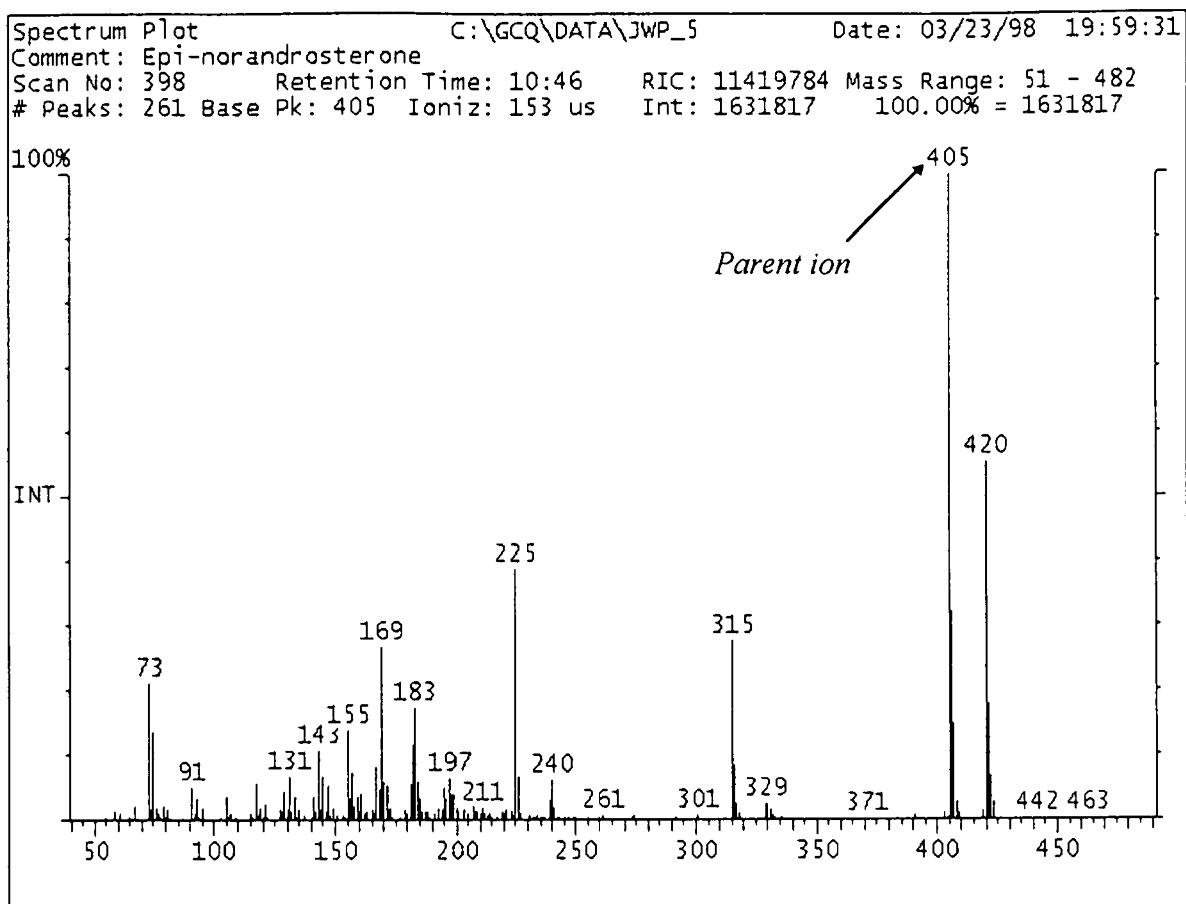

Figure 55 EI-GC/MS mass spectrum of the trimethylsilyl derivative of a epi-norandrosterone standard using the ion-trap detector

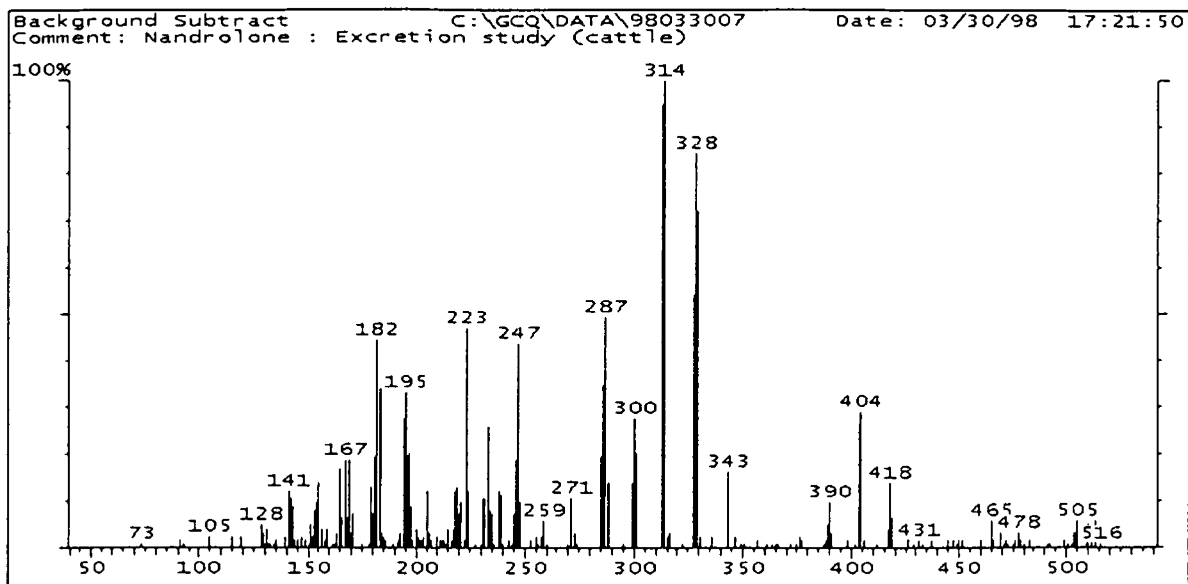

Figure 56 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of nandrolone in cattle

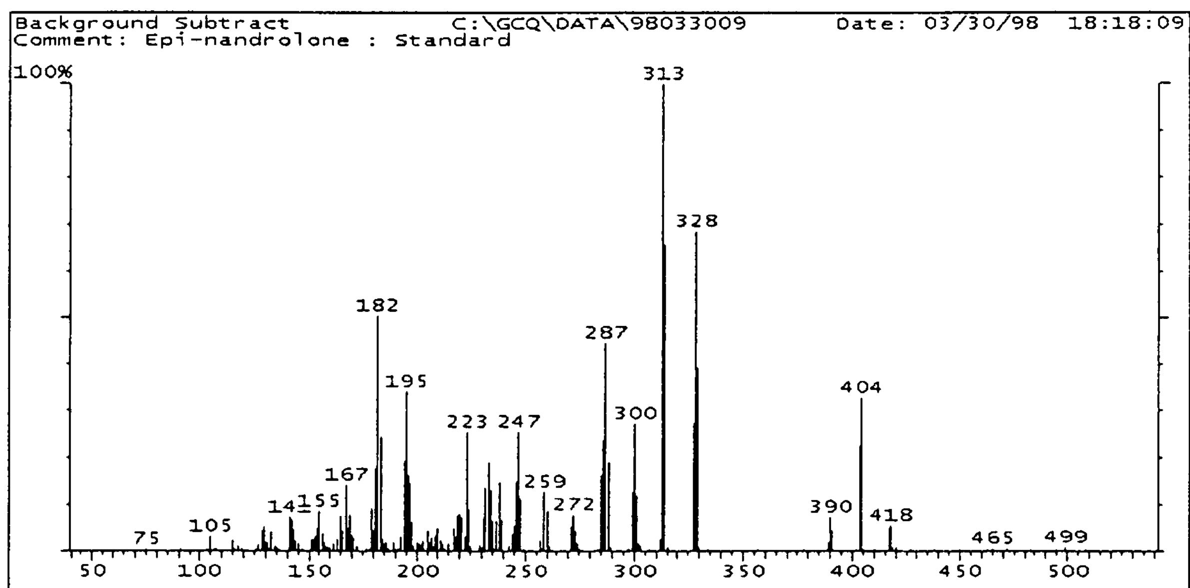

Figure 57 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of epi-nandrolone

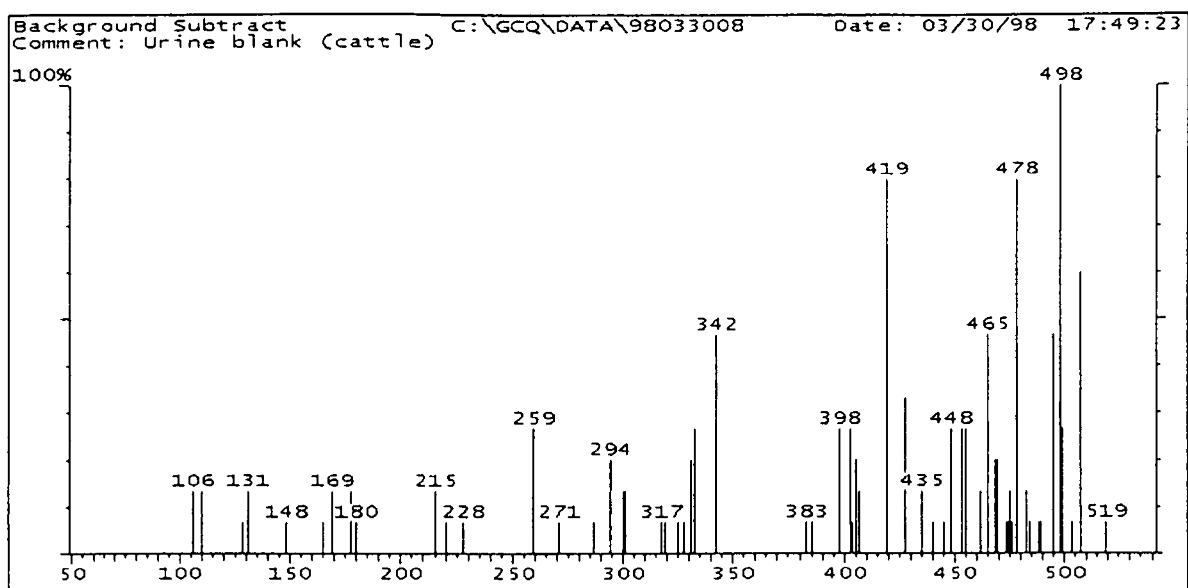

Figure 58 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

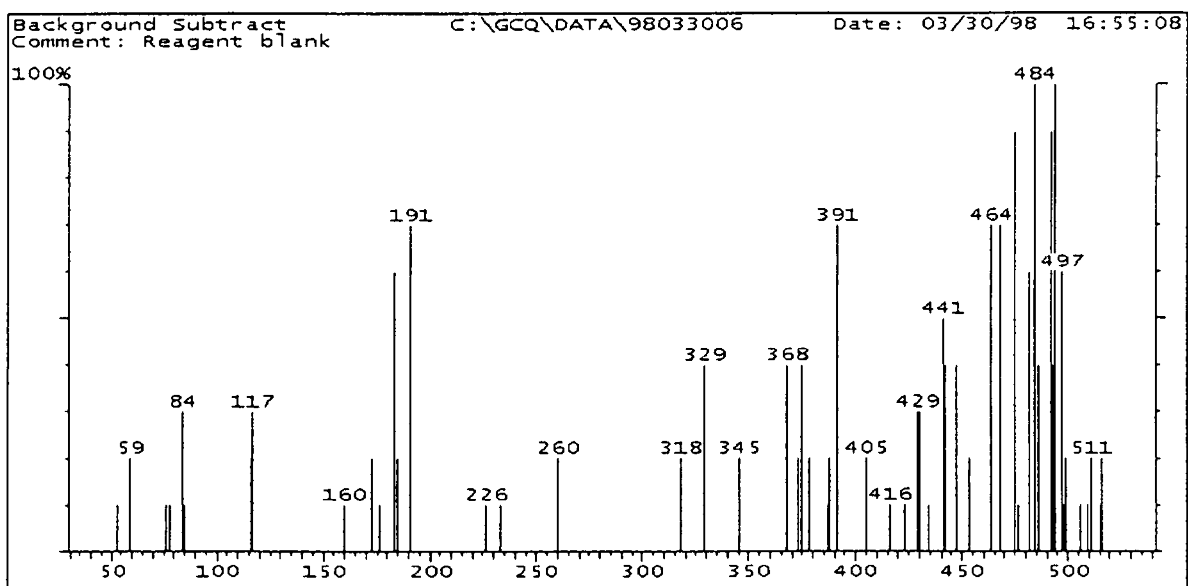

Figure 59 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

4.5.3.2 Swine

The data on the confirmation of epi-norandrosterone (a main metabolite of nandrolone in swine) in the urine of swine are presented as follows:

- Figure 60 (page 114) Excretion study of nandrolone in swine [subject C-1, urine collected on day 2 after treatment was stopped]
- Figure 61 (page 114) Epi-norandrosterone standard
- Figure 62 (page 115) Urine blank
- Figure 63 (page 115) Reagent blank

The retention time of epi-norandrosterone was determined as 10.46 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 0.728.

The background subtracted mass spectra are shown at 10.46 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is unequivocal proof that the excretion study is positive for nandrolone.

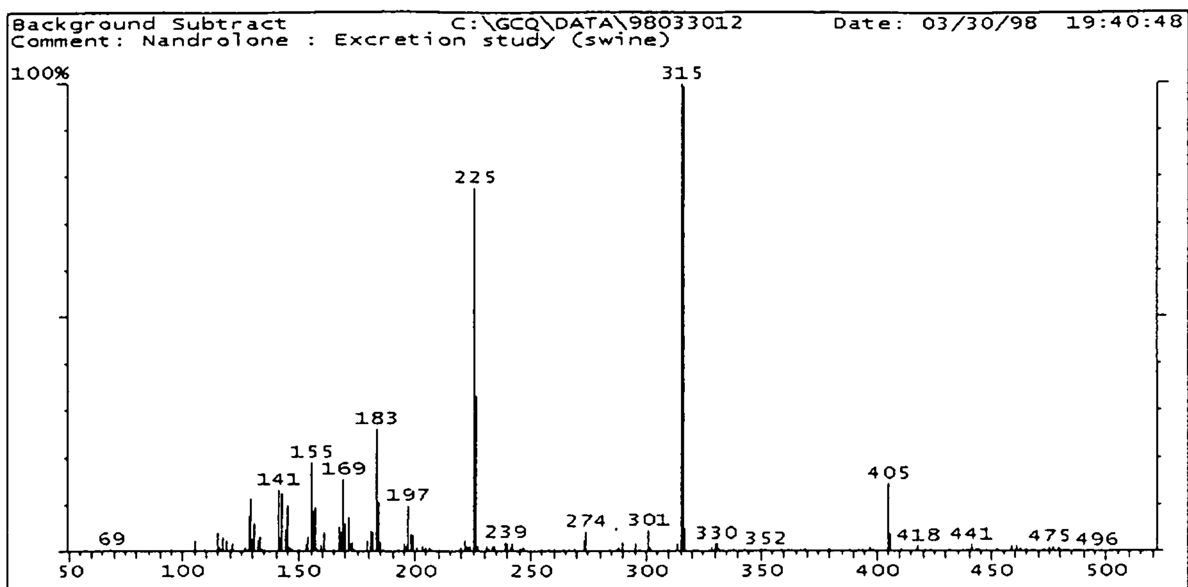

Figure 60 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of nandrolone in swine

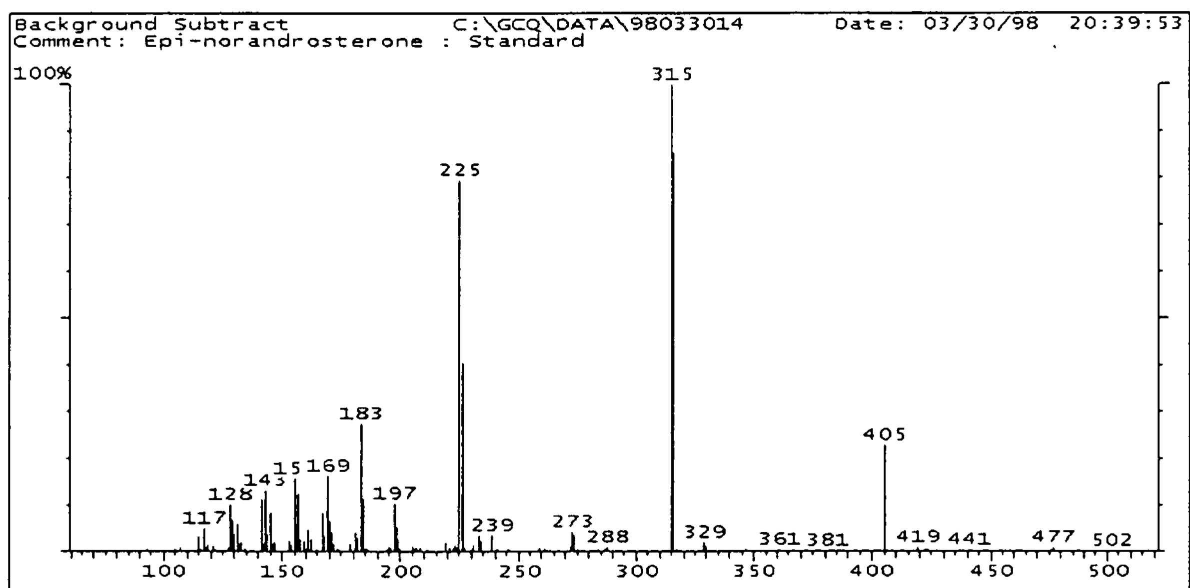

Figure 61 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of epi-norandrosterone

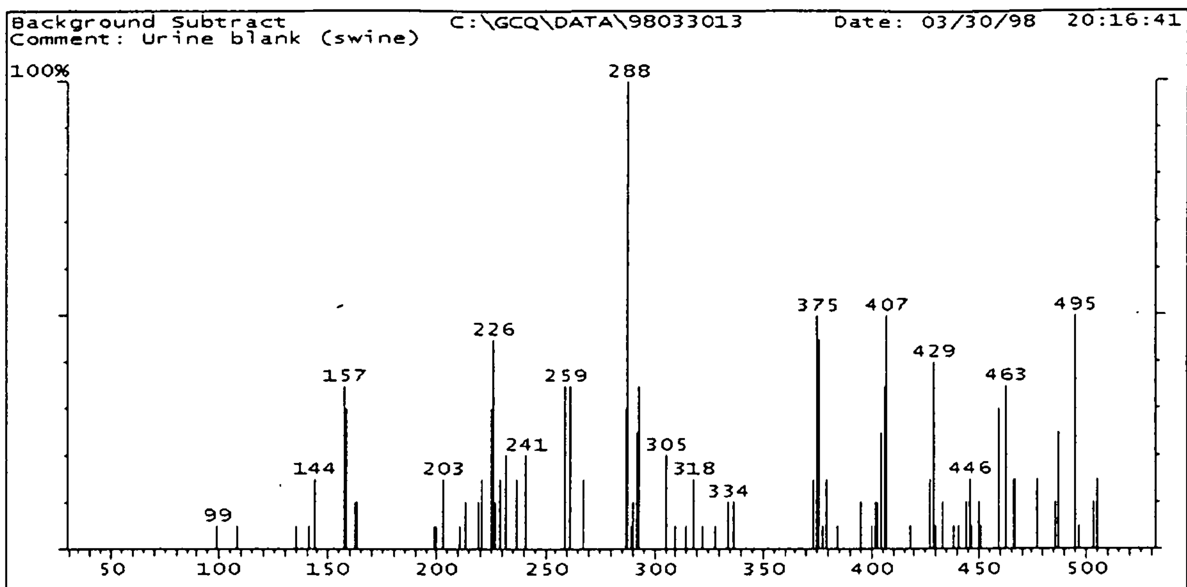

Figure 62 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

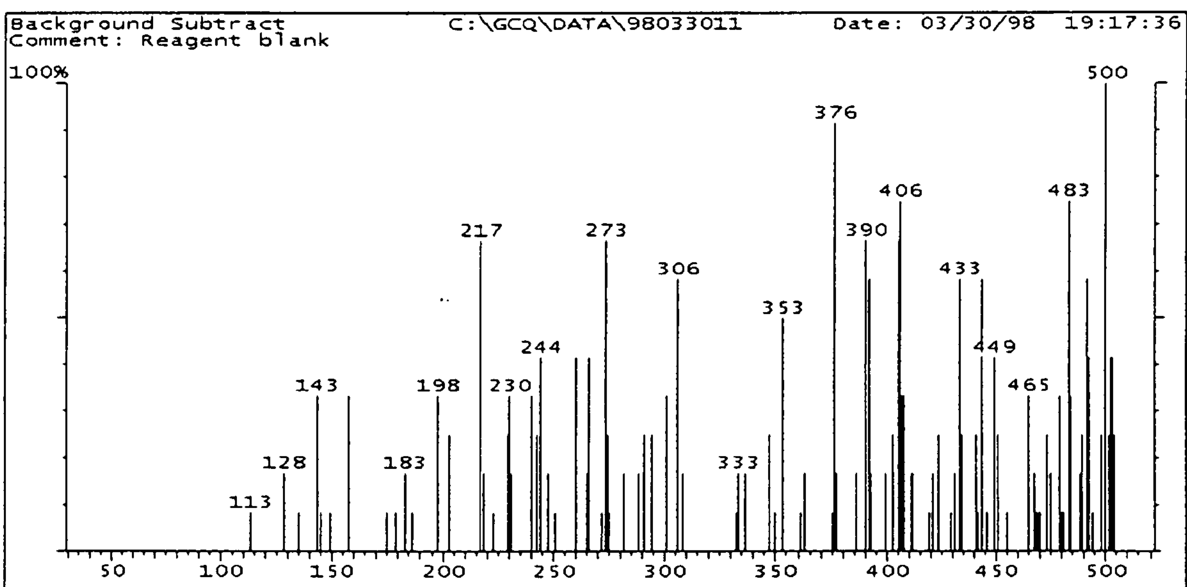

Figure 63 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

4.5.4 ZERANOL

A full-scan mass spectrum of a zeranol standard, using the ion-trap detection, is shown in Figure 64 (page 117). The ion with m/z=433, used as parent ion in the full-scan MS-MS spectrum, is indicated. A full-scan mass spectrum of a taleranol (zeranol metabolite in cattle and swine) standard, using the ion-trap detection, is shown in Figure 65 (page 118). The ion with m/z=433, used as parent ion in the full-scan MS-MS spectrum, is indicated.

4.5.4.1 Cattle

The data on the confirmation of zeranol in the urine of cattle are presented as follows:

- Figure 66 (page 119) Excretion study of zeranol in cattle [subject C-3, urine collected on day 11 after treatment was stopped]
- Figure 67 (page 119) Zeranol standard
- Figure 68 (page 120) Urine blank
- Figure 69 (page 120) Reagent blank

The retention time of zeranol was determined as 16.02 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 1.088.

The background subtracted mass spectra are shown at 16.02 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is unequivocal proof that the excretion study is positive for zeranol.

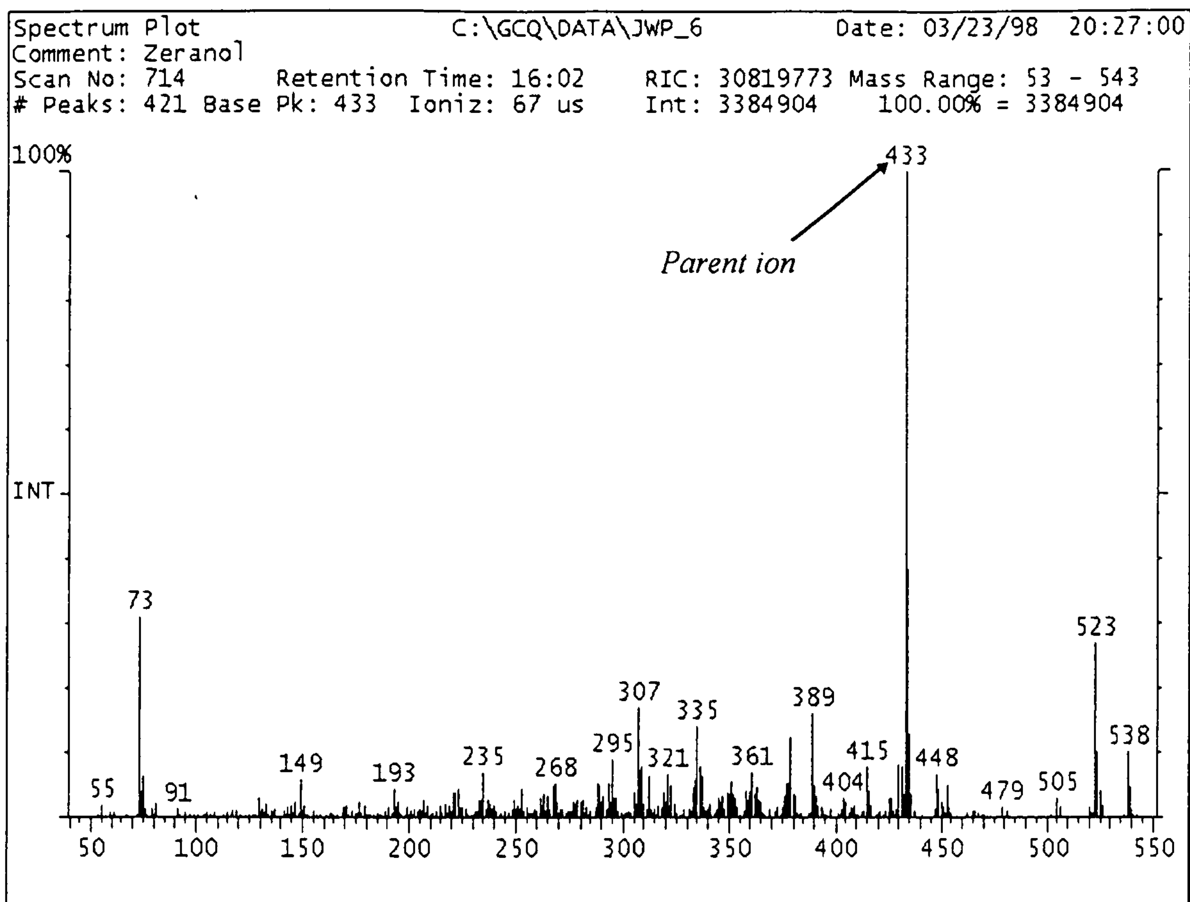

Figure 64 EI-GC/MS mass spectrum of the trimethylsilyl derivative of a zeranol standard using the ion-trap detector

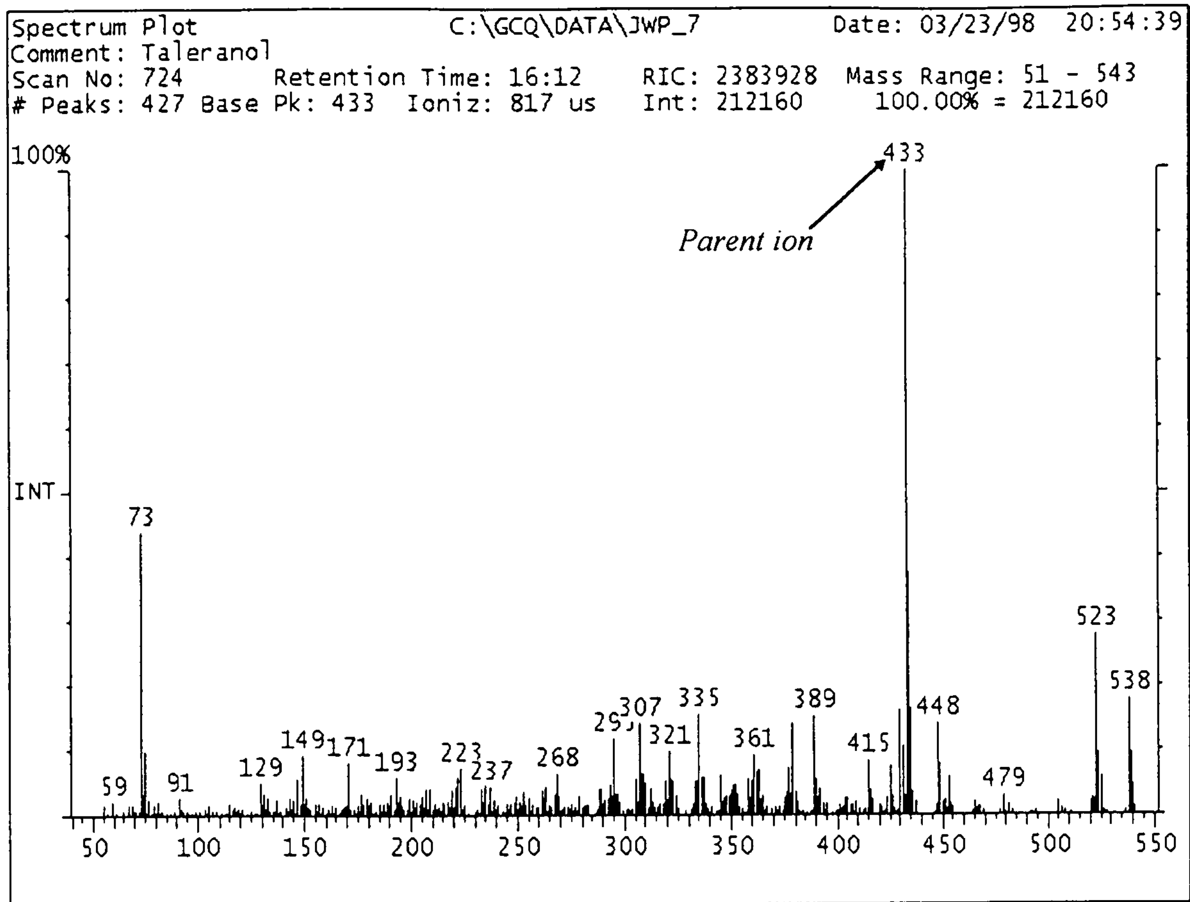

Figure 65 EI-GC/MS mass spectrum of the trimethylsilyl derivative of a taleranol standard using the ion-trap detector

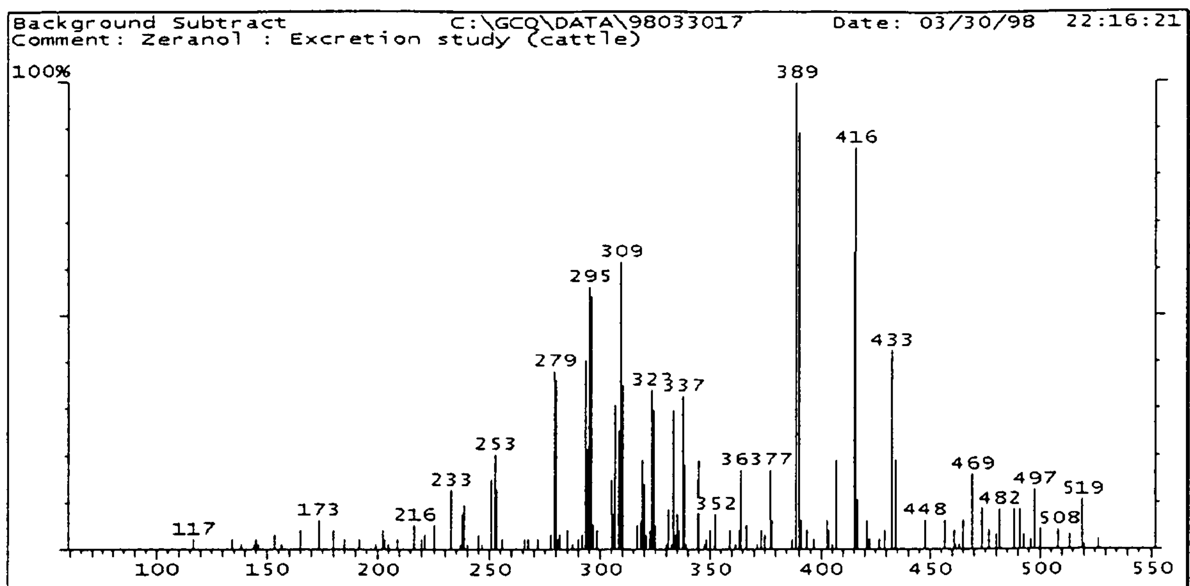

Figure 66 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of zeranol in cattle

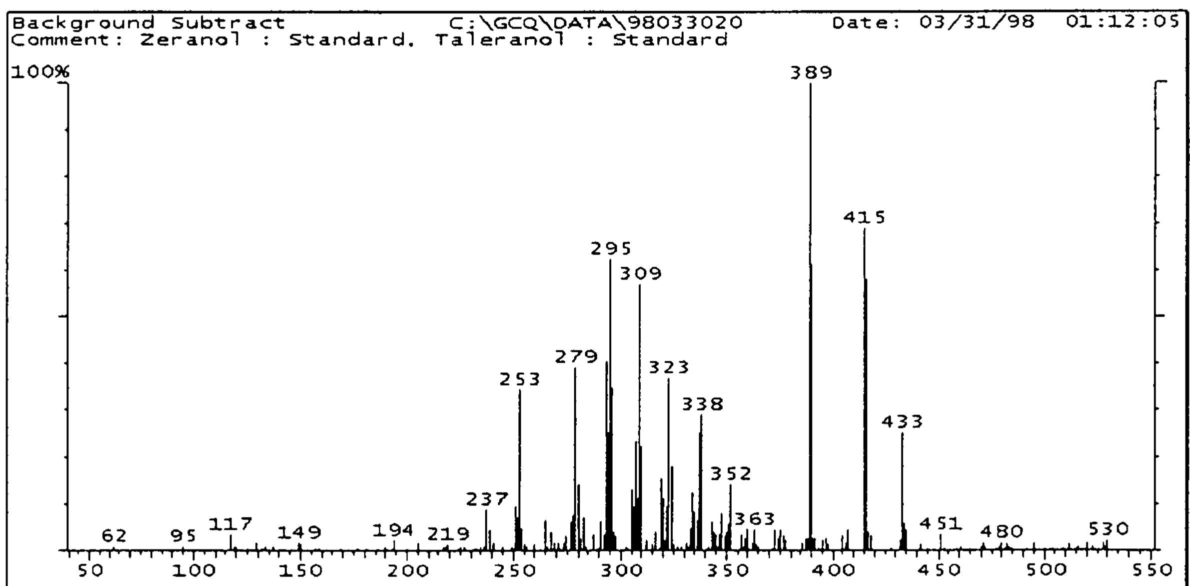

Figure 67 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of zeranol

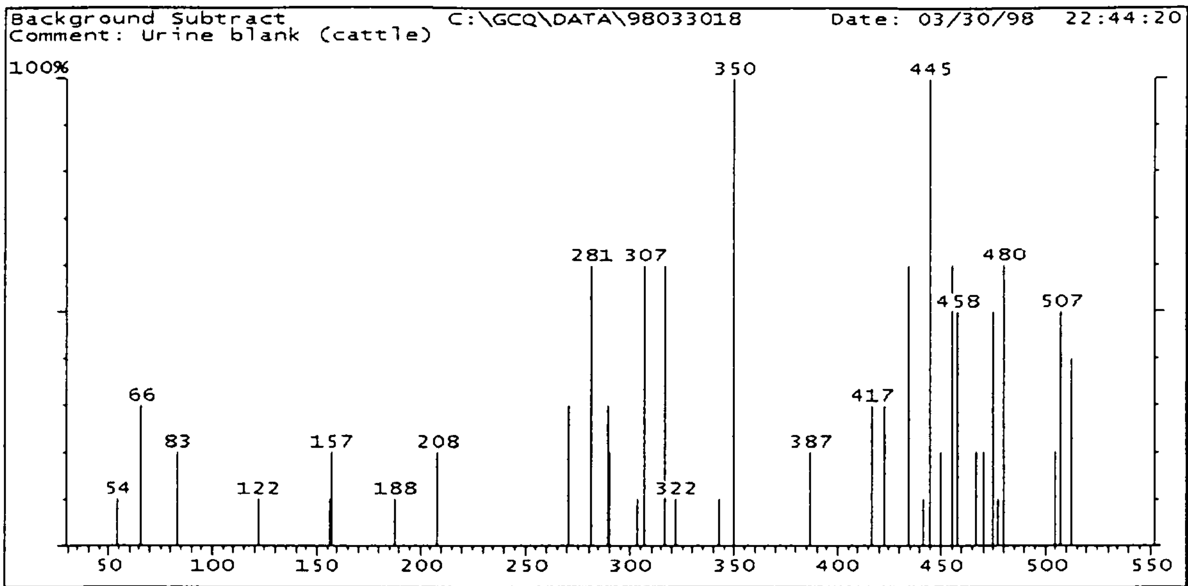

Figure 68 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

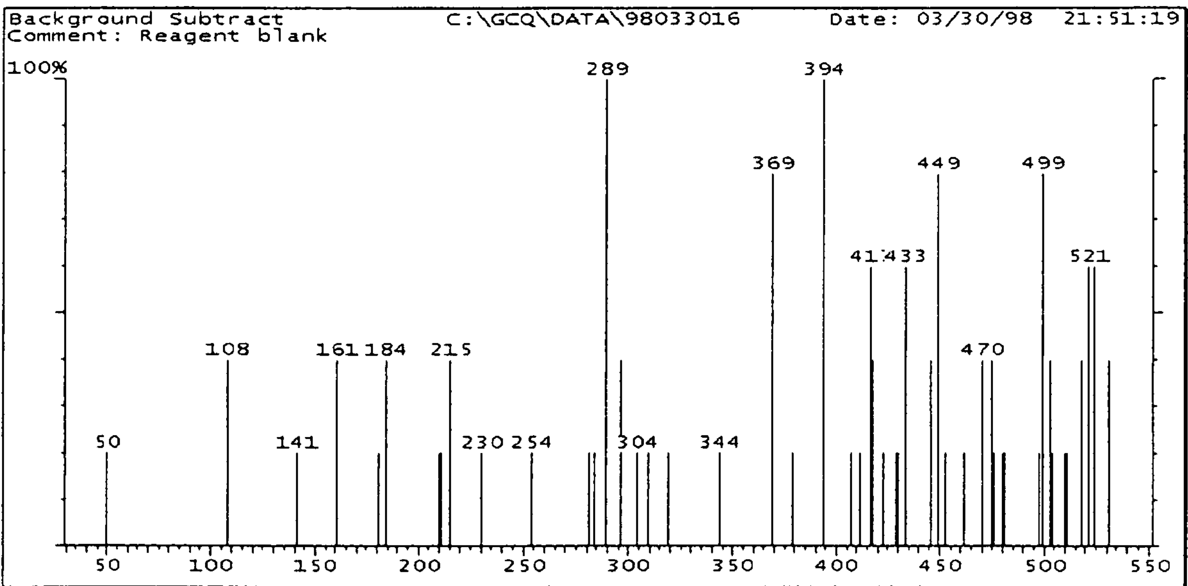

Figure 69 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

Taleranol (a metabolite of zeranol) could also be detected in the urine of cattle, and the data are presented as follows:

- Figure 70 (pagè 122) Excretion study of zeranol in cattle [subject C-3, urine collected on day 11 after treatment was stopped]
- Figure 71 (page 122) Taleranol standard
- Figure 72 (page 123) Urine blank
- Figure 73 (page 123) Reagent blank

The retention time of taleranol was determined as 16.12 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 1.126

The background subtracted mass spectra are shown at 16.12 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is even more proof that the excretion study is positive for zeranol.

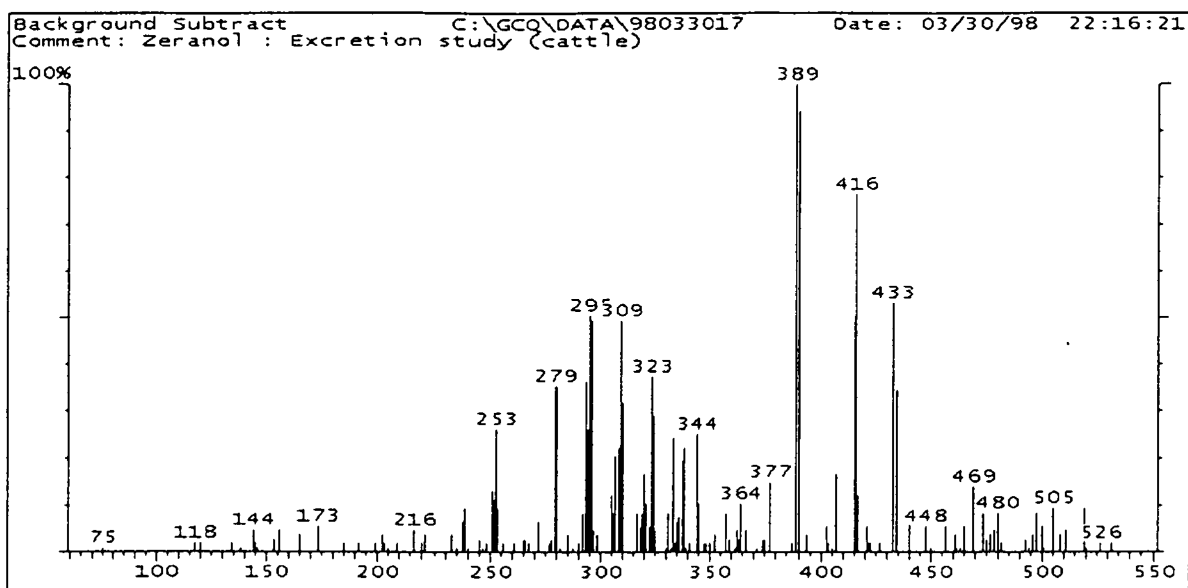

Figure 70 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of zeranol in cattle

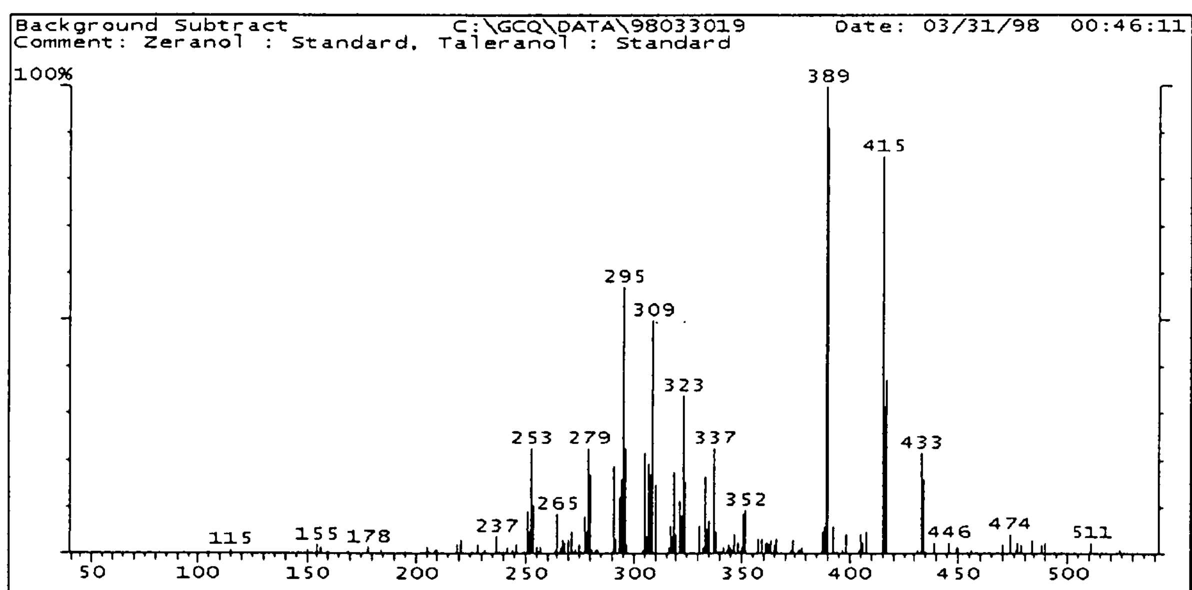

Figure 71 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of taleranol

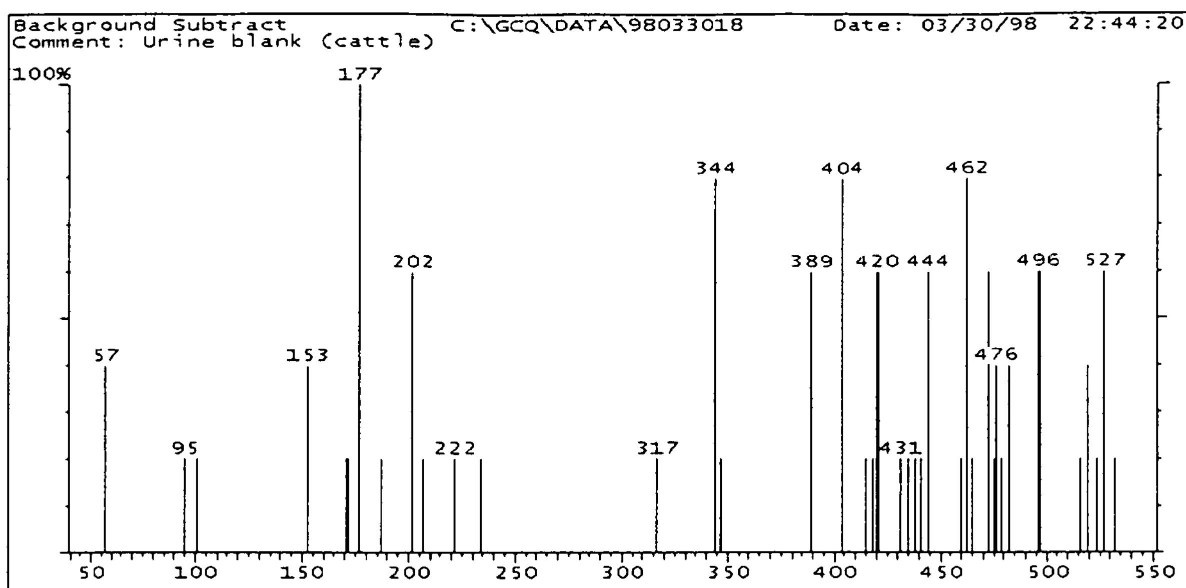

Figure 72 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

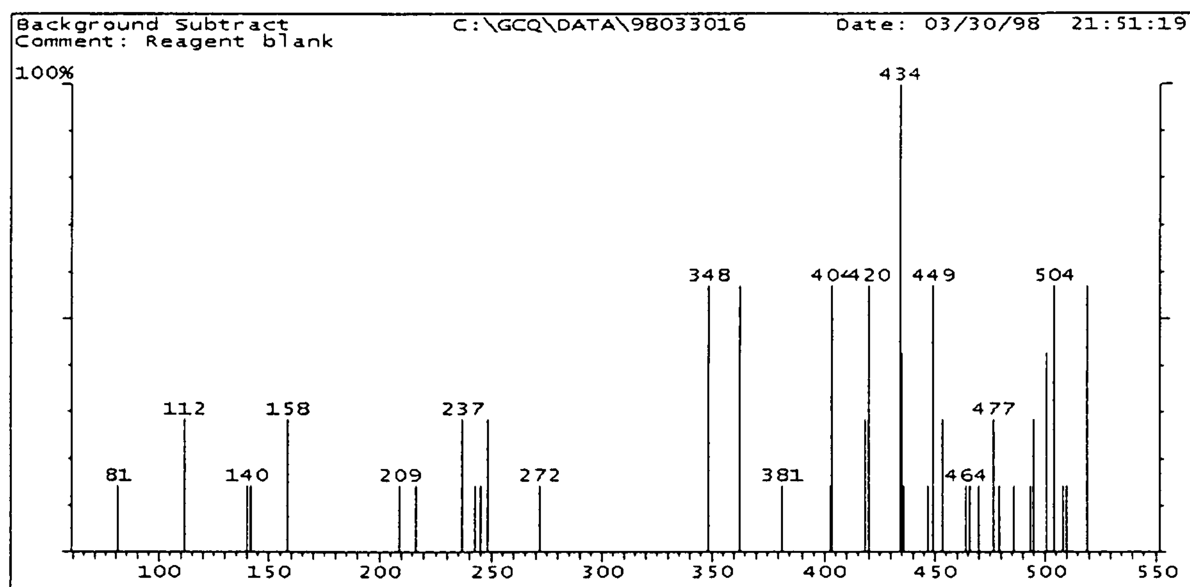

Figure 73 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

4.5.4.2 Swine

The data on the confirmation of zeranol in the urine of swine are presented as follows:

- Figure 74 (page 125) Excretion study of zeranol in swine [subject S-3, urine collected on day 11 after treatment was stopped]
- Figure 75 (page 125) Zeranol standard
- Figure 76 (page 126) Urine blank
- Figure 77 (page 126) Reagent blank

The retention time of zeranol was determined as 16.02 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 1.088.

The background subtracted mass spectra are shown at 16.02 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is unequivocal proof that the excretion study is positive for zeranol.

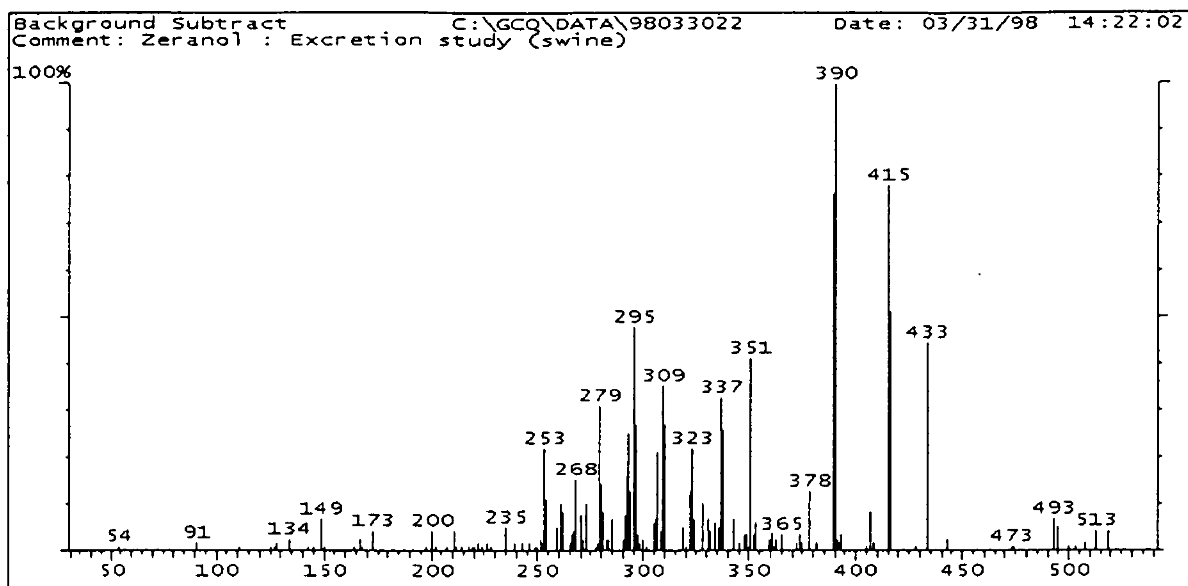

Figure 74 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of zeranol in swine

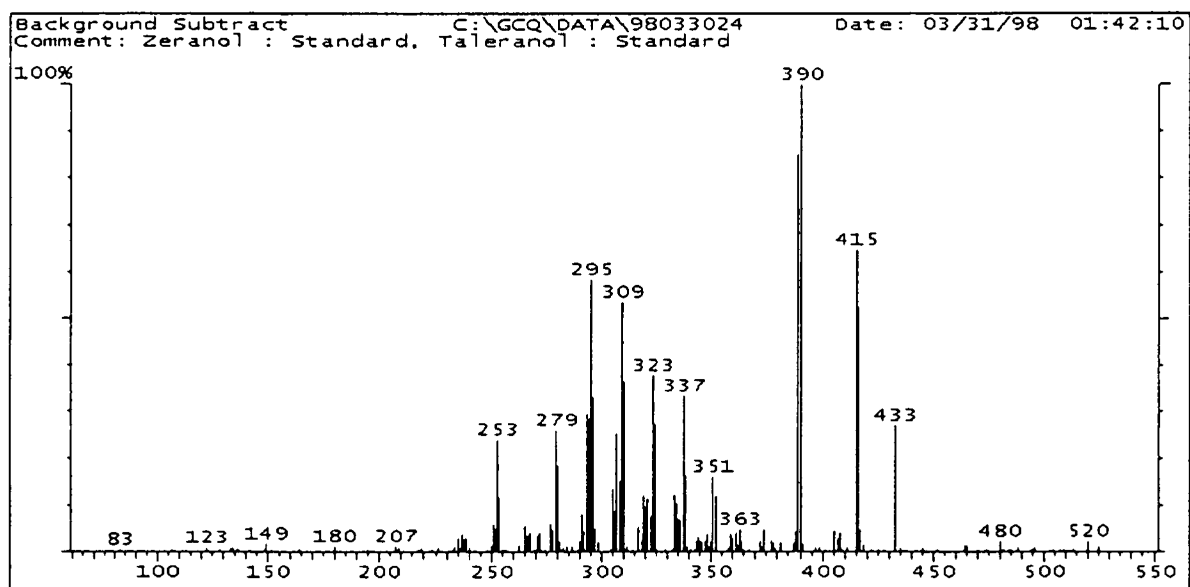

Figure 75 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of zeranol

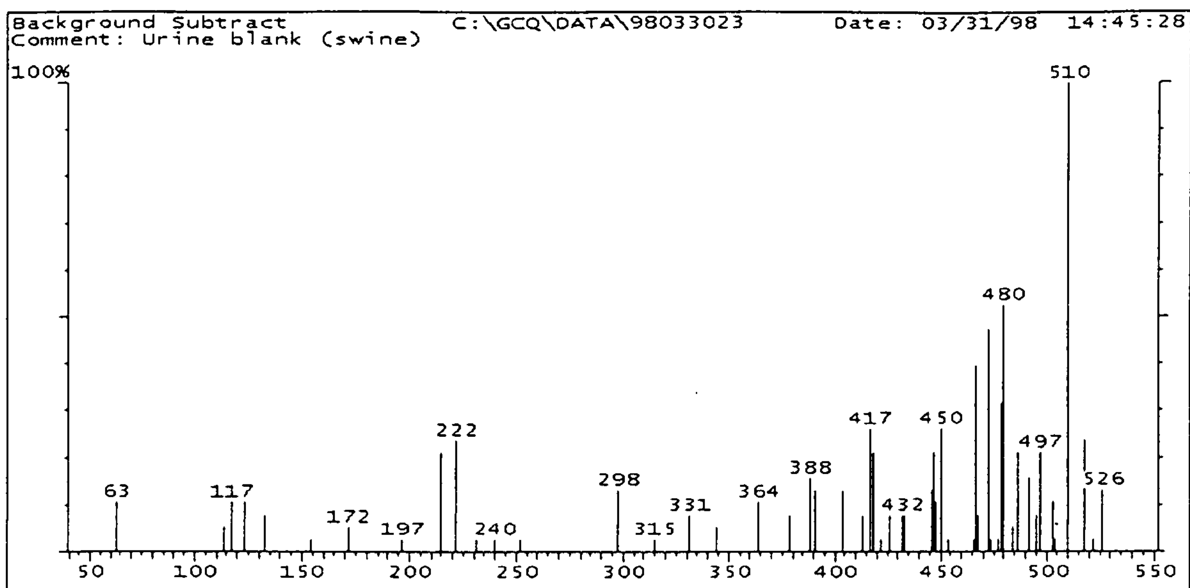

Figure 76 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

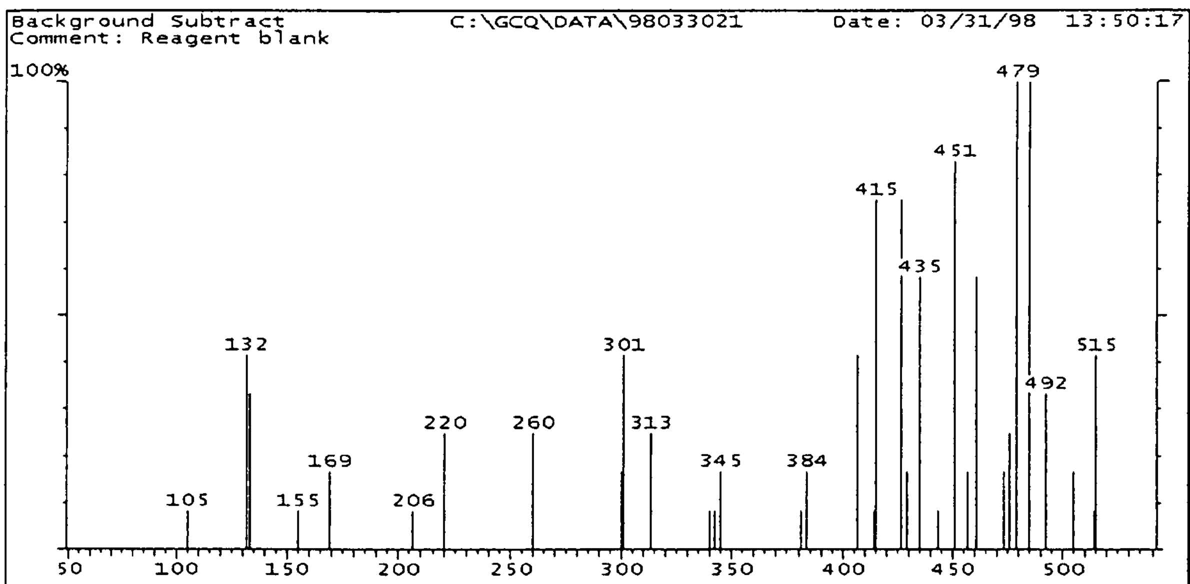

Figure 77 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

Taleranol (a metabolite of zeranol) could also be detected in the urine of swine, and the data are presented as follows:

- Figure 78 (page 128) Excretion study of zeranol in swine [subject S-3, urine collected on day 11 after treatment was stopped]
- Figure 79 (page 128) Taleranol standard
- Figure 80 (page 129) Urine blank
- Figure 81 (page 129) Reagent blank

The retention time of taleranol was determined as 16.12 minutes with a relative retention time (with respect to 17α-methyltestosterone as internal standard) of 1.126

The background subtracted mass spectra are shown at 16.12 minutes. The great correlation between excretion study and standard can clearly be seen in terms of characteristic product ions (resulting from ionization of the parent ion) and their relative abundance ratios. The relative retention times are also in close agreement.

It is also clear that no correlation exists between the standard and the urine blank, or between the standard and the reagent blank. This is even more proof that the excretion study is positive for zeranol.

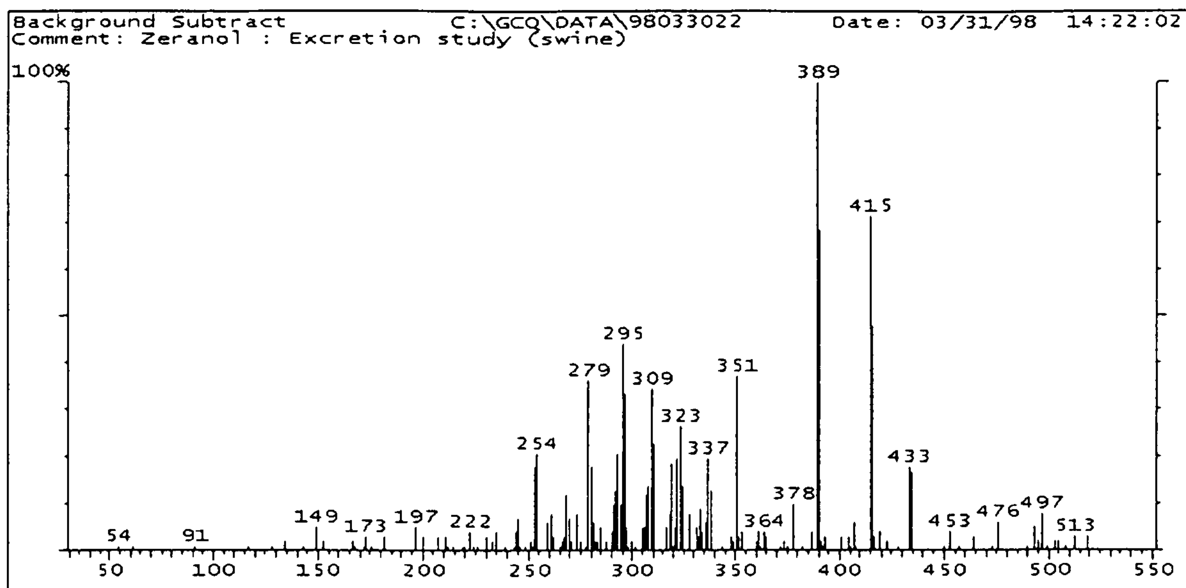

Figure 78 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of an excretion study of zeranol in swine

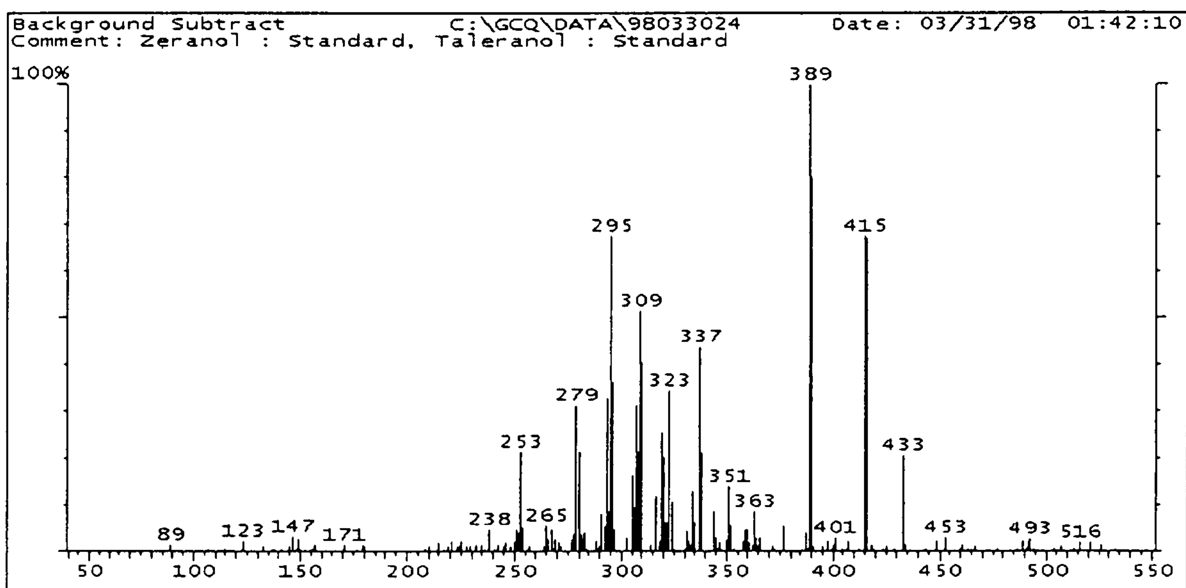

Figure 79 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a standard of taleranol

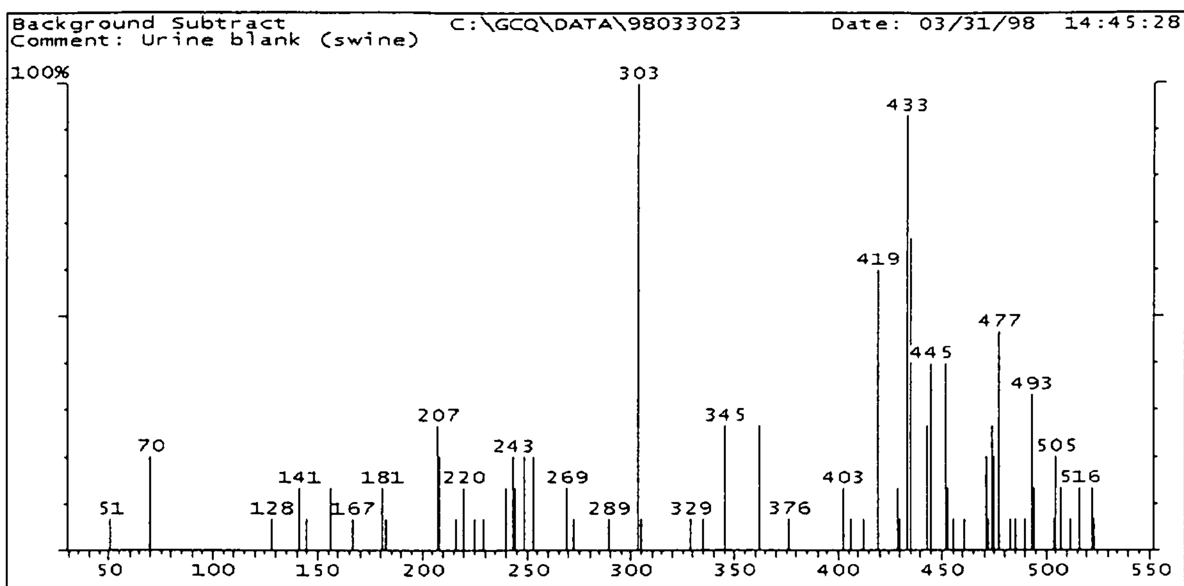

Figure 80 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a urine blank

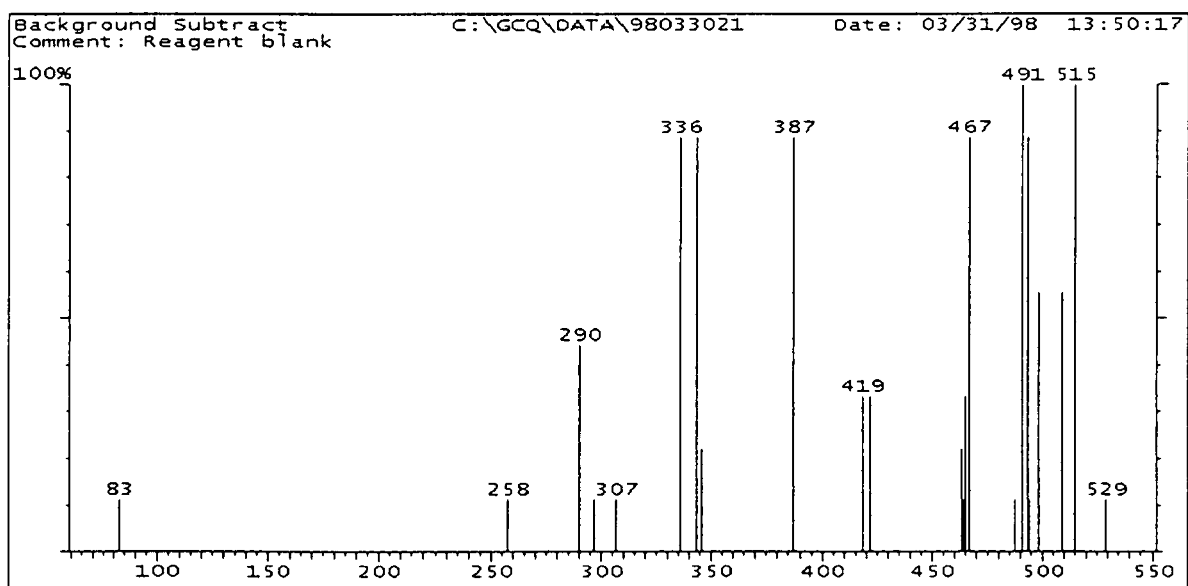

Figure 81 Full scan MS-MS mass spectrum of the trimethylsilyl derivative of a reagent blank

4.6 STABILITY OF ANALYTES

The aim of the stability studies was to investigate the stability of the analytes in urine from cattle and swine under various temperature storing conditions. Urine obtained from the previous excretion studies were stored frozen, at room temperature and in direct sunlight.

4.6.1 CLENBUTEROL

4.6.1.1 Swine

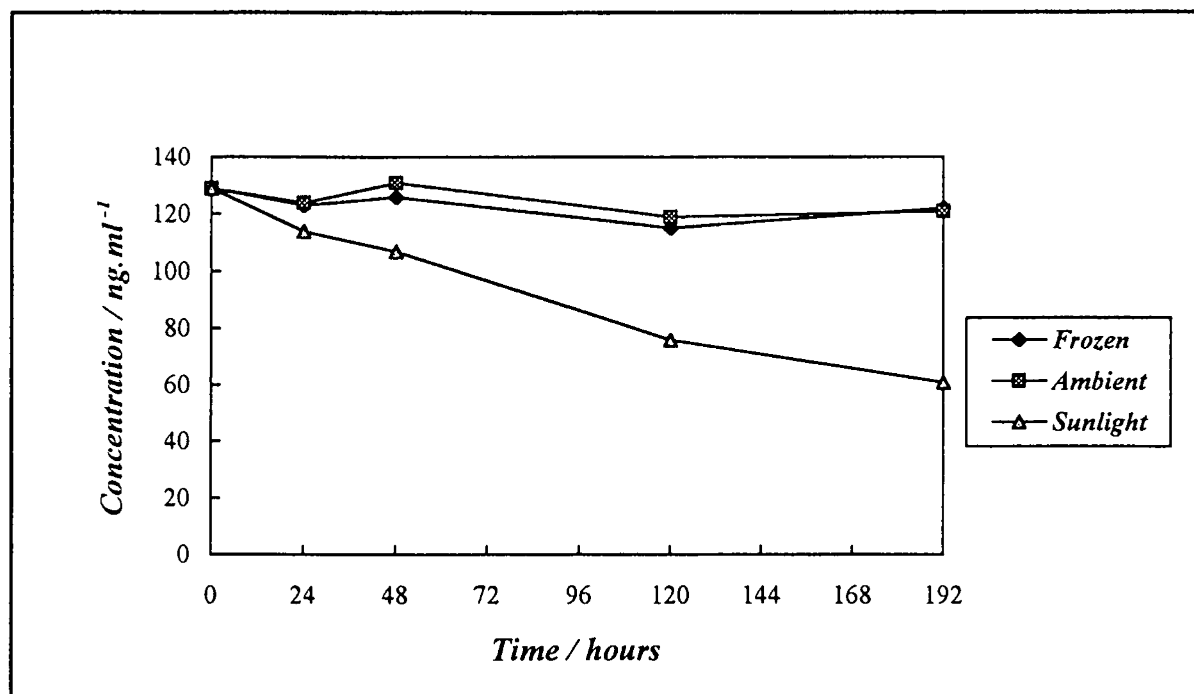

Figure 82 Concentration of clenbuterol in urine of swine *versus* time when a test sample is stored under different conditions

The data on the stability of clenbuterol in the urine from swine under various storing conditions, are presented in Figure 82 above. Clenbuterol was quantified (refer section 3.13.3, page 55) by constructing linear calibration curves over the expected concentration range.

The concentrations of the samples that were stored frozen and at room temperature remained constant over the observation period. The concentrations of the samples that were stored in direct sunlight decreased with about 50% over a period of 192 hours.

4.6.2 DIETHYLSTILBESTROL

4.6.2.1 Cattle

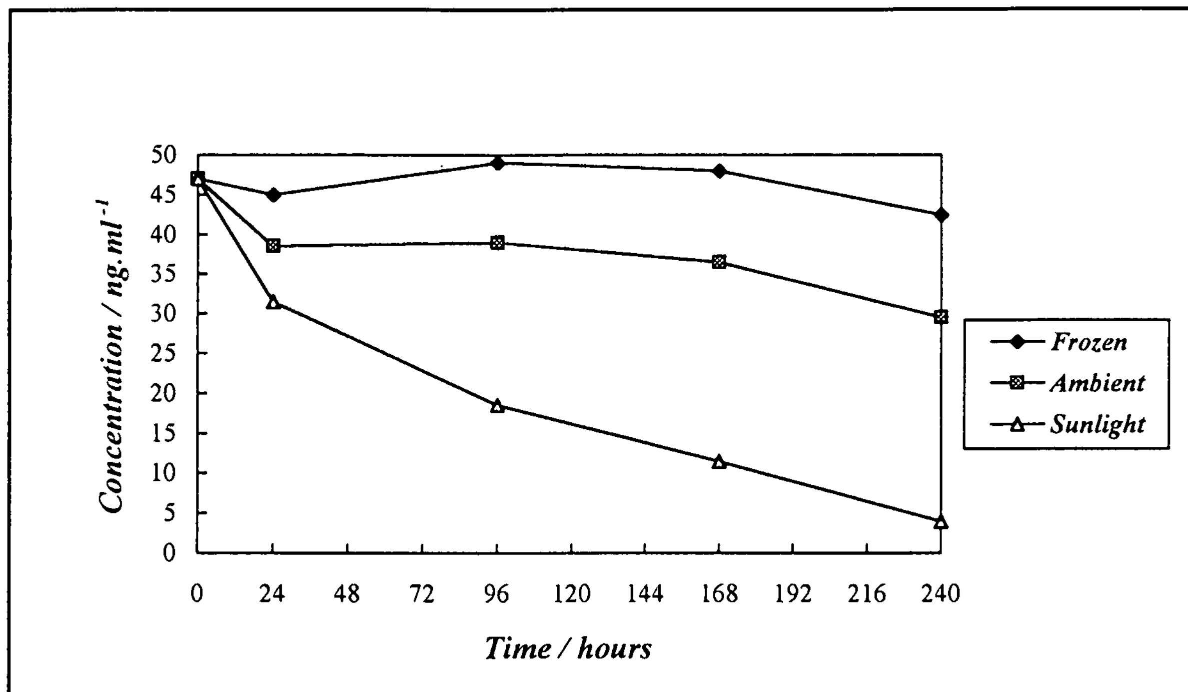

Figure 83 Concentration of diethylstilbestrol in urine of cattle *versus* time when a test sample is stored under different conditions

The data on the stability of diethylstilbestrol in the urine from cattle under various storing conditions, are presented in Figure 83 above. Diethylstilbestrol was quantified (refer section 3.13.3, page 55) by constructing linear calibration curves over the expected concentration range.

The concentrations of the samples that were stored frozen remained constant over the observation period. The concentrations of the samples that were stored at room temperature decreased with about 30% over a period of 240 hours.The concentrations of the samples that were stored in direct sunlight decreased with about 90% over the same period.

4.6.2.2 Swine

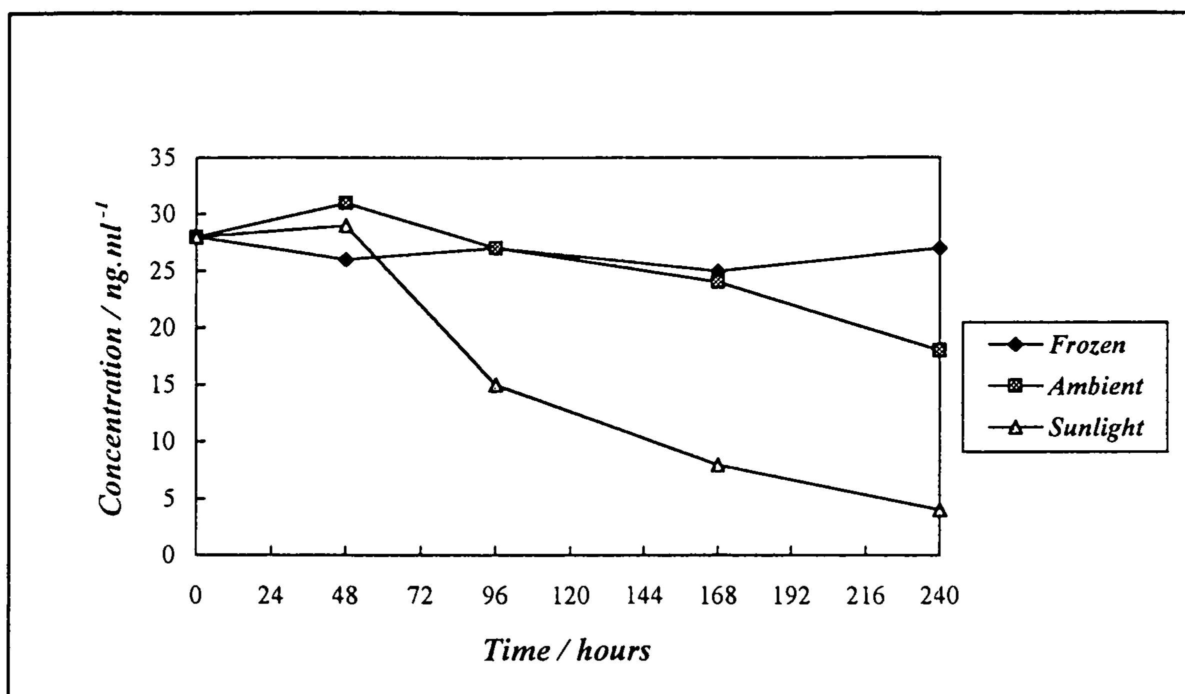

Figure 84 Concentration of diethylstilbestrol in urine of swine *versus* time when a test sample is stored under different conditions

The data on the stability of diethylstilbestrol in the urine from swine under various storing conditions, are presented in Figure 84 above. Diethylstilbestrol was quantified (refer section 3.13.3, page 55) by constructing linear calibration curves over the expected concentration range.

The concentrations of the samples that were stored frozen remained constant over the observation period. The concentrations of the samples that were stored at room temperature remained constant for 168 hours and then decreased with about 25% over the next 72 hours. The concentrations of the samples that were stored in direct sunlight decreased with about 80% over a period of 240 hours.

4.6.3 NANDROLONE

4.6.3.1 Cattle

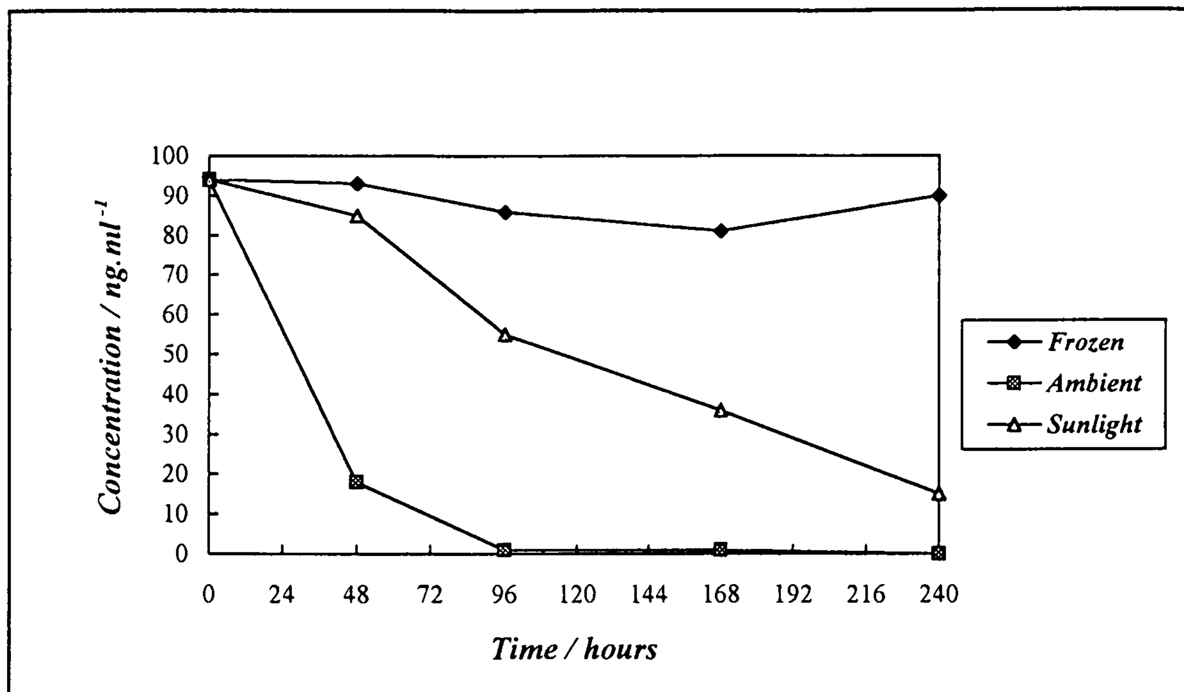

Figure 85 Concentration of 17α-19-nortestosterone in urine of cattle *versus* time when a test sample is stored under different conditions

The data on the stability of 17α-19-nortestosterone (nandrolone metabolite in cattle) in the urine from cattle under various storing conditions, also previously published [van der Merwe and Pieterse, 1994], are presented in Figure 85 above. 17α-19-Nortestosterone was quantified (refer section 3.13.3, page 55) by constructing linear calibration curves over the expected concentration range.

The concentrations of the samples that were stored frozen remained constant over the observation period. About no 17α-19-nortestosterone could be detected in the samples that were stored at room temperature after 96 hours, and the concentration of the samples that were stored in direct sunlight decreased with about 80% over a period of 240 hours.

Previous stability studies of 17α-19-nortestosterone in cattle have also shown that urine samples stored at ambient temperature were more unstable than those stored in direct sunlight. Although these results are against the general expectation, the studies were done on different excretion study samples on three different occasions and the same results were obtained every time.

PRESERVATION

In an attempt to decelerate the decomposition of 17α-19-nortestosterone in the urine of cattle when stored at ambient temperature, preservation was investigated. Sodium azide is normally used as preservative, thimerosal is a antibacterial/antifungal (topical) and 1,4-dithiothreitol (also known as Cleland's reagent) a protective agent for SH groups (thiols).

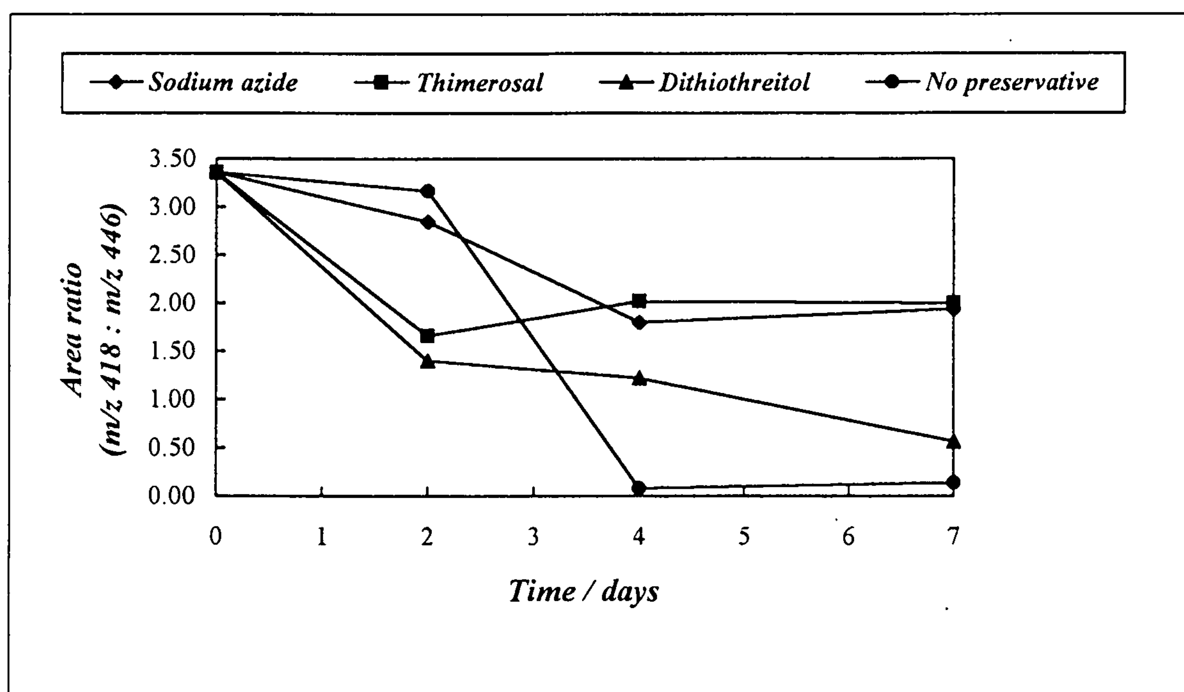

Figure 86 Area ratio of 17α-19-nortestosterone in urine of cattle *versus* time after different preservations

It can be seen from Figure 86 above that although preservation can not be used as an alternative to freezing, it does however have a decelerating effect on the decomposition of 17α-19-nortestosterone. Sodium azide and thimerosal seems to be more effective than dithiothreitol.

4.6.4 TRENBOLONE

4.6.4.1 Cattle

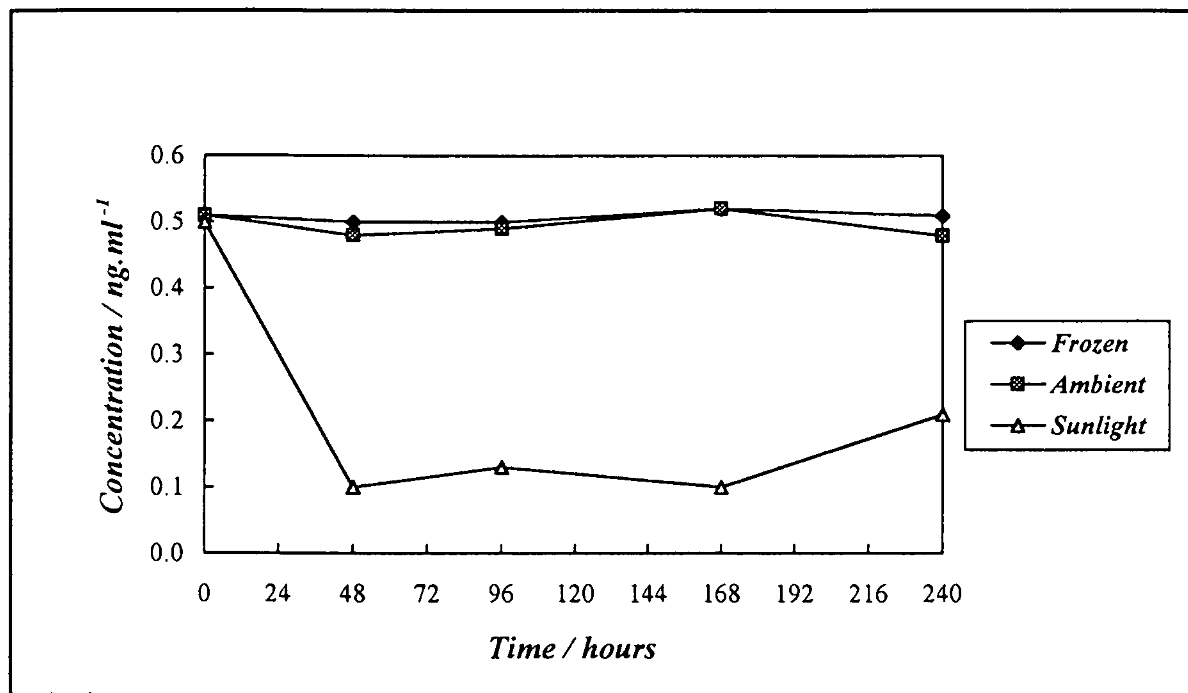

Figure 87 Concentration of 17α-trenbolone in urine of cattle *versus* time when a test sample is stored under different conditions

The data on the stability of 17α-trenbolone (trenbolone metabolite in cattle) in the urine from cattle under various storing conditions, are presented in Figure 87 above. 17α-Trenbolone was quantified (refer section 3.13.3, page 55) by constructing linear calibration curves over the expected concentration range.

The concentrations of the samples that were stored frozen and at ambient temperature remained constant over the observation period of 240 hours but the concentrations of the samples that were stored in direct sunlight decreased with about 60% over the same period.

This is in agreement with our data previously published [van der Merwe and Pieterse, 1994].

4.6.5 ZERANOL

4.6.5.1 Cattle

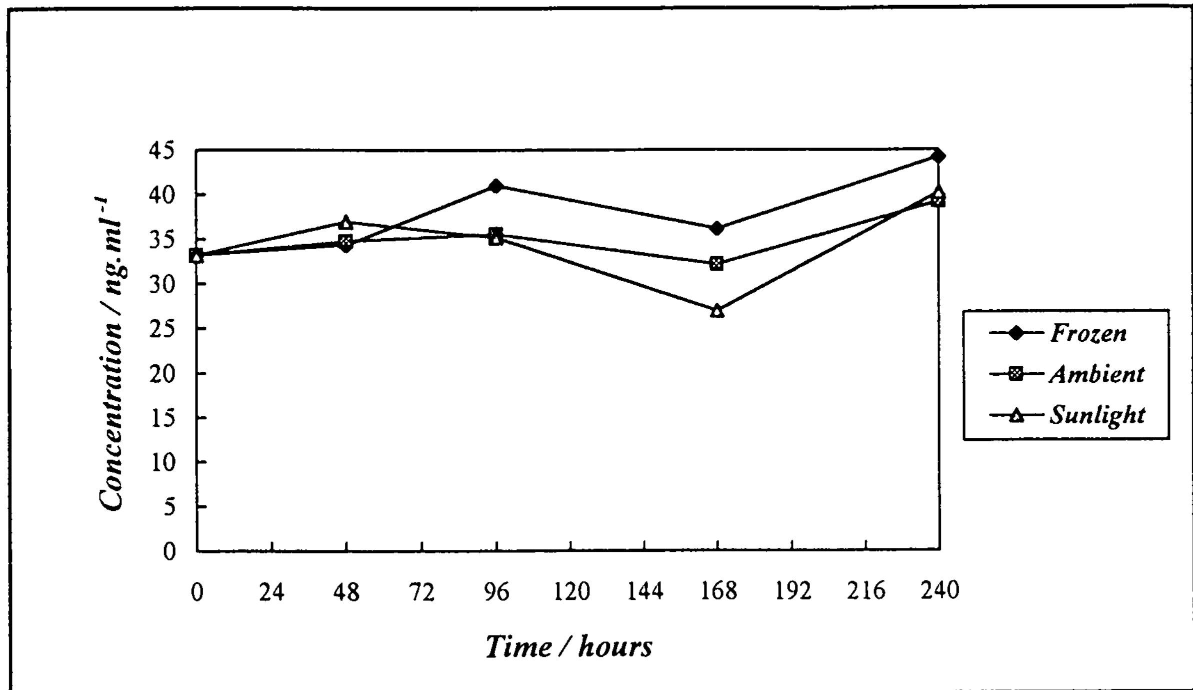

Figure 88 Concentration of zeranol in urine of cattle *versus* time when a test sample is stored under different conditions

The data on the stability of zeranol in the urine from cattle under various storing conditions, also previously published [van der Merwe and Pieterse, 1994], are presented in Figure 88 above. Zeranol was quantified (refer section 3.13.3, page 55) by constructing linear calibration curves over the expected concentration range.

Whether samples containing zeranol are stored frozen, at room temperature or in direct sunlight, there is no significant variation in the concentration of zeranol in the test sample for a period of at least 240 hours.

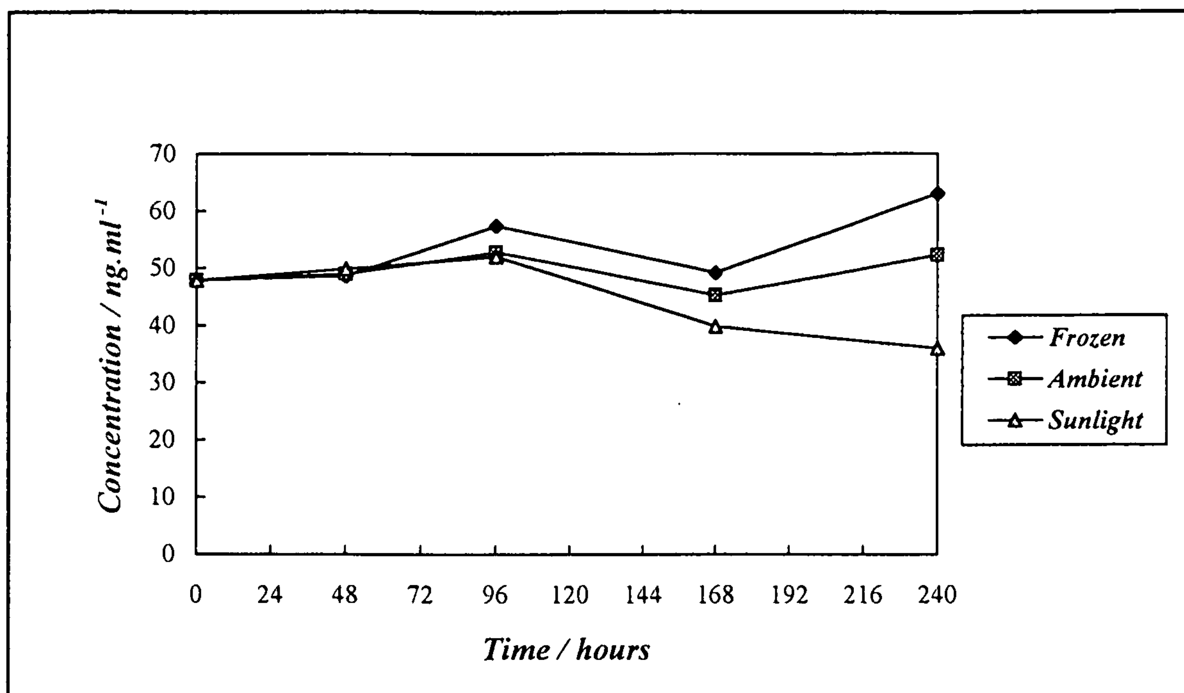

Figure 89 Concentration of taleranol in urine of cattle *versus* time when a test sample is stored under different conditions

The data on the stability of taleranol (zeranol metabolite in cattle) in the urine from cattle under various storing conditions, also previously published [van der Merwe and Pieterse, 1994], are presented in Figure 89 above. The calibration curve for zeranol was used to quantify taleranol in the test samples.

As for zeranol, there is also no significant decrease in the concentration of taleranol in the test sample whether stored frozen, at room temperature or in direct sunlight for a period of at least 240 hours.

4.6.5.2 Swine

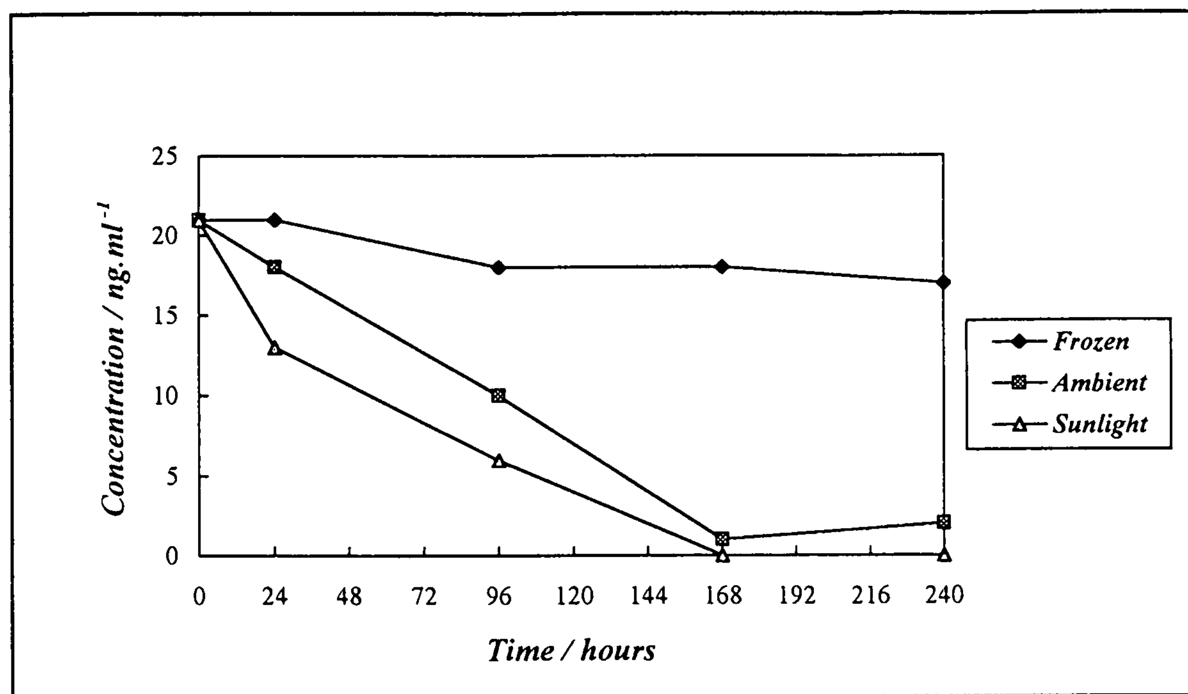

Figure 90 Concentration of zeranol in urine of swine *versus* time when a test sample is stored under different conditions

The data on the stability of zeranol in the urine from swine under various storing conditions, are presented in Figure 90 above. Zeranol was quantified (refer section 3.13.3, page 55) by constructing linear calibration curves over the expected concentration range.

The concentrations of the samples that were stored frozen remained constant over the observation period of 240 hours. The concentrations of the samples that were stored at ambient temperature and those that were stored in direct sunlight decreased so much that about no zeranol could be detected after 168 hours.

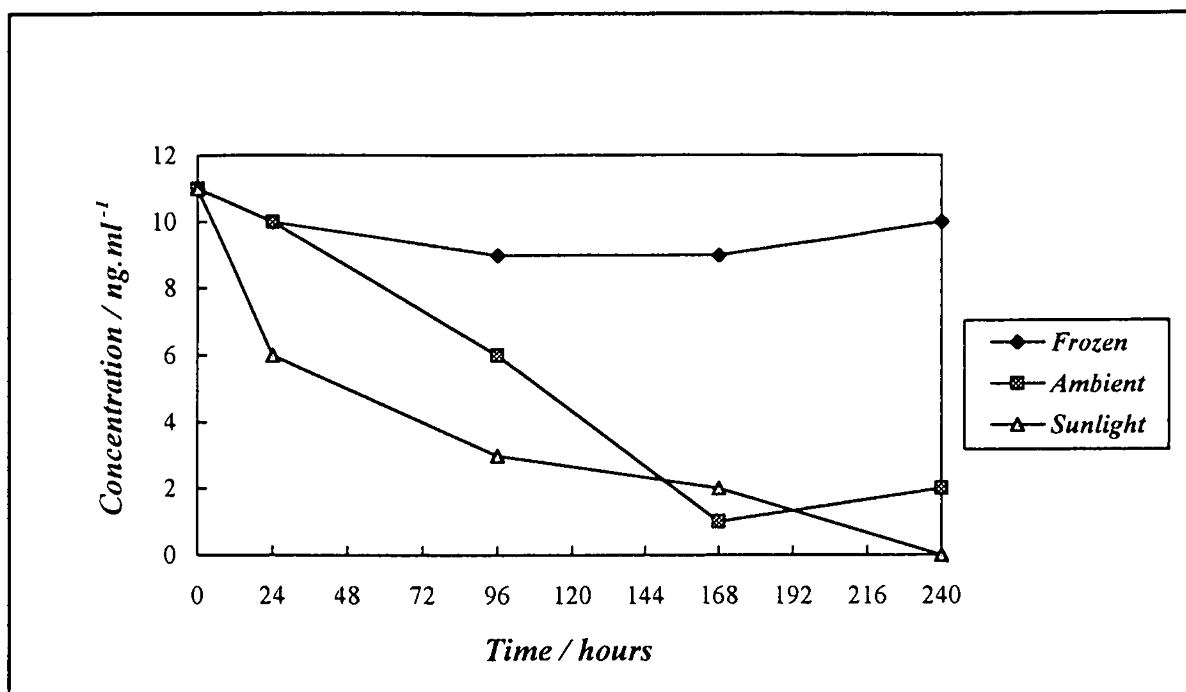

Figure 91 Concentration of taleranol in urine of swine *versus* time when a test sample is stored under different conditions

The data on the stability of taleranol (zeranol metabolite in cattle) in the urine from swine under various storing conditions, are presented in Figure 91 above. Taleranol was quantified (refer section 3.13.3, page 55) by constructing linear calibration curves over the expected concentration range.

The concentrations of the samples that were stored frozen remained constant over the observation period of 240 hours. The concentrations of the samples that were stored at ambient temperature decreased with about 80% over the same period, and about no taleranol could be detected after 240 hours.

The results of all these stability studies indicate that urine samples should be frozen as soon as possible after collection by inspectors at the different farms, to avoid false negative results when residue analysis are to be performed on these samples.

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ABSTRACT

Anabolic steroids and/or growth promoters are used to improve growth rate and feed conversion efficiency of livestock. The residues of these anabolics, which are present in the meat, may have a pharmacological activity due to oral bioavailability, and pose a risk to the consumer.

Certain anabolics can be given legally to farm animals in some countries, but are banned in most others because of their proved or alleged toxic and/or carcinogenic properties. The use of these substances is completely forbidden within the European Community (EC).

Before meat products can be exported to any member state of the EC, it is compulsory for the exporting country to have a monitoring programme to test for illegal use of these anabolic substances. The necessity to test for illegal use or to determine residue levels after legal use, has led to a strong interest in developing analytical methods for the identification and confirmation of anabolic agents in biological samples.

The objectives of this study were to develop suitable analytical methods with a view to identify residues of some growth-promoting veterinary drugs in the urine of cattle and/or swine, to confirm the presence of these veterinary drugs unequivocally in the urine and to examine the stability of these drugs in urine under different environmental storing conditions.

The excretion of clenbuterol, diethylstilbestrol, nandrolone, trenbolone and zeranol from cattle and/or swine were studied. A thorough literature study was done on the published analytical methods as well as the metabolism and pharmacokinetics of these drugs in cattle and/or swine. Reference standards were used to develop a GC-MS screening method for the identification of these drugs and/or their metabolites in the urine of cattle and/or swine.

Recoveries of 61-99% and detection limits of 0.9-2.1 ng/ml were obtained for the different analytes with the developed analytical method.

Trials were conducted in which these drugs were administered to cattle and/or swine. Urine samples were collected at regular time intervals and stored immediately at -20°C until time of analysis.

Reference standards were also used for the development of GC-MS-MS analytical methods to confirm the presence of these drugs and/or their metabolites in the urine of cattle and/or swine. Confirmation of the substances in urine was done by obtaining a MS-MS spectrum of the extract and comparing this with the MS-MS spectrum of a reference standard. The MS-MS spectra was obtained by using the ion-trap technique.

Urine samples are often collected at different farms and transported to the laboratory for residue analysis. Although it is standard procedure to freeze samples immediately after collection, it is not always possible. The results of this study show that the analytes contained in urine samples that were stored frozen remained stable for at least 10 days. If urine samples are stored at ambient temperature, concentrations of the analytes can decrease with as much as 30% after 10 days. Epi-nandrolone (metabolite of nandrolone in cattle) could however not be detected in the urine samples after 4 days. If urine samples are stored in direct sunlight, concentrations of the analytes can decrease with as much as 90% after 10 days.

It can be concluded that a method was developed to identify residues of some growth-promoting veterinary drugs and/or their metabolites in the urine of cattle and/or swine, and to confirm the presence of these drugs unequivocally in the urine. It can further be concluded that urine samples should be frozen as soon as possible after collection to prevent false negative results.

OPSOMMING

Anaboliese steroïede en/of groei promoters word gebruik om groeitempo en voeromsettingsdoeltreffendheid van lewende hawe te verbeter. Die residue van hierdie middels, wat in die vleis voorkom, mag 'n farmakologiese effek hê as gevolg van orale biobeskikbaarheid, en 'n risiko vir die verbruiker inhou.

Sekere anaboliese middels kan tans wettig aan diere toegedien word in sommige lande, maar in die meeste ander lande word dit verbied as gevolg van die middels se bewese of beweerde toksiese en/of karsinogeniese eienskappe. Die gebruik van hierdie middels is totaal verbode binne die Europese Gemeenskap (EG).

Voordat vleisprodukte na enige lidland van die EG uitgevoer kan word, moet daardie uitvoerland 'n moniteringsprogram in plek hê om te toets vir die onwettige gebruik van hierdie anaboliese middels. Die noodsaaklikheid om te toets vir die onwettige gebruik van hierdie middels, of om residu vlakke te bepaal na wettige gebruik, het die belangstelling geprikkel om analitiese metodes te ontwikkel vir die identifisering en bevestiging van anaboliese middels in biologiese monsters.

Die doelstellings van hierdie studie was om geskikte analitiese metodes te ontwikkel waarmee residue van sekere anaboliese middels in die urien van beeste en/of varke aangetoon kan word, om die teenwoordigheid van hierdie middels onteenseglik in die urien te bewys en om die stabiliteit van hierdie middels te ondersoek onder verskillende omgewings bewaartoestande.

Die uitskeiding van clenbuterol, diëtielstilbestrol, nandroloon, trenboloon en zeranol is in beeste en/of varke bestudeer. 'n Deeglike literatuurstudie is gedoen oor gepubliseerde analitiese metodes asook die metabolisme en farmakokinetika van hierdie middels in beeste en/of varke. Verwysingsstandaarde is gebruik om 'n GC-MS siftingsmetode te ontwikkel vir die identifisering van hierdie middels en/of hulle metaboliete in die urien van beeste en/of varke.

Herwinnings van 61-99%, en deteksielimiete van 0.9-2.1 ng/ml is verkry vir die verskillende analiete met die analitiese metode wat ontwikkel is.

Proewe is uitgevoer waarin hierdie middels aan beeste en/of varke toegdien is. Urienmonsters is gereeld versamel en dadelik gestoor by -20°C totdat dit geanaliseer is.

Verwysingstandaarde is ook gebruik om GC-MS-MS analitiese metodes te ontwikkel vir die onteenseglike bewys van die teenwoordigheid van hierdie middels en/of hulle metaboliete in die urien van beeste en/of varke. Bevestiging van die middels in urien is gedoen deur 'n MS-MS spektrum van die ekstrak te verkry en dit te vergelyk met die MS-MS spektrum van 'n verwysingstandaard. Die MS-MS spektra is verkry deur die ioon-vangs tegniek te gebruik.

Urienmonsters word gewoonlik by verskillende plase versamel en daarna versend na die laboratorium vir residu analise. Alhoewel dit standaard praktyk is om urienmonsters dadelik te vries nadat dit versamel is, is dit nie altyd prakties moontlik nie. Die resultate van hierdie studie toon dat analiete in urienmonsters wat dadelik gevries word, stabiel bly vir minstens 10 dae. Indien urienmonsters by kamertemperatuur gestoor word, kan die konsentrasies van die analiete met soveel as 30% afneem na 10 dae. Epi-nandroloon (metaboliet van nandroloon in beeste) kon egter na 4 dae nie meer in die urienmonsters aangetoon word nie. Indien die urienmonsters in direkte sonlig gestoor word, kan die konsentrasies van die analiete met soveel as 90% afneem na 10 dae.

Die gevolgtrekking kan gemaak word dat 'n metode ontwikkel is om residue van sekere groei promoters en/of hulle metaboliete in die urien van beeste en/of varke aan te toon, en om die teenwoordigheid van hierdie middels onteenseglik in die urien te bewys. 'n Verdere gevolgtrekking kan gemaak word dat urienmonsters so gou as moontlik gevries moet word nadat dit versamel is, om vals negatiewe resultate te voorkom.