Investigation of potential cardiovascular effects of the Pheroid® delivery system in conscious rats

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Dissertation submitted in fulfilment of the requirements for the degree Master of Science in Pharmaceutical Science at the North West University

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Graduation: May 2018

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If you are not willing to be a fool, you can't become a master

–Jordan Peterson

DECLARATION

I, Riaan van Wyk declare herewith that this dissertation (**Investigation of potential cardiovascular effects of the Pheroid® delivery system in conscious rats**), which I herewith submit to the North-West University is in compliance with the requirements set for the degree: M.Sc. Pharmaceutical Science is my own work and has not already been submitted to any other university.

I would hereby like to acknowledge the following people for their contributions to this study:

- Professor Faans Steyn, from the statistical consultation services on the Potchefstroom campus of the North-West University, for the performance of the statistical analysis of the processed data.
- Ms Katrin Christ, (Boehringer Ingelheim, Germany) for kindly offering her time and expertise to help during the surgical instrumentation of the telemetry transmitters into the rats.
- Dr Theunis Cloete, for always being available for questions and guidance regarding formulations and calculations.
- The Boehringer group, Mr Werner Mayer, Mr Florian Krause, Mr Michael Markert and Dr Eric Martel for offering their time in allowing me to study their setup and showing me the ropes of cardiovascular telemetry.
- Mr Lesley Masetle, for Mastersize analysis of my Pheroid® formulations.
- Dr Matthew Glyn and Dr Adrienne Leussa for confocal analysis of my Pheroid® formulations.
- Veterinary technologist, Mr. Kobus Venter, for always being available on short notice for helping me during my experiments with the rats.

PERSONAL ACKNOWLEDGMENTS

I would humbly like to thank God for hearing all my prayers during the course of this study and for granting me the ability to do anything in His name. It was His grace that replenished my reserves when I felt tired. Nehemiah 8:10 “Do not grieve, for the joy of the Lord is your **strength**”.

I would hereby like to acknowledge the following people who influenced the success of this study on a very personal level:

Professor Rose Hayeshi for her guidance and whose door was always open for my numerous questions.

Mr. Cor Bester, thank you for always being available to help during the course of my study and for being one of the best travel partners.

Dr Janke Kleynhans, thank you for always being available whenever I had a question, however numerous it may have been.

To each of my fellow students, thank you for creating a fun work environment and always providing support in each other’s studies.

To my parents, who I know you have been praying for my future. Thank you for all your support.

To Duné, thank you for being there, for unending love, support, attention and a lot of (healthy) snacks while I was working, you were my greatest motivation.

To my co-supervisor, Professor Anne Grobler, thank you for your charismatic inputs in my study, your passion for science is truly inspiring.

Professor Brian Guth, my supervisor, thank you for always being 5 minutes away via email, even though you were halfway across the globe. Your valued revisions were always a mere few days away and for that I am thankful. I am honoured that someone with your expertise in cardiovascular safety pharmacology could supervise my study.

ABSTRACT

Title

Investigation of potential cardiovascular effects of the Pheroid® delivery system in conscious rats

Aim:

The aim of this study was to evaluate the possible cardiovascular effects of the Pheroid® drug delivery system and to determine the possible central nervous effects of its combination with cannabidiol (CBD) in the conscious rat.

Introduction:

This project utilizes the principles of safety pharmacology to evaluate the cardiovascular safety of the Pheroid® drug delivery system using a telemetry-based acquisition system and the central nervous safety of a Pheroid®-CBD combination using a standard open-field test. The Pheroid® is a colloidal drug delivery system comprised of a dispersed oil phase of omega 3 and omega 6 fatty acids emulsified in N_2O saturated water. Pheroid® boasts a range of pharmaceutical applicability, among others for the enhancement of therapeutic efficacy due to better drug exposure, reduced cytotoxicity and faster onset of action. A demonstration of its lack of unwanted physiological effects could open the opportunity for further pharmaceutical applications.

Methods:

Eight male rats were instrumented with telemetry transmitters of which six were subsequently used to determine the safety of single intravenous (IV) and oral administration of the Pheroid® delivery system on cardiovascular parameters; including arterial blood pressure, heart rate and the ECG. The reference, positive control compounds were selected as follows: a single intraperitoneal dose of clonidine (0.03 mg/kg) and an oral dose of theophylline (30 mg/kg). For the assessment of the central nervous safety of a Pheroid®-CBD formulation, an open-field test was conducted on 18 male rats divided into three groups of six: CBD (10 mg/kg), pro-Pheroid®-CBD (10 mg/kg) and a control.

Results:

No significant effects of Pheroid® on cardiovascular parameters were observed at any time following oral or intravenous administration. The expected effects of clonidine and theophylline could be demonstrating confirming the sensitivity of the experimental model to detect changes in

the cardiovascular parameters measured. No behavioural effects were reported after assessment of the Pheroid®-CBD formulation on central nervous function.

Discussion:

This project has demonstrated that Pheroid® and its combination with CBD have no effects on cardiovascular or central nervous function respectively. These data showing cardiovascular and central nervous safety are supportive for the use of Pheroid® for various pharmacological applications.

Keywords:

Blood pressure; ECG; Heart rate; Pheroid®; Pre-Clinical Drug Development Platform; Rat; Safety pharmacology; Telemetry

UITTREKSEL

Title

Investigation of potential cardiovascular effects of the Pheroid® delivery system in conscious rats

Doel:

Die doel van hierdie studie was om die moontlike kardiovaskulêre effekte van die Pheroid®-dwelmafleweringsisteem te evalueer en om die moontlike sentrale senuwee-effekte van die kombinasie met kannabidiol (KBD) in die bewuste rot te bepaal.

Agtergrond:

Hierdie projek gebruik die beginsels van veiligheidsfarmakologie om die kardiovaskulêre effekte van die Pheroid®-dwelmafleweringsisteem te evalueer met behulp van 'n telemetrie-gebaseerde verkrygingsisteem en om die effekte van ‘n Pheroid®-KBD kombinasie op die sentrale senuweestelsel te bepaal deur middel van ‘n standaard oopveldtoets. Die Pheroid® is 'n kolloïdale dwelmafleweringstelsel wat bestaan uit 'n verspreide olie fase van omega 3 en omega 6 vetsure wat in N_2O versadigde water geëmulsifeer word. Pheroid® spog met 'n verskeidenheid farmaseutiese toepassings, onder meer vir die verbetering van terapeutiese effektiwiteit as gevolg van beter geneesmiddelblootstelling, verminderde sitotoksisiteit en vinniger aanvang van aksie. 'n Demonstrasie van sy gebrek aan ongewenste fisiologiese effekte kan die geleentheid bied vir verdere farmaseutiese toepassings.

Metodes:

Agt manlike rotte was met telemetrie-senders toegerus, waarvan ses later gebruik was om die veiligheid van enkel intraveneuse (IV) en orale toediening van die Pheroid® afleweringsisteem op kardiovaskulêre parameters te bepaal; insluitend arteriële bloeddruk, harttempo en die elektrokardiogram (EKG). Die positiewe kontroles was soos volg: 'n enkele intraperitoneale dosis klonidien (0.03 mg/kg) en 'n orale dosis teofillien (30 mg/kg). Vir die evaluering van die sentrale senuweestelsel effekte van 'n Pheroid®-CBD formulering, is 'n oopveldtoets uitgevoer op 18 manlike rotte wat verdeel was in drie groepe van ses: CBD (10 mg/kg), pro- Pheroid®-CBD (10 mg/kg) en 'n kontrole.

Resultate:

Geen wesenlike effekte van Pheroid® op kardiovaskulêre parameters is op enige stadium waargeneem na orale of intraveneuse toediening nie. Die verwagte effekte van klonidien en

teofillien kan aantoon dat die sensitiwiteit van die eksperimentele model bevestig word om veranderinge in die gemete kardiovaskulêre parameters op te spoor. Geen gedragseffekte is gerapporteer na die evaluering van die Pheroid®-CBD-formulering op sentrale senuwee-funksie nie.

Gevolgtrekking:

Hierdie projek het getoon dat Pheroid® geen effekte op hartfunksie het na orale en intraveneuse toediening nie. Die kombinasie van Pheroid® met CBD het geen effekte op sentrale senuwee-funksie getoon nie. Hierdie data wat die veligheid van Pheroid® op die kardiovaskulêre- en sentrale senuweestelselsisteme toon, ondersteun die gebruik van Pheroid® vir verskeie farmakologiese toepassings.

Sleutelterme:

Bloeddruk; EKG; Harttempo; Pheroid®; Pre-Clinical Drug Development Platform; Rot; Telemetrie; Veiligheidsfarmakologie

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ABBREVIATIONS

%	Percentage
®	Registered
°C	Degrees Celcius
µm	Micrometre
API	Active pharmaceutical ingredient
BT	Body temperature
CLSM	Confocal laser microscopy
CPMP	Committee for Proprietary Medicinal Products
D(0.1)	10% of particles in the sample are smaller than the indicated size
D(0.5)	50% of particles in the sample are smaller than the indicated size
D(0.9)	90% of particles in the sample are smaller than the indicated size
DBP	Diastolic blood pressure
ECG	Electrocardiogram
FDA	Food and Drug Administration
GLP	Good Laboratory Practise
HR	Heart rate
ICH	International Conference on Harmonization
MBP	Mean blood pressure
MIC	Minimum inhibitory concentration
ml	Millilitre
mV	Millivolts
N_2O	Nitrous oxide

NaCl	Sodium Hydrochloride
nm	nanometre
NWU	North-West University
PCDDP	Pre-Clinical Drug Development Platform
Rpm	Revolutions per minute
SA	South Africa
SBP	Systolic blood pressure
™	Trademark
USA	United States of America
Wt. %	Weight percentage

CHAPTER 1: STUDY JUSTIFICATION, AIM AND OBJECTIVES

The lack of cardiovascular safety of therapeutic agents is one of the largest contributors to drug attrition in the pharmaceutical industry. More specifically, only ~20% of cardiovascular drugs are licensed after successful phase III clinical trials and ~45% of drug withdrawals after approval are attributed to unexpected cardio-toxicity (Hutchinson & Kirk, 2011; Stevens & Baker, 2009).

The field of safety pharmacology is concerned with drug safety assessments and is formally defined by the International Conference on Harmonisation (ICH) S7A guideline as those studies that investigate any potential adverse pharmacodynamic effects of a substance on physiological systems in relation to exposure in the therapeutic range or above. Indeed, regulatory agencies such as the ICH and the Japanese Guidelines for Nonclinical Studies of Drugs Manual incorporated guidelines on safety assessments in order to detect adverse events early in drug development and thereby reduce the risk of failure later in development.

While the Japanese guideline recommends a much more comprehensive safety assessment on physiological systems, the ICH S7A focuses on the cardiovascular, respiratory and central nervous systems, collectively defined as the "core battery" (Andrade *et al*., 2016). With regards to safety assessments of the core battery, the ICH recommends the use of stress free, conscious animals for *in vivo* cardiovascular assessments of novel compounds, resulting in the widespread use of implantable telemetry technology such that the cardiovascular endpoints of interest can be collected without the use of anaesthesia or restraint of the animals.

In many cases, drug delivery systems are used as ancillary components of therapeutic products to ameliorate problems commonly associated with therapeutic drugs, such as poor efficacy, low biodistribution and intolerable dosage regimens. For these delivery systems to be pharmaceutically applicable, they need to conform to specific prerequisites. Ideally, a delivery system should be biocompatible, biodegradable, stable with an appropriate shelf-life, and most importantly, it should be safe (De Jong & Borm, 2008; Lian & Ho, 2000).

The delivery system under investigation is the Pheroid® drug delivery system which is a stable, lipid-based emulsion of submicron structures called Pheroids. Like most colloids, Pheroid® consists of a dispersion medium (continuous phase) and a dispersed phase (Pheroids) that can be customized as required in terms of morphology, size, structure and function (Grobler, 2009). The system incorporates the ethyl esters of omega-3 and omega-6 fatty acids and has been shown to successfully entrap both hydrophilic and hydrophobic compounds (Du Plessis *et al*., 2010; Steyn *et al*., 2011). A few attributes of Pheroid® contributing to its pharmaceutical applicability include the enhancement of therapeutic efficacy, reduced cytotoxicity, faster onset of

action and the ability to be administered using several routes of administration (du Plessis *et al*., 2010; Grobler *et al.,* 2008). Due to its pharmaceutical relevance, Pheroid® is being investigated in an assortment of applications ranging from oncology to infectious disease (Chinembiri *et al*., 2015; Gerber *et al*., 2008; Grobler *et al*., 2014; Steyn *et al*., 2011). It is for this reason that the safety of Pheroid® must be evaluated, with specific reference to its potential cardiovascular and central nervous effects.

Therefore, the aim of this study was to determine the potential cardiovascular and central nervous effects of the Pheroid® in the conscious rat using a telemetry-based cardiovascular data acquisition system and an open-field test, respectively.

To achieve this aim, several objectives were defined:

1) Surgical implantation of telemetry transmitters into six male Sprague-Dawley rats.
2) Establishment and validation of the telemetry system using two reference compounds with known cardiovascular effects (clonidine and theophylline).
3) Determining the cardiovascular effects of an oral and intravenous administration of Pheroid® in conscious rats.
4) Determining the effect of an oral pro-Pheroid®-CBD formulation on the central nervous system using open field technology in conscious rats.

Chapter 2 is a literature review, focusing on safety pharmacology, its history and role in drug development, cardiovascular telemetry. The pharmacology of clonidine and theophylline as reference compounds is then summarized, followed by an in-depth look at the Pheroid® delivery system. Chapter 3 is concerned with the steps taken to establish and validate the telemetry system, including the cardiovascular effects induced by the reference compounds. Chapter 4 describes the physical characterisation of the Pheroid® formulations used for the cardiovascular evaluation, while the actual cardiovascular assessment of Pheroid® is described in Chapter 5 (in article format). Chapter 6 is also written as a manuscript and details the evaluation of a pro-Pheroid®-CBD formulation on the central nervous system. The conclusion to this study is summarised in Chapter 7, along with future prospects.

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CHAPTER 2: LITERATURE REVIEW

2.1 Safety pharmacology

2.1.1 Introduction

Safety pharmacology is defined by the ICH S7A guideline as those studies that investigate any potential adverse pharmacodynamic effects of a substance on physiological systems in relation to exposure in the therapeutic range or above. These systems include the cardiovascular, respiratory and central nervous system, which is commonly referred to as the "core battery" (Andrade *et al*., 2016). Other physiological systems commonly affected by adverse drug effects may also include renal and gastrointestinal systems depending on the intended mechanism of action of the new drug. Despite their potential clinical relevance, such effects may not be observed in routine toxicological studies, as toxicology studies are intended to detect pathophysiological effects following multiple administration of a new drug. The observation that toxicity studies were rarely able to detect these effects on organ function prompted the drug development community to start conducting directed physiological safety testing.

This gave rise to a change in focus with respect to drug development, where physiological safety assessments were conducted separately from regulatory toxicity assessments. In order to better understand how safety pharmacology came to be such an important part of drug safety assessments one can look at certain events that prompted its implementation and shaped its development. The outcome of safety pharmacology studies proved to be crucial, given that adverse effects that went undetected could not only lead to mortality, but also greatly limit profits by restricting a drug's introduction into the commercial market. It is by this fact that safety pharmacology's role in drug development was secured (Guth, 2007).

2.1.2 History of safety pharmacology

The idea of safety pharmacology originated in the 1970's when Professor Gerhard Zbinden realised that the routine toxicity studies of the time lacked the ability to identify novel compounds with the potential to cause adverse pharmacodynamic effects on physiological systems (Bass *et al*., 2015). They did, however, entrust toxicologists with the safety studies necessary to avoid histopathological adverse effects. These toxicological studies typically involved haematology, histopathology and autopsy of the organs. The lack of attention during routine toxicology studies to drug-induced functional disturbances of organ systems was highlighted by Professor Gerhard Zbinden. Zbinden, who is considered the "father of safety pharmacology", went on to suggest the importance of functional toxicology in preclinical drug development in three respects. Firstly, certain toxic responses are not always due to biochemical reactions regardless of the dosage,

but may very well be due to functional organ responses with specific reference to cardiovascular and neurological systems. Secondly, measurements of functional organ effects may be detected much earlier in drug development due to the fact that such effects may occur after only single administration of a drug and at much lower dosages than are typically used in a toxicity study. Lastly, morphological damage to organs systems may be due to primarily functional effects such as vasodilation, vasoconstriction and cardiac overstimulation (Zbinden, 1984). Professor Zbinden emphasised the problem that pharmacodynamic safety of novel small molecules was only considered to be supplementary in the drug discovery process despite its potential importance. Thankfully, Zbinden's concerns were recognized and over time pharmaceutical companies as well as several regulatory agencies recognized the important role of safety pharmacology in drug discovery (Bass *et al*., 2015).

2.1.3 Regulatory guidelines of safety pharmacology

Kinter and Dixon (1995) who, in an attempt to fulfil the diverse research requirements of their customers, developed key physiological focus points for safety pharmacology studies. Among them is the Core Battery (related to cardiovascular, central nervous and respiratory systems), Ancillary (Behavioural, gastrointestinal and autonomic systems) and Special Safety Pharmacology studies (auditory and ocular functions) (Kinter & Dixon, 1995; Kinter & Valentin, 2002).

This approach recommending a more comprehensive safety pharmacological evaluation was adopted by the Japanese Guidelines for Nonclinical Studies of Drugs Manual, issued in 1995 (Anonymous, 1995). The Japanese guideline recommended not only specific safety testing on the cardiovascular, respiratory and central nervous systems, but also on smooth muscle, digestive system and the autonomic nervous system (Guth, 2007; Kinter & Valentin, 2002). The safety studies related to these systems were assigned under "category A", and were expected to be addressed before a drug was introduced into the clinical trial phases in Japan (Bass *et al*., 2015). The Japanese guideline also required that anesthetised animals be used for category A studies, specifically for cardiovascular and respiratory systems, with the anesthetised dog being the model of choice for these studies (Guth, 2007; Kinter & Valentin, 2002). While the use of anesthetised animal models has their advantages, such as the allowance of more invasive and sensitive techniques, which result in more thorough evaluations on haemodynamics, it was soon realised that anaesthesia could have its own haemodynamic effects, specifically on ventricular repolarisation (Bachmann *et* al., 2001; Guth, 2007). Apart from the fact that anaesthesia could have an impact on haemodynamics, it is commonly accepted that the use of stress free, conscious animals would provide a better understanding of what the physiological effects of a specific compound would be.

The ICH S7A guideline was issued in 2001, which recommends the use of unanesthetised, conscious animals for *in vivo* cardiovascular, respiratory and central nervous studies. The ICH S7A also came with three objectives for safety pharmacology studies. The first is the identification of compounds with undesirable pharmacodynamic properties relevant to human safety (risk identification). Secondly, any adverse pharmacodynamic effects found in toxicology or clinical studies, should be further evaluated using such studies (risk assessment). Lastly, the pharmacological mechanism of any identified adverse pharmacodynamic effects, needs to be further investigated in order to better assess the associated risk (risk mitigation) (ICH S7A, 2001). With regards to the first objective of risk identification, the "core battery" was specified by the ICH S7A as the primary focus for pharmacological safety profiling (Andrade *et al*., 2016). An unintended problem with this guideline was that as other physiological systems, like the renal and gastrointestinal systems, that could also be the target of adverse drug effects, tended to be ignored. As a result, the more comprehensive safety assessments on all relevant systems, as recommended by the older Japanese guideline, were often neglected during routine safety testing.

A few years later after the introduction of the ICH S7A guideline, the ICH introduced another guideline for preclinical studies. The ICH S7B guideline was introduced in 2005, which aimed to reduce the risk of ventricular arrhythmia, specifically torsade de pointes, by identifying compounds that delay ventricular repolarisation (QT interval prolongation) (ICH S7B, 2005). The ICH S7B specifically targeted agents that could affect the human potassium channels conducting the current IKr as the most common cause for prolongation of the QT interval (ICH S7B, 2005).

While the ICH S7A and S7B guidelines were not as thorough as the Japanese guideline in recommending safety testing of physiological systems other than those specified in the "core battery", they did however bring a range of new and useful safety testing strategies into use. For one, the ICH S7B recommended that cardiovascular drug safety studies be conducted using conscious, stress free animals to reduce stress-related artefacts. Secondly, there was the introduction of *in vitro* electrophysiological testing, commonly used early in the lead optimisation phase to screen for compounds with the potential to interact with hERG (Guth, 2007). Though the Japanese and ICH guidelines have their respective attributes and advantages to good safety pharmacology, they should be used as they were intended, as general guidelines. Indeed, safety pharmacology studies need to be tailored to the needs of the specific drug in development.

2.1.4 The role of safety pharmacology in drug development

The results of safety pharmacology studies, together with multiple dosing toxicology studies make up the preclinical safety assessment required to qualify a new drug for clinical testing. This

includes not only the active ingredient, but all vehicles and excipients needed to improve bioavailability. Safety pharmacology outcomes can be included into toxicology studies to some extent, but some potential adverse effects of a compound are best detectable by safety pharmacology studies but may not be detected using conventional toxicity studies (Luft & Bode, 2002). A rationale for performing safety pharmacology studies early in drug development is to terminate the early preclinical development of drugs that have unwanted adverse effects. The sooner the development of such compounds is terminated, the sooner alternative drug candidates can be identified. Most of these resources are spent in identifying drugs that are safe for human use, and "not of bringing drugs to the market, but in stopping drugs going to the market." (Pugsley *et al*., 2008). Therefore, safety pharmacology studies are intended to be carried out prior to phase 1 clinical trials in order to prevent any unwanted or even potentially life-threatening adverse effects of drugs. Preclinical safety pharmacology studies serve a range of purposes, but identifying compounds with adverse physiological effects helps reduce the risk of failure later in the drug discovery process (Redfern *et al*., 2002). This is supported by an evaluation of the reasons for drug attrition. The lack of drug safety accounted for about 30% of all failed drug development programs (Hornberg *et al*., 2014). A survey of 12 pharmaceutical companies including data from 150 potential new drugs found adverse effects in clinical trials in 82 cases leading to termination of their development programs. These adverse effects included liver toxicity, and adverse effects on the cardiovascular and the central nervous system (Hornberg *et al*., 2014; Olson *et al.,* 2000). In other studies, it was shown that cardiovascular safety and liver toxicity were the main causes of the termination of ~75% of the drugs that were withdrawn from the US market during 1975–2007, and over half of the 21 drugs terminated between 1991–2007 (see Figure 1) (Hornberg *et al*., 2014; Stevens & Baker, 2009). From these studies it may be concluded that one of the physiological systems most frequently affected adversely is the cardiovascular system (Bass et al., 2004).

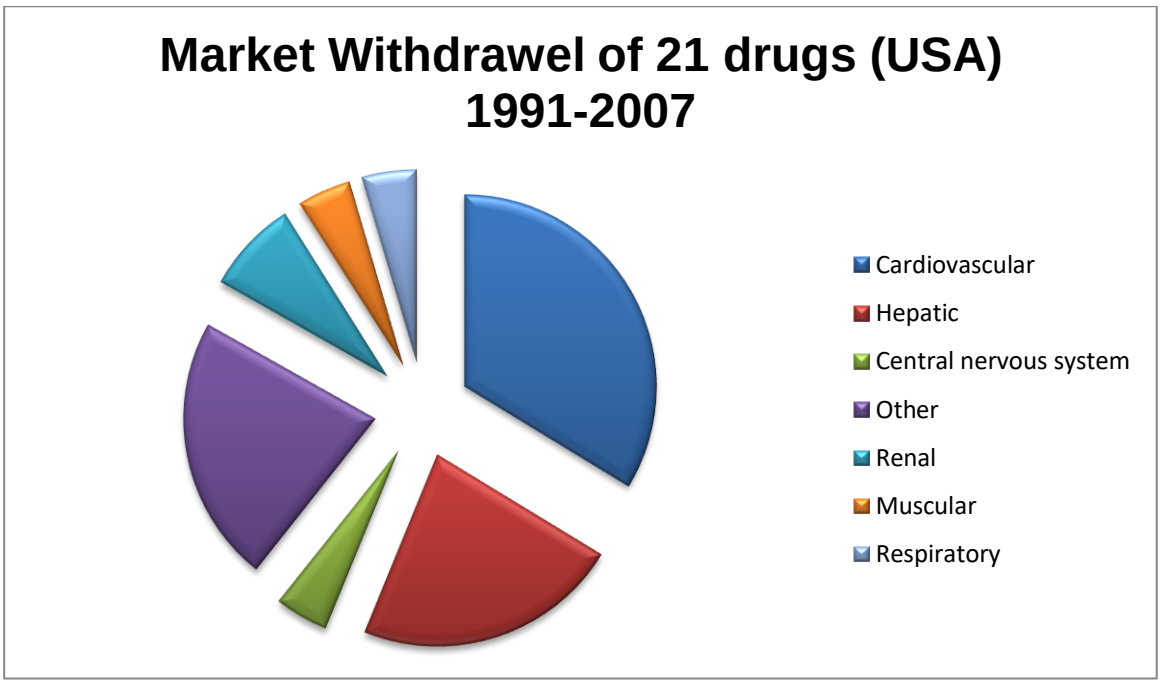

Figure 1: Physiological systems responsible for drug attrition based on adverse drug events (Adapted from Hornberg *et al*., 2014)

2.2 Cardiovascular telemetry

2.2.1 Introduction

The quality of physiological data is superior when the animal subjects are unstressed. This would also provide a superior baseline to which one could compare when introducing certain experimental conditions or events. Furthermore, the reduction of pain and distress promotes animal welfare and good science (Morton *et al.,* 2003). This is supported by the ICH S7A, and resulted in the use of appropriate test systems including the modified Irwin test, open field technology, whole-body plethysmography and telemetry systems for cardiovascular studies in dogs, pigs, and small rodents (Guth, 2007; Kinter & Valentin, 2002).

The evaluation of cardiovascular parameters using a telemetry-based approach refers to the remote detection and measurement of parameters such as heart rate, blood pressure or body temperature through the use of radio transmission of the data (Guth, 2007)

However, implantable telemetry is not the only means of collecting physiological data. Other, more non-invasive methods exist to measure cardiovascular parameters such as tail-cuffs for blood pressure and surface electrodes for ECG parameters (Kramer *et al*., 2001). These methods do, however, require that the animal be restrained which is associated with increased heart rate and blood pressure (Irvine *et al*, 1997).

2.2.2 Wireless telemetry

The pursuit of a stress-free, unrestrained and free-moving animal model with reduced human-animal intervention, lower risk of infection and higher quality data was the motivation for the development of a wireless telemetry system.

The very first concepts leading to cardiovascular telemetry as we know it today arose in the late 1950's when Dean Franklin designed the ultrasonic Doppler flowmeter, a device which harnessed the concept of the Doppler Effect to quantify blood flow based on backscattering signals from flowing red blood cells. On July 1, 1960, this device was successfully used to measure blood flow in the abdominal aorta of a dog (Sarazan & Schweitz, 2009). Three years later, with the help of Dr Robert van Citters, Franklin designed a telemetered Doppler Flowmeter, which they used at their San Diego hospital laboratory to record blood flow from a Boxer dog exercising outside the building, while Franklin received the signals on the second floor. While implantable pressure transducers were used at the time for the measurement of blood pressure, Van Citters and Franklin were adamant to investigate the possibility of a telemetered fully implantable pressure transducer system. With the help of Eph Konigsberg, they developed miniaturized implantable blood pressure gauges, which were later aptly named Konigsberg pressure transducers (Sarazan & Schweitz, 2009). These devices were used in their research when van Citters and Franklin instrumented baboons and giraffes in Kenya, Africa in 1965. In 1981, an electrical engineer by the name of Brian Brockway (founder of Data Sciences, 1984) developed a miniaturized, fully implantable telemetry pressure device for conscious rats based on the principles used in Konigsberg transducers (Brockway *et al*., 1991; Sarazan & Schweitz, 2009). This rat model required no restraint to measure arterial blood pressure, systolic blood pressure, diastolic blood pressure and heart rate. It was the technological innovation by Franklin and Citters that laid the foundation for the development of fully implantable cardiovascular telemetry devices for the measurement of physiological parameters as we know them today.

Although wireless telemetry systems have existed for almost over 50 years, it was only recently that commercially affordable and reliable implantable telemetry products have been developed and introduced into the market by various companies such as Data Sciences International (DSI), St. Paul, MN 55126; Biomedic Data Systems (BMDS), Seaford, DE 19973 and Star Medical, Arakawa-ku, Tokyo 116, Japan (Delaunois *et al*., 2009; Kramer & Kinter, 2003).

Kramer & Kinter (2003) best describe the implantable telemetry system as a combination of implantable airtight pressure transducers, a power source and a transmitter that detects changes in biological parameters within a conscious animal and transmits them using a radio frequency to an external remote receiver. The implantation of the transmitter requires a high level of surgical

skill and it is attached into the peritoneal cavity. The positive and negative electrodes for the ECG are affixed subcutaneously (Kramer *et al*, 1993 in the Lead II configuration of the standard limb-lead system, that is, the negative electrode at the right shoulder and the positive electrode near the lower left chest (Kharidia & Eddington, 1996). This configuration is reported to have fewer artefacts due to abdominal muscle activity, and it offers a strong signal of the QRS complex (Kramer *et al*., 2001). For the measurement of blood pressure, a gel-filled catheter is inserted into the abdominal aorta, but it could also be inserted into the carotid or femoral artery (Kramer & Kinter, 2003). The remote receiver unit, including an antenna which is typically placed near or inside the animal's cage, collects and digitizes the ECG and blood pressure signals and sends them to a data acquisition system which, in turn, can be used to evaluate the data as desired by the user (Kinter & Johnson, 1999).

Implanting a transmitter into an animal is an invasive procedure. Possible pain or discomfort can be adequately dealt with given the correct procedures for anaesthesia and analgesia. The advantages of implantable telemetry far outweigh disadvantages. The advantages associated with implantable telemetry are listed in Table 1 below.

Table 1: Benefits associated with telemetry-based data acquisition

Benefits of implantable telemetry
Higher quality data (less variability) allows for the use of fewer animals (refinement).
Time-dependent changes in parameters can be documented due to being able to collect data for longer periods
The instrumented animals are allowed natural movement in their home cage without physical restraint.
The levels of stress are reduced since the animals are not tethered to any external measurement apparatuses.

(Adapted from Morton *et al*., (2003))

2.3 The pharmacology of reference compounds

2.3.1 Introduction

Kramer & Kinter (2003) describes various points to consider when validating an implantable telemetry system. This includes the calibration of the sensor, confirmation that the signals are appropriately received by the data acquisition system and verifying that the data processing programs is functioning in accordance with the user specifications. The telemetry system may also be validated by verification of the expected physiological responses to the administration of acute pharmacological agents with known physiological effects. In the following section, the

pharmacological action of two reference compounds is discussed. These compounds are theophylline and clonidine.

2.3.2 Theophylline

In 1886, Henry Hyde Salter, an asthmatic, noted that after a strong cup of coffee, his asthma was improved. This was later attributed to the caffeine, a weak phosphodiesterase (PDE) inhibitor, in his coffee which had bronchodilating effects (Boswell-Smith *et al*., 2006). Theophylline (CAS ID: 58-55-9), an analogue of caffeine, is a non-selective PDE inhibitor that produces vasodilation of the systemic circulation, increases heart rate and increases cardiac contractility (Delaunois *et al*., 2009). Theophylline's bronchodilating effects were first described in the early 1920's and it was widely used for acute asthmatic symptoms during the 1930's (Weinberger, 1984). It is probably best known for its stimulatory effects on respiratory function and has been used to treat apnoea and chronic obstructive pulmonary diseases for over 6 decades.

2.3.2.1 Pharmacology and physiological effects of theophylline

Theophylline is a strong PDE inhibitor, therefore, to better understand its mechanism of action, the pharmacology of PDE inhibition is of relevance. During the activation of the sympathetic nervous system, catecholamines (norepinephrine and epinephrine) are released and bind to adrenoceptors found on outer cell membranes; beta1-adrenoceptors are located specifically in the heart. These receptors are coupled to membrane bound Gs-proteins that activate the enzyme adenylyl cyclase to form cAMP from intracellular adenosine triphosphate (ATP). The increase of cAMP is then responsible for the increase of heart contractility and heart rate (Lezoualc'h *et al*., 2016). PDEs inhibit the action of cAMP and are widely expressed in the airways, vasculature and heart and are crucial regulators of cAMP-mediated responses (Boswell-Smith *et al*., 2006). This would suggest that the inhibition of PDEs in the heart would prevent cAMP breakdown and therefore increase cardiac contractility and heart rate by elevating cAMP levels. The latter is supported by the findings of Sun *et al* (2007) who reported a significantly higher heart rate of PDE knockout (KO) mice compared to the heart rate of wild type (WT) mice.

In the blood vessels, cAMP plays a different role. The elevation of intracellular cAMP causes the relaxation of smooth muscle. This is due to the fact that myosin light chain kinase is directly inhibited by the release of cAMP. Myosin light chain kinase is the enzyme responsible for the phosphorylation of myosin and leads to contraction of smooth muscle. Therefore, the inhibition of PDE would further inhibit myosin light chain kinase and decrease smooth muscle contractility and produce vasodilation (Levick, 2003).

From what is known of PDE inhibitors and their effects on cardiac and vascular function, theophylline mimics the effects of β-agonists and would be expected to increase heart rate, cause vasodilation and increase myocardial contractility, making it a suitable compound to use as a reference drug.

2.3.3 Clonidine

The potent hypotensive effects of clonidine (CAS ID: 4205-90-7) in humans has made it a common treatment of patients with hypertension (Conway & Jarrot, 1980; Reid, 1981). Apart from clonidine's ability to lower blood pressure, it is has strong sedative effects due to its interaction with α-adrenoceptors located in the central nervous system.

2.3.3.1 Pharmacology and physiological effects of clonidine

Alfa-2 receptors (α_2), expressed both centrally and peripherally play crucial roles in regulating the transfer of information through a negative feedback mechanism mediated specifically by the neurotransmitter, norepinephrine (Langer, 2015). The α_2-receptor's inhibitory effect on norepinephrine release is credited to the inhibition of Ca^{2+} entry through calcium channels, which is also dependent on G-protein activation (Langer, 2008). The α_2-receptors can be found in a variety of places, among others in the blood vessels and the sympathetic terminals where they play crucial roles in the regulation of both the cardiovascular and the autonomic nervous systems by mediating vasoconstriction and inhibition of norepinephrine release. These receptors are also found in the central nervous system where their activation leads to sedation, reduced sympathetic outflow and an increase in cardiac vagal activity, which in turn leads to a decrease in cardiac output and heart rate (Ebert *et al*., 2000).

Clonidine, a potent α_2-adrenoceptor agonist, which acts both post- and presynaptically produces bradycardia and hypotension by reducing central and peripheral sympathetic activity and inhibit peripheral norepinephrine release via peripheral presynaptic receptors (Langer, 2008). After the inhibition and immediate decrease of the sympathetic nervous system, clonidine induces a brief increase in blood pressure due to the activation of the α_2-adrenoceptors, but this is short-lived, as the reduction of sympathetic tone leads to a decrease in blood pressure and heart rate (Khan *et al*., 1999). Another physiological effect of the α-sympathomimetic-drug clonidine is a decrease in body temperature (Tsoucaris-Kupfer & Schmitt, 1972). This is also thought to be due to the inhibitory effects that clonidine has on the release of norepinephrine, which is involved in thermoregulation (Ozawa *et al*., 1977).

From what is understood of clonidine's complex pharmacology, one would expect to see bradycardia, hypotension and hypothermia. These effects are more or less the opposite from

what one would expect from theophylline (increased heart rate), which makes both clonidine and theophylline interesting compounds to validate the sensitivity of the telemetry system.

2.4 The open-field test

2.4.1 Introduction

When assessing the effects of a compound on the central nervous system, the ICH S7A recommends evaluating motor coordination, behavioural changes and sensory reflexes, among others. For the evaluation of these endpoints, the most common functional observation tests available are the modified Irwin's test and the open-field test. In this study an open field test was used.

2.4.2 Behavioural parameters

The origin of the open-field test was in the 1930's with Calvin Hall, who used the defecation pattern of the animals as a measure of anxiety (Walsh & Cummins, 1976). Open-field tests have been expanded to include overall movement, rearing frequency, lethargy, time spent without movement, etc. For the assessment of behavioural effects, distance travelled is often used as it can be quantified easily. These behavioural traits are often a response to certain introduced variables, such as the introduction of foreign objects to the field, transference of the animal to the field, or exposure to a pharmacological test compound. The quantification of the aforementioned parameters gives an indication of whether a compound would induce an exploratory behaviour, such as a psychostimulant, or decrease activity (i.e. a sedative effect).

2.4.3 Open-field apparatus

The most common open-field system used is typically box-shaped, although circular shaped arenas were also used in the past. The field is crossed by photoelectric beams, which, when interrupted by the rat's movement, registers the movement of the animal as well as the frequency of rearing (Castagné *et al*., 2013). However, the use of such specialised cages is not always necessary, as most open field cages are simple boxes, with video cameras mounted above the floor of the arena. The captured videos are then analysed by video tracking software, which records movement parameters. The relative ease of this method is due to the fact that it is fully automated and allows for a sensitive analysis.

2.5 The Pheroid® drug delivery system

2.5.1 Introduction to drug delivery systems

Drug delivery systems may be used to enhance one or more of a drug's properties, including solubility, stability, specificity, efficacy, tissue targeting or even the controlled release of therapeutic compounds within a biological system (Vonarbourg *et al*., 2006). Ideally, a delivery system should be biocompatible, biodegradable, stable with an appropriate shelf-life, the incorporation and release of the drug should be controlled and, most importantly, it should be safe (De Jong & Borm, 2008; Lian & Ho, 2000). Drug delivery systems have been able to impact the field of oncology, immunology, cardiology, and endocrinology. There are many drug delivery systems patented and used commercially such as lipids, polymers and even drug releasing implants. The systems most frequently used commercially are colloids and consist commonly of micro-emulsions, liposomes, noisomes or nanoparticles (Grobler, 2009; Kreuter, 2014)

2.5.2 The Pheroid® delivery system

The Pheroid® delivery system is an emulsion of stable, lipid-based submicron structures called Pheroids. Like other colloids, Pheroid® consists of a dispersion medium (continuous phase) and a dispersed phase. The latter can be customized in terms of morphology, size, structure and function to fit the needs of the user (Grobler, 2009). The system, which incorporates the ethyl esters of essential fatty acids emulsified in nitrous oxide (N_2O) water, has been shown to successfully entrap both hydrophilic and hydrophobic compounds (Du Plessis *et al*., 2010; Steyn *et al*., 2011). The system has been shown to be stable for at least two years (Slabbert *et al*., 2011).

Based on various studies, Pheroid® is able to enhance the absorption, efficacy or targeted delivery of its entrapped active ingredients. Thereby, it is able to enhance the therapeutic action of a compound by improving the control of size, charge and hydrophilic-lipophilic characteristics of pharmacologically active compounds (Grobler, 2009; Slabbert *et al*., 2011; Steyn *et al*., 2011).

2.5.3 Structural characteristics and functions of the Pheroid® system

The Pheroid® structures can be customized to accommodate the physical properties of a given compound, which led to the development of different types of Pheroid® (Grobler, 2009).

The Pheroid® can specifically be customized into three different structures:

- Lipid-bilayer vesicles
- Micro-sponges

- Depots/reservoirs

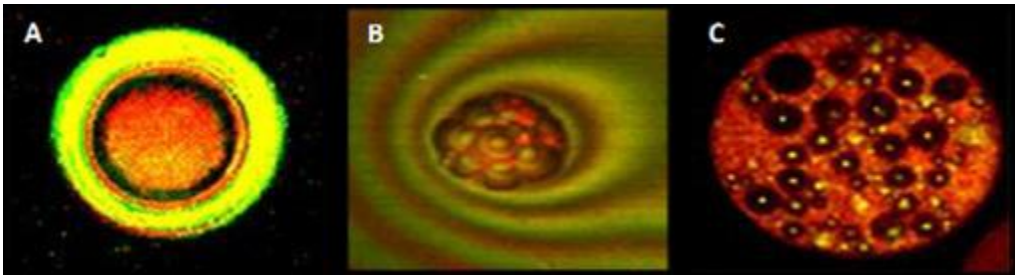

Figure 2: Confocal laser scanning micrographs of different Pheroid® formulations A) Vesicle, B) pro-Pheroid®, C) micro-sponge (Reprinted from Grobler (2009) with permission from the author)

Much like liposomes, Pheroid® vesicles also have lipid bilayers, but they do not contain cholesterol or phospholipids. Another unique component to the Pheroid® vesicles is the N_2O gas phase associated with the fatty acid dispersed phase. The N_2O gas is both water- and fat-soluble and it provides a unique advantage in that its presence allows free lateral movement of hydrophobic and hydrophilic compounds in the cell membrane (Grobler, 2009).

As shown in Figure 2 above, the micro-sponges are literally sponge-like structures, 0.5–5 nm in size and porous in nature which is particularly suitable for the delivery and transport of lipid-soluble compounds. These entities are also capable of supporting combination therapies, in which one compound may be entrapped within the interior volume, and the other inside the sponge like surfaces (Grobler, 2009; Uys, 2006).

The sizes of reservoirs, or depots, depends on the amount of pro-Pheroid® present in the formulation. The pro-Pheroid® is a precursor of the Pheroid® that excludes a water phase. This makes it an especially useful carrier for molecules that are unstable in aqueous environments. The principle of pro-Pheroid® relies on the fact that Pheroid® vesicles form upon contact with an aqueous media, be it an external source or intestinal fluid. If any active pharmaceutical ingredient (API) were to be present during this process, they would be entrapped within the newly formed Pheroid® vesicles (Grobler, 2009; Grobler *et al.*, 2014). The difference between Pheroid® and pro-Pheroid® in composition is shown graphically in Figure 3.

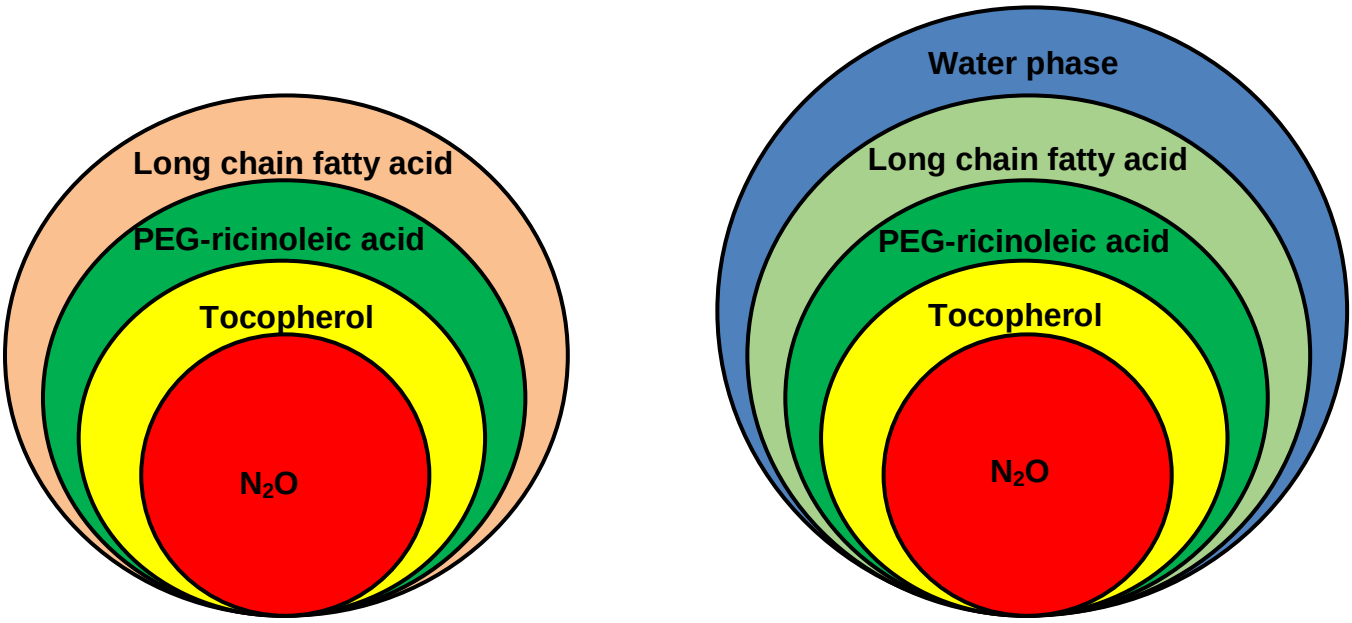

Figure 3: Graphic illustration of the difference between pro-Pheroid® (left) and Pheroid® (right) (Adapted from Grobler (2009) with permission from the author)

2.5.4 Components of Pheroid®

The Pheroid® is composed of mainly three phases, aqueous, oil and gas (N_2O).

The aqueous phase of the Pheroid® system is usually sterile water. The oil phase is composed of omega-3 and omega-6 fatty acids.

2.5.4.1 Essential fatty acid

The human body is incapable of producing essential fatty acids and so we have evolved to obtain them from our diet. However, Western diets have been shown to be deficient in certain fatty acids, which may have contributed to the pathogenesis of various diseases such as cardiovascular and autoimmune diseases as well as cancer (Simopoulos, 2002). The Pheroid® incorporates vitamin F ethyl ester in its composition; an essential fatty acid comprised of ethylated polyunsaturated fatty acids like omega-6 (linoleic acid), omega-3 (α-linolenic acid) and oleic acid but excludes arachidonic acid (AA). These fatty acids are specifically formulated in the *cis*-formation to be biocompatible with the fatty acids typically found in the human body (Grobler, 2009). These essential fatty acids generally serve to maintain energy homeostasis and membrane integrity within the cell, regulation of the body's immune response through the use of prostaglandins or leukotrins and to some extent, modulation of apoptosis (Grobler, 2009).

2.5.4.2 Kolliphor

Surfactants are stabilizing agents used during the production of lipid-based, water-insoluble delivery systems such as nano-emulsions and nano-particles (Raina *et al*., 2015). They act by lowering the surface tension between the dispersed phase (in this case, Pheroid®) and the continuous phase which allows the stable formation of the nanostructure. This is achieved via their amphiphilic nature that interacts both in a lipophilic manner with the lipid nano-structures and in a hydrophilic manner with the aqueous dispersion medium (Leonardi *et al.,* 2014). Both Kolliphor EL® and Kolliphor RH-40® (formerly known as Cremaphor) are non-ionic surfactants which are used during the formulation of Pheroid®. They are generally used in the pharmaceutical industry in conjunction with formulations of lipid-based delivery systems such as Pheroid® to increase their bioavailability (Berthelsen *et al*., 2015). Kolliphor RH-40® and Kolliphor EL® are formulated by combining castor oil and ethylene oxide in different ratios; however, the castor oil used for Kolliphor RH-40® is hydrogenated while that of Kolliphor EL® is not (Berthelsen *et al*., 2015).

2.5.4.3 DL-α-Tocopherol

DL-α-Tocopherol, also known as Vitamin E, is a lipid soluble antioxidant used in Pheroid® that plays a key role in neutralizing free radicals produced by any oxidation that may occur within the system (Buettner, 1993). It is classified as a “chain-breaking” antioxidant as it directly restores oxidizing radicals associated with the chain reaction that leads to membrane lipid damage (Buettner, 1993; Sano *et al*., 1997).

2.5.4.4 Nitrous oxide (N_2O)

A unique aspect of the Pheroid® system is the incorporation of nitrous oxide (N_2O), which is distributed throughout both the continuous- (aqueous) and dispersed (lipid) phase. The incorporation of N_2O into the system is known to serve at least three functions:

- It ensures the miscibility of the fatty acids dispersed throughout the dispersal medium (Grobler, 2009).
- It improves the stability of the formed vesicle Pheroid® structures (Grobler, 2009; Uys, 2006).
- It contributes to the assembly process of the Pheroids (Uys, 2006).

Nitrous oxide is used as a supplement to anaesthetics in general medical procedures, but also as an analgesic together with local anaesthesia during obstetric and dental procedures (Barr *et al*.,

1999). N_2O is able to move freely through the epidermal and dermal layers of the skin due to its hydrophilic and lipophilic nature. According to the Meyer-Overton rule, the potency of an anaesthetic is directly related to lipid solubility (Einarsdottir & Caughey, 1988). This explains the accumulation of N_2O at the lipid-rich membranes leading to an increase in cell membrane fluidity (Grobler, 2009).

In a comprehensive toxicity profiling of Pheroid® in Sprague-Dawley rats and BALB/c mice by Kleynhans (2018), it was concluded that Pheroid® showed no indication of toxicity following intravenous and oral administrations of both acute and subchronic dosing. The maximum dose of Pheroid® administered to both rodent models were 2000 mg/kg, while that of pro-Pheroid® was 50 mg/kg. Furthermore, pro-Pheroid® did not harbour any mutagenic effects following the AMES test for mutagenicity, while Pheroid®, along with its constituents, demonstrated no mutagenicity in the presence of cytochrome P450 enzymes and therefore harboured no effect on the structural integrity of cellular DNA. (Kleynhans, 2018).

2.5.5 Pharmaceutically suitable features of Pheroid®

Various studies on the Pheroid® system have concluded that it offers a variety of characteristics that contributes to its attractiveness for use as a pharmaceutical delivery system. A few of these attributes are discussed in the following section.

2.5.5.1 Rapid onset of action

Research on the Pheroid® system has shown that it has the ability to rapidly cross physiological barriers while carrying an API. As a result, the API is delivered much faster to its site of action, leading to an increase in time of action and inherently, faster relief of symptoms (Grobler, 2009).

2.5.5.2 Increased bioavailability of active compounds

Pheroid has been shown to increase the bioavailability of therapeutic agents. For example, in a study by Matthee (2007) to investigate the possible increase in efficacy of anti-tuberculosis drugs entrapped in Pheroid® in CD57 inbred mice, only 60% of the prescribed dosage of rifampicin was entrapped in pro-Pheroid® but this still yielded a 205% increase in bioavailability compared to the 100% dosage of the commercial.

2.5.5.3 Increase in therapeutic efficacy

A study was also performed in humans with the entrapment of rifampicin in Pheroid®. This resulted in a 60% increase in therapeutic efficacy in comparison to a standard formulation (Grobler, 2009). In an *in vitro* study by Langley (2007), it was investigated whether Pheroid®

would increase the efficacy of various antimalarial drugs. The study concluded that after the entrapment of mefloquine, artemether and artesunate in Pheroid®, the efficacy was enhanced by 314%, 254% and 238% respectively (Langley, 2007). Increases in the efficacy of active compounds might allow for a decreased dosage or dosing frequency and thus, improve patient compliance. This also could lead to the reduction in unwanted side effects, without reducing the potency of the administered compound (Grobler, 2009).

2.5.5.4 Reduced cytotoxicity

Some drugs may have toxicity due to cellular damage. The Pheroid®'s composition is part of the natural biochemical pathway, and is therefore thought to be non-toxic. It has the ability to reduce the side effects caused by its entrapped active compounds such as the membrane damage caused by miconazole nitrate (Grobler, 2009).

2.5.5.5 Alleviation of immune responses

Many new drugs are peptides or proteins. However, these molecules may initiate an immune response from the body. Since the fatty acids of the Pheroid® system are found frequently in the modern diet, it does not induce an immune response. Additionally, the Pheroid® has the ability to mask the entrapped proteins or peptides from the body's immune system, thus reducing an immune response that may be initiated (Grobler, 2009).

2.5.5.6 Reduced minimum inhibitory concentrations (MIC)

The minimum inhibitory concentration (MIC) can be defined as the lowest possible concentration of an antimicrobial, which will inhibit the visible growth of a bacterium or microorganism after an overnight incubation period (Andrews, 2001). In the study by Langley (2007), the MIC of antimalarial drugs, especially chloroquine, was reduced dramatically after their entrapment in Pheroid®. The reduction in MIC could enable the use of less active compounds while still achieving therapeutic efficacy. This in turn reduces treatment and manufacturing costs and potential side effects.

While Pheroid® boasts of a range of attractive pharmaceutical attributes, it needs to meet certain quality criteria to ensure its efficacy. The characterisation of Pheroid® and their related analytical techniques are described in the next section.

2.5.6 Pheroid® characterization

In order to comply with quality control regulations, the Pheroid® and pro-Pheroid® formulations need to be thoroughly characterised after their preparation. This is done using confocal laser scanning microscopy, particle size distribution and zeta-potential measurement.

2.5.6.1 Confocal laser scanning microscopy of the Pheroid® vesicles

The morphological characteristics of the Pheroid® vesicles, as well as their size, are analysed by confocal laser scanning microscopy (CLSM). A CLSM is comprised of an optical system, a scanning device, a light source (laser) and a detection system. CLSM has several advantages over other microscopic techniques, including a reduction of artefacts by elimination of background information from the focal plane using a spatial pinhole and better depth-of-field control (White *et* al., 1987). The latter allows the creation of high quality three-dimensional images by capturing two-dimensional images of the fluorescence-emitting sample at different focal depths.

One of the features of CLSM that proves useful for drug delivery system analysis is its ability to determine entrapment efficacy (EE) by confirmation of an entrapped compound or entity within the internal structure of the particle. The EE can be defined as the percentage of the compound in the formulation that ends up entrapped within the vesicles (Song *et al*., 2008).

Pheroid® vesicles possess a lipid bilayer, which makes Nile Red, a lipophilic, vermeil phenoxazine dye, a good choice for fluorescent labelling through its interaction with the lipid molecules of the Pheroid® formulations. When Nile Red is bound to the lipid bilayer, the lipid bilayer of the Pheroid® fluoresces with a red colour. The aqueous core of the vesicle is not bound to Nile red and therefore appears dark. The Nile red fluorophore marker has an excitation/emission wavelength of 515–560/ >590 nm (Greenspan *et al*., 1985) while the CLSM is equipped with three lasers, with excitation wavelengths of 405, 488 and 543 nm, respectively. The fluorescent light emissions are therefore collected for three different wavelength bands, above 650 nm, 540–640 nm, and 485-–545 nm (du Plessis *et al.,* 2010). These three images are then combined to provide an indication of whether the morphology of the Pheroid® structures meets the standard requirements, that is, an adequate size ranging from 200 nm to 2 µm.

2.5.6.2 Zeta potential measurement of the Pheroid® vesicles

The zeta potential can be defined as the quantitative difference in electrical charge on the surface of a particle and its surrounding environment or the solution in which it is suspended (Hunter, 2013). All lipid-based systems possess a charge on their surface that interacts with the environment, contributing to the stability of its structure. The stability of an emulsion is a crucial

parameter that needs to be taken into account, especially when active compounds are entrapped and transported within the particles. The Zeta potential is measured with the Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). A particle is considered stable if its zeta potential is high, defined as being greater than ±25 mV, where the repulsive forces exceed the attractive forces (Crooke, 2007; Roland *et al*., 2003). Therefore, if the attractive forces exceed the repulsive forces, and the zeta potential is low (0–±25 mV), the system becomes unstable and the particles aggregate.

2.5.6.3 Particle size and particle size distribution of the Pheroid® vesicles

The stability of an emulsion can be characterised by the rate at which the particles coalesce or aggregate within the emulsion. According to Stoke's law, the square of a particle's diameter is directly proportional to the velocity at which the particle moves within the emulsion (Robins, 2000). The size and distribution of these particles can give a quantitative indication of the rate at which the emulsion aggregates. Therefore, by reducing the size of the droplets, the stability of the emulsion can be increased by reducing particle aggregation and gravitational separation (Huang *et al*., 2001; McClements, 2015). Particle size and distribution is calculated on the basis that particles of different size have a distinctive light scattering pattern when a laser beam focuses on the particle. The scattered light is indirectly proportional to the size of the particle, i.e. the greater the angle at which the light refracts, the smaller the particle (Sochan *et al*., 2012). In a number of studies using poorly soluble drugs, it was shown that a reduction in particle size would lead to an increase in both dissolution rates and bioavailability (Liversidge & Cundy, 1995). The particle size is therefore a crucial parameter to ensure quality and function. The Pheroid® vesicles can range from 200 nm to 2 µm, but the size of the Pheroid® vesicles decrease when the mixing rate during the emulsification process is increased (Grobler, 2009; Uys, 2006). This allows a more controlled formulation of Pheroid® particle size. The particle size and particle size distribution of the Pheroid® vesicles are calculated using the Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom).

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CHAPTER 3: ESTABLISHMENT AND STANDARDISATION OF THE TELEMETRY-BASED CARDIOVASCULAR TESTING SYSTEM IN THE RAT

3.1 Introduction

The use of stress-free, undisturbed animals improves the quality of measured physiological data due to a reduction in the variability of measurements. The implementation of a fully implantable cardiovascular telemetry system has allowed the use of unrestrained animals to measure cardiovascular parameters including as arterial blood pressure (BP), heart rate (HR), and ECG parameters (such as PR and QT intervals) as well as the core body temperature (BT) (Popova *et al*., 2017). The North-West University (NWU) has recently acquired such a telemetry system (Data Sciences International DSI, St. Paul, MN 55126) for the purpose of identifying possible adverse drug effects during preclinical studies.

After it had been established, this system was validated to determine its ability to measure changes in the above-mentioned cardiovascular parameters. Six rats surgically implanted with telemetry transmitters (HD-S11) were administered single doses of two reference compounds (theophylline and clonidine) in a three-way crossover design. These reference compounds induce known effects on the cardiovascular function of rats. Theophylline, at a dose of 30 mg/kg oral (p.o.), is a bronchorelaxant and vasodilator which induces changes in cardiovascular function including an increase in HR, BP and BT. Clonidine, at a dose of 0.3 mg/kg intraperitoneal (i.p.), is an antihypertensive drug and its hypotensive action is mediated by its interaction with α-adrenoceptors. The decrease in blood pressure is accompanied by a fall in HR (Conway & Jarrot, 1980).

3.2 Materials

All specialised surgical equipment was kindly donated by Boehringer Ingelheim Pharma GmbH & Co. KG. The telemetry transmitters (HD-S11), receivers and base stations were purchased from Data Sciences International (DSI), St. Paul, MN 55126. The reference compounds, theophylline (CAS: 58-55-9) and clonidine (CAS: 4205-91-8), were purchased from Sigma Aldrich South Africa.

3.3 Experimental design and animals

3.3.1 Experimental animals

Eight male Sprague-Dawley rats (8-9 weeks old, body weight approximately 370 g, bred at the PCDDP, NWU, Potchefstroom) were instrumented in order to have two extra rats, should anything go wrong during or post-surgery. In other studies using the same strain of rat and the same telemetry system, it was shown that this animal number (N=6) is sufficient to detect biological changes in cardiovascular parameters (Delaunois *et al*., 2009). An ethics approval for the use of rats was obtained from the local authority (NWU-AnimCareREC: NWU-00346-15-A5).

3.3.2 Housing conditions

The rats were held in the NWU Vivarium and were fed a standard laboratory rodent diet with access to food and water *ad libitum*. The housing consisted of type II long individual ventilated cages (IVC) with corn-cob bedding. The IVC's underwent 60 air changes per hour through HEPA filters with 22 °C ± 1 °C and 55% ± 10% humidity. A day-night cycle of 12 hours was maintained using fluorescent tubes.

3.3.3 Experimental design and test compounds

Six rats were used for the validation study in a three-way crossover study design. Hence, each animal acted as its own control and sufficient time between treatments was ensured (~5 half-lives) to eliminate potential carryover effects. Theophylline has a half-life of 3.6 ± 0.9 hours in Wistar rats (Marin *et al*., 1986) whereas the half-life of clonidine in rats is 3 hours (Cho & Curry, 1969).

The rats received all three treatments in a volume of 200 µl, shown in Table 2 and Figure 4.

Table 2: Dosage regimen of reference and control compounds

Compound	Route of administration	Dosage
Control (Saline)	Oral (p.o.)	0 mg/kg
Theophylline	Oral (p.o.)	30 mg/kg
Clonidine	Intraperitoneal (i.p.)	0.3 mg/kg

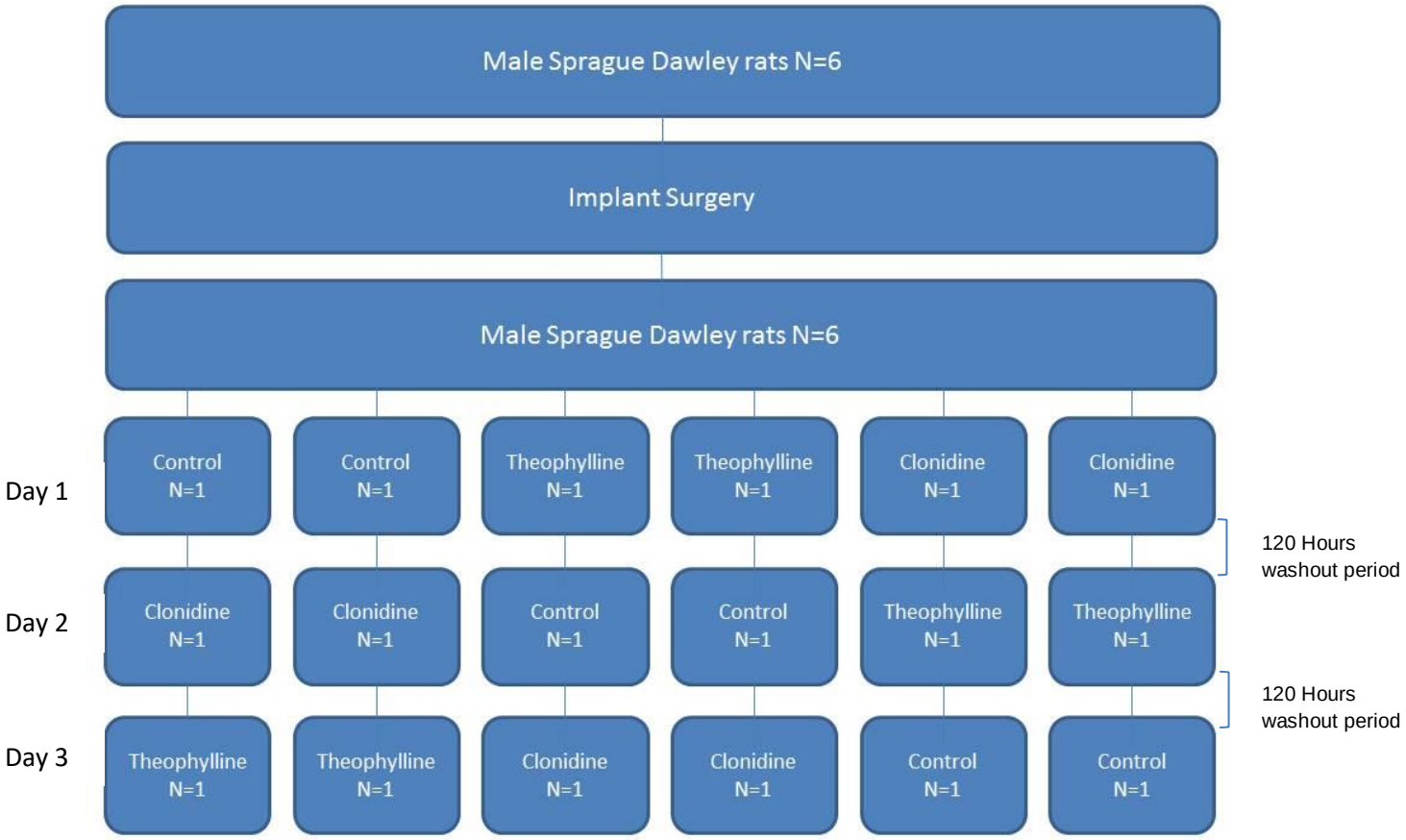

Figure 4: Experimental design of the telemetry validation study

During each of the experimental days, the rats were placed onto the receivers while still inside their individual home cages. Recording of pre-dose values was initiated as soon as each of the six transmitters was switched on using a magnet and the radio transmission confirmed using a low AM radio frequency. The recording of the pre-dose data lasted 2 hours in order to obtain a stable baseline. During this baseline period, no one was allowed inside the laboratory, to ensure that the rats and the collected data were not disturbed.

3.4 Telemetric system

The telemetry system and its software (Ponemah Physiological Platform) were purchased from Data Sciences International (DSI), St. Paul, MN 55126. The system consisted of eight telemetry receivers (of which only six were used), an Ambient Pressure Reference (APR) (which compensates for changes in the atmospheric pressure by providing dynamic corrections), a data acquisition system (desktop computer) and a matrix which was used to manage communication between the implanted transmitters and the data acquisition system. This system was used to collect data on BP, ECG and BT. The different transmitters were each assigned to a specific telemetry receiver a week before they were surgically implanted into the rats. At this time, the

transmitters were switched on in order to check the stability of the signal and manually calibrated by ensuring the zero-offset value for barometric parameters was equal to zero. It was therefore important to know and record which transmitter was implanted in which animal, as each animal would have its own specific transmitter and receiver.

3.5 Anaesthesia and surgical procedure

3.5.1 Anaesthesia and analgesia

All surgical procedures were performed under aseptic conditions. The rats were kept under anaesthesia for the duration of the procedure and the surgeries were performed by a veterinary technician under supervision of the veterinarian of the NWU Vivarium. The rats were fasted for 12 hours prior to surgery but had access to water *ad libitum*. On the day of surgery, each rat was weighed and put into an inhalation cage 30 min prior to surgery and anaesthetised with 2.5% isoflurane with 1 l/min Oxygen flow. The respiration rate and reflexes of the rat were carefully observed and as soon as it was determined that the rat was adequately anaesthetised, its abdomen and throat were shaved. Thereafter, Metacam (1 mg/kg) and Temgesic (0.03 mg/kg) were administered subcutaneously to alleviate any pain. The transmitter was prepared by removing any air bubbles inside the catheters. The rat was then cleaned and sterilised with Kodan™ antiseptic spray and placed onto the surgical field, with its nose placed inside the inhalation facemask. The facemask and surgical field is illustrated in Figure 5 below.

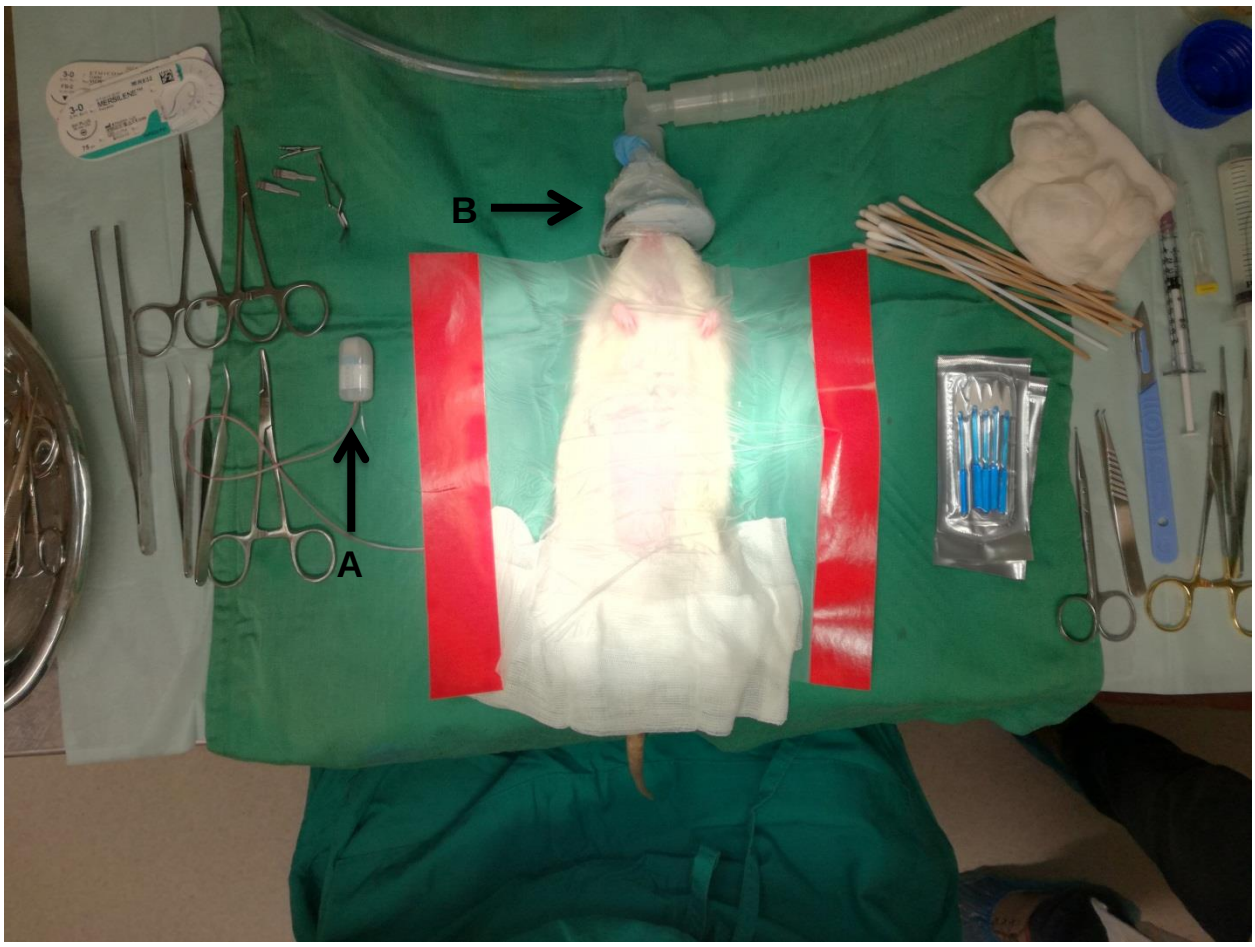

Figure 5: Surgical field and instruments used to perform anaesthesia: A) Telemetry transmitter; B) Airtight apparatus feeding 2.5% isoflurane and 1 L/min oxygen to the animal.

To prevent the animal's eyes from drying out during surgery, vitamin A ointment was applied to its eyes.

3.5.2 Cannulation of abdominal aortic artery

After a further confirmation of there being a surgical plane of anaesthesia, a 5-6 cm incision was made 0.5 cm below the sternum. The linea *alba* was opened with scissors and haemostats were used to open the abdomen. A gauze pad soaked in 0.9% NaCl solution (saline) was used to move the intestines to the side in order to locate the abdominal aorta. The aorta was then isolated using cotton-tipped swabs soaked in saline. The abdominal cavity was regularly dampened with warm saline to prevent the tissue from drying out.

After the aorta was isolated, a small haemostat was place on the aorta under the kidneys and another was placed at the aortic bifurcation. An arched cannula was used to puncture the aorta, directly followed by insertion of the catheter behind the cannula into the aorta. Tissue glue (one

drop) was used to close the vessel. The upper haemostat was then removed. If there was any blood loss from the aorta, additional tissue glue was added. When any bleeding had stopped, the second haemostat was removed. The gauze was removed from the abdominal cavity and the intestines were put back into place. The transmitter body was stitched to the rectus *abdominis* muscle layer using 75cm Mersilene® silk. The ECG leads were then tunnelled through the abdominal wall using a large cannula as shown in Figure 6 below.

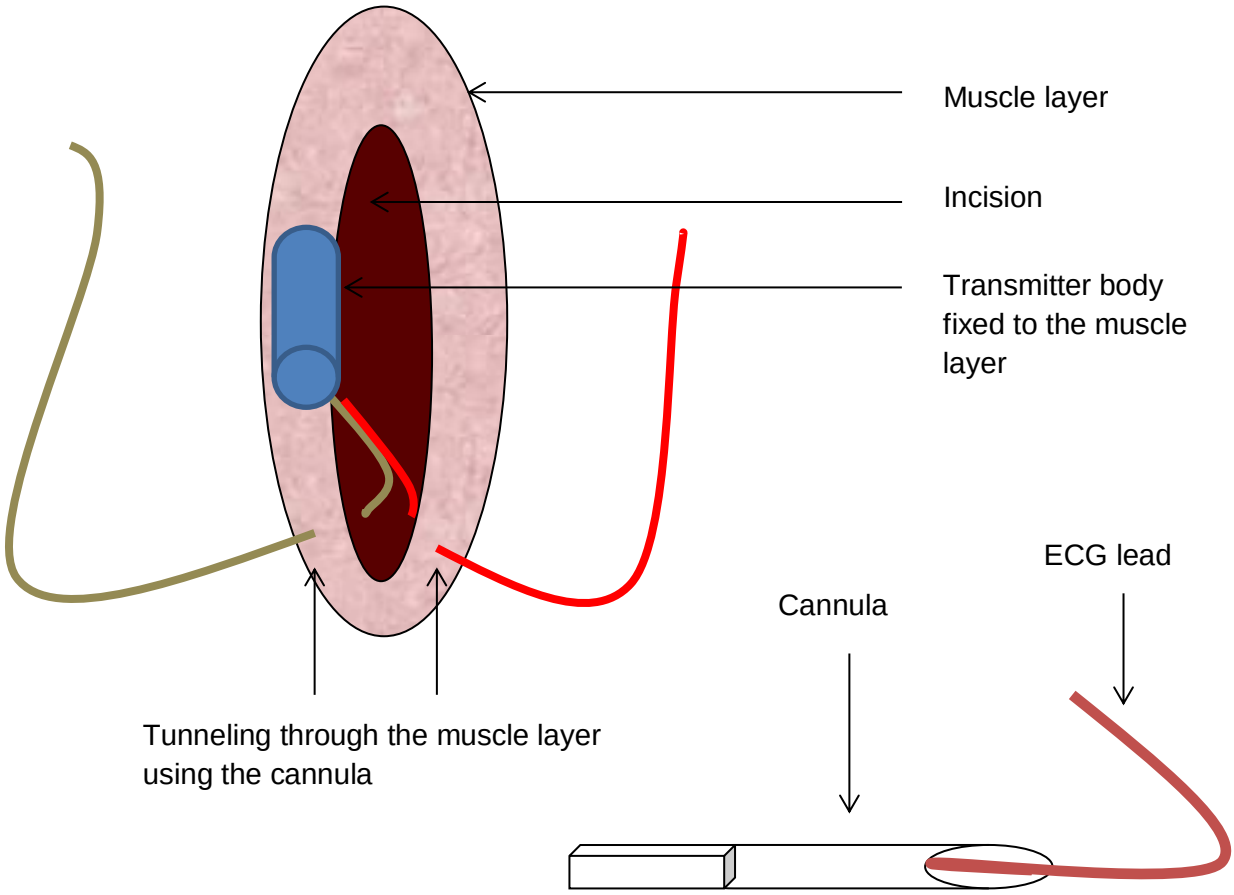

Figure 6: Cannulation of ECG leads through the abdominal muscle layer

After the ECG leads were tunnelled through the muscle layer with the cannula, the muscle layer was closed using 70 cm Vicryl® silk.

3.5.3 Attaching the ECG leads

In order to attach the ECG electrodes, a small incision was made into the connective tissue under the skin to reveal the muscle layer straight over the sternum. The red (positive) ECG lead was then tunnelled under the skin to the muscle layer over the sternum. A loop was created at the end of the ECG lead by removing 1 cm of insulation plastic with a scalpel and stretching the

uninsulated part up to 2 cm. After formation of the loop, it was closed using the 75 cm Mersilene® silk thread. Using a cannula, the looped ECG lead was tunnelled into the muscle layer over the sternum and attached to the muscle at the entrance of the incision with a single stitch. The rest of the cable was brought under the skin and closed. To attach the second (negative) ECG lead, a small incision was made in the skin over the trachea, revealing the muscle layer. The ECG lead was tunnelled to the trachea by using a tunnelling tube and after forming the same loop as the first ECG lead, it was attached over the trachea, bringing the muscle layer over the attached loop as shown in Figure 7.

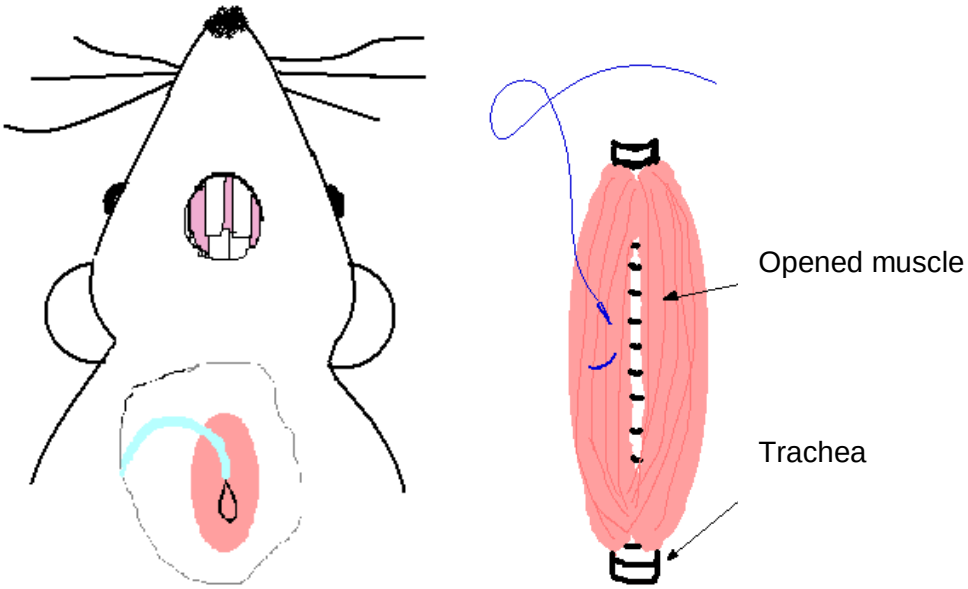

Figure 7: Attachment of ECG loop to the trachea

After the ECG leads were affixed to the respective throat and abdominal regions, the skin surrounding the incisions were closed using 45 cm Vicryl® stitches

3.5.4 Postsurgical medication and acclimatisation

For the following three days after surgery, each rat received 0.03 mg/kg Temgesic and 1 mg/kg Metacam SC for pain management and for 14 days post-surgery, the animals were monitored in accordance with Sop_Viv_Eqp_Opr 9: DSI Telemetry, Operation and Surgical Procedure. The observation focussed on the primary and secondary wounds of the animal as well as the general well-being including weight, mobility, posture and food and water intake. The rats were allowed a 14-day recovery period before they were acclimatised to the telemetry laboratory. During the acclimatisation, the rats were singly placed onto the telemetry receivers (in their home cages) for 2 hours every day for one week before the start of the experiment.

3.6 Statistical analysis

The data were exported to Excel 2016 where the average of all the parameters for each test substance was calculated at 15 min intervals. The percentage change after compound administration (0–480 min) relative to baseline (-120-0 min) for all parameters, with the exception of systolic (SBP) and diastolic blood pressure (DBP) was then calculated and expressed as mean ± S.E.M. The baseline for each parameter was expressed as “100%”, while the systolic and diastolic blood pressure was represented as absolute values. A two-way analysis of variance (ANOVA) with factors as sequence and rats within sequence, was performed on the mean-values over time for the % change from baseline. Thereafter, a three-way repeated measures ANOVA with factors phase, treatment and time (as repeated measures) was then performed where a $P < 0.05$ was considered a significant effect.

3.7 Results and discussion

3.7.1 Introduction

Several studies have reported the cardiovascular effects of clonidine and theophylline in the rat or dog (Bailey *et* al., 2011; Delaunois *et al*., 2009; Meehan *et al*., 1995). In the rat theophylline induced an increase in mean arterial BP, HR and BT, while clonidine induced a decrease in HR and BT, but had no significant effect on mean arterial BP. The validation of the telemetry system in this study relied on its ability to detect cardiovascular changes after single dose administrations of theophylline (30 mg/kg p.o.) and clonidine (0.3 mg/kg i.p.).

3.7.2 Mean arterial blood pressure

The effects of theophylline (30 mg/kg p.o.) and clonidine (0.3 mg/kg i.p.) on mean arterial BP are illustrated graphically in Figure 8. Clonidine induced an immediate increase in arterial BP, which later (about 300 min post-dosing) dropped below the control level. Theophylline did not affect arterial BP and was therefore no different than treatment with vehicle.

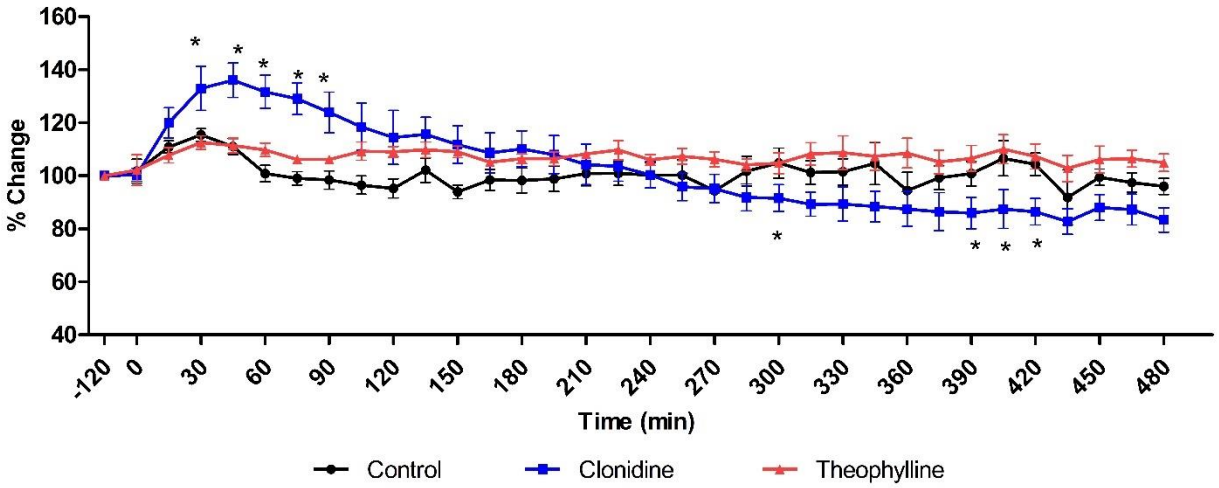

Figure 8: The effect of theophylline and clonidine on mean arterial blood pressure versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

3.7.3 Systolic blood pressure

The effects of clonidine and theophylline on SBP are illustrated in Figure 9. The administration of clonidine causes a transient increase in SBP relative to control, which dropped below the control level about 300 min post dosing. Theophylline had no effect on SBP.

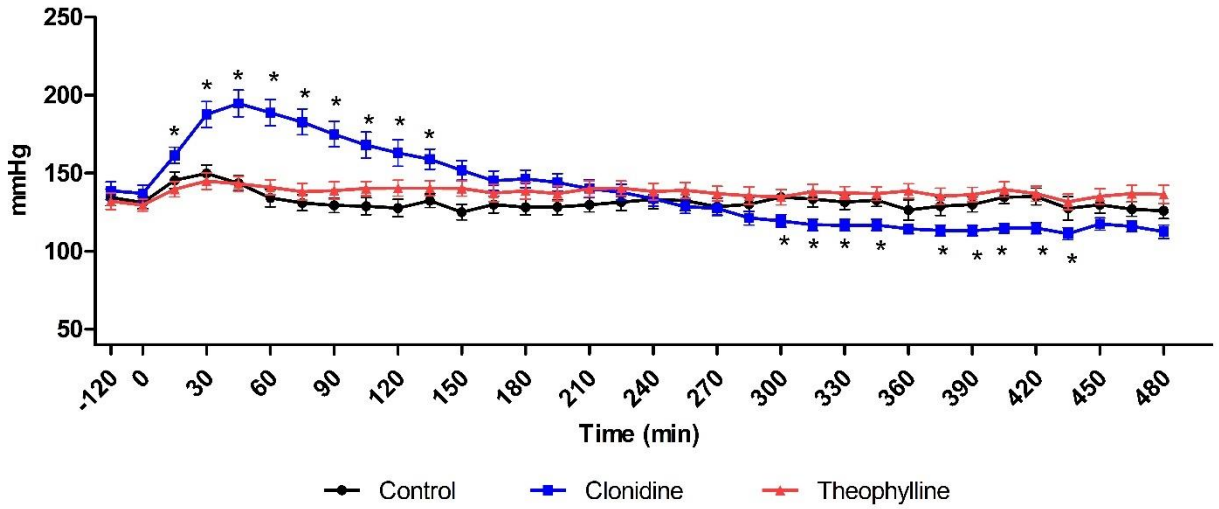

Figure 9: The effect of clonidine and theophylline on systolic blood pressure versus control. Values are presented as absolute values and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

3.7.4 Diastolic blood pressure

Theophylline and clonidine's effect on DBP is depicted in Figure 10. The administration of clonidine causes a transient increase in DBP relative to control, which dropped below the control level around 300 min post dosing

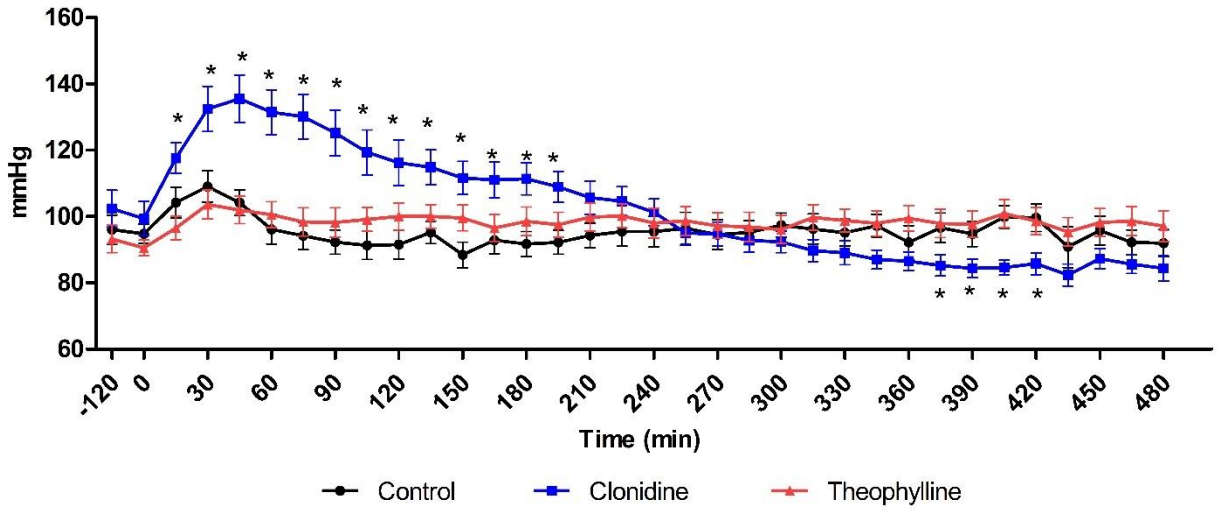

Figure 10: The effect of clonidine and theophylline on diastolic blood pressure versus control. Values are presented as absolute values and depicted as mean ± SEM (N=6). * $P < 0.05$ versus the control group.

3.7.5 Heart rate

The effects of clonidine and theophylline on HR are illustrated in Figure 11. After treatment with the vehicle (saline), there was no effect on HR except for a slight, transient increase shortly after treatment, attributable to handling of the animals. This was also clearly observed after theophylline administration, but this increase in HR lasted up to 450 min before returning to control thereby demonstrating a clear drug-induced effect. With clonidine, an immediate decrease in heart rate was observed that reversed partially at 180 min post-dosing.

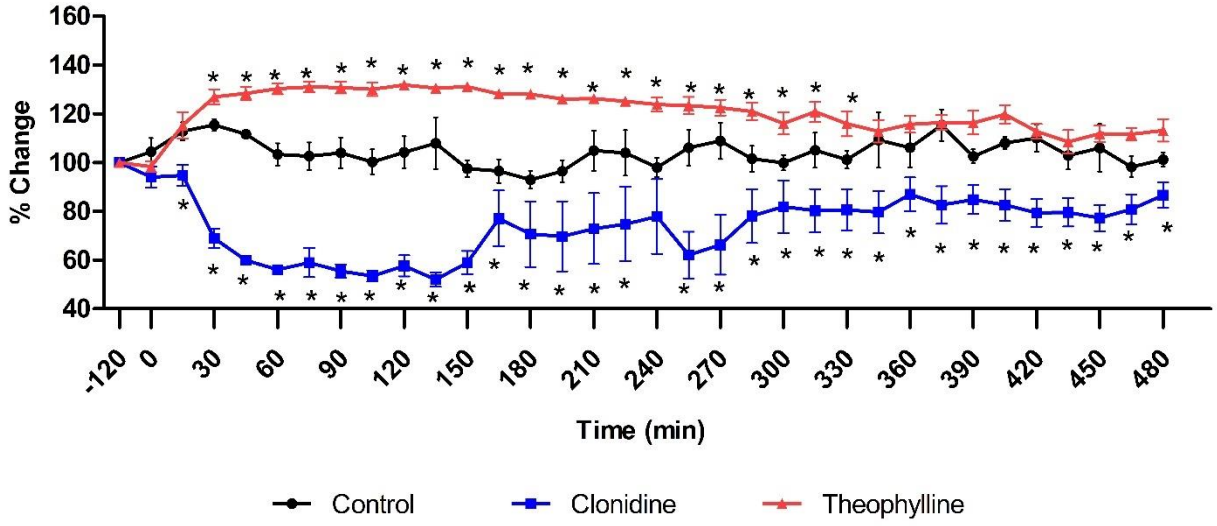

Figure 11: The effect of theophylline and clonidine on heart rate versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

3.7.6 Body temperature

The effect of clonidine and theophylline administration on BT is illustrated in Figure 12. After the administration of clonidine, a very clear decrease in BT was observed while theophylline had no effect.

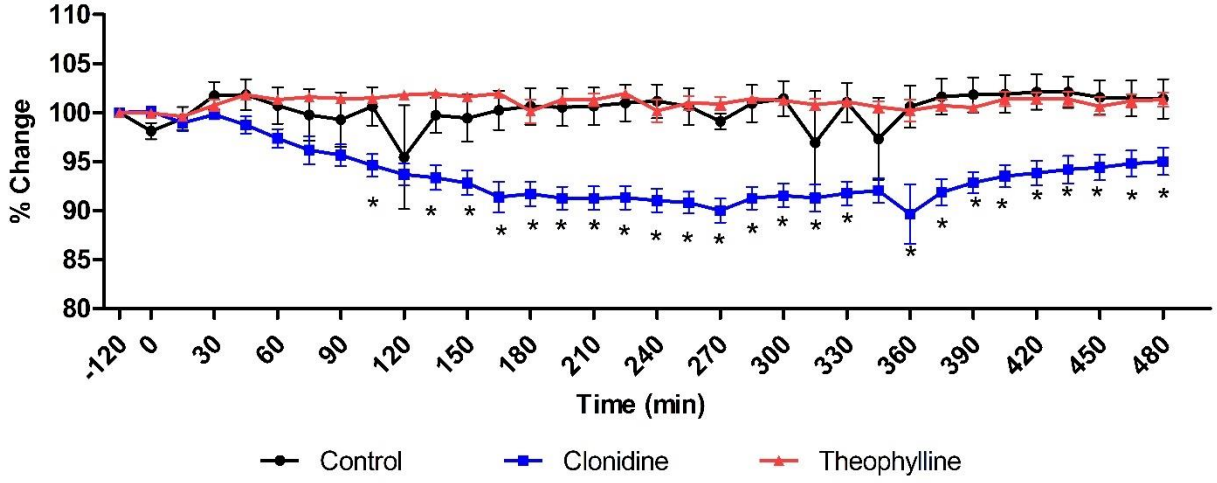

Figure 12: The effect of theophylline and clonidine on body temperature versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

3.7.7 PR Interval

The effects of clonidine and theophylline on the PR interval are illustrated in Figure 13. The administration of clonidine induced a significant increase in PR interval while a slight decrease in PR interval was observed after theophylline administration.

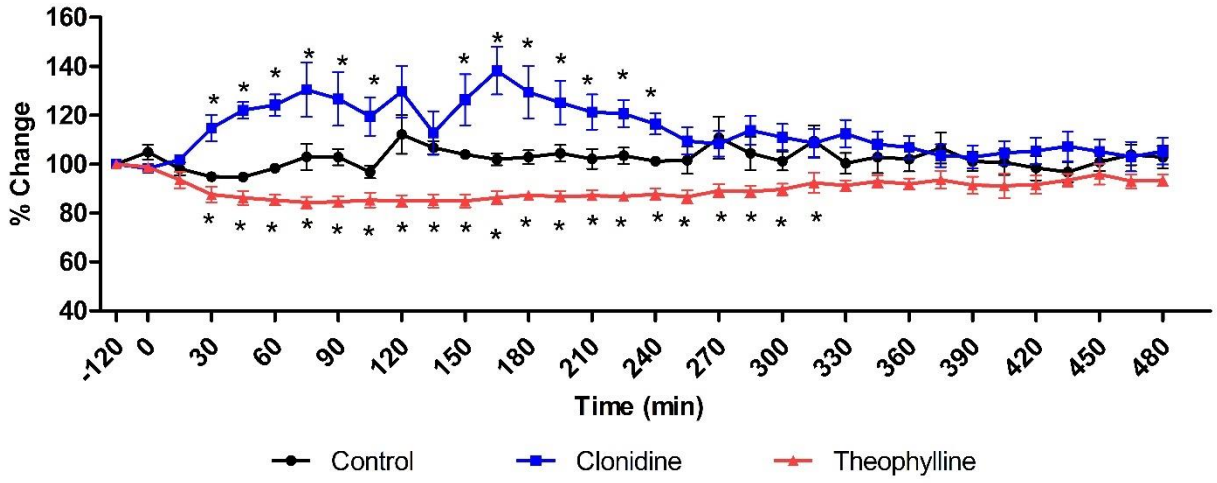

Figure 13: The effect of theophylline and clonidine on PR interval versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N = 6). * P < 0.05 versus the control group.

3.7.8 QT interval

Figure 14 illustrates the effects of clonidine and theophylline on the QT interval. Both caused significant increases in QT interval almost immediately after administration. These effects reversed at around 360 min after administration but remained significantly higher than the control up to 480 min.

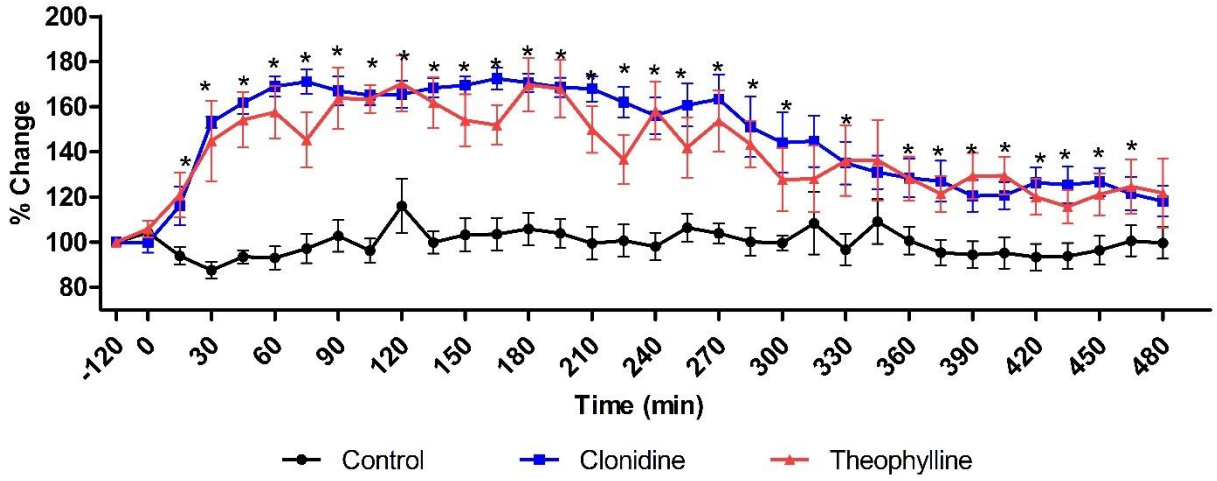

Figure 14: The effect of theophylline and clonidine on QT interval versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N = 6). * P < 0.05 versus the control group.

3.8 Discussion

The telemetry system used in this study was established and validated at the DST/NWU Preclinical Drug Development Platform Vivarium. The system was able to detect cardiovascular changes induced by clonidine and theophylline in conscious rats. The effects observed were, however, not completely consistent with the effects reported in other studies, (Delaunois *et al*., 2009; Meehan *et al*., 1995). For example, theophylline (30 mg/kg) had no effect on BT in the present study, while Delaunois *et al* (2009) reported an increase in BT using the same dose. The substantial BT changes seen with clonidine in this study, however, indicates that the system is capable of detecting changes if they occur. Delaunois et al (2009) also found that 30 mg/kg of theophylline had no effect on MBP, though they did report a significant increase in MBP after a much lower administration of theophylline (10 mg/kg). Clonidine, as an antihypertensive drug, was expected to decrease the MBP, but this was only observed starting about 300 min post-dosing, prior to which an increase in mean arterial BP was observed. The concomitant decrease in HR is also consistent with a transient pressure effect of clonidine in this model. This may be explained in part by clonidine's role as a strong α_2-adrenoceptor agonist, which leads to the inhibition of sympathetic tone. Therefore, after the activation of the α_2-adrenoceptors by clonidine, a transient increase in MBP is observed, followed by a broader decrease in MBP and HR (Khan *et al*., 1999). This correlates well with the findings reported here and the observations of Delaunois *et al* (2009) which suggested that the hypotensive effects of clonidine could only be seen in spontaneously hypertensive rat models (SHR) or anesthetized rats, due to clonidine's pharmacology as an α_2-adrenoceptor -agonist.

3.9 Conclusion

The telemetry system successfully detected haemodynamic changes induced by the administration of clonidine and theophylline. It was therefore concluded that the system could be used to investigate the possible cardiovascular effects of Pheroid.

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CHAPTER 4: CHARACTERIZATION OF PHEROID® FOR CARDIOVASCULAR ASSESSMENT

4.1 Introduction

The chemical and structural composition of Pheroid® is described in Chapter 2. This chapter summarises the manufacturing procedure and characterization of three Pheroid® formulations with different weight percentage (wt. %) oil phases as used in this study. While all the formulations were used for cardiovascular assessment, two of them were administered intravenously for cardiovascular assessment and were therefore filtered through 0.2 µm filters to avoid the risk of embolisms.

4.2 Materials

The materials used for the manufacturing of Pheroid® is summarized Table 3. Deionised water for all formulations was prepared in the Pilot Plant of the DST/NWU Preclinical Drug Development Platform.

Table 3: Raw materials used for the manufacturing of Pheroid® formulations

Raw material	Batch no.	Manufacturer/supplier
Vitamin F ethyl ester CLR	3174053	Chemipo, South Africa
Kolliphor® EL	84109716 KO	BASF Chemicals, South Africa
dl-α-Tocopherol	UT 16030037	Chempure, South Africa
Incromega E3322	000106107	Croda, South Africa
Medical Nitrous oxide gas	-	Afrox, South Africa

4.3 Manufacturing procedure of Pheroid® formulations

4.3.1 4% Pheroid®

Pheroid® (4 wt. % oil phase) was prepared by mixing Kolliphor® EL (1.17 g), vitamin F ethyl ester (2.77 g) and Incromega E3322 (0.05 g). The mixture was then heated to 70 °C and allowed to cool down to 55 °C at which point dl-α-tocopherol (0.01 g) was added. This mixture constituted the oil phase (4 wt. %) of the Pheroid®. Deionised water saturated with N_2O gas was heated to 70 °C and the heated oil mixture was then added to the heated N_2O saturated water (96 wt. %). This mixture was then homogenized at 13500 rpm until cooled to ≤ 40 °C and gassed with N_2O under 200 pKa for three days.

4.3.2 10% Pheroid®

10% Pheroid® is comprised of 10 wt. % oil phase and 90 wt. % N_2O gassed purified water. The 10% Pheroid was prepared by mixing Kolliphor® EL (2.94 g), vitamin F ethyl ester (7 g) and Incromega E3322 (0.05 g) and heated to 70 °C. When the mixture reached 55 °C, dl-α-tocopherol (0.01 g) was added. This mixture constituted the oil phase (10 wt. %) of the Pheroid®. Deionised water saturated with N_2O gas was heated to 70 °C and the heated oil mixture was then added to the heated N_2O saturated water (90 wt. %). The mixture was homogenized at 13500 rpm until it cooled down to ≤ 40 °C and gassed with N_2O under 200 pKa for three days.

4.3.3 pro-Pheroid®

The pro-Pheroid® formulation is 100 wt. % oil phase and is devoid of the N_2O water phase. Pro-Pheroid® was prepared by mixing Kolliphor® EL (BASF Chemicals, South Africa) (29.12 g), vitamin F ethyl ester (Chemipo, South Africa) (69.83 g) and Incromega E3322 (0.05 g). The mixture was heated to 70 °C and allowed to cool down to 55 °C at which point dl-α-tocopherol (Chempure, South Africa) (1 g) was added to the mixture. The mixture was then gassed with N_2O under 200 pKa for four days.

4.4 Characterization of Pheroid® formulations

4.4.1 Particle size and particle size distribution

4.4.1.1 Apparatus

The particle size and particle size distribution of all Pheroid® formulations was determined by laser diffraction with the use of a Malvern Mastersizer 2000 analyser (Malvern Instruments Ltd., Worcestershire, United Kingdom). The equipment was switched on 30 min prior to the actual measurements to allow the laser to stabilise. At this point, the Pheroid® samples were moderately mixed to ensure even distribution of the sample. The Mastersizer was pre-programmed with measurement protocols specifically for Pheroid® formulations to increase the consistency and accuracy of the measurements.

4.4.1.2 Experimental procedure

As soon as the laser was stabilised, it was aligned with deionised water. The sample was then slowly added and stirred at 1500 rpm until an obscuration rate of 10–20% was achieved. The refractive indices of the Pheroid® sample and the dispersion medium (deionised water) were 1.481 and 1.33 respectively. The data was expressed as D(0.1), D(0.5) and D(0.9), where D(0.5) represents the total volume size of the particles at which 50% of the sample is smaller than the

indicated size and 50% is larger than the indicated size. D(0.9) is a representation of the total volume size of the particles at which 90% of the sample is smaller than the indicated size and 10% is larger than the indicated size while D(0.1) represents the total volume size of the particles at which 10% of the sample is smaller than the indicated size and 90% is larger than the indicated size. Each sample was measured in triplicate.

4.4.1.3 Results

Pheroid® particles typically have sizes ranging from 200 nm to 2 µm, but for the purpose of this study, the 4% and 10% Pheroid® samples were filtered through 0.2 µm filters in an attempt to reduce the particle size for intravenous administration. Despite the filtration, Pheroid® structures were still present, which could be attributed to the dynamic equilibrium of Pheroid® in that its structures are constantly forming and dividing. The complete Mastersize analysis of the four Pheroid® formulations with D(0.1), D(0.5) and D(0.9) can be found in Annexure A. Table 4 contains the D(0.5) particle size distribution (mean ± SD) of all Pheroid® formulations. Fifty percent of the particles in the 4% Pheroid® (filtered) formulation were larger than 0.43 µm ± 0.002 and 50% of the particles were smaller than 0.43 µm ± 0.002. With regards to the 10% Pheroid® (filtered) formulation, 50% of the particles were larger than 0.2 µm ± 0.001 and 50% of the particles were smaller than 0.2 µm ± 0.001.

Table 4: Particle size distribution of the Pheroid® formulations used in this study

Pheroid® formulation	**D(0.5) (µm)**
Pro-Pheroid®	0.21 µm ± 0.002
4% Pheroid® (unfiltered)	0.5 µm ± 0.018
4% Pheroid® (filtered)	0.43 µm ± 0.002
10% Pheroid® (filtered)	0.2 µm ± 0.001

4.4.2 Confocal laser scanning microscopy (CLSM)

4.4.2.1 Apparatus

The particle size and morphological characteristics of the Pheroid® formulations were visualised using a Nikon D-Eclipse C1 confocal laser scanning microscope (CLSM) with a DXM 1200 digital camera. This analytical imaging technique has the ability to reduce artefacts by eliminating background information from the focal plane (such as out of focus emissions) with the use of a spatial pinhole while providing better depth-of-field control (White *et al*., 1987). The latter allows the creation of high-quality three-dimensional images by capturing two-dimensional images of the fluorescence-emitting sample at different focal depths.

4.4.2.2 Experimental procedure

Fifty µl of the Pheroid® sample was stained with 2 µl Nile (Red Molecular Probes, Thermo Fisher Scientific Inc., USA). After a 15 min incubation period in the dark, 20 µl of the mixture was placed on a microscope slide and covered with a coverslip for examination. The CLSM was equipped with three lasers, with excitation wavelengths of 405, 488 and 543 nm respectively. The fluorescent light emissions were collected for three different wavelength bands; above 650 nm, 540-640 nm, and 485-545 nm. These three images were then combined into one image to give an indication of the Pheroid®'s morphology.

4.4.2.3 Results

The CLSM micrographs for each Pheroid® formulation are shown in Figure 15–18. These images represent the merged fluorescence detected in the following wavebands: 500–530 nm and 568–642 nm.

4.2.2.3.1 pro-Pheroid®

The CLSM micrograph of the pro-Pheroid® formulation is shown in Figure 15. The sample was analysed in five aliquots to determine the average pro-Pheroid® concentration and size. The results reported the pro-Pheroid® concentration within the sample to be 4.01 x 10^{10}/ml and had an average particle size of 0.67 µm ± 0.04.

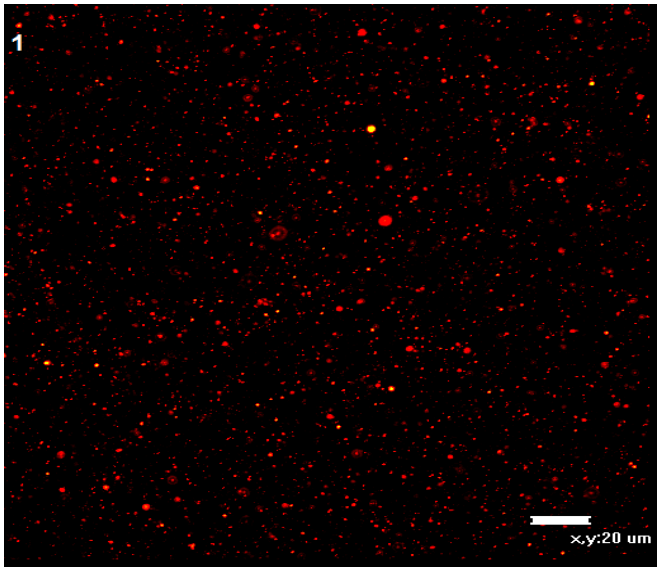

Figure 15: Confocal laser scanning microscopy micrograph of pro-Pheroid®

4.2.2.3.2 4% Pheroid® (unfiltered)

The CLSM micrograph of the unfiltered 4% Pheroid sample (Figure 16) shows clear Pheroid® structures, with an average particle size of 0.45 µm ± 0.03, which was somewhat smaller than that of pro-Pheroid®. The average concentration was 2.3 x 10^{10}/ml. These results were obtained by calculating the average of five aliquots of the sample.

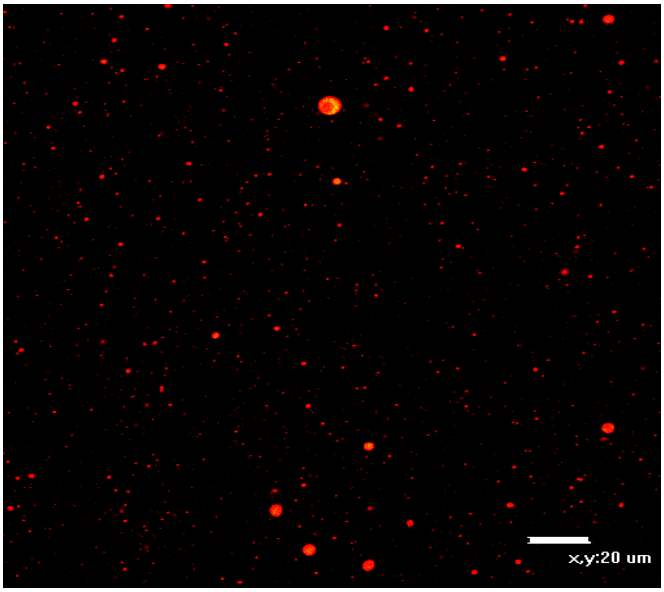

Figure 16: Confocal laser scanning microscopy micrograph of 4% Pheroid® (unfiltered)

4.2.2.3.3 4% Pheroid® (filtered)

The CLSM micrograph of the 4% Pheroid® sample filtered through a 0.2 µm filter is shown in Figure 17. The results of the confocal analysis were determined by calculating the average of five aliquots of the sample. The average concentration and particle size of the sample was 8.1 x 10^{10}/ml and 0.64 µm ± 0.09 respectively, proving to be the adequate size for intravenous administration.

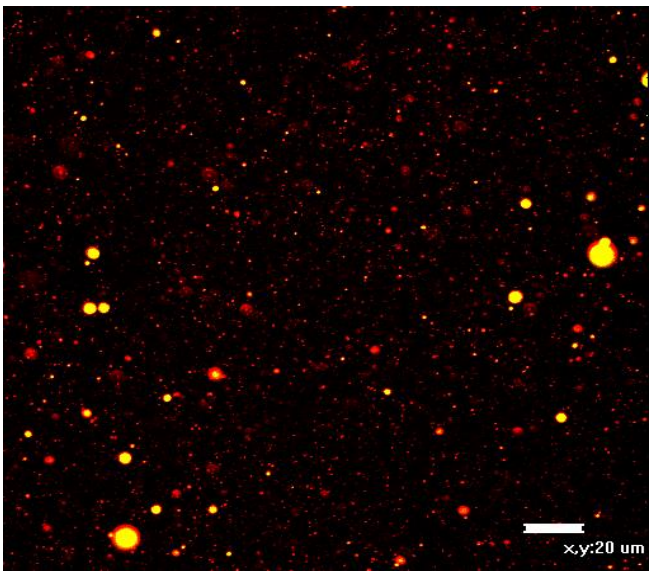

Figure 17: Confocal laser scanning microscopy micrograph of 4% Pheroid® (filtered)

4.2.2.3.4 10% Pheroid® (filtered)

The 10% Pheroid® sample was also filtered through a 0.2 µm in preparation for intravenous administration. Figure 18 is a CLSM micrograph of this sample and the confocal results was determined by calculating the average of five aliquots of the sample. The average Pheroid® concentration was 3.42×10^{10}/ml with an average particle size of 0.73 µm ± 0.23, proving adequate for intravenous administration.

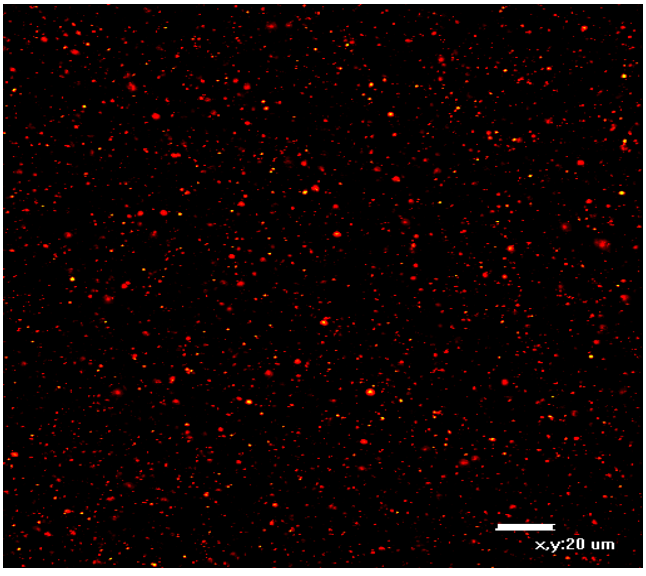

Figure 18: Confocal laser scanning microscopy micrograph of 10% Pheroid® (filtered)

4.4.3 Zeta potential

4.4.3.1 Apparatus

To determine the stability of the formed Pheroid® vesicle structures, the zeta potential was measured with a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, United Kingdom). The apparatus was highly sensitive to the presence of foreign particles in the cell such as air bubbles; therefore, much care was taken to avoid bubble formation by gently tapping the side of the cell.

4.4.3.2 Experimental procedure

In order to warm up the apparatus, it was switched on 30 min prior to analysis. During this time, a 5000x dilution of the sample was prepared by diluting 100 µl Pheroid® sample in 10 ml deionised water. Thereafter, 200 µl of this mixture was diluted once more in 10 ml water and mixed using a pipette to give a 5000x dilution. To measure the zeta potential, the cell was rinsed with 1 ml of the dilution and emptied before injecting 1 ml once again into the cell for analysis. Care was taken to avoid bubble formation by first injecting half of the sample and once the sampled reached the halfway mark, the cell was then rotated and filled with the other half. The cell was closed with two stoppers and placed in the measurement chamber to start the zeta potential analysis. Each sample was measured in triplicate.

4.4.3.3 Results

Figure 19 summarizes the average zeta potential and standard deviation (mean ± SD) for each Pheroid® formulation. The pro-Pheroid® and 4% Pheroid® (unfiltered) had relatively low zeta potentials of (-12 mV ± 0.55) and (-17.7 mV ± 2.54) respectively, and therefore tended to be more unstable. The filtered 4% and 10% Pheroid® proved to be the most stable of the four formulations, with zeta potentials of (-37.8 mV ± 1.8) and (-27.23 mV ± 2.06) respectively.

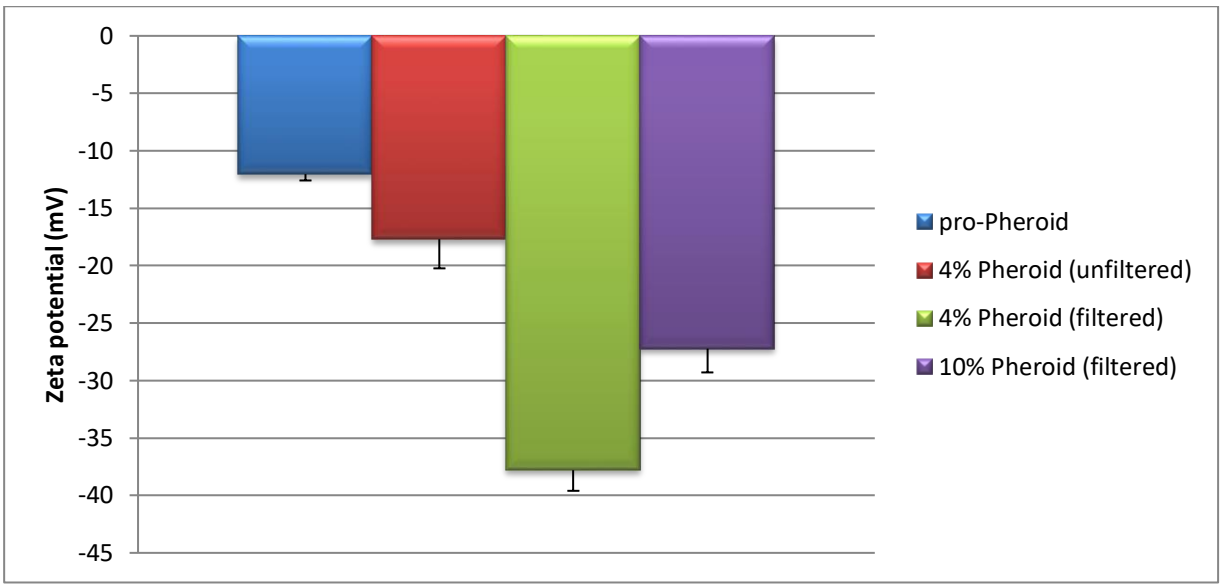

Figure 19: The average zeta potential (mean ± SD) in mV of each Pheroid® formulation

4.5 Conclusion

While the Pheroid® has been proven to enhance the application of oral drug administrations, its use in intravenous administrations is limited; due to its nature as an oil-based emulsion. Indeed, for intravenous administration of emulsions, the oil droplets must be < 5 µm to avoid adverse effects such as pulmonary embolisms (Hörmann & Zimmer, 2016; Koster *et al.,* 1996). By filtration of two Pheroid® formulations through a 0.2 µm filter, it is deemed safe for cardiovascular assessment via intravenous administration. This is supported by the results obtained from confocal analysis, showing average particle sizes of 0.64 µm ± 0.09 and 0.73 µm ± 0.23 for 4% and 10% Pheroid® respectively. The fact that there was still Pheroid® structures present after filtration, especially those larger than 0.2 µm remains unknown. One could speculate that this is due to the fact that the Pheroid® structures are in dynamic equilibrium, constantly forming and dividing (Uys, 2006). This is also supported by Grobler (2009) who attributes this to the fluidity of the Pheroid® structures brought on by N_2O gas.

Characterisation of the Pheroid® formulations has shown them to be up to standard with respect to manufacturing specifications, with the exception of the relatively low zeta potential of pro-Pheroid® and the unfiltered 4% Pheroid® (-12 mV ± 0.55 and -17.7 mV ± 2.54 respectively). According to Roland *et al* (2003), this is an indication of poor stability, which could lead to particle aggregation and possibly an increase in particle size. However, this was not seen in the confocal analysis, which reported an adequate particle size of 0.67 µm ± 0.04 for pro-Pheroid® and 0.45 µm ± 0.03 for the unfiltered 4% Pheroid®. This could be attributed to the fact that the samples

were analysed for zeta potential shortly after manufacturing and were not allowed to stand in storage for too long.

With respect to quality control regulations, the quality of the Pheroid® formulations was deemed acceptable for the subsequent cardiovascular assessment.

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CHAPTER 5: THE CARDIOVASCULAR SAFETY OF PHEROID® IN THE CONSCIOUS RAT

This chapter was written as a manuscript for submission to the Journal of Pharmacological and Toxicological Methods. For ease of reading, all images are placed in consecutive order with their respective descriptions and captions. See Annexure B for the applicable Author Information Pack.

Title: The cardiovascular safety of Pheroid® in the conscious rat

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Abstract

Introduction: The Pheroid® is a colloidal drug delivery system comprised of a dispersed oil phase of omega 3 and omega 6 fatty acids emulsified in N_2O saturated water. Pheroid® has pharmaceutical applicability for the enhancement of therapeutic efficacy due to better drug exposure, reduced cytotoxicity and faster onset of action. The purpose of this paper was to evaluate the possible cardiovascular effects of the Pheroid® drug delivery system in the conscious rat. **Methods:** Six male rats instrumented with telemetry transmitters were used in a three-way crossover design. The effects of single intravenous (IV) and oral (p.o.) administration of the Pheroid® delivery system on cardiovascular parameters including arterial blood pressure (BP), heart rate (HR) and the ECG were measured. As positive controls, a single intraperitoneal dose of clonidine (0.03 mg/kg) and an oral dose of theophylline (30 mg/kg) were also tested. **Results:** No significant effects of Pheroid® on cardiovascular parameters were observed at any time following oral or IV administration. The expected effects of clonidine and theophylline could be demonstrated confirming the sensitivity of the experimental model to detect changes in the cardiovascular parameters measured. **Discussion:** We have demonstrated that Pheroid® has no effects on cardiovascular function following both oral and IV administration. These data showing cardiovascular safety are supportive for the use of Pheroid for various pharmacological applications.

Keywords: Blood pressure; ECG; Heart rate; Pheroid®; Pre-Clinical Drug Development Platform; Rat; Safety pharmacology; Telemetry

Abbreviations:

PCDDP: Pre-Clinical Drug Development Platform

ICH: International Conference on Harmonization

MBP: Mean blood pressure

SBP: Systolic blood pressure

DBP: Diastolic blood pressure

HR: Heart rate

BT: Body temperature

ECG: Electrocardiogram

API: Active pharmaceutical ingredient

1. Introduction

The Pheroid® drug delivery system is a colloidal drug delivery system comprised of a dispersed oil phase of omega 3 and omega 6 fatty acids emulsified in N_2O saturated water. Pheroid® vesicles are formed upon contact of the fatty acids with the N_2O-saturated water. These vesicles are micron and submicron sized structures which entrap both hydrophobic and hydrophilic compounds and their morphology can be altered to change their release characteristics by using different methods of formulation (Grobler *et al*., 2008). The Pheroid® can also be formulated without a water phase, which makes it a more applicable carrier for molecules unstable in aqueous environments. This formulation is called pro-Pheroid®, and its principle relies on the fact that Pheroid® vesicles form upon contact with an aqueous media, be it an external source or intestinal fluid. If any active pharmaceutical ingredients (API) were to be present during this process, they would be entrapped within the newly formed Pheroid® vesicles (Grobler *et al.*, 2014). Pheroid® has a range of attributes contributing to its pharmaceutical applicability such as enhancement of therapeutic efficacy due to better drug exposure, reduced cytotoxicity and faster onset of action (du Plessis *et al*., 2010; Grobler *et al.,* 2008).

Pheroid® is also being investigated in a wide range of disease applications such as oncology and infectious disease (Chinembiri *et al*., 2015; Gerber *et al*., 2008; Grobler *et al*., 2014; Steyn *et al*., 2011). For more information on its applications, see Slabbert *et al*. (2011); and Steyn *et al*. (2011). Because of its various potential clinical applications as a delivery system, its cardiovascular safety has to be determined. For this purpose, a telemetry-based system was used to determine the cardiovascular effects of Pheroid® in conscious rats.

2. Methods

2.1 Animals

This study and the use of animals were approved by the NWU-AnimCareREC: NWU-00346-15-A5. Six male Sprague-Dawley rats of 8-9 weeks of age with a mean body weight of 370 g were used (PCDDP, North-West University, Potchefstroom, South Africa). The rats were housed singly in type II long individual ventilated cages (IVC) (Techniplast S.P.A., Buguggiate, Italy) with corn-cob bedding. The IVC's underwent 60 air changes per hour through HEPA filters with an environment of 22 °C ± 1 °C and 55% ± 10% humidity. A day-night cycle of 12 hours was maintained. The rats were fed a standard laboratory rodent diet (NutritionHUB, Stellenbosch, South Africa) and had access to food and water *ad libitum.*

2.2 Surgery

One week before the surgery, six physiological data transmitters (HD-S11, Data Sciences International, St. Paul, MN 55126) were each assigned to a specific telemetry receiver (Receiver system RPC-1, Data Sciences International, St. Paul, MN 55126). All surgical procedures were performed under aseptic conditions. On the day of the surgery the rats were anaesthetized in an inhalation cage with 2.5% isoflurane and 1l/min O_2. The abdomen and throat were shaved, and the rats were treated subcutaneously with Metacam (1 mg/kg) and Temgesic (0.03 mg/kg) for analgesia. The rat was placed on a heated pad with its nose placed inside an inhalation mask with 2.5% isoflurane and 1l/min Oxygen flow. A 5 cm incision was made 0.5 cm below the sternum. The *linea alba* was opened with scissors and a gauze pad soaked in 0.9% NaCl solution was used to isolate the aorta from the intestines. Once the aorta had been isolated using cotton-tipped swabs, two haemostats were used to close the blood flow of the arteries between the kidneys and the aortic bifurcation. The aorta was punctured with a sterile arched cannula, followed by insertion of the gel-filled catheter into the aorta. The cannula was removed, and the aorta sealed using tissue adhesive (Histoacryl®, Boehringer Ingelheim Pharma GmbH & Co. KG). The transmitter unit was stitched to the muscle layer of the abdominal cavity using 75cm Mersilene® silk. To attach the two ECG electrodes, a small incision was made into the connective tissue under the skin to reveal the muscle layer straight over the sternum. One ECG (positive) lead was tunnelled under the skin to the muscle layer over the sternum. The ECG lead was attached to the muscle layer over the sternum with a single stitch. The rest of the cable was brought under the skin and closed. To attach the second, negative ECG lead, a small incision was made in the skin over the trachea, revealing the muscle layer. The ECG lead was tunnelled to the trachea by using a tunnelling tube and was attached over the trachea, bringing the muscle layer over the ECG lead. After the ECG leads were fixed to the respective throat and abdominal regions, the skin surrounding the incisions were closed using 45 cm Vicryl® suture. The rats received a subcutaneous dose of Temgesic (0.03 mg/kg) and Metacam (1 mg/kg) for three days following the-surgery. The rats were allowed 14 days to recover from the surgery during which time their weight and food intake was monitored by a veterinarian. Thereafter, the rats were placed singly onto their respective telemetry receivers (inside their home cages) for 2 hours every day for one week for acclimatization to the experimental conditions.

2.3 Experimental design

2.3.1 Validation of the telemetry system

To validate the telemetry system, all six rats received an oral dose of 200 µl 0.9% NaCl solution (control) and two reference compounds on separate days: 0.3 mg/kg (i.p.) clonidine (Sigma-

Aldrich, Johannesburg, South Africa 1645) and 30 mg/kg (p.o.) theophylline (Sigma-Aldrich, Johannesburg, South Africa 1645) were used to determine the system's ability to detect their known cardiovascular effects.

2.3.2 Oral administration of Pheroid®

Using the same crossover design, the cardiovascular effects of an oral dosage of Pheroid® were determined by administration of two Pheroid® formulations: pro-Pheroid® and 4% Pheroid® along with 0.9% NaCl solution as control. All test compounds including the control were administered at 200 µl neat (p.o.).

2.3.3 Intravenous administration of Pheroid®

Two Pheroid® formulations, 10% and 4% Pheroid® along with a control were intravenously administered to the six rats via the tail vein at a dosage of 200 µl. Because the Pheroid® is a lipid-based emulsion, the samples were filtered through a 0.2 µm filter immediately prior to administration to reduce the risk of pulmonary embolism.

2.4 Pheroid® formulations

The Pro-Pheroid® formulation is the same formulation as the 4% Pheroid® but is devoid of the N_2O water phase. Pro-Pheroid® vesicles were prepared by mixing Kolliphor® EL (BASF Chemicals, South Africa) (29.12 g), vitamin F ethyl ester (Chemipo, South Africa) (69.83 g) and Incromega E3322 (0.05 g). The mixture was then heated to 70 °C and allowed to cool down to 55 °C at which point dl-α-tocopherol (Chempure, South Africa) (1 g) was added to the mixture. The mixture was then gassed with N_2O under 200 pKa for four days. Two Pheroid® formulations with different weight percentage oil phases were also prepared. 4% Pheroid® (4 wt. % oil phase) was prepared by mixing Kolliphor® EL (1.17 g), vitamin F ethyl ester (2.77 g) and Incromega E3322 (0.05 g). The mixture was then heated to 70 °C and allowed to cool down to 55 °C at which point dl-α-tocopherol (0.01 g) was added. This mixture constituted the oil phase (4%) of the Pheroid® vesicles. Purified water saturated with N_2O gas was heated to 70 °C and the previously mentioned heated oil mixture was then added to the heated N_2O saturated water (96 wt. % N_2O gassed purified water). This mixture was then homogenized at 13500 rpm until the mixture cooled down to ≤ 40 °C and gassed with N_2O under 200 pKa for three days. 10% Pheroid® is comprised of 10 wt. % oil phase and 90 wt. % N_2O gassed purified water. The 10% Pheroid was prepared by mixing Kolliphor® EL (2.94 g), vitamin F ethyl ester (7 g) and Incromega E3322 (0.05 g) and heated to 70 °C. When the mixture reached 55 °C, Dl-α-tocopherol (0.01 g) was added. This mixture constituted the oil phase (10 wt. %) of the total formulation. Purified water saturated with N_2O gas was heated to 70 °C and the previously

mentioned heated oil mixture was then added to the heated N_2O saturated water (90 wt. %). This mixture was then homogenized at 13500 rpm until it cooled down to ≤ 40 °C. The mixture was then gassed with N_2O under 200 pKa for three days.

2.5 Characterization of Pheroid®

Pheroid® vesicles have particle sizes ranging from 200 nm to 2 µm. The mean particle size and particle size distribution of the formulated Pheroid® vesicles were determined by light scattering with a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). The particle size analyser's laser was positioned in line with deionized water that contained a Pheroid® sample. The samples were stirred continuously to maintain homogeneity while the scattering intensity was captured at 90° and 25 °C. The morphological characteristics of the Pheroid® vesicles were analysed by confocal laser scanning microscopy (CLSM). The CLSM images were taken with a Nikon D-Eclipse C1 confocal laser scanning microscope with a DXM 1200 digital camera and a 10 pm pinhole. Nile Red was used for fluorescent labelling of the Pheroid® vesicles. The Nile red fluorophore marker has an emission wavelength of between 640 and 650 nm while the CLSM is equipped with three lasers, with wavelengths 405, 488 and 543 nm respectively. The fluorescent light emissions were collected for three different wavelength bands, above 650 nm, 540-640 nm, and 485-545 nm. The Pheroid®, being a lipid-based delivery system, possesses a charge on its surface that interacts with the environment, contributing to the stability of its structure. The stability of the Pheroid® vesicles was determined by measuring their Zeta potential using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). A particle is generally considered stable if its zeta potential is larger than ±25 mV where the repulsive forces exceed the attractive forces (Crooke, 2007; Roland *et al*., 2003).

2.6 Data acquisition

The Ponemah Physiological Platform (V5.20) acquisition program [Data Sciences International (DSI), St. Paul, MN 55126] was used. One day prior to surgery, the transmitters were switched on to check the signal quality and assigned to a specific receiver. The transmitters were also checked to ensure the zero-offset value for each parameter was equal to zero. The telemetry system generated data on mean blood pressure (MBP), systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate (HR), body temperature (BT) and the ECG parameters PR- and QT intervals. The data for each parameter was averaged over 15 min intervals and the sampling rate was 1000 Hz for ECG, 1 Hz for temperature and 500 Hz for blood pressure. The signals from the transmitters were received by six RPC-1 receivers and transmitted to a DSI data exchange matrix. An ambient pressure reference APR-1 was also integrated into the system to compensate

for ambient blood pressure changes, while the signals from the transmitters were evaluated using the Ponemah V5.20 acquisition program.

2.7 Statistical analysis

The derived data was exported to Excel 2016 where the average of all the parameters for each test substance was calculated at 15 min intervals. The percentage change after compound administration (0–480 min) relative to baseline (-120-0 min) for all parameters, with the exception of systolic and diastolic blood pressure was then calculated and expressed as mean ± S.E.M. The baseline for each parameter was expressed as "100%", while the systolic and diastolic blood pressure was represented as absolute values. A two-way analysis of variance (ANOVA) with factors as sequence and rats within sequence, was performed on the mean-values over time for the % change from baseline. Thereafter, a three-way repeated measures ANOVA with factors phase, treatment and time (as repeated measures) was then performed where a $P < 0.05$ was considered a significant effect.

3. Results

3.1 Pheroid® formulations characterization

The average particle size, zeta potential and particle size distribution (mean ± SD) are summarized in Table 1. The filtered 10% Pheroid® vesicles were the largest with an average particle size of 0.73 µm ± 0.23, while the pro-Pheroid®, filtered 4% Pheroid® and unfiltered 4% Pheroid® vesicles were 0.67 µm ± 0.04, 0.64 µm ± 0.09 and 0.45 µm ± 0.03, respectively. The pro-Pheroid® and 4% Pheroid® (unfiltered) had relatively low zeta potentials of - 17.7 mV ± 2.54 and -12 mV ± 0.55 respectively, while the 4% Pheroid® (filtered) and filtered 10% Pheroid® had higher zeta potentials of -37.8 mV ± 1.8 and -27.23 mV ± 2.06 respectively, making them the more stable of the four formulations. D(0.5) is a representation of the total volume size of the particles at which 50% of the sample is smaller than the indicated size and 50% is larger than the indicated size.

Table 1: Particle size, zeta potential and particle size distribution of Pheroid® vesicles in different formulations

Formulation	Average particle size (µm)	Zeta potential (mV)	D(0.5) (µm)
Pro-Pheroid®	0.67 µm ± 0.04	-12 mV ± 0.55	0.21 µm ± 0.002
4% Pheroid® (unfiltered)	0.45 µm ± 0.03	-17.7 mV ± 2.54	0.5 µm ± 0.018
4% Pheroid® (filtered)	0.64 µm ± 0.09	-37.8 mV ± 1.8	0.43 µm ± 0.002
10% Pheroid® (filtered)	0.73 µm ± 0.23	-27.23 mV ± 2.06	0.2 µm ± 0.001

3.2 Effects of clonidine and theophylline

The cardiovascular parameters that were most affected by the two reference compounds and the changes they induced are shown in Figures 1–4, which depict the changes in hear rate, PR interval, QT interval and body temperature, respectively. Immediately after the administration of clonidine, a transient increase in mean BP was observed, but at about 300 minutes post-administration, pressure decreased slightly relative to baseline and the control (results not shown). Theophylline did not have any significant effects on MBP

Figure 1 illustrates the effect of an administration of clonidine and theophylline on heart rate. Due to the handling of the rats, a small increase was observed in HR immediately after administration for both the theophylline and control group but not clonidine. This could be attributed to its potent pharmacology as an antihypertensive drug and the fact that it was administered intraperitoneally. The administration of clonidine was followed by an immediate decrease in heart rate, while theophylline had increased the heart rate. Both effects were statistically significant, when assessed from compound administration to 480 minutes post treatment.

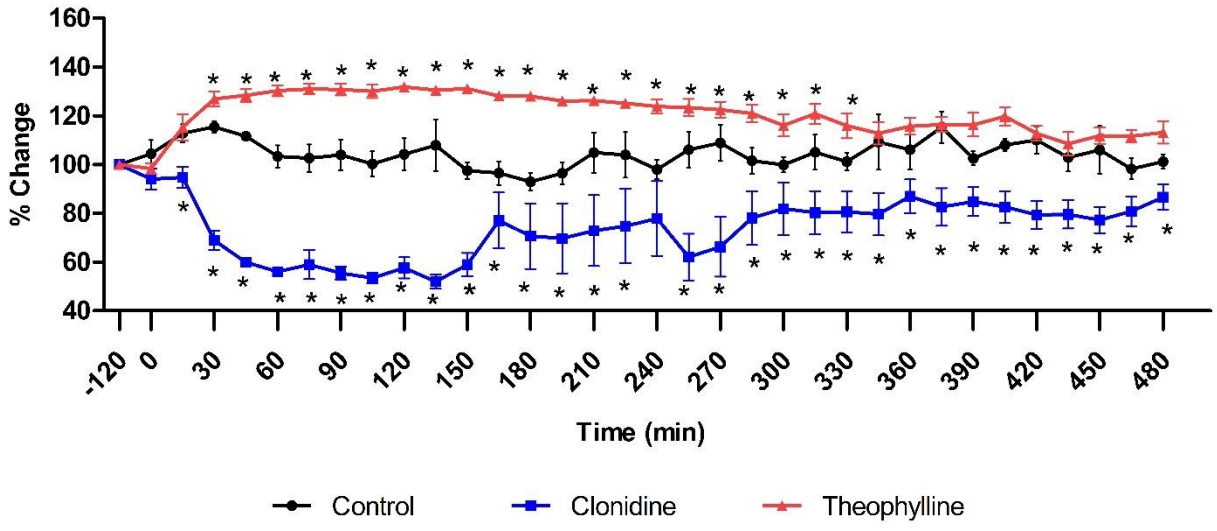

Figure 1: The effect of theophylline and clonidine on heart rate versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * $P < 0.05$ versus the control group.

The administration of clonidine was followed by a significant increase in PR interval, while theophylline caused a significant decrease in PR interval (see Figure 2).

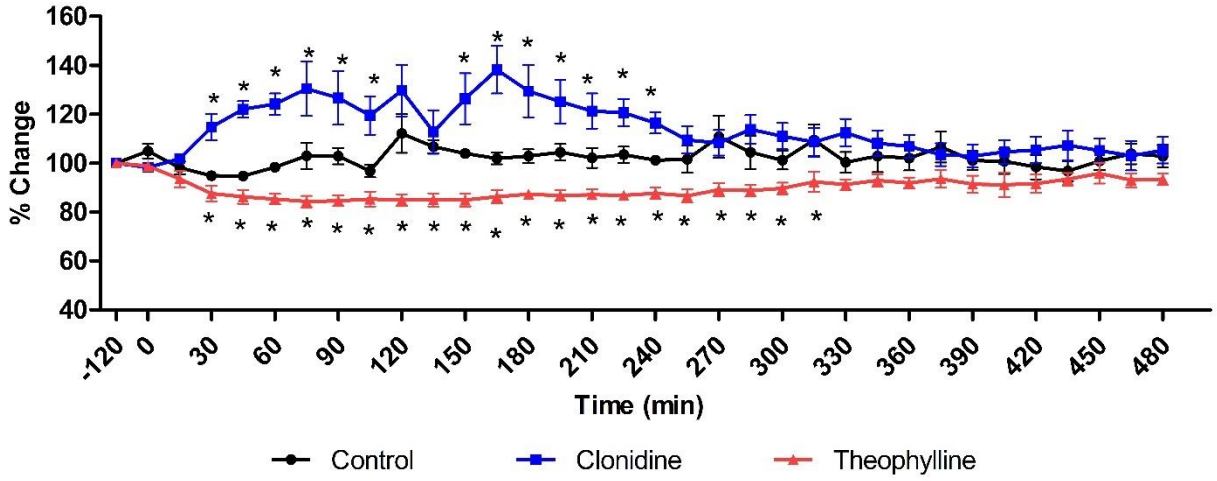

Figure 2: The effect of theophylline and clonidine on PR interval versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N = 6). * P < 0.05 versus the control group.

Both clonidine and theophylline had caused a significant increase in QT interval (see Figure 3 below).

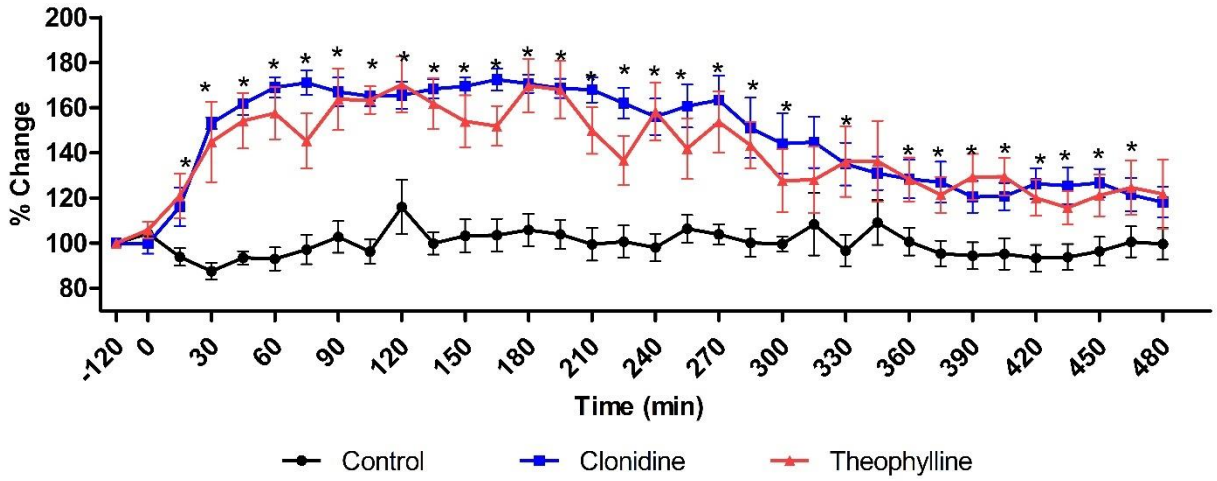

Figure 3: The effect of theophylline and clonidine on QT interval versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N = 6). * P < 0.05 versus the control group.

Figure 4 illustrates the effect of the reference compounds on body temperature. While theophylline had no effect, clonidine decreased the body temperature substantially and throughout the entire measurement period.

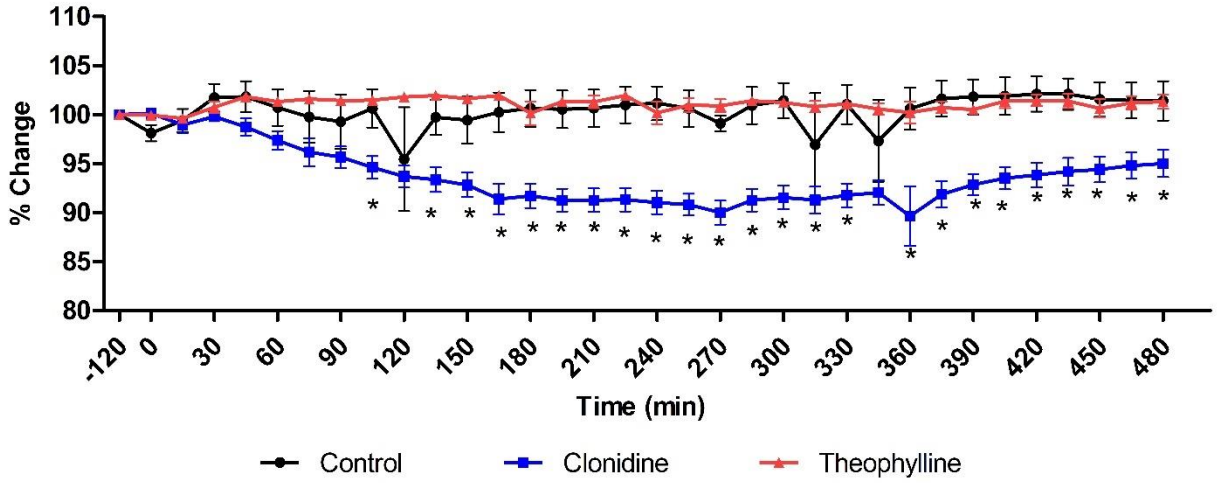

Figure 4: The effect of theophylline and clonidine on body temperature versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

3.3 Oral administration of Pro-Pheroid® and 4% Pheroid®

The effects of an oral administration of Pheroid® on arterial BP, SBP, DBP, HR, PR interval, QT interval and body temperature are presented in Figures 5–11 respectively. None of the oral Pheroid® formulations had any effect on the arterial blood pressure (see Figure 5 below).

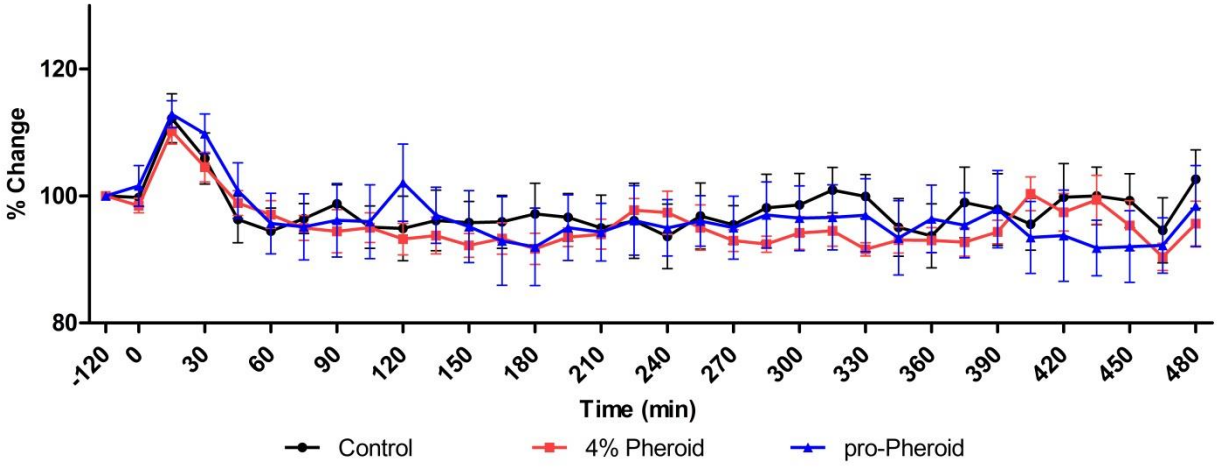

Figure 5: The effect of an oral administration of 4% Pheroid® and pro-Pheroid® on arterial blood pressure versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

The effects of the oral Pheroid® formulations on SBP are depicted in Figure 6 below. No effects were seen.

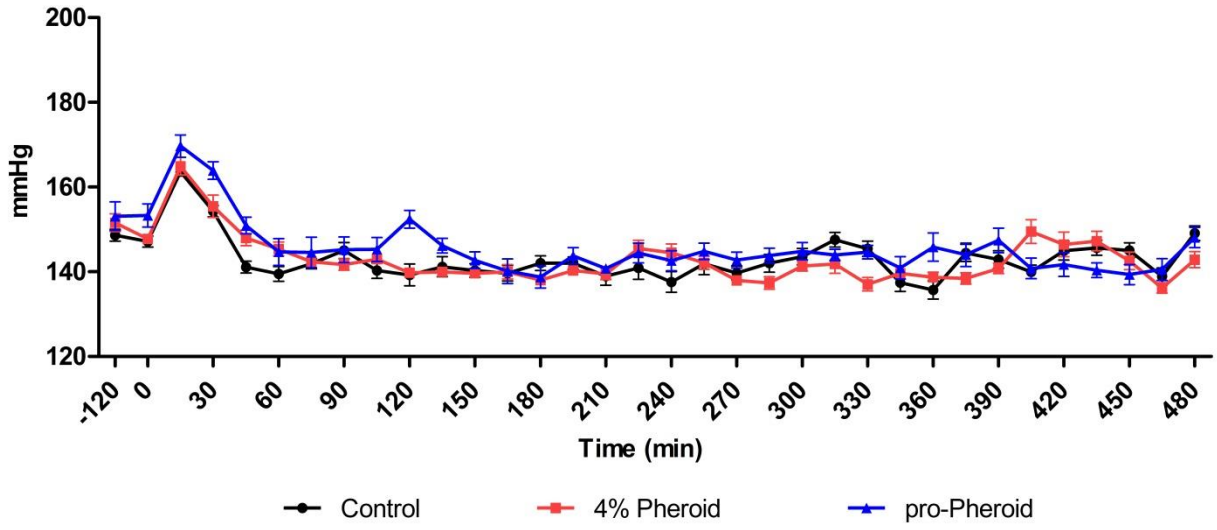

Figure 6: The effect of an oral administration of 4% Pheroid® and pro-Pheroid® on systolic blood pressure versus control. Values are presented as absolute values and depicted as mean ± SEM (N=6). * $P < 0.05$ versus the control group.

Figure 7 illustrates the effects of an oral administration of Pheroid® on DBP. Though the pro-Pheroid® formulation was associated with a higher DBP, the DBP was elevated prior to this treatment such that the administration of the pro-Pheroid® did not cause an increase in relationship to the pre-treatment values.

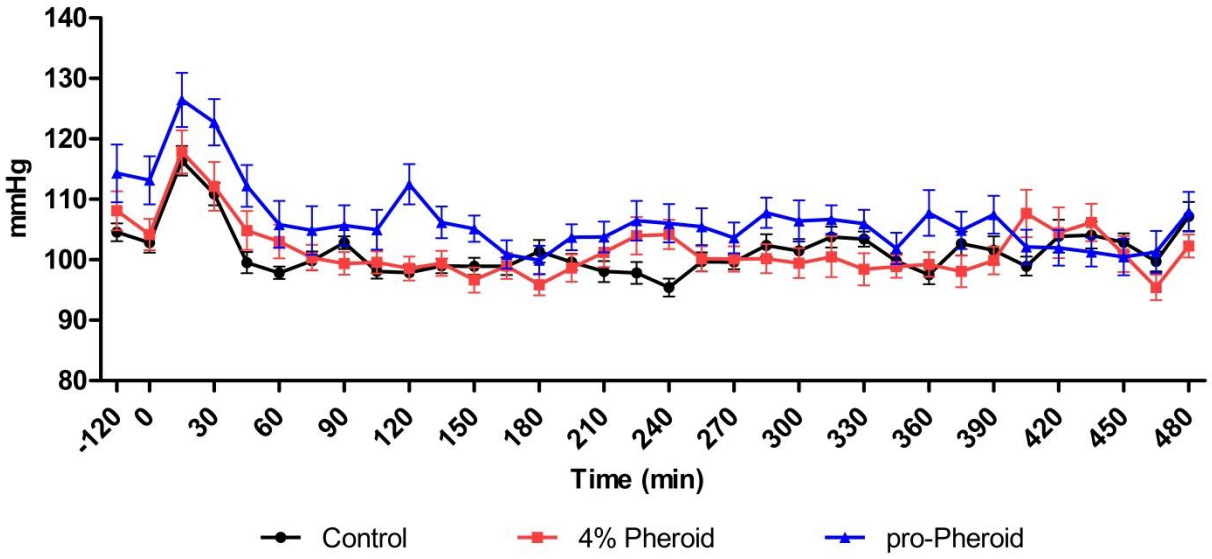

Figure 7: The effect of an oral administration of 4% Pheroid® and pro-Pheroid® on diastolic blood pressure versus control. Values are presented as absolute values and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

No effects on heart rate were seen after the oral administration of Pheroid® (see Figure 8 below.)

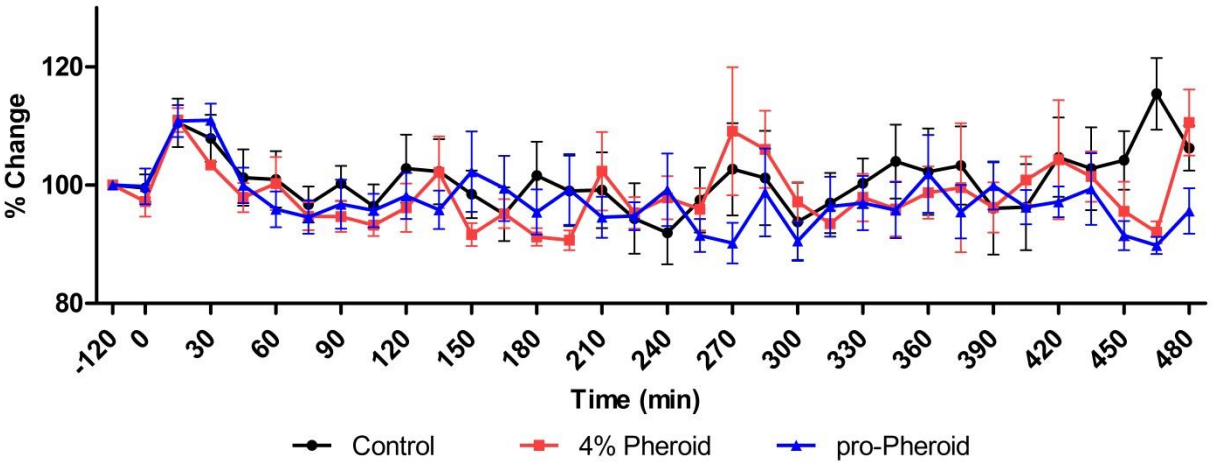

Figure 8: The effect of an oral administration of 4% Pheroid® and pro-Pheroid® on heart rate versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

Figure 9 shows the effect of the oral administration of Pheroid® on PR interval. Both the 4% Pheroid® and pro-Pheroid® treatments were not different from control.

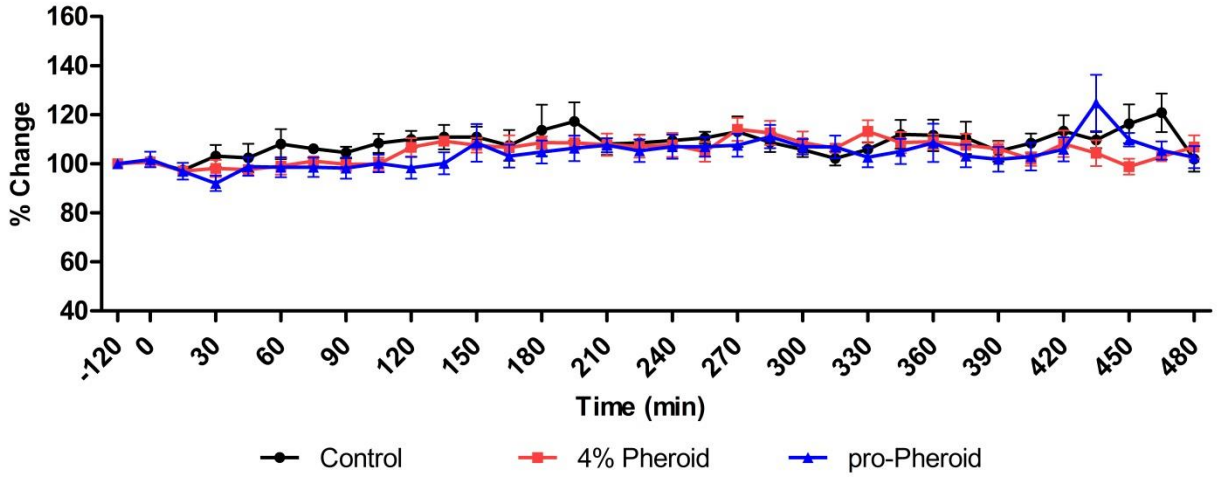

Figure 9: The effect of an oral administration of 4% Pheroid® and pro-Pheroid® on PR interval versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

Figure 10 shows the effects of an oral administration of 4% Pheroid® and pro-Pheroid® on the QT interval versus control. No effects were seen.

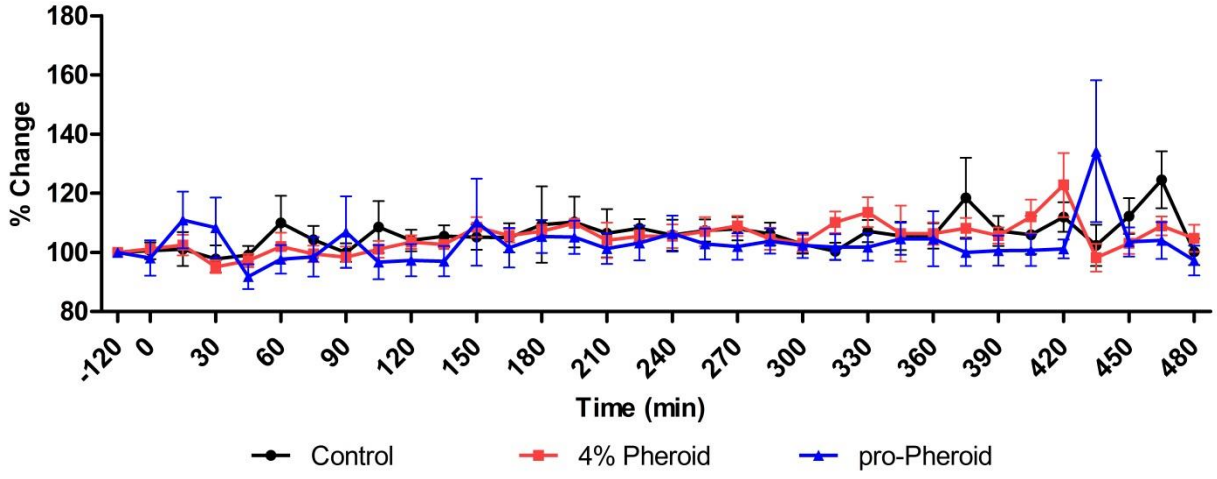

Figure 10: The effect of an oral administration of 4% Pheroid® and pro-Pheroid® on QT interval versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

The oral administration of 4% Pheroid® and pro-Pheroid® had no effect of BT when compared to the control (see Figure 11).

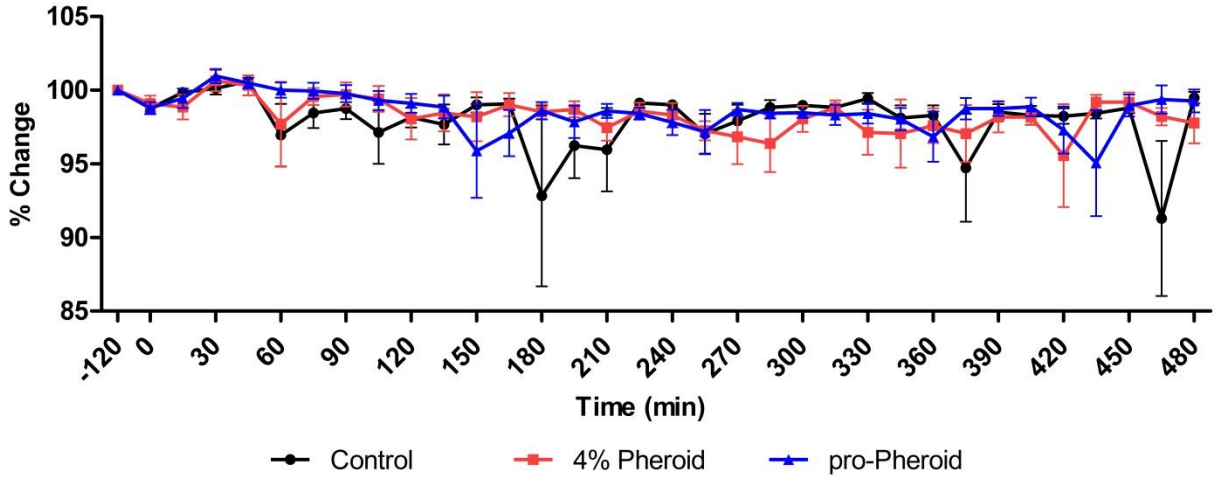

Figure 11: The effect of an oral administration of 4% Pheroid® and pro-Pheroid® on body temperature versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

3.4 Intravenous administration of 4% and 10% Pheroid®

The effects of an intravenous administration of 4% and 10% Pheroid® on arterial BP, SBP, DBP, HR, PR interval, QT interval and body temperature are summarized in Figure 12-18 respectively. None of the formulations had any significant effects on any cardiovascular parameters.

Figure 12 below illustrates the lack of effects on arterial BP after an intravenous administration of 4% Pheroid® and 10% Pheroid®.

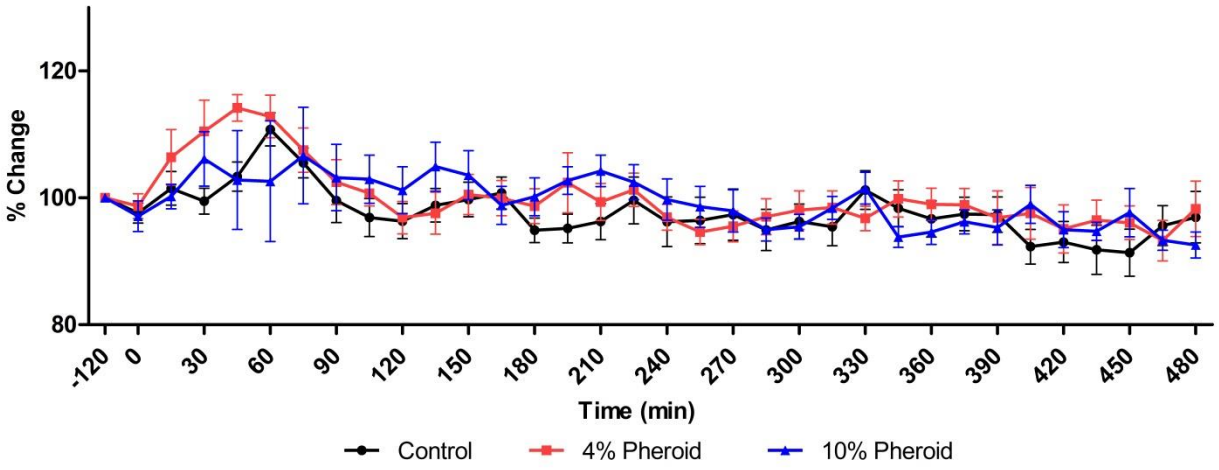

Figure 12: The effect of an intravenous administration of 4% Pheroid® and 10% Pheroid® on arterial blood pressure versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

The intravenous administration of both 4% Pheroid® and 10% Pheroid® had no effect of SBP versus control (see Figure 13).

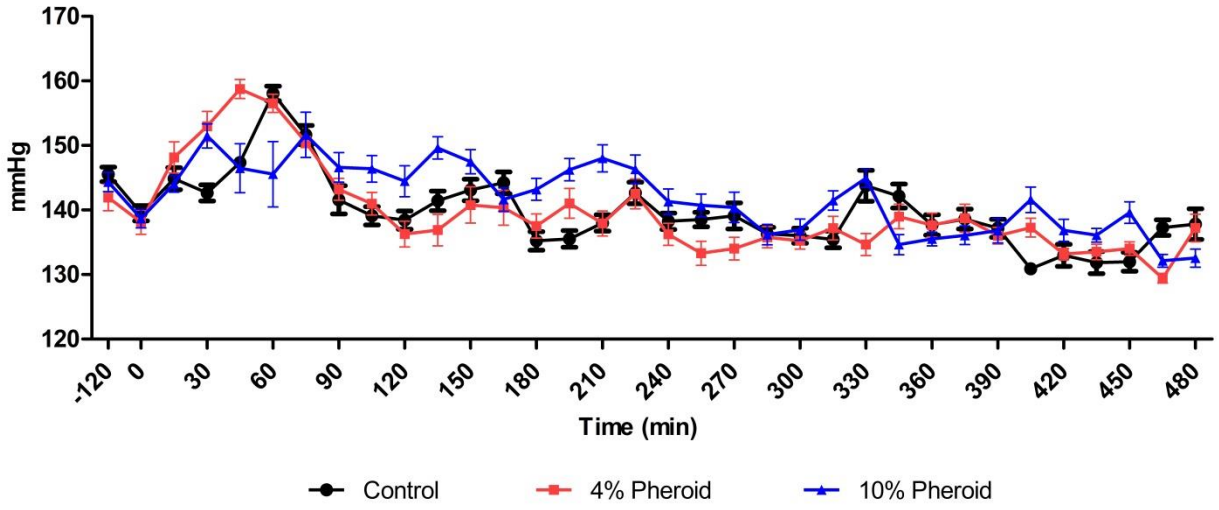

Figure 13: The effect of an intravenous administration of 4% Pheroid® and pro-Pheroid® on systolic blood pressure versus control. Values are presented as absolute values and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

The intravenous administration of 4% Pheroid® caused a transient increase in DBP immediately after administration, which dropped to around 75 min after administration. No effects of 4% Pheroid® or 10% Pheroid® on diastolic blood pressure were found (see Figure 14).

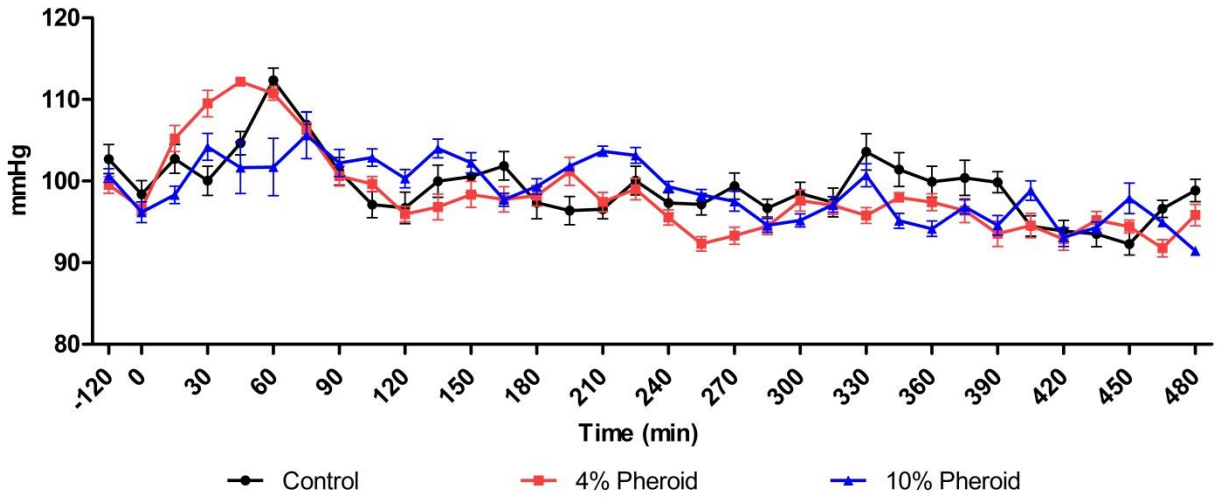

Figure 14: The effect of an intravenous administration of 4% Pheroid® and 10% Pheroid® on diastolic blood pressure versus control. Values are presented as absolute values and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

Neither the 4% Pheroid® or 10% Pheroid® had any effect on HR after intravenous administration (see Figure 15)

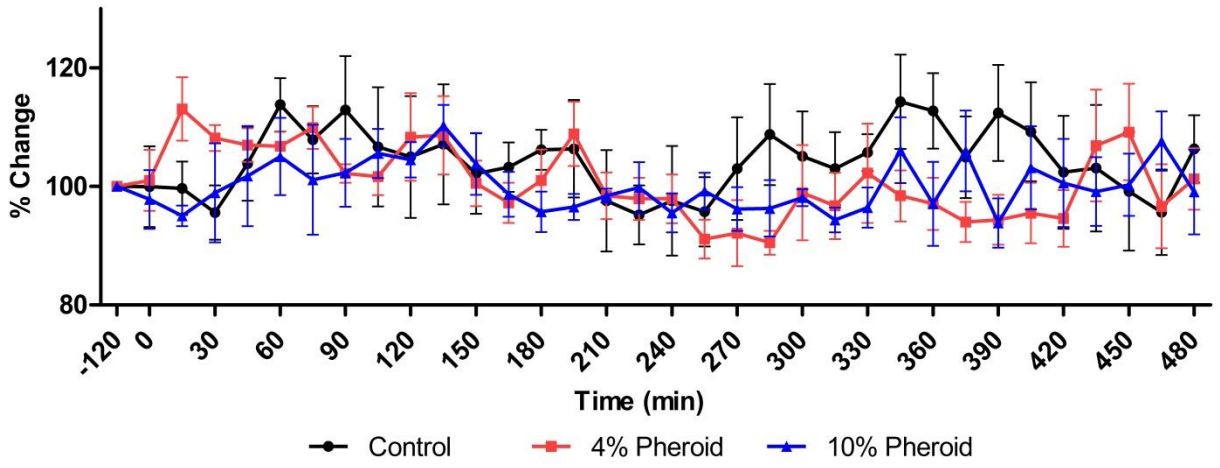

Figure 15: The effect of an intravenous administration of 4% Pheroid® and 10% Pheroid® on heart rate versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

No effects were seen on PR interval after the intravenous administration of 4% Pheroid® and 10% Pheroid® (see Figure 16)

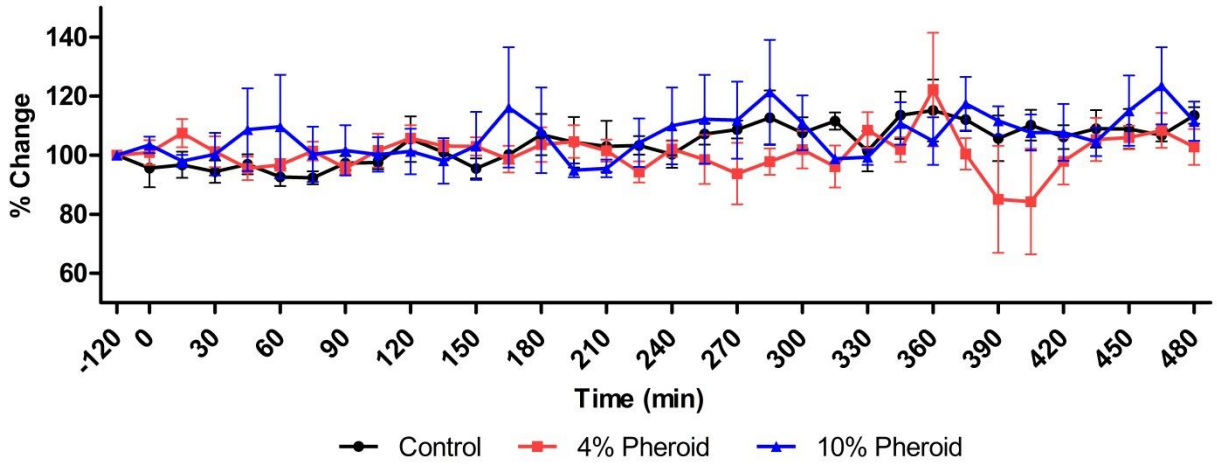

Figure 16: The effect of an intravenous administration of 4% Pheroid® and 10% Pheroid® on PR interval versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * $P < 0.05$ versus the control group.

After administration (IV), the 10%Pheroid® seemed to increase the QT interval transiently, but by 120 min following administration it had returned to the control level suggesting that it may not have been a treatment-related effect. In general, QT interval prolongation is a consequence of the inhibition of the hERG channel either by pharmaceuticals or congenital disease (Crumb and Cavero, 1999). Based on Pheroid®'s composition and the effect being only transient, it is highly unlikely that the 10% Pheroid® is a hERG blocker. Nonetheless, this was an unexpected finding with no observable explanation. No significant effects were seen on QT interval after the intravenous administration of 4% Pheroid® (see Figure 17).

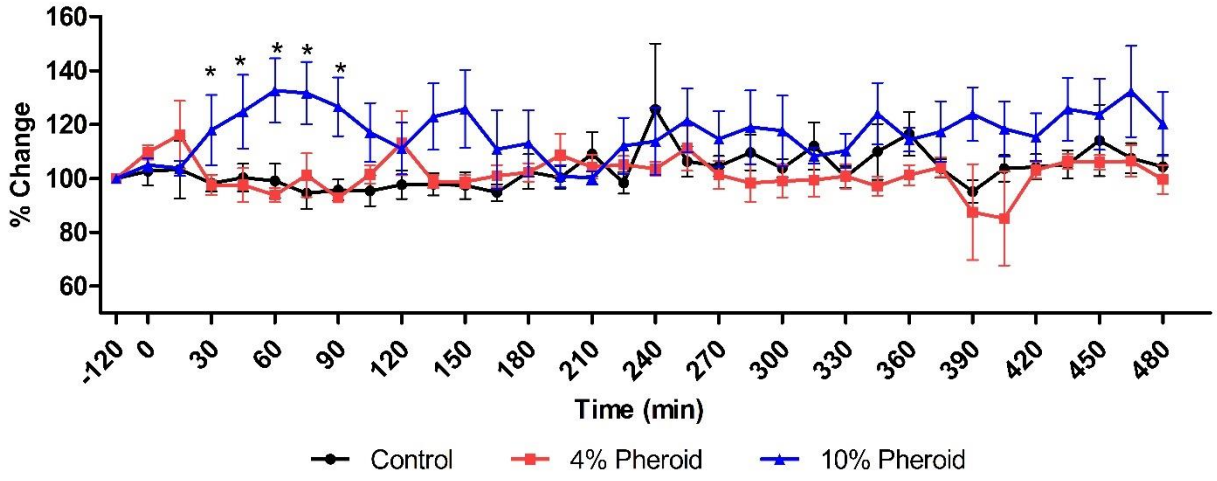

Figure 17: The effect of an intravenous administration of 4% Pheroid® and 10% Pheroid® on QT interval versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

Figure 18 below illustrates the effect of an intravenous administration of 4% Pheroid® and 10% Pheroid® on BT. No significant effects were observed.

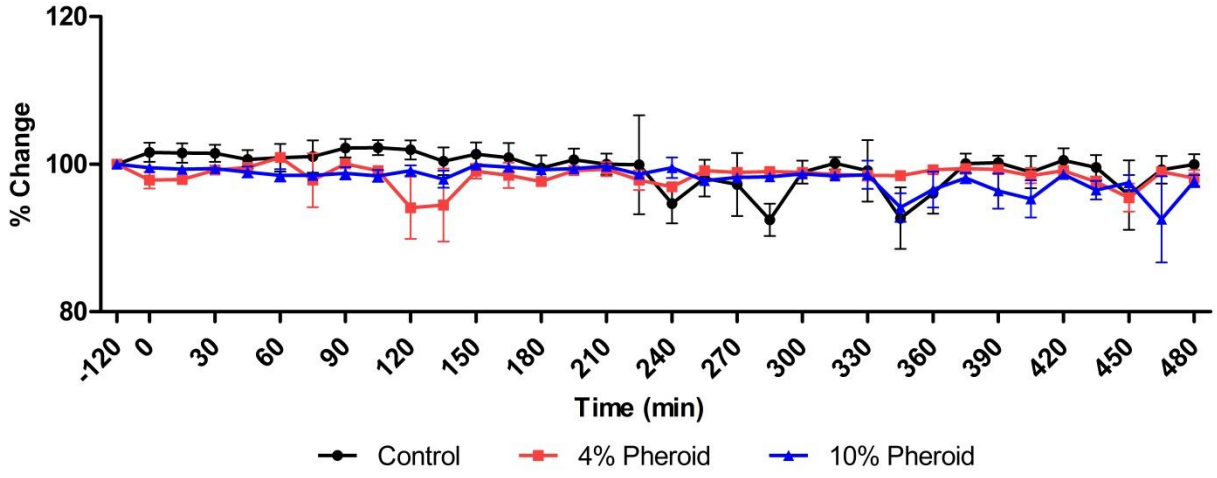

Figure 18: The effect of an intravenous administration of 4% Pheroid® and 10% Pheroid® on body temperature versus control. Values are presented as % change from baseline and depicted as mean ± SEM (N=6). * P < 0.05 versus the control group.

4. Discussion

The purpose of this study was to assess the possible cardiovascular effects of the Pheroid® drug delivery system given by oral and intravenous administration routes.

By using clonidine and theophylline as references, we were able to establish the model's sensitivity to detect haemodynamic changes. While we expected to see hypotensive effects of clonidine, no significant decrease in BP was observed. This could be explained in part by clonidine's pharmacology as an α_2-agonist, where hypotensive effects of the drug could be observed only in spontaneously hypertensive rat models (SHR) or anesthetized rats (Delaunois *et al*., 2009). Nonetheless, bradycardic and hypothermic effects were clearly observed with clonidine along with prolongation of both PR and QT intervals. The second reference compound, theophylline, was expected to have a much different cardiovascular profile than clonidine. Theophylline was administered initially at a dose of 10 mg/kg (p.o.) but was later increased to 30 mg/kg (p.o.). Even so, theophylline increased BP to a small extent, but this was still not statistically significant. A very clear increase in HR was observed after the administration of theophylline along with a decreased PR interval and prolonged QT interval. One could argue that the effect in the rat is only slight, due to the fact that the ion channel and repolarization mechanism of the rat is different to that of man (Anonymous, 1995; Tashibu *et al*., 2005).

The reported changes in cardiovascular function brought on by clonidine and theophylline were satisfactory to validate the telemetry system in the present study. We also have to mention the recurring appearance of an increased HR immediately after the administration of test compounds, regardless of their pharmacology. However, this effect was not observed in the rats receiving clonidine, instead inducing an immediate decrease in HR after administration which could be attributed to its potent pharmacological action as an antihypertensive drug administered intraperitoneally.

Though the sensitivity of the system was successfully established, it did not detect any significant hemodynamic effects of the Pheroid® formulations. Given its newly confirmed cardiovascular safety, more applications of Pheroid® could be considered without the restraint of safety issues. In one example, Pheroid® is investigated in a future phase 1 clinical human trial which aims to determine the effect of a Pheroid®-cannabidiol (CBD) formulation on the bioavailability of CBD. Other *in vivo* studies using Pheroid® include the enhancement of bioavailability and efficacy of various antimalarial drugs, salmon calcitonin and anti-tuberculosis drugs (Chelopo-Mgobozi, 2018; du Plessis *et al*., 2010; du Plessis *et al*., 2014; du Plessis *et al*., 2015; Steyn *et al*., 2011).

Possible future studies using Pheroid® may include its role in the prevention and treatment of various cardiovascular diseases. Indeed, Pheroid® is composed of omega-3 and omega-6 fatty acids. The former being known to be positively associated with the prevention of diabetes mellitus (type 1), asthma and even coronary heart disease (Hu *et* al., 2002; Simopoulos, 2008; Kris-Etherton *et al*, 2002). This is further supported by the Diet and Reinfarction Trial (DART) clinical trial that focussed on the effects of omega-3 in the prevention of CHD and described a reduction

of mortality in males due to myocardial infarction of up to 29% after a recommended increase of +/- 700 mg/day of omega-3 (Burr *et al*., 1989). Omega-6 fatty acids could also play a key role in the reduction of low-density lipoprotein (LDL) cholesterol, thereby lowering the risk of coronary heart disease (Mensink & Katan, 1992). Pheroid®'s role in the prevention of cardiovascular disease should be further investigated.

5. Conclusion

In this study we demonstrated the lack of cardiovascular effects of the Pheroid® delivery system in the conscious rat following both oral and intravenous administration.

Acknowledgements:

We would like to thank Katrin Christ and Cor Bester for performing all surgical procedures.

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Declarations of interest: None

Funding:

This work did not include any specific grant from funding agencies in the public, commercial, or not-for-profit sector.

CHAPTER 6: DETERMINATION OF THE EFFECTS OF A PHEROID®-CANNABIDIOL FORMULATION ON OPEN FIELD BEHAVIOUR IN RATS

Title

Determination of the effects of a Pheroid®-cannabidiol formulation on open field behaviour in rats.

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Abstract

Introduction: The purpose of this paper is two-fold; to evaluate the central nervous safety of the Pheroid® drug delivery system in combination with cannabidiol (CBD) in conscious rats in preparation for its possible use in clinical trials, and to determine whether the Pheroid® would enhance the behavioural effects of CBD on the central nervous system. **Methods:** 18 male rats were divided into three groups of six and were used in a crossover design to determine the effects of treatment using an open-field test. The treatments tested included CBD (10 mg/kg), pro-Pheroid®-CBD (10 mg/kg) and a saline control. **Results:** No significant effects on central nervous function was observed after treatment with the Pheroid®-CBD formulation on central nervous function. **Discussion:** We have demonstrated that the Pheroid®-CBD formulation does not enhance the effects of a pure CBD administration and in itself has no behavioural effect on central nervous function, thereby ensuring its safety for further pharmaceutical applications.

Keywords: Cannabidiol; Pheroid®; Pre-Clinical Drug Development Platform; Rat; Safety pharmacology

Abbreviations:

µm: Micrometre

AUC: Area under the curve

CBD: Cannabidiol

CLSM: Confocal laser microscopy

C_{max}: Maximum concentration

CNS: Central nervous system

D(0.5): 50% of particles in the sample are smaller than the indicated size

ICH: International Conference on Harmonization

Ml: Millilitre

mV: Millivolts

N_2O: Nitrous oxide

NaCl: Sodium Hydrochloride

nm: nanometre

NWU: North-West University

NWU: North-West University

PCDDP: Pre-Clinical Drug Development Platform

Rpm: Revolutions per minute

SA: South Africa

™: Trademark

Wt. %: Weight percentage

1. Introduction

The purpose of this paper is to evaluate the central nervous safety of a novel drug delivery system in combination with CBD in animals in preparation for its use in clinical trials. This drug delivery system is the Pheroid®, a system of which pharmaceutical applications are patented by the North West University (NWU). The Pheroid® drug delivery system was created to enhance the absorption of pharmacological and biological compounds by entrapping hydrophilic, hydrophobic and amphiphilic compounds in its nano- and micro-vesicles (Grobler, 2013). The pro-Pheroid® used in this study is a precursor format of the Pheroid® and was developed for oral administration and forms its vesicles *in vivo* post-administration (Grobler, 2013). It is important to note that the Pheroid® can also be administered orally, but the pro-Pheroid® is more suitable for encapsulation and therefore was selected for this study.

Due to its poor water solubility, CBD has been reported as having a low oral bioavailability of approximately 9% (Cherniakov *et a*., 2017). Therefore, this study was conducted to support a future phase 1 clinical trial in which the bioavailability of various Pheroid®-cannabinoid formulations will be determined. In a previous study, the Pheroid® has been proven to increase the bioavailability of cannabinoids in rodents (Cloete, unpublished). Since the bioavailability of the Pheroid®-CBD formulations will soon be investigated in humans, any potential adverse effects of Pheroid on physiological function should be determined beforehand. Cannabidiol (CBD) is a non-psychomimetic member of the cannabinoid family, which constitutes around 40% of the *Cannabis sativa* plant extract (Bergamaschi *et al.,* 2011). Cannabinoids are considered to be responsible for most of the pharmacological effects associated with the use of the cannabis plant, due to its agonistic action on CB_1 and CB_2 cannabinoid receptors. These receptors are distributed throughout the body, most notably in the central nervous system, heart, arteries and other tissues such as the spleen, gastrointestinal and urinary tracts and the reproductive system (Grotenhermen, 2004). Therefore, cannabinoids have the potential to affect multiple physiological systems. The effects of cannabinoids on the central nervous system have been under investigation for quite some time (Zuardi, 2008) and there is evidence to support that CBD is involved in complex processes of the cannabinoid system (Scuderi *et al*., 2009). These processes include the processing of memories, pain modulation, motor coordination and neuroprotection and this is due to cannabinoid's direct interaction with a number of neurotransmitters such as dopamine, serotonin, glutamate, norepinephrine and acetylcholine. The therapeutic effects of cannabinoids in spastic and movement disorders are related to their interactions with dopaminergic, GABAergic and glutamergic neurotransmitters (Grotenhermen, 2004).

In an unpublished study conducted in rodents (Cloete, unpublished), the oral bioavailability of different CBD preparations, including a pro-Pheroid®-CBD formulation, was determined and

compared with commercially available CBD oils. By comparing the pharmacokinetic profiles of the different formulations, the study found that the pro-Pheroid®-CBD formulation had higher CBD levels in the plasma compared to the other test formulations (Cloete, unpublished) (unpublished). Given the increase in CBD plasma levels when used with Pheroid®, it is of interest to test whether the pharmacological responses to cannabinoids are also affected.

To better understand and investigate the effects of a pro-Pheroid®-CBD formulation on the central nervous system, the locomotor activity metre test (open field) was selected, which is based on the studies by Boissier and Simon (1965). This test measures the effect of a substance on locomotor activity, whether it augments exploratory behaviour (such as a psychostimulant) or decreases activity (i.e. a sedative effect).

2. Methods

2.1 Animals

This study and the use of animals were approved by the NWU-AnimCareREC: NWU-00154-18-S5. Eighteen male Sprague-Dawley rats of 8-12 weeks of age were randomly assigned into three groups (PCDDP, North-West University, Potchefstroom, South Africa). The rats were housed in pairs in type II long individual ventilated cages (IVC) (Techniplast S.P.A., Buguggiate, Italy) with corn-cob bedding. The IVC's underwent 60 air changes per hour through HEPA filters with an environment of 22 °C ± 1 °C and 55% ± 10% humidity. A day-night cycle of 12 hours was maintained. The rats were fed a standard laboratory rodent diet (NutritionHUB, Stellenbosch, South Africa) and had access to food and water *ad libitum.*

2.2 Experimental design

The 3 groups of rats were exposed to each intervention, thereby lowering variability and increasing statistical power. To habituate the rats to the open-field chambers, each rat was placed in the open-field arena for 10 min one day prior to testing. Each rat was acclimated and tested in the same arena for the duration of the study. An adequate (~5 half-lives) washout period of 120 hours was provided between interventions, such that the duration of the study was three weeks. The experimental design is shown in Table 1.

Table 1: Experimental design and test groups for the open-field assessment of pro-Pheroid®-CBD

	Week 1	Week 2	Week 3
Group 1	Control	CBD-Saline	pro-Pheroid®-CBD
Group 2	CBD-Saline	Control	pro-Pheroid®-CBD
Group 3	pro-Pheroid®-CBD	CBD-Saline	Control

2.3 Test compounds and treatment

A pharmacokinetic study by (Cloete, unpublished) determined that the pro-Pheroid®-CBD formulation has a T_{max} of 2.9 ± 2.84 hours. Therefore, three hours prior to testing, each rat was given an oral administration of either saline (control), CBD-saline (10 mg/kg), as supported by Linge *et al*. (2016) and Scuderi *et al*. (2009), or pro-Pheroid®-CBD (10 mg/kg), such that the testing was conducted at the expected time of maximal concentration of CBD. The administered test compounds and their respective dosages are shown in Table 2.

Table 2: Dosage of the administered compounds

Treatment	Dose of CBD	Maximum volume per body weight (ml/kg)
pro-Pheroid®-CBD	10 mg/kg	200 µl
CBD-Saline	10 mg/kg	200 µl
0.9% Sodium Chloride Solution	0 mg/kg	200 µl

2.4 Open-field apparatus

The open-field arena consisted of three black boxes (100 cm x 100 cm x 100 cm), each with an open top. As each group consisted of six rats and each arena housed a single rat; each test was run in two consecutive sessions, i.e. three rats per session, per day. Each arena had a video camera mounted above the arena floor, which covered the entire arena. Each session was started by placing the rat inside the arena and ended after 6 min. The first minute of the recording was not evaluated; therefore, each measurement session lasted for 5 min and the video recording was evaluated at a later time.

2.5 Behavioural measures

For the purpose of this study, the total distance travelled (in meters) was measured.

2.6 pro-Pheroid®-CBD formulation

The pro-Pheroid®-CBD formulation was prepared by first formulating pro-Pheroid®. Pro-Pheroid® was prepared by mixing Kolliphor® EL (7.18 g) (BASF Chemicals, South Africa), vitamin F ethyl ester (Chemipo, South Africa) (17.23 g). The mixture was then heated to 70 °C and allowed to cool down to 55 °C at which point Dl-α-tocopherol (Chempure, South Africa) (1.15 g) was added to the mixture. To achieve a 10 mg/kg pro-Pheroid®-CBD dosage, 1.7 g pro-Pheroid® was weighed and allowed to cool. When the latter reached room temperature, 0.3 g CBD powder (BGM Pharmaceuticals, South Africa) was added and mixed well. The mixture was then gassed with N_2O for four days under 200 pKa.

2.7 Characterisation of pro-Pheroid®-CBD

The mean particle size and particle size distribution of the pro-Pheroid®-CBD vesicles used in this study were determined by a light scattering technique with a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). The particle size analyser's laser was positioned in line with deionized water that contained a Pheroid® sample. The samples were stirred continuously to maintain homogeneity while the scattering intensity was captured at 90° and 25 °C. The morphological characteristics of the pro-Pheroid®-CBD vesicles were analysed by confocal laser scanning microscopy (CLSM). The CLSM images were taken with a Nikon D-Eclipse C1 confocal laser scanning microscope with a DXM 1200 digital camera and a 10 pm pinhole. Nile Red was used for fluorescent labelling of the Pheroid® vesicles. The Nile red fluorophore marker has an emission wavelength of between 640 and 650 nm while the CLSM is equipped with three lasers, with wavelengths 405, 488 and 543 nm respectively. The fluorescent light emissions were collected for three different wavelength bands, above 650 nm, 540-640 nm, and 485-545 nm. Pro-Pheroid®, being a lipid-based delivery system, possesses a charge on its surface that interacts with the environment, contributing to the stability of its structure. The stability of the pro-Pheroid®-CBD vesicles was determined by measuring their Zeta potential using a Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, United Kingdom). A particle is generally considered stable if its zeta potential is larger than ±25 mV where the repulsive forces exceed the attractive forces (Crooke, 2007; Roland *et al*., 2003).

2.8 Statistical analysis

Potential differences between the three treatments were determined by one-way analyses of variance (ANOVA) using GraphPad Prism (version 5.02). Post-hoc analyses were executed using Tukey's honest significance difference (HSD), where $P < 0.05$ was considered a significant effect.

3. Results

3.1 pro-Pheroid®-CBD characterization

The average particle size, particle size distribution and zeta potential (mean ± SD) are summarized in Table 3. The pro-Pheroid®-CBD formulation had an average particle size of 1.68 µm ± 1.12 as determined by confocal laser scanning microscopy and a zeta potential of -32.73 ± 3.12 mV, which is very stable (see Annexure C). D(0.5) is a representation of the total volume size of the particles at which 50% of the sample is smaller than the indicated size and 50% is larger than the indicated size.

Table 3: Particle size, zeta potential and particle size distribution of pro-Pheroid®-CBD vesicles

Formulation	Average particle size (µm)	Zeta potential (mV)	D(0.5) (µm)
Pro-Pheroid®-CBD	1.68 µm ± 1.12	-32.73 ± 3.12	26.94 ± 0.11

The pro-Pheroid®-CBD vesicles were relatively large, with some Pheroids almost 20 µm in diameter (see Figure 1). CLSM analysis determined the pro-Pheroid®-CBD formulation to have an average Pheroid concentration of 2.32 x10 10/ml. These measurements were determined by calculating the average of five measurements. The CLSM analysis of the pro-Pheroid®-CBD formulation was deemed satisfactory.

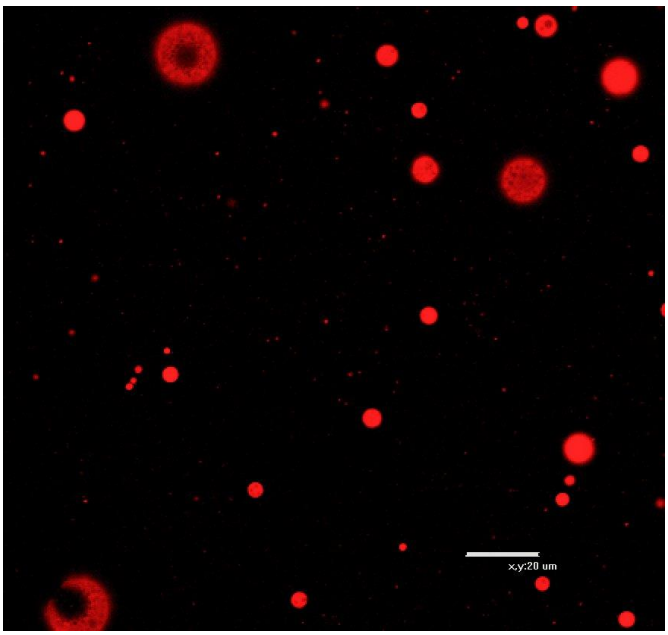

Figure 1: Confocal laser scanning microscopy micrograph of pro-Pheroid®-CBD vesicles

3.2 Open field results

Figure 2 shows the effect of pro-Pheroid®-CBD (10 mg/kg) on distance travelled in comparison to CBD-Saline (10 mg/kg) and the control group. The values are presented as the average distance travelled in meters (mean ± S.E.M). The pro-Pheroid®-CBD formulation (17.4 ± 1.63) had a slightly decreased distance travelled in comparison to both the groups receiving the control (18.87 ±2.41) and CBD-Saline (18.29 ± 1.86) but this was not judged to be significant since post-hoc comparisons between the three groups (Tukey's HSD) revealed no significant differences between any of the groups ($P > 0.05$).

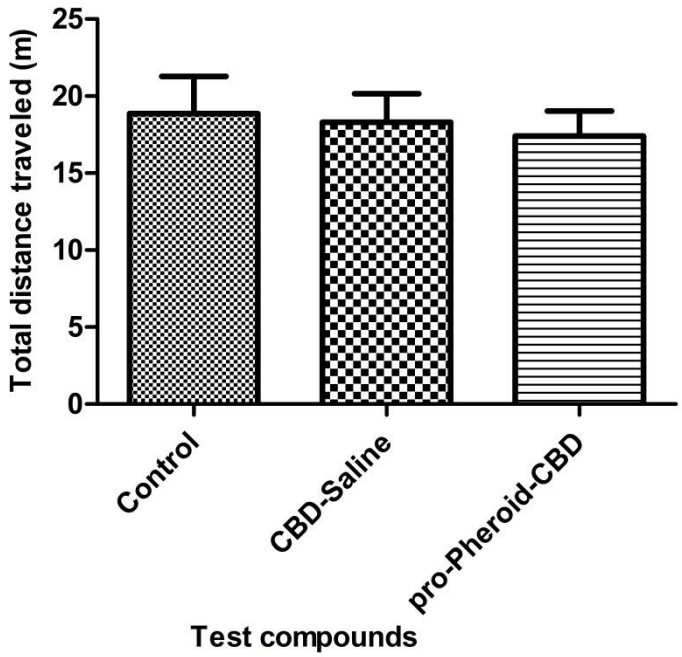

Figure 2: The effects of pro-Pheroid®-CBD (10 mg/kg) on the total distance travelled in comparison to CBD-saline (10 mg/kg) and the control in different groups of rats (n = 6).

4. Discussion

Cloete (unpublished) was able to show that a pro-Pheroid®-CBD formulation (20 mg/kg) increased the bioavailability of CBD in rats. Therefore, it was expected that the pro-Pheroid®-CBD formulation would have an exaggerated pharmacological effect when compared to the rats receiving CBD-Saline and control. However, this was not the case, as all the reported effects such as the decrease in distance travelled of the rats which received CBD-Saline and pro-Pheroid®-CBD was statistically insignificant. Indeed, this observation was first documented in the 1970's (Zuardi, 2008). This is also supported by Crippa *et al* (2004), which reported reduced hypothalamic activity in their human subjects receiving CBD. Furthermore, in 1981, a CBD clinical trial by Carlini & Cunha (1981) reported high levels of somnolence, which further supports its sedative effects. We attribute the lack of effects seen with CBD to the relatively low dosage of CBD used in this study (10 mg/kg).

5. Conclusion

Though Cloete (unpublished) was able to show that the Pheroid® could improve the bioavailability of CBD (C_{max} = 157.04 ± 66.58) compared to other commercial products, it was not able to enhance the observed effects in open-field assessments. However, this lack of effects still

supports its use in clinical trials, as it harboured no effects on central nervous function. A limitation of this study is that we did not do our own analysis of the CBD levels in the blood plasma to confirm sufficient absorption.

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CHAPTER 7: CONCLUSION AND FUTURE PROSPECTS

An in-depth safety profile of novel compounds and drug products, that is, the active ingredient as well as any excipient used, is required during preclinical drug development. One of the most important aspects to investigate is the potential for cardiovascular side effects. This was the rationale in assessing the Pheroid® drug delivery system for potential cardiovascular effects. For this purpose, we used a telemetry-based data acquisition system that allows an evaluation of cardiovascular function in stress free, conscious animals. For the purpose of validating the telemetry system used, a three-way crossover design was used, to demonstrate the detection of effects caused by two reference compounds, theophylline and clonidine. The use of such an experimental design allowed the use of a modest number of animals, while still maintaining statistical power to detect changes in the cardiovascular parameters measured. These changes included theophylline-induced increases in heart rate, prolongation of the QT-interval and a decrease in PR-interval, while clonidine decreased the heart rate and body temperature and prolonged both the PR- and QT-intervals. Three Pheroid® formulations, administered via oral and intravenous routes, were evaluated on cardiovascular function. The precursor form, pro-Pheroid® (100 wt. % oil phase) and the standard 4% Pheroid® (4 wt. % oil phase) were administered orally, while the same 4% Pheroid® and a 10% Pheroid® (10 wt. % oil phase) were administered intravenously. None of the formulations had any effects on cardiovascular parameters, regardless of administration route.

In preparation of a future phase 1 clinical trial (in which the bioavailability of CBD in combination with Pheroid® will be evaluated) and to determine whether Pheroid® would enhance the behavioural effects of CBD, we made use of an open-field test system to measure general movement as an index of behavioural response. For this purpose, we used CBD as a positive control, which did not have any effect. The effect of the pro-Pheroid®-CBD formulation was equivalent to CBD-Saline and could therefore be concluded to have no behavioural effect on central nervous function, and by extension, safe for further application.

Based on these data from the conscious rat, it can be concluded that Pheroid® is devoid of any cardiovascular effects while the pro-Pheroid®-CBD formulation is free of any potential effects on the central nervous system. In retrospect, the CNS evaluation should have included a Pheroid® group as control, instead of saline. This would potentially have given a better indication of the central nervous safety profile of Pheroid®. Regardless, the central nervous safety of the pro-Pheroid®-CBD formulation supports its use in future clinical trials.

Although the data on the central nervous safety of Pheroid® was lacking, the newly reported cardiovascular safety profile of Pheroid® supports further expansion of its pharmaceutical

applications. Indeed, Pheroid® is investigated in a range of applications, including a future phase 1 clinical human trial which aims to determine the effect of a Pheroid®-cannabidiol formulation on the bioavailability of CBD. Other studies include the *in vivo* enhancement of bioavailability and efficacy of various antimalarial drugs (du Plessis *et al*., 2015; Steyn *et al*., 2011). Also, due to its relative low bioavailability, a nasal spray containing salmon calcitonin in combination with Pheroid® was investigated *in vivo* which reported a potential increase in the absorption of salmon calcitonin, thereby potentially increasing the efficacy of the treatment of osteoporosis (du Plessis *et al*., 2010). Another *in vivo* study by Chelopo-Mgobozi (2018) was concerned with the investigation of a PLGA nanoparticle-Pheroid® formulation to potentially improve the bioavailability and efficacy of two anti-tuberculosis drugs, rifampicin and isoniazid. Due to its promising potential, the physiological safety profile of Pheroid was evaluated, to eliminate the risk of adverse effects on cardiovascular and central nervous function; this will support the use of Pheroid in other studies for the enhancement of bioavailability and efficacy of pharmaceutical agents.

Limitations of this study and future prospects:

- While this study was concerned with the cardiovascular evaluation of Pheroid, and the central nervous safety of a pro-Pheroid®-CBD formulation, we did not do a central nervous evaluation of Pheroid® on its own. Future studies could include this assessment in preparation of a full safety profile of Pheroid® using various tests. These tests include the Irwin test which provides an in-depth analysis of the behavioural effects induced by Pheroid® and the rotarod test which would provide more information on the effects of Pheroid® on motor coordination.

- While determining the central nervous effects of a Pheroid®-CBD formulation in rats, the CBD concentrations in the blood plasma was not measured. This could have confirmed whether the CBD was absorbed as expected.

- The use of a rat model for the evaluation of drug-induced effects on hERG channel (ventricular repolarization) is a limitation of this study, since the IKr channel and repolarization mechanisms of the rat is different to that of man. The rat model is however not necessarily an unsuitable model for the detection of all drug-induced effects on the ECG. For example, drug-induced effects on the sodium channels responsible for depolarization as well as calcium channels could be detected in the rat model, should they occur. As such the evaluation of the ECG in the rat is of value even if hERG mediated effects are not detected.

- Future prospects might include the establishment and validation of a whole-body plethysmography system for the respiratory assessment of Pheroid®.

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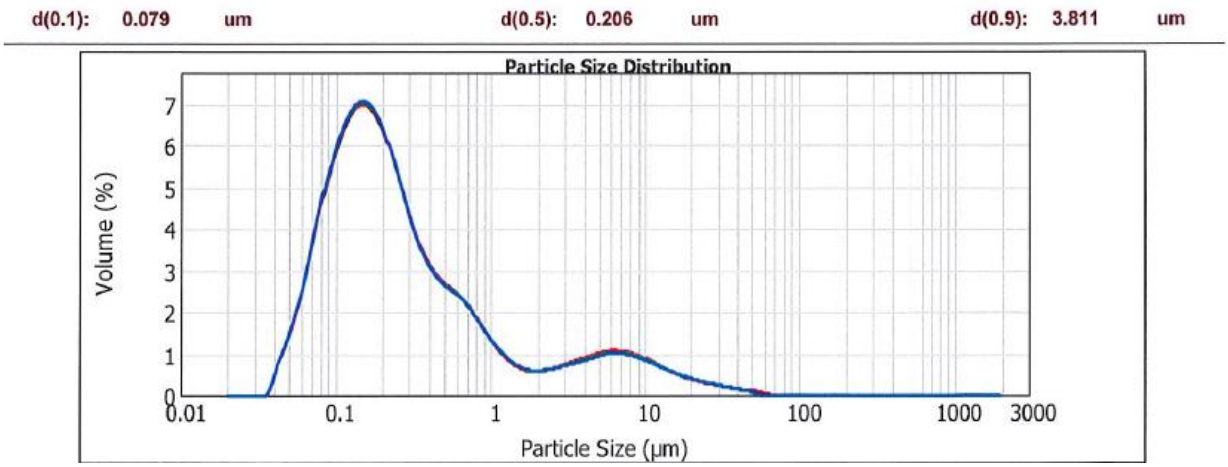
ANNEXURES

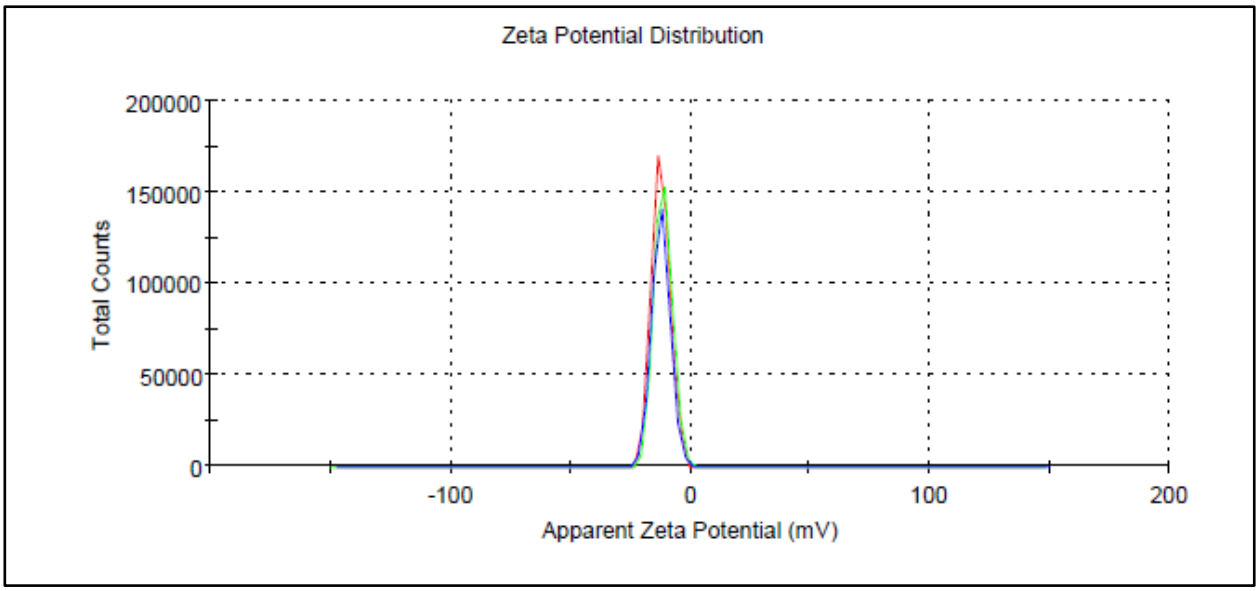
Annexure A: Mastersize and zeta potential analysis of Pheroid® formulations for cardiovascular assessment.

A.1.1 Pro-Pheroid® particle size distribution

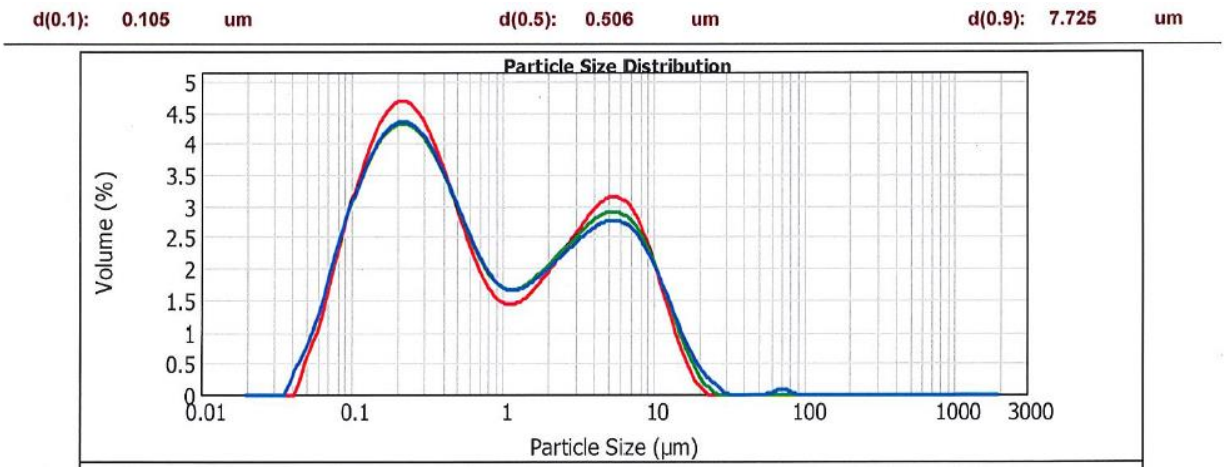


A.1.2 Pro-Pheroid® Zeta potential

Zeta Potential (mV): -12.3

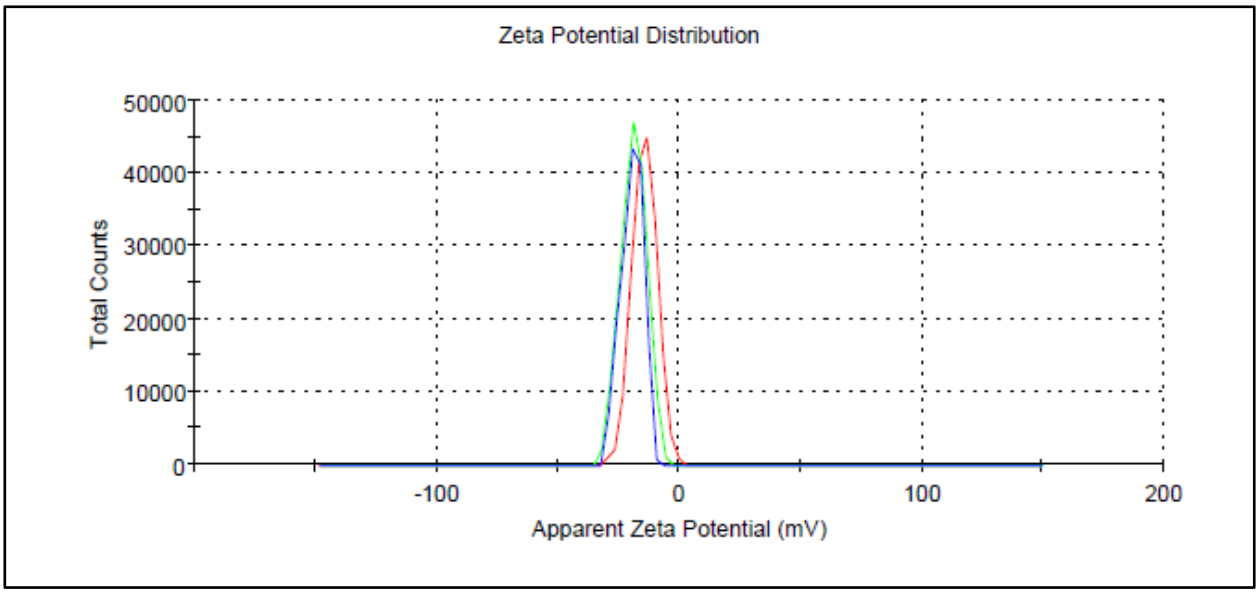


A.2.1 4% Pheroid® (unfiltered) particle size distribution

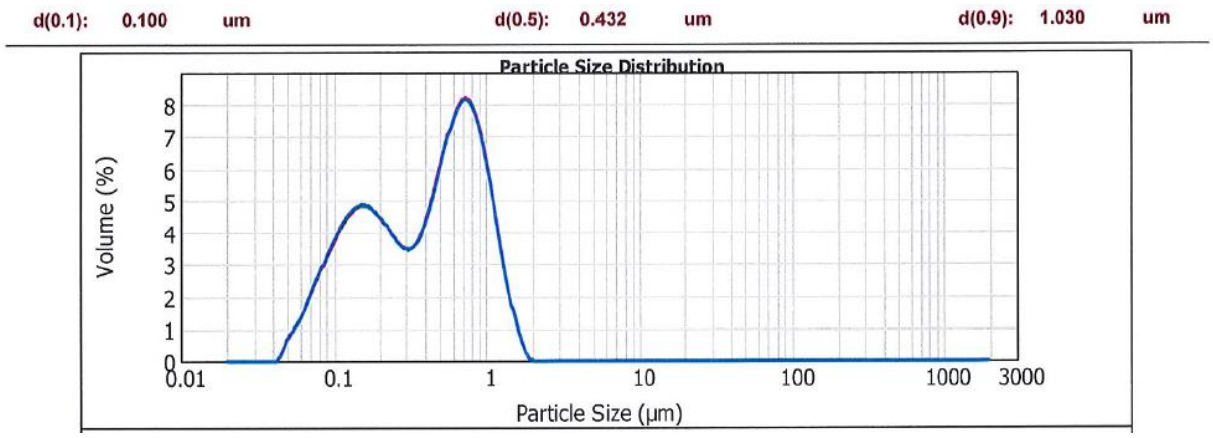


A.2.2 4% Pheroid® (unfiltered) Zeta potential

Zeta Potential (mV): -14.3

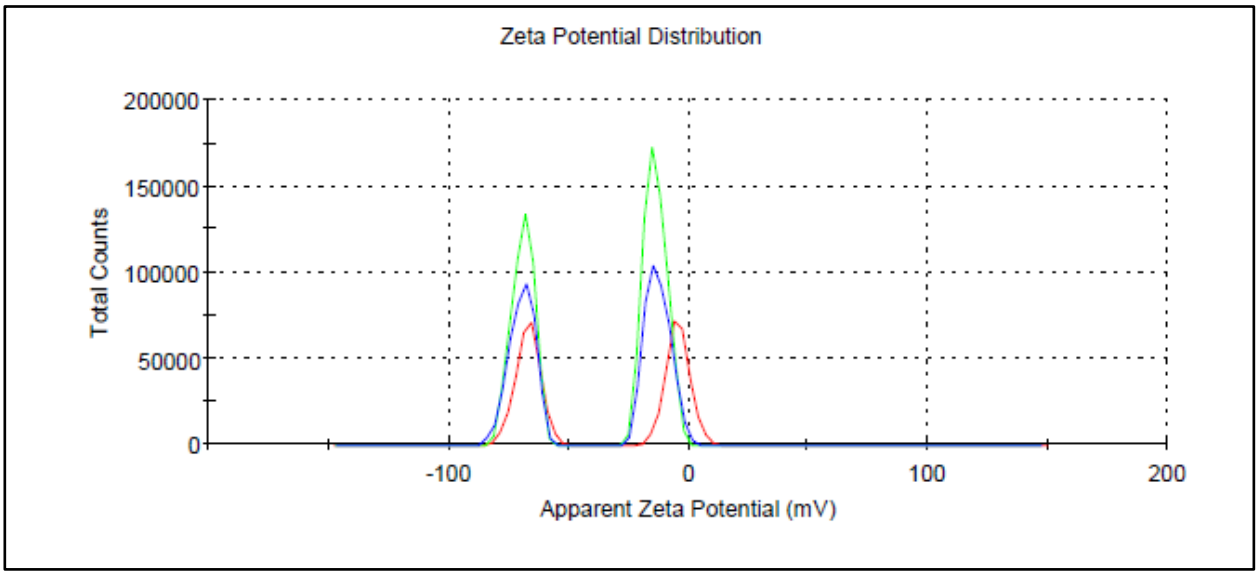


A.3.1 4% Pheroid® (filtered) particle size distribution

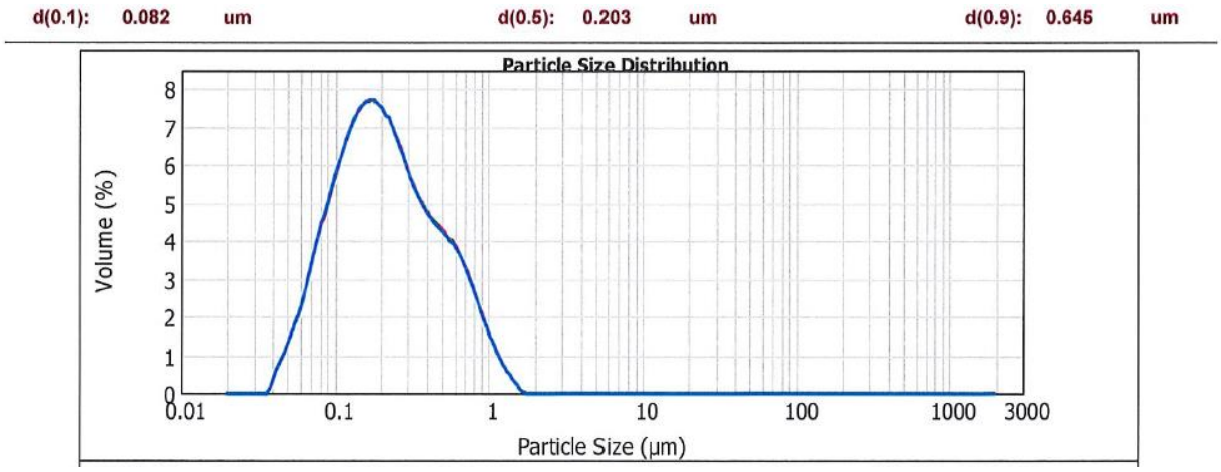


A.3.2 4% Pheroid® (filtered) Zeta potential

Zeta Potential (mV): -36.0

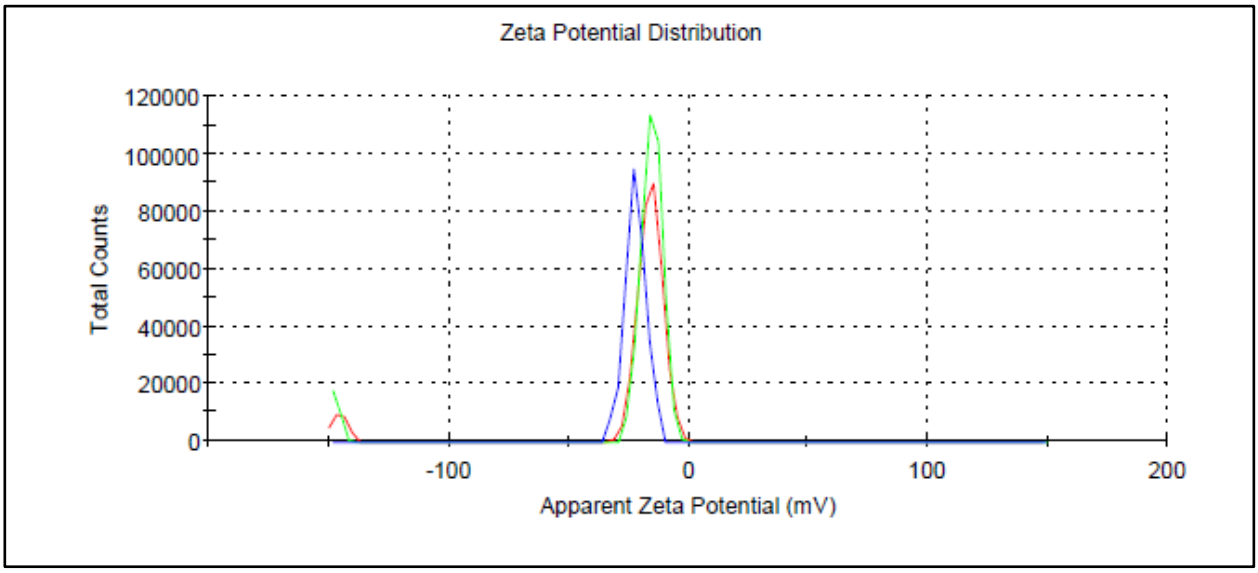


A.4.1 10% Pheroid® (filtered) particle size distribution



A.4.2 10% Pheroid® (filtered) Zeta potential

Zeta Potential (mV): -25.3



Annexure B: Author guidelines for the journal of pharmacological and toxicological methods

JOURNAL OF PHARMACOLOGICAL AND TOXICOLOGICAL METHODS

AUTHOR INFORMATION PACK

DESCRIPTION

Journal of Pharmacological and Toxicological Methods publishes original articles on current methods of investigation used in **pharmacology** and **toxicology**. Pharmacology and toxicology are defined in the broadest sense, referring to actions of drugs and chemicals on all living systems. With its international editorial board and noted contributors, *Journal of Pharmacological and Toxicological Methods* is the leading journal devoted exclusively to experimental procedures used by **pharmacologists** and **toxicologists**.

Benefits to authors

We also provide many author benefits, such as free PDFs, a liberal copyright policy, special discounts on Elsevier publications and much more. Please click here for more information on our author services.

Please see our Guide for Authors for information on article submission. If you require any further information or help, please visit our Support Centre.

AUDIENCE

Pharmacologists, Toxicologists, Biochemists.

IMPACT FACTOR

2017: 2.269 © Clarivate Analytics Journal Citation Reports 2018

ABSTRACTING AND INDEXING

EMBiology
BIOSIS
Chemical Abstracts
Current Contents/Life Sciences
EMBASE
MEDLINE®
International Pharmaceutical Abstracts

Scopus

GUIDE FOR AUTHORS

INTRODUCTION

Journal of Pharmacological and Toxicological Methods publishes articles on methods used in pharmacology, safety pharmacology and toxicology. *Journal of Pharmacological and Toxicological Methods* is the leading international journal devoted exclusively to the elaboration and validation of experimental methods.

Please visit our Pharmacology Author Resources page for guidance on manuscript preparation.

Types of paper

The *Journal of Pharmacological and Toxicological Methods* publishes papers in a range of categories:

"Research paper": description and characterization of a new or modified disease model, method, technique, apparatus or approach to analysis of data. This may include interspecies comparisons that contrast drug actions from a model relevance perspective; "Short communication" detailing simple modifications of an existing model, method, technique, apparatus or approach to analysis of data; "How To" articles that provide step-by-step guidance on the execution of a specific technique; "Appraisal of state-of-the-art" or "Historical review" of particular models, methods, techniques or apparatus; "Methods in drug discovery" - perhaps the most important category. Here, by showing how a method was used to select a drug that was eventually found to be clinically effective, the author establishes validation of the method.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:

• E-mail address

• Full postal address

All necessary files have been uploaded:

Manuscript:

• Include keywords

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

Graphical Abstracts / Highlights files (where applicable)

Supplemental files (where applicable)

Further considerations

• Manuscript has been 'spell checked' and 'grammar checked'

• All references mentioned in the Reference List are cited in the text, and vice versa

• Permission has been obtained for use of copyrighted material from other sources (including the Internet)

• A competing interests statement is provided, even if the authors have no competing interests to declare

• Journal policies detailed in this guide have been reviewed

• Referee suggestions and contact details provided, based on journal requirements

For further information, visit our Support Centre.

BEFORE YOU BEGIN

Ethics in publishing

Please see our information pages on Ethics in publishing and Ethical guidelines for journal publication.

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Human and animal rights

If the work involves the use of human subjects, the author should ensure that the work described has

been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; Uniform Requirements for manuscripts submitted to Biomedical journals. Authors should include a statement in the manuscript that informed consent was obtained for experimentation with human subjects. The privacy rights of human subjects must always be observed.

All animal experiments should comply with the ARRIVE guidelines and should be carried out in accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the authors should clearly indicate in the manuscript that such guidelines have been followed.

Declaration of interest

All authors must disclose any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work. Examples of potential competing interests include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding. Authors must disclose any interests in

two places:

1. A summary declaration of interest statement in the title page file (if double-blind) or the manuscript file (if single-blind). If there are no interests to declare then please state this: 'Declarations of interest: none'. This summary statement will be ultimately published if the article is accepted.
2. Detailed disclosures as part of a separate Declaration of Interest form, which forms part of the journal's official records. It is important for potential interests to be declared in both places and that the information matches. More information.

Submission declaration and verification

Submission of an article implies that the work described has not been published previously (except in the form of an abstract, a published lecture or academic thesis, see 'Multiple, redundant or concurrent publication' for more information), that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere in the same form, in English or in any other language, including electronically without the written consent of the copyright holder.

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manuscript has been accepted. While the Editor considers the request, publication of the manuscript will be suspended. If the manuscript has already been published in an online issue, any requests approved by the Editor will result in a corrigendum.

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Please write your text in good English (American or British usage is accepted, but not a mixture of these). Authors who feel their English language manuscript may require editing to eliminate possible grammatical or spelling errors and to conform to correct scientific English may wish to use the English Language Editing service available from Elsevier's WebShop.

Submission

Our online submission system guides you stepwise through the process of entering your article details and uploading your files. The system converts your article files to a single PDF file used in the peer-review process. Editable files (e.g., Word, LaTeX) are required to typeset your article for final publication. All correspondence, including notification of the Editor's decision and requests for revision, is sent by e-mail.

Additional information

Manuscripts will be evaluated by a member of the board of editors of the Journal, and a panel of international reviewers. An editorial decision will normally be made within 4 weeks of receipt of the manuscript, and publication will normally take place within 3 months of final acceptance.

PREPARATION

Note that papers focused on the actions of drugs are beyond the scope of the journal.

Specific instructions for regular articles & short communications:

Introduction

This must outline the reason for the study and justify the approach taken.

Methods

This section should be sufficiently detailed to permit the reader to replicate the study. It should be a full recipe, with step by step instructions. We prefer the bulk of the descriptions in prose, but tables summarising sequences of procedures are a good accompaniment to the text. Subcomponents of the method that have been described in detail in the literature should be described in full, but appropriate citation of the original source method is mandatory.

Results

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This section should be concise and must not contain repetition of the methods. Data in the text must not replicate data in tables or figures. SI units must be used.

Discussion

The potential value of the data to pharmacological or toxicological or safety pharmacology research methods must be clearly explained, with appropriate reference to existing methods and their limitations. This section must not contain paragraphs dealing with topics that are beyond the scope of the study. Use subheadings for clarity.

Specific instructions for "How To" articles:

“How To" articles provide step-by-step guidance on the execution of specific techniques.

Introduction

For "How To" articles, this section will be very brief, and will simply identify the therapeutic area, the goal of the method, and give mention to published alternatives descriptions if available. It is unlikely that many papers will be cited in this section.

Methods

This section should be sufficiently detailed to permit the reader to replicate the study. It should be a full recipe, with step by step instructions. We prefer the bulk of the descriptions in prose, but tables summarising sequences of procedures are a good accompaniment to the text. Subcomponents of the method that have been described in detail in the literature should be described in full, but appropriate citation of the original source method is mandatory.

Methods

This section should be sufficiently detailed to permit the reader to replicate the study. It should be a full recipe, with step by step instructions. We prefer the bulk of the descriptions in prose, but tables summarising sequences of procedures are a good accompaniment to the text. Subcomponents of the

method that have been described in detail in the literature should be described in full, but appropriate citation of the original source method is mandatory.

Results

This section should be concise and must not contain repetition of the methods. Data in the text must not replicate data in tables or figures. SI units must be used.

Discussion

The potential value of the data to pharmacological or toxicological research methods must be clearly explained, with appropriate reference to existing methods and their limitations. This section must not contain paragraphs dealing with topics that are beyond the scope of the study.

Specific instructions for "Appraisal of State of the Art"-articles:

Appraisal articles are reviews about the current best models. The article should identify and describe the current best model, and discuss the evidence (or lack of) to support this. A good model should demonstrably detect drugs that work (or cause adverse effects) in humans, and demonstrably have few false positives or negatives. This evidence should be presented. The review should contrast the current best model with other available but inferior models, thereby illustrating why one is the state of-the-art model.

Text to be divided into sections according to author choice.

Specific instructions for "Historical review" articles:

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Historical reviews can be more personal and less formal. Senior figures in a field may have decades of experience with models, methods, techniques of apparatus. It is of immense value and interest to the research community to learn the history of the development of a model, understand why one model was abandoned and another developed, and get insight into the thinking behind a model, and the impact of good and bad models in drug development in a particular field. In addition, in many fields many models are still used that are transparently inadequate - it would be of great value to obtain a candid expose from an experienced practitioner as to why this might be, including insight into personal perspective as it changed over the years.

Text to be divided into sections according to author choice

Specific instructions for "Methods in drug discovery" articles:

These articles are specifically intended to showcase, for the wider audience, exactly how methods were applied by a pharmaceutical company in the preclinical development of one of their own drugs. While it is appreciated that companies may wish to keep certain information confidential, it would be of great interest to the reader to be able to understand the logic (or lack of) behind the choice of each preclinical test used in the drug's development. Demonstration that use of a series of models, methods, techniques and apparatus gave rise to a drug that works in man is the closest we can get to a methods validation - a much neglected area. The reader would also benefit by having revealed the logic behind the decision-making that allowed the successful drug to proceed in development, while analogues and other compounds were dropped. Consideration of whether this decision was making based on proof, or on the exercise of judgment, or a mixture of both will be of value to the author as well as the reader as it will identify weak areas in the process for future improvements in pharmacological and toxicological methods.

Text to be divided into the following sections

Brief overview of evidence that drug X is now established as being clinically effective Original hypothesis that triggered the search for a drug if type X Preclinical models used in defining drug X's properties (subsections in sequence, explaining logic behind choice) Outcome of tests (subsections in sequence, explaining logic behind successive decision making) Conclusions

Specific instructions for all articles:

Essential title page information for all articles

Title. Please be concise and informative. Titles are used in information-retrieval systems so avoid abbreviations. **Author names and affiliations.** Please type the family name last (e.g., Michael J Curtis). Type the authors' affiliation addresses below the names. Use letters in lower-case superscript immediately after the author's name and in front of the appropriate address if there are multiple addresses. Provide the full postal address of each affiliation, including the country

name, and, the e-mail address of each author. **Corresponding author.** Clearly indicate the corresponding author and ensure their telephone and fax numbers (with country and area code) are provided. **Present/permanent address.** If an author has moved since the work described in the article was done, or was visiting at the time, a “Present address" (or "Permanent address") may be indicated as a footnote to that author's name. Superscript Arabic numerals are used for such footnotes.

Abstract

This must not exceed 250 words and should be subdivided into four subsections headed: Introduction,

Methods, Results, Discussion.

Keywords

Immediately after the abstract, provide a maximum of 10 keywords (one of which should be "methods") in alphabetical order, using British spelling and avoiding general and plural terms and multiple concepts (avoid, for example, "and" , " of"). These keywords will be used for indexing purposes.

Abbreviations

Be sparing with abbreviations: only abbreviations firmly established in the field may be eligible.

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Peer review

This journal operates a single blind review process. All contributions will be initially assessed by the editor for suitability for the journal. Papers deemed suitable are then typically sent to a minimum of two independent expert reviewers to assess the scientific quality of the paper. The Editor is responsible for the final decision regarding acceptance or rejection of articles. The Editor's decision is final. More information on types of peer review.

Acknowledgements

Collate acknowledgements in a separate section at the end of the article before the references and do

not, therefore, include them on the title page, as a footnote to the title or otherwise. List here those individuals who provided help during the research (e.g., providing language help, writing assistance or proof reading the article, etc.).

Formatting of funding sources

List funding sources in this standard way to facilitate compliance to funder's requirements:

Funding: This work was supported by the National Institutes of Health [grant numbers xxxx, yyyy]; the Bill & Melinda Gates Foundation, Seattle, WA [grant number zzzz]; and the United States Institutes of Peace [grant number aaaa]. It is not necessary to include detailed descriptions on the program or type of grants and awards. When funding is from a block grant or other resources

available to a university, college, or other research institution, submit the name of the institute or organization that provided the funding.

If no funding has been provided for the research, please include the following sentence: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Math formulae

Please submit math equations as editable text and not as images. Present simple formulae in line with normal text where possible and use the solidus (/) instead of a horizontal line for small fractional terms, e.g., X/Y. In principle, variables are to be presented in italics. Powers of e are often more conveniently denoted by exp. Number consecutively any equations that have to be displayed separately from the text (if referred to explicitly in the text).

Footnotes

Footnotes should be used sparingly. Number them consecutively throughout the article. Many word processors can build footnotes into the text, and this feature may be used. Otherwise, please indicate the position of footnotes in the text and list the footnotes themselves separately at the end of the article. Do not include footnotes in the Reference list.

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Electronic artwork

General points

• Use uniform lettering and sizing of your original artwork, and select a font as large as possible

• Use sans serif fonts in your illustrations such as: Arial, Helvetica, Tahoma or Calibri and use only one font throughout the figures

• Number the illustrations according to their sequence in the text

• Use a logical naming convention for your artwork files

• Do not put legends in the figures

• Size the illustrations close to the desired dimensions of the published version

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If your electronic artwork is created in a Microsoft Office application (Word, PowerPoint, Excel) then please supply 'as is' in the native document format. Regardless of the application used other than Microsoft Office, when your electronic artwork is finalized, please 'Save as' or convert the images to one of the following formats (note the resolution requirements for line drawings, halftones, and line/halftone combinations given below):

EPS (or PDF): Vector drawings, embed all used fonts.

TIFF (or JPEG): Color or grayscale photographs (halftones), keep to a minimum of 300 dpi.
TIFF (or JPEG): Bitmapped (pure black and white pixels) line drawings, keep to a minimum of 1000 dpi.
TIFF (or JPEG): Combinations bitmapped line/half-tone (color or grayscale), keep to a minimum of
500 dpi.

Please do not:

• Supply files that are optimized for screen use (e.g., GIF, BMP, PICT, WPG); these typically have a low number of pixels and limited set of colors;
• Supply files that are too low in resolution;
• Submit graphics that are disproportionately large for the content.

Color artwork

Please make sure that artwork files are in an acceptable format (TIFF (or JPEG), EPS (or PDF), or MS Office files) and with the correct resolution. If, together with your accepted article, you submit usable color figures then Elsevier will ensure, at no additional charge, that these figures will appear in color online (e.g., ScienceDirect and other sites) regardless of whether or not these illustrations are reproduced in color in the printed version. **For color reproduction in print, you will receive information regarding the costs from Elsevier after receipt of your accepted article**. Please indicate your preference for color: in print or online only. Further information on the preparation of electronic artwork.

Figure captions

Ensure that each illustration has a caption. Supply captions separately, not attached to the figure. A caption should comprise a brief title (**not** on the figure itself) and a description of the illustration. Keep text in the illustrations themselves to a minimum but explain all symbols and abbreviations used.

Tables

Please submit tables as editable text and not as images. Tables can be placed either next to the relevant text in the article, or on separate page(s) at the end. Number tables consecutively in accordance with their appearance in the text and place any table notes below the table body. Be sparing in the use of tables and ensure that the data presented in them do not duplicate results described elsewhere in the article. Please avoid using vertical rules and shading in table cells.

References

Citation in text

Please ensure that every reference cited in the text is also present in the reference list (and vice versa). Any references cited in the abstract must be given in full. Unpublished results and personal

communications are not recommended in the reference list, but may be mentioned in the text. If these references are included in the reference list they should follow the standard reference style of the journal and should include a substitution of the publication date with either 'Unpublished results' or 'Personal communication'. Citation of a reference as 'in press' implies that the item has been accepted for publication.

Web references

As a minimum, the full URL should be given and the date when the reference was last accessed. Any further information, if known (DOI, author names, dates, reference to a source publication, etc.), should also be given. Web references can be listed separately (e.g., after the reference list) under a different heading if desired, or can be included in the reference list.

Data references

This journal encourages you to cite underlying or relevant datasets in your manuscript by citing them in your text and including a data reference in your Reference List. Data references should include the following elements: author name(s), dataset title, data repository, version (where available), year, and global persistent identifier. Add [dataset] immediately before the reference so we can properly identify it as a data reference. The [dataset] identifier will not appear in your published article.

References in a special issue

Please ensure that the words 'this issue' are added to any references in the list (and any citations in the text) to other articles in the same Special Issue.

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Most Elsevier journals have their reference template available in many of the most popular reference management software products. These include all products that support Citation Style Language styles, such as Mendeley and Zotero, as well as EndNote. Using the word processor plug-ins from these products, authors only need to select the appropriate journal template when preparing their AUTHOR INFORMATION PACK 1 Aug 2018 www.elsevier.com/locate/jpharmtox 11 article, after which citations and bibliographies will be automatically formatted in the journal's style. If no template is yet available for this journal, please follow the format of the sample references and citations as shown in this Guide. If you use reference management software, please ensure that you remove all field codes before submitting the electronic manuscript. More information on how to remove field codes.

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List: references should be arranged first alphabetically and then further sorted chronologically if necessary. More than one reference from the same author(s) in the same year must be identified by

the letters 'a', 'b', 'c', etc., placed after the year of publication.

Examples:

Reference to a journal publication:

Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2010). The art of writing a scientific article. *Journal of Scientific Communications, 163*, 51–59. https://doi.org/10.1016/j.Sc.2010.00372.

Reference to a journal publication with an article number:

Van der Geer, J., Hanraads, J. A. J., & Lupton, R. A. (2018). The art of writing a scientific article. *Heliyon*, *19*, e00205. doi:10.1016/j.heliyon.2018.e00205.

Reference to a book:

Strunk, W., Jr., & White, E. B. (2000). *The elements of style.* (4th ed.). New York: Longman, (Chapter

4).

Reference to a chapter in an edited book:

Mettam, G. R., & Adams, L. B. (2009). How to prepare an electronic version of your article. In B. S.

Jones, & R. Z. Smith (Eds.), *Introduction to the electronic age* (pp. 281–304). New York: E-Publishing

Inc.

Reference to a website:

Cancer Research UK. Cancer statistics reports for the UK. (2003). http://www.cancerresearchuk.org/

aboutcancer/statistics/cancerstatsreport/ Accessed 13 March 2003.

Reference to a dataset:

[dataset] Oguro, M., Imahiro, S., Saito, S., Nakashizuka, T. (2015). *Mortality data for Japanese oak wilt disease and surrounding forest compositions*. Mendeley Data, v1. https://doi.org/10.17632/

xwj98nb39r.1.

Reference to a conference paper or poster presentation:

Engle, E.K., Cash, T.F., & Jarry, J.L. (2009, November). The Body Image Behaviours Inventory-3:
Development and validation of the Body Image Compulsive Actions and Body Image Avoidance Scales.
Poster session presentation at the meeting of the Association for Behavioural and Cognitive Therapies,
New York, NY.

Journal abbreviations source

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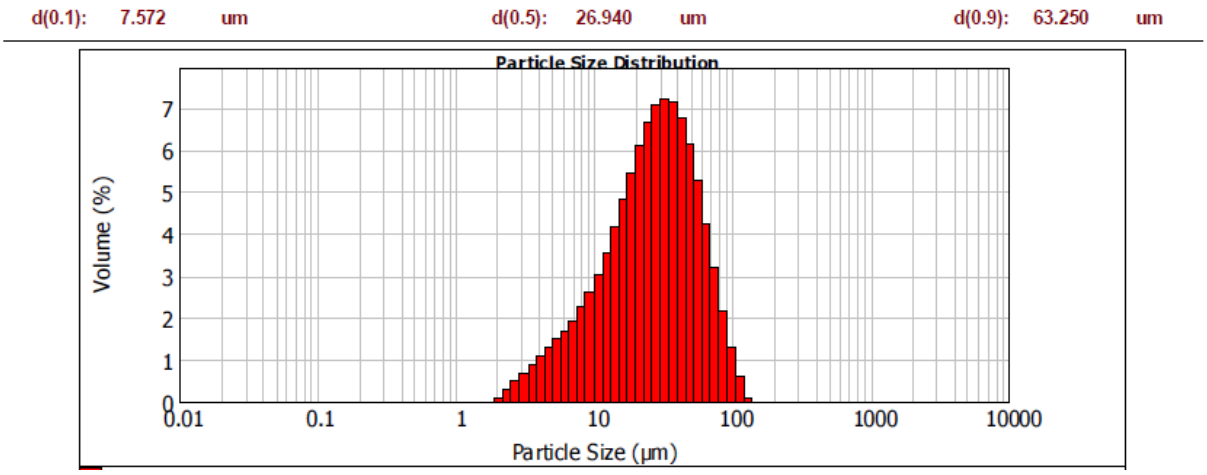
Annexure C: Mastersize and zeta potential analysis of Pheroid®-CBD formulation for central nervous assessment.

C.1.1 Pheroid®-CBD particle size distribution



C.1.2 Pheroid®-CBD Zeta potential

Zeta Potential (mV): -33.0