Effects of Specific Equine Babesiosis Treatments on Equine Oro-caecal Transit Time as measured by the Lactose $^{13}$C-Ureide Breath Test
Gedruckt mit der Genehmigung der Tierärztlichen Fakultät
Der Ludwig-Maximilians-Universität München

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Tag der Promotion: 08. Februar 2008
Dedicated to my parents
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<tr>
<td>AP</td>
<td>action potential</td>
</tr>
<tr>
<td>AP$^{13}$C</td>
<td>atom percentage $^{13}$C</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BMR</td>
<td>basal metabolic rate</td>
</tr>
<tr>
<td>Bwt</td>
<td>body weight</td>
</tr>
<tr>
<td>CF-IRMS</td>
<td>continuous flow isotope ratio mass spectrometry</td>
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<tr>
<td>CFT</td>
<td>complement fixation test</td>
</tr>
<tr>
<td>cm</td>
<td>centimetre</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CO$_2$</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>d</td>
<td>day</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ECL</td>
<td>enterochromaffin-like</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>GI</td>
<td>gastrointestinal</td>
</tr>
<tr>
<td>H$_2$</td>
<td>hydrogen</td>
</tr>
<tr>
<td>H$_2$BT</td>
<td>hydrogen breath tests</td>
</tr>
<tr>
<td>HCL</td>
<td>hydrochloride</td>
</tr>
<tr>
<td>IFA</td>
<td>indirect fluorescent antibody</td>
</tr>
<tr>
<td>IM</td>
<td>intramuscular</td>
</tr>
<tr>
<td>IV</td>
<td>intravenous</td>
</tr>
<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
<tr>
<td>LD$_{50}$</td>
<td>lethal dose at which 50% of subjects die</td>
</tr>
<tr>
<td>LU</td>
<td>lactose ureide</td>
</tr>
<tr>
<td>LUBT</td>
<td>lactose $^{13}$C-ureide breath test</td>
</tr>
<tr>
<td>NH$_3$</td>
<td>ammonia</td>
</tr>
<tr>
<td>NSAID</td>
<td>non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>MCFA</td>
<td>medium chain fatty acid</td>
</tr>
<tr>
<td>m</td>
<td>metre</td>
</tr>
<tr>
<td>M/MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>mg</td>
<td>milligram</td>
</tr>
<tr>
<td>MgClO$_4$</td>
<td>Magnesium perchlorate</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>ml</td>
<td>millilitre</td>
</tr>
<tr>
<td>MMC</td>
<td>migrating myoelectric complex</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge</td>
</tr>
<tr>
<td>n</td>
<td>number</td>
</tr>
<tr>
<td>NDIRS</td>
<td>non dispersive infrared spectrometry</td>
</tr>
<tr>
<td>$^{13}$C-OABT</td>
<td>$^{13}$C-octanoic acid breath</td>
</tr>
<tr>
<td>OCTT</td>
<td>oro-caecal transit time</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>Pee Dee Belemnite</td>
</tr>
<tr>
<td>PDR</td>
<td>percentage dose recovery</td>
</tr>
<tr>
<td>pH</td>
<td>negative logarithm of hydrogen ion concentration</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RMS</td>
<td>root mean square</td>
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SLZ/SP test</td>
<td>sulphasalazine/sulphapyridine test</td>
</tr>
<tr>
<td>SA</td>
<td>surface area</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>sRNA</td>
<td>subunit ribonucleic acid</td>
</tr>
<tr>
<td>( t_{1/2} )</td>
<td>time to recover 50% of total cumulative dose recovered</td>
</tr>
<tr>
<td>( t_{\text{lag}} )</td>
<td>duration of lag phase (prior to maximal breath enrichment)</td>
</tr>
<tr>
<td>( t_{\text{max}} )</td>
<td>time from ingestion of the substrate to the time of maximal breath enrichment</td>
</tr>
<tr>
<td>( \mu g )</td>
<td>microgram</td>
</tr>
<tr>
<td>VCO(_2)</td>
<td>CO(_2) production</td>
</tr>
<tr>
<td>( y )</td>
<td>year(s)</td>
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Equine babesiosis is one of the most common infectious tick-borne diseases in southern Africa (Heerden 1996; de Waal and Van Heerden 2004). The parasites *Theileria equi* and *Babesia caballi* cause acute, subacute or chronic diseases in Equidae (Phipps and Phipps 1996; Ribeiro et al. 1999; de Waal et al. 2004) or neonatal babesiosis or abortion in mares (Heerden 1996; de Waal et al. 2004). The disease leads to haemolytic anaemia (Schein 1988; Phipps et al. 1996; de Waal et al. 2004) and death can occur (de Waal et al. 2004).

Imidocarb dipropionate is an effective treatment to control the disease in Equidae (Schein 1988; Brüning 1996; Donnellan et al. 2003b; de Waal et al. 2004; Vial and Gorenflo 2006). Systemic side effects including depression, intestinal hypermotility, colic and lateral recumbency may occur (Adams 1981; Phipps et al. 1996).

Additional administration of atropine (Heerden 1996; de Waal et al. 2004) or glycopyrrolate (Donnellan et al. 2003a; Donnellan et al. 2003b) is reported to minimise or prevent the side effects.

Atropine is an anticholinergic drug that inhibits gastrointestinal motility (Ducharme and Fubini 1983; Adams 2001).

Glycopyrrolate, also an anticholinergic agent, has some advantages over atropine sulphate as it has an increased water solubility and thus does not cross the blood-brain barrier (Donnellan et al. 2003b). In addition, its duration is shorter than that of atropine as it has a shorter half-life.

The induced lactose $^{13}$C-ureide breath test (LUBT) has been developed by Heine (Heine et al. 1995) and validated against enterocolonic scintigraphy for the measurement of oro-caecal transit time (OCTT) in man (Wutzke et al. 1998; Geypens et al. 1999).
enterocolonic scintigraphy is not possible, the test has been validated in vitro for the measurement of OCTT, and has been found to be superior to the previously described hydrogen breath test (Sutton 2003). The induced LUBT was concluded to be a valid alternative to scintigraphy for measurement of OCTT in humans (Geypens et al. 1999; Geypens 2000) and suitable for this purpose in horses (Sutton 2003).

The objectives of this study were as follows:

- to improve knowledge of the specific effects of imidocarb treatment on oro-caecal transit time in horses

- to quantify the effects of premedication with atropine or glycopyrrolate on small intestinal motility

- to develop/validate a clinical protocol for treatment of babesiosis that results in minimal gastrointestinal complications
CHAPTER 2

LITERATURE REVIEW

This review details aspects of equine babesiosis including treatment, with emphasis on imidocarb, and pharmacological aspects of atropine and glycopyrrolate. It continues with a brief overview on the gastrointestinal anatomy of the horse and stable isotope breath tests with the lactose $^{13}$C-ureide breath test in particular. Further, certain aspects of stable isotopes and isotope ratio mass spectrometry are detailed.

2.1 EQUINE BABESIOSIS

2.1.1 Historical background

De Waal and Van Heerden’s (2004) review mentions that Wiltshire in 1883 described the first recorded case of equine babesiosis in South Africa, referred to as “anthrax fever”. A similar condition was observed and named “biliary fever” in the Cape by Hutcheon. Nunn regarded it as the “bilious form” of African horse sickness. However, Hutcheon considered biliary fever to be a different disease. The same disease was described as equine malaria in West Africa. In 1901 to 1902 it was demonstrated by Theiler that biliary fever is not identical to African horse sickness, but may occur concurrently to it (de Waal et al. 2004). An organism called Piroplasma equi was found by Laveran in 1901 (Schein 1988; de Waal et al. 2004; Uilenberg 2006), having examined blood smears from South African horses (Schein 1988; de Waal et al. 2004) from Theiler. After some controversy over the years about the classification of this organism, it was eventually placed in the genus Theileria (de Waal et al. 2004; Uilenberg 2006). Koch in 1904 identified two morphologically different equine Babesia spp. in Zimbabwe, which were later demonstrated by Nuttall and Strickland as being causative agents of equine babesiosis (de Waal et al. 2004). The name Piroplasma caballi was suggested for
the larger of the two parasites (Schein 1988; de Waal et al. 2004). They also demonstrated that no cross-reaction exists between these two parasites.

For many years little effort was made to determine which Babesia species caused the clinical signs of infection. Over time however, distinct differences in morphology, life cycle, vectors and susceptibility to chemotherapy became evident between the two parasites (de Waal et al. 2004).

Holbrook (1969) reviews that equine piroplasmosis was diagnosed as a mixed infection of Babesia caballi and Theileria equi in Florida, following the importation of 30 ponies from Cuba. In 1961, a parade horse of the Miami Police Department was diagnosed with Babesia caballi. In 1962, when the disease occurred in most of southern Florida, the tropical horse tick Dermacentor nitens was proven to be a vector. Since 1962, equine babesiosis has occurred regularly in Florida, which has lead to a build-up of infection within the vector and host population. By 1969 the disease had reached a state of stability in southern Florida, with most of the horses being low-level carriers (Holbrook 1969).

2.1.2 Background

Equine babesiosis, also known as biliary fever or equine piroplasmosis, is one of the most common infectious tick-borne diseases in southern Africa (Heerden 1996; de Waal et al. 2004). The intra-erythrocytic protozoa Theileria equi and Babesia caballi cause an acute, subacute or chronic disease in Equidae (Phipps et al. 1996; Ribeiro et al. 1999; de Waal et al. 2004). It also causes neonatal babesiosis and abortion in mares (Heerden 1996; de Waal et al. 2004). Horses, donkeys and zebras are all susceptible to both Theileria equi and Babesia caballi infections, although clinical cases occur mostly in horses (Schein 1988; Heerden 1996; de Waal et al. 2004). In general, Babesia caballi infections are clinically milder and
LITERATURE REVIEW


A lymphocytic stage of schizogony of *Theileria equi* in the mammalian host prior to the intra-erythrocytic stages has led to discrepancies in the classification of this parasite (Phipps *et al.* 1996; de Waal *et al.* 2004) and phylogenetic studies using small subunit ribosomal RNA (sRNA) suggest a distinct paraphyletic group for *Theileria equi* (Brüning 1996). Uilenberg (2006) suggests a classification to the genus *Theileria* for *Theileria equi*, due to the existing extra-erythrocytic multiplication, which often results in four merozoites and the occurrence of only transstadial transmission (Uilenberg 2006).

Equine babesiosis is one of the major reasons for the prohibition of importation of horses from South Africa into other countries (de Waal *et al.* 2004).

2.1.3 Aetiology and life cycle

The genus *Babesia* belongs to the phylum Apicomplexa, class Sporozoasida, subclass Coccidiasina, order Euococcidiorida, suborder Piroplasmorina and family Babesiidae (de Waal *et al.* 2004). *Babesia equi* has, after some years of controversy, been reclassified as *Theileria equi* (Mehlhorn and Schein 1998). The life cycle of *Babesia* spp. can be separated into three stages. The initial gamogony is a sexual stage in which gametes format and fuse inside the gut of an infected tick. This stage is followed by sporogony, an asexual reproduction in the salivary gland of the intervertebrate host. It is further followed by merogony, an asexual dividing stage inside the vertebrate host’s erythrocytes or in the case of *Theileria equi* also inside the lymphocytes (Vial *et al.* 2006).
LITERATURE REVIEW

2.1.3.1 Theileria equi

*Theileria equi* is smaller than *Babesia caballi* (Mehlhorn *et al.* 1998; de Waal *et al.* 2004) and unlike other *Babesia* spp. has a stage of schizogony in the vertebrate host’s lymphocytes (Schein 1988; Brüning 1996; Uilenberg 2006). The parasite develops within five days to sporozoites in the salivary gland of the infected tick vector (Schein 1988; de Waal *et al.* 2004). This occurs after the infected adult tick has attached to a vertebrate host (Schein 1988; de Waal *et al.* 2004; Uilenberg 2006). Transstadial transmission occurs by injection of the infected saliva into the host. In this case the infected tick loses its *theilerial* infection after transmission (Uilenberg 2006).

Twelve to 14 days after infected ticks first attach, merozoites are released to invade erythrocytes. Trophozoites, dividing forms and merozoites occur in the intra-erythrocytic stage. The merozoites occur either as two or four, in a maltese cross formation and are seen as pyriform parasites within the erythrocyte (Schein 1988; Vial *et al.* 2006).

2.1.3.2 Babesia caballi

The development of *Babesia caballi* is found exclusively in the vertebrate host’s erythrocytes (Schein 1988; Brüning 1996; de Waal *et al.* 2004). Within the host’s red blood cells, the pyriform merozoites are often found as pairs with an acute angle (Schein 1988; de Waal *et al.* 2004). This parasite causes usually a very low parasitaemia of less than 0.1 per cent of infected erythrocytes (de Waal *et al.* 2004).

2.1.4 Distribution

*Theileria equi* and *Babesia caballi* occur in Spain (Camacho *et al.* 2005), Portugal, France, Belgium, Poland, in large parts of the former USSR (Joyner *et al.* 1981) and Italy (Schein 1988; de Waal *et al.* 2004). Babesiosis occurs also in Asia (Donnelly *et al.* 1980), the Middle East (Hailat *et al.* 1997) and is endemic in China (Xu *et al.* 2003) and in Mongolia.
(Boldbaatar et al. 2005). Furthermore it has been reported from the Caribbean (Rampersad et al. 2003) and is distributed in Africa (Gummow et al. 1996; Heerden 1996; de Waal et al. 2004), Central (Madden and Holbrook 1968; Carbrey et al. 1971) and South America (Ribeiro et al. 1999), Florida (Holbrook 1969) and some regions in Australia (Schein 1988; de Waal et al. 2004).

It is generally thought that the geographical distribution of equine babesiosis is dependent on the distribution of the requisite tick vectors (Donnellan et al. 2003b; de Waal et al. 2004). Gummow et al. (1996), on the other hand, reported no correlation between the prevalence of equine babesiosis and the distribution of tick vectors in South Africa (Gummow et al. 1996).

Dermacentor species transmit only Babesia caballi in Central Europe, Central Asia (Phipps et al. 1996) and Florida (Frerichs et al. 1969), whereas Rhipicephalus evertsi evertsi transmit both Theileria equi and Babesia caballi in Africa (Heerden 1996; Phipps et al. 1996) and only Theileria equi in South Africa (de Waal et al. 2004). Rhipicephalus bursa is reported to be a vector of both parasites in Southern Europe and Asia Minor (Phipps et al. 1996). Rhipicephalus sanguineus has been reported to transmit Theileria equi and Babesia caballi and Rhipicephalus turanicus Theileria equi in South Africa (de Waal and Van Heerden 2004).

Hyalomma species transmit Theileria equi and Babesia caballi in Southern Europe, Middle, Near and Far East and in North Africa (Phipps et al. 1996). Hyalomma truncatum is responsible for the transovarial transmission of Babesia caballi in Southern Africa (Donnellan et al. 2003b; de Waal et al. 2004).

Furthermore, Boophilus microplus is reported to transmit Theileria equi to susceptible animals (Guimaraes et al. 1998; de Waal et al. 2004).

Northern Europe, the UK, Eire and New Zealand are among the few regions from where the disease has not been reported (Donnelly et al. 1980; Phipps et al. 1996).
2.1.5 Infection

Transmission of *Babesia* species occurs via the bite of an infected tick and transmission of the infective stage to the mammalian host via the salivary fluid (Phipps *et al.* 1996; Uilenberg 2006; Vial *et al.* 2006). The transmitting tick is only infective a few days after attachment and after maturation of the sporozoites within it (Uilenberg 2006). Depending on the vector tick, the incubation period varies between two to ten, and up to 21 days (Mehlhorn *et al.* 1998). *Theileria equi* follows only a transstadial transmission (Phipps *et al.* 1996; Mehlhorn *et al.* 1998; Uilenberg 2006). *Babesia caballi* infection can be transmitted transstadially by *Rhipicephalus evertsi evertsi* and transovarially by *Hyalomma truncatum*. Furthermore, an infection can occur after inoculation of infected blood into susceptible animals, as well as after the use of contaminated hypodermic needles. However, mechanical transmission by haematophagous insects has yet to be proven (Donnellan *et al.* 2003b; de Waal *et al.* 2004). *In utero* infection of equine fetuses by both *Babesia* species has been reported (Schein 1988; Phipps *et al.* 1996; de Waal *et al.* 2004).

2.1.6 Pathogenesis

*Theileria equi* is highly pathogenic and can infect up to 80% of erythrocytes (Mehlhorn *et al.* 1998). Progressive anaemia is characteristic of *Theileria equi* infections (de Waal *et al.* 2004). Varying degrees of thrombocytopenia, hypophosphatemia, hypoferronaemia and hyperbilirubinaemia are accompanied by peaks in parasitaemia (de Waal *et al.* 1987; de Waal *et al.* 2004). *Babesia* parasites depend on the red blood cells for their energy supply and the increased uptake of phosphorus by the erythrocytes may be responsible for the hypophosphatemia and the infected erythrocyte’s fragility (de Waal *et al.* 2004). In humans, hypophosphatemia has lead to adenosine triphosphate (ATP) depletion, which predisposes to development of haemolysis. In severely affected horses, haemolysis may lead to haemoglobinaemic nephrosis and uraemia. Concurrent infections, such as African horse
sickness and verminosis can complicate equine babesiosis and thus may lead to disseminated intravascular coagulopathy (de Waal et al. 2004).

Pathogenesis in Babesia caballi infections on the other hand is related to accumulation of parasitised erythrocytes in small blood vessels and capillaries (Schein 1988; de Waal et al. 2004). An acute hypotensive state may possibly cause acute death. Lung oedema has been associated with hyperacute infections and death in horses, as well as development of laminitis in some cases with gastrointestinal stasis.

Both parasites often cause subclinical infections, which present with a decrease in packed cell volume and platelet count (de Waal et al. 2004).

2.1.7 Clinical signs

The seasonal activity of the adult stages of vectors predisposes to a higher frequency of clinical signs during summer months (de Waal et al. 2004). Clinical signs occur after an incubation period of five to 30 days following the bite of an infected tick (Holbrook 1969; Schein 1988; Phipps et al. 1996; de Waal et al. 2004).

Babesia caballi infections are usually clinically inapparent and seldom lead to severe anaemia. Occasionally, it contributes to chronic inappetence, poor performance, loss of body weight, pale-pink mucous membranes, mild tachycardia and splenomegaly (de Waal et al. 2004).

A rare and peracute form results in horses being found either dead or moribund, and has been reported from South Africa (de Waal et al. 2004). The acute form is characterised by fever, anorexia, depression, icterus, haemolytic anaemia, petechial haemorrhages of the mucosal membranes and elevated respiratory and pulse rates (Schein 1988; Phipps et al. 1996; de Waal et al. 2004). Haemoglobinuria, an often described symptom of babesiosis in other species, is less frequently observed in equine piroplasmosis (Schein 1988; Phipps et al. 1996). In addition to the aforementioned symptoms, subacute cases may present with varying degree
of anorexia, weight loss, elevated or normal rectal temperature, increased pulse and respiratory rates, colic, constipation followed by diarrhoea, splenomegaly, and sometimes haemoglobinuria. The mucous membranes may vary from pale-pink or pale-yellow to bright yellow. Mild inappetance, poor performance, weight loss, splenomegaly and occasionally pale-pink mucous membranes and mild tachycardia are seen in chronic stages (de Waal et al. 2004). Strenuous exercise may predispose horses to the clinical manifestation of the disease (Hailat et al. 1997).

Weakness at birth or the rapid onset of listlessness as well as development of anaemia, severe icterus, malaise, fever and petechiae soon thereafter, characterise neonatal babesiosis in foals (de Waal et al. 2004).

Nervous signs are seldom seen in equine babesiosis, but ataxia, generalised trembling and mild to moderate tonic-clonic spasms have been reported from young foals infected with *Theileria equi*. Further complications of equine babesiosis are acute renal failure, colic and enteritis, pneumonia, loss of fertility in stallions and abortion in mares (de Waal et al. 2004).

2.1.8 Pathology

Clinical pathology reveals a decrease in haemoglobin concentration, erythrocyte and platelet count (de Waal et al. 2004; Camacho et al. 2005), as well as neutropaenia and lymphopaenia in acute cases (de Waal et al. 2004). Total serum bilirubin, urea, aspartate aminotransferase (AST), creatine kinase (CK), γ-glutamyltransferase (GGT) and lactate dehydrogenase (LDH) can, particularly in *Theileria equi* infected horses, present increased levels and indicate haemolytic anaemia and centrilobular degeneration and necrosis of hepatocytes (Camacho et al. 2005).

Macroscopically, equine babesiosis presents signs due to associated haemolytic anaemia and icterus. Common post mortem findings in acute infections include subcutaneous oedema of the lower body and extremities, serous exudates in all body cavities, splenomegaly (Schein
1988; Phipps et al. 1996; de Waal et al. 2004), as well as hepato- and renomegaly (Schein 1988; Phipps et al. 1996). Extensive kidney damage, including degeneration of the tubular epithelium with deposition of haemoglobin in the renal tubules, centrilobular necrosis and cellular infiltration of the sinusoids are often noted. Pronounced jaundice of serous membranes and pulmonary oedema are more prominent in Babesia caballi than in Theileria equi infections, whereas general lymphadenopathy has been observed in the latter (Schein 1988; Phipps et al. 1996).

Aborted foetuses and neonatal foals present anaemia and moderate to severe icterus, petechiae on visceral and serosal surfaces, hydrothorax, congestion and oedema of the lung, spleno- and hepatomegaly (de Waal et al. 2004).

2.1.9 Diagnosis

Diagnosis of equine babesiosis can be made from a combination of clinical signs, examination of stained blood smears and identification of the organism using light microscopy, serology, subinoculation of blood into a susceptible animal (Frerichs et al. 1969; Phipps et al. 1996; de Waal et al. 2004), polymerase chain reaction (PCR) (Rampersad et al. 2003; Alhassan et al. 2005; Vial et al. 2006) or xenodiagnosis (Frerichs et al. 1969; de Waal et al. 2004). Due to the low parasitaemia, the demonstration of the parasite in thin blood smears is often very difficult (Heerden 1996; Phipps et al. 1996; de Waal et al. 2004). Thick blood smears are therefore a recommended technique for parasite detection, particularly with low parasitaemia (de Waal et al. 2004). The complement fixation test (CFT) is of preferred use (Frerichs et al. 1969; Schein 1988; Brüning 1996; de Waal et al. 2004), helping to differentiate between Babesia caballi and Theileria equi infections (Madden et al. 1968; Weiland 1986). CFT in combination with the indirect fluorescent antibody (IFA) test is preferred for detection of carrier and chronic cases (Tenter and Friedhoff 1986; Weiland 1986) and for the purpose of screening horses before exportation to countries which are free from the disease (Schein...
1988; Phipps et al. 1996; Brüning 1996; de Waal et al. 2004). However, limitations of both CFT (Phipps et al. 1996) and IFA tests (de Waal et al. 2004) include the occurrence of false-positive and false-negative results. Another problem is the occurrence of cross-reactions in the CFT and IFA test (Joyner et al. 1981; Tenter et al. 1986; Weiland 1986; Brüning 1996). Cross reactions were not reported in an earlier study of Babesia spp. carrier horses using IFA test (Madden et al. 1968).

More recently enzyme-linked immunosorbent assays (ELISA) have been used in experimentally infected horses, but serological cross-reactions also have been encountered (Weiland 1986; de Waal et al. 2004). New ELISA tests based on recombinant antigens (Katz et al. 2000) and specific monoclonal antibodies have been developed for the detection of both parasites, but although the sensitivity appears to be better than the CFT, further validation studies are recommended (de Waal et al. 2004).

DNA probes have also been developed for the direct detection of Babesia spp. (Phipps et al. 1996), which can also detect parasite DNA in the blood of infected ticks (Brüning 1996). Parasitaemia of Theileria equi equivalent to less than 0.0025% is detectable with DNA probes (Posnett et al. 1991). Recent advances in the in vitro culture method of equine babesiosis parasites have lead to the identification of Theileria equi and Babesia caballi in microscopically and serologically negative horses. It is a very sensitive method, as parasitaemias as low as 10^{-10}% may be detected (de Waal et al. 2004).

Most recently a single-round and multiplex PCR method was developed for the simultaneous detection of Theileria equi and Babesia caballi, which appears to be a very promising tool for the routine laboratory diagnosis of equine babesiosis (Alhassan et al. 2005).

Equine babesiosis commonly occurs secondary to diseases or infections and therefore the possibility of an underlying disease or infection should always be kept in mind (Heerden 1996).
The differences in drug sensitivity between *Theileria equi* and *Babesia caballi* make an accurate diagnosis of the specific parasite involved essential when choosing therapy (de Waal *et al.* 2004).

2.1.9.1 Complement fixation test (CFT)

The complement fixation test (CFT) follows the principle of fixation of complement during the reaction between specific antigen and antibody. After using a known amount of complement, a remaining unfixed amount can be detected by a separate antigen-antibody reaction and thus the complement fixing activity of the original serum can be measured. It is assessed by estimation of lysis as a percentage, i.e. 0%, 25%, 50%, 75% and 100%. These values are accorded as follows: 4+ to 2+ (positive result), 1+ (trace) or a complete haemolysis as a negative result (Brüning 1996).

The CFT is highly specific for *Babesia caballi*. A reaction of at least 1+ at serum dilution of 1:80 or greater is considered as evidence of an acute infection in a horse that has been exposed to the parasite within the previous six months. Late stages of the carrier state present a 2+, 3+ or 4+ reaction at serum dilution of 1:5. In a study done by Frerichs *et al.* (1996), antibodies did not appear until six to eight days after the parasite was observed on blood smears, then increased rapidly and reached a peak at 30 to 45 days. Positive CFT titer persisted between three and 15 months longer than the carrier state (Frerichs *et al.* 1969; Joyner *et al.* 1981) and up to 24 (Brüning 1996) or even 28 months in the case of *Theileria equi* (Joyner *et al.* 1981). However, false negative results were presented in another study (Tenter *et al.* 1986).

2.1.9.2 Indirect fluorescent antibody test (IFAT)

In the indirect fluorescent antibody test (IFAT), parasite antigens are bound to glass slides and allowed to react with the test sera. After adding and binding of a fluorescein-labelled anti-
species serum, bound antibodies are visible under ultra-violet light. Sera are considered positive by presenting strong fluorescence at any dilution of 1:80 and above. This test is considered to be more sensitive than the CFT, as parasites can be detected between three to 20 days post infection and also during the latent phase of infection (Tenter et al. 1986; Weiland 1986; Brüning 1996).

2.1.9.3 Enzyme-linked immunosorbent assay (ELISA)

The optimised enzyme-linked immunosorbent assay (ELISA) uses either monoclonal antibodies or recombinant proteins (Brüning 1996; de Waal et al. 2004). In this test, antibodies in test sera compete with either the monoclonal antibody for the antigenic site in the merozoites or recombinant proteins from the parasite. A reduced colour reaction indicates positive titres. These tests are highly specific and sensitive (Brüning 1996).

2.1.9.4 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is used to amplify a sequence of DNA. Initially parasitic DNA has to be extracted and the resulting purified DNA is used as a template for subsequent PCR amplification. Specific primers for Babesia caballi and Theileria equi are necessary to test for the presence of DNA of these parasites. Following the PCR the presence and quantity of amplified DNA is tested using gel electrophoresis (Rampersad et al. 2003; Alhassan et al. 2005).

2.1.9.5 DNA probes

Extracted DNA from a blood sample is cut by restriction enzymes into several pieces. A specific DNA probe for the parasite is radiolabelled, incubated and hybridised with the extracted DNA. Subsequently autoradiography can be performed to screen for parasitic DNA (Posnett et al. 1991).
2.1.10 Differential diagnosis

Equine influenza, equine encephalosis and equine infectious anaemia have to be considered clinically as differential diagnoses for equine babesiosis. Equid herpesvirus-1 infection or iso-erythrolytic neonatal icterus must be distinguished in new-born foals. The former also has to be differentiated in cases of abortion in mares (de Waal et al. 2004).

2.1.11 Immunity

Horses can remain carriers for up to four years after an infection with *Babesia caballi* or probably life-long after a *Theileria equi* infection (Holbrook 1969; Schein 1988; Phipps et al. 1996; de Waal et al. 2004). As a reservoir for infection of ticks, these animals play an important role in the epidemiology of equine babesiosis (Holbrook 1969; Phipps et al. 1996; Donnellan et al. 2003b; de Waal et al. 2004).

Gummow et al. (1996) reported a higher prevalence of antibodies against *Babesia caballi* in colts than in fillies (Gummow et al. 1996).

Maternal antibodies can be detected in foals up to five (Heerden 1996; de Waal et al. 2004) or six (Phipps et al. 1996) months of age. Foals are probably protected by passively-acquired immunity and non-specific factors during the first six to nine months of age (de Waal et al. 2004). Infective tick bites usually produce only subclinical diseases during this time, with constant superinfections leading to a stable immunity (Phipps et al. 1996).

In a survey in Brazil, Ribeiro et al. (1999) found antibodies in horses of all ages, although less prevalent in foals of six months of age or younger (Ribeiro et al. 1999).

Cross-immunity has yet to be reported (Schein 1988; Donnellan et al. 2003b; de Waal et al. 2004) but simultaneous infection with both species may occur (Donnelly et al. 1980).
2.1.12 Treatment of equine babesiosis

*Theileria equi* parasites are generally more resistant to treatment than *Babesia caballi* (Kuttler 1980; Schein 1988; Kuttler 1988; Lindsay and Blagburn 2001). Different drugs such as quinuronium derivates, acridine derivates and bisazo dyes such as trypan blue are reported to be effective (Kuttler 1988; Brüning 1996; de Waal *et al.* 2004). Less effective results are also reported from treatments of *Theileria equi* with tetracycline and oxytetracycline hydrochloride or parvaquone, an anti-theilarial drug (de Waal *et al.* 2004). The anti-theilarial compounds buparvaquone (Zaugg and Lane 1989) and parvaquone (Kuttler *et al.*, 1987) failed to eliminate the infection in *Theileria equi* infected splenectomised carrier horses (Kuttler *et al.* 1987; Zaugg and Lane 1989). A recent study by Kumar *et al.* (2003) tested different anti-theilarial (parvaquone and burparvaquone) and anti-malarial (artesunate and arteether) drugs as well as imidocarb against an Indian strain of *Theileria equi* in donkeys. Imidocarb and a combination of arteether and burparvaquone were of similar efficacy in controlling *Theileria equi*, with reduced side effects from the drug combination compared to imidocarb alone (Kumar *et al.* 2003). Another new approach in treatment is drugs that target biosynthetic pathways of plasmid-derived organelles (Vial *et al.* 2006). Triclosan, belonging to the class of synthetic 2-hydroxydiphenyl ethers with broad-spectrum microbiotical activity, is an example of this group (Bork *et al.* 2003). This inhibitor had effective growth inhibiting effect on *Babesia caballi* and *Theileria equi* in an *in vitro* study (Bork *et al.* 2003; Vial *et al.* 2006). This points to a critical role played by type II fatty acid biosynthesis within these parasites (Vial *et al.* 2006).

Aromatic diamidines and related compounds, including diminazene diaceturate, amicarbalide diisethionate and imidocarb dipropionate have been shown to be more efficient babesiacides (Schein 1988; Brüning 1996; de Waal *et al.* 2004). Repeated treatments may be required for the control of *Theileria equi* infections as most of these compounds cannot sterilise this...
infection (Carbrey et al. 1971; Kumar et al. 2003; Donnellan et al. 2003b; de Waal et al. 2004).

Severity of the disease and success of treatment may also depend on protozoal strain differences and susceptibility of the affected animal (Brüning 1996). Therefore, in severe cases of babesiosis, supportive therapy might be necessary. This may include combinations of blood (Heerden 1996) with or without fluid infusion, administration of vitamins (Kuttler 1988), good nutrition (Phipps et al. 1996), antibiotics, polyionic electrolyte infusion, oral laxatives and essential phospholipids (Brüning 1996; de Waal et al. 2004).

Treatment should never aim to sterilise the infection in endemic areas (Heerden 1996), unless horses are to be moved from an endemic area into one free of disease (Kuttler 1988; de Waal et al. 2004).

Although donkeys were successfully vaccinated against Theileria equi in a recent study (Kumar et al. 2002), no vaccines for equine babesiosis are currently available (Brüning 1996; de Waal et al. 2004). Therefore control of the disease in endemic areas involves strategic exposure to infected ticks of young foals and subsequent regular anti-tick prophylaxis during their entire life (Heerden 1996; Brüning 1996). Highly sophisticated intensive management systems can probably eradicate the vector ticks completely and hence control the disease (Heerden 1996), but leave the animals with naïve immunity susceptible to subsequent reinfection (Vial et al. 2006).

2.1.13 Imidocarb

Although there are different babesiacidal drugs, imidocarb dipropionate is the drug of choice
Imidocarb dipropionate is an aromatic diamidine. The chemical formulae 1,3-bis[3-(4,5-dihydro-1H-imidazol-2-yl)phenyl]urea (PubChem CID 21389 2007) or [3,3’ bis-(2-imidazolin-2-yl) carbanilide dipropionate] (Lindsay et al. 2001) are published. It has a widespread distribution in body fluids and tissue (Belloli et al. 2002). A study performed on a small sample size showed that imidocarb crosses the equine placenta and reaches concentrations in the foetus similar to the dam (Lewis et al. 1999). Plasma concentration of imidocarb was not still detectable twelve hours after administration of 2.4 mg/kg bwt (Belloli et al. 2002). It is recommended to be administered intramuscularly or subcutaneously, but not intravenously (Vial et al. 2006). The precise mode of action against piroplasmosis is not yet clearly understood (Simpson and Neal 1980a; Kuttler 1988; Brüning 1996; Vial et al. 2006). It is thought to involve the compound’s combination with nucleic acids, causing partial uncoiling and denaturation of the DNA double helix of the parasite. This leads to dilation of the nuclea cisterna, karyorrhexis, cytoplasmic vacuolation, inhibition of food vacuole formations and ribosomal diminuation (Simpson et al. 1980a; Kuttler 1988; Brüning 1996). Simpson et al. (1980) reported intra-erythrocytic crystallisation of haemoglobin in imidocarb treated horses infected with Theileria equi, but concluded it as of no practical consequence, since only 1 to 2% of parasitised erythrocytes contained crystals (Simpson et al. 1980b).

Imidocarb also inhibits the entry of inositol into the parasitised erythrocyte and therefore leads to “starvation” of the parasite (Vial et al. 2006).

The therapeutic dose of imidocarb dipropionate is between 2 to 4 mg/kg body weight, with two doses given 24 hours apart (Phipps et al. 1996). A 2 mg/kg body weight dose administrated at a 24-hour interval is extremely effective against Babesia caballi (Frerichs and Holbrook 1974; Vial et al. 2006). Belloli et al. (2002) suggest a plasma concentration lower than 0.3 to 0.25 µg/ml of imidocarb as sufficient (Belloli et al. 2002). The commonly
recommended dosage is 2.4 mg/kg body weight administered intramuscularly on two consecutive days. This treatment sterilises *Babesia caballi* infection and clinically eliminates *Theileria equi* infections (Phipps et al. 1996). In one study using imidocarb dihydrochloride at a dosage of 4 mg/kg body weight administered four times at 72-hour intervals, the drug was able to clear the *Theileria equi* infection in 13 of 14 horses. However, all eight donkeys in the study died and severe side effects such as salivation, restlessness, gastrointestinal hypermotility and colic occurred in the treated horses (Frerichs et al. 1973). This result may be explained as a consequence of differing drug susceptibility to different *Babesia* strains as a similar treatment regimen failed to clear a European strain of *Theileria equi* infection within carrier horses in another study (Kuttler et al. 1987). This is supported by Schetters et al. (1997), who studied European and South African strains of *Babesia canis* in dogs and demonstrated that geographically different isolates reveal different pathology (Schetters et al. 1997).

It is recommended to divide the treatment into two equal doses given 30 minutes apart to minimise the side effects of therapy (Kuttler 1988; Heerden 1996).

2.1.14 Efficacy and side effects of different anti-parasitic agents

Sterilisation of *Babesia caballi* infections is effectively done by several drugs, but the elimination of *Theileria equi* is very difficult (Brüning 1996; de Waal et al. 2004). Diminazene diaceturate, amicarbalide and imidocarb sterilise *Babesia caballi*. However, diminazene diaceturate causes swelling and necrosis at the injection sites. Toxic doses may result in respiratory distress, depression and other signs of intoxication (Brüning 1996; de Waal et al. 2004).

The only report of elimination of *Theileria equi* was from a trial with imidocarb treatment, used at a dosage rate of 4 mg/kg body weight (Frerichs et al. 1973) and at 10 mg/kg body
weight at 72-hour intervals. However, severe side effects such as colic, diarrhoea and anorexia sometimes leading to death were observed (Carbrey et al. 1971; de Waal et al. 2004).

Meyer et al. (2005) focussed particularly on renal and liver function when examining six ponies following four intramuscular doses of 4 mg/kg imidocarb dipropionate given at 72-hour intervals. They reported local irritation at the injection sites, which was also reported by Kumar et al. (2003), plus mild colic, depression and anorexia in some animals. Temporary mild renal decompensation was also a feature, but no significant deleterious effect on hepatic function was noted (Meyer et al. 2005).

Even at the usual intramuscular dosage of 2.4 mg/kg body weight, colic may occur due to the cholinergic properties of this drug. Frerichs and Holbrook (1974) observed mild salivation and slightly increased gastrointestinal motility even at a dosage of 2 mg/kg body weight (Frerichs et al. 1974). Their study animals, on the other hand, did not show any local irritation at the injection site even though up to 8 mg/kg body weight of imidocarb dipropionate was used (Frerichs et al. 1974).

Additional administration of atropine (Heerden 1996; de Waal et al. 2004), or glycopyrrolate (Donnellan et al. 2003a; Donnellan et al. 2003b) or splitting the calculated dosage of imidocarb into two given 30 minutes apart, is reported to minimise or prevent the side effects (Kuttler 1988; Heerden 1996). The toxic dose of imidocarb dipropionate in horses is close to 10 mg/kg body weight, while the LD₅₀ is about 16 mg/kg body weight for 21 days, administered intramuscularly twice at an interval of 48 hours (Adams 1981; de Waal et al. 2004). Mortalities are due to hepatic and renal necrosis, occurring within six days after the first injection (Adams 1981).

Imidocarb is a carbamate, causing reversible inhibition of cholinesterase (Donnellan et al. 2003b). Intramuscular injection of imidocarb can cause local irritations such as acute
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haemorrhagic necrosis of the skeletal muscle with clear fascial oedema and mineralised myositis, described as post mortem findings (Adams 1981).

The toxicity of imidocarb dipropionate is related to its cholinergic (Donnellan et al. 2003a; Vial et al. 2006) plus direct nephrotoxic and hepatotoxic effects (Donnellan et al. 2003b). In horses, dose-dependent nephrotoxicity and hepatotoxicity is seen, with cortical tubular necrosis, hepatocellular necrosis and pronounced periportal hepatocellular swelling. These findings are similar to the toxic effects of imidocarb in dogs (Abdullah et al. 1984) and goats, in which species it may cause immediate transient excessive salivation and diarrhoea, followed by anorexia, dyspnoea, recumbency and death due to acute renal failure and uraemia caused by renal tubular necrosis. The hepatotoxic action of imidocarb has only minimal effect on goats (Corrier and Adams 1976). The recommended dosage of 4 mg/kg IV for dogs and goats, resulted in transient salivation for both species and diarrhoea in dogs (Abdullah and Baggot 1983). A study in goats reported that alterations in the disposition kinetics of imidocarb can occur as a result of fever and is dependent on the causative agent (Abdullah and Baggot 1986).

Horses receiving imidocarb dipropionate at a dosage of 4 mg/kg body weight may present a systemic reaction, including depression, excessive sweating, extreme restlessness, salivation, lacrimation, miosis, serous nasal discharge, dyspnoea, violent persistalsis and excessive gut motility with frequent defaecation, colic and lateral recumbency lasting between 30 minutes and four hours (Adams 1981; Phipps et al. 1996). The symptoms could be minimised by giving half the calculated dose twice, 15 to 30 minutes apart (Frerichs et al. 1973). Abortion was not reported from Adams’ study (Adams 1981). Blood urea nitrogen, serum aspartate aminotransferase, serum sorbitol dehydrogenase and serum creatine phosphokinase concentrations are also reported to be increased after an imidocarb treatment (Adams 1981).
After treatment at the dosage of 2.4 mg/kg body weight, only mild transient reactions such as salivation, diarrhoea and abdominal discomfort are generally seen (Heerden 1996). Resistance to imidocarb dipropionate has been introduced in laboratories and some acute phases of babesiosis have not responded to this drug. Residue problems have led to the unavailability of imidocarb dipropionate in some areas, including Europe (Vial et al. 2006).

2.2 ATROPINE AND GLYCOPYRROLATE

As an alternative to splitting the administered dose of imidocarb dipropionate, side effects may be minimised by the prior administration of either atropine sulphate (Heerden 1996) or glycopyrrolate at a dose of 0.0025 mg/kg i.v. or 1ml/80 kg bwt i.v. (Donnellan et al. 2003a; Donnellan et al. 2003b).

2.2.1 Atropine sulphate

In a review of cholinergic drugs, Adams (2001) described atropine as a parasympatholytic drug that prevents acetylcholine from exerting its characteristic effects on structures innervated by postganglionic parasympathetic nerves. This muscarinic blocking agent is an alkaloid, extracted from the Belladonna plants. Chemically, it consists of the organic base tropine, which is bonded through an ester linkage to tropic acid.

Atropine interacts with muscarine receptors of effector cells and by its occupation prevents acetylcholine from binding to the receptor. Large amounts of acetylcholine or other cholinomimetic drugs can push atropine aside by competitive binding. Different parts of the body have different susceptibilities to the drug. Therefore salivary and cholinergic sweat glands need only a small amount of atropine to be affected, whereas the heart, gastrointestinal tract and urinary tract require larger dosages (Adams 2001).
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Cardiovascular effects of atropine include an increased pulse rate and a raised cardiac output. The blood pressure either remains unchanged or increases slightly. Atropine, when given in a large dose, is directly depressant to the myocardium and also causes cutaneous vascular dilatation (Adams 2001).

By inhibiting the contractile effects of cholinergic nerve impulses, atropine also causes a relaxation of the smooth muscles of the gastrointestinal (GI) tract. It clearly decreased the myoelectrical and myomechanical activities in the jejunum and pelvic flexure in a study using impedance monitoring of equine intestinal motility (Lamar et al. 1984). Atropine induces a significant decrease in the amplitude of contractions, but not their frequency in the equine jejunum (Malone et al. 1996). This action has been used in the treatment of intestinal spasm and hypermotility (Adams 2001), although Ducharme and Fubini (1983) recommend that it should not be used for the relief of gastrointestinal spasm (Ducharme et al. 1983). Roberts and Argenzio (1986) suggest also that the use of atropine in colic and diarrhoea is contraindicated (Roberts and Argenzio 1986).

Atropine has also shown to delay gastric emptying of liquids in man and to reduce antroduodenal motor activity (Imbimbo et al. 1990). Secretions from the GI tract and from the salivary glands will be markedly reduced by atropine. By administration of exceedingly high doses, atropine may also decrease gastric secretion (Adams 2001). Donnellan et al. (2003) reported in their study a significant reduction of gastrointestinal motility in horses after co-administration of atropine (Donnellan et al. 2003a). Williams et al. (2000) showed in their study that even topical and subconjunctival atropine application decreases gut motility (Williams et al. 2000).

In the respiratory tract, atropine administration leads to a decreased secretion and bronchodilatation, which also gives temporary symptomatic relief from acute dyspnoea in
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horses with recurrent airway obstruction (Adams 2001). It can be used as a diagnostic test in the diagnosis of heaves (Robinson et al. 2001). Atropine also affects the ocular, urinary and central nervous systems (CNS), where it causes mydriasis and cycloplegia, urine retention, and mania and excitement, respectively. It is therefore contraindicated in the presence of increased intraocular pressure from acute angle glaucoma (Adams 2001). Mydriasis and possibly CNS stimulation may be seen as atropine crosses the blood-brain barrier (Donnellan et al. 2003b).

Clinically, atropine can be used to control smooth muscle spasm; to antagonise excessive cholinergic stimulation, in conjunction with morphine to reduce salivary secretion (Adams 2001); to prevent bradycardia and the second degree heart block in horses induced by xylazine (Adams 2001) or romifidine (Gasthuys et al. 1990), and to decrease the dose requirement of dobutamine in horses anaesthetised with detomidine and halothane (Weil et al. 1997). Atropine can also facilitate ophthalmoscopic examination, although it has been superceded by shorter-acting compounds. Furthermore it is used in preventing or breaking down adhesions between lens and iris and is also an essential antidote to anticholinesterase intoxication (Adams 2001).

Species are less susceptible to the toxic effects of atropine when it is given orally. Herbivora are usually more resistant to atropine toxicity than Carnivora. Clinically, an atropine poisoning case may present with dry mouth, thirst, dysphagia, constipation, mydriasis, tachycardia, hyperpnoea, restlessness, delirium, ataxia and muscle trembling. In severe cases, convulsions, respiratory depression and respiratory failure lead to death (Adams 2001).

One of the major aims of this study was to quantitate the effect of atropine administration on equine orocaecal transit time, and to study its interaction with imidocarb dipropionate on this
parameter. The effect of atropine on transit of solid phase material has not previously been determined in the horse, and was investigated in this study using the lactose $^{13}\text{C}$-ureide breath test, a novel test involving stable isotope technology.

2.2.2 Glycopyrrolate

Glycopyrrolate, a quaternary nitrogen compound, is also an anticholinergic agent but has some advantages over atropine sulphate and has found attention for preanaesthetic use in veterinary medicine (Singh et al. 1996; Adams 2001). It has an increased water solubility and thus does not cross the blood-brain barrier (Donnellan et al. 2003b). The tachycardic response associated with muscarinic block at the sinoatrial node seems to be a lesser problem with glycopyrrolate such that a dose of 2.5 µg/kg was reported not to influence the heart rate (Singh et al. 1997a). In addition, ocular effects are not seen with glycopyrrolate, as it does not cross the blood-brain barrier (Singh et al. 1997a).

In comparison to atropine sulphate, the reduced secretions within the respiratory tract and lesser penetration of the blood-brain barrier also make this drug a safer alternative (Adams 2001). Horses given glycopyrrolate prior to imidocarb administration showed the same gastrointestinal motility as a saline control group in a recent study (Donnellan et al. 2003a) when estimated according to ultrasonographic assessment of large bowel motility. Hence prior administration of glycopyrrolate may be preferable to atropine for minimising or preventing the side effects associated with imidocarb treatment.

Singh et al. (1996) evaluated the effect of glycopyrrolate as premedication to xylazine/ketamine-induced anaesthesia. They reported a significantly reduced cardio-vascular dysfunction by using glycopyrrolate and a delayed return to normal gastrointestinal motility without causing any side effects (Singh et al. 1996).
The effects of glycopyrrolate (Singh et al. 1997a) and atropine (Roberts et al. 1986) are dose dependent.

In this study, the effects of imidocarb dipropionate on equine intestinal motility, and the relative effects of atropine or glycopyrrolate premedication were quantified using stable isotope breath test technology. Pharmacological effects on oro-caecal transit time were studied using the induced lactose $^{13}$C-ureide breath test (LUBT) (Geypens et al. 1999; Sutton et al. 2002; Sutton 2003). The primary objective of this study was to determine the optimum premedication strategy for equine babesiosis in order to minimise the gastrointestinal complications associated with treatment.

Semi-quantitative studies of gastrointestinal motility using transabdominal ultrasonography have suggested that the effect of imidocarb on gut motility is best ameliorated by premedication with glycopyrrolate (Donnellan et al. 2003a; Donnellan et al. 2003b). As non-invasive quantitative stable isotope tests have now been validated for the measurement of equine oro-caecal transit time, this study aims to quantify the effects of treatment modalities on this specific parameter of intestinal transit.

2.3. BRIEF OVERVIEW OF EQUINE GASTROINTESTINAL ANATOMY AND PHYSIOLOGY

2.3.1 The equine stomach

The single chambered equine stomach is relatively small and has a physiological capacity of eight to 15 l. It lies with its largest part in the left dorsoproximal cranial abdomen and is bordered by the spleen, diaphragm, liver, jejunum, descending colon, the diaphragmatic flexure of the large colon, the right ascending colon and the pancreas (Nickel et al. 1979).
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It consists of four histologically different regions of mucosal lining. The *Pars proventricularis* (Nickel *et al.* 1979) is the oral half, similar to the oesophagus and lined by squamous mucosa without any glandular structure. There is the absence of active transport processes. The *margo plicatus* marks the border between the squamous mucosa and the narrow strip of glandular mucosa that presents the cardiac gland region (Merritt 1999) or *Pars glandularis* (Nickel *et al.* 1979). Although numerous somatostatin-immunoreactive cells can be found there, playing an important rule in endogenous control of gastric acid secretion, little is known about the function of this glandular region. Along the greater curvature and up the sides to where the junction with the cardiac gland mucosa occurs, there is the fundic gland region. This gastric region includes parietal cells secreting hydrochloric acid (HCL) and zymogen cells producing pepsinogen, as well as enterochromaffin-like (ECL) cells that may secrete histamine in addition to proven serotonin production. The gastric antrum is lined by the pyloric gland region, which is the main source of gastrin, produced by G-cells. Furthermore somatostatin and serotonin are produced there by D- and ECL-cells, respectively (Merritt 1999).

2.3.2 The small intestine

The stomach empties into the small intestine, which consists of the duodenum, jejunum and ileum with a length of 1 m, 25 m and 70 cm, respectively. The duodenum lies next to the liver and traverses between the right lobe of the liver and the ascending colon along the caecal base. At the duodenojejunal flexure the duodenum ends at the jejunum which is held in position by the mesenteric root. The jejunal tubes are mixed with the tubes of the descending colon in the left dorsal abdomen. The jejunum ends at the ileum in the left caudal abdomen, moves into the right caudal abdomen and ends with the *Ostium ileale* into the caecum (Nickel *et al.* 1979).
Myoelectrical recording has shown that the small intestinal migrating myoelectrical complex (MMC), a pattern of electrical activity, consists of three phases that last approximately 90 min and reoccur cyclically in horses. Unlike other species, MMC activity is not disrupted by *ad libitum* feeding. Phase I is a quiet period, showing no action potential (AP) activity. Phase II presents intermittent AP activity and is that period during which most of the transit of intestinal content occurs. Phase III has the most intense and continuous AP activity and is most prominent in the equine duodenum (Merritt 1999).

Brush border enzyme activity is responsible for the hydrolysis of disaccharides in the equine small intestine. The lactase enzyme neutral β–galactosidase declines in activity from birth to three years of age and disappears completely thereafter, leading to an inability of lactose digestion and absorption in adult horses (Roberts 1975).

2.3.3 The caecum and large colon

The caecum fills a major part of the right abdomen and has an average length of 1 m and a capacity of 16 to 68 l, with an average of 33 l, consisting of a caecal head, caecal body and caecal apex with several haustra. Through the *Ostium caecocolicum* it ends into the ascending colon (Nickel *et al.* 1979).

Ross *et al.* (1986) observed a haustra-to-haustra mixing movement involving the caecal body and caudal caecal base once every four minutes. This coordinated contraction, starting in the caecal body, was reported to push ingesta forward into the caecal base, and from there via the caecocolic orifice into the right ventral colon (Ross *et al.* 1986).

A pacemaker region, supposedly located in the caecal body near the apex, is thought to be responsible for caecal emptying and initiation of aboral peristaltic activity of the right ventral colon. Existing retropulsion of the right ventral colon, independent of caecal activity, was confirmed in a study investigating normal motility of the caecum and the right ventral colon.
(Ross et al. 1986) suggesting the presence of a further discrete pacemaker region for colonic activity.

Simmons and Ford (1990) reported a liquid flow from the caecum of 54.2 ± 1.89 l/d and from the large colon of 49.9 ± 1.25 l/d in ponies. The capacity of the caecum and large colon were 7.01 ± 0.8 (SE) and 17.71 ± 3.7 (SE), respectively (Simmons and Ford 1990).

Bacterial isolates from the equine caecum presented as 50.9% gram-negative rods, 22.8% gram-positive rods and 21.9% gram-positive cocci (Maczulak et al. 1985). Only 18% of these isolates were reported to use urea for growth and urease activity was detected in 2 of 114 isolates only. An explanation for the low number of ureolytic bacteria isolated was that either a low number of ureolytic bacteria might exist in the equine caecum, or that urease activity is related to the caecum wall and thereby excluded due to the sampling procedure of this study (Maczulak et al. 1985).

The ascending colon has a length of 3 to 4 m and a capacity of 55 to 130 l and lies in a U-formation in the abdominal cavity. It leads from right caudoventral to cranial, building the diaphragmatic flexure, towards the left, from cranial to caudal. There it bends backwards again, building the ventral and dorsal parts of the pelvic flexure before leading into the right dorsal colon. Near the 17th to 18th thoracic vertebra it ends at the transverse colon, a short part of the colon which pulls towards the craniodorsal left abdomen and leads into the descending or small colon. The small colon (2.5 to 4 m long) lies in the left craniodorsal abdomen and later ends close to the pelvis at the short rectum (Nickel et al. 1979).

The circular and longitudinal muscle layers of the equine colon are directly coupled electrically. They act as a pacemaker in the area of the pelvic flexure for retropulsion-propulsion events (Sellers et al. 1982). However, this pacemaker theory is questioned by other authors, who found out that there is a greater activity at the pelvic flexure area and the left ventral colon during a fed than a fasted state (Merritt et al. 1995).
2.3.4 Large colon and caecal impaction

Both caecal and colonic neuronal deficit has been reported as a cause of disordered motility of these sections of intestine, resulting in chronic or recurrent impactions. Schusser et al. (2000) reported that caecal neuronal density was decreased in those horses that suffered from recurrent caecal impaction, and suggested an hereditary basis (Schusser et al. 2000). NSAIDs have also been associated with large colon as well as caecal impaction by suppressing prostaglandin synthesis. A selective COX-2 inhibitor is suggested to preserve COX-1 activity and therefore maintain constitutive prostaglandin levels and intestinal contractility, but further research is needed (White 2000).

2.4 Stable Isotope Breath Tests

2.4.1 Background and Principles

Stable isotope breath tests provide an indirect and non-invasive means of testing gastrointestinal function. The main general principle of breath tests is that a metabolic reaction or a physiological process can be studied by measuring the rate of appearance of metabolites of a labelled test substrate in exhaled breath. This metabolite can either be hydrogen gas, produced by bacterial fermentation, or carbon dioxide from endogenous metabolism (Halliday and Rennie 1982). The choice of $^{13}$C labelled substrate determines the specific physiological process that is studied. The rate of increase in $^{13}$C:$^{12}$C ratio in exhaled breath provides an indirect measure of the physiological process under study, as long as this is the major rate-limiting stage in the metabolism of the specific $^{13}$C-labelled isotope administered. The dose, the $^{13}$C enrichment of the substrate and the degree of metabolic CO$_2$ production all influence the enrichment of $^{13}$C during a breath test (Stellaard and Geypens 1998).
Breath tests are used extensively in human gastroenterology for evaluation of small intestinal bacterial overgrowth and lactose intolerance (Bracher and Baker 1994; Romagnuolo et al. 2002), carbohydrate malabsorption, assessment of gastrointestinal motility and investigation of food digestibility (Bracher et al. 1994), effects of drugs on oro-caecal transit time (OCTT) (Staniforth 1987), pancreatic and hepatic function (Koletzko et al. 1997; Kalivianakis et al. 1997; Romagnuolo et al. 2002) and for diagnosis of Helicobacter pylori (Koletzko et al. 1997; Romagnuolo et al. 2002). However, the evaluation of liver and pancreatic function by specific breath tests is yet to be recommended for routine clinical diagnostic use (Romagnuolo et al. 2002).

2.4.2 ¹³C-Octanoic Acid Breath Test (¹³C-OABT)

The ¹³C-octanoic acid breath test (¹³C-OABT) has been reported for the evaluation of gastric emptying rate in dogs (Wyse et al. 2003), ponies (Wyse et al. 2001) and horses (Sutton 2003) in addition to its routine diagnostic use in people (Schwabe et al. 1964; Ghoos et al. 1993; Maes et al. 1994; Choi et al. 1998; Van Den Driessche 2001). The underlying principle of the ¹³C-OABT is that, following ingestion of a ¹³C-octanoate labelled test meal, the rate of increase in expiratory ¹³C:¹²C ratio forms an indirect measure of the rate of solid phase gastric emptying of that meal (Ghoos et al. 1993). ¹³C-octanoic acid is a medium chain fatty acid (MCFA). After leaving the stomach and subsequently the intestinal mucosa, MCFAs follow the portal venous system and reach the liver rapidly. After entering the Krebs cycle, ¹³CO₂ will be generated that equilibrates with the body bicarbonate pool, undergoes respiratory pulmonary exchange and causes a consequent increase in expiratory ¹³C:¹²C ratio (Bach and Babayan 1982). Measurement of this change in expiratory ¹³C:¹²C ratio can be used to provide an indirect measure of the rate of gastric emptying as this has been demonstrated to be the major rate-limiting determinant of this process. Metabolism of the ¹³C-octanoate otherwise proceeds at a constant rate in the healthy individual.
Lee et al. (2000) more recently validated the $^{13}$C *Spirulina platensis* breath test against the gold standard technique of scintigraphy in man as being a suitable indirect method for determination of gastric emptying rate (Lee et al. 2000b). They further investigated a simplified approach of the $^{13}$C-octanoic acid breath test for the measurement of gastric emptying in diabetics (Lee et al. 2000a). Recently, a promising preliminary study was published using natural $^{13}$C and the cheaper non-dispersive infrared spectrometry-analysis in man (Jonderko et al. 2005) for the assessment of gastric emptying rate.

### 2.4.3 Measurement of Oro-Caecal Transit Time

The symptoms of many gastrointestinal disorders may be secondary to small intestinal dysmotility. Techniques have been developed to assess oro-caecal transit time (OCTT) as an indirect measure of small intestinal transit. Gastroenterocolonic scintigraphy is used as the “gold standard” for measurement of OCTT in humans. The application of gastroenterocolonic scintigraphy is not possible in horses due to the large size of the equine abdomen and imaging difficulties.

OCTT is the time required to travel from the mouth to the arrival of the head of the column of ingesta in the caecum, and depends on the gastric emptying rate as well as the small intestinal transit time (Miller et al. 1997).

Grain ingestion may be responsible for a delay in gastrointestinal transit in horses (Lopes et al. 2004). Caloric content of a test meal shows influence only on gastric emptying. Gastric emptying and small intestinal transit seem to compensate each other, as a slow gastric emptying is not followed by a slow small intestinal transit in healthy subjects (Geypens 2000). The protozoal population of the caecum is not affected by the diet (Moore and Dehority 1993).
2.4.3.1 Hydrogen Breath Tests (H$_2$BT)

Hydrogen breath tests (H$_2$BT) have been performed in cats for evaluating the effect of age (Papasouliotis et al. 1998) or sedation (Sparkes et al. 1996) on OCTT, and also in horses (Murphy et al. 1998; Sutton 2003), ponies (Wyse et al. 2001), rats (Brown et al. 1987) and humans (Camboni et al. 1988; Sciarretta et al. 1994; Miller et al. 1997; Casellas and Malagelada 1998) for the assessment of this parameter. The principle of the H$_2$BT is based on the fact that carbohydrates not digested in the small intestine will be broken down in the large colon by intestinal bacteria. Hydrogen (H$_2$) is produced by this large intestinal microbial fermentation and subsequently exhaled in measurable quantities in breath, providing an indication of the arrival of undigested material in the caecum (Levitt 1969). The H$_2$BT is reported to be poorly reproducible (Ladas et al. 1989) and some H$_2$ non-producers were found, in which OCTT could not be determined effectively (La Brooy et al. 1983; Ladas et al. 1989; Sutton et al. 2000). The H$_2$BT produced significant shortening of OCTT in healthy humans, caused transit disorders and was therefore unreliable in OCTT measurements. In addition, Murphy (1998) noted that the lactulose H$_2$ breath test is highly variable in the measurement of OCTT in ponies and recommended further research until the test could be reliably applied for examination of gastrointestinal (GI) function (Murphy et al. 1998). Miller et al. (1997) showed that lactulose slowed gastric emptying, but significantly increased small intestinal motility and decreased OCTT (Miller et al. 1997).

Overall, the H$_2$BT has been used for the indirect measurement of OCTT, but has been shown to be inferior to other breath test techniques.

2.4.3.2 Sulphasalazine/Sulphapyridine (SLZ/SP) Test

The sulphasalazine/sulphapyridine (SLZ/SP) test has been used for the measurement of OCTT in man (Kellow et al. 1986; Staniforth 1989), dogs (Papasouliotis et al. 1995) and horses (McGreevy and Nicol 1998). Sulphasalazine (SLZ) is produced by the azo-bond
coupling of sulphapyridine with 5-aminosalicylic acid. It cannot be absorbed in the small intestine as it requires bacterial cleavage of the azo-bond in the large intestine. Once released in the large intestine, the sulphapyridine (SP) is rapidly absorbed and undergoes hepatic conjugation to various metabolites (Kellow et al. 1986). The SLZ/SP test requires the collection of multiple blood samples and subsequent analysis by high performance liquid chromatography. Using the SLZ/SP test, equine OCTT was reported to be prolonged in crib-biting individuals if these individuals were prohibited from performing their stereotypical behaviour (McGreevy et al. 1998). McGreevy et al. (2001) reported also when using the SLZ/SP test that equine OCTT was relatively reduced in crib-biting horses compared with normal individuals (McGreevy et al. 2001). Further disadvantages of the SLZ/SP test for equine use are that no standard technique for OCTT measurement has yet been validated against this test, and that pharmacokinetics and therapeutic indices of SP have yet to be established in the horse.

The fact that multiple blood sample collections are required, and that analytical techniques are difficult, makes the SLZ/SP test less attractive for routine measurement of equine OCTT.

Both the H$_2$BT and the SLZ/SP test have been used for the measurement of equine OCTT. However, both have a number of potential problems and require further validation. Gastroenterocolonic scintigraphy has been adopted as the gold standard technique for OCTT assessment in humans. This technique has limited accuracy in the horse, and a true “gold standard” for the non-invasive measurement of equine OCTT has yet to be accepted.

2.4.3.3 Induced Lactose $^{13}$C-Ureide Breath Test (LUBT)

The induced lactose $^{13}$C-ureide breath test (LUBT) was first proposed by Heine et al. (1994) to be a suitable test for measurement of human OCTT (Heine et al. 1994). The LUBT reflects the time interval between the ingestion of the isotope and the subsequent rise of $^{13}$CO$_2$:$^{12}$CO$_2$. 

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in breath (Wutzke et al. 1997). Heine et al. (1995) concluded that the glycosyl $^{13}$C-ureide
breath test reflected intestinal transit time, and that it acted as a specific marker for the action
of colonic microbial flora (Heine et al. 1995). Previous studies have compared the LUBT with
the H$_2$BT in man (Wutzke et al. 1997) and horses (Sutton et al. 2000) and found the LUBT to
be both more sensitive and more specific for OCTT measurement. Lactose $^{13}$C-ureide is
synthesised by the acid-catalysed condensation of lactose and urea (Morrison et al. 2000).
The lactose moiety may be cleaved in the small intestine to yield galactose and glucose $^{13}$C-
ureide, but the glucose-urea bond itself cannot be cleaved by intestinal brush border enzymes
(Ruemmele et al. 1997). Rather, the bond is cleaved by intestinal microflora in ruminants
(Merry et al. 1982), humans (Heine et al. 1994) and also horses (Sutton et al. 2000).
Excellent correlation was reported between the LUBT and scintigraphy in humans (Wutzke et
al. 1997; Geypens et al. 1999). Using the LUBT a good repeatability was found within
individuals, and daily dietary variations had little effect on test results (Heine et al. 1995). In
horses the LUBT was found to be a superior test to the H$_2$BT in terms of both sensitivity and
repeatability (Sutton et al. 2000; Sutton 2003). In vitro studies in the horse have also indicated
that the cleavage of the glucose $^{13}$C-ureide moiety and subsequent hydrolysis of $^{13}$C-urea is
relatively restricted to the large intestinal flora. Hence the $^{13}$C-LUBT should provide a
relatively specific indicator of oro-caecal transit in the healthy horse (Sutton 2003). Hence, it
was elected to use the LUBT for assessment of the effects of the different pharmacological
compounds on OCTT in this study.

2.4.4 Factors influencing Oro-Caecal Transit Time

Dehydration has been shown to delay gastric emptying but has no significant influence on
OCTT and intestinal motility in humans (van Nieuwenhoven et al. 2000).

OCTT is determined by gastric emptying and small intestinal transit as well as by ileal
emptying effects. Constipation is therefore a factor that delays OCTT (Geypens 2000).
In a human study, mouth-to-anus transit time, assessed by radio-opaque markers, showed no difference in age (Metcalf et al. 1987), whereas gastric, small intestinal and colon transit time studied by scintigraphy were found to be influenced by age and gender, with women having a slower gastric transit time (Graff et al. 2001). They further observed an acceleration of gastric emptying and small intestinal transit time with aging (Graff et al. 2001). The female menstrual cycle was not implicated in a study (Degen and Phillips 1996), gender bias was detailed elsewhere, where men had a shorter transit time for the whole colon (Metcalf et al. 1987; Degen et al. 1996).

2.4.5 Advantages

As a stable non-radioactive isotope is used, there are no radiation hazards as is potentially the case with the use of radioactive isotopes (Halliday et al. 1982; Koletzko et al. 1997; Romagnuolo et al. 2002). The stable isotope breath tests are also easy to perform, painless for the patient and different breath tests can be performed simultaneously (Koletzko et al. 1997). Exhaled breath can easily be collected and respiratory mask measurements produced good results in veterinary medicine, as tested in swine (McKirnan et al. 1986) and ponies (Orr et al. 1975).

Only tracer amounts of substrate are necessary (Koletzko et al. 1997; Stellaard et al. 1998), or substrates that are naturally enriched with $^{13}$C in the presence of a low background $^{13}$C diet (Jonderko et al. 2005). Substrates are usually given orally (Rating and Langhans 1997) and breath samples can be stored at room temperature for up to eleven weeks (Schoeller et al. 1977). Therefore this test has scientific and clinical value particularly in neonates, children, young adults and in pregnant women (Halliday et al. 1982; Koletzko et al. 1997) and for repeated testing (Koletzko et al. 1997; Rating et al. 1997; Swart and van den Berg 1998).
2.4.6 Disadvantages

The main disadvantage is the natural background level of environmental $^{13}$C, against which estimation of isotope label must be made (Halliday et al. 1982; Koletzko et al. 1997; Romagnuolo et al. 2002). This background is approximately 1.1% in the case of $^{13}$C (Halliday et al. 1982; Koletzko et al. 1997) and depends on the composition of C$_3$ and C$_4$ plant material (Stellaard et al. 1998), as well as geographical situation and nutrition (Koletzko et al. 1997). There is also the high cost of the isotope-labelled substance, as well as the necessity for high-technology measurement equipments (Halliday et al. 1982; Wutzke et al. 1997). An infrared spectroscopic technique has been developed to exchange the costly isotope ratio mass spectrometer, but a lower sensitivity and large sample volumes (Savarino et al. 1999) as well as the impossibility of automatic analysis of high sample numbers make this equipment only interesting for small gastroenterological centres (Savarino et al. 1999; Romagnuolo et al. 2002).

A further disadvantage of stable isotope breath tests is that any breath test has the same assumption of a substrate being metabolised to $^{13}$CO$_2$, after intake of the $^{13}$C tracer. An increase of $^{13}$CO$_2$ above baseline level is said to provide an indirect measurement of the function investigated (Rating et al. 1997). For this to be true, the metabolic or physiological function being studied must be the major rate-limiting step in the exhalation of $^{13}$CO$_2$ rather than a combination of metabolic determinants (Swart et al. 1998). This might not be the case in diseased animals (Rating et al. 1997; Wyse et al. 2004). Since only the end product $^{13}$CO$_2$ is measured, there is no information on all the pools and fluxes through which the labelled substrate and its metabolites have to pass (Rating et al. 1997).

Another disadvantage is that changes in the basal exhalation of $^{13}$CO$_2$ during the test will have an impact on the breath test calculation. Moreover, the basal metabolic rate (BMR) and the actual endogenous CO$_2$ production, which in most instances is unknown, is required for an exact calculation of percentage recovery of the specific stable isotope over time (Rating et al.
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In many clinical centres for humans, the BMR is now estimated indirectly, and allows more accurate assessment of total exhaled recovery of the ingested \(^{13}\text{C}\)-isotope to be made. Routine estimation of BMR is not yet available in horses, and so information on the percentage digestion and metabolism of \(^{13}\text{C}\)-compounds in this species is less accurate.

The hydrogen breath test that has been reported for assessment of OCTT in both humans and animals also has some disadvantages. Although Murphy et al. (1998) had satisfactory reproducibility in their analysis of duplicate samples (Murphy et al. 1998), it has been reported that the earlier used lactulose hydrogen breath test was poorly reproducible within (La Brooy et al. 1983; Ladas et al. 1989) or between subjects studied (La Brooy et al. 1983) and sedated cats (Sparkes et al. 1996). The overestimation of OCTT that occurs by the use of the hydrogen lactulose breath test, if the mean hydrogen concentration is within 15 ppm, can be reduced by a mean value of 10 min if a hydrogen threshold increment of 5 ppm is chosen to represent the arrival of the test meal at the caecum. This improves the correlation between the lactulose hydrogen breath test and scintigraphy to \(r = 0.90\) for measurement of OCTT (Sciarretta et al. 1994).

Pressman et al. (1987) used a \(^{14}\text{C}\) lactulose breath test to measure small intestinal transit time in man. They reported that besides the small within subject variation, there is only moderate accuracy for the test due to a delayed recovery of \(^{14}\text{CO}_2\) in breath. They also observed an increase of \(\text{CO}_2\) in subjects within 30 min. This is long before lactulose entered the colon and might be due to degradation of lactulose by aerobie bacteria in the small intestine (Pressman et al. 1987).

A study comparing the lactulose hydrogen breath test with scintigraphy for measurement of OCTT, proved that although both tests correlated well, neither of them is accurate for the measurement of small intestine transit time. This is because lactulose accelerates small intestinal transit and slows gastric emptying and scintigraphy produces a wide range of OCTT measurements, making it difficult to assess delayed small intestinal transit (Miller et al.)
1997). In scintigraphic assessment of OCTT, the arrival of 10% of the radioactive isotope in the caecum is generally taken to represent OCTT (Geypens et al. 1999; Geypens 2000; Van Den Driessche 2001), thus not allowing for prolonged retention of the majority of the radiolabelled meal in the small intestine.

2.4.7 Disadvantages in veterinary medicine

Despite these apparent drawbacks, breath tests using the $^{13}$C stable isotope of carbon have recently become more available in district hospitals and commercial laboratories (Swart et al. 1998).

Certain fundamental deficiency in the basic knowledge that is necessary for the analysis of breath tests make the use of them in veterinary medicine still difficult and therefore often confines them to research laboratories. The exact physiological origin of markers i.e. hydrogen, has yet to be established in animals, the effects of respiratory changes on the concentration of exhaled compounds, as well as the reference ranges for most of the markers for healthy and diseased animals have yet to be described (Wyse et al. 2004).

2.4.8 Isotopes of carbon and their measurements

An isotope is a chemical atom that contains the identical number of protons in its nucleus as an atom of its position in the periodical table of elements, but the number of neutrons in the nucleus is different. This leads to a different mass and different chemical, physical and biochemical behaviour (Koletzko et al. 1997).

Carbon has eight isotopes, with six radioactive, unstable isotopes ($^{9}$C, $^{10}$C, $^{11}$C, $^{14}$C, $^{15}$C, $^{16}$C) and two stable, non-radioactive $^{12}$C and $^{13}$C isotopes (Lefebvre 1985).

In this study, $^{13}$C is used, having a natural abundance of approximately 1.1% (Svejcar et al. 1993; Rating et al. 1997). This naturally occurring isotope can either be of (bio-) chemical or natural origin (Stellaard et al. 1998). Its use for clinical diagnostic and research is safe and
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without any adverse side effects and is measured by isotope ratio mass spectrometry (Koletzko et al. 1997).

The ratio of $^{13}\text{C}:^{12}\text{C}$ in a sample is always measured against a universal reference. This is usually the Pee Dee Belemnite limestone standard (PDB) in case of carbon originating substrates (Craig 1953; Craig 1957; Lefebvre 1985; Wolfe 1992; Stellaard et al. 1998). The results are expressed in $\delta^{13}\text{C}$, which is the ratio of these ratios (Lefebvre 1985):

$$\delta^{13}\text{C} = 10^3 \times \frac{(^{13}\text{C} / ^{12}\text{C})_{\text{sample}}}{(^{13}\text{C} / ^{12}\text{C})_{\text{standard}}} - 1 \text{ (Lefebvre 1985)}$$

The isotope content of grain and other primary plant material varies between different geographic areas (Nakamura et al. 1982).

$^{13}\text{C}$-labelled substrates can be produced either synthetically or obtained from naturally occurring substances that are isolated from animal or plant material (Swart et al. 1998).

A comparison test between 15 participating laboratories in Europe revealed that all produced $^{13}\text{C}$ enrichment values within the specification of continuous flow isotope ratio mass spectrometers (Stellaard et al. 1998).

2.4.9 Error rates

An error that might be introduced into a $^{13}\text{CO}_2$ breath test is due to the changes in the endogenous $^{13}\text{CO}_2:^{12}\text{CO}_2$ ratio that varies due to substrate and dose. Small doses, as used in tracer studies, might only produce a small signal that could be drowned by fluctuations in the endogenous $^{13}\text{C}:^{12}\text{C}$ ratio.

This error can be empirically corrected by measuring the change in endogenous breath $^{13}\text{CO}_2:^{12}\text{CO}_2$ by performing the breath test following the prescribed protocol, but in the
absence of labelled substrate. The measured changes in the $^{13}\text{CO}_2:^{12}\text{CO}_2$ ratio relative to the baseline sample are then subtracted from the results of the breath test with labelled substrate for each point of time (Schoeller et al. 1984).

### 2.4.10 Influence of exercise

Although exercise other than extreme exertion does not influence gastrointestinal transit time itself in man (van Nieuwenhoven et al. 1999), ponies and donkeys (Pearson and Merritt 1991), it leads to a decrease in dry matter digestibility as well as reduction in mean retention time (Pagan et al. 1998). A change in the mix of endogenous substrates that is oxidised for energy will change the source of substrate for energy metabolism, and is therefore likely to change the natural enrichment of expired CO$_2$. This can result in an increase in background enrichment (Schoeller et al. 1977; Wolfe et al. 1984), influencing measurement of oro-caecal transit time (which depends on assessment of small increases in exhaled $^{13}\text{CO}_2$).

Heavy exercise, on the other hand, rapidly decreases the $^{13}\text{C}:^{12}\text{C}$ ratio of breath CO$_2$ initially, before it then slowly increases (Schoeller et al. 1984). This may be explained by the withdrawal of carbohydrates from the bicarbonate pool during the transition from rest to exercise. Another reason might be the increased lipid utilisation that was not reflected in the respiratory exchange due to no equilibrium of gas exchange in the transition state. The decrease could also be due to a kinetic isotope effect that was expressed during the transition period when bicarbonate kinetics were not at equilibrium (Schoeller et al. 1984).

### 2.4.11 CO$_2$ production

The carbon dioxide production rate (VCO$_2$) at the time of a breath test is determined by the metabolic rate at that time. Since this rate is influenced by exercise, subjects should rest for the duration of breath tests (Swart et al. 1998) as CO$_2$ production is required to calculate percentage $^{13}\text{C}$ recovery, which provides the indirect measure of transit time.
A constant body pH throughout a $^{13}$C tracer study is important also, as the acid/base balance is an important factor affecting the bicarbonate elimination kinetics and therefore the breath CO$_2$ (Leese et al. 1994).

The actual CO$_2$ production has to be known to calculate an accurate % $^{13}$C elimination (in the percentage of $^{13}$C administered) (Rating et al. 1997). It is preferential to measure this actual CO$_2$ production, but if this can not be performed, the VCO$_2$ can be calculated from the basal metabolic rate (BMR) (Amarri et al. 1998).

In humans, use of an estimate of 9 mmol/kg per hour of endogenous CO$_2$ production is recommended for calculations in any person, irrespective of body shape and age (Rating et al. 1997).

### 2.4.12 Influences on $^{13}$C enrichment

Fat oxidation starts in humans after an eight hour fast. Because of the lower natural $^{13}$C enrichment of lipids compared to that of carbohydrates, the baseline $^{13}$CO$_2$ exhalation will decrease when oxidation shifts from carbohydrates to lipids. This mechanism causes the $^{13}$CO$_2$ exhalation to drop below baseline during more prolonged breath test regimens (Rating et al. 1997) and correction must be made for this phenomenon.

When a fasting subject receives carbohydrates, the low enrichment of expired CO$_2$ that is caused by the oxidation of fat during fasting, will increase, as carbohydrates will be used for the physiologically favoured glucose oxidation (Wolfe et al. 1984). Ingestion of subsequent meals during a breath test period increases breath $^{13}$CO$_2$ abundance due to a shift in substrate oxidation, affects the variability within subjects (Jones et al. 1985) and increases the OCTT (Priebe et al. 2004).

Therefore, in order to avoid any metabolic disturbances or undue variability, it is recommended that the test subjects fast overnight before the test and throughout the test (Schoeller et al. 1977).
2.4.13 Test meal

The validation of a stable isotope breath test requires the selection of a suitable test meal that should be low in natural background $^{13}$C (Boutton 1991). Furthermore a hay diet is more suitable, as diets containing grain influence heart rate, rectal temperature and could delay gastrointestinal transit time (Lopes et al. 2004).

The composition of the test meal with respect to energy density and fat content is important as it influences gastric emptying and therefore the time when the substrate will be available for metabolic processes to be studied (Swart et al. 1998).

The natural abundance of $^{13}$C in food is approximately 1.1% (Morrison et al. 2000), which reflects the enrichment or depletion of $^{13}$C relative to $^{12}$C due to isotope fractionation (Schoeller et al. 1980).

The variation of natural abundance of $^{13}$C in food depends on the plant and the pathway used for photosynthesis. The Calvin-Benson or so called C$_3$ pathway involves C$_3$ plants such as beet root or potatoes, which utilise a three-carbon intermediate to fix atmospheric CO$_2$ into plant material. C$_4$ plants such as maize, sugarcane and related grasses use a four-carbon intermediate for this process. This is called the Hatch-Slack or C$_4$ pathway (Hatch and Slack 1970). C$_4$ plants are significantly more enriched with $^{13}$C and therefore animals, which have ingested these plants will present a higher increase of $^{13}$C within their tissue (Halliday et al. 1982). Tropical plants are high in C$_4$ (Lefebvre 1985).

Therefore test meals for stable isotope breath tests generally have a low level of C$_4$ plant material unless the natural diet (and body reserves) of the test subject are high in $^{13}$C.

Driessche (2001) reported that liquid test meals do not reveal any abnormalities in patients with gastrointestinal disorders and recommends only the use of solid test meals (Van Den Driessche 2001). Solid phase test meals allow a more physiological assessment to be made of genuine motility disorders that might not be apparent in the liquid phase.
2.5 CONTINUOUS FLOW ISOTOPE RATIO MASS SPECTROMETRY (CF-IRMS)

2.5.1 Background

Historically, mass spectrometers were mainly used for physical studies of terrestrial isotopes (Brenna et al. 1997), in chemistry research laboratories and petroleum and pharmaceutical industries (Matern and Magera 2001).

In this study, a continuous-flow isotope ratio mass spectrometer (CF-IRMS) was used. These high-precision spectrometers have a gas chromatograph interfaced with an isotope ratio mass spectrometer, which increases sensitivity and speed of analysis (Brenna et al. 1997).

CF-IRMS are robust and simple, with staff having basic chromatography experience being able to operate it with ease (Preston and McMillan 1988).

2.5.2 Principle of a CF-IRMS

Once the sample is introduced into a spectrometer, the sample containers are loaded into an auto sampler, into which a double, concentric needle is inserted. The needle is flushed with helium before insertion and the carrier flow of helium is further used to flush out the sample gas from the container. After passing through a drying tube, containing MgClO$_4$ for removing water vapour, the gas reaches a packed column gas chromatograph, in which CO$_2$ is separated from other gases. The purified CO$_2$ pulses to the mass spectrometer inlet (Prosser et al. 1991), where the target isotope of interest is ionised and bombarded by electrons (Bier and Matthews 1982; Matern et al. 2001), so that some molecular ions become positively charged (Wolfe 1992). These ions reach the mass spectrometer and the magnet inside deflects them according to their mass to charge (m/z) ratio (Klein and Klein 1985; Wolfe 1992; Van Den Driessche 2001; Matern et al. 2001). The masses 44, 45 and 46 (^{12}C^{16}O$_2$, ^{13}C^{16}O^{18}O and ^{12}C^{16}O^{18}O, respectively) are collected by a triple collector (Prosser et al. 1991; Wolfe 1992; Van Den Driessche 2001), so that both the $^{13}$C:$^{12}$C and $^{18}$O:$^{16}$O ratios can be calculated for the CO$_2$ (Craig 1957).
Jonderko et al. (2005) have investigated the recently developed and cheaper non-dispersive infrared spectrometry (NDIRS) by analysing breath samples (Jonderko et al. 2005). Braden et al. (1999) have compared the NDIRS with the isotope ratio mass spectrometry for analysis of $^{13}\text{C}:{^{12}\text{C}}$ ratio and found that both spectrometers produced excellent and comparable results. This new spectrometer might in future be a low-cost and easy-to-operate alternative (Braden et al. 1999).

### 2.6 LACTOSE $^{13}\text{C}$-UREIDE BREATH TEST FOR ORO-CAECAL TRANSIT TIME MEASUREMENT

#### 2.6.1 Background

The induced lactose $^{13}\text{C}$-ureide breath test (LUBT) has been developed by Heine (Heine et al. 1995) and validated against the predicate method of enterocolonic scintigraphy for the measurement of OCTT in man (Wutzke et al. 1998; Geypens et al. 1999). Geypens et al. (1999; 2000) reported an excellent correlation between the LUBT and scintigraphy for OCTT measurement ($292 \pm 58$ min and $283 \pm 53$ min; $P = 0.0001$), and concluded that the breath test was a valid alternative to scintigraphy for OCTT measurement (Geypens et al. 1999; Geypens 2000). Wutzke et al. (1997) also compared the LUBT to the older lactulose hydrogen breath test and reported that the former had both greater sensitivity and specificity for OCTT measurement (Wutzke et al. 1997). In human gastroenterology the LUBT was also investigated in infants that were not yet weaned (Van Den Driessche et al. 2000; Van Den Driessche 2001; Christian et al. 2002a).

#### 2.6.2 Principle

The basis of a breath test for OCTT measurement is the ingestion of a non-absorbable carbohydrate, which gets fermented by microbes in the caecum and then found as an increase
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in exhaled hydrogen (Wyse et al. 2004). Lactose ureide is an ideal tracer for the evaluation of OCTT, as the sugar-urea bond is inert to intestinal enzymes and also has no osmotic effect on gut motility (Ruemmele et al. 1997). Roberts (1975) reported horses older than three years having a lactase enzyme deficiency and therefore being lactose intolerant (Roberts 1975), which makes them suitable for the LUBT.

Lactose $^{13}$C-ureide is a tasteless (Geypens et al. 1999) glycosyl ureide that is synthesised by the acid-catalysed condensation of lactose and urea (Morrison et al. 2000).

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**Figure 2.6.2.1:** 4-O-(β-D-galactopyranosyl)- β- D-glucopyranosyl ureide (LACTOSE UREIDE), * denotes the position of $^{13}$C enrichment (Morrison et al. 2003)

The underlying principle of the LUBT is the enzymatic splitting of the stable isotope labelled lactose-ureide (LU) by intestinal microbes to NH$_3$ and $^{13}$CO$_2$. The latter can be measured in exhaled breath samples when the isotope reaches the caecum (Mohr et al. 1999).

Ingestion of LU is followed by bacterial cleavage of the glycosyl-ureide bond in the large intestine (Heine et al. 1995; Geypens 2000), exclusively by bacterium *Clostridium innocuum* (Wutzke et al. 1998; Mohr et al. 1999). Subsequent hydrolysis of the $^{13}$C-urea moiety results in the production of a detectable increase in $^{13}$C:$^{12}$C output from the body bicarbonate pool. Hence, mass spectrometric analysis of the $^{13}$C:$^{12}$C ratio in expiratory breath after ingestion of
the labelled test meal provides an indirect measure of the rate of OCTT (Heine *et al.* 1995; Geypens 2000). This test recently has been validated *in vitro* for the measurement of equine OCTT and used *in vivo* to provide estimation of small intestinal transit times (Sutton 2003).

Premedication with unlabelled LU the day prior to the test leads to a higher and steeper $^{13}$CO$_2$ enrichment during the test (Wutzke *et al.* 1997; Geypens 2000; Morrison *et al.* 2003). This is due to the induction of microbial enzyme activity (Wutzke *et al.* 1998). Without induction on the day prior to the test with unlabelled LU, a significant percentage of test subjects will not show sufficient increase of $^{13}$CO$_2$ in breath to allow unambiguous interpretation of the LUBT (Geypens 2000). The metabolism of the tracer molecule is much slower in the absence of induction (Geypens 2000; Van Den Driessche 2001).

However, in humans, premedication of 1g unlabelled LU two hours prior to the labelled test meal resulted in a shortening of transit time to 2.5 hours due to enzymatic activity (Heine *et al.* 1994). Morrison *et al.* (2003) also observed a shortening of OCTT after premedication of unlabelled substrate in some subjects (Morrison *et al.* 2003).

The principle of the LUBT for measurement of OCTT is shown in Figure 2.6.2.2:

**Figure 2.6.2.2:** The principle of the LUBT for measurement of OCTT (Sutton 2003)
2.6.3 Disadvantages of LUBT

The LUBT is not suitable for use in infants prior to weaning, as they do not have the necessary bacterium *Clostridium innocuum* to split the glycosyl bond. Small intestinal overgrowth also limits the application of the LUBT (Van Den Driessche *et al.* 2000; Van Den Driessche 2001), as a positive breath signal would occur earlier than expected (Ruemmele *et al.* 1997).

The expense of the IRMS-analysis, high cost of the lactose-[^13C]ureide and the LU pretreatment for the induction of enzyme activity are among the disadvantages of the LUBT (Wutzke *et al.* 1997).

Variability between individuals evaluated for OCTT was described, attributed to the differences in the metabolic activity of the colonic microflora (Jackson *et al.* 1999).

In this study, the effects of imidocarb dipropionate on equine OCTT, and the relative effects of atropine or glycopyrrolate premedication were quantified using stable isotope breath test technology. Pharmacological effects on OCTT were studied using the lactose[^13C]-ureide breath tests (Geypens *et al.* 1999; Sutton 2003). The primary study objective was to determine the optimum dosing strategy for equine babesiosis in order to minimise the gastrointestinal complications associated with treatment.

Semi-quantitative studies of gastrointestinal motility using transabdominal ultrasonography have suggested that the effect of imidocarb on gut motility is best ameliorated by premedication with glycopyrrolate (Donnellan *et al.* 2003a). Since non-invasive quantitative stable isotope tests have now been validated for the measurement of equine OCTT, it is aimed in this study to quantify the effects of treatment modalities on this specific parameter of intestinal transit. The objectives of this study are as follows:
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- to improve knowledge of the specific effects of imidocarb treatment on oro-caecal transit time

- to quantify the effects of premedication with atropine or glycopyrrolate

- to develop/validate a clinical protocol for treatment of babesiosis that results in minimal gastrointestinal complications

2.7 HYPOTHESIS

- When administered without premedication imidocarb dipropionate at 2.4 mg/kg IM will result in clinical signs of abdominal pain and colic in a proportion of horses.

- Treatment with imidocarb dipropionate at 2.4 mg/kg IM will result in increased small intestinal motility, and a reduction in oro-caecal transit time as demonstrated by the lactose $^{13}$C-ureide breath test.

- As atropine has been proven previously to decrease equine small intestinal motility, premedication of horses with atropine or glycopyrrolate will reduce the changes in intestinal transit rate caused by imidocarb dipropionate.

- Premedication with atropine or glycopyrrolate will reduce the incidence of clinical side effects seen with imidocarb treatment alone.

- Premedication with atropine will result in a greater delay in small intestinal transit than seen with both the control and glycopyrrolate premedication groups.
CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 STUDY DESIGN

The preliminary part of the study entailed measurements of basal $^{13}$C:$^{12}$C expiratory ratio in the study animals under experimental conditions, to ensure that this parameter remains constant. The method of breath collection and analysis was performed as described below. This part of the study trials also ensured that the test meal and stable isotope substrate was palatable to all the study animals.

The study was thereafter divided into a randomised treatment trial. The effects on gut motility of three drug therapies (imidocarb dipropionate 2.4 mg/kg IM with saline IV; imidocarb dipropionate 2.4 mg/kg IM with atropine 0.035 mg/kg IV; imidocarb dipropionate 2.4 mg/kg IM with glycopyrrolate 0.0025 mg/kg IV) were tested against a saline control (saline IM with saline IV). The following drugs were used: imidocarb dipropionate (Forray 65®, Schering-Plough (Pty) Ltd, Isando, R.S.A.), atropine sulphate (Atropine 10®, Bayer Animal Health Division, Isando, R.S.A.), glycopyrrolate (Robinul®, Pharmacare Limited, Port Elizabeth, R.S.A.) and saline (Sodium Chloride 0.9%, Renalcare Service (Pty) Ltd, Midrand, R.S.A.). The intramuscular injection followed the intravenous injection immediately in each individual. Throughout the trial the same batches of drugs were used.

The relative effects of the compounds on oro-caecal transit time (OCTT) were measured in six healthy horses using the induced lactose $^{13}$C-ureide breath test (LUBT). Each horse received one treatment per week in randomised order, with a one-week wash out period between different drug therapies. All subjective clinical variables were assessed by the primary investigator.
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Since factors such as dietary composition, volume and consistency, exercise and physiological stress are known to affect equine gastrointestinal motility, all attempts were made to minimise these variables. Temporal effects on gut function were eliminated by the randomised nature of the study design.

3.2 STUDY ANIMALS

The study was performed on six healthy adult equidae (one Thoroughbred gelding, three Thoroughbred mares, one Basuto pony gelding and mare each), selected from the Equine Research Centre herd, Faculty of Veterinary Science, Onderstepoort, South Africa. Their range of age was four to 13 years (mean 8.2) and their body weight ranging from 339 to 545 kg (mean 460 kg).

Four horses and three ponies were used for the basal trial, however, one pony was later withdrawn from the study after consistently refusing the test meal.

3.2.1 Animal selection

The selected animals were in good body condition and had no recent history of colic. Five weeks prior to the trial, blood samples were collected by jugular vein puncture into potassium ethylenediaminetetraacetic acid (EDTA) and serum tubes (Vacutainer®, Becton Dickenson, SA Scientific, Randburg, R.S.A.). Diagnostic blood tests were done, consisting of total blood cell count, examination for blood parasites and biochemical analysis. The latter included total serum protein, albumin, globulin, albumin-globulin ratio, aspartate aminotransferase, gamma glutaryltransferase, urea and creatinine serum concentrations. The blood parameters were evaluated at the Section of Pathology, Department of Medicine, Faculty of Veterinary Science, Onderstepoort, South Africa.
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A general physical examination and faecal examination for Nematode egg count and Cestode egg recovery was performed. The Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, Onderstepoort, South Africa carried out the helminthological evaluation. One horse tested positive and therefore was administered an Ivermectin-Praziquantel anthelmintic (Equimax® oral paste, Virbac (Pty) Ltd, Halfway House, R.S.A.) one month prior to the trial. All other subjects were dewormed with Fenbendazole (Panacur®, Intervet (Pty) Ltd, Isando, R.S.A.) in February 2005 and showed negative helminthological results.

3.2.2 Management of test subjects

All subjects were brought in from pastures and stabled in individual loose boxes two weeks prior to the trial at the stables of the Equine Research Centre, Faculty of Veterinary Science, Onderstepoort, South Africa. They were bedded on wooden shavings and were fed hay and lucerne twice daily. Two weeks prior to the trial as well as from Friday to Sunday of each week during the study, subjects were allowed on the pasture during day time. This was done to reduce stress and the risk of impaction colic. Any exercise for the period of the study was otherwise restricted to minimise metabolic influences on gastrointestinal motility.

One horse presented colic signs, due to an impaction of the pelvic flexure, once between the test days. This horse was therefore stabled for a period of 24 hours at the Equine Clinic of the Faculty of Veterinary Science, Onderstepoort, South Africa and treated with a two-hourly drenching using Epsom salts initially and then a technical oil - water mixture.

All subjects were stabled and fasted 14 hours prior to the test as this eliminates or reduces baseline shifts in $^{13}$C abundance (Schoeller et al. 1980).
3.2.4 Ethics and welfare

The procedures detailed in this thesis were approved in advance by the Animal Use and Care Committee and the Research Committee, Faculty of Veterinary Science, Onderstepoort, South Africa.

The method used for assessment of gastrointestinal motility in this study was entirely non-invasive and generally extremely well tolerated by the horses. Collection of each breath sample took approximately two minutes, during which time the horse breathed through the Aeromask® (Trudell Medical International, Ontario, Canada). The stable isotopes are safe and have been used extensively in human and veterinary medicine without detrimental effect.

Although some studies have indicated that imidocarb dipropionate has dose-dependent hepato- and nephrotoxicity in a number of species, a recent study concluded that even sterilisation protocol doses of imidocarb (4 mg/kg IM every 72 h) had minimal effect on function of either of these organs (Meyer 2002; Meyer et al. 2005). A considerably lower dose of imidocarb was required in this study compared to the other study (Meyer 2002; Meyer et al. 2005).

3.3 TEST MEAL

The test meal consisted of 150g oats, 100g wheat bran and 200ml water for horses and 75g oats, 50g wheat bran and 100ml water for ponies. This contained 2.895 MJ for horses and 1.448 MJ for ponies, based on the digestible energy (D.E.) contents of 12.1 and 10.8 MJ/kg dry matter for oats and wheat bran, respectively (Frape 2004). Lactose 13C-ureide (Bell College of Technology, Hamilton, Scotland) (2.7 mg/kg bwt β-lactosyl 13C-ureide dihydrate) was incorporated into egg white, which was cooked in a microwave for approximately 60 seconds until firm, then homogenised and thoroughly mixed into the test meal.
3.3.1 Calculation of the dose
The required dose is usually between two to 20 times the minimum detectable dose, which depends on the occurring fluctuation and is at least twice the standard deviation. This would be 1.4 per mil as the usually fluctuation occurs within the standard deviation of 0.72 per mil (Schoeller et al. 1977). Previous studies recommend an ideal dose of labelled lactose ureide (LU) of 3 mg/kg bwt and 15 mg/kg bwt for the unlabelled LU for the LUBT in horses (Sutton 2003). In human studies the dose of unlabelled LU varies between 500 mg for children (Van Den Driessche 2001) and 1 g for adult (Wutzke et al. 1998; Geypens et al. 1999), and 250 mg for children (Van Den Driessche 2001) and from 500 mg (Geypens et al. 1999) to 1 g (Wutzke et al. 1998) of $^{13}$C-LU for adult.

In this study 30 mg/kg bwt of unlabelled LU were fed 18 hours before the test to induce enzymatic activity of the colonic bacteria. The labelled LU was fed at a dose of 2.7 mg/kg bwt.

3.4 LACTOSE $^{13}$C-UREIDE BREATH TEST
The induced lactose $^{13}$C-ureide breath test (LUBT) was used to measure OCTT and further specific parameters of small intestinal transit rate. 

$T_{1/2}$ indicates the time needed for half of the marker molecule to be emptied from the stomach, transported to the caecum, metabolised by bacteria, taken up in the blood bicarbonate pool and excreted in breath as carbon dioxide. This parameter thus describes more than oro-caecal transit of the head of the chyme (Geypens 2000).

3.4.1 Breath test protocol
The horses were fasted for 14 hours prior to the tests to ensure an empty stomach. Attempts were made to minimise all possible external/environmental factors on intestinal transit rates.
and alterations in VCO$_2$ by fasting and resting animals before and during the trial. Two basal breath samples were collected 60 and 0 min before test meal ingestion to estimate the basal $^{13}$C production. Thereafter, samples were taken in duplicate at 30 to 60 min intervals for 12 hours.

The time at which the test meal was given, was determined as 0 min.

Immediately after the test meal had been eaten, each horse was injected with the selected drug combination. One intravenous and one intramuscular injection was given in a blinded manner (saline IV/saline IM; glycopyrrolate IV/imidocarb IM; atropine IV/imidocarb IM; or saline IV/imidocarb IM). Breath sample collection was then continued at 30 to 60 min intervals for 12 hours.

For the duration of the study period subjects were fed a constant diet of lucerne/teff hay without concentrates, and allowed access to grazing. On the day before each breath test was to be performed the horses were stabled to minimise environmental influences on gut function. The subjects were stabled and maintained at rest for the duration of each test to minimise fluctuations in CO$_2$ production (VCO$_2$) and intestinal motility. Access to food and water was not allowed for the first ten hours of each study. Every horse was tested weekly for four weeks, to measure the relative effects of treatment on OCTT. Three horses were sampled at each trial day. Tuesdays and Wednesdays were designated as weekly data collection days. On the day before the test, horses were pre-fed with unlabelled LU to maximise bacterial enzymatic activity.

3.4.2 Collection of breath samples

Expiratory samples were collected using a previously validated technique (Sutton 2003). Prior to each sampling point a modified Aeromask® (Trudell Medical International, Ontario, Canada) was fitted to each horse’s muzzle, and the horse allowed to breathe through the
inspiratory and expiratory valves of the mask. As resting horses have irregular breathing
patterns with variable tidal volumes and flow rates (Slocombe et al. 1992) and a lower
respiratory frequency when using a mask (Evans and Rose 1988), each horse was allowed to
in- and exhale for several times before the collection bag (QuinTron®, QuinTron Instrument
Company, Wisconsin, USA) was attached. In this way we made sure that dead space of the
Aeromask® was filled with breath CO2. Expiratory samples were collected by attaching the
QuinTron® bag with one-way valve to the expiratory section of the mask. This collection
technique, which took approximately two minutes to perform, is found not to disturb the
horse’s breathing (Orr et al. 1975). The Aeromask® was removed between sampling events.
Breath samples were then, as described (Schoeller and Klein 1978), transferred via 20 ml
syringes and attached three-way stop cocks (both B. Braun Medical (PTY Ltd, Randburg,
R.S.A.) in duplicate to 10 ml Vacutainers® (Becton Dickenson, Lincoln Park, NJ, USA) and
stored at room temperature prior to analysis. Experiments have shown that samples stored in
this way remain stable for at least eleven weeks (Schoeller et al. 1978).

**Figure 3.4.2.1:** Equipment for duplicate breath collection.
**Figure 3.4.2.2:** Horse with Aeromask® and attached QuinTron® bag for breath collection.

### 3.5 MEASUREMENT OF $^{13}$C:$^{12}$C RATIO

Sample analysis was originally performed in South Africa by the stable light isotope unit of the Department of Archaeology, University of Cape Town. Vacutainers® containing the samples for analysis were sent in batches to the University of Cape Town by courier service. On analysis of the first samples, problems were evident in that no breath was found captured in the Vacutainer®. We then sent further samples to the department at Cape Town University via the land route as it was suggested that the breath might leak out during transport in an
airplane. After the same problem recurred, it was then decided to send duplicates of all samples for examination to Dr. Thomas Preston, Senior Lecturer in Isotope Biochemistry, Scottish Universities Environmental Research Centre, University of Glasgow, Scotland. All samples were successfully analysed without further incident.

Samples are usually measured as $^{13}\text{C}:{^{12}\text{C}}$ ratio against the Pee Dee Belemnite limestone (PDB) standard (Craig 1953; Craig 1957; Wolfe 1992). This standard refers to a Cretaceous belemnite from the Pee Dee formation in South Carolina (Craig 1957), with an actual isotopic ratio of 0.011273 (Craig 1953; Wolfe 1992). The $^{13}\text{C}$ enrichment is expressed as $\delta^{13}\text{C}_{\text{PDB}}$ “per mil” (‰), as defined by Craig (1953).

The $^{13}\text{C}:{^{12}\text{C}}$ content of each sample was measured relative to the international Pee Dee Belemnite limestone fossil standard ($\delta^{13}\text{C}_{\text{PDB}}$) by continuous flow isotope ratio mass spectrometry (CF-IRMS) using following equation that shows the relative difference in the ratio $^{13}\text{C}:{^{12}\text{C}}$ between the sample and the standard parts per thousand:

$$\delta^{13}\text{C}_{\text{PDB}} (\text{‰}) = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{PDB}}}{(^{13}\text{C}/^{12}\text{C})_{\text{PDB}}} \times 1000 \quad (\text{Craig 1953})$$

Where $^{13}\text{C}:{^{12}\text{C}}_{\text{PDB}}$ and $^{13}\text{C}:{^{12}\text{C}}_{\text{sample}}$ are the ratio of the standard and sample respectively.

Reference gases are analysed at the beginning of each batch run and every five to ten samples thereafter. Thereby, an amount of CO$_2$ of known isotopic composition and partial pressure is injected and analysed (Prosser et al. 1991). The CF-IRMS can measure with a precision of ~1.0 per mil for $^{13}\text{C}$, with 220 samples and standards being automatically analysed in ~18 hours (Prosser et al. 1991).
Before and after each horse’s samples of a trial day were run, an accuracy check was performed with the CF-IRMS automatically.

The natural abundance of $^{13}$C in an animal reflects the natural abundance of $^{13}$C in its diet. Most biological specimens have a smaller content of $^{13}$C than PDB, resulting in breath test samples having negative values (Lefebvre 1985; Klein et al. 1985). This indicates that the sample is “lighter”, i.e. contains less $^{13}$C than the standard gas. The values represent relative abundance variations only (Craig 1953).

According to recommendations (Slater et al. 2001), S.I. notation has been used and therefore $\delta_{\text{PDB}}^{13}$C was converted into the S.I. units of atom % $^{13}$C (AP$^{13}$C), using the following equation:

$$\text{AP}^{13}\text{C} = \frac{100}{1 + 1/(\delta/1000) + 1} \times R_{\text{PDB}}$$

(Slater et al. 2001)

Where $\delta$ is measured $\delta_{\text{PDB}}^{13}$C and $R_{\text{PDB}}$ is the isotope ratio of PDB = 0.0112372.

Although the S.I. unit atom % excess or mol% excess for enrichment are recommended units for tracer studies (Slater et al. 2001), parts per million (ppm) excess were used here, as very small levels of enrichment were among our results.

This was calculated using the formula:

$$\text{ppm excess}^{13}\text{C} = (\text{atom %})_E - (\text{atom %})_B \times 10^4$$

(Christian et al. 2002b; Sutton 2003)
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Where (atom %)\(_E\) is the measured abundance of the enriched breath sample after the test meal, and (atom %)\(_B\) is the average measured abundance of the baseline breath sample, before ingestion of the isotope tracer.

\[ VCO_2 \] is needed for the estimation of percentage dose recovery per hour (PDR/h) and there are differences in VCO\(_2\) between horses and ponies. Measurement of absolute VCO\(_2\) of each animal used in this study was not practical. Therefore two formulae were used for estimation of VCO\(_2\). Orr \textit{et al.} (1975) proposed a formula, based on measurements in resting, non anaesthetised ponies of mean mass 147 kg, where mean resting VCO\(_2\) was found to be equivalent to 0.156 (SE ± 0.010) l/min/m\(^2\), where pony body surface area = (10.5 x (bwt (kg) x 1000\(^{2/3}\)) / 10000 (Orr \textit{et al.} 1975). VCO\(_2\) in horses was found to be 2.84 (SD ± 0.42) ml/kg/min in a study comparing ventilation and gas exchanges in horses and cows (Gallivan \textit{et al.} 1989).

If subjects are fed a loading dose of unlabelled substrate, an absolute baseline correction can be calculated, using the following formula (Schoeller \textit{et al.} 1980):

\[
\text{Excess/deficit per }^{13}\text{C (mM)} = \frac{\text{mg unlabeled substrate}}{(\delta^{13}_{LD} - \delta^{13}_{CO2})^{M} R_{PDB} * n * 10^{-3}}
\]

Where M is molecular weight, n is number of labelled carbon positions and R\(_{PDB}\) is Pee Dee Belemnite limestone standard of 0.011273

If test meal contains different components, it is difficult to calculate the baseline correction and the empirical approach can be used. Therefore a baseline trial can be done, which follows the same protocol as the study except without feeding a labelled substrate. The \(^{13}\text{C}\) abundance for each time point is then subtracted from the one when the study with labelled substrate is
performed. A larger dose of labelled substrate will be needed as the empirical approach increases uncertainty of $^{13}$C breath test (Schoeller et al. 1980).

Irving multiplied baseline samples by their percentage CO$_2$ to obtain an estimate of CO$_2$ output, which was normalised using the subject’s weight or body surface CO$_2$ output. For the entire six hours kinetic measurement was obtained by averaging the hourly values (Irving et al. 1983).

Incremental changes in $^{13}$C abundance over baseline values were obtained by subtracting the mean of Delta per mil vs. PDB at -30 and -15 min from the subsequent Delta per mil vs. PDB values (Irving et al. 1983). This is the approach we used in hour study.

Finally the data were expressed as percentage dose recovery (PDR) of the labelled substrate per hour. The % administered isotope recovered as CO$_2$ was calculated with the following equation:

$$\text{PDR/h} = \left[ \frac{\text{VCO}_2 \times \text{breath}^{13}\text{C enrichment}}{\text{(mg dose / MW)} \times \text{dose enrichment} \times n} \right] \times 100$$ (Morrison et al. 2003)

Where VCO$_2$ is the CO$_2$ production rate, breath $^{13}$C enrichment is the $^{13}$C enrichment of breath of breath over baseline (ppm excess), mg dose is the amount of substrate administered (mg), MW is the molecular weight of substrate, dose enrichment is the $^{13}$C enrichment of substrate (ppm$^{13}$C), and n is the number of isotopically labelled atoms in the substrate molecule.

Cumulative PDR (PDR$_{\text{cum}}$) for the breath test was calculated by integrating the PDR values over the period of the test. PDR$_{\text{cum}}$ for a given time is the PDR$_{\text{cum}}$ of the previous point of
time plus the mean PDR of the time interval. This is multiplied by this time interval and is expressed in hours (Geypens 2000).

PDR$_{cum}$ for the breath test was calculated using the following equation:

\[
\text{Cum PDR} = \left[ (\text{PDR}_t + \text{PDR}_{t-1}) \times \Delta t (\text{h})/2 \right] + \text{cum PDR}_{t-1} \quad \text{(Geypens 2000)}
\]

By using the LUBT, OCTT has been defined as the point of time at which 3% of the cumulative dose recovery of the administered isotope has occurred in the exhaled breath. This was specifically calculated by GammaInv function using Microsoft Office® Excel 2003 (Microsoft Corporation, Redmond, USA) (Sutton 2003).

\[
\text{OCTT} = \text{GammaInv} (0.03; b + 1; 1/c)
\]

### 3.6 MODELLING AND STATISTICAL ANALYSIS

As recommended by Elashoff et al. (1982) curves were fitted separately for each individual subject using nonlinear least square regression (Elashoff et al. 1982).

The results of the breath tests were expressed as percentage (%) dose $^{13}$C-isotope recovered against time (PDR/hour). The $^{13}$CO$_2$ excretion curve was modelled using a non-linear power regression formula, developed for human stable isotope gastrointestinal data (Ghoos et al. 1993):

\[
y = at^b e^{-ct}
\]

Where $y$ is the percentage of the $^{13}$C dose recovered in breath per hour, $t$ is the time in hours and $a$, $b$ and $c$ are regression constants. The variable $a$ is the scaling factor related to the
amplitude of the peak, b relates to the ascending slope, c to the decay slope and d is a delay factor (Morrison et al. 2003).

To facilitate the fitting of multiple peaks, a further variable d was used. The equation that describes a peak now becomes (Morrison et al. 1998):

\[ y = a(t-d)^b e^{-c(t-d)} \]

The best model to the actual data was fitted using a least mean squares approach, using the Solver function of Microsoft Office® Excel 2003 (Microsoft Corporation, Redmond, USA).

Pattern of \(^{13}\)CO\(_2\) recovery from equine bicarbonate pool is multiphase in nature, with more than one rate-limiting step (Sutton 2003).

Further \(t_{1/2}\) is derived from this formula, which indicates the time to recovery of 50% of the total cumulative dose recovered. As this can be calculated from the integral of the model, the Gammainv function in Microsoft Office® Excel 2003 (Microsoft Corporation, Redmond, USA) was used:

\[ t_{1/2} = \text{Gammainv} (0.5; b+1; 1/c) \] (Sutton 2003)

\(T_{lag}\) is equivalent to that time at which recovery of \(^{13}\)C label becomes maximal rate in exhaled breath. It is calculated as following:

\[ t_{lag} = b/c \] (Sutton 2003)
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OCTT, $t_{\text{max}}$ and PDR could be calculated from the fitted curve. OCTT was taken as that point of time at which a cumulative 3% of total isotope recovery was achieved. $t_{\text{max}}$ was defined as the time from ingestion of the substrate to the time of maximal breath enrichment and was calculated by the following equation:

$$t_{\text{max}} = \frac{b}{c} + d \quad \text{(Morrison et al. 2003)}$$

Mean quantitative data was therefore gained in each study, allowing assessment of the relative effects of the different treatment strategies on OCTT. A linear model analysis of variance (ANOVA) was used to assess whether or not the different compounds cause a significant difference to OCTT as compared to each other and to the saline control tests. Paired $t$-tests with a confidence interval of 95% were also used to determine intra-individual significance of the different treatment groups. Linear model ANOVAs were performed using Minitab® for Windows, release 13 (Minitab Inc., State College, PA, USA). The paired $t$-tests were performed using SigmaStat 3.11 for Windows (Systat Software, Inc., Richmond, CA, USA). ANOVA has greater statistical power to detect treatment effects than other methods (Vickers and Altman 2001).

3.7 Clinical Monitoring

Clinical recordings were made from each subject at one hour prior to isotope ingestion and then one, two, three, five, seven, nine, eleven and twelve hours thereafter and recorded in tabular form. Parameters to be recorded included mucous membrane colour, capillary refill time, heart and respiratory rate, temperature, intensity and frequency of intestinal borborygmi on the left dorsal, left ventral, right dorsal and right ventral quadrant, faecal output and consistency and presence or absence of signs consistent with abdominal pain (e.g. flank
METHODS AND MATERIALS

watching, pawing ground, lying down). When signs of pain became marked, the horses were monitored closely over the entire period of discomfort.
CHAPTER 4

CLINICAL CHANGES IN SIX HEALTHY HORSES FOLLOWING SPECIFIC BABESIOSIS TREATMENT

4.1 INTESTINAL MOTILITY

Borborygmi were evaluated on a scale from zero to four, with zero being no and four normal gut motility.

The clinical examinations during the trial revealed an increase in borborygmi for the imidocarb with saline group (mean 2.64, ± SD 0.09). The imidocarb with atropine group on the other hand showed a decrease of gut motility (mean 1.86, ± SD 0.06) compared to the saline control group (mean 2.44, ± SD 0.09). The imidocarb with glycopyrrolate group presented an only mild decrease in borborygmi compared to the saline control group (mean 2.22 and 2.44, ± SD 0.10 and 0.09, respectively). Paired t-tests showed that imidocarb produced a significant increase in hindgut borborygmi, compared with the saline control test, whereas atropine and glycopyrrolate medication produced a significant decrease in this parameter (p < 0.001).

4.2 SIGNS OF COLIC AND DEFAECATION

Four of six horses in the imidocarb and saline group only presented colic symptoms as in lying down, pawing, rolling and looking at the flank from between 30 min to five hours after injection of imidocarb.
These horses also showed severe diarrhoea between one to three hours after injection of imidocarb. Two horses had additional watery diarrhoea between two and three hours after drug administration. The number of defaecation was 23 times after imidocarb with saline injection, compared to twelve to 15 times in all other groups.
**Figure 4.2.2:** Horse showing colic after administration of imidocarb with saline. Note the marks of diarrhoea on the wall.

Colic was not observed in the study group horses when imidocarb was administered concurrently with either atropine or glycopyrrolate.

### 4.3 HEART AND RESPIRATORY RATE

The average heart rate was highest in the horses administered imidocarb with atropine (mean 47.62 per minute, ± SD 6.09) and also increased in the imidocarb with glycopyrrolate group (mean 38.42 per minute, ± SD 5.37). The imidocarb with saline group had a slightly elevated heart rate (mean 36.02 per minute, ± SD 5.08) and the heart rate of the saline control group was lowest (mean 33.75 per minute, ± SD 1.32). The significant heart rate increase in the imidocarb with atropine group (p < 0.001) was first noticed 30 min after drug administration and returned in four of six horses back to normal after further 30 min and ranged between 44 and 96 beats per minute. The other two horses had an elevated pulse lasting until three hours.
after injection. A significantly increased heart rate was also noted in five of six horses of the imidocarb with glycopyrrolate group. This increase lasted from 30 to a maximum of 90 minutes after drug administration. The duration of elevated rates was comparably shorter than in the imidocarb with atropine group.

The respiratory rate was significantly higher in the imidocarb with saline group (mean 16.37 per minute, ± SD 2.76, p < 0.001), compared to all other groups (mean range 12.80 – 13.16 per minute, ± SD 1.48 - 2.65). Those observations correlated in duration with the presentation of colic symptoms in the imidocarb with saline group.

Table 4.3.1: Mean and ± SD of clinical parameters for each drug group.

<table>
<thead>
<tr>
<th></th>
<th>Imidocarb/ Atropine</th>
<th>Imidocarb/ Saline</th>
<th>Imidocarb/ Glycopyrrolate</th>
<th>Saline/ Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Borborygmi</strong></td>
<td>Mean: 1.86</td>
<td>2.64</td>
<td>2.22</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>± SD: 0.06</td>
<td>0.09</td>
<td>0.10</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Heart rate</strong></td>
<td>Mean: 47.62</td>
<td>36.24</td>
<td>38.42</td>
<td>33.78</td>
</tr>
<tr>
<td>(Beats/min)</td>
<td>± SD: 6.09</td>
<td>5.08</td>
<td>5.37</td>
<td>1.32</td>
</tr>
<tr>
<td><strong>Respiratory rate</strong></td>
<td>Mean: 13.16</td>
<td>16.37</td>
<td>12.83</td>
<td>12.80</td>
</tr>
<tr>
<td>(Breaths/min)</td>
<td>± SD: 2.65</td>
<td>2.76</td>
<td>1.81</td>
<td>1.48</td>
</tr>
</tbody>
</table>

Figure 4.3.1: Means of heart rates
Figure 4.3.2: Means of respiratory rates

Drug combinations: Drug 1 = Imidocarb + atropine; drug 2 = imidocarb + saline; drug 3 = imidocarb + glycopyrrolate; drug 4 = saline control group

Table 4.3 2: P-values of clinical parameters for each drug group (confidence interval 95%)

<table>
<thead>
<tr>
<th>P-values</th>
<th>Drug 1 vs. saline control</th>
<th>Drug 2 vs. saline control</th>
<th>Drug 3 vs. saline control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Borborygmi</td>
<td>p &lt; 0.001</td>
<td>p &lt; 0.001</td>
<td>p = 0.401</td>
</tr>
<tr>
<td>Mean heart rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Beats/min)</td>
<td>p &lt; 0.001</td>
<td>p = 0.006</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Mean respiratory rate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Breaths/min)</td>
<td>p = 0.024</td>
<td>p &lt; 0.001</td>
<td>p = 0.939</td>
</tr>
</tbody>
</table>

Drug combinations: Drug 1 = Imidocarb + atropine; drug 2 = imidocarb + saline; drug 3 = imidocarb + glycopyrrolate
4.4 INCIDENT BETWEEN TRIAL DAYS

One horse suffered from an impaction of the pelvic flexure on a Sunday between trial weeks three and four. This followed treatment with imidocarb-saline combination of therapies. For the duration of the colic, this horse was therefore stabled at the Equine Clinic of the Faculty of Veterinary Science, Onderstepoort, South Africa. The horse coliced only mildly and the rectal examination revealed an impaction that was relatively small and softish. Therefore and to avoid any interference with the trial itself, the horse was only drenched initially with Epsom salts and water and then two-hourly with an oil-water mixture. Any other medication did not occur and the horse was walked and trotted alternately every hour for ten to 15 minutes. Sixteen hours later the horse started passing soft manure. It was then started on small portions of fresh grass, followed by soaked teff and lucerne and continued from Tuesday onwards with the normal trial procedures.

4.5 PRELIMINARY CLINICAL CONCLUSIONS

Colic signs were commonly observed in those horses treated with a combination of imidocarb and saline only, and this was accompanied by a relatively high incidence of diarrhoea and a significant increase in large intestinal borborygmi. These results suggest that imidocarb had a direct parasympathomimetic effect on large intestinal motility in the study subjects, with consequent frequent propulsive contractions, diarrhoea and colic. Pain in these subjects was evidenced by an increase in respiratory rate not seen when imidocarb was accompanied by medication with either atropine or glycopyrrolate.

Both parasympatholytic agents atropine and glycopyrrolate reduced the effect of imidocarb on inducing diarrhoea, colic and increased respiratory rate, and would both therefore be considered beneficial in the clinical setting when treating babesiosis with imidocarb.
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However, atropine caused a prolonged elevation of cardiovascular rate, suggesting that this compound may have greater side effects than glycopyrrolate.

No marked cardiovascular or gastrointestinal effects were observed in those horses treated with the intravenous and intramuscular saline injections only. This suggests that the study procedure, and collection technique itself, was not accountable for any of those clinical signs attributed to pharmacological intervention.
CHAPTER 5

LACTOSE $^{13}$C-UREIDE BREATH TEST FOR THE EFFECTS OF SPECIFIC BABESIOSIS TREATMENTS ON EQUINE ORO-CAECAL TRANSIT TIME

5.1 DELTA VALUES

The maximal increase in $^{13}$C abundance, expressed as delta values, ranged between the subjects between -15.71 and -13.30. The maximal delta value for each horse is shown in Table 5.1.1:

<table>
<thead>
<tr>
<th>Max C delta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse 1</td>
</tr>
<tr>
<td>Range:</td>
</tr>
</tbody>
</table>

5.2 $^{13}$C RECOVERY CURVES

The $^{13}$C recovery curves for the induced lactose $^{13}$C-ureide isotope breath test were plotted for each individual. Curves of two subjects are shown as an example in Figures 5.2.1 – 5.2.8. Time in hours is plotted along the x-axis, and percentage dose recovery per hour of the stable isotope (PDR/hr) is shown along the y-axis.
Figure 5.2.1: Horse 7, week 1, Imidocarb 2.4 mg/kg with atropine 0.035 mg/kg

OCTT = 10.463 hours

Figure 5.2.2: Horse 7, week 2, Imidocarb 2.4 mg/kg with saline

OCTT = 4.914 hours
Figure 5.2.3: Horse 7, week 3, Saline control
OCTT = 7.731 hours

Figure 5.2.4: Horse 7, week 4, Imidocarb 2.4 mg/kg with glycopyrrolate 0.0025 mg/kg
OCTT = 5.506 hours
Figure 5.2.5: Horse 3, week 1, Imidocarb 2.4 mg/kg with saline
OCTT = 6.980 hours

Figure 5.2.6: Horse 3, week 2, Saline control
OCTT = 5.287 hours
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Figure 5.2.7: Horse 3, week 3, Imidocarb 2.4 mg/kg with glycopyrrolate 0.0025 mg/kg
OCTT = 5.314 hours

Figure 5.2.8: Horse 3, week 4, Imidocarb 2.4 mg/kg with atropine 0.035 mg/kg
OCTT = 7.087 hours

5.3 INTESTINAL PARAMETERS

When OCTT was prolonged, as was the case with atropine, the mathematical curve fitting was difficult. In such cases the OCTT might be less accurate as it was largely based on the mathematical calculation using the least mean square method. This was due to the incomplete recovery of $^{13}$C at the end of breath sample collection. However, manual assistance of the
LUBT RESULTS

Solver function of Microsoft Office® Excel to find the minimum root mean square (RMS) values for the model led to successful results to the recovery data in each case.

The mean (± SD) measurements for OCTT, t_{1/2}, t_{lag}, t_{max} for the different drug combinations were: Imidocarb 2.4mg/kg with atropine 0.035 mg/kg OCTT 12.98 h ± 8.22 h, t_{1/2} 27.91 h ± 22.16 h, t_{lag} 16.75 h ± 11.58 h, t_{max} 24.89 h ± 19.24 h; Imidocarb 2.4 mg/kg with saline OCTT 5.85 h ± 1.74 h, t_{1/2} 11.36 h ± 3.25 h, t_{lag} 7.29 h ± 2.03 h, t_{max} 10.85 h ± 2.99 h; Imidocarb 2.4 mg/kg with glycopyrrolate 0.0025 mg/kg OCTT 6.10 h ± 1.01 h, t_{1/2} 12.19 h ± 2.88 h, t_{lag} 7.66 h ± 1.21 h, t_{max} 11.03 h ± 2.25 h and saline OCTT 5.77 h ± 1.67 h, t_{1/2} 14.19 h ± 3.55 h, t_{lag} 7.70 h ± 1.65 h, t_{max} 11.64 h ± 2.63 h.

A summary of OCTT data is shown in Table 5.3.1 and individual mean OCTT parameters are presented in Tables 5.3.2.

Table 5.3.1: Mean OCTT parameters for each drug group using $^{13}$C-LUBT (n = 6).

<table>
<thead>
<tr>
<th></th>
<th>Imidocarb/ Atropine (h)</th>
<th>Imidocarb/ Saline (h)</th>
<th>Imidocarb/ Glycopyrrolate (h)</th>
<th>Saline Control (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean OCTT</td>
<td>12.98</td>
<td>5.85</td>
<td>6.10</td>
<td>5.77</td>
</tr>
<tr>
<td>±SD OCTT</td>
<td>8.22</td>
<td>1.74</td>
<td>1.01</td>
<td>1.67</td>
</tr>
<tr>
<td>Mean t_{1/2}</td>
<td>27.91</td>
<td>11.36</td>
<td>12.19</td>
<td>14.19</td>
</tr>
<tr>
<td>±SD t_{1/2}</td>
<td>22.16</td>
<td>3.25</td>
<td>2.88</td>
<td>3.55</td>
</tr>
<tr>
<td>Mean t_{lag}</td>
<td>16.75</td>
<td>7.29</td>
<td>7.66</td>
<td>7.70</td>
</tr>
<tr>
<td>±SD t_{lag}</td>
<td>11.58</td>
<td>2.03</td>
<td>1.21</td>
<td>1.65</td>
</tr>
<tr>
<td>Mean t_{max}</td>
<td>24.89</td>
<td>10.85</td>
<td>11.03</td>
<td>11.64</td>
</tr>
<tr>
<td>±SD t_{max}</td>
<td>19.24</td>
<td>2.99</td>
<td>2.25</td>
<td>2.63</td>
</tr>
</tbody>
</table>
**Table 5.3.2:** OCTT parameters of each subject using the $^{13}$C-LUBT. Weekly parameters are shown as well as the mean and SD (n = 4) for each subject. Data from week 3 of subject 4 and 6 were withdrawn. The different drug combinations are shown in different colors: Saline = black, imidocarb/atropine = red, imidocarb/saline = green, imidocarb/glycopyrrolate = blue.

<table>
<thead>
<tr>
<th>Horse 1</th>
<th>OCTT (h)</th>
<th>$t_{1/2}$</th>
<th>$t_{lag}$</th>
<th>$t_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>3.35</td>
<td>18.72</td>
<td>6.35</td>
<td>11.95</td>
</tr>
<tr>
<td>Week 2</td>
<td>6.09</td>
<td>17.47</td>
<td>8.84</td>
<td>14.75</td>
</tr>
<tr>
<td>Week 3</td>
<td>5.39</td>
<td>14.44</td>
<td>7.63</td>
<td>12.45</td>
</tr>
<tr>
<td>Week 4</td>
<td>9.28</td>
<td>12.25</td>
<td>10.14</td>
<td>11.99</td>
</tr>
<tr>
<td>Mean (h)</td>
<td>6.03</td>
<td>15.72</td>
<td>8.24</td>
<td>12.79</td>
</tr>
<tr>
<td>± SD</td>
<td>2.46</td>
<td>2.93</td>
<td>1.62</td>
<td>1.33</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Horse 7</th>
<th>OCTT (h)</th>
<th>$t_{1/2}$</th>
<th>$t_{lag}$</th>
<th>$t_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>10.46</td>
<td>15.79</td>
<td>11.93</td>
<td>15.12</td>
</tr>
<tr>
<td>Week 2</td>
<td>4.91</td>
<td>8.27</td>
<td>5.72</td>
<td>7.46</td>
</tr>
<tr>
<td>Week 3</td>
<td>7.73</td>
<td>16.31</td>
<td>10.00</td>
<td>14.94</td>
</tr>
<tr>
<td>Week 4</td>
<td>5.51</td>
<td>10.83</td>
<td>6.70</td>
<td>9.19</td>
</tr>
<tr>
<td>Mean (h)</td>
<td>7.15</td>
<td>12.83</td>
<td>8.56</td>
<td>11.68</td>
</tr>
<tr>
<td>± SD</td>
<td>2.52</td>
<td>3.88</td>
<td>2.91</td>
<td>3.94</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Horse 3</th>
<th>OCTT (h)</th>
<th>$t_{1/2}$</th>
<th>$t_{lag}$</th>
<th>$t_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>6.98</td>
<td>10.40</td>
<td>7.93</td>
<td>10.00</td>
</tr>
<tr>
<td>Week 2</td>
<td>5.29</td>
<td>11.17</td>
<td>6.50</td>
<td>8.88</td>
</tr>
<tr>
<td>Week 3</td>
<td>5.31</td>
<td>11.47</td>
<td>6.93</td>
<td>10.45</td>
</tr>
<tr>
<td>Week 4</td>
<td>7.09</td>
<td>12.75</td>
<td>8.63</td>
<td>12.01</td>
</tr>
<tr>
<td>Mean (h)</td>
<td>6.17</td>
<td>11.81</td>
<td>7.47</td>
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<tr>
<td>± SD</td>
<td>1.00</td>
<td>0.69</td>
<td>0.95</td>
<td>1.30</td>
</tr>
</tbody>
</table>
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### Table 1: OCTT (h) for Horse 4

<table>
<thead>
<tr>
<th>Week</th>
<th>OCTT (h)</th>
<th>t_{1/2}</th>
<th>t_{lag}</th>
<th>t_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>7.11</td>
<td>12.67</td>
<td>8.65</td>
<td>12.00</td>
</tr>
<tr>
<td>Week 2</td>
<td>27.47</td>
<td>63.86</td>
<td>36.82</td>
<td>57.17</td>
</tr>
<tr>
<td>Week 3</td>
<td>4.80</td>
<td>9.69</td>
<td>6.11</td>
<td>8.96</td>
</tr>
<tr>
<td>Mean (h)</td>
<td>13.13</td>
<td>24.03</td>
<td>13.74</td>
<td>26.04</td>
</tr>
<tr>
<td>± SD</td>
<td>12.48</td>
<td>22.30</td>
<td>11.10</td>
<td>27.00</td>
</tr>
</tbody>
</table>

### Table 2: OCTT (h) for Horse 5

<table>
<thead>
<tr>
<th>Week</th>
<th>OCTT (h)</th>
<th>t_{1/2}</th>
<th>t_{lag}</th>
<th>t_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>10.60</td>
<td>34.90</td>
<td>16.23</td>
<td>28.17</td>
</tr>
<tr>
<td>Week 2</td>
<td>7.51</td>
<td>11.83</td>
<td>8.71</td>
<td>11.32</td>
</tr>
<tr>
<td>Week 3</td>
<td>8.83</td>
<td>16.35</td>
<td>10.89</td>
<td>15.38</td>
</tr>
<tr>
<td>Week 4</td>
<td>6.86</td>
<td>14.54</td>
<td>8.88</td>
<td>13.30</td>
</tr>
<tr>
<td>Mean (h)</td>
<td>8.45</td>
<td>19.40</td>
<td>11.18</td>
<td>17.04</td>
</tr>
<tr>
<td>± SD</td>
<td>1.65</td>
<td>10.49</td>
<td>3.51</td>
<td>7.60</td>
</tr>
</tbody>
</table>

### Table 3: OCTT (h) for Horse 6

<table>
<thead>
<tr>
<th>Week</th>
<th>OCTT (h)</th>
<th>t_{1/2}</th>
<th>t_{lag}</th>
<th>t_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 1</td>
<td>5.64</td>
<td>10.19</td>
<td>6.75</td>
<td>9.12</td>
</tr>
<tr>
<td>Week 2</td>
<td>4.18</td>
<td>9.04</td>
<td>5.45</td>
<td>8.23</td>
</tr>
<tr>
<td>Week 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 4</td>
<td>5.05</td>
<td>8.90</td>
<td>6.12</td>
<td>8.45</td>
</tr>
<tr>
<td>Mean (h)</td>
<td>4.96</td>
<td>9.37</td>
<td>6.11</td>
<td>8.60</td>
</tr>
<tr>
<td>± SD</td>
<td>0.74</td>
<td>0.71</td>
<td>0.65</td>
<td>0.47</td>
</tr>
</tbody>
</table>

### 5.4 Significance of Results

Analysis of variance (ANOVA) was used to calculate the significance of the drug treatments on the measured parameters of gastrointestinal motility. The drug combinations were assumed to have significant difference to the saline controls, if the p-value was < 0.05. OCTT, t_{max}, t_{1/2}, t_{lag}.
t\textsubscript{lag} had the values p = 0.017, p = 0.030, p = 0.045 and p = 0.019 respectively. The p-values for each horse and different weeks were all above 0.05. This indicates that the results of this study were influenced significantly only by the different drug combinations, and not by any temporal effects during the study period. The p-values are shown in Table 5.4.1.

Table 5.4.1: P-values of different factors.

<table>
<thead>
<tr>
<th></th>
<th>P\text OCTT</th>
<th>P\text t\textsubscript{1/2}</th>
<th>P\text t\textsubscript{lag (10%)}</th>
<th>P\text t\textsubscript{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>0.20</td>
<td>0.27</td>
<td>0.185</td>
<td>0.20</td>
</tr>
<tr>
<td>Weeks</td>
<td>0.18</td>
<td>0.20</td>
<td>0.154</td>
<td>0.16</td>
</tr>
<tr>
<td>Drugs</td>
<td>0.017</td>
<td>0.05</td>
<td>0.019</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Furthermore the differences between each drug combination for OCTT, t\text{max}, t\textsubscript{1/2} and t\textsubscript{lag} were evaluated for significance using the $t$–test, or in cases where the results were not normally distributed the Mann – Whitney Rank Sum Test.

5.4.1 Effects on OCTT

For OCTT the p-values were significant for Imidocarb 2.4 mg/kg with atropine 0.035 mg/kg (drug 1) versus Imidocarb 2.4mg/kg with saline (drug 2), drug 1 versus Imidocarb 2.4 mg/kg with glycopyrrolate 0.0025 mg/kg (drug 3) and drug 1 versus saline (drug 4) with p = 0.009, 0.017 and 0.016 respectively. Drug 2 versus drug 3 (p = 0.768), drug 2 versus drug 4 (p = 0.944) and drug 3 versus drug 4 (p = 0.700) showed no significance, but the power of these tests was below 0.8, which shows a likelihood of non detection of significant differences when one might actually exist there.

5.4.2 Effects on $t$\text{max}

Only the p-value (p = 0.030) of the comparison of drug 1 versus drug 3 presented significance for $t$\text{max}. Drug 1 vs. drug 2 (p = 0.082), drug 1 vs. drug 4 (p = 0.095), drug 2 vs. drug 3 (p =
0.696), drug 2 vs. drug 4 (p = 0.493) and drug 3 vs. drug 4 (p = 0.687) were above significance. Also these results present a power of the test of below 0.8, which suggests interpretation with caution. Furthermore no great differences in the median values between each group were presented in the latter values. This difference could be explained with the random sampling variability.

5.4.3 Effects on \( t_{1/2} \)

\( t_{1/2} \) had no significance in any of its p-values, which could be either due to the low power of these test or the small difference in the median values between two groups. The p-values of drug 1 vs. drug 2, drug 1 vs. drug 3, drug 1 vs. drug 4, drug 2 vs. drug 3, drug 2 vs. drug 4 and drug 3 vs. drug 4 are 0.101, 0.116, 0.209, 0.651, 0.201 and 0.329 respectively.

5.4.4 Effects on \( t_{\text{lag}} \)

The p-values for \( t_{\text{lag}} \) were significant for drug 1 vs. drug 2 (p = 0.017), drug 1 vs. drug 3 (p = 0.030) and drug 1 vs. drug 4 (p = 0.032) and not significant for drug 2 vs. drug 3 (p = 0.710), drug 2 vs. drug 4 (p = 0.727) and drug 3 vs. drug 4 (p = 0.966). The power of the tests with insignificant p-values was also in this case below 0.8 and the differences in median values between two groups were also not great.

The p-values are presented in Table 5.4.2. OCTT, \( t_{1/2} \), \( t_{\text{lag}} \) and \( t_{\text{max}} \) are presented in comparison between each drug combination in Figures 5.4.1.

**Table 5.4.2**: P-values of comparison between drug combination groups.

<table>
<thead>
<tr>
<th>Drug comparison</th>
<th>P OCTT</th>
<th>P ( t_{1/2} )</th>
<th>P ( t_{\text{lag}} ) (10%)</th>
<th>P ( t_{\text{max}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1 vs. D2</td>
<td>0.009</td>
<td>0.101</td>
<td>0.017</td>
<td>0.082</td>
</tr>
<tr>
<td>D1 vs. D3</td>
<td>0.017</td>
<td>0.116</td>
<td>0.030</td>
<td>0.030</td>
</tr>
<tr>
<td>D1 vs. D4</td>
<td>0.016</td>
<td>0.209</td>
<td>0.032</td>
<td>0.095</td>
</tr>
<tr>
<td>D2 vs. D3</td>
<td>0.768</td>
<td>0.651</td>
<td>0.710</td>
<td>0.696</td>
</tr>
<tr>
<td>D2 vs. D4</td>
<td>0.944</td>
<td>0.201</td>
<td>0.727</td>
<td>0.493</td>
</tr>
<tr>
<td>D3 vs. D4</td>
<td>0.700</td>
<td>0.329</td>
<td>0.966</td>
<td>0.687</td>
</tr>
</tbody>
</table>

Significant differences are highlighted in red.
**LUBT RESULTS**

**Figures 5.4.1:** Comparison of drug combinations with OCTT, $t_{\text{max}}$, $t_{1/2}$ and $t_{\text{lag}}$.

*Drug 1: Imidocarb with atropine; Drug 2: Imidocarb with saline; Drug 3: Imidocarb with glycopyrrolate; Drug 4: Saline control*

**5.5 PRELIMINARY CONCLUSIONS ON EFFECT OF DRUG COMBINATIONS ON PARAMETERS OF GASTROINTESTINAL TRANSIT**

Using the induced lactose $^{13}$C-ureide breath test it was shown in this section of the study, that concurrent medication of imidocarb-treated horses with atropine resulted in a significant prolongation of the oro-caecal transit time of the chosen test meal. Prolongation of other
parameters, including caecal half-transit time, also occurred but these changes were not significant due to the wide variance in these parameters.

Contrary to expectation, imidocarb dipropionate itself did not result in significant reduction in the parameter of OCTT when compared with saline controls. Similarly, combined treatment of horses in this study with both imidocarb dipropionate and glycopyrrolate did not result in a significant reduction in oro-caecal transit or caecal half-emptying time when compared with the saline controls. Those horses treated with the atropine/imidocarb combination did have significant prolongation of OCTT when compared to those treated with imidocarb and the shorter acting parasympatholytic agent, glycopyrrolate. This latter finding suggests that the different parasympatholytic agents have different mechanisms of action on the equine small intestine, an important clinical finding that has not previously been recognised.
6.1 EFFECT OF SPECIFIC BABESIOSIS TREATMENT ON EQUINE ORO-Caecal Transit Time

6.1.1 Validation of study methods and protocol

The effect of imidocarb administration alone and in combination with atropine and glycopyrrolate on oro-caecal transit time (OCTT) was assessed using the lactose $^{13}$C-ureide breath test (LUBT). The LUBT is a valid method for measurement of OCTT in man (Wutzke et al. 1997) and horses (Sutton et al. 2000). Although horses older than three years may be lactose intolerant (Roberts 1975), this has not been shown to be a rate-limiting factor in vitro for the digestion of lactose $^{13}$C-ureide labels (Sutton 2003). It was shown in a previous study that the lactose ureide (LU) bond is exclusively split by Clostridium innocuum (Wutzke et al. 1998; Mohr et al. 1999). Furthermore it was reported that the fermentation of lactose ureide is restricted to the caecum in horses and that prior exposure to lactose $^{12}$C-ureide increases the rate of microbial lactose $^{13}$C-ureide digestion significantly (Sutton 2003) and therefore increases the appearance of tracer in breath (Morrison et al. 2003). In addition, as there is no osmotic effect on motility (Ruemmele et al. 1997), LU is therefore an ideal tracer for measuring OCTT.

A minimum of 14 hours of prior exposure to lactose $^{12}$C-ureide was shown to maximise subsequent $^{13}$CO$_2$ production rate, and this was considered due to maximal induction of the requisite bacterial activity in the caecum (Sutton 2003).

The horses were stabled from two days prior to breath collection study days and were not exercised at all for the entire duration of the trial to avoid increase of background enrichment.
of expired CO₂ due to changes in the metabolic state (Schoeller et al. 1977; Wolfe et al. 1984).

Unlabelled lactose ureide was fed a day prior to the ingestion of the labelled isotope test meal, as it results in a greater $^{13}$CO₂-enrichment, due to the induction of microbial enzyme activity (Wutzke et al. 1998). The horses were starved overnight to decrease the baseline isotope ratio of breath CO₂ (Schoeller et al. 1977; Schoeller et al. 1984). Furthermore no food intake was allowed during the trials as an earlier study found it to shorten OCTT (Priebe et al. 2004).

Duplicate expiratory breath samples were collected from each horse using a modified Aeromask® (Trudell Medical International, Ontario, Canada). Each sampling collection took about two minutes. Horse’s breathing (Orr et al. 1975) as well as respiratory and heart rate are not influenced by respiratory gas collection masks in non exercising horses (Evans et al. 1988).

Four different drug combinations were tested (imidocarb 2.4 mg/kg IM with atropine 0.035 mg/kg IV, imidocarb 2.4 mg/kg IM with saline IV, imidocarb 2.4 mg/kg IM with glycopyrrolate 0.0025 mg/kg IV and saline IM with saline IV) in a randomised, double blinded trial on six clinically healthy subjects (two ponies, four horses). The doses of the different drugs chosen in this study were based on reports taken from various earlier studies (Kuttler 1980; Singh et al. 1996; Heerden 1996; Phipps et al. 1996; Singh et al. 1997a; Singh et al. 1997b; Sutton et al. 2002; Belloli et al. 2002; Donnellan 2006).

Imidocarb is the drug of choice for equine babesiosis (Donnellan et al. 2003b) at a recommended dosage of 2.4 mg/kg bwt by intramuscular administration (Belloli et al. 2002).

6.1.2 Discussion of study results

Imidocarb did not produce a significant decrease in equine oro-caecal transit time (OCTT), when compared with the co-administration of glycopyrrolate group or the saline control group. This result was surprising as the cholinergic acting imidocarb could be expected to
DISCUSSION AND CONCLUSION

hasten transit time due to an increase in circular and longitudinal smooth muscle activity (Malone et al. 1996). Donnellan (2006) on the other hand, using transrectal ultrasonographic measurement of intestinal motility, did not find an increase in intestinal contractions in the imidocarb group (Donnellan 2006), which could explain our findings in the horses given imidocarb with saline. Donnellan’s study assessed intestinal motility using ultrasonography after administration of four different drug therapies (imidocarb 2.4 mg/kg IM with saline IV, imidocarb 2.4 mg/kg IM with atropine 0.02 mg/kg IV, imidocarb 2.4 mg/kg IM with glycopyrrolate 0.0025 mg/kg IV and saline IM with saline IV). It was clearly proven in this current study that the co-administration of atropine increased OCTT significantly compared to the other drug combinations and saline group. Donnellan’s (2006) similar study found a decrease in intestinal contractions in the atropine group (Donnellan 2006). A decrease of intestinal contractions could lead to chyme not being forwarded and therefore leading to an increased OCTT.

The power of our statistical calculation was low for the results with a p-value above 0.05. This indicates a likelihood of not detecting a significant difference when one actually exists. It is possible that as the number of subjects used for this study was low, a higher number might have shown clearly interpretable results. Furthermore, the number of collected breath samples for the drug combination imidocarb with saline and imidocarb with atropine were additionally minimised as two horses had to be excluded from a single trial day. This was due to the breath test results as well as the clinical observation of heart rate, faecal output and borborygmi that strongly led to the suspicion of the blindly administered drug combination of imidocarb with saline and imidocarb with atropine having been mixed up in these two horses.
DISCUSSION AND CONCLUSION

6.1.3 Potential confounding factors in study interpretation

Human studies have shown that gender (Degen et al. 1996; Graff et al. 2001) and age (Graff et al. 2001) have an influence on intestinal transit time. Although the exact effect of these parameters is not known in horses, each subject in this study acted as its own control, thus eliminating the effects of any such confounding factors.

The accuracy of $^{13}$C isotope breath tests depends on a constant basal $^{13}$CO$_2$ production during the test period. Equine diet, particularly the natural background $^{13}$C of that diet (Morrison et al. 2000) and exercise (Rating et al. 1997) can both influence this basal $^{13}$CO$_2$ production by metabolic fluctuation. Therefore horses were starved before and during the trial and not exercised for the entire trial period. It has been shown elsewhere that dehydration does not influence OCTT (van Nieuwenhoven et al. 2000). However, another factor that might have influenced the results of this study is the fact that the horses used were allowed to graze on pasture during the wash out period between the days of breath sample collection. This factor might have influenced gut motility, as it represented changes in both dietary grass intake and relative exercise levels. We decided before trial to take this risk, as we assumed this influence might only be a minor component and also tried to find a balance in preventing colic due to impaction and a stable daily routine for the horse with minimal effects on gut motility. The prevention of impaction colics seemed very important as the study was done in May and June which correlates with the drought season in the north of South Africa, during which a peak of colics due to impaction can be clinically observed in equine practice (personal communication, Dr. Sutton). The progressive effect of any such confounding factor on the longitudinal results of the study was minimised also by the active randomisation of the drug therapies for each horse with time (see Appendix A).
Grazing during the study period between trial days might have increased the consumption of C₄ enriched tropical plants such as the Kikuyu grass that is common on pasture in this area. This likely led to an increase of natural background $^{13}\text{C}$ (Craig 1957; Morrison et al. 2000) in expiratory breath. The baseline $^{13}\text{C}$ abundance expressed as delta values ranged from -21.49 to -16.90. The maximal increase in $^{13}\text{C}$ abundance between the subjects was found to be between -13.30 and -15.71. This represents relatively close values for baseline and maximal increase measurements. However, increases in breath $^{13}\text{CO}_2$ were measured against the $^{13}\text{CO}_2$ baseline output in each case, measured before the isotope substrate was fed. Initial measurement, taken in the test subjects under test conditions, after ingestion of the test meal without enrichment by the isotope, showed a tendency for baseline $^{13}\text{C}$ production to fall with time under test conditions. The cause for this was not clear but may have been caused by the metabolism of the test meal which was lower in $^{13}\text{C}$ content than residual carbohydrate stores in the test subjects. In order to limit this variable from future studies it would be necessary to either measure basal $^{13}\text{C}$-output in a larger number of individuals over time, and to plot its mean change under test conditions, or to supplement the test meal with a much higher dose of $^{13}\text{C}$-isotope so as minimise the effect of any fluctuation in background output. This latter solution is the most straightforward, but was too expensive for the means of this study.

It has been proposed in a study to measure individual CO₂ production (VCO₂) for calculating an accurate percentage dose recovered (PDR) (Amarri et al. 1998). As this was impractical here, VCO₂ was calculated in this study, based on reported studies in ponies (Orr et al. 1975) and horses (Gallivan et al. 1989).

The point of rise of concentration in exhaled breath, was the same in other breath test studies using either direct plotting or cumulative sum plotting of PDR/h exhaled breath data (Brown
DISCUSSION AND CONCLUSION

et al. 1987; Sutton 2003). Therefore only the commoner approach of direct plotting was used in this study, with use of least means squares plots to fit the power regression curve.

Although several digestive processes might be part of the OCTT, our results seemed to have been described very well by a one-curve model. Therefore we did not apply an elsewhere described two-curve model fit to our data (Vickers et al. 2001; Christian et al. 2002b).

6.2 PHYSIOLOGICAL INTERPRETATION OF CLINICAL INFORMATION OBSERVED DURING THE STUDY

In this study four horses developed colic signs, including pawing, lying down and rolling after administration of imidocarb with saline. This was expected to occur as colic (Frerichs et al. 1973; Frerichs 1977; Meyer et al. 2005) and also diarrhoea was observed in earlier studies in both horses (Carbrey et al. 1971) and goats (Corrier et al. 1976). The symptoms started 30 min after drug administration and lasted up to five hours and correlated to the occurrence of watery diarrhoea. Phipps (1996) and Adams (1981) reported similar findings for the duration of colic signs (Adams 1981; Phipps et al. 1996) after administration of imidocarb. Corrier (1976) observed in his study in goats the diarrhoea to last between three to five hours (Corrier et al. 1976). The duration of systemic side effects could be associated with a high plasma concentration of imidocarb. Belloli et al. (2002) found imidocarb to be rapidly absorbed and to be detectable in the plasma concentration ten minutes after administration with a following plateau level for two hours and subsequent fast distribution but slow elimination phase (Belloli et al. 2002). Adams (1981) also found imidocarb to increase rapidly after administration and to decline over the following two to four hours (Adams 1981).

Another study reported mild diarrhoea and abdominal discomfort in horses given lactose (Roberts 1975). Lactose itself does not seem to have caused any of these in our study as only
some subjects given imidocarb with saline presented these symptoms, and the quantity of lactose produced by digestion of the labelled isotope was extremely small.

In contrast to Donnellan’s study we did not observe any signs of colic after administration of imidocarb with atropine (Donnellan 2006). This might be explained by the higher dose of atropine used in our study (0.035 mg/kg IV) and could lead to the assumption that a higher dose of atropine than 0.02 mg/kg (Donnellan 2006) would be necessary to prevent side effects of imidocarb administration. On the other hand the dose of atropine should not be too high either, as another study assessing the side effects of atropine administration, observed colic symptoms due to atropine administration starting from 0.044 mg/kg bwt (Ducharme et al. 1983).

One horse in the study suffered colic due to an impaction of the pelvic flexure. This occurred more likely due to the limited exercise and environmental influences than due to side effects of atropine administration, as the horse had only received atropine 17 days prior to the colic.

On auscultation of gastrointestinal motility a significant increase in borborygmi was noted in the group that had imidocarb alone. This confirms the finding of other studies, where the use of imidocarb caused “violent peristalsis” (Adams 1981) or hypermotility of the gastrointestinal tract (Frerichs et al. 1973; Frerichs et al. 1974). Atropine is known to reduce gastrointestinal motility (Ducharme et al. 1983; Roberts et al. 1986; Imbimbo et al. 1990; Adams 2001; Sutton et al. 2002), even when administered only by topical or subconjunctival application (Williams et al. 2000) and to delay gastric emptying (Imbimbo et al. 1990; Sutton et al. 2002). In this study the imidocarb with atropine group presented a significant decrease in borborygmi and the imidocarb with glycopyrrolate group presented gut sounds similar to the saline group. Glycopyrrolate also has a dose dependent (Roberts et al. 1986; Singh et al. 1996) effect on decreasing gut motility (Singh et al. 1997a; Singh et al. 1997b). Therefore
when given with imidocarb the increasing effect of imidocarb and the decreasing feature of
glycopyrrolate seem to level each other.

The administration of imidocarb with atropine led to a tachycardia for up to three hours. This
finding is in agreement with another study that evaluated effects of atropine on end-organs
(Mirakhur and Dundee 1980). The heart rate of the imidocarb with glycopyrrolate group was
increased only for one hour and not at all increased in one horse of the latter group. This is a
common and sometimes even desired finding after the use of atropine (Gasthuys et al. 1990;
or glycopyrrolate (Singh et al. 1996; Singh et al. 1997a; Singh et al. 1997b; Adams 2001).
Singh et al. (1996) found an increase in heart rate after administration of 2.5 µg/mg bwt
glycopyrrolate as premedication for anaesthesia (Singh et al. 1996), or as a premedication for
sedation with xylazine (Singh et al. 1997b). However, in another of their studies using the
same dose no change in heart rate occurred (Singh et al. 1997a).

Imidocarb’s adverse effects might be due to excessive cholinergic action (Abdullah et al.
1984) on the large intestine.

### 6.3 CONCLUSION

The breath test collection technique was easy to perform and excellently tolerated by the
horses used in this study. The ability of atropine and glycopyrrolate to prevent the side effects
of imidocarb administration and the effect on oro-caecal transit time (OCTT) of all these
drugs were assessed in this study. No significant changes in OCTT were noted with imidocarb
with glycopyrrolate or imidocarb alone. Atropine on the other hand showed an increase in
OCTT. However, both atropine and glycopyrrolate improved the clinical side effects of
imidocarb. Atropine showed more inhibitory effects on the gastrointestinal tract, reducing
borborygmi compared to glycopyrrolate. Also the heart rate was increased for a longer
duration with atropine than with the use of glycopyrrolate.
DISCUSSION AND CONCLUSION

The fact that glycopyrrrolate ameliorated the clinical side effects of imidocarb, namely diarrhoea and colic with increased intestinal borborygmi, without significantly changing OCTT, suggests that its predominant mechanism of action in the horse (at 0.0025 mg/kg IV) is on the large intestine. Conversely atropine (at 0.035 mg/kg IV) was shown to increase OCTT in this study, in addition to significantly decreasing intestinal borborygmi and prolonging caecal half-transit time. This latter finding suggests that atropine has potent effects on both equine small and large intestine. As imidocarb alone did not cause significant reductions in the rate of oro-caecal transit of the chosen test meal, it must be concluded from this study that its side effects result principally from direct stimulation of large intestinal hypermotility, colic and diarrhoea, rather than modulation of small intestinal motility.

This method of evaluating effects on equine gastrointestinal transit time could be applied to further studies. The emphasis could be put on different parts of the gastrointestinal tract, using different substrates. It would be desirable for the future, if further research in this field would be done. Although LU and IRMS analysis are expensive techniques, this non-invasive modality will hopefully be of future diagnostic importance in the investigation of cases with recurrent colic and in clinical pharmacology trials.
# APPENDIX

## APPENDIX A: RANDOMISATION OF TREATMENT PROTOCOL

<table>
<thead>
<tr>
<th>Injection Protocol</th>
<th>Horse 1</th>
<th>Horse 7</th>
<th>Horse 3</th>
<th>Horse 4</th>
<th>Horse 5</th>
<th>Horse 6</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>First week</td>
<td>A</td>
<td>C</td>
<td>B</td>
<td>D</td>
<td>C</td>
<td>A</td>
<td>ABCDAC</td>
</tr>
<tr>
<td>Second week</td>
<td>D</td>
<td>B</td>
<td>A</td>
<td>C</td>
<td>D</td>
<td>B</td>
<td>ABCDBD</td>
</tr>
<tr>
<td>Third week</td>
<td>B</td>
<td>A</td>
<td>D</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>ABCDAB</td>
</tr>
<tr>
<td>Fourth week</td>
<td>C</td>
<td>D</td>
<td>C</td>
<td>B</td>
<td>A</td>
<td>D</td>
<td>ABCDCD</td>
</tr>
</tbody>
</table>

A Saline control  
B Imidocarb 2.4 mg/kg IM / saline IV  
C Imidocarb 2.4 mg/kg IM / Atropine 0.035 mg/kg IV  
D Imidocarb 2.4 mg/kg IM / Glycopyrrolate 0.0025 mg/kg IV
Equine babesiosis is an infectious tick-borne disease that leads to haemolytic anaemia. The parasites *Theileria equi* and *Babesia caballi* cause acute, subacute or chronic diseases in Equidae, neonatal babesiosis or abortion in mares and death can occur. Imidocarb dipropionate is the drug of choice to control the disease in Equidae. Due to its cholinergic properties, systemic side effects include depression, intestinal hypermotility, and colic and lateral recumbency may occur.

For avoidance of these undesired effects, prior administration of either atropine or glycopyrrolate is proposed to ameliorate side effects.

Atropine and glycopyrrolate are both anticholinergic drugs that inhibit gastrointestinal motility. Glycopyrrolate is preferred over atropine sulphate as it has increased water solubility and thus does not cross the blood-brain barrier.

Previous studies have validated the induced lactose $^{13}$C-ureide breath test (LUBT) against enterocolonic scintigraphy for the measurement of oro-caecal transit time (OCTT) in humans (Geypens *et al.* 1999) and the test has been validated *in vitro* for this measurement in horses (Sutton 2003). It was concluded to be a valid alternative to scintigraphy for OCTT measurements in humans (Geypens *et al.* 1999) and a superior technique to the H$_2$ breath production test in horses (Sutton 2003).

The aims of this study were to improve the knowledge of the specific effects of imidocarb treatment on equine OCTT, to quantify the effects of premedication with atropine or glycopyrrolate and to develop/validate a clinical protocol for treatment of babesiosis that results in minimal gastrointestinal complications.
The relative effects of three drug therapies (imidocarb dipropionate 2.4 mg/kg IM with saline IV (I/S); imidocarb dipropionate 2.4 mg/kg IM with atropine 0.035 mg/kg IV (I/A); imidocarb dipropionate 2.4 mg/kg IM with glycopyrrolate 0.0025 mg/kg IV (I/G)) were measured against a saline control (SC) (saline IM with saline IV) in a randomised treatment protocol.

The relative effects of these compounds on OCTT were measured in six healthy horses using the induced lactose $^{13}$C-ureide breath test (LUBT). Duplicate breath samples were taken and the $^{13}$C:$^{12}$C ratio of the breath samples was measured using continuous flow isotope ratio mass spectrometry (CF-IRMS). Sequential exhaled breath delta $^{13}$C-values were used to provide an estimate of OCTT.

The mean (± SD) measurements for OCTT for the different drug combinations were: I/A 12.98 h ± 8.22 h, I/S 5.85 h ± 1.74 h, I/G 6.10 h ± 1.01 h and SC 5.77 h ± 1.67 h. Only OCTT of I/A was significantly increased, compared to the other drug combinations. Mean OCTT in the horses treated with I/S showed a non significant tendency to decrease.

In our clinical observations I/S caused colic and diarrhoea in four of six horses. Colic symptoms were presented with pawing, looking at flank and rolling, lasting from 30 min to five hours after injection. Gut motility was auscultated on a scale from zero (none) to four (normal). It was increased (mean 2.64, ± SD 0.09) after administration of I/S and decreased (mean 1.86, ± SD 0.06) after I/A was given.

The average heart rate was highest in the horses administered I/A (mean 47.62, ± SD 6.09) and less so in the I/G group (mean 38.42, ± SD 5.37) as well as the I/S group (mean 36.24, ± SD 5.08). The heart rate of the SC group was lowest (mean 33.78, ± SD 1.32).

The respiratory rate was notable higher in the I/S group (mean 16.37, ± SD 2.76), compared to all other groups (mean range 12.80 – 13.16, ± SD 1.48 - 2.65).
SUMMARY

The breath test collection technique was easy to perform and excellently tolerated by the horses used in this study. I/S did not produce a significant decrease in OCTT, whereas atropine caused a significant increase in OCTT. However, both atropine and glycopyrrolate ameliorated the clinical side effects of imidocarb. Since atropine showed more inhibitory effects on the gastrointestinal tract, as well as causing an increased OCTT, glycopyrrolate is therefore preferable as pre-medication for imidocarb to minimise undesirable side effects. As imidocarb dipropionate treatment did not cause a significant decrease in equine OCTT, its principal effect on the equine gastrointestinal tract appears to be to increase large rather than small intestinal motility. Similarly, glycopyrrolate ameliorated the effects of imidocarb by reducing large intestinal effects without causing a significant delay in small intestinal (oro-caecal) transit, whereas atropine caused a significant increase in OCTT and reduction in intestinal borborygmi.

Imidocarb Dipropionat ist das Medikament der Wahl für die Equine Babesiose. Aufgrund seiner cholinergen Eigenschaften, können systemische Nebenwirkungen wie Depression, gastrointestinalen Hypermotilität, Diarrhoea, Kolik und Festliegen auftreten.

Eine zusätzliche Verabreichung von Atropin oder Glycopyrrolat ist zur Vermeidung der unerwünschten Nebenwirkungen empfohlen.

Atropin und Glycopyrrolat gehören zu der Gruppe der anticholinergen Pharmaka und haben daher eine hemmende Wirkung auf die gastrointestinale Motilität.

Glycopyrrolat ist gegenüber Atropin bevorzugt, da es eine erhöhte Wasserlöslichkeit aufweist und folglich die Blut-Hirn Schranke nicht überschreitet.

Frühere Studien haben den Laktose $^{13}$C-Ureid Atemtest mit der enterokolonischen Szintigrafie bezüglich Messungen von oro-zäkalen Transitzeiten in Pferden verglichen. Der Laktose $^{13}$C-Ureid Atemtest wurde gegenüber der Szintigrafie zu einer gültigen Alternative zur Messung von oro-zäkalen Transitzeiten erklärt.

Das Ziel unserer Studie lag darin, das Wissen über die spezifische Wirkung auf die oro-zäkale Transitzeit nach der Gabe von Imidocarb zu verbessern. Desweiteren wurde die Wirkung der Premedikation mit Atropin und Glycopyrrolat untersucht und ein Therapievorschlag für die Babesiose erarbeitet, der minimale gastrointestinale Nebenwirkungen auslöst.
In unserer randomisierten Studie wurden die Wirkungen von drei medikamentösen Therapiemöglichkeiten (Imidocarb Dipropionat 2,4 mg/kg IM und Kochsalzlösung IV (I/S); Imidocarb Dipropionat 2,4 mg/kg IM und Atropin 0,035 mg/kg IV (I/A); Imidocarb Dipropionat 2,4 mg/kg IM und Glykopyrrrolat 0,0025 mg/kg IV (I/G)) mit einer Kontrollgruppe (SC) (Kochsalzlösung IM und Kochsalzlösung IV) verglichen.

Um die ungefähre Wirkung dieser Medikamentenkombinationen auf die oro-zäkale Transitzeit zu messen, haben wir den Laktose $^{13}$C-Ureid Atemtest in sechs klinisch gesunden Pferden angewandt. Doppelte Proben der Ausatemluft wurden gesammelt und das Verhältnis von $^{13}$C:$^{12}$C wurde mittels CF-IRMS (continuous flow isotope ratio mass spectrometry) gemessen.

Im Mittel (± SD) waren die Messungen der oro-zäkalen Transitzeit für die verschiedenen Medikamentenkombinationen: I/A 12,98 h ± 8,22 h, I/S 5,85 h ± 1,74 h, I/G 6,10 h ± 1,01 h und 5,77 h ± 1,67 h für die SC-Gruppe. Verglichen zu den anderen Medikamentengruppen, war nur die oro-zäkale Transitzeit für die Gruppe I/A signifikant verlängert. Für die I/S Kombination konnte nur eine nicht signifikante Tendenz zu einer Verkürzung der oro-zäkale Transitzeit festgestellt werden.


Gastrointestinale Motilität wurde auskultiert und auf einer Skala von Null (keine) bis vier (normal) bewertet. Nach der Gabe von I/S wurde eine erhöhte (im Mittel 2,64; SD ± 0,09) Motilität festgestellt und nach der Verabreichung von I/A, eine verminderte (im Mittel 1,86; SD ± 0,06).
Die durchschnittliche Herzfrequenz war in der I/A Gruppe am höchsten (im Mittel 47,62, SD ± 6,09) und etwas niedriger in der I/G Gruppe (im Mittel 38,42; SD ± 5,37), wie auch in der I/S (im Mittel 36,24; SD ± 5,08). Die Herzfrequenzen der SC_Gruppe (im Mittel 33,78; SD ± 1,32) lagen am niedrigsten.

Die Atemfrequenz war, verglichen mit den anderen Gruppen (im Mittel 12,80 – 13,16, ± SD 1,48 – 2,65), in der I/S Gruppe merkbar erhöht (im Mittel 16,37, SD ± 2,76).

Die Sammlung der Atemgasproben war leicht durchführbar und wurde von den Pferden dieser Studie hervorragend toleriert.


REFERENCES


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Ref Type: Conference Proceeding


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ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Dr. David Sutton. He introduced me into stable isotope breath test technology and gave me the chance to be involved with it. Whether he lived in Pretoria or in Edinburgh, his patient explanations and his guidance through this entire project were exceptional. I also thank him for motivating me, his assistance and the cookies provided during the long trial days.

I would like to thank Prof. Alan Guthrie, who made this research project possible and therefore gave me the opportunity to stay in this beautiful country. I deeply appreciate that he provided me with staff and horses and allowed me to use the Equine Research Centre facilities.

I would like to thank Prof. Hartmut Gerhards for his support and advice in achieving the Dr. med. vet. degree and thank him very much for making the collaboration between the Ludwig-Maximilans University in Munich and University of Pretoria possible.

I have benefited greatly from the collaboration of Dr. Tom Preston, who analysed our samples with very short notice and was of great help in interpreting them.

I would like to thank Dr. Thireshni Chetty for administering the drugs and therefore making it possible to double blind this study. I further thank her for her cheerfulness, emotional support and friendship.

I would like to thank Ms. Stelles de Villiers for her enormous input. Her professionalism and organisatory skills made this study run very smoothly.

This study would not have been possible without the help of Ms. Claire Malic, Mr. Chris Joone and Mr. Chris Matjiane. I thank you all for working long hours, as well as for your pleasant company, good humour and excellent bakery.

I would like to thank Dr. Kenneth Joubert for his motivation, support and statistical assistance.
I thank the horses AJ, Blue, Sister, Caya, Hunter and Speed for their patience and compliance, through which this study was possible.

Financial support was generously provided from start-up research funds for new academics from the University of Pretoria, from Departmental research funds from the Department of Companion Animal Clinical Studies, as well as from the Faculty Research Fund and the Equine Research Centre. I am sincerely grateful for this support, as I would not have been able to perform this study without it.

My family’s support and encouragement during this trial is very much appreciated.

I particularly thank Soorya for providing intellectual, emotional and organisational support over this very long time and for making my stay in this country very pleasant.
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