

The residue kinetics and safety of R-salbutamol in ruminants

by

Marlien Prinsloo

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Stellenbosch University

Department of Animal Sciences, Faculty of AgriSciences

Supervisor: Prof L.C. Hoffman

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DECLARATION

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ABSTRACT

With an increased demand to supply food to an ever-increasing world population the application of new technologies to improve animal production efficiency has become a global priority. A balance must be found however between the production benefit of these technologies, animal welfare and human safety.

The purpose of the two studies undertaken was to determine the target animal tolerance of R-salbutamol when included in feed, the remaining residues in liver, kidney, muscle and fat following various withdrawal periods, and the withdrawal period at which animal tissue will be safe for human consumption.

To determine the withdrawal period, the residues detected in animal tissue must be compared to the Acceptable Daily Intake (ADI) of R-salbutamol in humans. As no extensive toxicological evaluation has ever been done for R-salbutamol, a complete evaluation of available toxicological data was performed. Repeat dose studies, carcinogenicity, genotoxicity, reproductive, foetal and acute toxicity data were evaluated to determine the ADI. No carcinogenicity, genotoxicity or reproductive toxicity were noted for R-salbutamol. Long-term effects were observed with a No Adverse Effect Level (NOAEL) of 12 mg/kg BWt. Foetal toxicity (teratology) were noted at high inclusion levels in rabbits (cranioschisis at 50 mg salbutamol/kg BWt) with a NOAEL of 10.75 mg/kg derived from a rat teratology study. The Acute Reference Dose (ARfD), derived from human studies, was however the lowest safe level with a NOAEL (also referred to as the point of departure (POD)) of 1mg R-salbutamol/kg BWt, due to the possible acute effects of R-salbutamol ingestion at higher inclusion levels. An ARfD of 3 µg/kg BWt were calculated and used as the upper limit of the ADI, which was determined to be 0 - 3 µg/kg BWt in humans.

The lamb feedlot study included 13 lambs; one lamb being slaughtered at the start of the trial as control. The remaining 12 lambs were treated with 21 mg R-salbutamol per day (0.5 mg/kg BWt) for 28 days. R-salbutamol was withdrawn from the feed 7 hours prior to slaughter for 6 of the lambs and withdrawn for 24 hours from the feed for the remaining 6 lambs. Liver, kidney, muscle and fat samples were collected at slaughter and analysed for parent and total salbutamol (parent + metabolites) residues. Heart rate, rectal temperature and respiratory rate were measured before the start of the medicated period (study day -1) and again on day 28 of the medicated period.

Heart rate did not differ between day -1 and 28, but respiratory rate and rectal temperature were both higher at day 28 compared to day -1 ($P < 0.05$). The increase in rectal temperature

was not clinically significant as it remained within normal parameters. The increase in respiratory rate in 7 of the 12 lambs was attributed to lower respiratory tract infection.

Parent salbutamol residues in lambs were the highest in the liver ($\pm 89\%$ of residues detected) at 7 hours withdrawal, followed by the kidney ($\pm 7\%$), fat ($\pm 2.5\%$) and muscle ($\pm 1\%$). The residue concentrations at 24 hours after withdrawal were less than 50% of the levels detected at 7 hours following withdrawal. Metabolism of R-salbutamol is rapid following absorption due to high pre-systemic metabolism as indicated by the high level of metabolites detected following hydrolysis of tissue samples with β -glucuronidase. At 7 hours after withdrawal, 44% of the total residues were present as parent salbutamol in the liver and 39% at 24 hours after withdrawal. In the kidney 28% and 30% of parent salbutamol were detected at 7 hours and 24 hours, respectively as a percentage of total salbutamol. Muscle had 47% parent salbutamol as a percentage of total salbutamol at 7 hours after withdrawal. The percentage reduced to 17% after 24 hours withdrawal. In fat, 11% of total salbutamol were present as parent salbutamol at 7 hours and 14% 24 hours after withdrawal.

The theoretical maximum daily intake of salbutamol for humans calculated from the lamb residue data is less than 10% (16.88 $\mu\text{g}/\text{day}$) of the acceptable daily intake (ADI) (180 $\mu\text{g}/\text{kg}$ BWt in adult human). Animal tissue from feedlot lambs fed R-salbutamol, according to label recommendations, with zero withdrawal in feed, will provide adequate consumer protection.

In a similar study performed in beef cattle one group of four cattle received non-medicated feed as the negative control group. Three groups of six cattle each received 150 mg R-salbutamol/head/day (0.42 mg/kg BWt) in feed for 44 days. One animal from the control group was slaughtered on study day 3. The three medicated groups were slaughtered on <12-, 24- and 48-hours following withdrawal of R-salbutamol from feed respectively together with one each of the control group. The control animals were included to show that there were no residues occurring naturally within the test herd. Liver, kidney, muscle and fat samples were collected and analysed for salbutamol residues. No residues were detected for total salbutamol (LOQ = 3 $\mu\text{g}/\text{kg}$) in any of the tissues. No visual adverse events were observed in the study animals and R-salbutamol was well tolerated in feedlot cattle.

From the data in this study MRL for R-salbutamol is therefore suggested to be set for liver at 300 $\mu\text{g}/\text{kg}$, muscle at 6.5 $\mu\text{g}/\text{kg}$, kidney at 40 $\mu\text{g}/\text{kg}$ and fat at 13 $\mu\text{g}/\text{kg}$. At these levels the daily intake of residue will still be below 20% of the ADI.

Further investigation into intravenous and oral pharmacokinetics of R-salbutamol in both lambs and cattle will be beneficial to better understand the absorption, distribution, metabolism and excretion of R-salbutamol in feedlot lambs and cattle.

OPSOMMING

Met die toenemende vraag na voedsel vir 'n steeds toenemende wêreldbevolking, het die toepassing van nuwe tegnologie om die doeltreffendheid van diereproduksie te verbeter, 'n wêreldwye prioriteit geword. Daar moet egter 'n balans gevind word tussen die produksie voordele van hierdie tegnologie, dierewelsyn en menslike veiligheid.

Die doel van die twee studies wat onderneem is, was om die veiligheid van R-salbutamol te bepaal in die teiken diere na voer insluiting, die oorblywende residue te bepaal in lewer, niere, spier en vet na aanleiding van verskillende onttrekkingsperiodes en die onttrekkingstydperk waar dierlike weefsel veilig sal wees vir menslike gebruik.

Om die onttrekkings periode te kan bepaal, moet die residue wat waargeneem is in die dierlike weefsel vergelyk word met die aanvaarbare daaglikse inname (ADI) vir R-salbutamol in mense. Geen ekstensiewe toksikologiese evaluasie is al ooit vir R-salbutamol gedoen nie. Gevolglik is 'n volledige evaluasie hier gedoen op die beskikbare toksikologiese data. Herhaalde dosis studies, karsinogenisiteit, genotoksisiteit, reprodktiewe, fetale en akute toksisiteitsdata is geëvalueer om die ADI te bepaal. Geen karsinogeniese, genotoksiese of reprodktiewe toksisiteit is vir R-salbutamol opgemerk nie. Langtermyn effekte is waargeneem met 'n Geen Negatiewe Effek Vlak (NOAEL) van 12 mg/kg LM. Fetale toksisiteit (teratologie) is op hoë insluitingvlakke aangetoon in konyne (kranioskise teen 50 mg salbutamol/kg LM) met 'n NOAEL van 10,75 mg/kg LM, wat afgelei is van 'n rot teratologie studie. Die akute verwysingsdosis (ARfD), afgelei van menslike studies, was egter die laagste veilige vlak met 'n NOAEL (ook verwys na as die Vertrek Punt (POD)) van 1 mg R-salbutamol/kg LM, as gevolg van die moontlike akute effekte as gevolg van inname van R-salbutamol. 'n ArfD van 3 µg/kg LM is bereken en gebruik as die boonste limiet van die ADI, vasgestel as 0 - 3 µg/kg LM in mense.

Dertien lammers is in die eerste studie ingesluit; een lam is aan die begin van die studie as kontrole dier geslag. Die oorblywende 12 lammers is vir 28 dae behandel met 21 mg R-salbutamol per dag (0.5 mg/kg LM). R-salbutamol is 7 uur voor slag by 6 van die lammers uit die voer onttrek en onttrek vir 24 uur voor slag in die oorblywende 6 lammers. Lewer-, nier-, spier- en vet monsters is by slag ingesamel en geanaliseer vir biointakte en totale salbutamol (biointakte + metaboliete) residue. Hartklop, liggaamstemperatuur en respirasietempo is voor die aanvang van die medikasie periode (studie dag -1) en weer op dag 28 van die medikasie periode gemeet.

Geen verskil is waargeneem in hartklop tempo tussen dag -1 en 28 nie, maar respirasietempo en liggaamstemperatuur was beide hoër op dag 28 in vergelyking met dag -1 ($P < 0.05$). Die toename in rektale temperatuur was nie klinies beduidend nie, aangesien dit binne normale parameters gebly het. Die toename in respiratoriese tempo in 7 van die 12 lammers is toegeskryf aan lae respiratoriese infeksie.

Biointakte salbutamol residue in lammers was die hoogste in lewer ($\pm 89\%$ van residue) by 7 uur onttrekking, gevolg deur nier ($\pm 7\%$), vet ($\pm 2,5\%$) en spier ($\pm 1\%$). Die residu konsentrasies 24 uur na onttrekking was minder as 50% van die vlakke wat op 7 uur na onttrekking gemeet is. R-salbutamol metaboliseer vinnig na absorpsie as gevolg van hoë pre-sistemiese metabolisme, soos aangedui deur die hoë vlak van metaboliet wat gemeet is na die hidroliese van weefselmonsters met β -glukoronidase. Sewe ure na onttrekking was 44% van die totale residue in die lewer teenwoordig as biointakte salbutamol en 39% op 24 uur na onttrekking. In nier is 28% en 30% van ouer salbutamol as 'n persentasie van die totale salbutamol onderskeidelik by 7 uur en 24 uur na onttrekking aangetref. Spier het 47% ouer salbutamol as 'n persentasie van totale salbutamol by 7 uur na onttrekking gehad. Die persentasie het gedaal na 17% na 24 uur onttrekking. In vet was 11% van die totale salbutamol teen 7 uur as ouer salbutamol teenwoordig en 14% 24 uur na onttrekking.

Die teoretiese maksimum daaglikse inname van salbutamol vir mense, bereken uit die data van die lam residu studie, is minder as 10% ($16.88 \mu\text{g}/\text{dag}$) van die aanvaarbare daaglikse inname (ADI) ($180 \mu\text{g}/\text{dag}$ vir 'n volwasse mens). Diereweefsel uit voerkraal lammers wat volgens die etiketaanbevelings R-salbutamol gevoer word, met 'n nul onttrekking in voer, sal dus sal voldoende beskerming van verbruikers bied .

In 'n soortgelyke studie wat in beeste uitgevoer is, het een groep van vier beeste nie gemedikeerde voer ontvang nie en gedien as die negatiewe kontrolegroep. Drie groepe van ses beeste elk het vir 44 dae $150 \text{ mg R-salbutamol} / \text{kop} / \text{dag}$ ($0.42 \text{ mg}/\text{kg LM}$) in voer ontvang. Een dier van die kontrolegroep is op studie dag 3 geslag. Die drie medikasie groepe is geslag op onderskeidelik <12, 24 en 48 uur na die onttrekking van R-salbutamol uit voer, saam met een elk van die kontrolegroep. Die kontrole diere is ingesluit om aan te toon dat daar geen residue binne die toets kudde voorkom nie. Lewer-, nier-, spier- en vet monsters is versamel en ontleed vir salbutamol residue. Geen residue is opgespoor vir totale salbutamol ($\text{LOQ} = 3 \mu\text{g} / \text{kg}$) in enige van die weefsels nie. Geen visuele nuwe-effekte is waargeneem in die studie diere nie, en R-salbutamol was dus goed verdra in voerkraalbeeste.

Uit die data in hierdie studie word dit voorgestel dat die Maksimum Residu Vlak vir R-salbutamol ingestel word vir lewer teen $300 \mu\text{g} / \text{kg}$, spier teen $6.5 \mu\text{g} / \text{kg}$, nier teen $40 \mu\text{g} / \text{kg}$ en vet teen $13 \mu\text{g} / \text{kg}$. Op hierdie vlakke sal die daaglikse inname van residu steeds onder 20% van die ADI wees.

Verdere ondersoek na binneaarse en mondelingse farmakokinetika van R-salbutamol by beide beeste en lammers sal voordelig wees om die absorpsie, verspreiding, metabolisme en uitskeiding van R-salbutamol beter te verstaan in voerkraalbeeste en lammers.

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DEDICATIONS

Dedicated to my daughters, Lize and Mieke. Remember you are never too old to learn.

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LIST OF ABBREVIATIONS

AA	Adrenergic Agonists
ADI	Acceptable Daily Intake
ADME	Absorption, Distribution, Metabolism and Excretion
ARfD	Acute Reference Dose
BWt	Body Weight
CF	Consumption Factor
C_{max}	The maximum plasma concentration achieved following oral dosage of the drug investigated
FDA	United States of America - Food and Drug Administration
GIT	Gastro Intestinal Tract
MCC	Medicines Control Council
MRL	Maximum Residue Limit
NOAEL	No Adverse Effect Level
OECD	Organisation for Economic Co-operation and Development
POD	Point of Departure
$t_{1/2}$	Half-life
T_{max}	the time to reach C_{max}
TMDI	Theoretical Maximum Daily Intake
UCL	Upper Confidence Level
UF	Uncertainty Factor
VICH	International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
β_2 -AA	Beta2-Adrenergic Agonists
β -AA	Beta-Adrenergic Agonists
β -AR	Beta-Adrenergic Receptors
DAFF	South African Department of Agriculture, Forestry and Fisheries

NOTES

The language and style used in this thesis is in accordance with the requirements of the *South African Journal of Animal Science*. This thesis represents a compilation of manuscripts, where each chapter is an individual entity and some repetition between chapters is therefore unavoidable.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Background

Livestock production is one of the major role players in agricultural economics, contributing to 40% of the South African agricultural gross domestic product (GDP). It employs 1.3 billion people and is the major source of livelihood for over a billion poor people (Steinfeld, et al., 2006).

Currently, meat contributes to one third of the protein intake of the world, but with an increase in population numbers to an estimated 9 billion in 2050 and income growth, food preferences are placing an increased demand on livestock production with a doubling in demand for meat predicted from 229 million tons in 1990/01 to a staggering 580 million tons in 2050 (UNPD, 2007). This will also have a significant impact on the environment and technologies in agriculture will have to be developed and employed to reduce the impact of livestock production on the environment by half to decrease the already significant impact it has (Steinfeld, et al., 2006).

On top of a growing demand for meat and pressure on reducing the environmental impact of increased livestock production, there is an ever-increasing competition between animals and humans for available land. Hence significant improvement in production efficiencies is one of the major drivers to provide for the ever-increasing need for more meat. One major production system that is growing internationally is intensive production systems such as feedlots that have started becoming more common for ruminants. Currently, technologies including steroidal hormone implants, ionophores and beta-adrenergic agonists are available in certain countries and are utilised to improve production efficiency of livestock production in these intensive production systems. However, more and more pressure from consumers is placed on the withdrawal of these products from livestock production systems, although the withdrawal of these products in the EU was “precautionary” in nature as authorities felt that it could not, with certainty, say that there was an absence of a health risk after performing risk assessments. The ban was therefore due to consumer pressure rather than scientific facts (Kerr & Hobbs, 2005).

Capper and Hayes (2012) studied the impact of the removal of growth enhancing technologies (GET) on the economy and environment in the United States. The reduction in production

efficiency with the withdrawal of GET would result in less meat being produced and would increase the required beef population size, to produce the 454 million kg meat shortfall, with 385 000 animals. Water consumption will increase with 20.14×10^6 litres, land use will increase by 265 000 ha and feedstuff consumption by 2.8 million tons. Subsequently, the manure output will increase with 1.8 million tons leading to an increase in carbon emissions of 714.5 t/454 million kg beef produced.

At the centre of the GET ban is the safety of the products in question and the need for scientific evaluation of the effect of such technologies on the health and safety of the consumer.

Beta-adrenergic agonists (β -AA) (non-hormonal growth enhancers) have been used in beef production over the past 20 years and was first introduced into the South African market in 1995 (Webb, Allen, & Morris, 2018). The first product registered was Zilmax (zilpaterol hydrochloride, MSD Animal Health). Following the introduction into the South African market, Zilmax has been registered in other countries including the United States (US), Canada and Mexico (Merck Animal Health, 2017).

Paylean™ was the first β -AA to be introduced into the US market for use in pigs in 1999 (FDA, 1999) and Optaflexx™ 45 (ractopamine hydrochloride, Elanco Animal Health) for use in beef cattle in 2003 (FDA, 2003). Ractopamine, as with all other beta-agonists, is banned for use in production animals for improved production efficiency in the EU (European Commission, 2005) and Mainland China (Meador & Wu, 2011), but has been determined safe by Regulatory Authorities in 27 countries, including the United States, Canada, Mexico, Japan, South Africa and South Korea.

R-salbutamol, a β -AA, was introduced into the South African market in 2014 for use in beef feedlots for production improvement and in 2017 for use in sheep feedlots as Salbutamate 10% by Animate Animal Health (43 Hornbill Road, Rooihuiskraal, Centurion, South Africa). Salbutamate 10% is the first β -AA to be registered for use in sheep feedlots in South Africa (Animate Animal Health, 2017). The benefit of the use of R-salbutamol, above that of especially zilpaterol, is that it does not have a negative effect on the tenderness of beef and lamb (Dávila-Ramírez, et al., 2013; De Klerk, 2016; Du Toit, 2017; Steenekamp, 2014; Webb, Allen, & Morris, 2018).

As part of the registration requirements for veterinary drugs used in production animals, of which the meat is intended for human consumption, residue studies in the target animals must be performed and these form part of this study (MCC, 2004; VICH, 2015). The aim of these investigations is to determine the levels of the marker residue, parent salbutamol, and total

salbutamol (parent + metabolite) in lambs and cattle treated with R-salbutamol during the feedlot finishing phase. An extensive safety risk assessment will also form part of this thesis to determine the Acceptable Daily Intake (ADI) for humans, which is the level of residues in animal tissue remaining after treatment, deemed safe for human consumption, and the subsequent determination of a Maximum Residue Limit (MRL) for R-salbutamol in lamb and cattle tissue (meat, liver, kidney and fat).

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Synthetic Beta-adrenergic agonists (β -AA) have been used for the treatment of asthma since the 1940's (Konzett, 1940). Since the development of the first synthetic β -AA, isoproterenol (isopropyl adrenaline), many new molecules have been investigated with varying degrees of efficacy and safety profiles.

With the ban in Europe of hormonal growth promoters in 1986, research was initiated to develop non-steroidal growth promoters and the use of β -AA were investigated as an alternative (Kuipers, Noordam, van Dooren-Flipsen, Schilt, & Roos, 1998). In the 1980's research was done on various β -AA with regards to performance improvement, including salbutamol, ractopamine, cimaterol, L 664,969 and clenbuterol (Mersmann, 1998; Ricks, Dalrymple, Baker, & Ingle, 1984).

2.2 History of Beta-Agonist development

The development of β -AA as a treatment for asthma started at the end of the 19th century following the observations by the physician, George Oliver, that ingestion of a sheep's adrenal gland caused radial artery constriction. Subsequent, research together with Edward Shafer confirmed a strong action of adrenal extract on the heart, blood vessels and skeletal muscles (Oliver & Shafer, 1895). Takamine (1901) as referenced by Tattersfield (2006), was the first person able to extract the active ingredient from the adrenal gland and named it adrenalin in his 1901 publication. It was subsequently marketed as Adrenalin by Parke, Davis & Company.

Initially in the 1890's, adrenalin was used for the treatment of asthma with little success, but Solomon Solis-Cohen (1900) persisted that it would work by increasing the dosage and so started the evolution of asthma treatments. In the early development of the use of adrenalin as asthma treatment it was noted that taking adrenalin via the oral route had little to no effect. By injecting adrenalin subcutaneously, or by inhaling adrenalin, it was later noted that some of the adverse events were reduced.

So, the research into synthesised β -AA started in earnest. The early research was focused on asthma in humans. In 1965, Cunningham presented the first data indicating that epinephrine and nicotine administration can lead to changes in pig growth (Cunningham,

1965). The first major commercial investigation was performed at American Cyanamid Co. in the early 1980's with research into the effect of clenbuterol in cattle (Ricks, Dalrymple, Baker, & Ingle, 1984). Subsequently, many other research projects were performed and reported on, with different β -agonists (e.g. salbutamol, ractopamine, cimaterol, L664,969) indicating variable responses in chickens, pigs, cattle and sheep (Mersmann, 1998).

2.3 β -Adrenergic Receptors

Cells have developed receptors to transmit signals from external stimuli into important physiological responses within the cell. Beta-adrenergic receptors (β -AR) are one of many highly specialised receptors (Ostrowski, Kjelsberg, Caron, & Lefkowitz, 1992) and together with α -adrenergic receptors, form part of a group of G protein-coupled receptors (GPCR) (Johnson, Smith, & Chung, 2014).

The existence of specific receptors on body cells that regulate either inhibiting or stimulatory responses within the body in reaction to the binding of neurotransmitters or drugs, were first described by John Newport Langley, a physiologist in Cambridge, during the late 1800's (Langley, 1875; Maehle, 2004). Paul Ehrlich described these transmitters to be selective (Limbird, 2004). It is not until 1948, however, that the existence of alpha (α) and beta (β) adrenergic receptors was described by Ahlquist (1948) with both an inhibitory and excitatory effect caused by adrenalin.

Alpha adrenergic receptors (α -AR) are found in the circulatory system on vascular smooth muscle and are responsible for controlling blood pressure. Two types of α -AR have been described, namely α_1 and α_2 (Reid, 1986). For the purpose of the further discussion, focus will be on the β -AR.

β -AR are present on the surface of almost every type of mammalian and avian cell which are targets for ligands, such as catecholamines, leading to various physiological processes when stimulated (Mersmann, 1998). β -AR's are glycoproteins within the membrane of certain types of cells within the body (De Blasi, 1989). The three subtypes of β -AR are:

- β_1 -AR,
- β_2 -AR,
- β_3 -AR.

The pharmacological and physiological responses of cells are determined by the specific ratio of these three β -AR subtypes present on that cell. The distribution and proportion of the 3

sub-types of β -AR differ between tissues types as well as between species in a specific tissue type (Mersmann, 1998). The β 2-AR is one of the most widely studied receptors (Ostrowski, Kjelsberg, Caron, & Lefkowitz, 1992) and the focus of this review.

β -AR contain seven transmembrane domains which are hydrophobic in nature as illustrated in Figure 2.1.

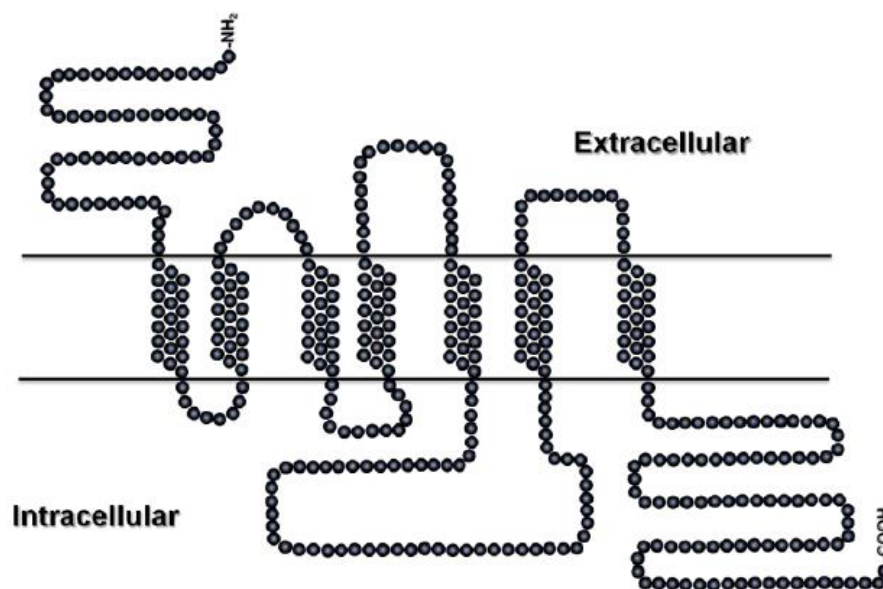


Figure 2.1: β -AR model (Johnson, Smith, & Chung, 2014)

β -ARs are made up of more than 400 amino acids and similarities between β -receptors in their amino acid make-up is approximately 50%, whereas these similarities between species within a specific sup-type is approximately 75% (Mills & Mersmann, 1995).

2.4 β -Adrenergic Agonists

Agonists are defined as compounds that bind to receptors and have a similar or more potent effect than the natural occurring mediator (Fiems, 1987). The neurotransmitter, norepinephrine and the adrenal medullary hormone, epinephrine, are the natural occurring physiological adrenergic agonists (AA) responsible for stimulating β -AR (Fiems, 1987). β -AA have a chemical similarity to ephedrine and nor-ephedrine in that they all share a basic chemical structure (Fiems, 1987).

Oral administration of some β -AA lead to a production improvement due to increases in muscle and decreases in fat accretion in cattle, pigs, poultry, and sheep. The exact mechanism of

action is not yet fully understood due to the complex nature of the functions controlled by β -AA (Mersmann, 1998).

The first possibility is that of the direct effect within fat and muscle cells (Mersmann, 1998). Other mechanisms should however also be considered such as the effect of the central nervous system on feed intake (Ordway, O'Donnell & Frazer, 1987), increased blood flow (Beermann, et al., 1987; Eisemann, Huntington, & Ferrell, 1988; Mersmann, 1989) to target organs and hormonal release (Beermann, et al., 1987). Specific focus should also be placed on the pharmacodynamic properties of individual molecules within the class of β -AA and the different effects that they may have between species as well as within species at different physiological ages and the effect of different diets thereupon (Mersmann, 1998).

Variability in the effect of different β -AA in different species could be attributed to different factors according to Mersmann (1998), which included the difference in agonistic effect between molecules in a specie due to the affinity of an agonist to the receptor, and/or the delivery mode of the agonist to the target tissue. β -AR may also be inactivated more rapidly by certain β -AA and some β -AA may have a higher affinity for certain β -AR sub-types. Specie differences in amount of β -AR on a specific target tissue may also lead to a difference in response.

2.4.1 Mode of action of β -AA

When a phenethanolamine or β -AA binds to the Beta-AR on the extracellular N-terminus of the receptor, the receptor is stimulated, and a conformational change is activated in the β -AR. This in turn allows for the binding of the G_S -protein (guanine nucleotide stimulating protein) to the intracellular loop of the receptor. The G_S -protein coupling activates adenylyl cyclase (AC) which transforms ATP (adenosine triphosphate) to cAMP (cyclic adenosine monophosphate). The increasing cAMP stimulates the phosphorylation of protein kinase A (PKA to pPKA) (De Blasi, 1989; Ostrowski, Kjelsberg, Caron, & Lefkowitz, 1992; Johnson, Smith, & Chung, 2014; Mersmann, 1998).

Depending on the specific tissue where the β -AR stimulation takes place the formation of pPKA will trigger different physiological processes (Figure 2.2).

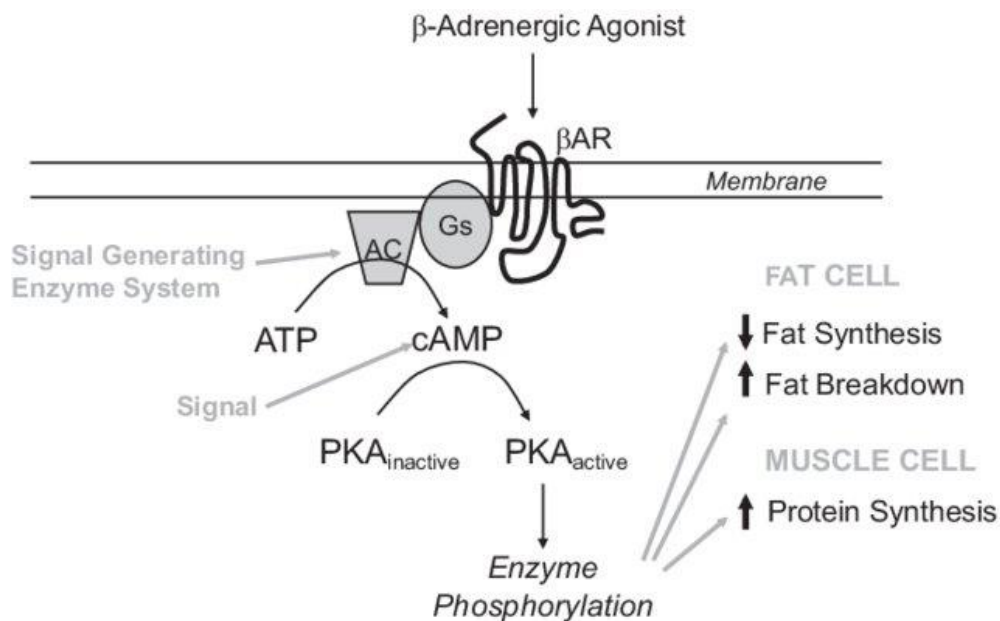


Figure 2.2: β -AR mechanism of action (Anderson, Moody, & Hancock, 2009)

In fat cells, the formation of pPKA stimulates the hydrolysis of triacylglycerol (TAG) through the activation of adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL) which leads to increased lipolysis (Ricks, Dalrymple, Baker, & Ingle, 1984; Johnson, Smith, & Chung, 2014; Mersmann, 1998).

In muscle cells, the increased levels of cAMP leads to the phosphorylation of adenosine monophosphates-activated protein kinase (AMPK) which in turn is positively correlated with an increase in mRNA levels in muscle fibers and involved in energy metabolism (Johnson, Smith, & Chung, 2014; Lynch & Ryall, 2008). Numerous studies have indicated that the increased muscle mass is due to increased mRNA levels in muscle in contrast to an increase in DNA as in the case of steroid hormone treatment (Walker, et al., 2010).

Contradicting work has been reported with regards to the reason for the increased muscle mass, with some researchers reporting an increase in protein accretion, some a decrease in protein degradation and in some instances, both. This seems to be specific to the animal species investigated and the type of β -AA used in the trials performed (Fiems, 1987; Moody, Hancock, & Anderson, 2000).

Protein accretion, and subsequent muscle growth, is associated with a change in the activity of calpastatin and calpains within the muscle of β -AA treated animals. An increase in calpain I activity was observed at low growth rates in non-responsive muscle types, but a decrease was reported at higher growth rates. As growth however increased, an increase in calpastatin and calpain II activity was observed (Bardsley, et al., 1992).

2.4.2 Differences in physiological activity of the different β -AA's

The physiological activity of β -AA is determined by the activity of the molecule at the receptor as well as how it is absorbed, the rate of metabolism and elimination and subsequent distribution to the target tissues. The chemical characteristic of the β -AA may also influence these parameters. Therefore, it is important to determine and investigate the pharmacokinetic properties of each of the β -AA molecules to determine the specific effect and subsequent toxicity of the molecule within the broader group of β -AA (Smith, 1998).

As in the case of steroid hormones, the physiological function and activity of a class of molecules cannot be over generalized. For example, estrogen, aldosterone and testosterone are all steroid hormones with similar chemical properties, they have very different physiological functions. Similarly, different β -AA differ in their physiological activity (Smith, 1998).

The biological activity of β -AA is determined by the aromatic ring attached to the β -carbon (Figure 2.3). Various substitutes are attached to the aromatic ring which in turn determines the half-life, route of metabolism as well as the efficacy of the molecule at receptor level. The structure of chemically synthesised β -AA is similar to the natural occurring adrenergic neurotransmitters norepinephrine and epinephrine except for the bulky group on the R-group of the aliphatic nitrogen (Figure 2.3) (Smith, 1998).

	Aromatic Substitution			Category	Example(s)
	A	B	C		
	-H	-OH	-H	Phenol	Ractopamine Ritodrine
	-OH	-H	-OH	Resorcinol	Fenoterol Terbutaline
	-OH	-OH	-H	Catechol	Isoproterenol Dobutamine
	-CH ₂ OH	-OH	-H	Saligenin	Salbutamol Salmeterol

Figure 2.3: General structure of β -AA and common substitutions on the aromatic rings. The R-group is a bulky group on the aliphatic nitrogen, usually an isopropyl, alkylphenol, alkylphenyl or t-butyl group. Labels *p*- and *m*- on the aromatic ring indicate the *para*- and *meta*- position relative to the beta-carbon (Smith, The pharmacokinetics, metabolism, and tissue residues of beta-adrenergic agonists in livestock, 1998).

Smith (1998) explained the intricate differences with regards to the chemical structures and subsequent efficacy of the different available β -AA in detail. In summary, the substitution at the β -carbon influences the longevity of the activity of a β -AA at the receptor in mammalian and avian species. β -AA with a 3-hydroxyl group on the aromatic ring (catechols) are metabolised at a very rapid rate by catecholamine O-methyl transferases (COMT) leading to limited effectiveness if dosed via the oral route.

The route of metabolism is also influenced by the nature of the substitution on the aromatic ring. β -AA were developed with different patterns of hydroxylation (grouped as the phenols, resorcinols and saligenins) to retain activity at the β -AR which are not affected by COMT but are rapidly metabolised in the liver and intestinal wall leading to a short half-life. These molecules include ractopamine and salbutamol (Smith, 1998).

Subsequently, new β -AA were developed that were still active at binding the β -AR but are more resistant to metabolism. These beta-agonists have halogen substitutions in the place of hydroxyl groups. These include molecules such as clenbuterol, cimaterol and mabuterol, salbutamol, ractopamine and zilpaterol (Morgan, 1990; Anderson, Moody, & Hancock, 2009). The differences in molecular structure for these β -AA are indicated below in Figure 2.4.

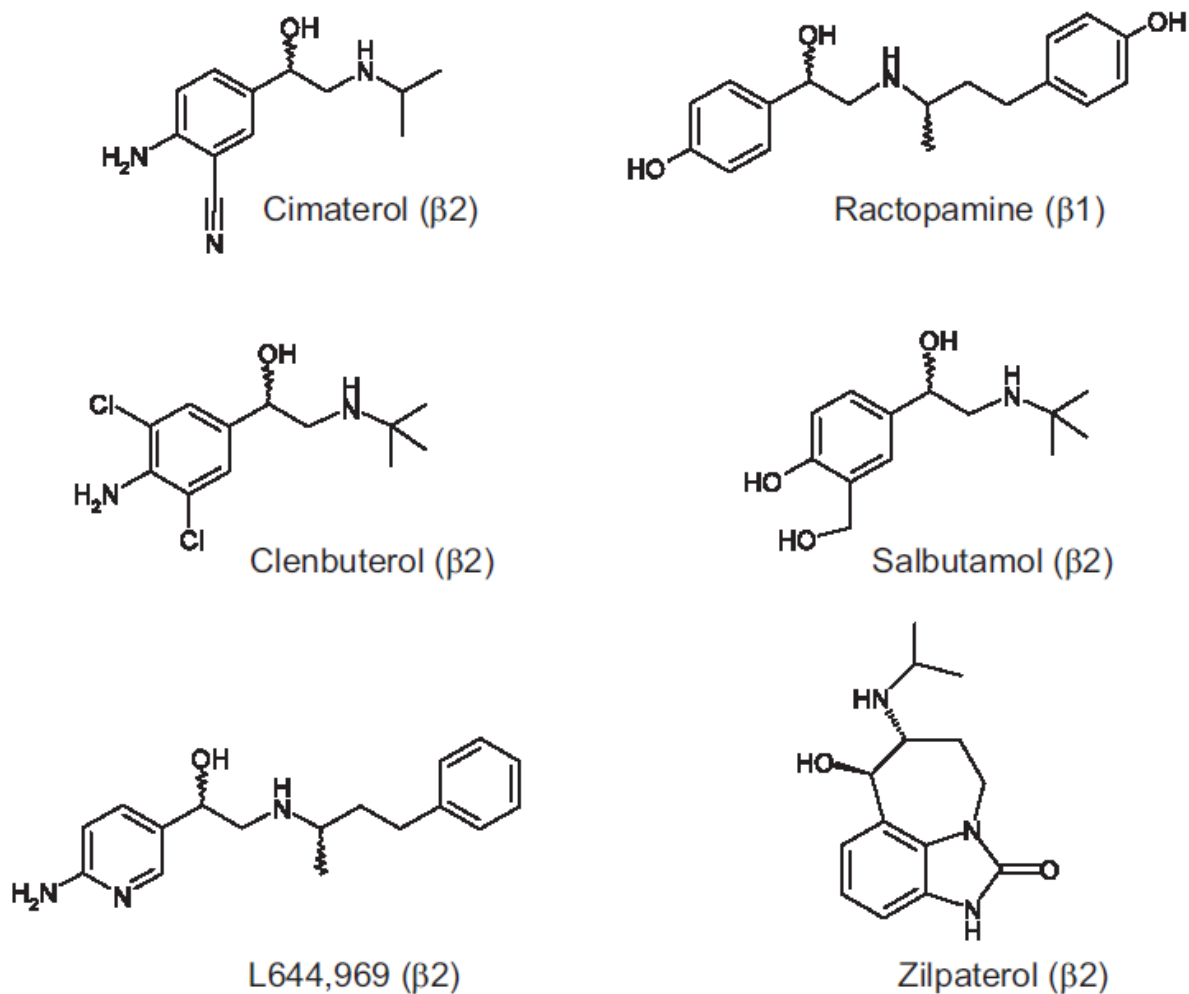


Figure 2.4: Molecular structure of some phenethanolamines. β -AR affinity is indicated in parentheses (Anderson, Moody, & Hancock, 2009).

Lipophilicity is also influenced by the hydrogen substitution on the aromatic portion. As an example, salbutamol has a much lower lipophilicity than salmeterol, which is lipid-soluble. The length of the N-arylalkoxyalkyl side chain differs dramatically between the two molecules with that of salmeterol being much longer than the side chain of salbutamol. The side chain of salmeterol has an affinity for the cell membrane in contrast with that of salbutamol which is too short to bind to the cell membrane. Therefore, salbutamol remains extracellular (Smith, 1998).

The β -carbon determines the chirality of the molecule, determining the dextrorotatory and levorotatory stereoisomers (Smith, 1998).

2.4.3 Enantio selectivity of β -AA with specific reference to salbutamol

Ruffolo (1991) indicated that the levorotatory stereoisomer is solely responsible for the biological activity of direct-acting β -AA.

Stereoisomers are structurally identical molecules with the only difference that the one is a mirror image of the other. The isomers for salbutamol are shown in Figure 2.5.

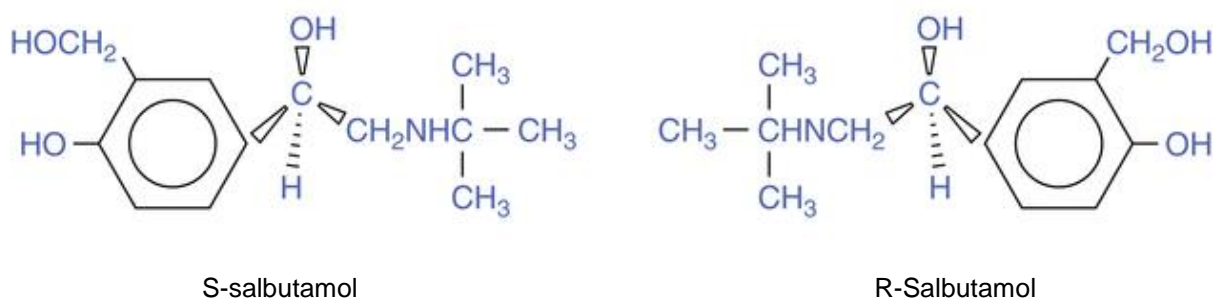


Figure 2.5: The S- and R-isomers for salbutamol (iKnowledge, 2015).

Research has indicated that racemic β -AA can cause airway hyper-reactivity in humans and animals and that this could be a secondary cause to β -AR desensitisation. The hyper-reactivity is indicated to be caused by the S-enantiomer of β -AA. The more active R-enantiomer is referred to as the eutomer and the less active S-enantiomer, the distomer (Waldeck, 1999).

Salbutamol is a rapidly-acting, potent non-catecholamine β_2 selective agonist. It binds reversibly to β_2 -AR which are located in several sites in the body, such as airway smooth muscle cells (causing relaxation), mast cells (inhibiting histamine release) (Russo et al., 2005) and elsewhere e.g. uterus (causing contractility), cardiovascular system (causing palpitations) and voluntary muscle (leading to tremors) (Anon, 1971; Health Products Regulatory Authority, 1983; Libretto, 1993).

The binding sites of interest for the application of R-salbutamol in production animals are adipose and lean muscle cells where binding results in repartitioning (Sumano, Ocampo, & Guteirrez, 2002).

It has been found that the desired bronchodilatory effect of salbutamol is a consequence of β_2 -AR stimulation by R-salbutamol (Hartley & Middlemiss, 1971). Similarly, the bronchodilatory effect of the β -AA, terbutaline (used as a bronchodilator in humans) was also found to be due to the R-enantiomer (Wetterlin, 1972).

S-salbutamol has virtually no affinity for β_2 -AR and as such no therapeutic activity in this context (Fawcett, et al., 2004). The side effects of racemic salbutamol administration are

commonly manifestations of β 2-AR stimulation: for example, fine muscle tremor, flushing, palpitations and sometimes tachycardia. S-salbutamol has no bronchodilating activity and has detrimental effects on airways, such as pro-inflammatory actions and may mask the desired bronchodilatory effects of the R-isomer when administered as part of a racemic mixture (Hansen, Yen, Klindt, Nelssen, & Goodband, 1997). It follows that a derivative of racemic salbutamol which is optically pure for the R-isomer would have superior activity with reduced side effects due to the absence of S-isomer interference.

R-salbutamol is more potent than an equivalent dose of racemic salbutamol (Hansen, Yen, Klindt, Nelssen, & Goodband, 1997). The Xopenex FDA approved label (Sepracor Inc., 1999) stated that “R-salbutamol has a 2-fold greater affinity for beta receptors than racemic salbutamol and a 100-fold greater affinity than S-salbutamol”. Thus, in isolation, R-salbutamol has a greater potency (without the competitive inhibition by the S-salbutamol for the receptor sites) and greater pharmacodynamic effects. This was also found to be the case with R-terbutaline having a 1000-fold more potent effect than S-terbutaline (Jeppsson, Johansson, & Waldeck, 1984). R;R-fomoterol was also found to be 1000-fold more potent than S;S-fomoterol and R;R-fomoterol also had an increased effect on the beat frequency of cilia in the tracheal mucosa with S;S-formoterol having no effect (Lindberg, Khan, & Runer, 1995). The same potency difference was observed between R-clenbuterol and S-clenbuterol (Waldeck & Widmark, 1985).

The improved efficacy of R-salbutamol in the absence of S-salbutamol has led to the development of Salbutamate 10% for use in animal production. The presence of only the R-isomer enables producers to only dose half of what the therapeutic dosage would have been for the racemic formulation. Fawcett et al. (2004) observed similar production improvements in chickens fed racemic salbutamol at 10 ppm vs. R-salbutamol at 5 ppm in feed. The chickens fed 10 ppm racemic salbutamol had higher tissue concentrations of salbutamol compared to the chickens fed 5 ppm and 10 ppm R-salbutamol (Table 2.1).

Table 2.1: Influence of diets containing R-salbutamol and racemic salbutamol on concentration of salbutamol residues in tissues of 42-day old chickens (Fawcett et al., 2004)

Diet	Concentration in tissue (mg/kg)			
	Plasma	Liver	Fat	Muscle
5 ppm R-salbutamol	7.6 ± 0.8	34.1 ± 4.9	5.7 ± 1.1	11.7 ± 1.2
10 ppm R-salbutamol	14.5 ± 2.7	65.5 ± 11.4	6.2 ± 1.2	21.8 ± 3.6
10 ppm Racemic Salbutamol	23.1 ± 2.5	173.2 ± 8.65	14.8 ± 0.3	29.8 ± 1.85

The reduction in the total dosage of R-salbutamol in comparison to racemic salbutamol therefore has the potential benefit for reduced residue levels in the tissue of treated animals.

2.4.4 Absorption, distribution, metabolism and excretion of β -AA

The half-life ($t_{1/2}$) of a substance is the time it takes for a substance to lose half of its pharmacological or physiological effect (Riviere, 2009).

Two classes of β -AA are found. Those with halogenated aromatic ring systems and those with hydroxylated aromatic ring systems. In general, the β -AA with halogenated aromatic ring systems are metabolized via conjugative oxidative pathways and subsequent longer plasma half-lives. β -AA with hydroxylated aromatic rings are only metabolized by conjugation and subsequently have short plasma half-lives (Smith, 1998).

β -AA with high oral bioavailability, long plasma half-lives, and relatively slow rates of elimination lead to high oral potencies in humans consuming animal tissue treated with β -AA (hereto further referred to as consumers). On the other hand, the β -AA with low oral bioavailability, short plasma half-lives, and rapid rates of elimination have low oral potencies in consumers as the shorter plasma half-life leads to much lower residues in edible tissue and a much lower risk to consumers (Smith, 1998).

Various studies have investigated the extent of absorption in humans and animals of various β -AA by monitoring the plasma levels after single exposure and long-term exposure to variable dosage levels. Most β -AA achieve peak plasma levels within 1 to 3 hours following oral administration in humans and animals (Morgan, 1990; Meyer & Rinke, 1991). Peak plasma levels of clenbuterol in calves were observed to increase with repeated dosing indicating accumulation of clenbuterol (Sauer, Limer, & Dixon, 1995). Similar peak plasma levels as with clenbuterol were observed when salbutamol was orally dosed in cattle, although the oral dose of salbutamol was several times higher than that of the oral clenbuterol dosage. No increase in plasma peak levels was observed after multi day dosing of salbutamol, indicating that salbutamol does not accumulate as in the case of clenbuterol (Pou, et al., 1992).

The extent of drug absorption should also be evaluated based on the extent of urinary excretion and not only be based on the plasma levels obtained. Radio-labelled clenbuterol was rapidly absorbed within an hour in calves following oral dosage and recovery in urine and the non-gastrointestinal components accounted for 76% of the radiolabel although less than

50% were excreted in the urine after 48 hours (Smith & Paulson, 1997). In contrast, in swine more than 88% of radiolabelled ractopamine HCl were excreted in the urine (Dalidowicz, Thomson, & Babbitt, 1992).

Although radiolabelled salbutamol studies have not been performed in food producing animals to the knowledge of the author, studies have been performed in rats, rabbits, dogs and humans. Sixty percent of radiolabelled salbutamol were absorbed by rats, 90% in humans and 65% in dogs. Differences between species were however observed in the amount of free salbutamol excreted in urine. Dogs excreted the majority (60 – 90%) of salbutamol in the free form, whereas 60% were excreted as free salbutamol and 40% as a glucuronide conjugate in rats within 24 hours. The glucuronide is formed mainly in the liver (Martin, Hobson, Page, & Harris, 1971).

Like all drugs, the extent of salbutamol systemic absorption depends upon the route of administration.

For asthma treatment in humans, the usual route of drug delivery is via inhalation or nebuliser. Research has shown that a portion of the drug will be swallowed when administered this way providing more data on the drug's fate after oral absorption (Ward & Schultz, 1995).

Salbutamol is rapidly absorbed after oral administration with most of the dose being excreted in urine as parent or metabolite in humans (80-100% over 24 hours). Almost complete absorption occurs when given extravascular in humans (Boulton and Fawcett, 2001). Ward *et al.* (2000) reports the slow oral absorption of R- and S-salbutamol after oral dosing (2 - 3 hours to peak) compared with a peak plasma concentration of 1 - 4 hours in racemic studies. Notably, S-salbutamol takes longer (t_{max}) to reach maximum plasma concentration (C_{max}) than when administered as part of the racemate (Price & Clissold, 1987; Ward, et al., 2000).

In one study, the bio-availability of R, S-salbutamol (as a mean of both enantiomers) following an oral dose was 39% compared to 42% as an inhaled dose. But, the bioavailability of pure R-salbutamol was lower following an oral dose (9%) than an inhaled dose (24%) (Ward, et al., 2000). The reduced bio-availability of R-salbutamol is thought to be due to competitive inhibition of metabolism by S-salbutamol and highlights the differences that exist when comparing chiral and non-chiral studies (Boulton & Fawcett, 1997).

Absorption is not the limiting factor for pharmacological activity of β -AA, but rather the rate of biotransformation and excretion is (Smith, 1998). Several researchers mention that R-salbutamol is subject to intestinal pre-systemic metabolism in the gut wall of humans by a yet

to be identified enzyme that does not exist in livestock animals, significantly affecting bioavailability in humans (Boulton & Fawcett, 1996a; Ward, et al., 2000)

Enantiomer inter-conversion also occurs in the gut probably due to acid-catalysed racemisation in the stomach, resulting in an insignificant inversion to the S-isomer after oral administration of pure R-salbutamol (Boulton & Fawcett, 1996a; Ward, et al., 2000). Later, Fawcett *et al.* (2004) reported that chickens on diets containing R-salbutamol gave rise to the drug in tissue with no evidence of racemisation.

Intravenous studies in rats show that racemic salbutamol crosses the blood-brain barrier thus explaining some of its central nervous system effects at high doses (Price & Clissold, 1987).

There is rapid absorption and distribution of salbutamol in humans. Plasma protein binding is not significant for either enantiomer and is dose dependant, ranging from 7 - 64% and the blood:plasma ratio is ~1 (Boulton & Fawcett, 2001). Tissue binding is not enantio-selective (steady state volumes of distribution are alike). Salbutamol has a prolonged distribution phase of longer than 2 hours (Boulton & Fawcett, 2001). The apparent volume of distribution in humans is 156L after intravenous administration (Price & Clissold, 1987) indicating extensive extra vascular uptake/distribution.

Most β -AA are metabolised by conjugation to form a major metabolite (i.e. they are uncomplicated by multiple metabolic pathways). This is also true for the metabolism of salbutamol and its enantiomers. But unlike other β 2-AA, R-salbutamol ("active" enantiomer) is metabolised faster than S-salbutamol (the "inactive" enantiomer) leaving a disproportionate amount of S-salbutamol in the system relative to the dose of parent drug (Boulton & Fawcett, 1996b; Ward & Schultz, 1995).

The metabolism of salbutamol differs in humans and animals. Sulphated conjugates (4'-sulphate) are the main metabolites in humans, whilst glucuronic acid conjugates (4'-glucuronide) are the main metabolites in rodents (rats and rabbits) and dogs (Boulton & Fawcett, 1996a; Walle, Pesola, & Walle, 1993). Furthermore, there is a marked interspecies variation in the extent of glucuronidation. For example, in dogs 30% and in rabbits 90%, of total urinary radioactivity has been recovered as glucuronide conjugate (Boulton & Fawcett, 1996a). This complicates comparative studies between different species. The conjugation of salbutamol at the *p*-hydroxyl group renders salbutamol with no metabolic activity. Therefore, the metabolite is physiologically inactive (Martin, Hobson, Page, & Harris, 1971).

It is worth noting that whilst the comparison of salbutamol metabolism in humans and in animals is not simple and direct, the clinical relevance of the differences has not been demonstrated. In humans, sulphate conjugation is catalysed by a highly stereo-selective monoamine form of phenolsulphotransferase. Research has demonstrated that salbutamol metabolism is indeed stereo-selective, with the two enantiomers competing for enzyme occupancy (Ward, et al., 2000). In chickens, the metabolism of racemic salbutamol is stereo-selective, with the two enantiomers competing for the enzymes of metabolism and exhibiting the same pattern observed in humans (Fawcett, et al., 2004). In contrast, Ward *et al* (2000) found that enantio-selective metabolism did not occur in the lungs and was confined to the gastrointestinal tract (GIT) and systemic circulation in humans. Nevertheless, there will be overexposure to S-salbutamol after intravenous and oral administration (the latter due to the extensive presystemic GIT metabolism in humans).

Sulphoconjugation occurs in the small intestine (presystemic) and in the liver (first-pass) in humans. Presystemic intestinal metabolism is the major route of elimination following oral administration in humans. The sulphate conjugate has no pharmacological activity (Boulton & Fawcett, 1996b; Ward, et al., 2000).

Hepatic metabolism accounts for around half the clearance of R-salbutamol from plasma, such that the plasma concentration of S-salbutamol at steady state during intravenous infusion of the racemate should be ± 1.5 times greater than R-salbutamol. Oral administration gives the highest difference in enantiomer plasma concentration (due to the presystemic gut metabolism). There is a reported 2.4-fold difference at steady state and 7.3-fold difference after a single oral dose (Boulton & Fawcett, 2001). In humans, the plasma concentration of the sulphate conjugate is around five times that of salbutamol, 1 - 5 hours after oral or inhalation dosing. The metabolite is virtually undetectable after intravenous administration (Price & Clissold, 1987). Studies show a 5 - 11-fold greater sulphoconjugation of R-salbutamol in different human tissues. The resulting blood levels of salbutamol consist predominantly of the inactive S-salbutamol, at least partially explaining why dose-response curves from salbutamol blood levels have never been successfully determined (Pesola & D'Costa, 2004).

There is thought to be a large enantiomeric selective first-pass effect after oral administration such that R-salbutamol is less bio-available than S-salbutamol (30% vs. -70%) (Boulton & Fawcett, 1997). Due to the selectivity of metabolism and excretion, S-salbutamol will predominate systemically.

Regardless of the route of administration, salbutamol and its metabolites are rapidly excreted in urine and faeces. The excretion of parent and metabolised salbutamol could potentially have an impact on environmental contamination. Steenekamp (2014) reported ten times lower levels of salbutamol residues excretion in faeces in comparison to zilpaterol when fed to cattle. The residues declined rapidly in faeces following a 3-day withdrawal period (Figure 2.6).

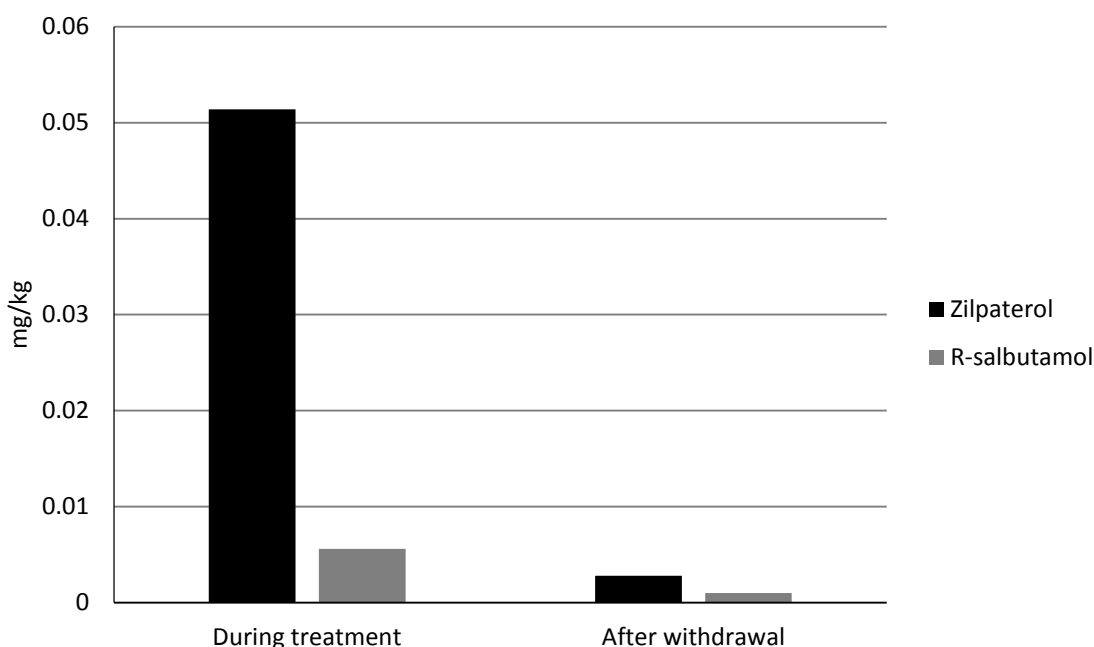


Figure 2.6: Zilpaterol and R-salbutamol residues in cattle faeces (Steenekamp, 2014)

Zhou (2017) found salbutamol to be sensitive to photo degradation and residues will subsequently not build up in the environment due to degradation by ultraviolet light.

An Italian study (Zucatto, et al., 2006) measured levels of pharmaceuticals in two rivers. Predicted environmental concentrations were calculated based on prescription figures and theoretically adjusted for the percentage of each drug excreted as a parent compound. The ratio of measured to predicted environmental concentrations of salbutamol was 0.12 in one river and 1.96 in the other. The detected levels were very low (1 – 8 ng/L) in comparison to other detected pharmaceuticals. Ofloxacin were detected at levels between 33 and 600 ng/L. Based on predicted environmental concentrations of salbutamol it was concluded that salbutamol was not a threat to the environment.

On a cellular level, it is believed that organic cation transporters are responsible for eliminating salbutamol and metabolites from cells (Boulton & Fawcett, 2001). Around 80% of a single oral

dose was recovered from urine in 24 hours and around 30% was the unchanged drug (Boulton & Fawcett, 1996b). This is comparable to the findings in intravenous excretion patterns where 75% of the dose was recovered in the urine (after 24 hours), 65% unchanged and 10% were metabolites (Price & Clissold, 1987). The different excretion pattern of unchanged drug in oral/inhalation and intravenous routes is probably due to the presystemic metabolism that occurs in the human gastrointestinal tract (Boulton & Fawcett, 1996b).

Unchanged salbutamol undergoes active tubular secretion (clearance is greater than creatinine). The renal clearance of the metabolite is less than parent salbutamol which may indicate that it is freely filtered i.e. not actively secreted (Price & Clissold, 1987).

According to Ward *et al* (2000), the half-life for the R-metabolite is shorter than the S-metabolite and similar to the parent clearance rate. Since the metabolic clearance of S-salbutamol is slower than that of R-salbutamol, accumulation of S-salbutamol will occur, resulting in a longer half-life for S-salbutamol compared to R-salbutamol. Terminal half-life was reported as 2.5 hours for R-salbutamol and 5 hours for S-salbutamol (Ward, et al., 2000). It is likely that the only stereo-selective renal clearance is active rather than selective tissue or plasma protein binding.

Typically, data for elimination half-life depends on several variables. For example, the dosage, route of administration, health of the study subjects and whether the value is calculated from urinary data. Price and Clissold (1987) reported that the elimination half-life of salbutamol in humans can range from 2.4 hours (intravenous) to 6.5 hours (for multiple oral doses). In contrast, another study found that in humans, the elimination rate of the 4'-O-sulphate metabolite is similar to the unchanged salbutamol after oral administration of the drug to healthy volunteers (Boulton & Fawcett, 2001). This suggested that the kinetics of elimination of the sulphate metabolite is formation rate limited. It is excreted shortly after it is formed due to its high polarity as shown by low plasma concentration and high recovery in urine (Boulton & Fawcett, 2001).

In rats and dogs, radio labelled salbutamol is rapidly cleared from all tissues. The liver and kidney are the only organs in which small amounts of radioactivity are detected 24 hours after oral administration (Pesola & D'Costa, 2004).

2.5 Human safety of β -AA use in production animals

The Organisation for Economic Co-operation and Development (OECD) guidelines for pre-clinical toxicological testing is based on internationally agreed testing methods to assess the safety of chemicals. These guidelines have been adopted by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) in the guideline for evaluation of the safety of residues remaining in production animals following the administration of veterinary drugs (Guideline #GL33). This guideline was developed by an expert working group consisting of representatives from regulatory bodies from Japan, United States of America (USA) and the European Union (EU). The South African Department of Agriculture, Forestry and Fisheries (DAFF) is an observer to the steering committee and encourages the use of the VICH guidelines (VICH, 2018).

These guidelines for the evaluation of the toxicology of veterinary drugs are in line with the guidelines for toxicological testing for pesticides, food additives and human medicine as set out by the OECD. The aim of the toxicological testing is to identify the no-observed adverse effect level (NOAEL) for the veterinary drug in question. The NOAEL can be defined as the “the highest experimental point that is without adverse effect” according to Dorato (2005). The lowest-observed adverse effect level (LOAEL) is therefore the lowest experimental point in a toxicological trial where an adverse effect is observed.

There is however no standard definition of what is viewed to be an adverse effect and the interpretation of whether an effect observed is potentially harmful to humans, lies with the toxicologist (Dorato & Engelhardt, 2005). This review will therefore take the interpretation of the researcher and other reviewers of the experimental work as guidance in determining the NOAEL for the specific toxicological study.

Depending on the veterinary drug in question, repeat dose studies, carcinogenic studies, reproductive toxicity studies, foetal developmental toxicity studies (teratology) and genotoxicity studies are evaluated as the basis of the safety evaluation to address the issue of “appreciable risk”. Additional studies, including neurotoxicity, immunotoxicity, pharmacological effects and the effect on human intestinal microflora can also be included based on the nature of the pharmaceutical in question. In the case of antibiotics used as veterinary pharmaceuticals, the effect of residues in animal tissue on human intestinal microflora would be a very important factor to determine the toxicology of the antibiotic in question (VICH, 2009). Due to the nature of β -AA, the toxicological studies of importance are as follows:

- repeat dose studies
- carcinogenic studies
- reproductive toxicity studies
- foetal developmental toxicity studies (teratology)
- genotoxicity studies

From each of these studies (performed at multiple dosage levels much higher than the anticipated routine dosage levels) the NOAEL is derived.

The aim of evaluation within the toxicological studies is to determine the safety of any residues ingested by humans due to the application of any veterinary drug in food producing animals and therefore the preferred route of application is oral administration in any *in vivo* toxicological study evaluated. For each specific toxicological investigation, preferred target species are identified (VICH, 2009).

The European Food Safety Authority (EFSA) undertakes amongst other things, safety evaluation for pesticides and other active substances based on requests for scientific advice from the European Commission (EC), European Member States and the European parliament. This work is undertaken by appointed Scientific Committees and Scientific Panels (EFSA, 2018). The Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) is responsible for evaluating the safety and/or efficacy of any additives used in animal feed. EFSA has done safety evaluations for two β -AA; zilpaterol and ractopamine (EFSA, 2009; EFSA, 2016).

Similarly, the Joint FAO/WHO Expert Committee on Food Additives (JECFA), consisting of expert scientific committees that perform risk assessments on food additive safety, has evaluated the safety and completed the risk assessments on ractopamine, zilpaterol and clenbuterol (JECFA, 2018). EFSA uses the risk assessments performed by JECFA when undertaking their safety evaluations.

A risk assessment has not been done for R-salbutamol use, as South Africa is the first country in the world to register the active ingredient for use in production animals. The aim of the following summary is to evaluate the safety and to do the risk assessment for R-salbutamol.

2.6 Setting of an Acceptable Daily Intake (ADI) or Acute Reference Dose (ARfD)

The end point of evaluating the toxicology of veterinary drugs is to determine the Acceptable Daily Intake (ADI) for the veterinary drug investigated. The ADI is then again used together

with the animal tissue residue data derived from residue depletion studies to determine the Maximum Residue Limit (MRL) in new investigational veterinary drugs. The ADI together with the MRL and the residue depletion data is used to determine the Withdrawal Period (WP) (also referred to as the Withholding Period), at which point the consumption of any animal tissue derived from animals fed the veterinary drug is safe for human consumption (MCC, 2004).

The ADI is defined by JECFA as “An estimate of the amount of a substance in food or drinking water, expressed on a body weight basis that can be ingested daily over a lifetime without appreciable risk (standard human = 60 kg BWt)”. This calculation would therefore only take the NOAEL values of the repeat dose studies, carcinogenicity, genotoxicity, reproductive and foetal toxicity studies into account.

JECFA considered that certain drug classes may have more acute effects (pharmacological or toxicological) due to residue intake (usually in one meal) and that the sub-acute and long-term studies used to determine the ADI may not be an accurate benchmark for toxicological evaluation. Therefore, depending on the nature of the compound in question, it may be of value to define an Acute Reference Dose (ARfD) from acute intake studies for certain compounds (JECFA, 2009).

The ARfD is only relevant when the calculated acute effect is lower than the ADI determined for the veterinary drug in question and where the ARfD of the veterinary drug in question is below 1 mg/kg BWt in humans (JECFA, 2016).

β -AA are known to have acute effects by increasing heart rate in humans and animals. These effects in humans and animals are however dose related and differ between the different molecules within the class. Fenoterol has been reported to have a more pronounced effect on the increase in heart rate than salbutamol or terbutaline and the effect on the increase in heart rate was dose related (Wong, Pavord, Williams, & al., 1990).

It would also appear that a tolerance develops rapidly to the effect of β 2-AA on the cardiovascular stimulating effect. Tachycardia is therefore only observed in infrequent users of β 2-AA (Bremner, Woodman, Burgess, & et.al., 1993; Wong, Pavord, Williams, & al., 1990).

Clenbuterol was one of the first β -AA to be investigated for use in animal production in the 1990's. American Cyanamid Company abandoned the development of clenbuterol as a productivity enhancer due to the residue toxicity in beef edible tissue (Johnson, Smith, & Chung, 2014).

Producers illegally using non-registered β -AA usually do so at higher levels than that tested in research trials and/or do not adhere to any withdrawal period that would deem the animal tissue produced safe. The incorrect use of these β -AA can be attributed to ignorance regarding the application of these molecules or the believe that higher inclusion levels would increase production efficiencies. This would lead to residue levels in especially the liver and kidney (of the animals produced) that could lead to adverse effects in humans consuming such animal tissue (food poisoning) (Johnson, Smith, & Chung, 2014).

In 1992 in Catalonia, Spain, 113 cases of clenbuterol food poisoning in humans related to the consumption of veal liver, from illegally fed clenbuterol to cattle, were reported. Of the 113 cases, 50% of the affected individuals presented with symptoms of tachycardia, nervousness, headaches, and myalgia and muscle tremors. Symptoms presented between 15 minutes to 6 hours after the ingestion of the liver and lasted between 90 minutes to 6 days (Salleras, Dominquez, Taberner, Moro, & Salva, 1995). In fact, between 1998 and 2002, 50 cases in four separate incidences of clenbuterol intoxication were reported in Portugal due to ingestion of lamb meat and beef meat and liver from animals fed clenbuterol, with symptoms ranging from tachycardia, distal tremors, nausea, epigastralgia, diarrhoea, fever, headaches and dizziness, myalgia and asthenia, hypertension, dry mouth and vomiting (Barbosa, et al., 2005). Many additional cases of acute clenbuterol food poisoning have been reported in France (Pulce, Lamaison, Keck, Bostvironnois, & Nicolas, 1991), Italy (Brambilla, et al., 1997; Maistro, Chiesa, Angeletti, & Brambilla, 1995) and China (Shiu & Chong, 2001) due to illegally fed clenbuterol in food production animals.

Based on these observations, β -AA residues in animal tissue are more likely to lead to acute effects in humans when ingesting animal tissue and therefore the ARfD for R-salbutamol together with the ADI need to be evaluated to determine the safety of R-salbutamol to humans.

The NOAEL is determined from the studies as discussed in section 2.7 and the lowest NOAEL value identified is used for the calculation of the ADI. It could then be summarised that the ADI is calculated to set a safe intake level in humans with regards to any long-term or chronic effects that a veterinary drug may have (VICH, 2009), whereas the ARfD is calculated to set a safe intake level in humans to prevent any acute effects that may arise from ingestion of residues via animal tissue derived from treated animals with the veterinary drug in question (JECFA, 2016).

2.7 Toxicity data on β -AA with specific focus on R-salbutamol and/or salbutamol

2.7.1 Repeat dose studies

Repeat dose studies are performed to determine the effect of repeated exposure (usually daily) at multiple levels expressed as mg/kg BWt to determine the effect of a veterinary drug and/or its metabolites after prolonged exposure and subsequently deriving a NOAEL (VICH, 2004a). According to the FDA and EU guidelines, only studies in rats are required, but in Japan studies in rodent and non-rodent species are required, with the rat as the accepted rodent species and the dog the accepted non-rodent species. Selection of the target species should however be evaluated in terms of the relevance of the human pharmacokinetics, pharmacodynamics and metabolism of the veterinary drug in question (VICH, 2004a; b).

Rats dosed orally for 4 months up to 50 mg salbutamol/kg BWt/day showed no histological changes and no evidence of toxicity was observed (Anon, 1971).

In a 4-month dog study where oral doses of 0.02 to 25 mg salbutamol/kg BWt/day were administered and dose related tachycardia were observed with the most noted effect one hour after administration. At the higher doses, skin flushing was observed (Anon, 1971). Similar results were observed with ractopamine at 0.002mg/kg BWt (EFSA, 2009), zilpaterol at 0.5 to 1 mg/kg BWt (EFSA, 2016) and salmeterol at 0.15 to 2 mg/kg BWt (Owen, Beck, & Damment, 2010). It is however suggested that dogs are more sensitive to beta-adrenergic substances than other species i.e. monkeys and therefore the dog data is not considered when determining the NOAEL value for β -AA (EFSA, 2009).

Cardiotoxicity was investigated in an oral study in rabbits at a dose of 150 mg salbutamol/kg BWt/day for 9 days. No cardiac histological lesions or cardiac toxicity were detected (Poynter & Spurling, 1971).

Yamada et al. (1977) investigated the effect of salbutamol in rats in a 1-month study at oral dosages of 17, 50 or 150 mg salbutamol/kg BWt/day. No changes were observed in electrocardiograms, blood biochemistry, urine analysis and haematology. Histological examination indicated a 13 - 27% increase in heart weight, muscle hypertrophy, interstitial oedema, congestion, focal myocardial necrosis and fibrosis as well as an increase in lung weight. These effects were however reversible after 1-month withdrawal of salbutamol and the findings were therefore not considered to be of toxicological significance but rather of pharmacological significance.

A range of subcutaneously administered β -AA cause myocardial necrosis due to the tachycardia which increases oxygen demand on the heart. The oxygen cannot be supplied to the heart as the coronary arteries are less perfused during shortened diastole. The severity of this effect varies depending on the number of beta-1 receptors stimulated (Stanton, Brenner, & Mayfield, 1969; Balazs, 1973).

An unpublished study by Bhide (2010a) in Sprague-Dawley rats at daily oral dosages of 0, 6, 12 and 24 mg R-salbutamol hydrochloride/kg BWt for 28 days led to reduced body weight in males in the 24 mg/kg BWt group. Increased relative weight of the adrenals were observed in the females from the 12 mg/kg BWt group. No related gross pathological or histological changes were seen, and the findings were not dose dependent and hence the findings were not considered to be of toxicological importance. No significant changes were observed in gross pathology or histopathology in the adrenals, aorta, brain, colon, duodenum, epididymis, eyes, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, oesophagus, ovaries, skeletal muscle, skin, mammary gland, spleen stomach, testes, thymus, thyroid, urinary bladder and uterus. The NOAEL value for this study was therefore determined to be 12 mg R-salbutamol/kg BWt.

In a similar study by Bhide (2010b) in Swiss Albino mice at daily dosages of 0, 6, 12 and 24 mg/kg BWt of R-salbutamol hydrochloride for 28 days, reduced body weights were observed in males in both the 24 mg/kg and 48mg/kg BWt groups. Also, an increased liver weight was observed in the 24 mg/kg BWt group and decreased kidney weights in the 24 and 48 mg/kg BWt groups. No significant changes were detected in gross pathology or histopathology in the adrenals, aorta, brain, colon, duodenum, epididymis, eyes, heart, ileum, jejunum, kidneys, liver, lungs, lymph nodes, oesophagus, ovaries, skeletal muscle, skin, mammary gland, spleen stomach, testes, thymus, thyroid, urinary bladder and uterus. Similar to the previous study by Bhide (2010a), the NOAEL for R-salbutamol can be set at 12 mg/kg BWt.

2.7.2 Carcinogenicity

To assess the carcinogenic potential of an investigational veterinary drug, VICH Guideline GL28 (2005) recommends a 2-year rat study and an 18-month mouse study.

Numerous studies with different β -AA have reported the formation of mesovarian leiomyomas in various rat strains and more specifically the Sprague-Dawley rat (Kelly, Marler, & Weikel, 1993; Nelson & Kelly, 1971; Nelson, Kelly, & Weikel, 1972). Mesovarian leiomyomas were also found in long-term rat studies performed with salbutamol with dose related increases in

the incidence of leiomyomas of the mesovarian. These tumours were however found to be benign (Jack, Poynter, & Spurling, 1983; Poynter, Harris, & Jack, 1978; Tabachnick, 1981). Jack *et al.* (1983) indicated that the formation of the mesovarian leiomyomas were a function of the pharmacokinetic mode of action of salbutamol and terbutaline and not due to DNA-reactivity. This was confirmed by the preventative action of concurrent administration of propranolol (a β -adrenergic blocker) in the development of the tumours.

The use of long-term studies in rats has however been questioned as indicator of carcinogenicity effects of investigational drugs in humans (MacDonald, 2004). Rat ovary anatomy also differs from that of woman and the occurrence of mesovarian leiomyomas in woman is very rare and no increase in the incidence thereof has been observed over the last 90 years with the use of beta-agonist as asthma treatment in humans (Poynter, Harris, & Jack, 1978).

Also, no similar development of mesovarian leiomyomas were seen in any long-term studies in mice, dogs, non-human primates or hamsters (Poynter, Harris, & Jack, 1978; Tabachnick, 1981). In an 18-month study with CD-1 mice, no evidence of tumour development was observed at oral doses up to 500 mg salbutamol/kg BWt and in a 22-month study in Golden Hamsters dosed up to 50 mg salbutamol/kg BWt, no tumour development was observed (Brambilla, Mattioli, Robbiano, & Martelli, 2013).

Rat carcinogenicity study data can therefore be excluded as a carcinogenicity indicator for humans (Jack, Poynter, & Spurling, 1983). Salbutamol is therefore not carcinogenic in humans. This is similar to the findings of the EFSA in their safety evaluation of both Ractopamine (EFSA, 2009) and Zilpaterol (EFSA, 2016).

2.7.3 Genotoxicity

Genotoxicity studies are performed to investigate any damage that a veterinary drug might have with respect to DNA and its fixation.

As outlined in VICH guideline GL23 (2014) the studies generally required to assess the genotoxic potential of an investigational new veterinary drug are as follows:

1. A test for gene mutation in bacteria
2. *In vitro* test for chromosomal effects in mammalian cells
3. *In vivo* test for chromosomal effects using rodent hematopoietic cells

Brambilla et al. (2013) reported that no genotoxicity was observed in any of the genotoxicity assays for racemic salbutamol as summarised in Table 2.2.

Table 2.2: Genotoxic effect of salbutamol

Test System	Results		Dose LED or HID
	Without e.m.s.	With e.m.s.	
<i>S. typhimurium</i> TA98, TA1537, TA1538, reverse mutation	-	-	NR
<i>E. coli</i> WP2, WP2 uvrA, WP67, reverse mutation	-	-	NR
<i>Saccharomyces cerevisiae</i> , forward mutation	-	-	NR
<i>Saccharomyces cerevisiae</i> , mitotic gene conversion	-	-	NR
Chromosomal aberrations, human lymphocytes <i>in vitro</i>	-	-	NR

LED, lowest effective dose; HID, highest ineffective dose; NR, not reported; without e.m.s., without exogenous metabolic system; with e.m.s., with exogenous metabolic system.

The genotoxicity of salbutamol has been evaluated as part of the New Drug Application (NDA 20-837) with the U.S. Food and Drug Administration (FDA) by Sepracor and evaluated in both the bacterial test for gene mutation, and the *in vitro* test for chromosomal effects in mammalian cells (Whitehurst, 1999).

The bacterial gene mutation test was conducted for NDA 20-837 and evaluated the ability of salbutamol, in the presence and absence of rat microsomal enzyme (S9), to induce reverse mutation at specific histidine loci in 5 *Salmonella typhimurium* test strains (TA1535, TA1537, TA98, TA100 and TA102) and at the tryptophan locus in *Escherichia coli* test strain WP2uvrA. A non-reproducible increase in reversion frequency was observed with strain TA100 and TA1537 (both with S9 only) and was not statistically significant nor dose dependent at concentrations ranging between 5000-10,000 µg/plate (Whitehurst, 1999).

An *in vitro* test for chromosomal effects was carried out in the Chinese hamster ovary (CHO)/HPRT mammalian cell forward gene mutation assay. The CHO cells were incubated in the presence and absence of S9 at 10 concentrations ranging from 0.167 - 5000 µg/ml. While R-salbutamol was shown to be cytotoxic at 5000 µg/ml, all average mutant frequencies were within negative control ranges (Sepracor Inc., 1999).

Libretto (1993) and Xopenex FDA Approved Extended Label (Sepracor Inc., 1999) note that salbutamol was not mutagenic *in vitro* against four bacterial strains with or without metabolic activations (liver microsomes) and at concentrations of up to 1 million times its effective concentration. No mutagenic activity was detected in Kada's reversion and recombination test or Zimmerman's' reversion and mitotic gene conversion test.

These original results point towards a favourable outcome in the battery of genotoxicity testing. A chemical's genotoxicity test results are recognized as an indicator of its carcinogenic potential since many carcinogens have a genotoxic mode of action (VICH GL28 / CVM 141). Hence a negative test battery supports the request that carcinogenicity tests may not be required. The CVM Guideline 141 further states that "because it is generally believed that non-genotoxic compounds exhibit a threshold dose for carcinogenicity and human exposure to residues of veterinary drugs is low, non-genotoxic compounds should not be routinely tested for carcinogenicity." In addition to the studies summarized above, results from non-radio-labelled tissue residue studies with R-salbutamol and its racemate demonstrate that the residue levels associated with chronic administration to livestock will be in orders of magnitude below the corresponding therapeutic dose in humans (Malucelli, Ellendorff, & Meyer, 1994). Furthermore, stereo-selective pharmacokinetic studies have demonstrated that in humans, the systemic availability of orally administered R-Salbutamol is very low due to extensive pre-systemic metabolism (Boulton & Fawcett, 1996b).

Lack of genotoxicity is often a good indication that carcinogenicity is unlikely. Coupled with a long history of use in humans for the control of asthma with no evidence of a carcinogenic link, there appears substantiated scientific evidence to suggest that R-salbutamol is not carcinogenic and to argue against the need for R-salbutamol specific carcinogenicity studies.

2.7.4 Reproductive and Foetal Toxicity (teratogenic effect)

No impairment of fertility was observed in a reproductive rat study at oral doses of up to 50 mg salbutamol/kg BWt/day (Sepracor Inc., 1999; Health Products Regulatory Authority, 1983). However, studies using radio labelled racemic salbutamol administered either intraperitoneally or intravenously in pregnant rats have shown some transfer of radio labelled material to the foetus equal to 10% of the maternal plasma radioactivity (Gardey-Levassort, Richard, Hauguel, Thiroux, & Olive, 1982). According to Hauguel et al. (1982), an increase in foetal plasma insulin levels were observed prior to an increase in blood glucose concentration after a single salbutamol injection of 600 µg/kg BWt at 21 days of gestation. A chronic daily infusion

of 600 µg/kg BWt from day 6 to 20 of gestation led to a decrease in the insulin-secreting ability of the foetus following a glucose injection to the pregnant mother. This however did not have any effect on the glycogen content of the placenta or foetal liver nor on birth weight.

Salbutamol may also delay preterm labour as it interferes with uterine contractility. Salbutamol is classified as Pregnancy Category C (risk cannot be ruled out). Salbutamol is used therapeutically in humans to delay onset of birth by reducing uterine contractions specially to prevent premature labour (Gummerus, 1981).

A single-generation embryo-foetal study was performed in New Zealand White Rabbits and no embryo or foetal toxicity was observed after R-salbutamol was administered orally at doses up to 25 mg/kg BWt/day during days 6 - 20 of pregnancy (Sepracor Inc., 1999; Whitehurst, 1999).

Results in studies performed on pregnant mice showed cleft palate malformations in 9.3 % of foetuses of females dosed subcutaneously at a dosage of 25 mg salbutamol/kg BWt/day and in 4.5% of foetuses from pregnant females dosed subcutaneously at 0.25 mg salbutamol/kg BWt/day. No cleft palate formation was observed when salbutamol was administered subcutaneously at 0.025 mg/kg BWt/day. However, 30.5% of foetuses from the positive control group (2.5 mg isoproterenol/kg BWt/day) presented with cleft palate. No statistical differences were noted between the incidence of cleft palate between the treatment and control groups. In identical trials where salbutamol was administered subcutaneously in pregnant rabbits and rats, no cleft palate was observed in foetuses (Sepracor Inc., 1999; Szabo, Difebbo, & Kang, 1975; Whitehurst, 1999).

It is important to note that the subcutaneous dosing in pregnant animals can lead to an increase in the output of endogenous steroids due to stress, which can lead to the increased incidence of cleft palate (Barlow, Morrison, & Sullivan, 1975). Cortisone, the endogenous steroid hormone secreted during stress, delays the rate at which the palate shelf moves during embryonic development and in doing so prevents the fusion of the shelves, leading to cleft palate (Walker & Fraser, 1957).

In pregnant rats dosed orally at dosages of 0.5, 2.32, 10.75 and 50 mg salbutamol/kg BWt/day no foetal abnormalities were observed. Neonatal mortalities were observed at the highest dosage level of 50 mg/kg BWt/day which was attributed to a lack of maternal care and not due to any direct effect of salbutamol (Health Products Regulatory Authority, 1983).

Cranioschisis were observed in 37% of foetuses from Stride Dutch Rabbits treated orally with 50 mg salbutamol/kg BWt/day (Sepracor Inc., 1999; Whitehurst, 1999)

Congenital abnormalities, such as cleft palate or limb deformation, have rarely been reported in the offspring of human patients being treated with salbutamol (Sepracor Inc., 1999; Whitehurst, 1999).

As the preferred species for toxicological evaluation is the rat in this instance and the route of exposure is oral, the NOAEL can be set at 10.75 mg/kg BWt/day from the study in pregnant rats for the teratological effect (Health Products Regulatory Authority, 1983).

2.7.5 Acute oral toxicity – single dose studies

Acute oral toxicity of racemic Salbutamol (R+S-salbutamol) results depend on whether the animal has been fed or fasted. Fed rats tolerate salbutamol and other β -AA better than when fasted. This can be attributed to the higher C_{max} (the maximum plasma concentration achieved following oral dosage of the drug investigated) and shorter T_{max} (the time to reach C_{max}) as a result of accelerated gastric emptying in fasted animals (the β -AA is absorbed more rapidly). The higher C_{max} will ultimately result in more pronounced acute effects (Kast & Nishikawa, 1983). For the purpose of determining the ADI for possible residues consumed by humans, only oral studies in fed animals will be considered as the residues in animal tissue consumed by humans will naturally always be part of a meal.

LD_{50} is usually determined in acute toxicity studies. The LD_{50} is defined as the level of drug or substance given to test subjects that is enough to kill 50% of the test population (Duffus, 2006).

A LD_{50} of 8600 mg salbutamol/kg BWt was reported by Kast and Nishikawa (1983) in orally dosed male Sprague-Dawley rats (8-week old) and 7600 mg salbutamol/kg BWt in females fed rats. A LD_{50} in excess of 2000 mg salbutamol /kg BWt was also reported in a trial with rats and mice (Anon, 1971).

According to the Globally Harmonized System of Classification and Labelling of Chemicals (GHS) a chemical with a LD_{50} between 2000 mg/kg BWt and 5000 mg/kg BWt is classified as Category 5 acute toxicity, with Category 1 representing the most hazardous and Category 5 the least hazardous with regards to acute toxicity. The LD_{50} of salbutamol indicates a category 5 classification for salbutamol.

It is important to note that the metabolism of salbutamol in animals differ to that in humans. Sulphate conjugation is the main route of metabolism of salbutamol in humans with the sulphate conjugate being metabolically inactive. The sulphate conjugation in humans takes place in the intestines (presystemic) and in the liver (first-pass). Of the ingested dose 48.4%

were excreted as the sulphate conjugate in humans whilst the bioavailability was found to be 50% of the oral dose (Morgan, Paull, Richmond, Wilson-Evered, & Ziccone, 1986). Metabolism of salbutamol is also different between animal species with major differences also being noted between animals. Salbutamol is metabolised by glucuronic acid conjugation (Martin, Hobson, Page, & Harris, 1971) in animals compared to sulphate conjugation in humans. Martin et al. (1971) investigated the difference in metabolism of salbutamol between species. In rats, 90% of the excreted salbutamol in urine was as the metabolite. In dog studies, between 0% and 30% of excreted salbutamol in urine was in the form of the metabolite. Therefore, using animal acute data for determination of human safety has little scientific merit. With the number of human studies performed with salbutamol it is therefore more pertinent to determine human safety based on the available human study data.

The other important aspect to note is the difference in enantio-selectivity of salbutamol (R- or S-). Most studies were performed using the racemic formulation. It is however clear that there are major differences in the metabolism as well as the effects of the enantiomers of salbutamol as discussed in section 2.4.3.

Evaluating the data on the enantiomers of salbutamol, it has been shown that the R-isomer is biologically active at the receptor site whereas the S-isomer is viewed as being inactive (Boulton & Fawcett, 1997).

In human trials, the plasma levels of R-salbutamol are much lower than that for S-salbutamol or the racemate dosed individually. However, the effect on heart rate after oral dosage of R-salbutamol is similar to that of the racemate when the racemate is dosed at double the level of R-salbutamol. A dose of 5 mg R-salbutamol results in a plasma C_{max} of ~2.5 ng/ml whereas 10 mg of the racemate translates into a plasma C_{max} of ~3.5 ng/ml R-salbutamol (Boulton & Fawcett, 1997).

It is therefore safe to assume that the possible adverse effects of R-salbutamol can be compared to that of the racemate in humans when investigating the safe intake levels for humans at equipotent dosages.

Boulton and Fawcett (1996b), gave seven healthy volunteers a single dose of 1.6 mg racemic salbutamol intravenously per person and 4 mg racemic salbutamol per person orally. Loading doses were given to the participants the day before the start of the oral phase of the study. Three 4 mg racemic salbutamol sulphate oral doses were ingested every 8 hours. At the start of the oral study, a fourth 4 mg oral dose were administered 8 hours following the last oral dose. The R- and S-salbutamol levels were determined in plasma and urine was collected at

specified time-points. At the same time-points, the heart rates were monitored. Figure 2.7 below summarises the data findings in the trial for heart rate and plasma levels for both intravenous and oral doses.

The C_{max} value for R-salbutamol when dosing the racemate at 4 mg per person was ~ 3.5 ng/ml plasma. The oral dose of racemic salbutamol caused a slight increase in heart rate of 10 ± 4 beats per minute which as expected, gradually decreased back to normal as the plasma level of salbutamol decreased. At a plasma level of ~ 1 ng/ml R-salbutamol there was zero effect on the heart rates in the volunteers. These results are similar to the findings of Morgan et al. (1986) where a 4 mg oral intake of racemic salbutamol increased heart rate by 10 beats per minute.

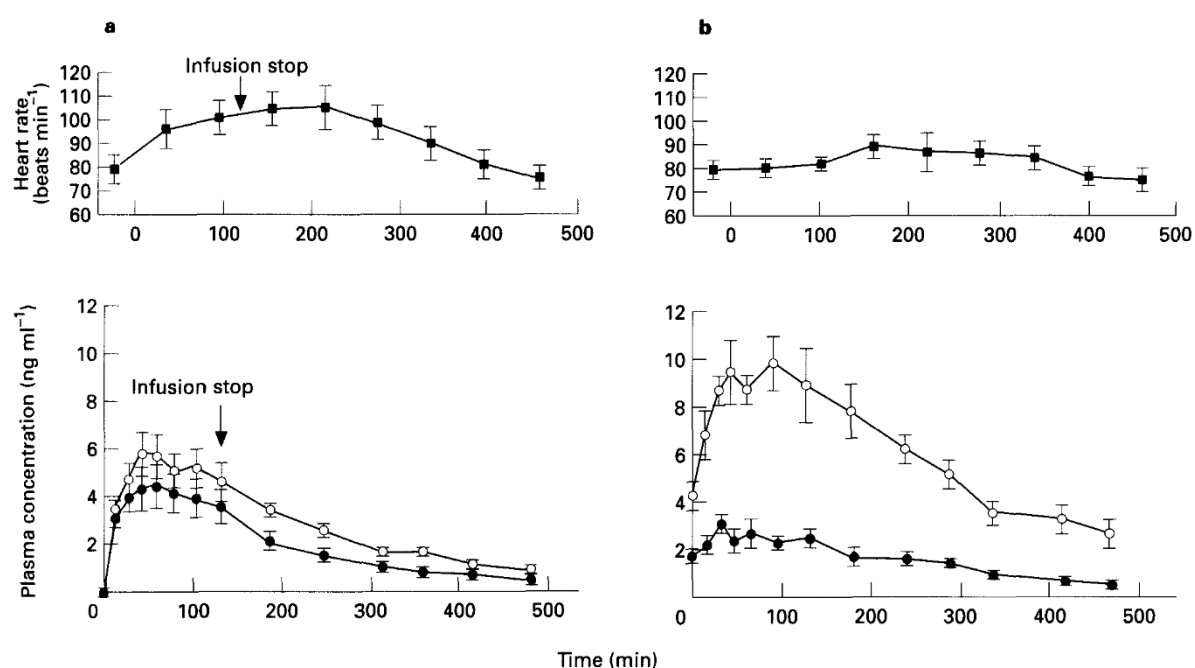


Figure 2.7: Mean (\pm s.e. mean) hourly heart rates (beats/min) (■) and plasma concentrations (ng/ml) for S- (○), and R- (●) salbutamol following (a) intravenous administration (1.6 mg) and (b) the fourth hourly oral dose (4.0 mg) of racemic salbutamol sulphate (Boulton & Fawcett, 1996b).

In a similar trial by Boulton and Fawcett (1997) comparing the pharmacokinetics of racemic salbutamol with that of R-salbutamol and S-salbutamol, lower plasma peak levels were observed for R-salbutamol, confirming the lower systemic bio-availability and shorter half-life of R-salbutamol as seen in Figure 2.8.

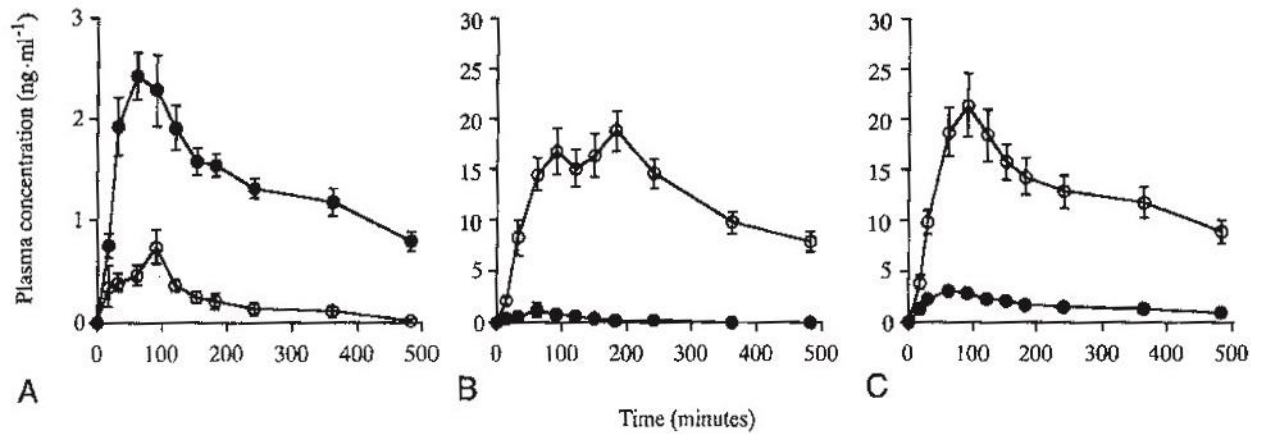


Figure 2.8: Plasma concentration-time profiles for R-salbutamol (●) and S-salbutamol (○) after oral administration of 5 mg R-salbutamol (A), 5 mg S-salbutamol and 10 mg racemic salbutamol (mean \pm SEM) (Boulton & Fawcett, 1997)

Due to the much higher dose of R-salbutamol in this study of 5 mg R-salbutamol and 10 mg racemic salbutamol per person compared to 4 mg racemic salbutamol per person in the previous study by Boulton and Fawcett (1971), the increase in heart rate was slightly more pronounced (Figure 2.9), but there were no significant differences between the R-salbutamol and racemic salbutamol treatments at most time points. Heart rate was back to normal at plasma levels of ~ 1 ng/ml, confirming the results from the previous study by Boulton and Fawcett (1971).

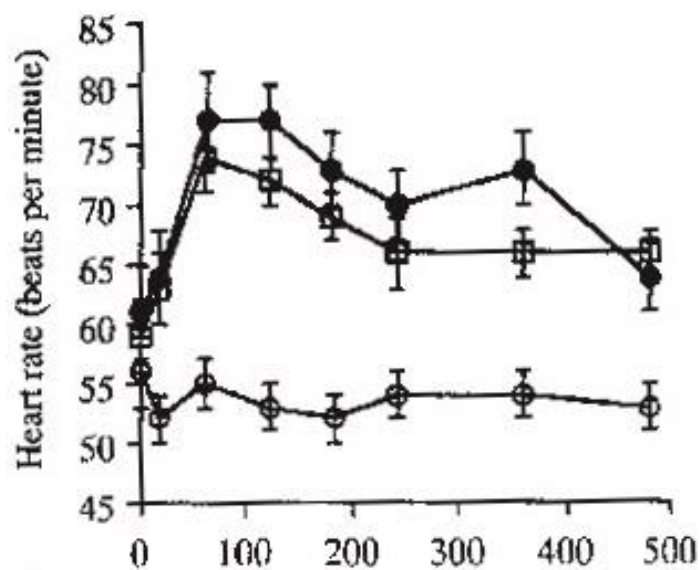


Figure 2.9: Pharmacodynamic response-time profile for heart rate after oral administration of 5 mg R-salbutamol (●), 5 mg S-salbutamol (○) and 10 mg racemic salbutamol (□) (mean \pm SEM) (Boulton & Fawcett, Pharmacokinetics and pharmacodynamics of single oral dose of Albuterol and its enantiomers in humans, 1997)

From the observations of Boulton and Fawcett (1996b), accepting a linear response between dose and plasma level, it can be calculated that an oral dose of \sim 1.6 mg racemic salbutamol will result in a maximum S-salbutamol plasma level of 4 ng/ml and R-salbutamol concentration of 1 ng/ml. At these levels, no clinical measurable heart rate effects were present. It should be noted that the racemate results in competition for elimination of R-salbutamol which is not the case when the pure R enantiomer is administered (Boulton & Fawcett, 1996b).

In the study by Boulton and Fawcett (1997), a 5 mg dose of R-salbutamol per person led to a C_{max} of \sim 2.5 ng/ml in plasma. Heart rate were back to normal at a plasma concentration of \sim 1ng/ml R-salbutamol. Applying the same calculation as above, this gives a value of 2 mg oral R-salbutamol as the safe dose for R-salbutamol in humans where no acute adverse effects will be noticeable. At these levels, no clinical measurable effects were present (i.e. heart rate, plasma glucose, and potassium and / or ECG changes). This equates to a NOAEL value for R-salbutamol of 33.33 μ g/kg BWt. Alternatively, a LOAEL (lowest adverse effect level) value of 66.66 μ g/kg BWt can be set from the above study with R-salbutamol at a single dose of 4 mg per person (average volunteer body weight = 75 kg in the Boulton and Fawcett study (1996b)).

2.7.6 Toxicological Conclusion

No genotoxicity or carcinogenesis were noted in the reported data above. This agrees with the conclusion of the FEEDAP panel that reviewed the safety of ractopamine. The FEEDAP panel did not consider ractopamine to be carcinogenic or genotoxic (EFSA, 2009). Similarly, EFSA in their evaluation of the JECFA report concluded that zilpaterol is also unlikely to be carcinogenic or genotoxic (EFSA, 2016).

No reproductive toxicity was noted for salbutamol and is in line with similar findings for zilpaterol (EFSA, 2016) and ractopamine (EFSA, 2009).

Congenital abnormalities were noted for salbutamol and a NOAEL for teratology can be set at 10.75 mg/kg BWt/day based on the oral dose study in pregnant rats (Health Products Regulatory Authority, 1983). This is similar to the NOAEL values for ractopamine of 15 mg/kg BWt set for teratological effects in pregnant rats (EFSA, 2009) and 0.2 mg/kg BWt for zilpaterol due to teratological effects in pregnant rats and rabbits (EFSA, 2016).

Repeat dose toxicity studies indicated a NOAEL of 12 mg salbutamol/kg BWt/day for R-salbutamol in Swiss Albino mice (Bhide, 2010b).

A NOAEL of 10.75 mg /kg BWt/day was determined from the teratological effects in foetuses from pregnant rats (Health Products Regulatory Authority, 1983).

2.8 Acceptable Daily Intake (ADI) Calculations

2.8.1 Standard Method of Calculation

The standard accepted method as drafted by the Committee for Veterinary Medicinal Products (CVMP), which forms part of The European Agency of the evaluation of Medicinal Products (EMA) (CVMP EMA, 2001), for the determination of an ADI is to first establish a NOAEL and thereafter determine a satisfactory uncertainty factor (UF). Uncertainty factors are applied when extrapolating data from the toxicity observed in laboratory animals to health risks in humans, this usually being a factor of 10 for interspecies difference and a factor of up to 10 for human variability (depending on the extent and quality of available human data) (Lozano & Trujillo, 2012). Additional uncertainty factors can be applied depending on the nature of the toxic effect. Toxic end points which has been given additional factors include, carcinogenicity, teratogenicity and where steep dose responses are seen above the NOAEL (Renwick, 1995).

Calculation of the ADI for R-salbutamol can therefore be as calculated from the NOAEL value of 10.75 mg/kg BWt/day as described in the rat maternal and foetal toxicity study (section 2.7.6). The only effect observed in this study (Health Products Regulatory Authority, 1983) was neonatal mortalities, at 50 mg/kg BWt/day, which was attributed to a lack of maternal care and not due to any direct effect of salbutamol. According to Renwick (1995) an additional uncertainty factor cannot be applied to one study due to an observed effect through a different route of administration (oral vs. subcutaneous) or between studies in different species (i.e. rabbits vs. rats). The importance of possible teratological effects can however not be ignored and an additional safety factor of 10 is suggested to be applied in this instance as pregnant mothers can potentially ingest residues of salbutamol in tissue from animals treated with R-salbutamol.

The formula used to calculate the ADI (expressed as mg/kg BWt/day) is as follows (CVMP EMEA, 2001):

$$ADI = \frac{NOAEL}{UF}$$
$$ADI = \frac{10.75 \text{ mg/kg/day}}{(10 \times 10 \times 10)}$$
$$= 0,011 \text{ mg/kg BWt/day}$$

The total ADI expressed on a body weight basis using 60 kg BWt as the standard adult human weight. The total ADI would therefore be 0.66 mg/day.

2.8.2 Alternative published ADI Calculation

In a report compiled by the Institute of Environment and Health (Holmes, et al., 2007) for the Department for Environment Food and Rural Affairs, UK Government, a NOAEL of 17 mg/kg BWt/day for cardiotoxicity from a one-month oral study in rats was used. To allow an assessment of the calculated racemic salbutamol intakes, a project-specific ADI of 0.017 mg/kg BWt/day was derived by dividing the NOAEL by a total uncertainty factor of 1000 to allow for intra- and inter-species variation with an additional factor of 10 due to the limited amount of data available in the review by Holmes et al. (2007).

2.8.3 FDA Adopted Alternative Method for ADI Calculation

The FDA has adopted a new method for calculating ADI for MRL determination based on actual human data. This method is based on the research of Matthews et al. (2004) who investigated the use of human data derived from pharmaceutical clinical trials to estimate the NOEL value of a molecule rather than relying on laboratory animal models. They believe that this data provides a more specific estimate of the toxic effects of molecules in humans than the historically used values extrapolated from animals with assumed safety factors applied. This investigated value is referred to as the Maximum Recommended Therapeutic Dose (MRTD). The values for the investigated molecules can be found in the FDA Maximum (Recommended) Daily Dose Database (Benz, 2013).

According to Matthews et al. (2004) the NOAEL is defined as MRTD/10. The MRTD for salbutamol was set at 0.533 mg/kg BWt/day.

Therefore:

$$\begin{aligned} NOAEL &= \frac{MRDT}{10} \\ &= \frac{0.533 \text{ mg/kg}}{10} \\ &= 0.053 \text{ mg/kg BWt/day} \end{aligned}$$

The uncertainty factor of species difference will not apply in this calculation as the NOAEL is derived from human data. A 10 times uncertainty factor should however be applied to account for individual differences.

$$\begin{aligned} ADI &= \frac{NOAEL}{10} \\ &= \frac{0.0533 \text{ mg/kg}}{10} \\ &= 0.0053 \text{ mg/kg BWt/day} \end{aligned}$$

The ADI for a 60kg adult human would therefore be 0.032 mg/day.

2.9 Acute Reference Dose Calculation

Since R-salbutamol would most probably have a greater acute than chronic effect. (Boulton & Fawcett, 1996b), it is important to calculate the Acute Reference Dose (ARfD) for R-

salbutamol. These acute effects are dose dependant and include tremors, tachycardia, heart palpitations and muscle cramps (Health Products Regulatory Authority, 1983).

The basic approach for the derivation of an ARfD is based on the identification of an appropriate point of departure (POD), or threshold, for the pharmacological or toxicological endpoint of concern. This is typically identified as a NOAEL or bench mark dose lower confidence limit (BMDL). The ARfD is determined by dividing this POD (or NOAEL) by an appropriate Uncertainty Factor (UF) (JECFA, 2016).

The ARfD can be reported as an amount of the substance expressed on a per person or body weight basis (e.g. mg/person or mg/kg body weight)

The formula for calculation of the ARfD is (JECFA, 2016):

$$ARfD = \frac{POD}{UF}$$

Where:

- POD is the point of departure or threshold for pharmacological or toxicological effects of concern (alternatively referred to as the NOAEL)
- UF is an uncertainty or safety factor, or series of factors that typically account for considerations such as animal to animal variability, interspecies extrapolation, quality of data, severity of response, etc.

In the EFSA (2016) evaluation of Zilpaterol, the ARfD was also chosen to be the most accurate indicator of human safety rather than the ADI calculated from chronic studies. The available data used were from a LOAEL set at 0.00076 mg/kg BWt derived from a study in asthmatic patients. The uncertainty factor applied was 10 for individual variability and a factor of 2 due to the use of a LOAEL value instead of a NOAEL value. The ARFD for zilpaterol were subsequently set at 0.00004 mg zilpaterol/kg BWt per day.

Similarly, the EFSA evaluation (2009) for ractopamine used a NOAEL value of 0.067 mg/kg BWt but due to the experimental weaknesses in the human trial assessed, an uncertainty factor of 50 (10 for individual variability and 5 for uncertainty of the data from the human trial) was applied. The ARfD for ractopamine were set at 0.001 mg ractopamine/kg BWt per day.

If the LOAEL value of 0.067 mg R-salbutamol/kg BWt as calculated in section 2.7.5 and the formula as used in the zilpaterol EFSA evaluation (2016) is applied the ARfD for R-salbutamol will be as follows:

$$ARfD = \frac{POD}{UF}$$

$$ARfD = \frac{0.067 \text{ mg/kg BWt}}{20}$$

$$ARfD = 0.003 \text{ mg/kg BWt}$$

Should we use the derived NOAEL as calculated in section 2.7.5 the factor of 2 for correction of data derived from a NOAEL value instead of a LOAEL can be excluded and only a factor of 10 need to be applied. The POD of R-salbutamol for the calculation of the ARfD is therefore 0.033 mg/kg BWt. The calculation is thus as follows:

$$ARfD = \frac{POD}{UF}$$

$$ARfD = \frac{0.033 \text{ mg/kg BWt}}{10}$$

$$ARfD = 0.003 \text{ mg/kg BWt}$$

The two methods of calculations calculated to the same value and therefore would indicate that the calculation of the NOAEL value from the Boulton and Fawcett (1997) trial is accurate.

2.10 ADI or ARfD?

The ADI's calculated from the long-term toxicity studies as well as the three additional published methods (CVMP EMEA, 2001; Holmes, et al., 2007; Matthews, Kruhlak, Benz, & Contrera, 2004) range from 0.011 – 0.53 mg/kg BWt.

As stated in section 2.6, the ARfD would be considered if it is lower than the ADI and below 1mg/kg BWt.

All the required criteria have been met as discussed above and as both the safe intake level for zilpaterol (EFSA, 2016) and ractopamine (EFSA, 2009) are also based on the ARfD, the ARfD of 0.003 mg/kg BWt (rounded as per JECFA standard procedure) will be used as the safe maximum intake for R-salbutamol. The ADI in humans for R-salbutamol can therefore be set at 0 - 3 µg/kg BWt using the ARfD as the upper limit of the ADI (JECFA expresses ADI as µg/kg BWt).

2.10.1 Human safety conclusion

Comparing the calculated ADI (derived from the ARfD) of that R-Salbutamol (<3 µg/kg BWt) with that of Zilpaterol (0 - 0.04 µg/kg BWt) (EFSA, 2016) and Ractopamine (0 - 1 µg/kg BWt) (EFSA, 2009) it is apparent that R-Salbutamol has a higher safety margin in humans than either Zilpaterol or Ractopamine.

2.11 Conclusion

The improved safety profile of R-salbutamol in comparison to currently available β-AA for use in food producing animals warrants further investigation into the residue levels of R-salbutamol in cattle and sheep to determine the MRL as well as determine a safe withdrawal period of R-salbutamol for use in cattle and sheep feedlot production.

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CHAPTER 3

R-SALBUTAMOL RESIDUE DEPLETION STUDY IN FEEDLOT LAMBS

ABSTRACT

This study investigated the residue kinetics and target animal tolerance of R-salbutamol in feedlot lambs. Group A consisted of one randomly selected lamb slaughtered as the negative control to indicate that there were no residues occurring naturally within the test flock. Group B and C (6 lambs each) were fed 21 mg R-salbutamol per day (0.5 mg/kg BWt) in feed for 28 days. Heart rate, respiratory rate and rectal temperature were recorded at day -1 and 28. R-salbutamol was withdrawn from the feed for 7 hours prior to slaughter (group B) and 24 hours prior to slaughter (group C). Liver, kidney, muscle and fat samples were collected and analysed for salbutamol residues (LC-MS/MS). No significant differences were observed in heart rate in the non-medicated and medicated period. Significant differences were observed for rectal temperature and respiratory rate between day -1 and 28. Rectal temperature differences were of no clinical significance and within the normal range. The difference in respiration rate were attributed to lower respiratory infection. No visual adverse events were observed in the study lambs. Salbutamol residues were highest in the liver, followed by kidney, muscle and fat at both withdrawal times. Almost ninety percent of all parent salbutamol residues were present in the liver. At 24 hours the average muscle residue was below the limit of quantification. Residue levels were more than 50% lower at 24 hours withdrawal compared to the levels at 7 hours. The theoretical maximum daily intake of salbutamol for humans is less than 10% of the acceptable daily intake. Therefore, in conclusion, the animal tissue from feedlot lambs fed R-salbutamol according to the prescribed dosage in feed, will provide adequate consumer protection at zero-day withdrawal.

3.1 Introduction

Beta-adrenergic agonists (β -AA) have been researched and used in the feedlot industry for more than 30 years (Merck Animal Health, 2017). Zilpaterol was the first beta-agonist to be investigated and registered for use as a growth enhancer in feedlot cattle. Today, Zilpaterol is widely used in sheep feedlots in South Africa under veterinary prescription, as no commercial zilpaterol product has been registered with the Fertilizer, Farm Feeds, Agricultural Remedies

and Stock Remedies Act of 1947 (Act 36 of 1947) as a stock remedy for use in feedlot lambs (Allen, 2015). In fact, as far as could be ascertained, no zilpaterol product is registered for use in feedlot lambs anywhere in the world. Zilpaterol has however been tested extensively in feedlot lambs and the efficacy as a growth enhancing technology has been confirmed (Estrada-Angulo, et al., 2008; López-Carlos, et al., 2010; López-Carlos, et al., 2011; Pringle, Calkins, Koochmaraie, & Jones, 1993; Vahedi, et al., 2014; Webb, Allen, & Morris, 2018). An official safety evaluation by the European Food Safety Authority (EFSA) has not been done on the residue kinetics of zilpaterol in lambs and no official withdrawal period has been determined in feedlot lambs. No published data on residue kinetics of Zilpaterol in sheep could be found.

A new selective β 2-AA, Salbutamate 10% (R-salbutamol sulphate – 100 g/kg) has been registered in South Africa (Reg. No. G4084 (Act 36 of 1947)) for feedlot cattle and lambs (Animate Animal Health, 2017). The need for a registered, safe β -AA growth enhancer for feedlot lambs was identified and subsequent production research has been done on the possibility for use in feedlot lambs (De Klerk, 2016; Du Toit, 2017). As part of the registration intent, research had to be completed with regards to the metabolism and residue kinetics of R-salbutamol in feedlot lambs.

Due to the varying half-life, metabolism and excretion between species and between molecules within the β -AA class, species specific research with regards to the metabolism and residue depletion is of utmost importance. According to the VICH Guidelines (VICH Steering Committee, 2015) the purpose of such trials is to demonstrate the depletion of the marker residue once the drug is withdrawn from treatment and to determine how long after withdrawal a regulatory safe residue level is reached. In the case of R-salbutamol, an international Maximum Residue Limit (MRL) has not been set and as part of this study the suggested MRL will be calculated to assist in determining the safe level in tissue for human consumption and subsequent safe withdrawal period.

3.2 Materials and Methods

The research project was approved by Animal Ethics – ARC: Animal Production Institute (Irene) (APEIC15/06) and handling and housing of all animals were conducted according to the South African National Standards 10386: 2008.

The trial was conducted at the Agricultural Research Council: Animal Production Institute facility (Irene, Gauteng, South Africa).

3.2.1 Animals

The animals used in this study were male, Dorper type lambs (4-5 months of age) with an average weight of 42.53 ± 1.14 kg at arrival. Animals were purchased from Cavalier Feedlot, Kameeldrift, Pretoria. Thirteen lambs were randomly allocated to one of three groups according to a randomised, parallel group design. The individual ovine were the experimental unit in this study.

3.2.2 Housing

Indoor stable units with individual metabolic stanchions were used during the trial. Stables were not environmentally controlled and environmental conditions fluctuated with ambient conditions. The lambs were able to socialise through visual interaction as the stanchions were next to each other and divided by metal bars. Physical interaction was however restricted.

The identification number of the lamb housed inside a stanchion, the study number, brand of feed and feed quantity were indicated on the outside of each stanchion. The size of each indoor stanchion was 1.5 m².

Routine practices were standardised across all animals. All lambs were at the study facility for 7 days prior to the start of the R-salbutamol administration.

Prior to the start of the trial the facility was washed and cleaned, and stanchions and the floor of the house were washed at the end of each day to ensure health and hygiene of the animals.

3.2.3 Feed and water

The rams purchased were already processed and fed a grower ration at the feedlot. Therefore, feed acclimatisation was not required once they arrived at the trial facility. A balanced, commercial sheep feedlot finisher ration, sourced from Cavalier Feedlot, Kameeldrift, Pretoria was fed (Table 3.1). Feed requirement adjustments were made daily by observing animal behaviour associated with feeding.

Feed and potable water were supplied in containers at the front of the stanchion.

A 250 g specimen of each batch of feed was collected and analysed to confirm that no levels of R-salbutamol were present in the feed received from Cavalier Feedlot.

Table 3.1: Nutrient composition of sheep feedlot finisher ration

Nutrient	Quantity (g/kg)
Crude Protein	160
Fat	50
Fibre	170
Moisture	120
Calcium	10
Phosphorus	2

3.2.4 Experimental Procedure

Two groups of six animals each received R-salbutamol orally in feed twice daily for 28 days.

Feeding of animals were according to Standard Operating Procedures: SR006 – Small Ruminants – Dispensing feed to animals in metabolic crates (Agricultural Research Centre, Irene). Lambs was fed twice daily at 8am and 4pm. During the acclimation phase the intake of each individual lamb was determined to ensure that each lamb consumed 100% of its daily feed. Leftover feed was weighed each afternoon and the next day's feed allocation was adjusted accordingly. Each animal received 21 mg R-salbutamol per animal per day (± 0.5 mg/kg body weight (BWt) at start weight). The daily dosage of R-salbutamol was divided in 2 equal doses and administered as a top-dressing to the feed for a period of 28 days. R-salbutamol was administered as a feed additive, as it will be marketed and sold as a feed additive for feedlot lambs. R-salbutamol was used at the intended dose, dosage frequency and duration required to imitate the sheep feedlot situation.

Personnel wore overcoats, impervious gloves and a dust mask during administration and handling of R-salbutamol and the medicated feed.

3.2.5 Animal handling

The lambs were handled by personnel in the stanchion during clinical examination. Animals were loaded on to a vehicle for transport to the on-site abattoir at the end of the study. The abattoir is situated on site at the ARC-API Irene. No other methods of restraint were applied.

3.2.6 Animal Observations

General health observations were performed daily throughout the the study. The aim of the general heath observation was to detect any health issues that may warrant veterinary care

or constitute withdrawal of animals from the study. These observations included, but were not limited to, habitus, appetite, colour of urine, colour and consistency of faeces, salivation, vomiting, skin lesions and an obvious change in general health condition.

Clinical examinations were performed once during the acclimatisation period 2 days prior to the start of the medication period (at 10:00 in the morning) and again on the last day of the medication period (at 8:45 in the morning). These examinations included heart rate, respiratory rate and rectal temperature. Clinical observations were performed in the station following a period with the animal to allow them to get used to the handler. Heart rate was determined by means of a stethoscope held on the left side of the thorax (low third intercostal space). Beats per minute were counted for 15 seconds, multiplied by 4 and recorded. Respiratory rate was determined by counting chest movement for a 15 second period and multiplying it by 4. Rectal temperature was measured with a digital thermometer placed in the rectum of the sheep following heart rate and respiratory rate monitoring.

The first observation was to determine the clinical parameters prior to the start of the R-salbutamol treatment and the observations at the end of the study, prior to withdrawal of R-salbutamol, were to determine if R-salbutamol had any effect on heart rate, respiratory rate and rectal temperature.

3.2.7 Feed withdrawal and slaughter schedule

The trial design is based on the VICH guideline GL48 (Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Marker residue depletion studies to establish product withdrawal periods) (VICH, 2015).

The guidelines applicable to products intended for zero-day tissue withdrawal require only one time-point with 6 animals at the time point between 3- and 12-hours following withdrawal. This constitutes zero-withdrawal. The zero-day tissue withdrawal intent were based on the cattle study (Chapter 4) where no residues were detected in any tissue following 12 hours withdrawal.

An additional 24 hours withdrawal point were included in the trial design to evaluate residue depletion should any residues be detected at the 7 hours withdrawal time point.

Group A, consisting out of 1 animal, did not receive R-salbutamol and was slaughtered after the acclimation period as negative control (study day +1). Group B and C, consisting out of six animals each receiving R-salbutamol, were slaughtered at 7 hours and 24 hours after withdrawal of R-salbutamol medicated feed, respectively. The carcasses of Group B and C

were disposed of at the abattoir and were not intended for human consumption. The control animal (group A) entered the food chain. No animals were removed after inclusion in the study.

The R-salbutamol medicated feed of groups B and C were removed on the morning of day 29 at 7 hours and 24 hours before slaughter, respectively. The feeding troughs were cleaned, and all the animals received non-medicated feed without R-salbutamol for the remainder of the period until they were slaughtered.

Slaughter schedule:

- Day 1 one control animal from Group A.
- Day +29 Group B animals (n=6) (7:38 – 7:58 hours (<12 hours) after last R-salbutamol consumption)
- Day +30 Group C animals (n=6) (24:47 – 24:58 hours (24 ± 2 hours) after last R-salbutamol consumption)

Specimens of muscle, kidney, liver and renal fat were collected at slaughter for determination of R-salbutamol residue levels.

3.2.8 Body weight (BWt)

The animals were weighed during acclimatization on study day -6 for the purposes of inclusion and allocation to study groups and again on the morning of R-salbutamol withdrawal (study day +29) to be able to determine the average mg /kg BWt intake of R-salbutamol.

3.2.9 Organ sampling for residue analysis

Specimens of the muscle, kidney, liver and renal fat were collected for determination of R-salbutamol concentrations on day +29 (7 hours withdrawal) and day +30 (24 hours withdrawal). The samples taken from the control animal slaughtered at the start of the medicated feed period (day +1) were used as the negative control during laboratory analysis to ensure that the method used for analysis were accurate for the determination of R-salbutamol in animal tissue. Negative control tissue samples were also used to spike samples in the lab to a known amount to determine accuracy of laboratory results.

Duplicate specimens of approximately 250 gram or as much as is physically possible, of muscle, kidney, liver and renal fat were collected from each animal. Extreme care was taken to avoid contamination of the tissues. Separate knives were used for each animal and the

operator(s) took precautions to remove any contaminated clothing and washed their hands between sampling of each animal.

If an organ became contaminated by touching another collected organ, the outside of both organs was washed under running potable water. Collected organ specimens were placed in labelled freezer bags of suitable size and sealed air tight at the time of slaughter. Labels indicate the tissue specimen, animal ID, study group, date of collection, collection time and the study number. Organ specimens were kept cool in polystyrene containers with ice and frozen within 24 hours after collection at $<-20^{\circ}\text{C}$. All organ specimens were dispatched to the analytical laboratory (ARC OVI Residue Laboratory, Pretoria) after the last sample collections.

Liver, muscle, kidney and fat samples of Test Group A, B and C were analysed for parent and total (parent + metabolites) salbutamol residues.

3.2.10 Analytical Method

Samples were analysed at the Agricultural Research Council (ARC) Residue Laboratory at Onderstepoort Veterinary Institute (OVI). Method validation was performed to demonstrate that the LC-MS/MS method was fit-for-purpose in the identification of R-salbutamol in animal tissue.

The correlation coefficient for the calibration curve of all tissues were >0.99 .

Racemic Salbutamol was purchased from Sigma Aldrich (Lot # SZB9016XV, 99.8% purity). HPLC grade solvents (Ammonium formate, Methanol (MeOH), trichloroacetic acid (TCA) Acetonitrile (ACN), β -glucuronidase) were supplied by Riedel-de Haën and Sigma Aldrich. HPLC-grade H_2O were obtained from an Elgastat UHQ water purifier system.

Solutions were made as follow:

- 1M Ammonium formate (dissolve 6.408 g in 100 ml H_2O)
- 5mM Ammonium formate (5 ml of 1M ammonium acetate to 1000ml of H_2O or MeOH)

A Kinetex 2.6 μ PFP 100 Å, 50 x 2.10 mm, analytical column was used during the analysis.

Ultra High Performance Liquid Chromatograph (UPLC) analysis was performed using a Shimadzu Nexera X2 analyser and mass spectrometry (MS) was performed using an ABSciex Triple quadruple mass spectrometer.

Aliquots of 5 ± 0.1 g of liver, muscle and kidney, and 1 ± 0.1 g of fat were placed in homogeniser tubes together with three blank samples and 7 spiked samples at 0.5, 1, 2, 5, 10, 20 and 50 ppb. Spiked samples were then vortexed for 30 seconds. Acetate buffer (1M,

200 μL , pH 5.2) together with 3.8 ml dH_2O was then added to the samples and tissue was homogenised for approximately 1.5 min. For the total residue detection method 50 μL β -glucuronidase was added to the sample, gently mixed and incubated at 37°C for 3 hours. For the samples analysed for parent salbutamol this step was omitted.

TCA 10% (100 μL) and ACN (10 ml) containing 1% (v/v) acetic acid were then added and vortexed. Thereafter, 4 g MgSO_4 and 1 g NaCl were added and thoroughly mixed for 1 min on the vortex. The samples were then frozen at this point at -20°C for 2 hours. Samples were then centrifuged at 9500 g for 10 min, ensuring the centrifuge remained at 4°C .

A total of 5 ml of the content of the tube was then transferred to a 50 ml tube containing 150 mg C_{18} and vortexed. The sample was centrifuged again at 9500 g for 10 min at approximately 4°C . The remaining fluid was carefully decanted into a test tube and dried completely at 50°C . The dried extract was then reconstituted with 300 μL 50% MeOH followed by filtration through 0.22 μm PTFE filters. The solution was transferred to a vial and injected on the LC-MS auto-sampler.

The Limit of Quantification (LOQ) for this method was determined to be 0.5 $\mu\text{g}/\text{kg}$.

3.2.11 Statistical Analysis

A primary statistical method to determine the withdrawal period from each of the body tissues is described in the guidelines on MRL's and Withdrawal Periods by the Medicines Control Council of South Africa (MCC) (MCC, 2004).

The protocol describes the conventional statistical methods based on a regression analysis to be applied for the determination of withdrawal periods. However, as two time points with six animals each were chosen as the design for the residue trial, as described in the MCC guidelines for zero withdrawal, no regression analysis was possible. The alternative method to determine the withdrawal period, as set out in the MCC guidelines, is to set the withdrawal period at the time point where the calculated Theoretical Maximum Daily Intake (TMDI) is below the Acceptable Daily Intake (ADI).

To calculate the TMDI, the average residue in each tissue (i.e. liver, muscle, kidney and fat), as determined at each withdrawal time point, is used and added to the product of the standard deviation (SD) multiplied with the food consumption factor (CF). The CF was described by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in their 34th report entitled "Evaluation of Certain Veterinary Drug Residues in Food" (JECFA, 1989).

The consumption factor for each tissue type is summarised in Table 3.2.

Table 3.2: Food Consumption Factors for each tissue type as defined by JECFA

Tissue	Daily Intake
Muscle	300 g
Liver	100 g
Kidney	50 g
Fat	50 g

A one-sided upper tolerance limit or upper confidence limit (UCL) is then calculated by adding the calculated theoretical intake of residues in the animal tissue and adding the product of the standard deviation (SD) with the g^{TM} factor.

$$UCL = \text{mean} + (g^{\text{TM}} \times SD)$$

Where:

g^{TM} = A factor for calculating a one-sided tolerance estimate of conformance with a given threshold and confidence level.

The g^{TM} factor is based on the number of animals used at each time point in the residue kinetic study and the accepted statistical significance of the data as well as the statistical confidence. MCC has accepted the use of a 95% statistical significance with a 90% statistical confidence (MCC, 2004). The table with the relevant g^{TM} factors is published in the MCC guidelines on MRL's and Withdrawal Periods (MCC, 2004).

Using the sample size ($N = 6$) in this trial at each time point, the g^{TM} parameter applicable for this trial equals 3.066.

All data is reported as the average \pm standard error of the mean (SEM) unless otherwise indicated.

3.3 Results and Discussion

As the feed used in the trial was spiked twice daily with the test item it had to be confirmed that the initial feed was free from R-salbutamol or any other β -AA. Feed was tested for R-salbutamol and zilpaterol and it was confirmed that the feed did not contain any beta-agonist.

The average body weight at the start of the trial (day -6) was 42.53 ± 1.14 kg and at the end of the trial (day +29) 48.72 ± 1.61 kg. The ADG for all animals in the trial was only 162 g/day,

which would be expected to be much lower than normal feedlot performance of 300 - 350 g/day, as animals were not fed *ad lib*.

There were no R-salbutamol related physical signs (tremors and restlessness) recorded during daily general health observations of animals in this study. Horses fed zilpaterol at 0.17 mg/kg BWt showed signs of profuse sweating, skeletal muscle tremors and restlessness 20 to 25 minutes after ingesting zilpaterol. These symptoms continued for a week following the zilpaterol ingestion (Wagner, Morstrom, Hammer, Thorson, & Smith, 2008).

Nasal discharge, that was observed in 2 of the 12 lambs at the start of the trial (day -6 to -4), and again at day 16, was associated with apparent ovine respiratory disease, as diagnosed by a veterinarian, and not considered to be related to R-salbutamol treatment.

Ocular discharge and depressed behaviour were observed in 6 of the 12 lambs from day -6 to -2, and again from day 11 to 28. This was diagnosed as infectious keratoconjunctivitis by a veterinarian.

The nasal discharge, depressed behaviour and ocular discharge can possibly be attributed to lower respiratory tract infection, caused by viruses, bacteria, or parasites (Scott, 2018)

Heart rate, respiratory rate and rectal temperature was measured at day -1 and Day 28 of the trial. There was no difference ($P > 0.05$) in the heart rate of the lambs measured at day -1 (111.83 ± 4.39 bpm) and day 29 (114.83 ± 3.79 bpm). This data is in alignment with the observations of Marchant-Forde et al. (2008) where no effect was observed on the heart rate of pigs fed different levels of R-salbutamol. Similar observations were made in 2 unpublished trials performed in Australia by the developers of R-salbutamol for production animal use (Chambers, 2007a, b).

This is in contrast with the effect on heart rate that was observed with the supplementation of various other longer acting beta-agonists in cattle, lambs, goats and pigs. Mersmann (1987) reported an increase in heart rate in pigs of between 36% and 83% for isoproterenol, 15% to 43% for dobutamine, and 43% to 46% for terbutaline. The increase of heart rate was dose related for all β -AA's used in these studies. Increased heart rate ($P \leq 0.05$) was also observed in cattle fed ractopamine and zilpaterol (Frese, 2015) and a similar effect on the increase in heart rate ($P \leq 0.05$) was observed in lambs fed cimaterol (Beerman, Hogue, Fishell, Dalrymple, & Ricks, 1986) and goats fed zilpaterol (Hatefi, et al., 2017).

Differences ($P \leq 0.05$) were however observed in the respiratory rate and rectal temperature of lambs between day -1 and day 28. Rectal temperature increased from $38.97 \pm 0.07^\circ\text{C}$ (day -1) to $39.5 \pm 0.16^\circ\text{C}$ (day 28). Normal rectal temperature in lambs is between 38.3°C and

39.9°C (Fielder, 2018a), therefore the measurements were within the normal range and not clinically significant.

The respiratory rate also increased from 47.92 ± 5.01 (day -1) to 81.33 ± 9.55 (day 28) breaths per minute after inclusion of R-salbutamol. Normal respiratory rate in lambs is between 16 and 34 breaths per minute (Fielder, 2018b). The respiratory rate on day -1 was slightly higher than normal, but respiratory rate at day 28 could be classified as hyperventilation.

Environmental temperature was not recorded.

The increase in respiratory rate was observed in 7 of the 12 lambs of which 6 of the 7 lambs showed clinical signs of possible lower respiratory tract infection. Increased respiratory rate is one of the clinical signs of lower respiratory tract infection and commonly affect sheep (Scott, 2018).

The 7 lambs with increased respiratory rate had an average respiratory rate of 106.14 ± 5.38 at day 28 in comparison to the remaining 5 lambs with a respiratory rate of 43.5 ± 5.03 breaths per minute. The increase in respiratory rate could therefore be attributed to the lower respiratory tract infection rather than the use of R-salbutamol.

An increase in respiratory rate, attributed to the use of β -AA, was observed in previous studies with the use of zilpaterol in cattle (Boyd, et al., 2015; Hales, et al., 2014) and goats (Hatefi, et al., 2017), as well as with the use of clenbuterol in milk replacer calves (Blum & Flueckiger, 1988).

Many studies have been performed where the total salbutamol residue (parent + metabolised salbutamol) is the target residue for detection. These analysis methods include hydrolyses of the sample with β -glucuronidase enzyme (in animal tissue) to extract the metabolised salbutamol fraction and report total salbutamol residues (parent + metabolites) in the target tissue. These methods are however developed to assist in determining the illegal use of beta-agonists in countries where their use is not approved (Chang, Chang, & Tsai, 2018; Montes Nino, et al., 2017; Yi-Po, et al., 2016).

Parent salbutamol was tested as the marker residue in the present investigation. As R-salbutamol is metabolised through glucuronide conjugation in animals and the metabolite is not active (Martin, Hobson, Page, & Harrison, 1971), the parent salbutamol fraction is considered for residue detection and is the marker residue for residue analysis. This is in accordance with the marker residues accepted for both zilpaterol (EFSA, 2016) and ractopamine (EFSA, 2009). However, to better understand the metabolism and excretion of R-salbutamol in this study, the analysis method was repeated on each sample with the

additional hydrolysis step to enable detection of total salbutamol residue (parent + metabolised salbutamol).

Table 3. summarises the parent salbutamol residue levels as determined for all tissue of animals slaughtered at the 7 hour and 24-hour time point as well as the negative control animal.

Table 3.3: Mean concentration (\pm standard deviation) of parent salbutamol in the tissue of lambs

Study Day	Group	Parent salbutamol ($\mu\text{g}/\text{kg}$ in tissue) \pm Standard Deviation			
		Muscle	Kidney	Liver	Fat
+1	A (Control)	<LOQ*	<LOQ	<LOQ	<LOQ
+29	B (7hrs)	0.79 \pm 0.80**	6.32 \pm 3.78	75.80 \pm 23.18	2.03 \pm 1.38
+30	C (24hrs)	0.44 \pm 0.47***	2.73 \pm 1.78	33.82 \pm 12.56	0.98 \pm 1.20****

*LOQ = limit of quantification (0.50 $\mu\text{g}/\text{kg}$ tissue)

** 3 of 6 samples tested <LOQ

*** 5 of 6 samples tested <LOQ

**** 2 of 6 samples tested <LOQ

Where the analysed value is below the LOQ (0.50 $\mu\text{g}/\text{kg}$) the value is taken as half the LOQ (0.25 $\mu\text{g}/\text{kg}$) to determine the average and standard deviation as per the MCC guidelines (2004) and the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) (VICH, 2015).

At both time points, the highest average residues of parent salbutamol in tissue were detected in the liver (85.4 - 93.7% after 7 hours and 83.2 - 94.2% after 24 hours withdrawal) followed by the kidney (4.7 - 10.4% after 7 hours and 0.8 - 12.7% after 24 hours withdrawal). Muscle had 0.3 - 2.4% after 7 hours and 0.4 - 4.3% after 24 hours withdrawal of all residues and fat had 1.3 - 3.8% after 7 hours and 0.6 - 5.1% after 24 hours withdrawal. Variation did occur between animals with regards to the distribution of percentage of total parent salbutamol in tissue but in all instances, liver had by far the highest residue level followed by kidney. Variation also occurred between animals at both time points with regards to whether fat or muscle tissue had the highest residue levels.

Three of the 6 muscle samples had no detectable residues at 7 hours after withdrawal, while 5 of the 6 samples at 24 hours after withdrawal had no detectable residues of parent salbutamol. All fat samples had detectable residue levels of parent salbutamol after 7 hours withdrawal, and two fat samples were below the LOQ after 24 hours withdrawal.

To determine the total fraction of parent salbutamol together with the major metabolite, samples were hydrolysed with β -glucuronidase as an additional step in the extraction as described in section 3.2.10. The total salbutamol for all 4 tissues is summarised in Table 3.3.

Table 3.3: Mean concentration (\pm standard deviation) of total salbutamol (parent + metabolite) in the tissue of lambs

Study Day	Group	Total salbutamol ($\mu\text{g}/\text{kg}$) \pm Standard Deviation			
		Muscle	Kidney	Liver	Fat
+1	A (Control)	<LOQ*	<LOQ	<LOQ	<LOQ
+29	B (7hrs)	3.33 \pm 2.66	15.93 \pm 16.01	181.27 \pm 74.8	19.03 \pm 9.49
+30	C (24hrs)	2.62 \pm 0.52	8.58 \pm 3.62	91.31 \pm 37.76	6.58 \pm 7.18

*LOQ = limit of quantification (0.50 $\mu\text{g}/\text{kg}$ tissue)

At 7 hours after withdrawal of R-salbutamol in feed, 41.8% of the total residues was present as parent salbutamol in the liver compared to total salbutamol (75.8 $\mu\text{g}/\text{kg}$ parent salbutamol vs. 181.27 $\mu\text{g}/\text{kg}$ total salbutamol). At 24 hours after withdrawal the amount of parent salbutamol reduced slightly to 37.0% of total salbutamol in the liver (33.82 $\mu\text{g}/\text{kg}$ parent salbutamol vs. 91.32 $\mu\text{g}/\text{kg}$ total salbutamol). The average total salbutamol at 24 hours after withdrawal (91.32 $\mu\text{g}/\text{kg}$ total salbutamol) was however reduced to 50% of that detected at 7 hours after withdrawal (181.27 $\mu\text{g}/\text{kg}$ total salbutamol) in the liver.

Less parent salbutamol was detected in the kidney as a proportion of total salbutamol at both withdrawal time points with 39.6%, and 31.7% of parent salbutamol as a percentage of total salbutamol detected at 7 hours (6.32 $\mu\text{g}/\text{kg}$ parent salbutamol vs. 15.93 $\mu\text{g}/\text{kg}$ total salbutamol) and 24 hours (2.73 $\mu\text{g}/\text{kg}$ parent salbutamol vs. 8.58 $\mu\text{g}/\text{kg}$ total salbutamol) respectively. The average total salbutamol at 24 hours after withdrawal (8.58 $\mu\text{g}/\text{kg}$ total salbutamol) was also reduced to almost half (54%) of that detected at 7 hours after withdrawal (15.93 $\mu\text{g}/\text{kg}$ total salbutamol) in the kidney.

Muscle had 23.8% parent salbutamol as a percentage of total salbutamol at 7 hours after withdrawal (0.79 $\mu\text{g}/\text{kg}$ parent salbutamol vs. 3.33 $\mu\text{g}/\text{kg}$ total salbutamol), but the percentage dropped to 16.9% after 24 hours withdrawal (0.44 $\mu\text{g}/\text{kg}$ parent salbutamol vs. 2.62 $\mu\text{g}/\text{kg}$ total salbutamol). The average total salbutamol at 24 hours following withdrawal (2.62 $\mu\text{g}/\text{kg}$ total salbutamol) was 78.5% of the total salbutamol detected at 7 hours after withdrawal (3.33 $\mu\text{g}/\text{kg}$ total salbutamol) in the muscle, but at very low levels as indicated in Table 3.3.

The concentration of total salbutamol declined more rapidly in fat than in any other tissue with only 34.6% of total salbutamol still present at 24 hours (6.58 µg/kg total salbutamol) after withdrawal in comparison to the levels at 7 hours after withdrawal (19.03 µg/kg total salbutamol). Parent salbutamol were the lowest as a percentage of total salbutamol in fat with 10.7% of total salbutamol present as parent salbutamol at 7 hours after withdrawal (2.03 µg/kg parent salbutamol vs. 19.03 µg/kg total salbutamol) and 14.9% of total salbutamol present as parent salbutamol at 24 hours after withdrawal (0.98 µg/kg parent salbutamol vs. 6.58 µg/kg total salbutamol).

As the MRL has not been set for R-salbutamol, the trial data can be used to determine a TMDI according to the MCC guidelines (MCC, 2004) from which a MRL can be derived. The formula is described in detail in section 3.2.11. The UCL is determined for each tissue type by adding the product of the SD with the g^{TM} factor. Table 3.5 describes the calculation for the UCL at 7 hours following withdrawal and Table 3.6 describes the calculations for the UCL at 24 hours withdrawal. The UCL for each tissue type is then multiplied by the relevant consumption factor to determine the TMDI (Table 3.7).

Table 3.5: UCL for parent salbutamol in liver, kidney, muscle and fat at 7-hours withdrawal

	Muscle	Liver	Kidney	Fat
Average Residue (µg/kg)	0.79	75.80	6.32	2.03
SD	0.80	23.18	3.78	1.38
g^{TM} (90/95)	3.066	3.066	3.066	3.066
UCL (90/95) (µg/kg)	3.25	146.87	17.91	6.25

Table 3.6: UCL for parent salbutamol in liver, kidney, muscle and fat at 24-hours withdrawal

	Muscle	Liver	Kidney	Fat
Average Residue (µg/kg)	0.44	33.82	2.73	0.98
SD	0.47	12.56	1.78	1.20
g^{TM} (90/95)	3.066	3.066	3.066	3.066
UCL (90/95) (µg/kg)	1.88	72.33	8.19	4.66

Table 3.7: Theoretical Maximum Daily intake calculated from parent salbutamol UCL in each tissue type at 7- and 24-hours following withdrawal

Time	UCL (90/95) ($\mu\text{g}/\text{kg}$)				Average intake after Consumption Factor (CF) is applied (μg)				TMDI ($\mu\text{g}/\text{person}$)
	Muscle	Liver	Kidney	Fat	Muscle (CF = 300g)	Liver (CF = 100g)	Kidney (CF = 50g)	Fat (CF = 50g)	
	7 hrs	3.25	146.87	17.91	6.25	0.98	14.69	0.90	
24 hrs	1.88	72.33	8.19	4.66	0.56	7.23	0.41	0.23	8.43

Figure 3.1 below illustrates the the calculated TMDI in comparison with the ADI ($\mu\text{g}/\text{person}/\text{day}$).

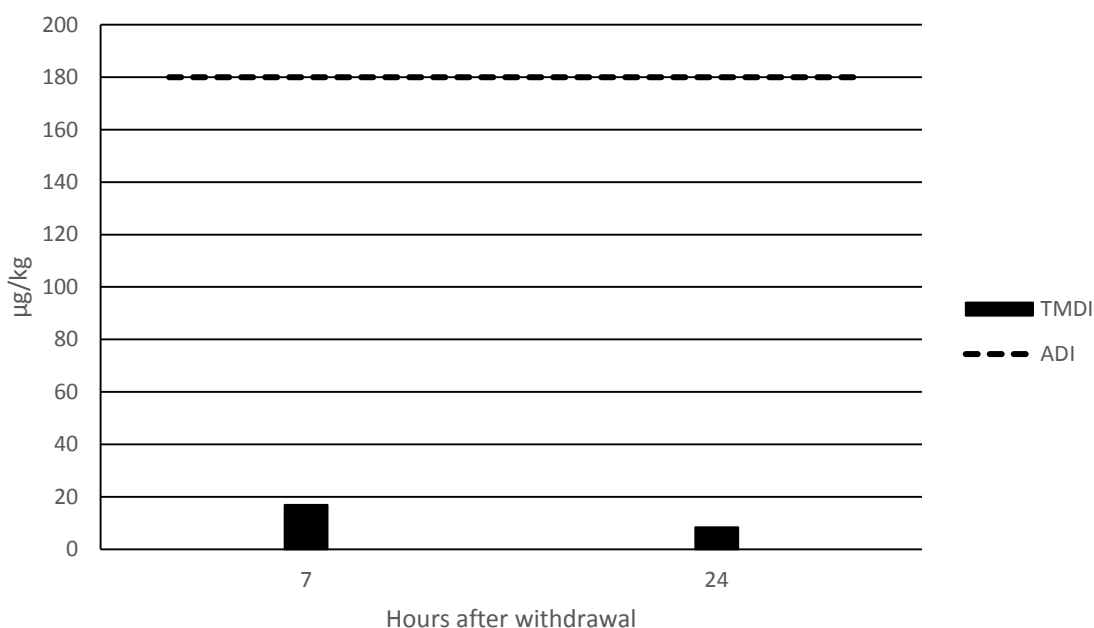


Figure 3.1: TMDI calculated for parent salbutamol in lambs

The withdrawal period is set at the time point where the TMDI is below the ADI. The TMDI for salbutamol is far below the ADI at both time points (Figure 3.1). The TMDI is 9.38% of the ADI at 7 hours and 4.68% of the ADI at 24 hours after withdrawal.

Similar calculations with residue data on zilpaterol in lambs (0.15 mg zilpaterol/kg BWt) indicated a TMDI at day zero of 462% of the ADI, 206% at day 2 and 22.9% at day 5. The

TMDI were equal to ADI at day 4 of the zilpaterol treatment. Also, a half-life for zilpaterol of 15.3 ± 1.8 hours were calculated for zilpaterol in lambs using urinary excretion values. According to pharmacological theory, this would indicate that all residues will be excreted from the lambs' body by 6.25 days (half-life x 10), although residues were still detected after 9 days in the lambs' liver, muscle and kidney. Day 0 to 5 residue excretion followed a linear trend, but the day 9 residue levels were slightly higher than the day 5 values (Shelver & Smith, D.J., 2006)

When the data from Shelver and Smith (2006) is compared to the published MRL for zilpaterol (EFSA, 2016), the liver and kidney values are below the MRL at 2 days of withdrawal. The residue in meat is only below the published MRL following 5 days of withdrawal.

Although a half-life has not been determined for R-salbutamol in lambs, the half-life in humans for R-salbutamol is calculated to be 2.00 ± 0.49 hours (Boulton & Fawcett, 1996b). If the half-life is similar in lambs this will indicate that all residues should be excreted from the body by 20 hours. Parent salbutamol residues are still detected beyond this point (Table 3.), although very low, which could indicate that residual and active R-salbutamol may still be absorbed from the gastro intestinal tract following withdrawal.

The TMDI is used as the starting point for proposing a MRL for any molecule used in food producing animals. The published MRL for Zilpaterol, when applying the food consumption factors, equates to 27.7% of the ADI for an adult human and that of Ractopamine equates to 20% of the ADI for an adult human.

The MRL for R-salbutamol is therefore suggested to be set for liver at 300 $\mu\text{g}/\text{kg}$, muscle at 6.5 $\mu\text{g}/\text{kg}$, kidney at 40 $\mu\text{g}/\text{kg}$ and fat at 13 $\mu\text{g}/\text{kg}$. At these levels the daily intake of residue will still be below 20% of the ADI.

3.4 Conclusion

R-salbutamol has no visual adverse effects on animals consuming the medicated feed and therefore R-salbutamol use in feedlot production can be confirmed to be safe for the target animal. Although the increase in rectal temperature were not clinically significant and the increase in respiratory rate could be attributed to lower respiratory infection, the effect of R-salbutamol use on rectal temperature and respiratory rate should be further investigated.

Salbutamol residues in ovine tissue fed R-salbutamol in their ration for 28 days, showed a rapid decline in residue levels from 7 hours after withdrawal to 24 hours after withdrawal. The TMDI calculated based on the residue levels detected, indicated that salbutamol residues

present in animal tissue following 7 hours withdrawal is far below the safe intake level/ADI calculated in humans. According to the MCC guidelines (MCC, 2004), any study determining a withdrawal period of less than 12 hours is considered to be equivalent to zero withdrawal. The withdrawal period for R-salbutamol in lambs can therefore be set at zero with confidence that ingestion of tissue from lambs fed R-salbutamol at the recommended dosage levels will provide adequate consumer protection.

At the determined UCL for the individual tissue, the intake level in animal tissue is far below the safe level/ADI in humans. Should the individual tissue contain the above UCL levels, humans will have to consume over 55 kg of meat, 1.2 kg liver, 10 kg of kidney or 28.8 kg of fat to have an intake equal to the safe level of R-salbutamol in humans, where no adverse events will be observed. For any acute pharmacological adverse event to occur, the tissue intake would have to be more than 10 times the ADI to have any possible acute effect in humans. Animal tissue derived from feedlot lambs fed R-salbutamol at the recommended inclusion level, with no withdrawal, is therefore deemed safe for human consumption.

3.5 References

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CHAPTER 4

R-SALBUTAMOL RESIDUE DEPLETION STUDY IN FEEDLOT CATTLE

ABSTRACT

This study investigated the residue and target animal tolerance of R-salbutamol in feedlot cattle. One group of four cattle received non-medicated feed as the control group A. Three groups each of six cattle received 150 mg R-salbutamol/head/day (0.42 mg/kg BWt) in feed for 44 days (group B, C and D). One animal from the control group was slaughtered on study day 3. Group B, C and D was slaughtered on <12-, 24- and 48-hours following withdrawal of R-salbutamol from feed respectively together with one each of the control group. The control animals were included to show that there were no residues occurring naturally within the test flock. Liver, kidney, muscle and fat samples were collected and analysed for salbutamol residues (HPLC-DAD). No residues were detected for total salbutamol (LOQ = 3 µg/kg) in any of the tissues except for one muscle sample at 48 hours withdrawal (37 µg/kg). This result was disregarded and viewed as an analytical anomaly. No visual adverse events were observed in the study animals and R-salbutamol was well tolerated in cattle.

4.1 Introduction

An ever-increasing demand for food production to feed a growing world population has led researchers to develop technologies to improve animal growth efficiency and subsequent increase in meat production from the limited land and water resources (Capper & Hayes, 2012; UNPD, 2007). These technologies have not been accepted by everyone as the solution to the increased food demand. The withdrawal of antibiotics in the European Union (EU) (European Commission, 2005) and pressure on the use of hormonal growth implants (Doyle, 2000) are but a few examples of technology under scrutiny by regulators and the consumers. Beta-adrenergic agonists (β -AA) have not been spared criticism and have been banned for use in food producing animals in 160 countries including the EU (Kerr & Hobbs, 2005) and China (Meador & Wu, 2011).

The reported cases (Barbosa, et al., 2005; Brambilla, et al., 1997; Maistro, Chiesa, Angeletti, & Brambilla, 1995; Pulce, Lamaison, Keck, Bostvironnois, & Nicolas, 1991; Salleras, Dominquez, Taberner, Moro, & Salva, 1995; Shiu & Chong, 2001) of clenbuterol related food poisoning due to consumption of animal tissue from animals illegally fed clenbuterol as

productivity enhancer has placed a negative light on the value of β -AA as a safe technology in the quest to improve food supply.

In August 2013 Tyson Foods Inc., the second largest processor and marketer of chicken, pork and beef in the world, announced that they will not buy any cattle fed Zilmax (zilpaterol hydrochloride, MSD Animal Health) stating their concerns with regards to the welfare of cattle fed zilpaterol (Reuters, 2013). Subsequently MSD Animal Health (formerly known as Merck Animal Health) suspended the sale of Zilmax in the United States (US) market (American Veterinary Medical Foundation, 2013). The impact on the withdrawal of Zilmax from the US market was estimated to lead to a reduction in meat production of 6% (Vance, 2013).

The evaluation of these technologies on the impact on animal welfare, as well as consumer safety is therefore of utmost importance. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has however evaluated the safety and use of zilpaterol and ractopamine and found it to be safe if used according to the prescribed dosing regime and adhering to correct withdrawal periods (EFSA, 2009; EFSA, 2016).

Prior to the registration of Salbutamate 10% (R-salbutamol sulphate, Animate Animal Health) for use in cattle, a residue study is required to determine the residue kinetics of R-salbutamol in beef cattle as well as the target animal safety. The growth efficiency of the animals in the trial were not of concern as feed intake was restricted and the animals would therefore not perform as expected in a normal growth efficiency trial.

4.2 Materials and Methods

The research project was approved by the Clinvet Animal Ethics Committee (AEC: CV09/665) and handling and housing of all animals were conducted according to the South African National Standards 10386: 2008.

The trial was conducted at ClinVet International (Pty) Ltd (Bainsvlei, Free State, South Africa).

4.2.1 Animals

The animals used in this study were feedlot type cattle (gender not specified) ranging in weight from 340 kg to 385 kg at arrival. Animals were purchased from Sparta Beef, Marquard, Free State.

Twenty-two cattle were randomly allocated to one of four groups according to a randomised, parallel group design. The individual bovine was the experimental unit in this study.

4.2.2 Housing

Animals were housed individually in indoor stable units with outside paddocks. Stables were not environmentally controlled and environmental conditions fluctuated with ambient conditions.

The identification number of the bovine housed inside a stable, the study number, study group brand of feed and feed quantity were indicated on the outside of each stable. The size of each indoor stable was 7.5 m² and the size of the outside paddock was 115.2 m² on the north-east and south-east aspect and 98.4 m² on the north-west and south-west aspect of the facility respectively.

Routine practices were standardised across all animals. All cattle were at the study facility for 14 days prior to the start of R-salbutamol administration.

4.2.3 Feed and water

The cattle purchased was already processed and fed a grower ration at the feedlot. Therefore, feed acclimatisation was not required once they arrived at the trial facility.

A balanced, commercial cattle feedlot grower ration, sourced from Nutrifeds, Bloemfontein, Free State was fed (Table 4.).

Table 4.1: Nutrient composition of cattle feedlot finisher ration

Nutrient	Quantity (g/kg)
Protein	140.3
Fat	71.4
Ash	50.9
NDF	210.5
ADF	67.5
Moisture	128.1
Calcium	6.5
Phosphorus	4.8

Feed intake was restricted during the trial to ensure that cattle consumed their allocated 150 mg R-salbutamol per day. Cattle were fed three times a day and each feed were spiked with 50 mg R-salbutamol during the medicated period. Animal behaviour associated with feeding was observed through the day and the allocated amount of feed was adjusted if it became

clear that feed intake requirements increased. Feed and free access to potable water was supplied in plastic and rubber troughs on the floor.

A 250 g specimen of each batch of feed was collected and analysed to confirm that no levels of R-salbutamol were present in the feed.

4.2.4 Experimental Procedure

One group of four cattle received non-medicated feed as the control group A. Three groups (group B, C and D) each of six cattle received R-salbutamol in feed three times a day for 44 days.

All the animals received feed without R-salbutamol during the 14-day non-medicated phase prior to the 44-day medicated (R-salbutamol) phase. During the non-medicated phase, feed was supplied in excess and the intake of individual cattle were determined. This was done to ensure that all feed allocated and medicated each day were consumed. If any feed was leftover, the feed was weighed in the afternoon and the next days feed allocation was adjusted accordingly. From day +1 group B, C and D was fed 1.5 g of Salbutamate 10%, equivalent to 150 mg R-salbutamol per animal per day (± 0.42 mg/kg body weight (BWt) at start weight), in feed daily for 44 consecutive days while the control group (group A) received non-medicated feed. The 44 days of treatment was equivalent to the period in commercial feedlots prior to marketing, referred to as the finishing stage before slaughter.

R-salbutamol was administered as a feed additive, as it is marketed and sold as a feed additive for feedlot cattle. R-salbutamol was used at the intended dose, dosage frequency and duration required to imitate the cattle feedlot situation.

Personnel wore overcoats, impervious gloves and a dust mask during administration of R-salbutamol.

4.2.5 Animal handling

The cattle were handled by personnel in a cattle crush during clinical examination and were loaded on a truck for transport to the local abattoir at the end of this study. No other forms of restraint were applied.

4.2.6 Animal Observations

General health observations were performed daily throughout the study. The aim of the general health observation was to detect any health issues that may warrant veterinary care or constitute withdrawal of animals from the study. These observations included, but were not limited to, habitus, appetite, colour of urine, colour and consistency of faeces, salivation, vomiting, skin lesions and any obvious change in general condition.

Clinical examinations were performed once during the non-medicated phase on day -11 of the study. These examinations included, but were not limited to, body condition, vital signs (heart rate, respiratory rate and rectal temperature), mucous membranes, eyes, motility, thoracic auscultation and skin condition. The aim of the clinical examination was to determine if all animals were healthy and suitable to be included in the study.

The intent of the trial was to determine the residue kinetics of R-salbutamol in cattle. Clinical examination following the start of the trial was not repeated as it is not required by VICH guideline GL48 (Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Marker residue depletion studies to establish product withdrawal periods) (VICH, 2015).

4.2.7 Body weight

The animals were weighed once during the non-medicated feeding period on day -11 of the study, with the weights being rounded up to the next 5 kilograms. Cattle were not weighed at the end of the study as the intent of the study was to determine the residue kinetics of R-salbutamol in cattle and growth would be restricted due to restricted feeding.

4.2.8 Feed withdrawal and slaughter schedule

The trial design was based on the VICH guideline GL48 (Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Marker residue depletion studies to establish product withdrawal periods). A minimum of four animals is required per time point and a minimum of three time points to allow for regression analysis. Higher number of animals per time point will improve statistical analysis to improve determination of the withdrawal period (VICH, 2015).

Group B, C and D, consisting of six animals each receiving R-salbutamol, were slaughtered at 12, 24 and 48 hours after withdrawal of R-salbutamol medicated feed, respectively. The

feeding troughs were cleaned, and all the animals received feed without R-salbutamol for the remainder of the study. The slaughter schedule is summarised in Table 4.2.

Table 4.2: Slaughter schedule applied to the cattle fed R-salbutamol or none

Study day	Description
3	one control animal from Group A
+45	Group B animals (n = 6) plus 1 control animal (group A) (11 hours 34 minutes to 11 hours 51 minutes (<12 hours) after last R-salbutamol consumption)
+46	Group C animals (n = 6) plus 1 control animal (group A) (22 hours 57 min to 23 hours 06 minutes (24 ± 2) last R-salbutamol consumption)
+30	Group D animals (n = 6) plus 1 control animal (group A) (46 hours 30 minutes to 46 hours 39 min (48 ± 2 hours) last R-salbutamol consumption)

4.2.9 Organ sampling for residue analysis

Specimens of the muscle, kidney, liver and renal fat were collected for determination of R-salbutamol concentrations on study days +3, +45 (<12 hours withdrawal), +46 (24 hours withdrawal) and +47 (48 hours withdrawal). Negative control tissue samples were also used to spike samples in the lab to a known amount to determine accuracy of laboratory results.

Duplicate specimens of approximately 250 g or as much as physically possible, of muscle, kidney, liver and renal fat were collected from each animal. Extreme care was taken to avoid contamination of the tissues. Separate knives were used for each animal and the operator(s) took precautions to remove any contaminated clothing and to wash hands between sampling of each animals.

If an organ became contaminated by touching another collected organ, the outside of both organs was washed under running potable water. Collected organ specimens were placed in labelled freezer bags of suitable size and sealed air tight at the time of slaughter. Labels indicate the tissue specimen, animal ID, study group, date of collection, collection time and the study number. Organ specimens were kept cool in polystyrene containers with ice and frozen within 24 hours after collection at -20°C or lower. All organ specimens were dispatched to the analytical laboratory (ARC OVI Residue Laboratory, Pretoria) after collection on day +46.

Liver, muscle, kidney and fat samples of Test Group A, B, C and D were analysed for total (parent + metabolites) salbutamol residues.

4.2.10 Laboratory method

The analysis of salbutamol in tissue was performed at the Onderstepoort Veterinary Institute, Residue Laboratory. The laboratory is the official laboratory in South Africa for residue monitoring of, among other, β -AA in animal tissue. The laboratory was the only laboratory in South Africa able to analyse animal tissue for salbutamol residues. At the time of the trial, the method applied for detection of all β -AA residues in animal tissue at the Residue Laboratory was high performance liquid chromatography (HPLC) method with a diode array detector (DAD).

The HPLC-DAD method for the assay of the salbutamol enantiomers in bovine liver and kidney was based on an enzymatic hydrolyses followed by an anion exchange clean-up. For bovine fat the method was based on extraction with tetrachloroethylene, liquid-liquid extraction with a potassium hydrogen phosphate buffer, followed by a cation exchange clean-up. For bovine muscle, the analytes were extracted with an acidified potassium hydrogen phosphate buffer followed by a cation exchange clean-up. Feed samples were extracted with a mixture of 0.02 M sulphuric acid and methanol (90:10). The supernatant was filtered and analysed.

The enzymatic hydrolyses was done by using β -glucuronidase, which meant that if any results were obtained it would be for both the parent and metabolised salbutamol fractions. This is not the standard practice as parent salbutamol should be the marker residue for monitoring purposes. At the time of the study this was however the standard method of analysis for β -AA, including zilpaterol and ractopamine.

The Limit of Quantification (LOQ) for the method was 3 $\mu\text{g}/\text{kg}$. The method was found to be selective for R- and S-salbutamol in fat, kidney, liver and muscle. The method accuracy was confirmed using spiked quality control samples, which was run with every batch. Repeatability was confirmed with the use of six replicates of each matrix at both 2 and 5 ppb, which was run with every batch.

As R-salbutamol is metabolised through glucuronide conjugation in animals and the metabolite is not active (Martin, Hobson, Page, & Harrison, 1971), the parent salbutamol fraction is usually considered for residue detection and is the marker residue for residue analysis. This is in accordance with the marker residues accepted for both zilpaterol (EFSA, 2016) and ractopamine (EFSA, 2009).

4.2.11 Statistical Analysis of Data

A primary statistical method to determine the withdrawal period from each of the body tissues is described in the guidelines on MRL's and Withdrawal Periods by the Medicines Control Council of South Africa (MCC) (MCC, 2004).

The protocol describes the conventional statistical methods based on a regression analysis to be applied for the determination of withdrawal periods. With all residue results below the LOQ statistical analysis were not possible.

To calculate the Theoretical Maximum Daily Intake (TMDI) from any residue results the average residue, as determined at each withdrawal time point, is used for each animal tissue (i.e. liver, muscle, kidney and fat) and multiplied with the food consumption factor (CF) as determined by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in their 34th report entitled "Evaluation of Certain Veterinary Drug Residues in Food" (JECFA, 1989).

The consumption factor for each tissue type is summarised in Table 4.3.

Table 4.3: Food Consumption Factors for each tissue type as defined by JECFA

Tissue	Daily Intake
Muscle	300 g
Liver	100 g
Kidney	50 g
Fat	50 g

A one-sided upper tolerance limit or upper confidence limit (UCL) is then calculated by adding the calculated theoretical intake of residues in the animal tissue and adding the product of the standard deviation (SD) with the g^{TM} factor.

$$UCL = \text{mean} + (g^{\text{TM}} \times SD)$$

Where:

g^{TM} = A factor for calculating a one-sided tolerance estimate of conformance with a given threshold and confidence level.

The g^{TM} factor is based on the number of animals used at each time point in the residue kinetic study and the accepted statistical significance of the data as well as the statistical confidence. MCC has accepted the use of a 95% statistical significance with a 90% statistical confidence (MCC, 2004). The table with the relevant g^{TM} factors is published in the MCC guidelines on MRL's and Withdrawal Periods (MCC, 2004).

Using the sample size ($N = 6$) in this trial at each time point, the g^{TM} parameter applicable for this trial equals 3.066.

4.3 Results and Discussion

As the feed used in the trial was spiked three times per day with the R-salbutamol, it had to be confirmed that the feed was free from R-salbutamol or any other β -AA. Feed was tested for R-salbutamol and zilpaterol and it was confirmed that the feed did not contain any beta-agonist.

The average body weight at the start of the trial was 356 kg. Animals were not weighed during the trial as the trial was not considered to be a growth performance trial due to restricted feeding.

There were no R-salbutamol related physical signs (tremors and restlessness), often associated with β -AA ingestion (Wagner, Morstrom, Hammer, Thorson, & Smith, 2008), recorded during the daily general health observations of the animals in this study.

Nasal discharge was recorded in two animals in group A (control group), one animal in group B and one animal in group C. Nasal discharge was similar in nature in all animals and was transient and resolved without treatment. Nasal discharge in feedlot cattle may be as a result of dust in the feed or may be associated with apparent bovine respiratory disease (Montgomery, et al., 2009; Taylor, Fulton, Lehenbauer, Step, & Confer, 2010).

All tissue residue results were below the limit of quantification (LOQ) of 3 $\mu\text{g}/\text{kg}$, except for one muscle sample at 48 hours (0.037 mg salbutamol/kg muscle).

This value is however ignored and viewed as a laboratory anomaly and disregarded from the data set according to the Dixon's Q-test.

$$Q_{\text{exp}} = 1$$

and therefore

$$Q_{\text{exp}} > Q_{\text{crit}} (\text{CL}=95\%) > 0.625 (N=6).$$

The theoretical maximum intake for humans consuming beef treated with R-salbutamol could therefore not be calculated from the study data. In a similar study Zhang, et al. (2017) investigated the residues of parent salbutamol and that of its metabolites in cattle following racemic salbutamol treatment at 0.15 mg/kg BWt for 21 days. Residues were detected in all tissue (liver, kidney, fat and muscle) at varying levels at zero withdrawal. Liver had the highest residue level, followed by kidney, fat and muscle (Table 4.4).

Table 4.4: Parent salbutamol residue concentration in cattle tissue at zero withdrawal ($\mu\text{g}/\text{kg}$) (Zhang, et al., 2017)

R-salbutamol ($\mu\text{g}/\text{kg}$ in tissue)			
Muscle	Kidney	Liver	Fat
13.92	32.20	53.69	31.16

Zhang et al. (2017) investigated the residues for salbutamol in the liver and kidney and determined that only 9.9% of the total salbutamol residues detected were parent salbutamol ($53.69 \mu\text{g}/\text{kg}$ parent salbutamol vs. $547.52 \mu\text{g}/\text{kg}$ total salbutamol) in the liver at zero-day withdrawal. In kidney, 35.5% of total salbutamol residues were present as parent salbutamol ($32.50 \mu\text{g}/\text{kg}$ parent salbutamol vs. $91.55 \mu\text{g}/\text{kg}$ total salbutamol) at zero-day withdrawal, indicating rapid metabolism of salbutamol in cattle. The average concentration of parent salbutamol in the liver of the cattle was lower ($53.69 \mu\text{g}/\text{kg}$) at zero withdrawal in cattle than the concentration of parent salbutamol reported in lambs ($75.23 \mu\text{g}/\text{kg}$) following 7 hours withdrawal (Chapter 3). However, total salbutamol (parent + metabolite) concentration in cattle ($547.52 \mu\text{g}/\text{kg}$) was three times higher in the liver after zero withdrawal compared to total salbutamol concentration detected in lambs ($181.27 \mu\text{g}/\text{kg}$) after 7 hours withdrawal (Chapter 3). Racemic salbutamol was used in the cattle trial, in comparison to the R-salbutamol in lambs. It has been shown in humans that R-salbutamol has a shorter half-life compared to the racemic mixture (Boulton & Fawcett, 1996). The difference in withdrawal time between the cattle and lamb study should be factored in when comparing this data, as salbutamol is rapidly excreted from the body following withdrawal. Zhang et al. (2017) reported a rapid decline in the urine concentration of parent salbutamol from day zero ($1654.4 \text{ ng}/\text{ml}$) to day-3 ($<\text{LOQ}$ of $0.5 \text{ ng}/\text{ml}$) following withdrawal.

The higher levels of salbutamol residues in the study by Zhang et al. (2017) in muscle, kidney and fat raises the question if salbutamol is metabolised differently in cattle compared to lambs. The difference in rate of metabolism in R-salbutamol vs. racemic salbutamol observed by Boulton and Fawcett (1997) would indicate that the residue profile may differ in R-salbutamol compared to racemic salbutamol. The low ratio of parent salbutamol to total salbutamol indicated a rapid metabolism of salbutamol (Zhang, et al., 2017).

The residue data from a trial performed by Fawcett et al. (2004) in chickens, confirms the stereo-selective metabolism of racemic salbutamol vs. R-salbutamol. At zero-withdrawal, chickens fed 10 ppm racemic salbutamol compared to 5 ppm R-salbutamol had much higher R-salbutamol residues levels present in liver (55.2 µg/kg vs 34.1 µg/kg), fat (7.2 µg/kg vs. 5.7 µg/kg) and leg muscle (14.2 µg/kg vs. 11.7 µg/kg), confirming the competition between the 2 enantiomers for the enzymes for metabolism (Boulton & Fawcett, 1997).

In a residue study performed with zilpaterol fed at 0.15 mg/kg BWt for 4 days in cattle, residues were reported in the liver, muscle and kidney at 24 hours withdrawal, however, no residues were detected at 48 hours withdrawal in the muscle and none at 96 hours withdrawal in the liver and kidneys (Boison, Ramos, Samders, Chicoine, & Scheid, 2013). This is in contrast with a study by Morris (1997) where residues were still detected in muscle after a 7-day withdrawal in cattle fed zilpaterol for 50 days at 0.15 mg/kg BWt. The difference in the time following withdrawal of zilpaterol and salbutamol where residues are still detected in muscle, indicates a difference in the rate of metabolism between the two molecules and therefore a difference in the half-life of the two β-AA's. Shelver and Smith (2006) determined the half-life of zilpaterol to be 15.3 ± 1.8 hours in sheep. No pharmacokinetic work has been performed with R-salbutamol in cattle and sheep to date.

The total average daily intake for humans consuming beef tissue from R-salbutamol treated cattle can be calculated using the data reported by Zhang (2017), by using the food consumption factors for each tissue as set out by JECFA (2000) described in Chapter 3. The calculation is summarised in Table 4.5.

Table 4.5: Calculation of average daily intake of salbutamol in humans at zero-day withdrawal

Tissue	Average parent Salbutamol concentration (µg/kg) *	Food consumption factors	Average residue intake (µg/person)
Muscle	13.92	300 g	4.18
Liver	53.69	100 g	5.37
Kidney	32.20	50 g	1.61
Fat	31.16	50g	1.56
Total salbutamol intake per day in humans			12.72

* Data from Zhang et al. (2017)

This average human intake value calculated from the cattle data is slightly higher than the TMDI calculated from salbutamol residue data in lambs (Chapter 3). The possible explanation for the difference is that the result in cattle was obtained following a zero-day withdrawal vs. a 7-hour withdrawal in the lamb study. The average intake per day for humans calculate to

7.33% of the Average Daily Intake (ADI) of 180 µg, derived from the Acute Reference Dose (ARfD for R-salbutamol, which indicates that at zero withdrawal, the residue levels in bovine tissue will be safe for human consumption.

The study of Zhang, et al. (2017) was however performed using racemic salbutamol and not R-salbutamol. As previously indicated, R-salbutamol metabolises much quicker and is less bioavailable than the racemic molecule (Boulton & Fawcett, 1996b), this could possibly explain the lack of residues detected in the cattle treated with R-salbutamol.

The inability to detect any salbutamol residues in the current study can be attributed to either the possible quicker metabolism and excretion of R-salbutamol in cattle, or the use of the HPLC-DAD method. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is currently the preferred method for β-AA detection used for zilpaterol (EFSA, 2016) and ractopamine (EFSA, 2009) residue detection as it is more sensitive and specific than the HPLC-DAD method (Marquet, 2002). At the time (2010) this was however the only method available at the Agricultural Research Council Residue Laboratory at Onderstepoort. The residues detected in the salbutamol residue study in sheep can be attributed to either a difference in the metabolism rate of R-salbutamol in sheep, or the use of LC-MS/MS, instead of the HPLC-DAD method.

4.4 Conclusion

R-salbutamol use in feedlot cattle is safe for use in the target animal as no physical adverse events were observed with the addition of R-salbutamol in the feed.

No residues were detected in the cattle R-salbutamol residue study possibly due to either the faster metabolism of R-salbutamol in cattle, or the less reliable and specific method of HPLC-DAD used during sample analysis. The data from a residue study performed using racemic salbutamol in cattle indicates that the residues present in beef after treatment with salbutamol is safe for human consumption.

The lack of method sensitivity in the analysis for salbutamol residues in the current study warrants a repeat of the study using a more sensitive analysis method (LC-MS/MS) as well as analysing samples for both parent and total salbutamol.

The possibility of the quicker metabolism and excretion of R-salbutamol in cattle warrants pharmacokinetic studies of R-salbutamol in cattle to determine the half-life and elimination kinetics.

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CHAPTER 5

GENERAL CONCLUSION AND RECOMMENDATIONS

The aim of these investigations was to determine the levels of the marker residue, parent salbutamol, and total salbutamol (parent + metabolite) in lambs and cattle treated with R-salbutamol during the feedlot finishing phase.

5.1 Human Safety

A comprehensive human risk assessment was done for R-salbutamol and a subsequent Acute Reference Dose (ARfD) of 3 µg/kg BWt for humans was determined to be the upper safe intake level where no acute adverse events would be observed. The ARfD were used as the upper limit for the Acceptable Daily Intake (ADI), which was set at 0 - 3 µg/kg BWt. In comparison with the ADI for zilpaterol of 0 - 0.04 µg/kg BWt (EFSA, 2016) and the ADI for ractopamine of 0 - 1 µg/kg BWt (EFSA, 2009), R-salbutamol can therefore be classified as the safer of these beta-agonist for use in animal production, with a high safety margin in humans.

5.2 Lamb Residues

In the lamb residue study (Chapter 3), the highest concentration of residues was detected in the liver, followed by the kidney, muscle and fat. Parent and total salbutamol residues in the liver, kidney, muscle and fat declined rapidly from 7 hours to 24 hours after withdrawal of R-salbutamol in the feed. Less than half of the residues detected in all the tissue analysed were parent salbutamol indicating rapid metabolism and excretion of R-salbutamol after ingestion. This also suggests a low oral bioavailability for R-salbutamol with a large portion of active parent salbutamol being metabolised in the gut lining and liver through the first pass effect. Further investigation into the pharmacokinetics of oral vs. intravenous administration of R-salbutamol in feedlot lambs will shed more light on the absorption, distribution, metabolism and excretion (ADME) of R-salbutamol.

The Theoretical Maximum Daily Intake (TMDI) for humans calculated from the lamb residue data in the liver, kidney, muscle and fat indicated that the residues in lamb tissue after 7 hours withdrawal of R-salbutamol is far below the ADI calculated for humans. The TMDI is 8.98% of the ADI at 7 hours after R-salbutamol withdrawal. It is therefore safe to recommend a zero withdrawal for Salbutamate 10% (R-salbutamol sulphate) when used according to the label recommendations.

No adverse pharmacological effects were detected visually in lambs administered R-salbutamol, suggesting that R-salbutamol is safe for use at the recommended inclusion levels in feedlot lamb diets. There was no difference in heart rate between the non-medicated and medicated period. There was a significant difference in the respiratory rate following R-salbutamol inclusion, possibly due to lower respiratory tract infection. The significant increase in rectal temperature of the lambs was not of clinical significance as it remained within the normal range of rectal temperature for sheep. Heart rate would normally be expected to increase with β -agonist administration as it is a cardio active drug. The effect of R-salbutamol on the increased respiratory rate and rectal temperature should however be investigated further.

5.3 Cattle Residues

In the cattle residue study (Chapter 4), no residues were detected at <12, 24 or 48 hours after withdrawal. The HPLC-DAD method used to determine the residues is not the preferred method for beta-agonist analysis. Further investigation into residue depletion in cattle using the LC-MS/MS method is therefore recommended.

Residue levels in a cattle study by Zhang et al. (2017), medicated with racemic salbutamol, indicates rapid metabolism of salbutamol in cattle as is the case with lambs. The difference in the ratio between parent salbutamol and total salbutamol may indicate difference in the rate of metabolism of salbutamol between cattle and sheep. The study by Zhang et al. (2017) used racemic salbutamol and it is known that R-salbutamol metabolises faster than S-salbutamol and R-salbutamol is less bioavailable compared to S-salbutamol in humans (Boulton & Fawcett, 1996b). Determination of the absorption, distribution, metabolism and excretion (ADME) of R-salbutamol through pharmacokinetic studies of oral vs. intravenous administration of R-salbutamol in feedlot cattle is therefore required.

No observable adverse events were noted during the trial period, although this cannot be used to conclude that R-salbutamol is safe for the target animal. Physical monitoring of animals is required in future studies on the use of R-salbutamol in cattle. Nonetheless, as animal welfare has become an important factor for consumers, the effect of R-salbutamol on the heart rate, respiratory rate and rectal temperature should be further investigated to determine if R-salbutamol has a similar effect in cattle compared to observed effects with the use of other β -AA.

5.4 Maximum Residue Limit (MRL)

From the lamb study, the MRL for R-salbutamol in animal tissue can safely be set at 6.5 µg/kg in meat/muscle, 300 µg/kg in liver, 40 µg/kg in kidney and 13 µg/kg in fat. These values would equate to a TMDI of almost 9% of the ADI for R-salbutamol in comparison to the MRL values for zilpaterol that equates to a TMDI at 30% of ADI (EFSA, 2016) and Ractopamine with a TMDI at 25% of ADI (EFSA, 2009).

5.5 Final Conclusion

The results of this study indicate that R-salbutamol, used according to label recommendations, is safe for both the target species (feedlot lambs and cattle) as well as for humans consuming beef and lamb tissue from animals treated with R-salbutamol with a zero-day withdrawal.

5.6 References

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