UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Synthesis of cardiotonic steroids from 14C-(4C)-labelled cholesterol in rat models of hypertension

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, for the degree of Master of Science in Medicine

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Declaration

I, Hannah Bint Ebrahim Mullah, declare that this dissertation is my own unaided work, except where stated. It is being submitted for the degree of Master of Science in Medicine in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg. The work contained in this dissertation has not been submitted for any degree or examination in this university or any other university.

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Hannah Bint Ebrahim Mullah, on this ___1st___ day of ___November_____ 2022

Professor GP Candy (Supervisor)

Date: 1st November 2022

Dedication

I want to dedicate this dissertation to my parents, Ebrahim and Fahmida, for their support, prayers, finances and unconditional love. To my colleagues and friends, Anza, Sarhana, Madalitso and Brenda, for helping me in times of need and being there every step. To my supervisor, Professor Candy, for giving me this opportunity, guiding me through difficulties, making this period worthwhile and being the best supervisor that guided me through difficulties, making this period valuable and the best supervisor anyone could have asked for. And lastly, to Ezzat for making this journey adventurous, being my partner in crime, helping me overcome obstacles, and being with me through it all.

Abstract

Background. Hypertension a significant risk factor for cardiovascular diseases and organ failure. Recent studies have shown associations between the gut microbiome and hypertension marked by a low species abundance and diversity of the gut microbiome in hypertension. Furthermore, the eradication of *Helicobacter pylori* resulted in significant blood pressure reductions. This data links the role of the gut microbiome and the presence of *H. pylori* to hypertension. However, the proposed mechanism is not understood. This study hypothesised that endogeneous cardiotonic steroids (CTS) which modify Na^+-K^+ ATPase activity and affect arterial pressure, are formed from bile acids, and mediated by the gut microbiome by a presently unknown pathway.

Aim. To demonstrate the conversion of ^{14}C-(C4)-cholesterol to bile acids and CTSs in rat models of hypertension.

Methods. Ethics approval was obtained for the study (AREC Clearance: 2018/09/42/C). Radioactively labelled ^{14}C-(C4)-cholesterol was administered once-off through oral gavage in Spontaneous Hypertensive Rats (SHR) and Wistar Kyoto rat models of hypertension and blood pressure was monitored for 21 days. Faecal samples were collected daily. Bile acids and CTS were extracted with methyl-tert-butyl ether and separated by Ultra-High Performance Liquid Chromatography with mass-spectrometry used for their identification. Standards were available for only a few primary, secondary and CTS compounds.

Results. The findings of this study were 1) that the ^{14}C-(C4)-label was poorly incorporated into primary and secondary bile acids and was not detected in CTS compounds; 2) bufalin was detected in some, not all, of the SHR samples but was absent in the WKY rat samples; 2) digitoxin was detected in both WKY and SHR samples; and 3) correlation of blood pressure increase in WKY, not SHR rats, and in female rats, with the total proportion of measured hydrodeoxycholic acid and lithocholic acid.

Conclusion. Other ^{14}C- or preferably ^{13}C-primary bile acids should be tested to improve conversion to secondary bile acids and CTSs. Measurement of the full bile acid and CTS profiles will elucidate the spectrum of CTSs present in faecal material and will suggest important pathways in the biosynthesis of these compounds. However, this study provides a valuable platform on which future studies can be based when determining the importance of the microbiome in hypertension.

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List of Abbreviations:

3βHSD	3β-hydroxysteroid dehydrogenase
ABP	Ambulatory blood pressure
ACE	Angiotensin converting enzyme
Akt	Protein Kinase B
amu	Atomic mass unit
ATP	Adenosine triphosphate
BP	blood pressure
CA	Cholic acid
CAS	Central Animal Service
CDCA	Chenodeoxycholic acid
cpm	Counts per minute
CTSs	Cardiotonic steroids
DBP	Diastolic blood pressure
DCA	Deoxycholic acid
DSS	Dahl salt-sensitive
EDLFs	Endogenous digitalis-like factors
EGFR	Epidermal growth factor receptor
EO	Endogenous ouabain
ERK	Extracellular regulated kinase
FC	Free cholesterol
FXR	Farnesoid X receptor
GF	Germ-free
GFR	Glomerular filtration rate
GPCRs	G-protein coupled receptors
HPLC	High-pressure liquid chromatography
LC/MS	Liquid chromatography and Mass spectrometry
LCA	Lithocholic acid
LMIC	Low-and middle income countries
m/z	Mass-charge ratio
MBG	Marinobufagenin
MCA	Muricholic acid

MS	Mass spectrometry
MTBE	Methyl-tert-butyl-ether
Na^+-K^+ ATPase	Sodium-potassium ATPase pump
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NO	Nitric oxide
OLC	Ouabain-like compounds
Q-TOF	Quadrupole time-of-flight
RAAS	Renin-angiotensin-aldosterone system
ROS	Reactive oxygen species
RT	Retention times
SBP	Systolic blood pressure
SHR	Spontaneously hypertensive rats
SHRSP	Spontaneously hypertensive stroke-prone rats
SPE	Solid phase extraction
SRD5β	Progesterone 5β-reductase
SSR	Salt-sensitive rats
UDCA	Ursodeoxycholic acid
UP	University of Pretoria
WHO	World Health Organisation
WKY	Wistar Kyoto

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Chapter 1: Literature review

1.1 Hypertension

According to the World Health Organisation (WHO) Global Burden of Disease Study, a rise in blood pressure, referred to as hypertension, is the leading cause of mortality and morbidity worldwide (Campbell *et al*., 2014). Hypertension is a leading risk factor for cardiovascular diseases, including ischaemic heart disease, remodelling or hypertrophy of the heart and heart failure, stroke, aneurysms, kidney failure and metabolic dysfunction (Oparil et al., 2018). Furthermore, both globally and in the low- and middle-income countries (LMIC), the burden of these diseases have increased since the year 2000 (Mills et al. 2020; World Health Organization. 2021). Approximately one-third of the world's population is diagnosed with high blood pressure (Makridakis and DiNicolantonio, 2014) with South Africa ranked within the top ten LMIC in the prevalence of hypertension (Sarki et al. 2015; World Health Organization. 2021).

The prevalence of hypertension is the highest in economically developing countries, particularly in the sub-Saharan African region (Cappuccio and Miller, 2016). Furthermore, 24.4% of the population in South Africa have been diagnosed with hypertension, costing the economy an estimated 7.5% of the healthcare expenditure (Dougherty et al. 1996). Although the highest portion of healthcare expenditure remains directed towards antiretroviral therapy, non-communicable disease deaths exceed that of HIV/AIDS, with cardiovascular disease being the leading cause (Schutte, 2019).

Currently, the causes of hypertension are believed to be multifaceted and arise from a combination of genetic, environmental, as well as behavioural causes. The central factors underlying this disease include a high dietary salt intake and low dietary potassium intake, ageing, stress and a sedentary lifestyle (Bolívar, 2013). Conditions associated with hypertension include central obesity, type-2 diabetes, high fasting blood glucose, and dyslipidemia with an atherogenic, prothrombotic state, and pro-inflammatory state. These are all known and related cardiovascular risk factors due to similar underlying mediators, mechanisms and pathways (Figure 1.1; Alshehri, 2010). The co-occurrence of several of these conditions and risk factors has been termed 'metabolic syndrome' (Swarup *et al*., 2021).

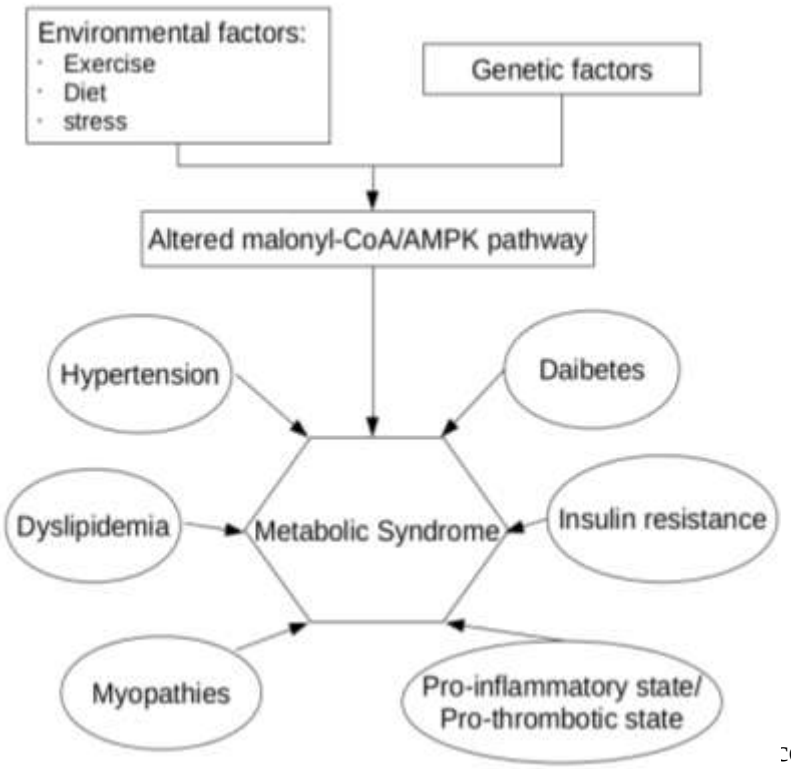


Figure 1.1: A combination of genetic and environmental factors leads to type-2 diabetes, central obesity, hypertension, atherogenic dyslipidemia, prothrombotic state, pro-inflammatory state, and high fasting blood glucose. These are all known cardiovascular risk factors related to similar underlying mediators, mechanisms and pathways. Their co-occurrence is also known as 'metabolic syndrome' (adapted from Alshehri, 2010).

1.2 Factors affecting and determining blood pressure

1.2.1 Nitric oxide

Nitric oxide (NO) is produced from the amino acid L-arginine and is an essential arterial vasodilator, hence a regulator of blood pressure. Oral administration and intravenous infusion of L-arginine in humans prevented platelet-aggregation (Adams *et al*., 1995), led to improved endothelium-dependant vasodilation (Clarkson *et al*., 1996), and decreased monocyte adhesion to the endothelium (Adams *et al*., 1997). However, these data need to be interpreted with caution as the concentration of L-arginine utilised in these studies significantly exceeded physiological concentrations by 3-8 fold (Bode-Böger *et al*., 1998). A decrease in NO bioavailability may cause endothelial dysfunction, reducing vasodilation and increasing blood

pressure (Hermann *et al*., 2006). Changes in the concentration of endothelial NO can occur due to a decrease in L-arginine concentrations, reduced arginine bioavailability, or decreased uptake of L-arginine by the regulation or blocking of its cationic transport system (Luiking *et al*., 2012). Therefore, arginine and the NO pathway significantly maintain vascular homeostasis under normal physiological conditions.

1.2.2 Salt

Diets rich in salt are known to promote hypertension through a mechanism which is not fully understood and is thought to be multifactorial. The integration of the actions of multiple organ systems, including the kidneys, regulates sodium excretion and plays a role in the pathophysiology of hypertension. In mammals, blood pressure regulation and fluid body maintenance are regulated by renal sodium handling (Yang and Xu, 2017).

The mechanism of renal sodium handling is regulated by the renin-angiotensin-aldosterone system (RAAS). It comprises the enzyme renin and its product, angiotensin II and the hormone, aldosterone. Renin is an enzyme released from the kidneys into the bloodstream in response to low blood pressure. It cleaves a protein circulating in the blood, angiotensinogen, to angiotensin I. After that, the angiotensin-converting enzyme (ACE), converts angiotensin I to angiotensin II. Angiotensin II causes vasoconstriction of the arterioles, leading to increased blood pressure (Britannica, 2017). This hormone is usually associated with the "fight or flight" response, increasing cardiac output, vasoconstriction of the arterioles and release of renin.

Furthermore, it triggers the release of aldosterone from the adrenals and vasopressin from the pituitary glands. Aldosterone and vasopressin both cause the kidneys to retain sodium, while aldosterone also causes the excretion of potassium. The increased sodium results in water retention, increasing blood volume and blood pressure (MSD manual, 2021). Atrial natriuretic peptide counteracts this response, inhibiting the RAAS by blocking the release of renin in response to high blood pressure. (Fountain and Lappin, 2020). These processes regulate the glomerular filtration rate, measuring how well the kidneys filter excess fluid and waste products (Dalal *et al*., 2021).

The elucidation of the roles of the renin-angiotensin-aldosterone, vasopressin and sympathetic nervous systems has enhanced our understanding of renal sodium handling and blood volume and pressure regulation. In landmark experiments, de Wardener *et al*. (1961)

suggested the existence of an endogenously circulating "Third Factor", which inhibited the ubiquitous Na^+-K^+ ATPase pump. The Na^+-K^+ ATPase pump is an active ion transporter of Na+ and K+ ions across the plasma membranes of eukaryotic cells, facilitating renal sodium reabsorption and potassium excretion, and thus plays a critical role in the homeostasis of these cations (Castañeda-Bueno *et al*., 2012).

The Na^+-K^+ ATPase pump is a heterodimeric protein consisting of two non-covalently linked α and β subunits. The catalytic α-subunit contains the binding sites for ATP and other ligands, including inhibitors, while the β subunit is essential for protein assembly and membrane delivery (Liu and Shapiro, 2007). Adenosine triphosphate (ATP) is required by the Na^+-K^+ ATPase pump to move Na+ out of cells and inwardly directed K^+ to maintain intracellular Na^+ and K^+ concentrations. The ion gradient generated can keep other passive co-/counter-transporters operating to maintain intracellular ion homeostasis and nutrient uptake (Hauck and Frishman, 2012).

Subsequent studies have identified a number of endogenous digitalis-like factors (EDLFs) or cardiotonic steroids which modify the Na^+-K^+ ATPase pump activity (Table 1.1). These compounds have been discussed in detail in subsequent sections, and their synthesis was the focus of this MSc dissertation.

1.3 Endogenous digitalis-like factors (EDLFs)/Cardiotonic steroids (CTSs)

Cardiotonic steroids (CTS) are cardioactive steroids that play essential roles in health and disease. Recent discoveries, which include the specific identification of endogenous CTSs in humans and delineating an alternative mechanism by which CTSs can signal through the Na^+/K^+-ATPase, have increased the interest in this field substantially. Although CTSs were first considered and necessary in the regulation of renal sodium transport and arterial pressure, recent work implicates these hormones in the regulation of cell growth, differentiation, apoptosis, and fibrosis, the modulation of immunity and carbohydrate metabolism, and the control of various central nervous functions as well as behaviour (Bagrov *et al.*, 2009).

1.4 Structure of CTSs

The main structural features of CTSs are (Cornelius *et al*., 2013; Morsy, 2017; Khalaf *et al*., 2018; El-Seedi *et al*., 2019):

i. In common with all steroids and sterols, a 17-carbon cyclopentanoperhydrophen anthrene fused 4-ring nucleus, consisting of three cyclohexane rings (A, B, and C) and a cyclopentane D ring structure (Figure 1.2 (A)). In CTSs, this aglycone nucleus is structurally distinct to other steroids due to its unique set of fused rings system. This is seen by the way rings AB are *cis*-fused to rings CD, whereas rings BC are trans-fused, giving it a characteristic 'U' shape (Figure 1.5 (B);

ii. a five or six-membered unsaturated lactone ring at position C-17 defines the class of the CTS – the cardenolides (5-membered) and bufadienolides (6-membered), respectively, and plays an essential role in receptor binding (Figure 1.2 (B));

iii. the characteristic C14-beta-hydroxyl group and hydroxyls at positions C3-β-hydroxy is where glycoside group(s) may be attached. Hydroxyl groups can also occur at the C12- and C16- positions, influencing the partitioning of the CTSs into the aqueous media, as well as affecting their duration of action (Figure 1.2 (B));

iv. a glycoside side chain (sugar moiety) may be attached to the C-3 aglycone steroid core (Figure 1.2). The glycoside source causes the size of the CTS and its degree of unsaturation to vary. The sugar moiety consists of a variable number of sugar molecules, from 1-4, attached to the C3β-OH group. L-rhamnose, D-glucose, D-digitoxose, D-digitalose, D-digginose, D-sarmentose, L-vallorose, and D-fructose are the main sugars used. Predominantly, these sugars exist in the β-conformation. The kinetics and lipophilic character of the entire glycoside depend on the presence of acetyl groups on the sugar. Moreover, this variability of sugar molecules and hydroxyl groups causes differences in the polarity of the CTSs, affecting their half-life, degree of absorption, and time to maximal effect (El-Seedi *et al*., 2019; Morsy, 2017; Cornelius *et al*., 2013).

1.5 Cardenolides

Cardenolides are C23-steroids with methyl groups at C-10 and C-13 and consist of an unsaturated 5-membered lactone ring at C-17 (Figure 1.2). They are mostly isolated from plants including the following families: Apocynaceae, Asparagaceae, Asteraceae,

Brassicaceae, Celastraceae, Combretaceae, Crassulaceae, Euphorbiaceae, Fabaceae, Malvaceae, Moraceae, Ranunculaceae, Scrophulariaceae, and Solanaceae (El-Seedi *et al*., 2019). These CTSs are synthesised from a cholesterol-derived C21 steroid, usually progesterone, and contain a 14β-hydroxyl group (Kreis and Müller-Uri, 2010). A study by Dobler *et al*. (2015) also identified some cardenolides from insects, which acquire these CTSs through a sequestration process. This process involves the specific and selective uptake and accumulation of plant toxins for defensive purposes, having gained the ability by co-evolving with cardenolide-containing plants (Dobler *et al*., 2015). Moreover, some cardenolides have also been isolated from bodily fluids and are believed to be produced endogenously in humans. The most commonly identified cardenolides include digoxin, digitoxin and ouabain (El-Mallakh *et al*., 2019).

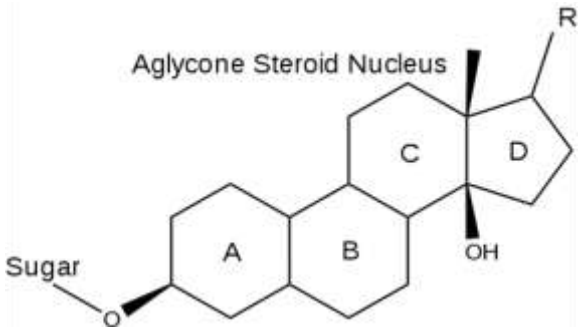
1.6 Bufadienolides

Bufadienolides are C24-steroids contain a 6- membered, double unsaturated lactone ring at C-17 and β-hydroxyl at C-3 (Figure 1.2) (Morsy, 2017). Both bufadienolides and their glycosides are toxic are associated with essential hypertension, pre-eclampsia, other cardiovascular diseases, and kidney disease (Puschett *et al*., 2010). Conversely, drug preparations containing bufadienolides from frog or toad skins have been used as a part of traditional medicine in treating congestive heart failure in the past (Qi *et al*., 2018). Furthermore, bufadienolides are also reported to be involved in many other biological activities, such as antineoplastic and cell growth inhibitory properties, as well as affecting the central nervous system (Qi *et al*., 2018). The most commonly identified bufadienolides include bufalin, marinobufagenin (MBG), proscillaridin A, and telocinobufagenin (Table 1.2., Pavlovic, 2014).

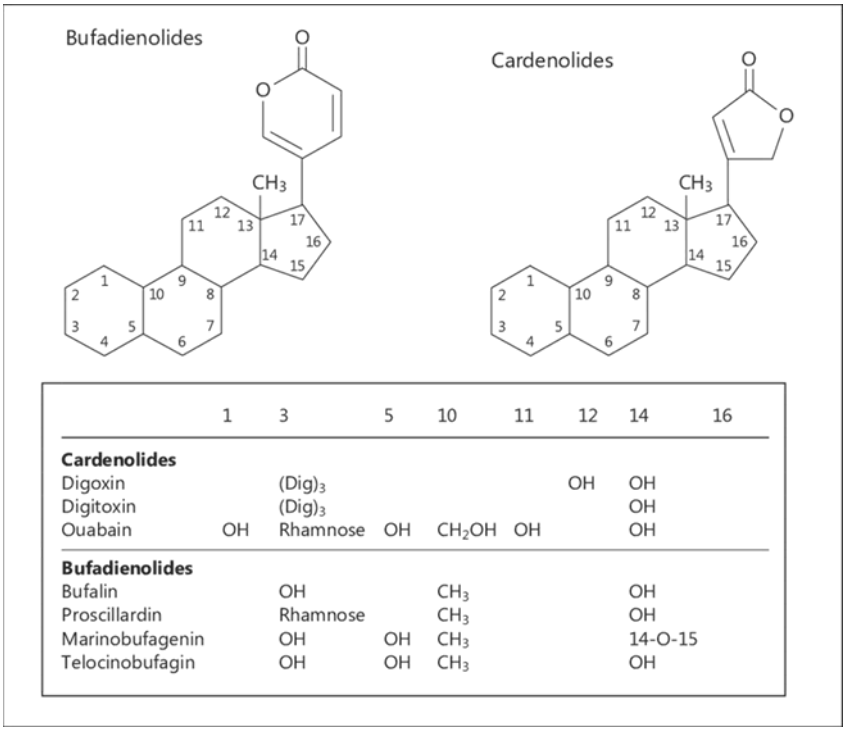
They have been isolated from both plants and animals. The main plant sources include the following families: Crassulaceae, Hyacinthaceae, Iridaceae, Melianthaceae, Ranunculaceae, and Santalaceae. Bufadienolide-containing plants tend to be toxic to livestock, creating agricultural problems (Steyn and van Heerden, 1998). They occur in animals, poisonous amphibians, including the cane toads of the genus *Bufo* (Bókony *et al*., 2019), and have been isolated from Photinus (fireflies) as well as snakes of the genus Rhabdophis (Steyn and van Heerden *et al*., 1998). Some of these CTSs have been identified in human blood and urine (Bagrov *et al*., 2009).

In amphibians, these CTSs are synthesised from cholesterol using the pathway of bile acid production (Siperstein *et al*., 1957; Bagrov *et al*., 2009) which includes cholesterol side-chain cleavage by CYP27A1, an enzyme expressed in the extra-hepatic tissues, including adrenal glands (Federova *et al*., 2015; Dmitrieva *et al*., 2000).

(A)



(B)



	1	3	5	10	11	12	14	16
Cardenolides								
Digoxin		$(Dig)_3$				OH	OH	
Digitoxin		$(Dig)_3$					OH	
Ouabain	OH	Rhamnose	OH	CH_2OH	OH		OH	
Bufadienolides								
Bufalin		OH		CH_3			OH	
Proscillardin		Rhamnose		CH_3			OH	
Marinobufagenin		OH	OH	CH_3			14-O-15	
Telocinobufagin		OH	OH	CH_3			OH	

Figure 1.2: **(A)** The main structural features of CTSs and **(B)** common chemical structures of bufadienolides and cardenolides as well as the substitutions on different positions of described bufadienolide or cardenolide (Pavlovic, 2014). Reproduced with permission from Karger Publishers, CCC License number: 5392901025616 (Oct 20, 2022).

1.7 Mammalian CTSs

A study conducted in 1991 by Hamlyn *et al*.(1991) identified a compound purified by mass spectroscopy in 300L of human plasma that was indistinguishable from ouabain (Hamlyn *et al*., 1991). Studies performed after that confirmed the existence of this compound in multiple bodily fluids and tissues, including digitalis-like compounds in cataractous lenses in humans (Lichtstein *et al*., 1993); a compound identical to MBG in the urine of patients after acute myocardial infarction (Bagrov *et al*., 1998); preeclampsia (Lopatin *et al*., 1999); elevated blood pressure and hypertension (Hauck and Frishman, 2012), cardiac remodelling and kidney disease (reviewed by Paczula *et al*., 2016).

Although most studies report the presence of a single CTS, mammalian plasma contains both ouabain-like and MBG-like immunoreactive compounds (Hamlyn *et al*., 1991; Ludens *et al*., 1991; Oda *et al*., 2001). Immunoreactivity to both MBG and marinobufotoxin (MBT) are at least two compounds that are secreted by adrenomedullary-derived cells in tissue culture (Yoshika *et al*., 2007). Compound/s of EDLFs that cross-react with a polyclonal anti-ouabain antibody have also been demonstrated in the hypothalamic and pituitary extracts of rats (Ferrandi *et al*., 1997) and from the hypothalamus of Dahl salt sensitive (DSS) rats, spontaneously hypertensive rats (SHR), and normotensive rats (Huang and Leenen, 1995).

Immunization against ouabain did not affect blood pressure in normotensive rats but reduced sodium excretion (Nesher *et al*., 2009). In contrast, antibodies against ouabain prevented the development of hypertension in DSS rats (Gomez-Sanchez *et al*., 1994). Furthermore, antibodies against MBG also lowered blood pressure in DSS rats (Federova *et al*., 2002) and antibodies against other glycosides have also reduced blood pressure in animal models (Buckalew, 2015).

Thus, EDLFs have been described in many biological fluids and have been produced in tissue culture. They play a role in salt-sensitive essential hypertension by binding to and inhibiting the Na^+-K^+ ATPase activity (Jaitovich and Bertorello, 2010). Although the synthesis of these compounds has been relatively well described in plants, biosynthesis in animals is less well described.

Table 1.1: Cardiotonic steroids and their physiological effects (Compiled for each compound and with courtesy from the National Center for Biotechnology Information; 2022 as referenced).

Cardiotonic steroid	**Physiological effects:**
Cardenolides	
Digoxin	• Inhibits the Na+-K+ adenoside triphosphatase (ATPase) pump; • Enhances cardiac contractility by increasing intracellular calcium; • Toxic at high concentrations and may lead to arrhythmias, decreased renal function and more. • Exhibits anti-tumour activities.
Digitoxin	• Inhibits the Na+-K+ adenoside triphosphatase (ATPase) pump; • Leads to an increase in intracellular sodium and calcium levels and a decrease in intracellular potassium levels; • Increased calcium precedes cell death and decreased potassium increases caspase activation and DNA fragmentation leading to apoptosis; exhibits anti-tumour activities. • Exerts similar toxic effects to digoxin.
Ouabain	• Inhibits the Na+-K+ adenoside triphosphatase (ATPase) pump. • Endogenous concentrations increase essential human hypertension and also induce it. • Exhibits anti-tumour activities.
Bufadienolides	
Bufalin	• Inhibits the Na+-K+adenoside triphosphatase (ATPase) pump; • Induces apoptosis by activating the transcription factor AP-1 via a mitogen-activated protein kinase (MAPK) pathway; • Has potential cardiotonic and antineoplastic activity. • Exhibits anti-tumour activities.
Marinobufagenin	• Inhibits the Na+-K+ adenosine triphosphatase (ATPase) pump; • Causes vasoconstriction; • Effects are similar to digitalis; • Found in the plasma and urine of human subjects with myocardial infarction, kidney failure and heart failure. • Exhibits anti-tumour activities.
Telocinobufagin	• Inhibits the Na+-K+ adenosine triphosphatase (ATPase) pump; • Increased concentration in plasma of human subjects with end-stage renal failure. • Exhibit anti-tumour activities.

1.8 Biosynthesis of CTSs

Cholesterol is the precursor of bile acids and steroid hormones. In the formation of steroid hormones, including CTSs, cholesterol is initially converted to pregnenolone, the precursor of all steroid hormones. This occurs through side chain cleavage of a six-carbon unit, eventually forming steroid hormones containing 21 or fewer carbon atoms (Berg *et al*., 2002). These compounds have slight structural differences and interact with specific receptor molecules, exerting differing genomic and non-genomic effects (Bagrov and Shapiro, 2008). In plants, the formation of CTSs mainly, cardenolides (Figure 1.3), likely evolved from the biosynthetic pathways of 24-alkyl phytosterols and endogenous plant steroid hormones rather than cholesterol (Hu *et al*., 2020). Earlier studies suggested they were synthesised from cholesterol, with pregnenolone being the intermediate (Kreis and Müller-Uri, 2010). However, despite decades of research, not all enzymes involved in the pathway have been described. Mitochondrial cytochrome P450-dependant sidechain cleaving enzyme leads to the formation of pregnenolone, and in animals, the enzyme is cytochrome P450scc, CYP11A (Agrawal *et al*., 2012). Yet, there is no evidence of such a P450 in plants and the conversion to pregnenolone appears to occur by an uncharacterized P450scc-like enzyme.

Cholesterol side-chain cleavage has been shown for synthesising endogenous ouabain by using radiolabelled hydroxycholesterol as a possible CTS precursor (Lichtstein *et al*., 1998). Pregnenolone is converted to isoprogesterone catalyzed by 3β-hydroxysteroid dehydrogenase (3βHSD) in the presence of NAD^+. After that, the isomerisation of the 5,6 double bond of isoprogesterone to progesterone is catalyzed by 3KSI. Progesterone is then converted to 5β-pregnane-3,20-dione by progesterone 5β-reductase (SRD5β). Furthermore, 3βHSD also catalyzes the reduction of 5β-pregnane-3,20-dione to 5β-pregnane-3β-ol-20-one in the presence of nicotinamide adenine dinucleotide phosphate (NADPH). Next, 5β-pregnane-3β-ol-20-one is converted to 5β-pregnane-3β,14β-diol-20-one and 5β-pregnane-3β,14β-21-triol-20-one, by unknown enzymes. Thus, it is also unknown which reaction occurs first: the pregnane 14β-hydroxylation or the 21-hydroxylation. The enzyme required for the synthesis of the lactone ring is malonyl coenzyme A:21-hydroxypregnane 21-O-malonyltransferase, converting 5β-pregnane-3β,14β-21-triol-20-one to 21-O-malonyl5β-pregnane-3β-14β-diol-20-dione (Hu *et al*., 2020). Enzymes required for adding sugar side-chains and modifying the aglycone functional groups still need to be identified (Figure 1.3; Züst *et al*., 2018).

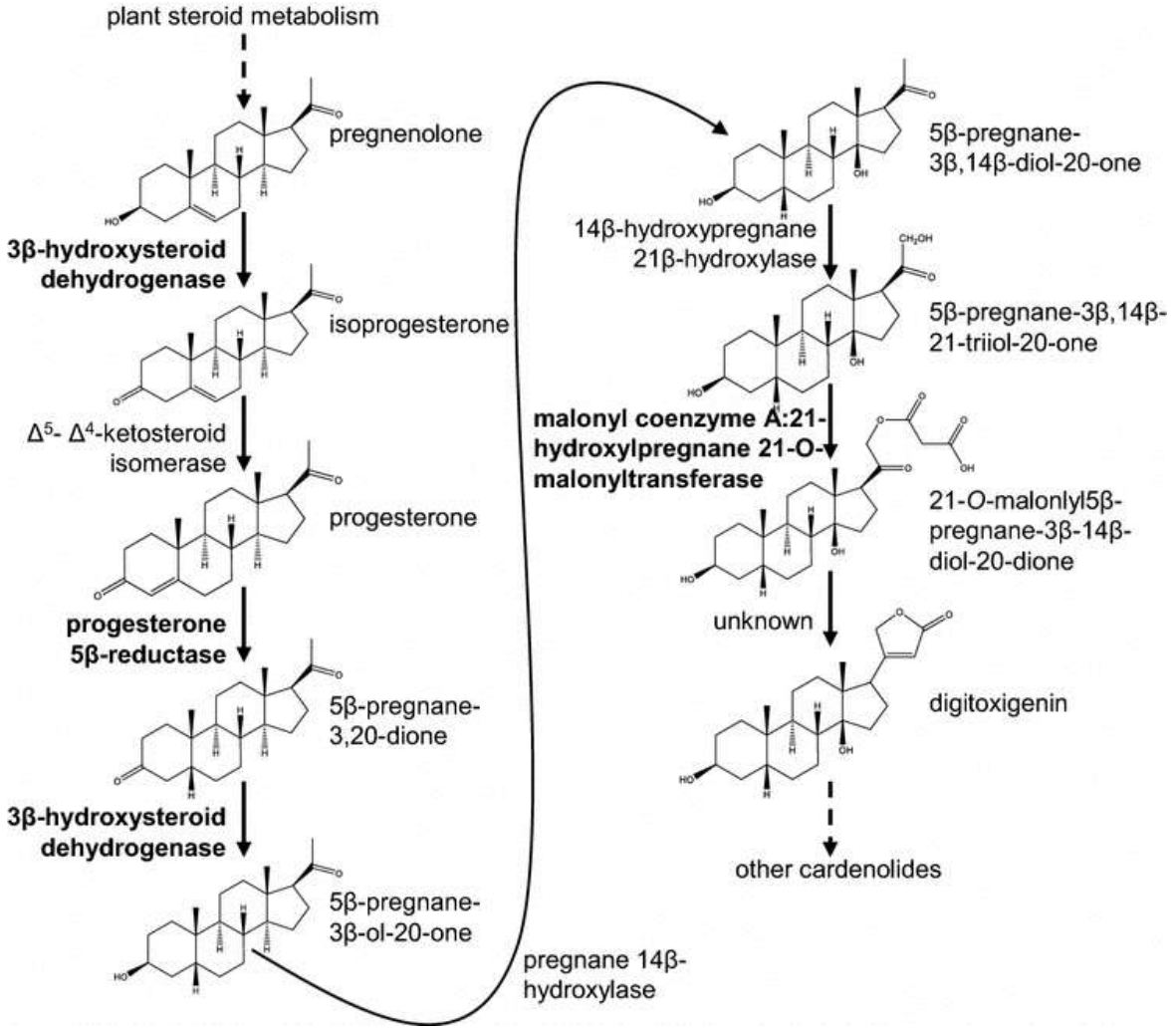


Figure 1.3: Biosynthesis of cardenolides in plants. Enzymes involved for which there is evidence are marked in bold (Züst *et al*., 2018; with permission from Springer Nature, 5386460018386).

1.8.1 Mammalian cell lines

Digitalis-like bioactivity could be synthesised in mammalian adrenal tissue from mammalian steroid pathway precursors (Perrin *et al*., 1997; Lichtstein *et al*., 1998; Qazzaz *et al*., 2004). Furthermore, physiological, and pharmacological stimuli were able to synthesise or cause the release of ouabain-like compounds (OLC) from the hypothalamus and adrenal tissues (Laredo *et al*., 1997). However, a pathway for the biosynthesis of CTSs in mammals has not been completely elucidated.

Bioinformatics analysis has deduced a hypothetical OLC biosynthetic pathway (Figure 1.4) using tissues from Milan hypertensive rats. There was significant overexpression for genes coding for the P450 catalysed side chain cleavage and Δ5-3β-hydroxysteroid dehydrogenase

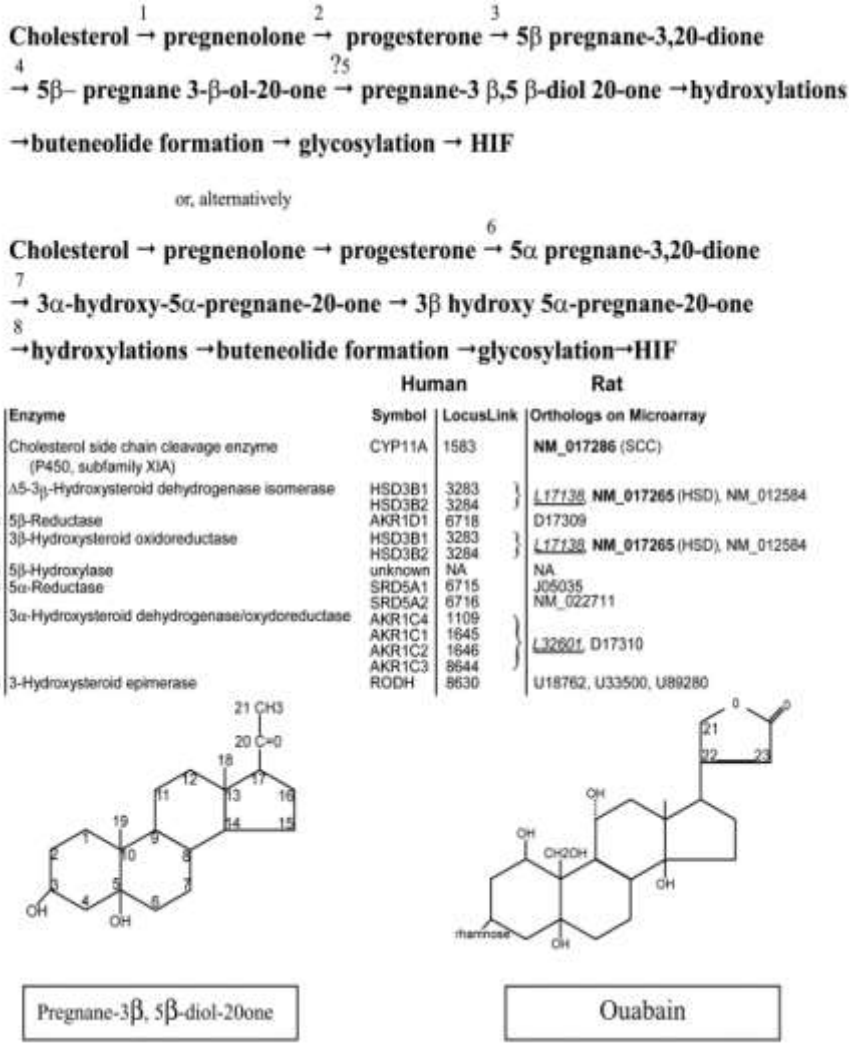


Figure 1.4: Hypothetical biosynthetic pathway for the conversion of cholesterol to endogenous cardiac glycosides. The relevant human genes and rat orthologues for the pathway enzymes were identified (Murrell *et al*., 2005; reproduced with permission from Karger Publishers, CCC License number: 5412980210430 (Oct 20, 2022)).

and isomerisation in the hypothalamus of Milan hypertensive rats as compared to their normotensive counterparts. The marked reduction in the production of inhibitory material of the Na^+-K^+ ATPase pump after the knockdown of Δ5-3β-hydroxysteroid dehydrogenase in rat brain suggested the established and classical synthetic pathways of steroid hormones, with the P450scc being considered to be the rate-limiting step (Murrell *et al*., 2005).

De novo biosynthesis of the CTSs, digoxin and its dihydro analogue was demonstrated in an adrenocortical cell-derived mouse tumour cell line. ^{14}C label from acetic acid and from

cholesterol were incorporated into the two digoxin-like materials. The study showed that cholesterol was the major precursor of these compounds and that the cellular machinery required for *de novo* synthesis of these compounds was present in adrenal cells. Therefore, this work in conjunction to other research suggests that the mammalian adrenal cortex mediated the biosynthesis and secretion of various cardiac glycosides. These observations were important as the ^{14}C-cholesterol used is stably ring-labelled. Thus, the presence of the radiolabel in the digoxin-like products could have only arisen from further metabolism of ^{14}C-cholesterol itself and not from another mechanism such as an isotope exchange etc. Furthermore, the necessary biosynthetic steps involved in the process were few as only three significant enzymatic reactions 1) the configuration inversion (*cis-trans-cis)* of carbon 5 and 14; 2) addition of a lactone ring at carbon 17; and 3) addition of the glycoside side chain, were required to convert progesterone/pregnenolone to the simplest cardiac glycoside. However, these significant enzymes have yet to be described (Qazzaz *et al*., 2004).

Doris and colleagues established that the production of bufadienolide CTSs by adrenocortical cells was not dependent on cholesterol side chain cleavage (Doris et al., 1989, 1994). It was also demonstrated using murine Y1 adrenocortical cells, that although cholesterol was required as a precursor in the *de novo* formation of bufadienolides, such as MBG, side chain cleavage of cholesterol to pregnenolone by P450scc was not involved (Doris *et al*., 1989, 1994; Dmitrieva *et al*., 2000, 2005).

Hamlyn *et al*. (2011) hypothesized that endogeneous ouabain (EO) might be synthesised via an aldosterone-like pathway, following the observation that angiotensin II and ACTH, and pregnenolone and progesterone, stimulate EO secretion (Hamlyn and Manunta, 2011). Preliminary work using bovine adrenal cortex primary cell cultures showed that the biosynthesis of EO was affected by 11β-hydroxylase inhibitors. In mammals, a 11β isomer of ouabain would be formed through the direct participation of 11-hydroxylase in EO synthesis. Additionally, a 11β-orientated hydroxyl group, *in vivo,* would spontaneously form a mixture of two 11-19 hemiketal isomers. During isolation, the isomers were most likely to be converted back to a single 11β isomer of ouabain. Thus, it raises the possibility that the physiological targets and actions of the hemiketals *in vivo* differ from the isolated form of EO because of the existence of an additional ring and reduced flexion of the steroidal A, B and C rings (Hamlyn *et al*., 2003).

Subsequently, a continuation of studies done by Doris and colleagues established that the production of bufadienolide CTSs by adrenocortical cells did not depend on cholesterol side chain cleavage (Doris *et al*., 1989, 1994). Later it was also demonstrated in murine Y1 adrenocortical cells that although cholesterol was required as a precursor in the *de novo* formation of bufadienolides, such as MBG, side chain cleavage of cholesterol to pregnenolone by P450scc was not involved (Dmitrieva *et al*., 2000, 2005).

A reduction of Δ^4-3-ketones had been thought to give rise to most animal steroid metabolites with a 3-hydroxyl substituent (Berg *et al*., 2002). Furthermore, cholestenone was not an intermediate in converting cholesterol to coprosterol, and it was likely that the cardiac lactones might form by a path that retained the cholesterol 3-β-hydroxyl group without the formation of a 3-ketone intermediate (Rosenheim and Webster, 1935). The CTSs may be synthesised from cholesterol via a "bile acid pathway" (e.g., from cholanates) without side chain cleavage (Chen and Osuch, 1969).

1.8.2 Bile acids

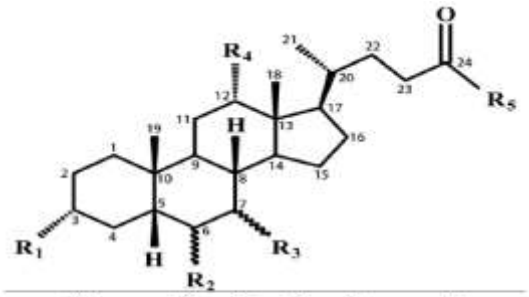
Homeostasis of cholesterol is maintained through dietary intake, *de novo* synthesis from acetyl CoA, and its conversion to bile acids (Figure 1.5(A); Chiang, 2013). Bile acids form mixed micelles with fatty acids and are thus lipid-carriers able to solubilise many lipids and cholesterol. A major mechanism of cholesterol elimination from the body to prevent accumulation is bile acids to solubilise cholesterol in bile (Chen and Cassaro, 2020).

Bile acids also regulate triglyceride, cholesterol, and glucose homeostasis, as well as energy expenditure by acting as ligands for G-protein coupled receptors (GPCRs) such as TGR5 (also known as GPBAR1, M-BAR, and BG37) and nuclear hormone receptors including farnesoid X receptor (FXR; also known as NR1H4). The activation of these receptors also plays a role in glucose metabolism, dyslipidemia, obesity, and liver steatosis. Thus, bile acids have a crucial role in metabolic diseases, which are often associated with hypertension (Xu, 2016).

The average healthy human bile acid composition consists of cholic acid (CA, ~40%) and chenodeoxycholic acid (CDCA, ~40% and deoxycholic acid (DCA, ~20%). In the intestine, the ratio of glycine:taurine-conjugated bile acids are ~3:1, and the bile acid pool is highly hydrophobic (Chiang, 2017). Bile acids are similar in structure to steroids and have a carboxyl side chain to which glycine and taurine may be conjugated (Figure 1.5(B); Monte *et*

al., 2009). The primary CA and CDCA and secondary bile acids are further transformed into other compounds in the gut by the gut microbiome (Ridlon *et al*., 2006).

(A)



BAs	R_1	R_2	R_3	R_4	R_5
CA	α-OH	H	α-OH	OH	OH
CDCA	α-OH	H	α-OH	H	OH
DCA	α-OH	H	H	OH	OH
LCA	α-OH	H	H	H	OH
UDCA	α-OH	H	β-OH	H	OH
HCA	α-OH	α-OH	α-OH	α-OH	OH
HDCA	α-OH	α-OH	H	H	OH
α-MCA	α-OH	β-OH	α-OH	H	OH
β-MCA	α-OH	β-OH	β-OH	H	OH
ω-MCA	α-OH	α-OH	β-OH	H	OH
MDCA	α-OH	β-OH	H	H	OH
Unconjugated					OH
Glycine conjugates					$NHCH_2CO_2H$
Taurine conjugates					$NHCH_2CH_2SO_3H$
Sulfated BAs	HSO_4				

(B)

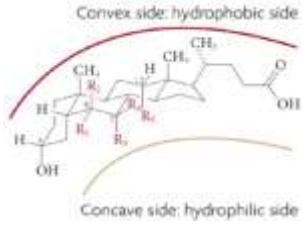


Figure 1.5: **(A)** Numbering of the chemical structure of main primary and secondary bile acids. **(B)** Mammalian bile acids have a 5β-configuration with α-hydroxyl groups at positions 3, 7 and 12 (Monte *et al*. 2009; reproduced with email permission from World Journal Gastroenterology). Compared to Figure 1.2(A).

Primary bile acids (Figure 1.5(A)) are synthesised in the liver from cholesterol and are stored in the gall bladder before being secreted into the small intestine (Berg *et al*., 2002). This occurs through two biosynthetic pathways: the "classical" and "alternative" pathways. In the "classical" pathway, cholic acid (CA) and chenodeoxycholic acid (CDCA) are synthesised in the liver via neutral sterol intermediates. This pathway is initiated by the enzymes CYP11A1 and CYP7A1 and is the main pathway in the synthesis of bile acids. , The rate-limiting enzyme of this "classical" pathway, is CYP7A1 which forms 7α-hydroxy-4-cholesten-3-one (C4) from cholesterol. CA is then formed after catalysis by CYP8B1 in a series of reactions.

C4 is finally converted to CDCA in the absence of CYP8B1 metabolism. The expression of CYP7A1 is regulated through negative feedback by CA levels. In mouse liver, CDCA may be converted to α- and β- muricholic acid (α/ β –MCA; Li and Chiang, 2014).

In the "alternative" pathway, acidic intermediate metabolites are formed in the liver, and the primary bile acid synthesised is CDCA (Monte *et al*., 2009). This pathway is controlled by the enzymes CYP27A1 and CYP7B1 and accounts for less than 10% of secondary bile acids produced. This pathway may become upregulated if the main pathway is affectedor inhibited. Once CA and CDCA are synthesised, they are amino conjugated and deconjugated by bacterial 7α-dehydroxylase in the distal intestine. Here, the bacterial microbiome converts them to secondary bile acids (figure 1.5(A)), deoxycholic acid (DCA) and lithocholic acid (LCA), respectively. Epimerisation of the 7α-hydroxylated groups in CDCA to 7β forms ursodeoxycholic acid (UDCA). Most of the LCA is excreted into faeces, while the rest is taken to the liver, rapidly conjugated by sulfonation and excreted into the bile. In the mouse intestine, CDCA is also converted by CYP3A1 and epimerase to the secondary bile acids, taurohyocholic acid, tauromurideoxycholic acid, α/β/ω-muricholic acids (α/β/ω-MCA), taurohyodeoxycholic acid (THDCA) and tauroursodeoxycholic acid (TUDCA) (Li and Chiang, 2014).

After secretion into the intestine, both the primary and secondary bile acids are reabsorbed from the intestine into the liver via the portal venous circulation at the sinusoidal and canalicular plasma membranes. This process occurs through primary (ATP-dependent), secondary (Na^+ gradient-dependent), and tertiary (OH^- or HCO_3^--dependent anion exchange) transport systems (Dawson *et al*., 2009). In this way, the bile acid pool is maintained as approximately 95% of the bile acids secreted into the bile are derived from the recirculating pool, and only 5% of the bile acids are lost in faeces. The bile acid pool consists of approximately 3g of bile acids and cycles 4-12 times daily through the enterohepatic circulation (Chiang, 2013).

1.8.3 Biosynthesis of cardiotonic steroids from bile acids

The enzyme CYP7A1 ("classical" pathway) is responsible for the side chain cleavage of cholesterol and is only expressed in the liver. In contrast, the enzyme CYP27A1 ("alternative" pathway) is expressed in the "extra-hepatic" tissues, including the adrenals. Therefore, it was hypothesised by Federova *et al*. that MBG, which is synthesised in non-hepatic tissues, is synthesised via the "acidic" bile acid pathway that uses the CYP27A1

enzyme to oxidize cholesterol into bile acids. The results from the study confirmed the hypothesis, and the following was revealed: in human trophoblast, and rat adrenocortical cells, post-transcriptional silencing of the CYP27A1 gene led to a 70% reduced expression of CYP27A1 mRNA, a 2-fold decrease in total bile acids, and a 67% decrease in MBG levels, as compared to non-treated cells or cells transfected with non-targeting siRNA.

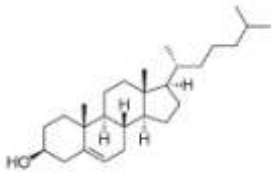
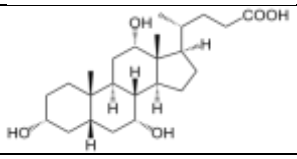
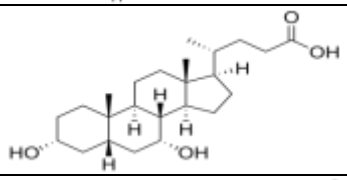
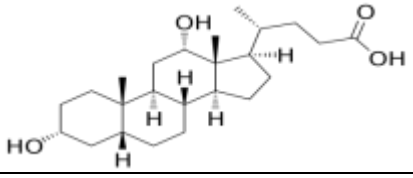
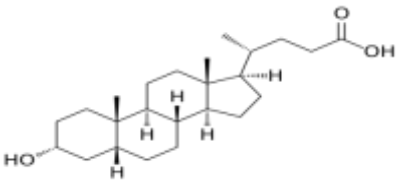
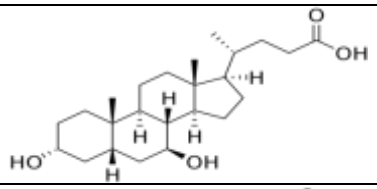
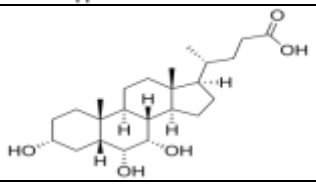
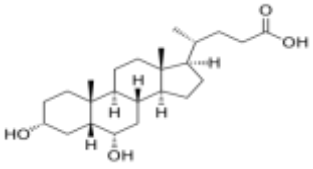
In contrast, MBG levels were not affected by the silencing of the CYP11A1 gene in either cell culture. However, progesterone production was halved in the human trophoblast cells, and in rat adrenocortical cells, there was a 90% reduction in corticosterone compared to cells transfected with non-targeting siRNA. Furthermore, a high salt diet in DSS rats increased systolic blood pressure (SBP), elevated plasma MBG levels, and increased adrenocortical CYP27A1 mRNA and protein. However, adrenocortical CYP11A1 mRNA and protein levels did not change (Federova *et al*., 2015).

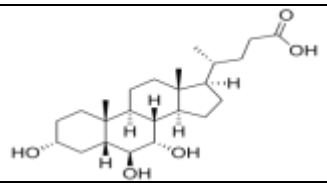
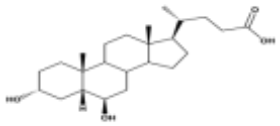
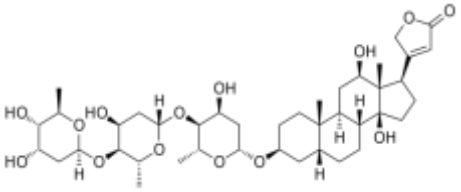
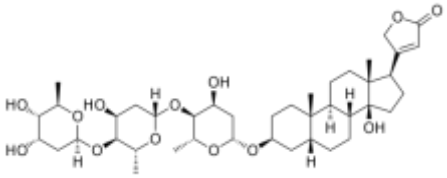
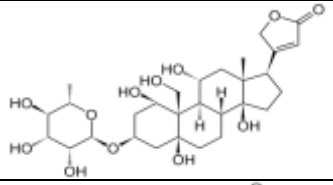
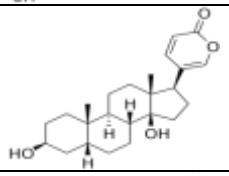
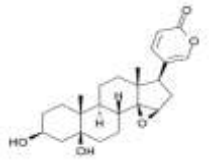
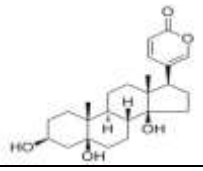
The expression of 3βHSD was 20 times higher in the ear gland of *B. bufo gargarizans* than in the liver. Thus, this enzyme was considered important in the biosynthesis of bufadienolides. Bbg-3βHSD cDNA, cloned from the ear-side parotid gland and a recombinant prokaryotic fusion expression plasmid was introduced into *E. coli* for prokaryotic expression. Subsequently, Bbg-3βHSD was shown to be able use pregnenolone and dehydroepiandrosterone, *in vitro*, when NAD^+ was used as the coenzyme (Xu *et al*., 2018). A recent study has hypothesised that bufadienolides are biosynthesised in the toad paratoid gland starting from cholesterol, with bile acids as the intermediate (Zhang *et al*., 2021). Previously, 7α-hydroxycholesterol was demonstrated to be a substrate of 3βHSD, and 7α-hydroxy-4-cholesten-3-one a substrate of SRD5β (Gonzales *et al*., 2018). SRD5β was cloned from the Bufonidae for the first time and the recombinant Bbg-SRD5β was expressed in *E. coli*. It was observed that progesterone could be reduced to 5β-dihydroprogesterone without substrate inhibition. Moreover, the catalytic rate was increased ~1.8 times by the mutant type, Bbg-SRD5β-Y132G as compared to the wild type Bbg-SRD5β. This showed that the Y132 site was essential for the catalytic activity of Bbg-SRD5β (Zhang *et al*., 2021).

Structurally, bufadienolides consist of an A/B *cis*, B/C *trans* and C/D *cis* configuration. Therefore, in the biosynthesis of bufadienolides, the steroidal A/B ring will be converted to the *cis*-configuration structure. The A/B ring *cis* fusion of bile acids is achieved by Δ^4 double bond reduction (Figure 1.5). Thus, 3βHSD and SRD5β may play an essential role in this process by converting the A/B ring *cis*-configuration structure (Zhang *et al*., 2021). The C-19

and C-21 steroid and bile acid biosynthesis in vertebrates involve the enzyme SRD5β. It can reduce the Δ^4 group of C-19 and C-20 steroids into 5β-stereoisomers (Chen and Penning, 2014).

Table 1.2: Chemical structures of cholesterol, primary and secondary bile acids and cardiotonic steroids mentioned in this thesis. Images courtesy of Pubchem.

Compound:	**Chemical structure:**
Cholesterol	
Primary bile acids: Cholic acid	
Chenodeoxycholic acid	
Secondary bile acids: Deoxycholic acid	
Lithocholic acid	
Ursodeoxycholic acid	
Hyocholic acid	
Hyodeoxycholic acid	

α/β/ω-muricholic acid	
Murideoxycholic acid	
Cardenolides: Digoxin	
Digitoxin	
Ouabain	
Bufadienolides: Bufalin	
Marinobufagenin	
Telocinobufagin	

The bioactivity of steroids is dependent on C-19 (Schiffer *et al*., 2019). Recently, two new 19-norbufadienolides with cardioactive activity were isolated from the venom of *Bufo Bufo gargarizans*. The results showed that these were lower in cytotoxicity while exhibiting more significant cardiotonic activity (Chen *et al*., 2018).

1.9 Mechanism of action of Cardionic Steroids

CTS have specific binding sites on the extracellular loops of the α subunit of the Na^+-K^+ ATPase and exert their effects via the 'ionic' and the 'signalling' pathways. In the ionic pathway (Figure 1.6), CTSs inhibit the Na^+-K^+ ATPase pump function, causing an increase in cytosolic Na^+ concentration and a decrease in cytosolic K^+ concentration. Cytosolic Ca^{2+} then increases, which activates various other pathways that lead to genomic and non-genomic effects such as increased vascular constriction and blood pressure. These actions form the basis for using digoxin and digitoxin in heart failure, where they lower the hypertensive effects of ouabain as they do not raise blood pressure (Song *et al*., 2014).

In comparison, the signalling pathway (Figure 1.6) includes the association of Src in a caveolar domain with the Na^+-K^+ ATPase. Src gets activated when CTSs bind to the Na^+-K^+ ATPase, which further activates the epidermal growth factor receptor (EGFR) and phospholipase C (PLC). This leads to further downstream events such as the generation of reactive oxygen species (ROS); activation of mitogen-activated protein kinase, additionally activating extracellular regulated kinase (ERK); activation of phosphoinositide-3 kinase (PI (3)K), leading to the activation of protein kinase B (Akt) and stimulation of endocytosis; activation of protein kinase C (PKC) through the activation of PLC. Similarly, these steps eventually lead to genomic and non-genomic effects (Bagrov and Shapiro, 2008).

In physiological conditions, they are considered significant in the regulation of the renal sodium transport and arterial pressure but may further regulate other molecular mechanisms and are implicated in other conditions such as preeclampsia, end-stage renal disease, congestive heart failure, and diabetes mellitus (Bagrov *et al*., 2009). The ouabain-sensitive sodium efflux rate constant and the Na^+-K^+ ATPase activity correlated significantly with the diastolic and systolic blood pressure ($p<0.001$; Morne *et al*., 1986). It has also been observed that in response to elevated sodium intake, the levels of CTSs, particularly ouabain, are significantly elevated in untreated patients with hypertension (Hauck and Frishman, 2012). In salt-sensitive hypertensive patients, the excessive levels of CTSs inhibited the Na^+/K^+-ATPase in the vasculature, leading to vasoconstriction. Additionally, their action on blood pressure was observed to be dependent on the activation of the renin-angiotensin system in rats (Rassler, 2010).

MBG concentrations are also elevated in heart and renal failure, idiopathic hyperaldosteronism (PA) and essential hypertension, where its concentrations were increased

and correlated with blood pressure (Gonick *et al*., 1998). MBG and novel endogenous digitalis, telocinobufagin (TCB), were identified in the plasma of patients with terminal renal failure by using high-resolution mass spectrometry (MS) and nuclear magnetic resonance (NMR) (Komiyama *et al*., 2005). In vivo, sodium loaded DSS rats observed that immune neutralisation of MBG by a specific antibody reduced blood pressure and renal sodium excretion (Federova *et al*., 2002, 2007). Furthermore, MBG concentrations increased with 24-hour ambulatory blood pressure (ABP) and were related to salt sensitivity in men but not women (Federova *et al*., 2015). Another study also showed that plasma MBG concentration correlated with 24h diastolic blood pressure in salt-sensitive hypertensive patients, and plasma MBG levels were reduced in response to dietary sodium restriction (Łabno-Kirszniok *et al*., 2021).

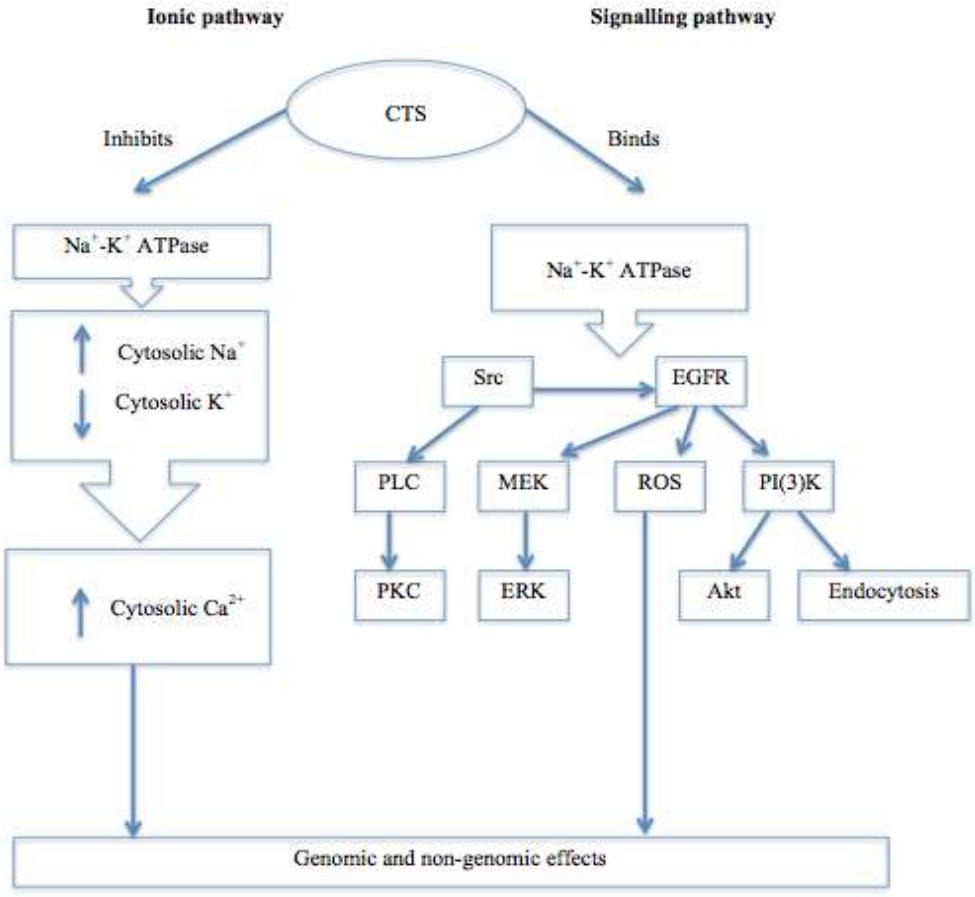


Figure 1.6: CTSs exert their effects on the Na^+-K^+ ATPase via two pathways: the 'ionic' and the 'signalling' pathway, leading to genomic and non-genomic effects (Adapted from Bagrov and Shapiro, 2008).

1.10 The gut microbiome and CTSs

The gut microbiome is involved in converting primary bile acids to secondary bile acids and other compounds (Ridlon *et al*., 2006). Hence, it has also been shown to have an increasing role in regulating metabolic pathways within a host and have a significant function in health and disease (Bull and Plummer, 2014). The distribution and abundance of bacteria and their metabolites are altered in cardiovascular diseases (Mendelsohn and Larrick, 2013). Thus, such gut microbiome dysbiosis may also play a role in the development of hypertension (Li *et al*., 2017).

Knowing that certain CTSs are synthesised from precursors such as cholesterol and bile acids and that the gut microbiome also uses these precursors to form various products, it is hypothesised that bacteria residing in the gut may also take part in the synthesis of certain CTSs. However, at present there is very little information on the presence or production of CTSs in the gut.

Several studies have described the presence of cardiotonic steroids in bile and faecal material. Kieval *et al*. (1986) demonstrated immunoreactivity to bufalin antibodies in human bile fractions, but without immunoreactivity to digoxin. Other studies have established the bile as a major route for the elimination of intravenously and orally administered digoxin and digitalis (IARC Working Group, 2013). Both digoxin and digitoxin are excreted into the bile, with up to thirty percent of a digoxin dose being excreted in this manner (Harrison & Gibaldi, 1976; Iisalo, 1977). Excretion is species and dependent on steroid structure in rats, up to 58% of an ouabain dose (Yang *et al*. 2009) undergoes biliary excretion, whereas rabbits and dogs excreted less than 5% of an administered ouabain dose within 12 hours (Russell, Klassen, 1973). Whereas ouabain and deslanatoside C are primarily excreted in the urine, K-strophanthoside is excreted in both bile and urine (Marzo *et al*. 1977). An important route of elimination in humans of an intravenously administered digoxin is the direct transport into the gut lumen by a P-glycoprotein, which also determines the uptake of orally administered digoxin from the intestine. The role of the P-glycoprotein in regulating digoxin concentrations appears to be significant (Drescher *et al*., 2003). Whereas between 50 and 70% of an administered digoxin dose may be recovered in the urine, faecal elimination accounts for about 15% (Marcus *et al*., 1964; Mayer *et al*., 1996).

Pharmacokinetic studies have shown CTS, and their metabolites can be detected in plasma (Wei, Yu *et al*., 2019; Wei, Hou *et al*., 2019), bile (Kieval *et al*. 1988; Ning *et al*., 2010; Zhu *et al*., 2013) and faecal material (Marcus *et al*., 1964; Mayer *et al*., 1996). After oral administration of the traditional Chinese medicine, Shexiang Baoxin Pill, a further study showed that the five major bufadienolides were widely distributed in mouse tissues, including the liver and intestine (Huang *et al*. 2015). An early study showed ^{14}C-(C4)-cholesterol was converted to marinobufagenin.

Unaltered cardiotonic steroids from an orally administered dose appearing in the intestine and faecal material from an oral dose of steroid would not be unexpected. However, data showing uptake, wide tissue distribution and appearance of metabolites in the bile and faecal material suggests the cardiotonic steroids may undergo many transformations analogous to bile acids in the liver and intestines. Importantly, as with bile acids (Ridlon *et al*., 2014; Gérard, 2014; Callender, 2022), the microbiome may play a significant role in the pharmacokinetics and toxicology of cardiotonic steroids (Dey, 2020).

Cholesterol and bile acid metabolism is altered in spontaneously hypertensive rats (Uchida *et al*., 1978). It is also known that some intestinal bacteria aid in degrading certain CTSs or other steroid derivatives to harmless products (Haiser *et al*., 2014). However, in hypertension, the abundance of specific bacterial species in the gut is altered (Yan *et al*., 2017), and consequently, the CTSs may not be degraded by the bacteria present. In addition, as discussed earlier, gut microbiota dysbiosis in chronic angiotensin II-infused conventional mice caused changes in plasma and faecal metabolites. Some of these metabolites could also act as precursors to produce CTSs, which could further establish a link between the gut microbiota mediating the formation of these CTSs.

Metabolites derived from cholesterol when observing for CTSs, particularly the upregulated faecal metabolites. These included taurodeoxycholate (TDC), taurohyodeoxycholic acid (THDCA), taurodeoxycholic acid (TDCA), and taurolithocholic acid (TLCA) (Cheema and Pluznick, 2019). It was not surprising to observe changes in these metabolites, as the gut microbiota and the host work together to achieve bile acid synthesis. Moreover, TDC decreased blood pressure in rats (Cheema and Pluznick, 2019).

Although studies have demonstrated the relationship between gut microbiota and hypertension, understanding the mechanisms linking the two is still lacking, one way this can

be explained is that the gut microbiota produces bile acids that act as hormones stimulating certain receptors and influencing pathways involved in blood pressure regulation. The gut microbiota metabolises cholesterol and primary bile acids. Cholesterol is converted to coprostanol and excreted in the faeces. The gut microbiota converts primary bile acids to over twenty secondary bile acid metabolites. The main bile acids in the gut of humans consist of deconjugation, oxidation, epimerisation of hydroxyl groups of C3, C7, and C12, 7-dehydroxylation, esterification and desulfation.

Spontaneously hypertensive stroke prone rats (SHRSP) have significantly decreased gut *Lactobacillus* compared to control Wistar Kyoto rats (WKY). Nine of 18 plasma bile acids were significantly decreased in SHRSP, with CA being reduced 77%, compared to the WKY. CA supplementation restored CA, and hyocholic acid concentrations to that of the WKY rats and systolic blood pressure decreased by 18±7mmHg. No such effect was observed in the WKY rats. Furthermore, the impaired vasodilation of the aorta induced by acetylcholine in SHRSP was improved. Supplementation restored the relative abundance of faecal *Lactobacillus* was also restored in the SHRSP. Thus, plasma CA levels may be critical to developing hypertension (Zhang *et al*., 2020).

Moreover, specific reductions in conjugated bile acids have been observed in hypertensive humans and rats. In addition, a decrease in the blood pressures of germ-free rats compared to germ-free conventionalised rats was seen through the conjugation of bile acids by the host alone and, in the absence, deconjugation step by microbiota. Moreover, using taurine to restore the conjugation of bile acids led to an increase in the availability of circulating conjugated bile acids as ligands. This improved the hosts' susceptibility to hypertension via bile acid nuclear and G-protein coupled receptors. Therefore, blood pressure homeostasis may be controlled by the host and its bacterial symbionts via the resulting pool of bile acid metabolites, including the downstream formation of CTSs (Chakraborty *et al*., 2020).

It has been documented that several microorganisms were involved in the 14α-hydroxylation of progesterone and other steroids. Several fungi have been shown to introduce a hydroxyl group to progesterone at the 14α-position and some other steroids. The 14α-hydroxyl configuration can then be chemically transformed to the 14β-hydroxy substituent usually found in CTSs (Shah *et al*., 2013).

1.11 *Helicobacter pylori* and CTSs

Helicobacter pylori (H. pylori) are gram-negative, spiral-shaped bacteria in the gastric mucosa. *H. pylori* infection is the leading cause of gastritis and is also associated with gastric cancer (Kusters *et al*., 2006). It infects more than 50% of the world's population. In the African population, the prevalence of *H. pylori* is between 60% based on serologic tests and 90% using the urea breath test (Bardhan, 1997). Recently, *H. pylori* have also been associated with hypertension, linked to a high salt diet (Testerman and Morris, 2014) and metabolic disease (Martin-Nuñez et al., 2021). However, the mechanism through which this occurs has not yet been established. Beevers *et al*. (2004) found that a statistically significant correlation existed between salt secretion and *H. pylori* infection. This was seen in older men and women and younger men, but not younger women (Beevers *et al*., 2004). Another study by Migneco *et al*. (2003) eradicated *H. pylori* in hypertensive patients, after which significant decreases in blood pressure, especially diastolic blood pressure values, were seen. These strains consisted of *cag* pathogenicity islands, and an association was seen between Cag-A positive strains of *H. pylori* and cardiovascular diseases, such as hypertension (Migneco *et al*., 2003).

It has also been observed that the presence of salt upregulates the expression of Cag-A (Loh et al., 2012) and further enhances its virulence and growth (Gancz *et al*., 2008). A high salt diet might also make it easier for *H. pylori* to enter the body by irritating the gastric mucosa (De Koster *et al*., 1994). *H. pylori* is also observed to be ubiquitous in African populations. Furthermore, a Sowetan study showed that most of these organisms were Cag-A positive (Segal *et al*., 2001). This could further explain the association between salt-sensitive hypertension in African patients.

L-arginine availability is essential for NO synthesis and NO is fatal to *H. pylori*. However, L-arginine substrate competition by the *H. pylori* arginase and its metabolism into polyamines may significantly dysregulate the host immune response to *H. pylori* infection and may further lead to endothelial dysfunction relating to hypertension (Chaturvedi *et al*., 2012). The virulence and growth of *H. pylori* is also enhanced in the presence of cholesterol (Jiménez-Soto *et al*., 2012), with the efficient conversion of cholesterol to glycosides (Wang *et al*., 2012). The bacterium also possesses the unique characteristic of steroid assimilation. It was demonstrated by Wunder *et al*. that *H. pylori* glycosylated retained free cholesterol (FC) to evade host immune systems by reinforcing its membrane lipid barrier. This allowed it to

resist the bacteriolytic action of the phosphatidylcholines (Wunder *et al*., 2006). Another similar research showed that 3β-OH and 3-OH steroids were selectively absorbed by *H. pylori,* where the former was glycosylated, and with or without glycosylation, both steroids were used as membrane lipid components (Shimomura *et al*., 2013). Therefore, this establishes that certain steroids are advantageous to the survival of *H. pylori.* This mechanism may also aid in the formation of CTS precursors.

These data may explain why eradicating *H. pylori* infection can result in significant blood pressure decreases in patients with hypertension (Migneco *et al*., 2003). Further research is required to investigate the mechanism and role of *H. pylori* in elevated blood pressure in hypertensive patients.

1.12 Detection of CTSs in biological samples

As reviewed above, many studies have determined the presence of EDLFs using non-specific immunological assays. Liquid chromatography with mass spectrometry (LC/MS) offers one of the best approaches to analysing these compounds, due to their ability to measure small compounds present in sub-microgram/molar per ml concentrations. This is achieved by combining liquid chromatography's separation capabilities based on retention on the stationary phase of the column and elution by the mobile phase. with mass spectrometry's mass analytical capabilities. Furthermore, multiple compounds can be detected simultaneously using ionisation techniques and mass analyser technologies (Pitt, 2009); (Houck and Siegel, 2015).

The compounds that leave the LC/HPLC column are ionised by the MS using electron ionisation sources. These ionised molecules are then accelerated through the instrument's mass analyser, separating the compound according to their different mass-to-charge (*m/z*) ratios. Ion detection and analysis occur on a computer, where the mass spectrum for each compound appears as peaks as a function of their *m/z* ratios. These compounds can then be identified, compared, and quantified using computer libraries of mass spectra for different compounds (Cramer and Humpf, 2012).

The significant advantages of using this type of system for quantitative analysis are the improved sensitivity and selectivity of trace concentrations of compounds. Furthermore, MS/MS can further fragment the ions after ionisation for detection of structurally related

isomers and, thus, differences in *m/z* ratios. Thermally unstable compound can also be detected because an ESI ion source can be used, which requires lower ionisation temperatures; moreover, tandem MS analysis decreases signal-to-noise ratios, enabling the sensitive detection of targeted compounds (Pitt, 2009).

1.12.1 Animal models of Hypertension

The SHR rat strain resulted from selective inbreeding of WKY rats with high blood pressure at Kyoto University in Japan. It has been reported that there are genetic differences between SHR colonies from different laboratories, the newly imported normotensive WKY rats from Charles River Laboratories in Germany might not be the most appropriate control for the SHR rats used in this study (Louis and Howes, 1990). Although these SHR rats were bred from stock animals at the University of the Witwatersrand's Central Animal Unit, blood pressures noted in the SHR rats were significantly higher than the WKY controls and therefore the latter serve as an appropriate control. Two Dahl salt sensitive rats were included in the pilot study to develop methodology as the WKY rats were not yet available and were excluded from the data analysis.

1.13 Problem statement

The source of the CTSs is unknown. They are produced from a cholesterol derived C_{21} steroid, usual progesterone in plants. Although not fully understood, CTSs in mammals are also shown to be synthesised using cholesterol as a precursor in the adrenals. If the present study demonstrates the conversion of ^{14}C-(C4)-cholesterol to bile acids and, ultimately, the CTSs, this suggests a novel pathway to produce CTSs. It would further highlight the gut microbiome's role in producing these compounds. It would be ethically difficult to justify this study in humans without prior animal studies being conducted to support the hypothesis and rationale for the study. Therefore, this study uses animal models.

It has been shown that the abundance of specific bacterial species in the gut is altered in populations with hypertension (Yan *et al.*, 2017). Additionally, the successful eradication of *H. pylori* resulted in significant blood pressure decreases (Migneco *et al.*, 2003). Thus, this data links the gut microbiome and the presence of *H. pylori* in the host to hypertension.

However, the proposed mechanism has not yet been elucidated. Furthermore, very little information is available on whether and how the gut microbiota may use bile acids to produce toxic compounds, such as CTSs. Prior research has shown:

1) The conversion of ^{14}C-(C4)-cholesterol to bile acids in rat models (Siperstein *et al*., 1957).
2) The conversion of ^{14}C-(C4)-cholesterol to marinobufagenin in toads with high residual remaining in the gut (Siperstein *et al*., 1957).
3) The conversion of bile acids to CTSs in the adrenal cortex (Fedorova *et al*., 2015).

Therefore, from the analysis of bile acids in faecal samples, the precursors to the CTSs can be identified from those which occur prior to the appearance of the CTSs. This would additionally explicate the relationship between the gut microbiome and *H. pylori* in the biosynthesis of this compound.

1.14 Hypothesis

It is hypothesised that CTSs are formed from secondary bile acids mediated by the gut microbiome. This occurs with the initial conversion of cholesterol to bile acids or other CTSs precursors. These precursors are then converted to secondary bile acids and recycled back to the liver, before being secreted back into the gut. The gut microbiome finally converts these to CTS compounds by a presently unknown pathway. Additionally, the gut microbiota dysbiosis reported in hypertensive patients may consequently not allow the degradation of CTSs to harmless products as has been demonstrated in toads. The circulating and reabsorbed CTSs in the plasma inhibit the activity of the Na^+-K^+ ATPase, affecting arginine transport and thus, blood pressure.

1.15 Aim

To demonstrate the conversion of ^{14}C-(C4)-cholesterol to labelled bile acids and CTSs in rat models of hypertension.

1.16 Objectives

- To determine the conversion ^{14}C-(C4)-cholesterol to labelled sterols in rat models of hypertension.
- To extract the bile acids as well as CTSs that may be present in the faeces throughout the experiment.
- Measure blood pressure changes in the rat models after administration of labelled ^{14}C-(C4)-cholesterol.
- At termination, cannulate the bile ducts of the rat models to sample the bile acids.
- To follow the disappearance of ^{14}C-(C4)-cholesterol, and appearance of labelled primary and then secondary bile acids and lastly, the CTSs using HPLC/MS.

Chapter 2: Materials and methods

Suppliers and manufacturers of consumable materials used in this study have been summarised in Appendix A.

Ethical Considerations. The study was obtained from the University of the Witwatersrand Animal Research Ethics Committee (Clearance certificate number: 2018/09/42/c; Appendix B) and carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). Modifications were later approved to add additional co-workers and expert individuals to perform certain procedures.

All studies using live rats were conducted rat models at the Central Animal Services (CAS) Unit of the University of the Witwatersrand at the Facullty of Health Sciences in Parktown, Johannesburg.

Biosafety Considerations. Approval (IBC 20180401; Appendix B) for the study for the use of the radioactive ^{14}C-(C4)-cholesterol was approved and obtained from the Institutional Biosafety Committee of the University of Witwatersrand to ensure the safety of investigators, co-workers, CAS and auxillary staff. Radioactive doses were administered by qualified personal and gloves were worn at all times.

2.1 Animal studies

A pilot study was undertaken as a proof of concept of the study. An available spontaneously hypertensive rat (SHR) female rat and Dahl salt sensitive (DSS) male rat were used which had been bred at the CAS Unit. The experiment used SHR rats (n=6 and 2 controls) with equal numbers of male and female rats, bred within CAS.

Subsequently, the ancestral strain of the SHRs, Wistar Kyoto (WKY) rats were imported from Charles River Laboratories (Charles River Laboratories, GmbH, Sulzfeld) in Germany. These were more appropriate to use as the comparative group than DSS rat. Approval for the import of the WKY rats was obtained from the Department of Agriculture and Fisheries, Pretoria, South Africa.

The first litters of rats bred at the CAS Unit with 6 WKY and 2 control WKY rats, with equal numbers of male and female rats, were used in the experiment..

For all experimental work (Figure 2.2), the rats were housed individually at CAS, on a 12-hour light cycle with the temperature maintained at a constant 30°C. All rats were fed the same standard CAS diet and the rats had unrestricted access to water. The rats were habituated to handling prior to carrying out any experimental work. Each week, all rat were weighed by the researcher and their health condition checked by the responsible qualified CAS veterinarian.

2.2 Blood pressure measurements of rat models

Blood pressure was recorded in the rat models using a tail cuff BP monitor (BIOPAC – NIPB250 Tail Cuff System) loaned from the School of Physiology, University orf the Witwatersrand (Figure 2.1). The environment's temperature was kept at ~30°C, and the rats were habituated to handling and the blood pressure recording procedure. Rats were placed in restrainers for 20 minutes to calm them down. The tail cuff was attached to their tails (part of the tails closest to the body). Tail cuff BP measurements were recorded before the administration of ^{14}C-(C4)-labelled cholesterol and then every week over 4 weeks until the end of the experimentation.

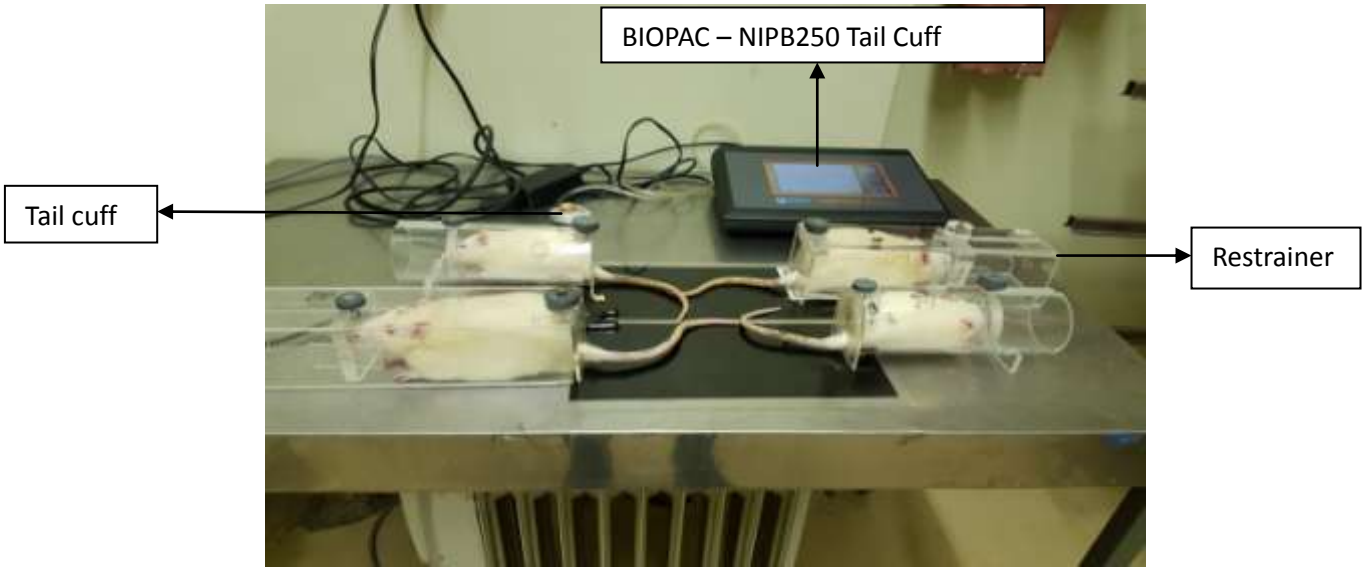


Figure 2.1: BIOPAC – NIPB250 Tail Cuff System. Rats were placed in restrainers for 20 minutes to calm them down. The tail cuff was attached to their tails (the part of the tail closest to the body). Blood pressure was measured similarly to automated BP machines.

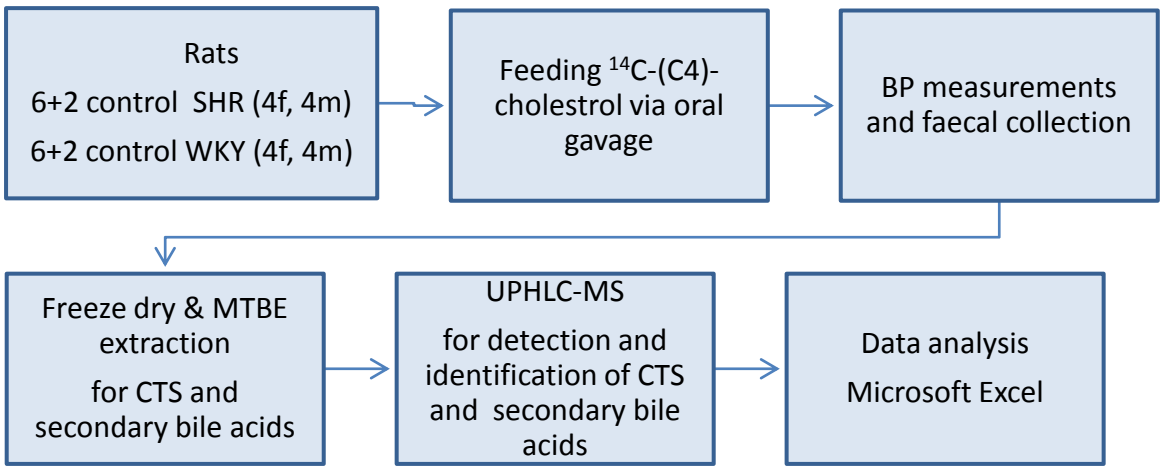


Figure 2 2: Schematic flow diagram of the experiment.

2.2 Administration of ^{14}C-(C4)-labelled cholesterol to rat models

The loss of the bile acids pool in faeces each day is approximately 5% (Chiang, 2013), and in the cold-blooded toad (20^{o}C), the conversion of ^{14}C-labelled cholesterol to marinobufagenin took 70 days (Siperstein *et al*. 1957). It was estimated that the conversion of the ^{14}C-(C4)-cholesterol to the bile acids and CTSs in a rat model of body temperature of 37^{o}C would take 21-30 days (=70 days divided by a factor of 2 for each 10^{o}C increase in body temperature). It was hypothesised that throughout the experimental period, labelled bile acids would be present in extracts, and the labelled CTSs would only be present in the days 19-25.

^{14}C-(C4)-labelled-cholesterol (0.279 µCi; 10 µl/rat) was diluted in 100 µl of canola oil (carrier) and administered once to the rat models by oral gavage (Figure 2.2).

2.2.1 Dose calculation

The radioactive dose of administered ^{14}C-(C4)-labelled cholesterol was less than background radiation and less than the level used in the routine patient diagnostic ^{14}C-Urea Breath Test (10 µCi) for *Helicobacter pylori* by the Scintillation Services Laboratory, 3rd floor of the Faculty of Health Sciences. Radioactivity was recorded as counts per minute (cpm) of the

administered dose in the extracted samples. A scintillation counter (Tricarb Vial-based Liquid Scintillation Counter; Perkin Elmer Corporation) was used to measure the radioactivity.

2.3 Collection of faecal samples and extraction

The rats were transferred to metabolic cages (1 rat per metabolic cage) each evening (when they were active) and returned to normal housing each morning for the duration of the experiment. The metabolic cages allowed for the sterile collection of faeces and urine, which were passed through the wired grid in the cages into collection tubes. Faecal material was collected from the metabolic cages each morning and frozen at -80^{o}C until bile acid and steroids extracted as described below. Standard safety and protective procedures were used when working with radioactivity. Animal bedding was changed, and housing and metabolic cages were washed daily, and wastewater was disposed of according to procedures for handling radioactive waste.

Rat faecal samples were collected from the metabolic cages each morning in Falcon tubes (50ml) for 27 days after the administration of ^{14}C-(C4)-labelled cholesterol. The faecal samples were stored at -80°C until use.

2.4 Collection of bile from rat models

Bile acid was collected on the day of termination. The rats were anaesthetised using ketamine/xylazine and barbiturate. The liver and common bile duct were exposed and a a 0.025mm needle inserted into the common bile duct. An extracorporeal bile-duct cannulation (BDC) bypass loop was attached to the needle and one millileter of bile was collected in a microtube over a period of 2 hours (Yang *et al*., 2017). Anaesthetisia was maintained using isofluraneProcedures were carried out by a veterinarian, Professor Kennedy or the Department of Physiology); Figure 2.3))

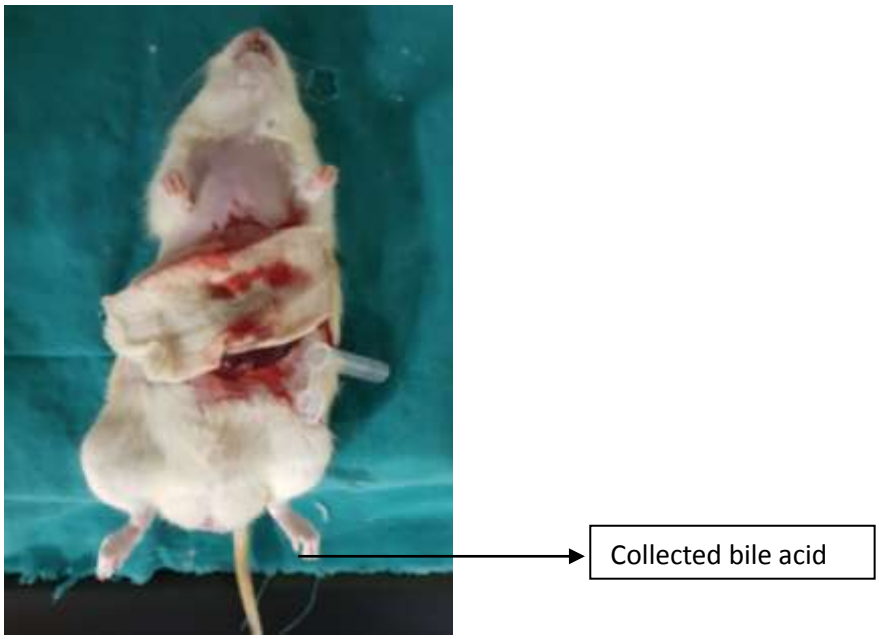


Figure 2.3: Bile acid collection. Rats were opened by the abdominal area under anaesthesia. A 0.025mm diameter injection needle girth was inserted into the bile duct. An eppendorf tube was inserted at the end of the loop. The procedure was performed in sterile clinical settings at the University CAS Unit.

The eppendorf were then centrifuged for 10 minutes at 10000g. The supernatants were then transferred into new separate microtubes and stored at -80°C for further analysis.

2.5. Extract clean-up procedure for analysis

Faecal samples were freeze-dried overnight (Figure 2.2). One gram (1 g) of dried material was placed into separate glass test tubes and extracted with methyl-*tert*-butyl ether (MTBE; 10ml), respectively. MTBE is an organic compound with the molecular formula $(CH_3)_3COCH_3$ and is a volatile, flammable, and colorless liquid that is sparingly soluble in water. The faecal material was homogenized first with a spatula and then placed in an ultrasonic bath for 2 minutes.They were then placed a rotatory shaker for an hour. The homogenized mixtures were then centrifuged for 10 minutes at 2000g. The supernatants were collected into new 10ml glass bottles and the solvent (MTBE) evaporated under a nitrogen stream. The dried extracts were reconstituted in MTBE (5ml). The individual days' samples were further grouped together in 3-day pools - days 1-3, 4-6, 7-9, 10-12, 13-15, 16-18, 19-21, 22-24 and 25-27 Finally, the samples were evaporated under nitrogen again before being reconstituted with 5ml of MTBE and stored at -20°C for the subsequent counting of radioactivity and UHPLC/MS analysis.

2.6 Measuring radioactivity of extractions

From each 3-day pooled faecal sample extract, 0.5ml was added to 4.5ml of scintillation fluid (PPO (2,5-diphenyloxazole) PerkinElmer Ultima Gold, PerkinElmer, Midrand, South Africa), in scintillation vials and measured after mixing. The radioactivity of the faecal extracts of the rats was measured as counts per minute (CPM) using a TRICARB 4810R scintillation counter (from Perkin Elmer, Ohio United States; obtained from Perkin Elmer (South Africa)).

2.7. Sample clean-up for UHPLC/MS analysis

a. MTBE sample extracts (1ml; Figure 2.2) were transferred from the 10ml glass bottles into 10ml glass test tubes, and the MTBE was evaporated under a stream of nitrogen to near dryness. Thereafter, 0.5ml of methanol was added to each test tube and sonicated in an ultrasonic bath for 5 minutes. This was done to homogenise the methanol with the residue precipitates in the test tubes. Immediately after, 0.5ml of distilled water was added, and the test tubes were swirled around to form a mixture which was then transferred to 1ml HPLC autosampler vials.

   The mixtures were drawn into a syringe and were filtered(disposable low binding 0.22µ pore size) into HPLC autosampler glass tube inserts (0.5ml) for analysis by Ultra-High-Performance Liquid Chromatography-MS Q-TOF as described below.

   Some samples from the preliminary pilot study analysis were spiked with CA and HDCA to confirm retention times.

b. The preliminary UHPLC chromatograms of the MTBE extracts prepared above resulted in many peaks with high background making analysis difficult. Further clean-up and concentration were attempted to reduce non-bile acid and non-CTS peaks. Samples from days 1-9, 10-18, and 19-25 were combined and evaporated under nitrogen and reconstituted with 5ml of ethyl-acetate.

   These samples were prepared by solid-phase extraction (SPE) using silica-based (Sep-Pak Vac 3cc/60mg) cartridges with a hold-up volume of 1ml. The cartridges in an extraction manifold were equilibrated with methanol (99.99%; 1ml) and then eluted with ethyl acetate (2ml) under gravity, to near dryness. The sample (5ml) was immediately added, and the waste collection tube was replaced. The sample was eluted to relative dryness ,and the eluent was discarded . The cartridge was then moved on top of the third collection tube and eluted with 2ml of ethyl

acetate, which was the hydrophilic bile acids and was labelled as such. Once again, the cartridge was moved on top of the fourth collection tube, and the second wash was done using 2ml of acetic acid (5%) in methanol to collect was the CTSs. To determine if any difference was seen with this process as compared to only having done the clean-up process, the clean-up process in (a) was applied to the eluents (waste, bile acids and CTSs) of days 19-25, which were then run on the LC/MS Q-TOF using the conditions in Table 2.1 and Table 2.2.

2.8 Preparation and run of internal standards and analytical method validation

Analytical standards of ^{14}C-(C4)-labelled cholesterol, bufalin, ouabain, digitoxin, cholic acid (CA) and hyodeoxycholic acid (HDCA) prepared in methanol at 1mg/ml concentrations and diluted to 1ppm in methanol/water 50:50 v/v just before injection on the HPLC/MS.

2.9 Profiling of extractions and data analysis

Samples were analysed at the Shimadzu South Africa (Pty) Ltd University of Johannesburg, Johannesburg). Samples were transported on dry ice to maintain sample integrity. Analysis of the extractions from the faecal samples of the pilot and main study was performed using a Shimadzu Nexera X3 Binary UHPLC system with two LC-40D XS pumps coupled to a Shimadzu LCMS-9030 Quadrupole Time of Flight (Q-TOF) detector (Shimazu, Kyoto, Japan). The column used was a 100mmx2.1µm Shim-pak Velox biphenyl column, and the flow rate was a constant 0.3ml/min the oven temperature was kept at 40^{o}C. Mobile phases and gradients have been tabulated in Table 2.1 and Quadrupole Time of Flight (Q-TOF) detector settings tabulated in Table 2.2. A SIL-40C XS autosampler was used.

Where bile acid and CTS standards were not available, approximate calculated or estimated values were input manually into the MS to identify the CTSs and the ^{14}C-(C4)-labelled cholesterol in the samples.

Table 2.1: Solvents and elution gradients for the separation of the bile acids and CTSs.

Bile acids			**Cardiotonic steroids**		
Time (min)	**A.Conc (%) [water; 0.1% formic acid 5mM NH4 formate]**	**B.Conc (%) [Methanol/0.1% Formic + 5mM NH4 formate acid]**	**Time (min)**	**A.Conc (%) [water; 0.1% formic acid 5mM NH4 formate**	**B.Conc (%) [Methanol/0.1% Formic + 5mM NH4 formate acid]**
0.00	65.0	35.0	0.00		
0.60	65.0	35.0	1.00	80.0	20.0
0.61	50.0	50.0	3.00	50.0	50.0
9.00	15.0	85.0	9.00	15.0	85.0
11.00	0.0	100.0	11.00	0.0	100.0
13.00	0.0	100.0	13.00	0.0	100.0
13.01	65.0	35.0	13.10	80	20.0
17:00	65.0	35.0	17.00	80.0	20.0

Table 2.2: Table 2.2: Q-TOF Mass spectrometer settings.

MS Parameter	Bile acids	Cardiotonic steroids
SIM Integrating Range(+/-)	20 ppm	20 ppm
MRM Integrating Range(+/-)	20 ppm	20 ppm
Q1 Transmission m/z Width(Custom)	10.0	10.0
Center	Off	Off
Threshold Coefficient	4	4
Detector Voltage	-0.20 kV Relative to the Tuning	-0.20 kV Relative to the
--Segment 1 Event 1--	File)	Tuning File)
Acquisition Mode	MS	MS
Polarity	**Negative**	**Positive**
Start Time :0.000 min	0.000	0.000
End Time :17.000 min	17.000	17.000
TOF Start m/z :300.0000	300	300
TOF End m/z :600.0000	600	800
Compound Name :		
ID	Off	Off
Event Time	0.500s	0.500s
Pulser Injection Times	994	994
Save Profile	Off	Off
Threshold	High	High
Interface Volt	-3.00 kV	4.00 kV
Corona Needle Volt	-3.50 kV	4.50 kV
Interface :	DUIS - ESI	DUIS - ESI
Nebulizing Gas Flow	2.0 L/min	2.0 L/min
Heating Gas	On	On
Heating Gas Flow	10.0L/min	10.0L/min
Interface heater	On	On
Interface Temperature	300^{o}C	300^{o}C
Desolvation Temperature	526^{o}C	526^{o}C
Sub-Interface Ionization Mode	ESI	ESI
Sub-Nebulizing Gas	Off	Off
Start of Data Acquisition, Diverter Valve	MS	MS
After Data Acquisition, Diverter Valve	No change	No change
Drying Gas	On	On
Drying Gas Flow	10 L/min	10 L/min
DL Temperature :250 C	250^{o}C	250^{o}C
Heat Block :400 C	400^{o}C	400^{o}C

2.10. Statistical analysis of data

Data was recorded in EXCEL (Microsoft Corporation) and imported into Statistica 14.0. (TIBCO Software In, California) For statistical analysis, peak areas of ^{14}C-(4C)-labelled bile acid or CTS, relative to that of total bile acids + CTS peak areas (where these were detected) was calculated to determine the proportion of label incorporated.

Data was recorded in Tables and graphs as mean±standard deviation. Comparison of the proportion of steroids between groups was determined with a Mann-Whitney U Test. Changes in steroid proportion over the duration of the experiment were determined using the Friedman test, ANOVA test, and Kendall coefficient of concordance test (Kendall's W). Associations between steroid proportions and blood pressures were reported as the Pearson correlation coefficient with the significance of the association with a p-value. A p-value of less than 0.05 was reported for all analyses as significant difference.

Chapter 3: Results

3.1. Overview

This project aimed to conversions of cholesterol to secondary bile acids and cardiotonic steroids (CTSs). As described in the previous section, two rats, 1 spontaneously hypertensive rat (SHR) female and 1 Dahl salt sensitive (DSS) male rat, were used in a pilot experiment to test the protocol. Sixteen SHR and Wistar Kyoto (WKY) rats (6 experimental rats and 2 control rats) from each strain, with equal numbers of males and females) were gavaged (experimental rats only) with ^{14}C-C4)-cholesterol. The control rats were not administered the labelled cholesterol. Blood pressure (BP) and weight were measured weekly with overnight faecal samples collected in metabolic cages. Two rats died during the study and their and BPs were excluded from the data. Bile acids and CTS were identified using UPHLC-MS and commercially available standards. Relative quantification was by measuring the analyte relative to total bile acids measured, or (bile acids+CTSs measured was reported.

3.1.1 Animal models

Six month old WKY (n=6) and nine month old SHR (n=6) and controls (n=2 for each group) and an equal number of males and females, weighing between 196-352 g and 172-287 g respectively, were used to determine the conversion of ^{14}C-(C4)-cholesterol to bile acids and other steroid molecules.

Table 3.1: Weights and blood pressures recorded for the WKY and SHR rats over 4 weeks following administration of ^{14}C-(C4)-cholesterol. Male vs female *p<0.05; WKY vs SHR #p<0.05; (#) p=0.052); sex comparisons between strains were not significant – see text.

Parameter	WKY rats		SHR rats	
	Females (n=3)	Males (n=3)	Females (n=4)[a]	Males (n=3)
Weight (g)	203.0±6.2	317.3±24.2*	182.3±8.1	179.0±8.5
Initial SBP (mm Hg)	145.5±1.4	145.6±1.9	182.2±4.8(#)	173.9±19.7#
Final SBP (mm Hg) (Day 28)	149.5±0.4	149.7±0.7	197.1±4.7(#)	199.6±0.2#
Initial DBP (mm Hg)	84.5±2.1	84.0±2.1	78.7±15.4	63.9±1.9
Final DBP (mm Hg) (Day 28)	86.7±0.4	86.0±3.2	88.0±16.3	79.5±8.2

[a]: SHR pilot female rat was included

3.2 Blood pressure readings

Blood pressure (BP) readings were taken before the administration of ^{14}C-(C4)-cholesterol and then weekly over a period of 4 weeks until the termination of the rats.

Dahl Salt Sensitive (DSS) rats (1 male & 1 female) were used in the pilot study while awaiting the delivery of the WKY rats from Germany. The blood pressures of these rats increased from initially SBP/DBP 176/96 to 199/107mm Hg in the male and SBP/DBP 134/69 to 170/77mm Hg in the female DSS rat, which was different to the other study rat strains. Hence, the data from the DSS rats were excluded from blood pressure analysis.

Systolic blood pressures were higher ($p<0.0001$) and diastolic pressures ($p=0.21$) similar in SHR rats compared to the WKY rats, resulting in a greater pulse pressure of 110±18mm Hg and 65±7mm Hg, respectively ($p<0.0001$). Systolic BP increased for both WKY ($p=0.023$) and SHR rats ($p=0.013$) over the experimental period. Diastolic BP was unchanged in WKY rats and increased in SHR rats ($p=0.023$; Table 3.1; Figure 3.1.). Small sample size did not allow statistical comparsions between sex (female or male) between strains.

3.3 Radioactivity of faecal extracts

Radioactivity in the 3 day pooled faecal extracts (of 24 days) decreased rapidly to a constant activity after nine days. In males, this residual activity was about 5% (30 cpm) of the initial activity (600 cpm; 5%), whereas in female rats, the residual activity was about 9% of the activity measure on days 1-3. The decrease of radioactivity in the faecal samples signifies the metabolism or excretion of ^{14}C-cholesterol, this aligns with our hypothesis (Figure 3.2.).

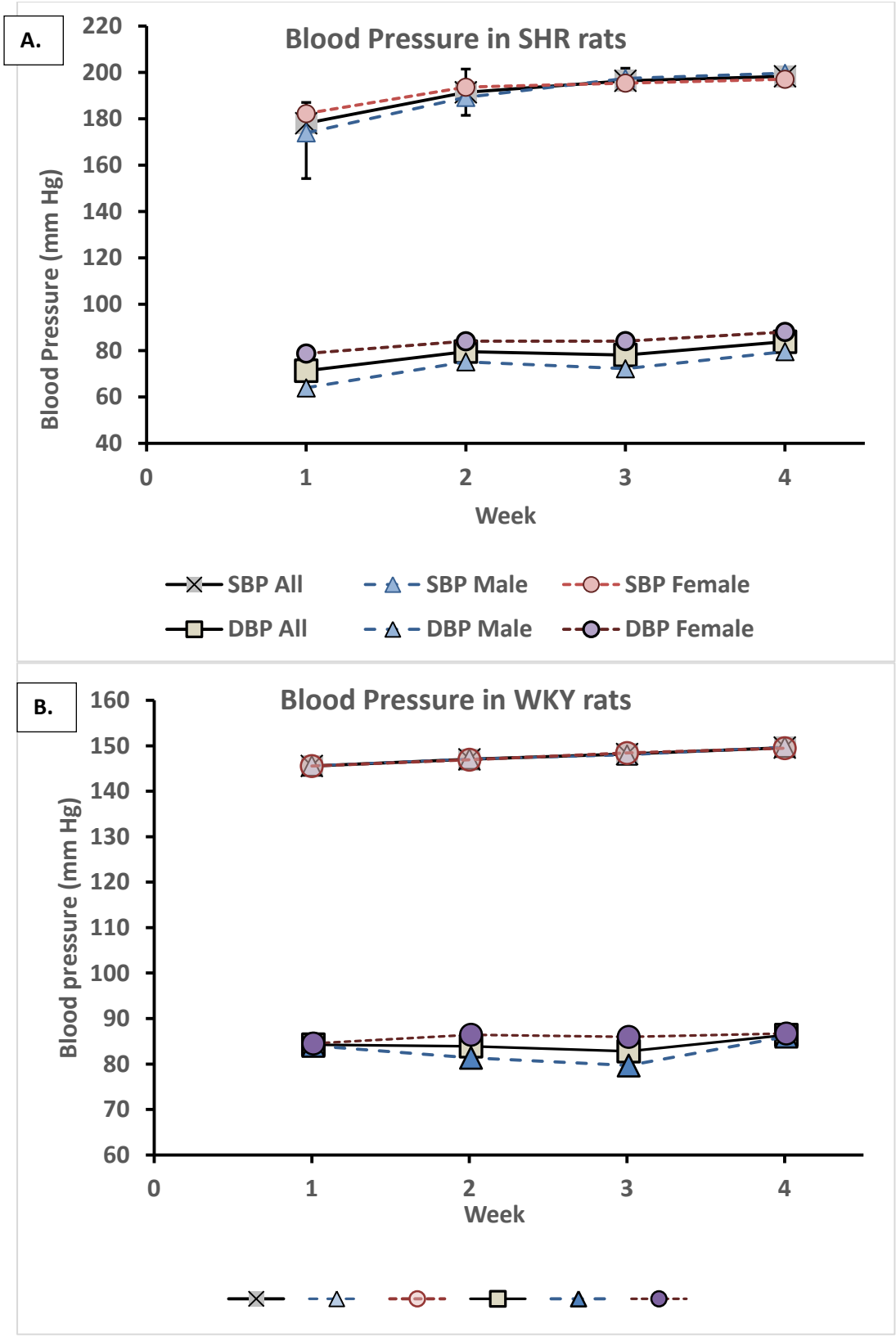


Figure 3.1: Blood pressure measures and changes recorded in A.) SHR rats and B.) WKY rats over the 24-day experiment. Blood pressures were similar in both male and female rats. Note the y axis scale is less in the WKY graph. SBP increased in both strains and in the WKY males ($p<0.05$) between weeks 1 and 4.

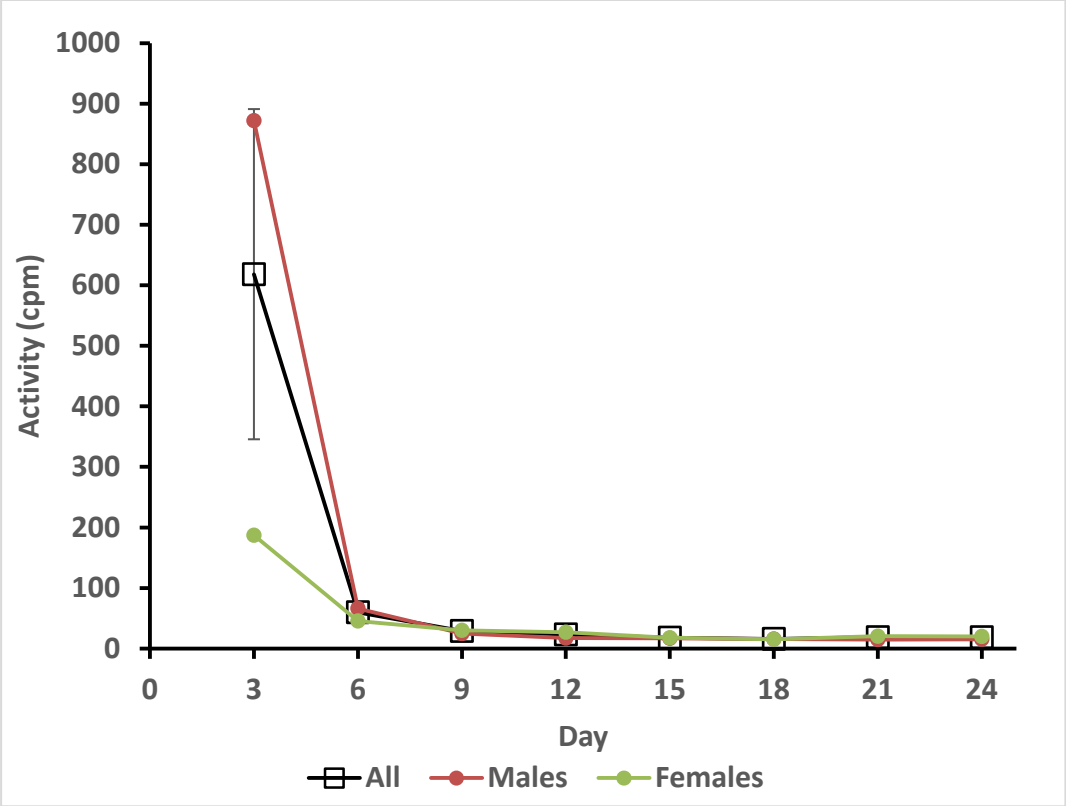


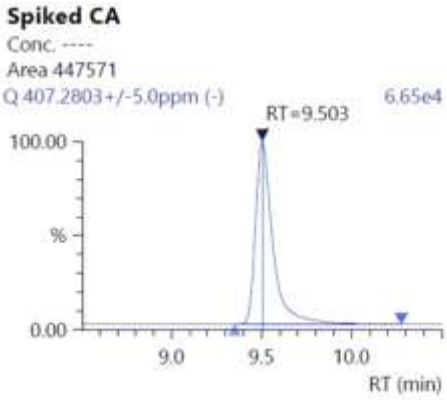
Figure 3.2: Radioactivity in the faecal material of SHR rats. Data as mean±SEM for all data (n=6) and means were shown for males (n=3) and females (n=3) without SEM.

3.4 UHPLC/Q-TOF analysis of the internal standards

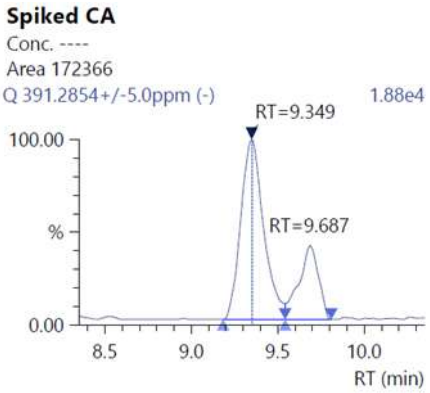
The chromatograms with retention times (RT) of bile acids (Figure 3.3) and CTSs (Figure 3.4) standards acquired by LC/MS Q-TOF were summarised in Table 3.1. The chromatograms of their ^{14}C-labelled counterparts were also acquired (Figure 3.5). A lithocholic (LCA) standard was not available and an approximate value was manually input into the MS to acquire a chromatograph.

Some bile acids and standards had multiple peaks, which were isomers of the same molecular weight and differing retention times because of the position of the hydroxyl groups. Several bile acids (chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and hyodeoxycholic acid (HDCA)) had very similar or identical molecular weights making peak assignment difficult.

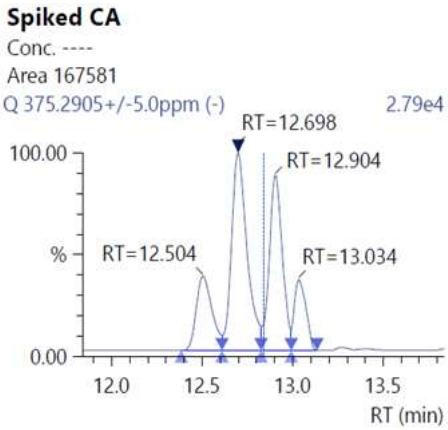
(A)



Cholic acid m/z 407.2803

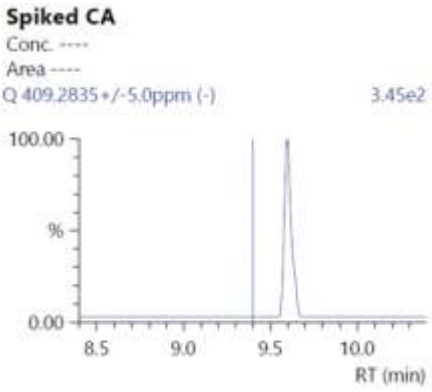


Hyodeoxycholic acid m/z 391.2854

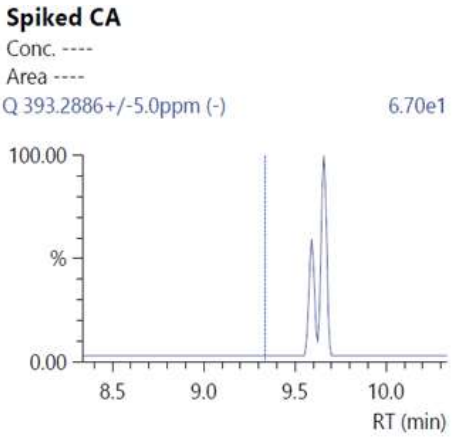


Lithocholic acid 375.2905

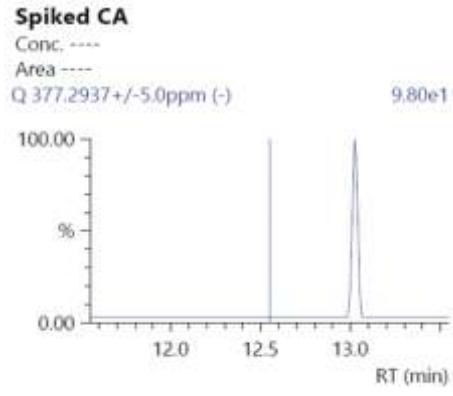
(B)



^{14}C-cholic acid m/z 409.2835



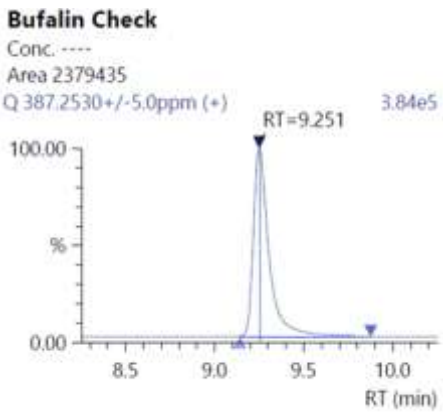
^{14}C-Hyodeoxycholic acid m/z 393.2886



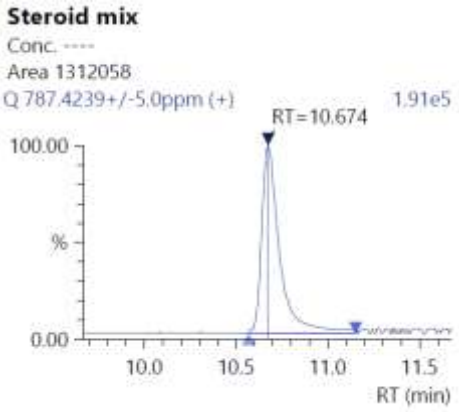
^{14}C-Lithocholic acid m/z 377.2937

Figure 3.3: **(A)** Chromatograms, m/z and retention times of bile acid (BA) standards acquired through LC/MS Q-TOF **(B)** ^{14}C-labelled equivalent BAs were generated from chromatograms. CA: cholic acid; HDCA: hyodeoxycholic acid; LCA: lithocholic acid.

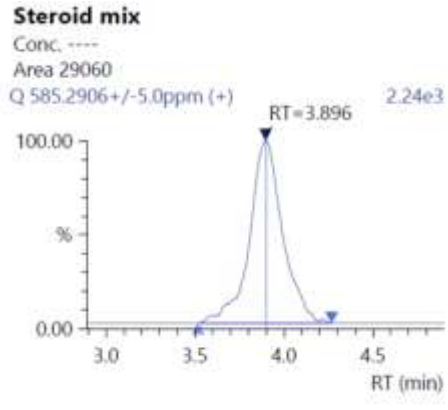
(A)



Bufalin m/z 387.2530

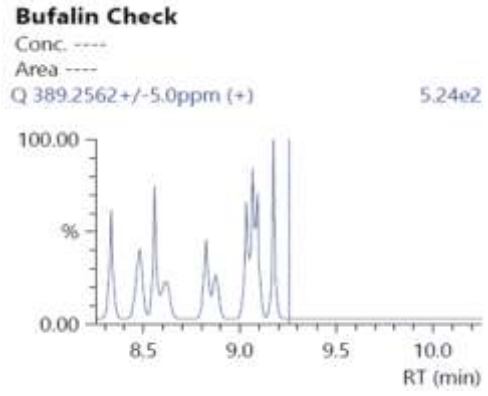


Ouabain m/z 585.2906

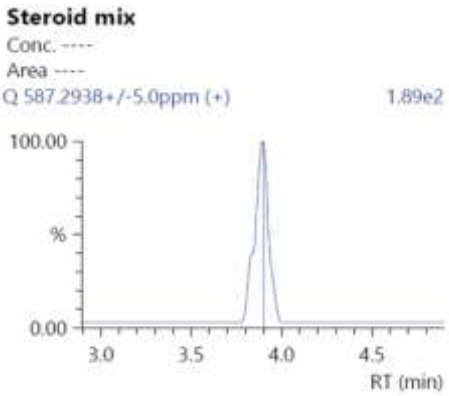


Digitoxin m/z 787.4739

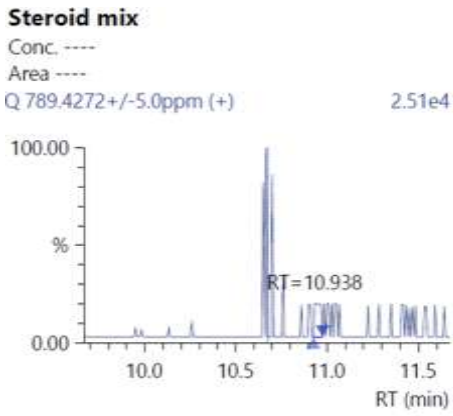
(B)



^{14}C-Bufalin m/z 389.2562



^{14}C-ouabain 587.2938



^{14}C-Digitoxin 789.4272

Figure 3.4: **(A)** Chromatograms with m/z, retention times of cardiotonic steroid standards acquired through UHPLC/MS Q-TOF: bufalin, ouabain and digitoxin and **(B).** ^{14}C-labelled equivalents were generated from the Shimadzu online database.

Table 3.2: Summary of retention times as mean±standard deviation [minimum – maximum] and m/z of standards using the UHPLC/QTOF with ion selective monitoring. Conditions have been described in the text.

	Retention time (min)	***m/z***
Cholesterol	10.062±0.010 [10.047 -10.089] (n=20)	369.3540
^{14}C-Cholesterol	10.069±0.007 [10.058 – 10.085]	371.3596
Primary bile acids		
Cholic acid	9.715±0.103 [9.661 – 10.105]	407.2953
^{14}C-Cholic acid	9.62	409.3676
Chenodeoxycholic acid	7.876±0.100 [7.580 – 8.065]	391.3200
^{14}C- Chenodeoxycholic acid	8.035±00..098 [7.860 – 8.188]	393.3200
Secondary bile acids		
Deoxycholic acid	7.018±0.066 [6.860 – 7.237	391.2845
^{14}C- Deoxycholic acid	7.025±0.097 [6.716 – 7.207]	393.2906
Hyodeoxycholic acid	9.349 & 9.687	391.2854
^{14}C-hyodexoycholic acid\	9.58 & 9.65	393.2886
Lithocholic acid	12.504, 78.698, 12.904 & 13.034	375.2905
^{14}C-Lithocholic acid	13.2	377.2937
Cardiotonic steroids		
Bufalin	9.251	387.2530
^{14}C-Bufalin		389.2562
Ouabain	3.896	585.2906
^{14}C-Ouabain		587.2938
Digitoxin	10.674	787.4239
^{14}C-digitoxin		789.4272

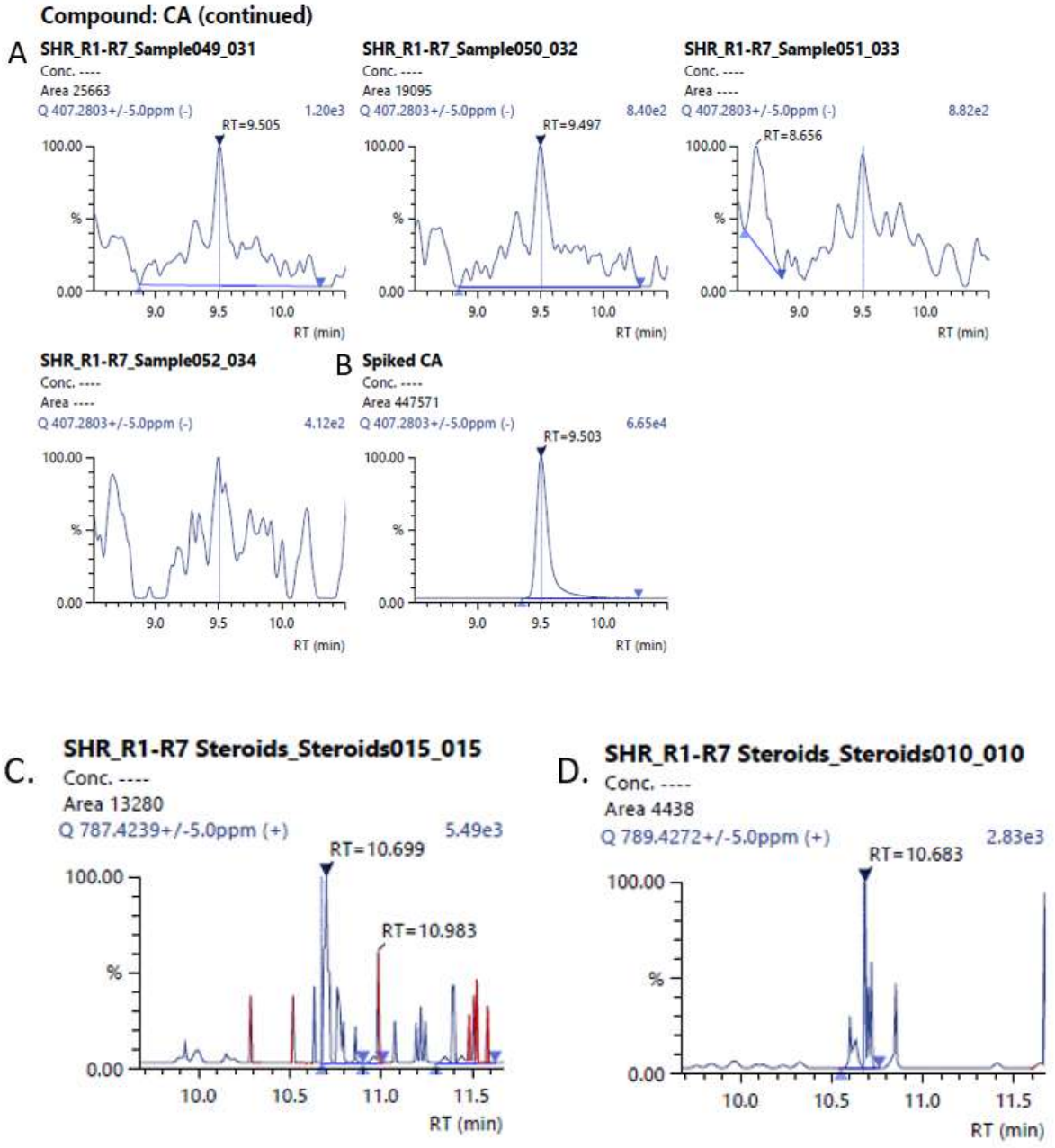


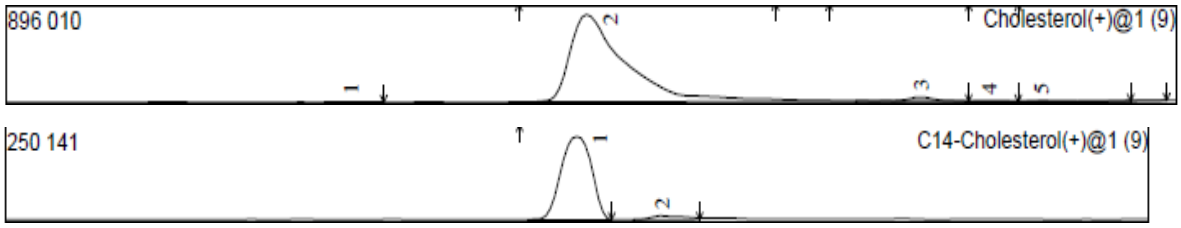
Figure 3 5: Selection of chromatograms with ion selective monitoring for A.) cholic acid (CA;m/z 407.2803; top 2 rows) showing the complexity of the sample. Cholic acid was not detected in many samples. B.) CA standard. Lower chromatograms C). Digitoxin (m/z :787.4239±5.0 ppm detected in the sample of a male SHR rat. D.) Detection of ^{14}C-digitoxin (m/z: 787.4272) detected in the same sample.

3.5 Profiling of extractions and data analysis using UHPLC/MS Q-TOF

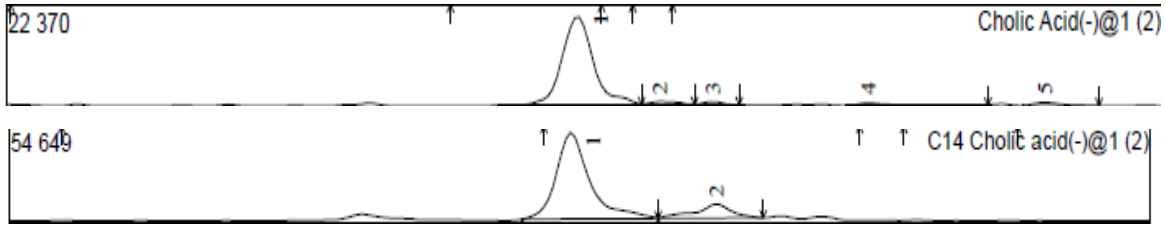
3.5.1 Analysis of bile acids and cardiotonic steroids

Chromatograms of the faecal extracts analysis for using HPLC and targeted ion-selective monitoring MS have been shown below (Figure 3.6.). The conversion of ^{14}C-(C4)-cholesterol to ^{14}C-labelled bile acids and CTSs was detected by monitoring peaks 2amu's heavier than the *m/z* of the standard (Table 3.2). As noted above, peak assignment was difficult with closely eluting peaks, ions being detected on the slope of overlapping peaks, peaks of similar molecular weight and the unavailability of specific standards (Figure 3.5.).

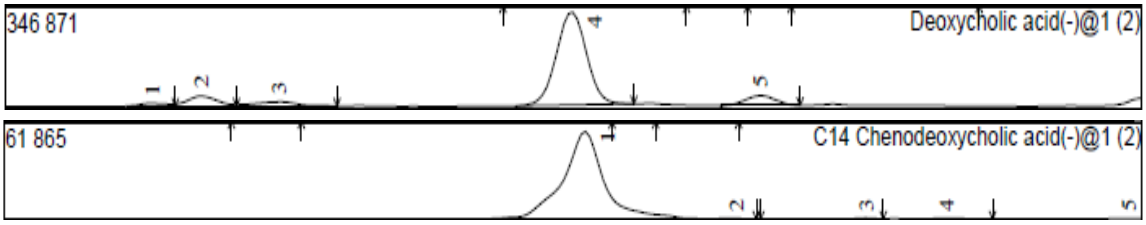
A. Cholesterol



B. Cholic acid



C. Chenodeoxycholic acid



D. Secondary bile acids, deoxycholic acid

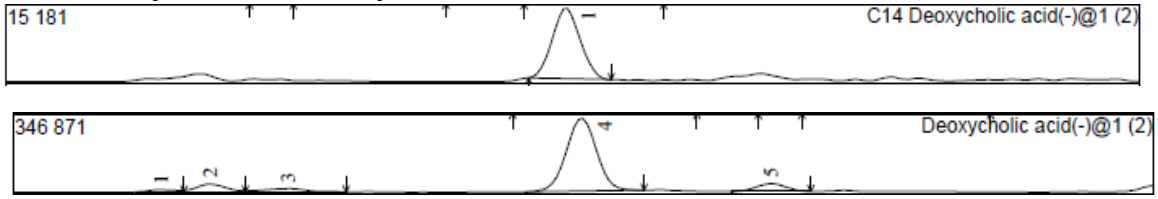


Figure 3.6: Typical UHPLC chromatograms of A.) cholesterol, B.) the primary bile acids, cholic acid, and C.) chenodeoxycholic acid, and D.)the secondary bile acid deoxycholic acid extracted from faecal material of a female SHR rat. Detection of the compounds was by Q-TOF with ion-selective monitoring. The ^{14}C-labelled compound is shown in the lower panel for each compound.

Table 3.3: Result output of peak areas and heights of ion-selective monitoring of cholic (upper panel), deoxycholic (middle panel) and ^{14}C-labeled deoxycholic acid (lower) for a SHR female (labelled as Rat 6 in this Table) and the initial test DSS male rat (titled as Rat 24 in this Table) for samples combined for days 1-3 (titled as 001) and days 4-6 (titled as 002). See Appendix A.1. for the EXCEL Table of this.

MS Summary(Compound)

ID#1 Compound Name: Cholic Acid m/z: 407.2953

Title	Sample Name	Sample ID	Ret. Time	Area	Height	Conc.
Blank 001 Bile Acids 001.lcd	Blank 001	Blank 001	9.845	917	257	0.000
Rat 6 001 Bile Acids 002.lcd	Rat 6 001	Rat 6 001	9.684	149290	19631	0.000
Rat 6 002 Bile Acids 003.lcd	Rat 6 002	Rat 6 002	9.692	50943	6721	0.000
Rat 6 003 Bile Acids 004.lcd	Rat 6 003	Rat 6 003	9.678	129957	17521	0.000
Rat 6 004 Bile Acids 005.lcd	Rat 6 004	Rat 6 004	9.684	100133	13157	0.000
Rat 6 005 Bile Acids 001.lcd	Rat 6 005	Rat 6 005	9.671	85728	12561	0.000
Rat 6 006 Bile Acids 002.lcd	Rat 6 006	Rat 6 006	9.672	92408	12112	0.000
Rat 6 007 Bile Acids 003.lcd	Rat 6 007	Rat 6 007	9.671	106737	14492	0.000
Rat 6 008 Bile Acids 004.lcd	Rat 6 008	Rat 6 008	9.737	377	100	0.000
Rat 6 009 Bile Acids 005.lcd	Rat 6 009	Rat 6 009	9.683	13014	1967	0.000
Blank 002 Bile Acids 006.lcd	Blank 002	Blank 002	9.675	452	120	0.000
Rat 24 001 Bile Acids 007.lcd	Rat 24 001	Rat 24 001	9.686	91931	12890	0.000
Rat 24 002 Bile Acids 008.lcd	Rat 24 002	Rat 24 002	9.679	7839	1171	0.000
Rat 24 003 Bile Acids 009.lcd	Rat 24 003	Rat 24 003	9.671	124228	15591	0.000
Rat 24 004 Bile Acids 010.lcd	Rat 24 004	Rat 24 004	9.674	117711	14699	0.000
Rat 24 005 Bile Acids 011.lcd	Rat 24 005	Rat 24 005	9.677	117369	15852	0.000
Rat 24 006 Bile Acids 012.lcd	Rat 24 006	Rat 24 006	9.664	135269	19047	0.000
Rat 24 007 Bile Acids 013.lcd	Rat 24 007	Rat 24 007	9.661	163502	21177	0.000
Rat 24 008 Bile Acids 014.lcd	Rat 24 008	Rat 24 008	9.844	355	94	0.000
Rat 24 009 Bile Acids 015.lcd	Rat 24 009	Rat 24 009	9.674	29107	4336	0.000
Blank 003 Bile Acids 016.lcd	Blank 003	Blank 003	10.105	324	86	0.000
Average			9.715	72266	9694	0.000
%RSD			1.061	80.112	78.677	0.000
Maximum			10.105	163502	21177	0.000
Minimum			9.661	324	86	0.000
Standard Deviation			0.103	57894	7627	0.000

ID#2 Compound Name: Deoxycholic acid m/z: 391.2845

Title	Sample Name	Sample ID	Ret. Time	Area	Height	Conc.
Blank 001 Bile Acids 001.lcd	Blank 001	Blank 001	6.860	2041	361	0.000
Rat 6 001 Bile Acids 002.lcd	Rat 6 001	Rat 6 001	7.018	2387830	322839	0.000
Rat 6 002 Bile Acids 003.lcd	Rat 6 002	Rat 6 002	7.019	184553	25538	0.000
Rat 6 003 Bile Acids 004.lcd	Rat 6 003	Rat 6 003	7.015	549923	74999	0.000
Rat 6 004 Bile Acids 005.lcd	Rat 6 004	Rat 6 004	7.013	305063	42021	0.000
Rat 6 005 Bile Acids 001.lcd	Rat 6 005	Rat 6 005	7.009	158939	18990	0.000
Rat 6 006 Bile Acids 002.lcd	Rat 6 006	Rat 6 006	7.010	472701	62556	0.000
Rat 6 007 Bile Acids 003.lcd	Rat 6 007	Rat 6 007	7.004	338980	45303	0.000
Rat 6 008 Bile Acids 004.lcd	Rat 6 008	Rat 6 008	--	--	--	N.D.(W/B)
Rat 6 009 Bile Acids 005.lcd	Rat 6 009	Rat 6 009	7.023	64460	9495	0.000
Blank 002 Bile Acids 006.lcd	Blank 002	Blank 002	7.237	880	232	0.000
Rat 24 001 Bile Acids 007.lcd	Rat 24 001	Rat 24 001	7.018	204091	29902	0.000
Rat 24 002 Bile Acids 008.lcd	Rat 24 002	Rat 24 002	7.023	50929	7117	0.000
Rat 24 003 Bile Acids 009.lcd	Rat 24 003	Rat 24 003	7.019	646921	87731	0.000
Rat 24 004 Bile Acids 010.lcd	Rat 24 004	Rat 24 004	7.012	172582	21315	0.000
Rat 24 005 Bile Acids 011.lcd	Rat 24 005	Rat 24 005	7.017	226335	30614	0.000
Rat 24 006 Bile Acids 012.lcd	Rat 24 006	Rat 24 006	7.007	208591	26551	0.000
Rat 24 007 Bile Acids 013.lcd	Rat 24 007	Rat 24 007	7.007	1080218	136651	0.000
Rat 24 008 Bile Acids 014.lcd	Rat 24 008	Rat 24 008	--	--	--	N.D.(W/B)
Rat 24 009 Bile Acids 015.lcd	Rat 24 009	Rat 24 009	7.006	73941	9974	0.000
Blank 003 Bile Acids 016.lcd	Blank 003	Blank 003	--	--	--	N.D.(W/B)
Average			7.018	396054	52899	0.000
%RSD			0.939	142.767	143.400	0.000
Maximum			7.237	2387830	322839	0.000
Minimum			6.860	880	232	0.000
Standard Deviation			0.066	565436	75858	0.000

ID#3 Compound Name: C14 Cholic acid m/z: 409.3676

Title	Sample Name	Sample ID	Ret. Time	Area	Height	Conc.
Blank 001 Bile Acids 001.lcd	Blank 001	Blank 001	9.703	1110	252	0.000
Rat 6 001 Bile Acids 002.lcd	Rat 6 001	Rat 6 001	9.707	412852	50618	0.000
Rat 6 002 Bile Acids 003.lcd	Rat 6 002	Rat 6 002	9.711	52701	7158	0.000
Rat 6 003 Bile Acids 004.lcd	Rat 6 003	Rat 6 003	9.703	122676	17675	0.000
Rat 6 004 Bile Acids 005.lcd	Rat 6 004	Rat 6 004	9.706	53543	7040	0.000
Rat 6 005 Bile Acids 001.lcd	Rat 6 005	Rat 6 005	9.695	136413	16883	0.000
Rat 6 006 Bile Acids 002.lcd	Rat 6 006	Rat 6 006	9.693	141448	19698	0.000
Rat 6 007 Bile Acids 003.lcd	Rat 6 007	Rat 6 007	9.693	122109	15132	0.000
Rat 6 008 Bile Acids 004.lcd	Rat 6 008	Rat 6 008	9.859	657	168	0.000
Rat 6 009 Bile Acids 005.lcd	Rat 6 009	Rat 6 009	9.723	12162	1688	0.000
Blank 002 Bile Acids 006.lcd	Blank 002	Blank 002	9.471	712	144	0.000
Rat 24 001 Bile Acids 007.lcd	Rat 24 001	Rat 24 001	9.706	202744	25756	0.000
Rat 24 002 Bile Acids 008.lcd	Rat 24 002	Rat 24 002	9.719	11569	1471	0.000
Rat 24 003 Bile Acids 009.lcd	Rat 24 003	Rat 24 003	9.703	202936	21929	0.000
Rat 24 004 Bile Acids 010.lcd	Rat 24 004	Rat 24 004	9.701	170362	20090	0.000
Rat 24 005 Bile Acids 011.lcd	Rat 24 005	Rat 24 005	9.691	218185	25852	0.000
Rat 24 006 Bile Acids 012.lcd	Rat 24 006	Rat 24 006	9.684	401524	40805	0.000
Rat 24 007 Bile Acids 013.lcd	Rat 24 007	Rat 24 007	9.677	328179	35515	0.000
Rat 24 008 Bile Acids 014.lcd	Rat 24 008	Rat 24 008	9.747	817	202	0.000
Rat 24 009 Bile Acids 015.lcd	Rat 24 009	Rat 24 009	9.695	42550	4862	0.000
Blank 003 Bile Acids 016.lcd	Blank 003	Blank 003	9.677	1106	278	0.000
Average			9.698	125541	14915	0.000
%RSD			0.664	104.411	99.189	0.000
Maximum			9.859	412852	50618	0.000

Conditions for separating and detecting bile acids and CTS were different (Tables 2.1 & Table 2.2) which made interpretation difficult as a result of having two separate analyses. In the absence of internal standards and calibration curves, bile acids and CTS coumpounds were calculated relative to that of total bile acids and CTSs detected. For reasons beyond the researcher's control (Covid and laboratory access), there were significant delays between obtaining initial studies to optimise UHPLC/Q-TOF conditions and results obtained when access to laboratories was permitted.

Figure 3.7 depicts the change in the relative ratio labelled to total bile acid, adjusted to the ratio of ^{14}C-labelled/total cholesterol. The primary bile acids were labelled in 1-3 days, whereas the label incorporation into the secondary bile acid, deoxycholic acid, as could be expected, only occurred from day 4-6) in a SHR female rat. Once labelled, the fraction of ^{14}C-label incorporated remained relatively constant. In the initial DSS male rat, the incorporation of the label into deoxycholic acid was not apparent.

In the main study, most bile acid, both primary and secondary, could not be detected in most samples (Table 3.3), except for HDCA and LCA. Furthermore, the equivalent ^{14}C labelled compounds were detected in a minority of the samples. Hence, only the relative quantities of secondary bile acids, HDCA to LCA in the SHR rats to WKY rats could be compared (Figure 3.8). No differences between sex or strain were found for the relative proportions of HDCA or LCA for each time (Mann Whitney U Test; $p>0.10$, except LCA at day 3 SHR vs WKY: $p=0.07$). No differences over time (Friedman ANOVA and Kendall coefficient of concordance test) for either SHR (HDCA: $p=0.60$; LCA: $p=0.12$) or in WKY rats (HDCA:$p=0.09$; LCA: $p=0.27$) were noted.

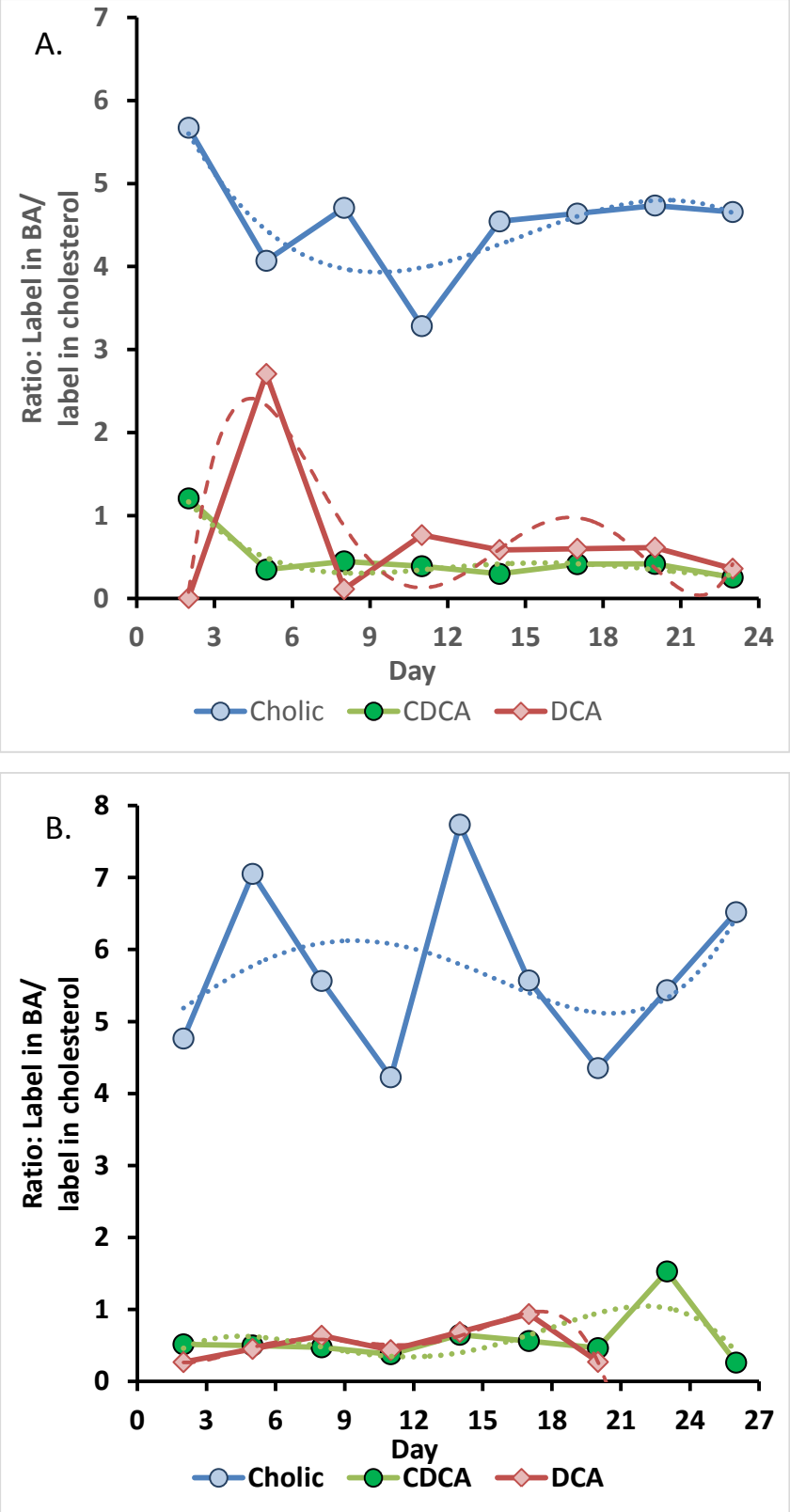


Figure 3.7: Ratios of measured ^{14}C-labelled bile acids /total bile acids relative to that of detected ^{14}C-labelled cholesterol/total cholesterol in a A.) a female SHR rat and a B.) pilot study DSS male rat over the duration of the experiment. Abbreviations: CDCA - chenodeoxychloic acid; DCA - deoxycholic acid.

Table 3.4: Output from the for the bile acids cholic acid (left) and the HDCA (right) for the main study with the SHR rats. Cholic acid (CA) was not detected in most samples whereas hyodeoxycholic acid (HDCA) was detected in most.

Compound: CA

Data Filename	Found RT	Area
SHR_R1-R7_Sample001_001	----	----
SHR_R1-R7_Sample002_001	----	----
SHR_R1-R7_Sample003_001	----	----
SHR_R1-R7_Sample004_002	----	----
SHR_R1-R7_Sample005_003	----	----
SHR_R1-R7_Sample006_004	----	----
SHR_R1-R7_Sample007_005	----	----
SHR_R1-R7_Sample008_006	9.493	18831
SHR_R1-R7_Sample009_007	----	----
SHR_R1-R7_Sample010_008	----	----
SHR_R1-R7_Sample011_009	----	----
SHR_R1-R7_Sample012_010	----	----
SHR_R1-R7_Sample013_011	----	----
SHR_R1-R7_Sample014_012	----	----
SHR_R1-R7_Sample015_013	9.508	31880
SHR_R1-R7_Sample016_014	9.506	27671
SHR_R1-R7_Sample017_015	9.501	6429
SHR_R1-R7_Sample018_016	----	----
SHR_R1-R7_Sample019_001	----	----
SHR_R1-R7_Sample020_002	----	----
SHR_R1-R7_Sample021_003	----	----
SHR_R1-R7_Sample022_004	----	----
SHR_R1-R7_Sample023_005	----	----
SHR_R1-R7_Sample024_006	----	----
SHR_R1-R7_Sample025_007	----	----
SHR_R1-R7_Sample026_008	----	----
SHR_R1-R7_Sample027_009	----	----
SHR_R1-R7_Sample028_010	----	----
SHR_R1-R7_Sample029_011	----	----
SHR_R1-R7_Sample030_012	----	----
SHR_R1-R7_Sample031_013	----	----
SHR_R1-R7_Sample032_014	9.528	21287
SHR_R1-R7_Sample033_015	----	----
SHR_R1-R7_Sample034_016	----	----
SHR_R1-R7_Sample035_017	----	----
SHR_R1-R7_Sample036_018	----	----
SHR_R1-R7_Sample037_019	----	----
SHR_R1-R7_Sample038_020	----	----
SHR_R1-R7_Sample039_021	----	----
SHR_R1-R7_Sample040_022	9.523	15656
SHR_R1-R7_Sample041_023	----	----
SHR_R1-R7_Sample042_024	----	----
SHR_R1-R7_Sample043_025	----	----
SHR_R1-R7_Sample044_026	----	----
SHR_R1-R7_Sample045_027	----	----
SHR_R1-R7_Sample046_028	9.528	112528
SHR_R1-R7_Sample047_029	----	----
SHR_R1-R7_Sample048_030	----	----
SHR_R1-R7_Sample049_031	9.505	25663
SHR_R1-R7_Sample050_032	9.497	19095
SHR_R1-R7_Sample051_033	----	----
SHR_R1-R7_Sample052_034	----	----
Spiked CA	9.503	447571

Compound: HDCA

Data Filename	Found RT	Area
SHR_R1-R7_Sample001_001	9.349	169328
SHR_R1-R7_Sample002_001	9.343	161361
SHR_R1-R7_Sample003_001	9.378	224242
SHR_R1-R7_Sample004_002	9.349	422323
SHR_R1-R7_Sample005_003	9.368	621717
SHR_R1-R7_Sample006_004	9.354	253486
SHR_R1-R7_Sample007_005	9.346	933264
SHR_R1-R7_Sample008_006	9.371	1796034
SHR_R1-R7_Sample009_007	9.350	227239
SHR_R1-R7_Sample010_008	9.372	250169
SHR_R1-R7_Sample011_009	9.359	97743
SHR_R1-R7_Sample012_010	9.365	90573
SHR_R1-R7_Sample013_011	9.364	333789
SHR_R1-R7_Sample014_012	9.380	1418707
SHR_R1-R7_Sample015_013	9.392	944706
SHR_R1-R7_Sample016_014	9.360	3857852
SHR_R1-R7_Sample017_015	9.361	53840
SHR_R1-R7_Sample018_016	9.366	148216
SHR_R1-R7_Sample019_001	9.408	306469
SHR_R1-R7_Sample020_002	9.393	210876
SHR_R1-R7_Sample021_003	9.390	525610
SHR_R1-R7_Sample022_004	9.385	198250
SHR_R1-R7_Sample023_005	9.398	213844
SHR_R1-R7_Sample024_006	9.375	685424
SHR_R1-R7_Sample025_007	9.378	161523
SHR_R1-R7_Sample026_008	9.395	236350
SHR_R1-R7_Sample027_009	9.371	51842
SHR_R1-R7_Sample028_010	9.383	171595
SHR_R1-R7_Sample029_011	9.387	129216
SHR_R1-R7_Sample030_012	9.350	209225
SHR_R1-R7_Sample031_013	9.411	1377102
SHR_R1-R7_Sample032_014	9.380	2067894
SHR_R1-R7_Sample033_015	9.356	127888
SHR_R1-R7_Sample034_016	9.372	259302
SHR_R1-R7_Sample035_017	9.391	106636
SHR_R1-R7_Sample036_018	9.378	89684
SHR_R1-R7_Sample037_019	9.366	111012
SHR_R1-R7_Sample038_020	9.385	108503
SHR_R1-R7_Sample039_021	9.384	23411
SHR_R1-R7_Sample040_022	9.368	1687993
SHR_R1-R7_Sample041_023	9.354	59064
SHR_R1-R7_Sample042_024	9.373	11318
SHR_R1-R7_Sample043_025	9.348	242045
SHR_R1-R7_Sample044_026	9.351	357128
SHR_R1-R7_Sample045_027	9.347	151913
SHR_R1-R7_Sample046_028	9.387	675833
SHR_R1-R7_Sample047_029	9.363	161107
SHR_R1-R7_Sample048_030	9.376	453787
SHR_R1-R7_Sample049_031	9.376	1025924
SHR_R1-R7_Sample050_032	9.365	805645
SHR_R1-R7_Sample051_033	9.376	590248
SHR_R1-R7_Sample052_034	9.364	426750
Spiked CA	9.349	172366

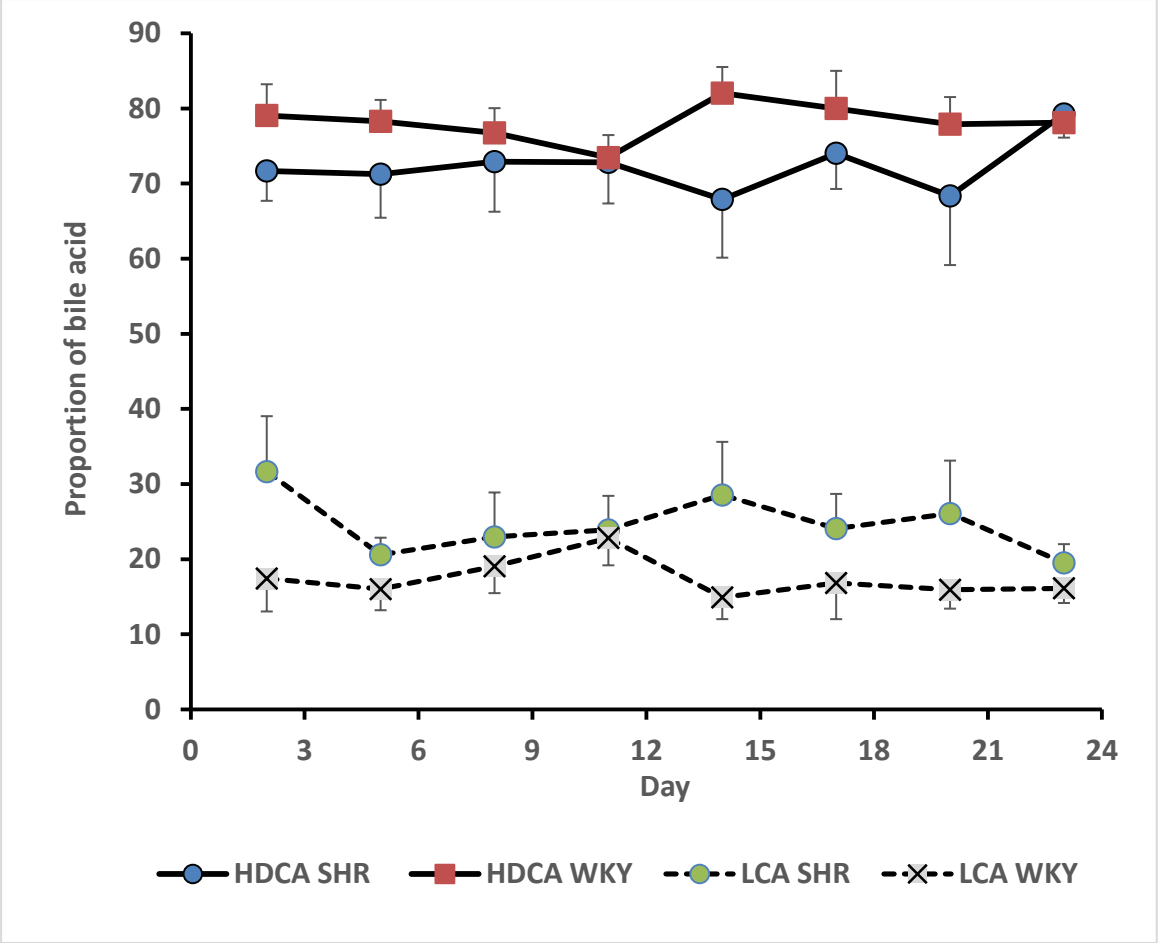


Figure 3.8: Relative proportion of secondary bile acids HDCA and LCA in SHR (n=7) and WKY rats (n=8) over the duration of the experiment. No differences between sex or strain were found for the relative proportions of HDCA or LCA for each time period (Mann Whitney U Test; $p>0.10$, except LCA at day 3 SHR vs WKY: $p=0.07$). No differences over time (Friendman ANOVA and Kendall coefficient of concordance test) for either SHR (HDCA: $p=0.60$; LCA: $p=0.12$) or in WKY rats (HDCA:$p=0.09$; LCA: $p=0.26$) were noted. Data as mean±SEM. Abbreviations: HDCA - hypdeoxycholic acid; LCA - lithocholic acid; SHR - spontaneously hypertensive rats; WKY - Wistar Kyoto rats.

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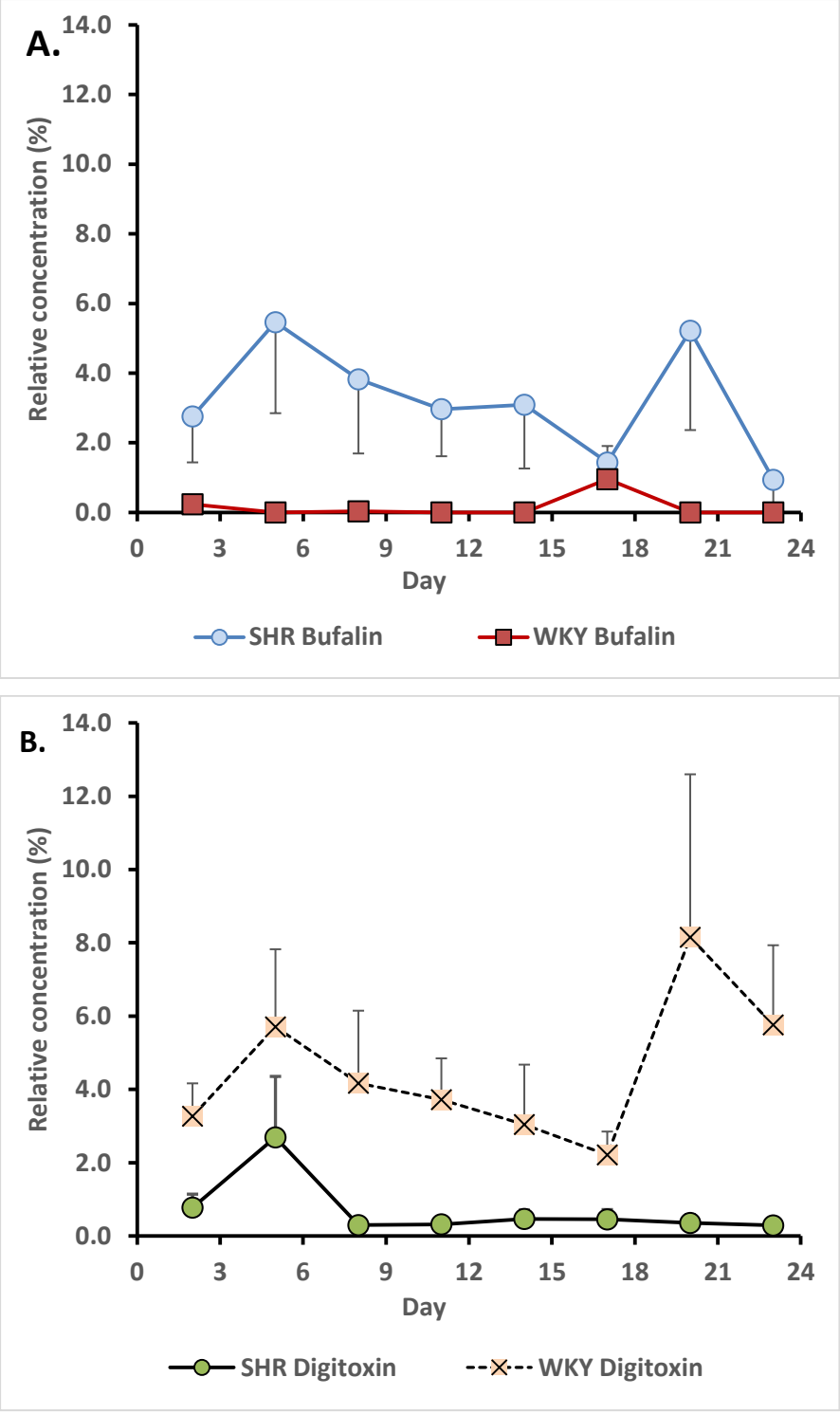


Figure 3.9: Time course of A.) bufalin and B.) digitoxin in SHR and WKY rats respectively over the 24 day experiment. Cardiotonic steroids (CTS) peak areas were expressed relative (total bile acid + CTS peak areas). Bufalin was detected in most SHR faecal samples and not detected in most WKY rat faecal samples and visa-versa. Hence, the few statistical differences have been mentioned in the text. Abbreviations: SHR - spontaneously hypertensive rats; WKY - Wistar Kyoto rats.

Incorporation of the ^{14}C-label into digitoxin or bufalin was not detected. The proportions of bufalin and digitoxin relative to total bile acids+CTSs detected in the sample, did not change significantly throughout the experiment (Friedman ANOVA and Kendall coefficient of concordance test) for SHR (bufalin: p=0.78; digitoxin: p=0.71) or in WKY rats (bufalin p=0.66; digitoxin p=0.26) (Figure 3.9.A and B).

The proportion of bufalin was higher in the SHR samples ($p<0.05$) except days 16-18 (p=0.11) and days 22-24 (p=0.053) (Figure 3.9.A) but was detected only in few WKY rats samples. Conversely, digitoxin was detected in all WKY samples and was absent in many SHR rats. The proportion of digtoxin was higher than that of the SHR rats throughout most of the experiment $p<0.005$ (Days 1-3: p=0.052; 4-6: p=0.09; 13-15: p=0.07; 18-20: p=0.053) (Figure 3.9.B).

Ouabain was not detected in any SHR or WKY samples.

3.5.2. Association of proportions of bile acids and cardiotonic steroids with blood pressure

Correlations were determined between blood pressure over the four week period and the relative proportions of bile acids or CTSs detected. In all rats, SBP and DBP correlated with the relative proportion of HDCA (SBP: r= -0.3706; p=0.0157, DBP: r=0.3773; p=0.0138) and with relative proportion of LCA (SBP: r= +0.3967; p=0.0093, DBP: r=-0.4064; p=0.0078) (Table 3.4.).

Blood pressure and WKY *vs* SHR rats.

Strain. WKY rats showed significant correlations with DBP and the relative proportions of the the secondary bile acids HDCA (r=0.3969; p=0.0271) and LCA (r=-0.3982; p=0.0265) (Table 3.4.). No such correlations were apparent in SHR rats (HDCA: r= +0.16; p=0.44, LCA: r= -0.22; p=0.28) (Figure 3.10.). No association was shown with SBP. However, in female rats, a significant relationship was found between SBP (-0.5929, p=0.0059) and DBP (+0.6181, p=0.0037) and the proportion of HDCA, and this was apparent in SHR female rats, although the small sample size was small.

Blood pressure and male vs female rats.

Female rats showed significant correlations of both SBP and DBP with the proportion of both HDCA (Figure 3.11. A) and LCA ((Figure 3.11. B; Table 3.4.)). Correlations in male SHR rats were not significant (Figure 3.11; stipple lines and Table 3.4).

Table 3.5: Pearson correlation coefficients (r) between blood pressures and sex in all rats and between WKY and SHR strains. P value shown in brackets below each number.

	All rats		**Sex Male**		**Sex Female**	
Bile acid & strain	**SBP**	**DBP**	**SBP**	**DBP**	**SBP**	**DBP**
HDCA						
All	**-0.3706 (0.0157)**	**+0.3773 (0.0138)**	+0.03 (0.91)	0.01 (0.97)	**-0.5929 (0.0059)**	**+0.6181 (0.0037)**
WKY	0.25 (0.18)	**0.6969 (0.0271)**	0.06 (0.85)	+0.37 (0.22)	0.44 (0.17)	0.48 (0.13)
SHR	-0.18 (0.36)	0.16 (0.44)	0.29 (0.41)	0.14 (0.70)	**-0.7567 (0.0183)**	**0.6811 (0.0434)**
LCA						
All	**+0.3967 (0.0093)**	**-0.4064 (0.0078)**	-0.02 (0.92)	-0.10 (0.65)	**0.6246 (0.0032)**	**-0.5828 (0.0070)**
WKY	-0.15 (0.41)	**-0.3982 (0.0265)**	-0.13 (0.68)	-0.24 (0.44)	-0.31 (0.35)	-0.48 (0.13)
SHR	0.22 (0.27)	-0.22 (0.28)	-0.16 (0.64)	-0.06 0.86)	0.64 (0.065)	-0.64 (0.065)

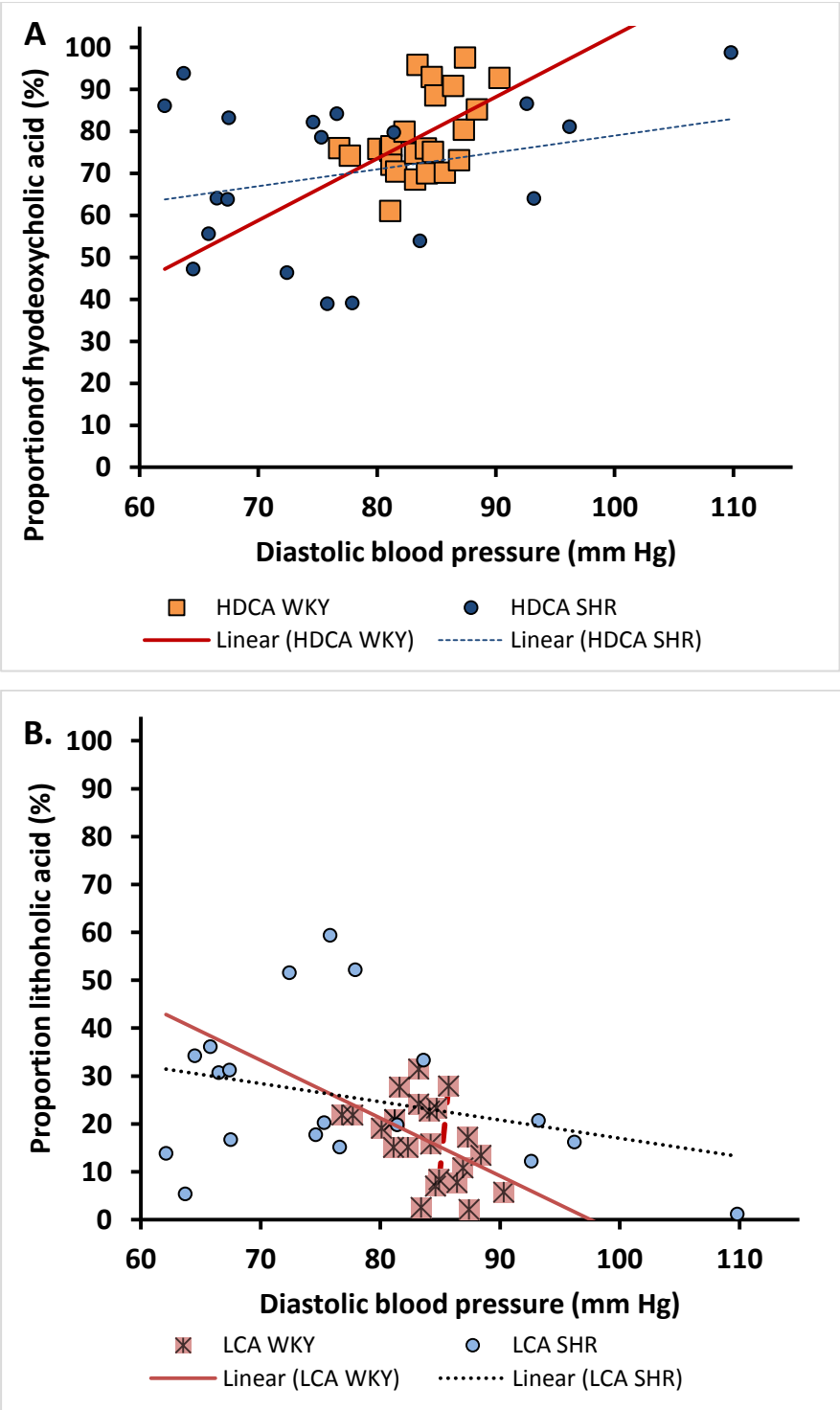
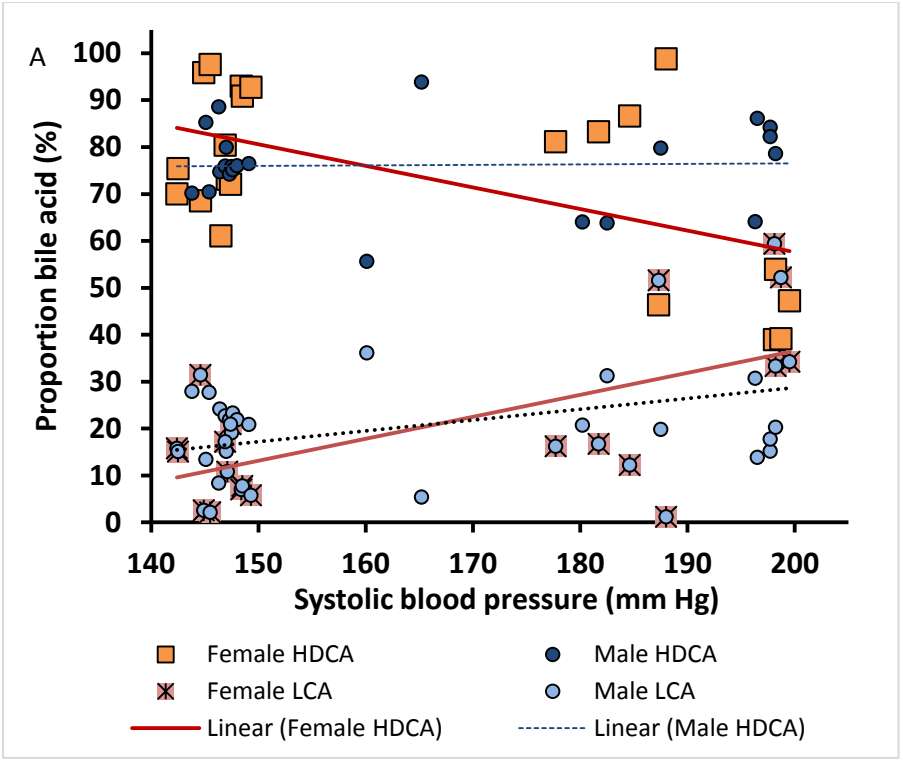


Figure 3.10: Relationship between diastolic blood pressure and secondary bile acids A.) hydodeoxycholic acid (HDCA) and B) lithocholic acid (LCA) in WKY and SHR rats. WKY rats showed significant correlations with the proportion of HDCA (bold lines: r=0.3969; p=0.0271) and LCA (r= -0.3982; p=0.0265). Correlations in SHR rats were not significant (stipple line, HDCA: r=0.16; p=0.44, LCA: r=-0.22; p=0.28). Abbreviations: SHR - spontaneously hypertensive rats; WKY - Wistar Kyoto rats.



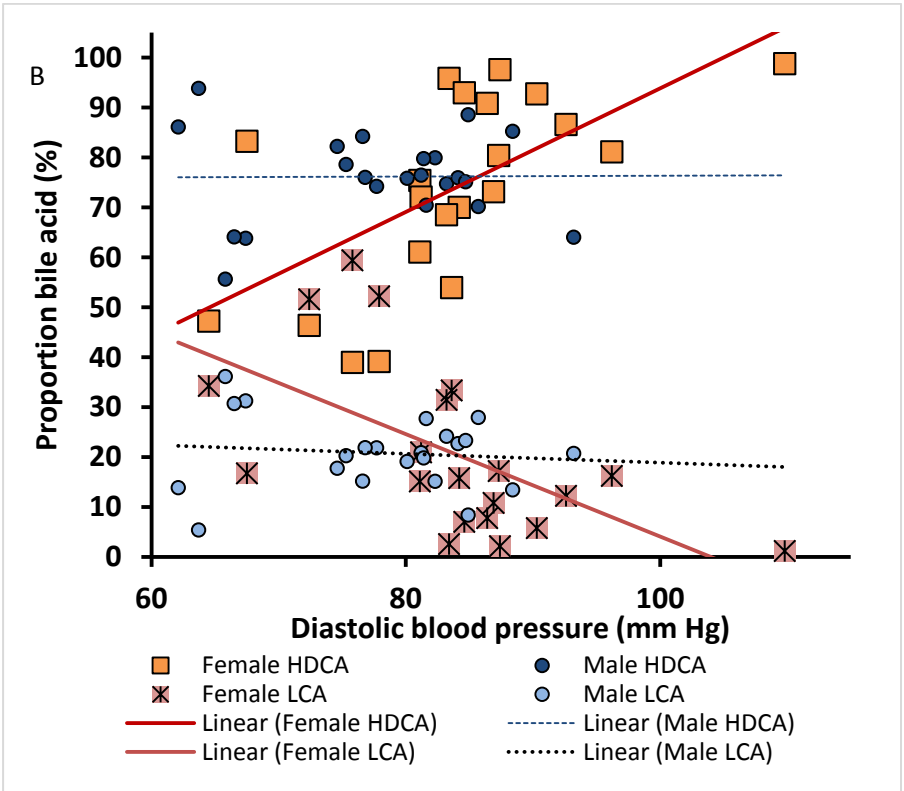


Figure 3.11: Relationship between A.) SBP and B.) DBP and the secondary bile acids HDCA and LCA in male and female rats. Female rats showed significant correlations of both A.) SBP (A) and B.) DBP with the proportion of HDCA and LCA (bold lines). Correlations in male SHR rats were not significant (stipple lines)—details of significance in Table 3.4. Abbreviations: SHR - spontaneously hypertensive rats; WKY - Wistar Kyoto rats; SBP – systolic blood pressure; DBP – diastolic blood pressure; HDCA - hypdeoxycholic acid; LCA - lithocholic acid.

Bufalin, digitoxin and blood pressure.

Bufalin was detected in some SHR samples and was almost absent in WKY samples and appeared not detected in rats with blood pressures SBP< 150 mm Hg (WKY rats). Conversely, digitoxin was detected in most WKY samples and was present at low proportions (<1.5%) in a few SHR samples. There appeared to be no association with either male or female rats.

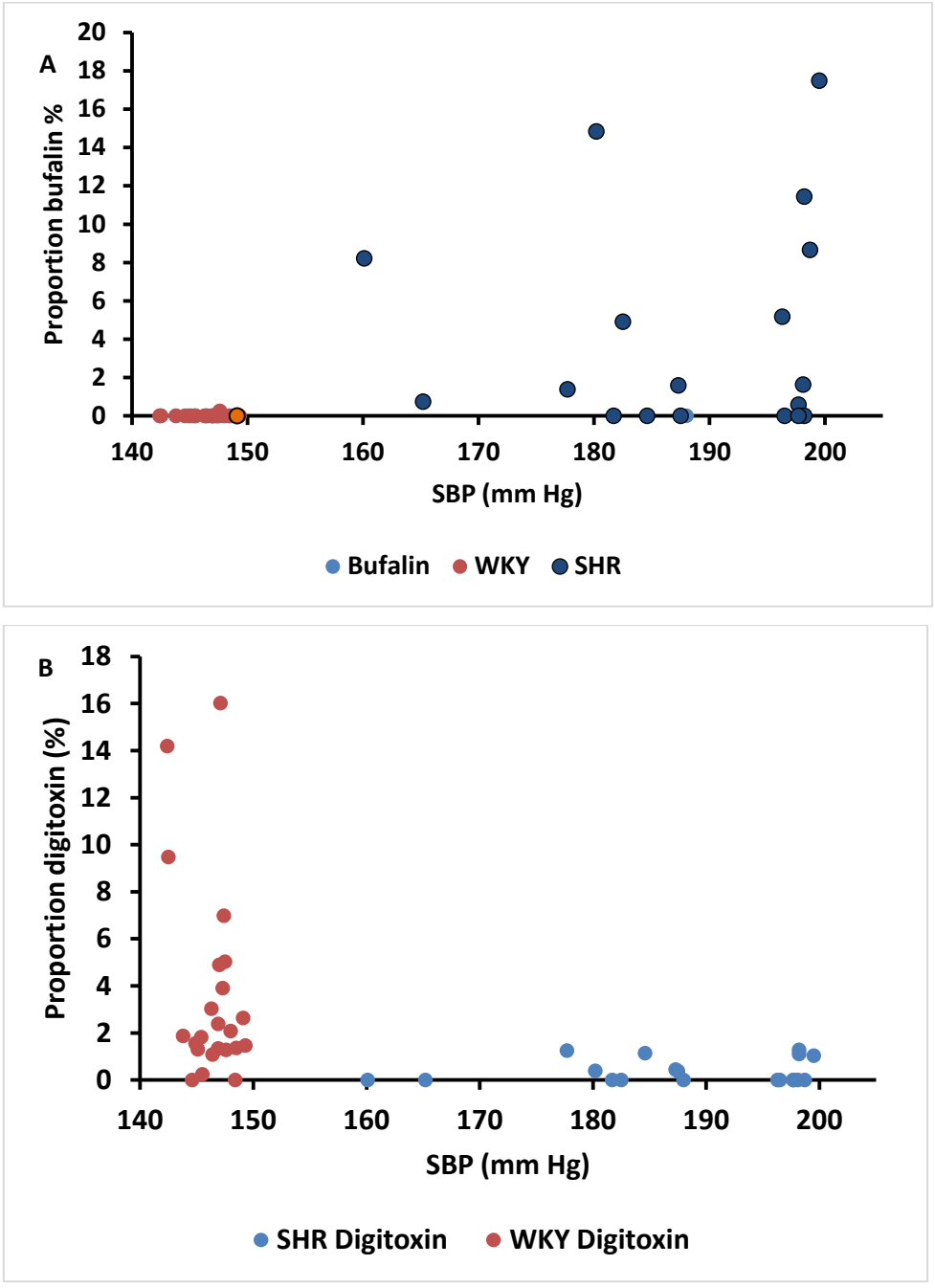


Figure 3.12: Scattergram of the relative proportion of A). bufalin and B.) digitoxin with SBP in SHR and WKY rats.

Chapter 4: Discussion

The aim of the study was to follow the conversion of ^{14}C-(C4)-labelled cholesterol to bile acids and cardiotonic steroids in SHR and WKY control rats. The main findings of this study were:

1)The presence of constant low levels of ^{14}C-label in faecal material with,

2) incorporation of label into determined primary and secondary bile acids.

3) detection of bufalin in certain SHR faecal samples and absence in WKY rats.

4) presence of digitoxin in most WKY rat faecal samples but only few, and at low concentrationsin SHR faecal samples; and

5) correlations of a.) only DBP and with the proportion of HDCA and LCA in WKY female rats b.) SBP and DBP with HDCA in SHR female rats.

4.1 Detection of bufalin and digitoxin in rat models of hypertension

^{14}C-(C4)- labelled cholesterol was used as the starting material in line with early studies in this field (Siperstein *et al*., 1957). This study found 'steady-state' ^{14}C-label in faecal material of an almost constant 5% of the dose in males and 9% in females but the reason for this apparent sex-based difference was not apparent.

The radiation of ^{14}C-(C4)-cholesterol radiation dose was calculated to be twice that administered to human patients per kilogram weight undergoing the ^{14}C-urea breath test for the stomach ulcer associated bacterium *Helicobacter pylori*. This amount of radioactivity administered to human patients is equivalent to less than that of a chest X-ray. Furthermore, the emitted beta-particle travels a short in air and the exposure to researchers would be minimal. The use of a radioactively labelled compound allowed the immediate monitoring of possible labelled compounds in the faecal matter, particularly in the latter part of the experiment when CTS production would be expected. Furthermore, monitoring of label in chromatographic separation procedures would have allowed alternative isolation of labelled compounds.

Follow-up studies should use non-radioactive ^{13}C labelled, or a deuterium labelled steroid to eliminate concerns of radiation exposure. Labelling at position 4 on the steroid fused cyclopentanoperhydro-phenanthrene 4-ring nucleus (Figure 1.4. and 1.5.) was appropriate to follow conversion of cholesterol to bile acids and the cardiotonic steroids. It would be of

interest to explore dual labelled starting material to elucidate conversions such as the side chain cleavage and lactone ring formation. Furthermore, although ^{14}C-labelled bufalin was detected in some samples, the conversion of cholesterol to the marinobufagenin was reported to be only about 2% (Siperstein *et al*., 1957) which may account for the lack of ^{14}C-labelled secondary bile acids and CTSs. Other starting steroid intermediates could be tested to improve the conversion to, and production of CTS compounds by the gut.

In toads, Siperstein *et al*. (1957) demonstrated that the bufadienolide MBG was synthesised from ^{14}C-cholesterol over 70 days to MBG (Siperstein *et al*., 1957). The biochemical transformations included cholesterol side chain degradation/replacement to give a doubly unsaturated lactone the incorporation of a tertiary hydroxyl group at C-5 (Siperstein *et al*., 1957). As pregnenolone was not an intermediate of the bufadienolides, it was concluded that cholesterol side chain cleavage did not occur (Siperstein *et al*., 1957; Garraffo and Gros, 1986).

Methyl-*tert* butyl ether has been reported to extract most lipid classes (Matyash *et al*. 2008) and was used in the present study. It has been suggested that a MTBE/methanol/water (10:3:2.5 (v/v/v)) mix may improve lipid extraction (Eggers and Schwudke *et al*. 2016) but this was not tested in the current study.

Losses in the main study may have arisen during the extraction on silica SPE cartridge as the post-sample elution with ethyl acetate was not run on the HPLC/MS. There were also significant delays between running the experiment and storage before analysis by HPLC/MS which might have contributed to sample degradation. In this regard, the half-life of CTSs, particularly bufalin (sigma-aldrich) is ~6 months, depending on storage conditions. Most of the samples were stored at -20°C and only analysed after a period of ~2 years. Purified bile acids can be stored at room temperature (see Study Limitations below).

4.1.1 Analysis of bile acids and CTSs

The method for bile acids was based on a Shimadzu technical application which was modified as outlined in the Methods in Chapter 2. The methods were developed with the kind assistance of technical HPC/MS support team (see Acknowledgements) from Shimadzu South Africa with the analyses undertaken on their equipment (see Study Limitations).

Although methods are available for the analysis of bile acids, including a kit from Shimadzu (Bile acid prep analysis HPLC_MS 2017_files; Shimadzu Corporation), we are unaware of

studies simultaneously determining both CTSs and bile acids. Indeed, in the present study the cardiotonic steroids were analysed with different solvent mobile phases with detection polarity in positive mode whereas the bile acids used negative mode.

Some bile acids and standards had multiple peaks which were isomers of the same molecular weight and because of the position of the hydroxyl groups had differing retention times. Several bile acids (chenodeoxycholic acid, deoxycholic acid and hyodeoxycholic acid) measured had very similar or identical molecular weights making peak assignment difficult without the use of MS/MS.

The analysis used UHPLC/MS with a targeted approach and did not profile all the bile acids or CTSs present in sample. Data analysis was limited to what was measured and a comparator was needed to eliminate errors arising from more concentrated and less concentrated samples. Therefore, the approach was to determine the proportion (% of area) of individual peak areas relative to total peak areas for the sample. This approach was limited when only few bile acids were measured. For example, in the case of the measurement of the two secondary bile acids HDCA and LCA in the samples, this resulted in the distinct and opposite trends observed for these bile acids and blood pressures (Figures 3.10. and 3.11. and Table 3.4).

4.1.2 Conversion of cholesterol to bile acids and CTSs

The proportion of radiolabelled cholesterol remained constant (SHR : 8.1±2.3%; WKY 7.6±2.5%) throughout the 27 days of the pilot study animals and was without any trend. Radioactivity suggests loss of ^{14}C-label in faecal matter between days 1-3 and days 4-6. (Figure 3.2) and it is not clear the fate of this label.

The proportion of ^{14}C-labelled cholic acid and chenodeoxycholic acid appeared to decrease in the SHR rat with the appearance of a secondary bile acid deoxycholic acid on days 4-6. These trends were not apparent in the WKY sample .

In the main study, the relative proportions of the secondary bile acids HDCA and LCA appeared to remain relatively constant throughout the measurement period (Figure 3.8.). The absence of radiolabel in many samples limited the interpretation of these observations.

4.1.3 Cardiotonic steroids in faecal matter

This appears to be the first report of the presence of cardiotonic steroids in faecal matter. Bufalin was detected in many SHR but in few WKY samples. The opposite was apparent for digitoxin which was detected in WKY samples and was present in only some SHR samples and at low levels (Figure 3.13.). As would be expected, the proportion of these CTSs was remained constant throughout study period (Figure 3.10.). These results confirm previous detection of bufalin and digitoxin in faecal matter from SHR samples from our laboratory and undertaken by Professor Duncan Cromarty at the University of Pretoria. This analysis also detected digoxin, marinobufagenin and telocinobufagenin and failed to detect ouabain. Thus, two laboratories have detected the presence of CTSs in faecal matter of rats and neither detected ouabain in these samples.

Immunoreactivity to bufalin in plasma has been reported to show some correlation with systolic blood pressure in humans and to show a similar relationship to that observed in the present study with digitoxin (Oda *et al*., 2001). Digitoxin has been reported to antagonise the actions of ouabain and it was interesting that in this study digitoxin was detected in faecal extracts of WKY rats and only low levels in the SHR rats (Figure 3.13; Manunta *et al*., 2000; Blaustein 2014).

Ouabain was not detected in any samples. Although Hamlyn *et al*. (1991) reported the presence of ouabain-like compound in human plasma, reports have since appeared demonstrating immunoactivity toward an ouabain-like substance in human plasma, which could not be confirmed by HPLC and other techniques (Lewis *et al*., 1994; Baecher *et al*. 2014).

Taken together, these data would be consistent with the hypothesis that an important source of the CTSs is the gut and that these CTS are transported into blood circulation.

4.2 Correlation of blood pressure, the proportion of HDCA and LCA and CTS

It was difficult to interpret data where bile acid and cardiotonic steroid were not present in all samples and a limited number of bile acids were detected and measured. The limitation of reporting relative proportions of HDCA and LCA has been discussed above.

The interesting data was the observation of digitoxin in WKY rats with lower blood pressures and the presence of bufalin in the SHR rats with higher blood pressures (Figure 3.13). As

discussed above, bufalin may correlate with systolic blood pressure in humans and show a similar relationship with digitoxin to that observed in the present study (Oda *et al*. 2001). Furthermore, digitoxin has been reported to antagonise the actions of ouabain (Manunta *et al*., 2000; Blaustein, 2014).

What remains to be determined is whether there are distinct profiles of CTSs in SHRs compared to WKY rats or whether this is simply an effect of blood pressure.

4.3 Study Limitations

The present study failed to detect ^{14}C-label incorporation into cardiotonic steroids. The absence of bile acid standards and the lack of access to instrumentation and laboratories during the Covid pandemic adversely affected the progress and outcomes of this study.

The study would not have been possible without the exceptional support of the staff at Shimadzu South Africa. Working with a commercial company was difficult as they had time constraints and many other commitments, which limited the access to the instrumentation and the scope of analysis that could be undertaken.

The use of non-radioactive ^{13}C-labelled primary or secondary bile acids, not cholesterol, and the use of a laboratory experiemced in the routine measurement of bile acids will greatly facilitate further studies. ^{14}C-labelled cholesterol was used to determined incorporation of label using instrumentation readily available in our Faculty. Furthermore, chromarographic separations could be undertaken to concentrate labelled fractions.

4.4 Conclusion

The important findings of this study following the conversion of 14C-(4)-labelled cholesterol in the rat models were the 1) incorporation of ^{14}C-label into primary and secondary bile acids; 2) detection of bufalin in some, not all, of the SHR samples and absence in the WKY rats; 3) presence of digitoxin in WKY rats with low levels in some SHR samples; and 4) correlation of blood pressure in WKY, not SHR rats, and in female rats, with the proportion of HDCA and LCA measured. The conclusions which can be made were limited as only certain bile acids and cardiotonic steroids were measured and detected. However, the study provides a valuable platform on which future studies can be based.

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Appendices

Appendix A: List of materials

Table A1: Laboratory consumables

Product:	**Manufacturer:**
5ml test tubes	Thermo Scientific™
10ml test tubes	Thermo Scientific™
10ml Falcon tubes	Thermo Scientific™
10ml bottles	PerkinElmer®
6ml Plastic scintillation vials	PerkinElmer®
Syringes	Shimadzu
Filters	Shimadzu
Silica-based SPE cartridges	Microsep
Autosampler vials	Shimadzu
0.5ml Autosampler vial glass tube inserts	Shimadzu

Table A2: Laboratory reagents

Product:	**Manufacturer:**
MTBE	Merck
Ultima Scintillation fluid	PerkinElmer® Midrand, South Africa
Methanol	Merck
Ethyl Acetate	Merck
Distilled water	Sigma-Aldrich
Acetic acid	Merck

Table A3: Chemicals

Product:	**Manufacturer:**
^{14}C-(C4)-cholesterol	PerkinElmer®
Bufalin	Sigma-Aldrich
Oaubain	Sigma-Aldrich
Digitoxin	Sigma-Aldrich
CA	Sigma-Aldrich
HDCA	Sigma-Aldrich

Appendix A.1.

ID#1 Compound Name: Cholic acid m/z: 407.2953									
	Title			**Sample Name**	**Sample ID**	**Ret Time**	**Area**	**Height**	**Conc.**
Blank 001	Bile Acids 00	1	.lcd	Blank 001	Blank 001	9.845	917	257	0.00
Rat 6 001	Bile Acids 00	2	.lcd	Rat 6 001	Rat 6 001	9.684	149290	19631	0.00
Rat 6 002	Bile Acids 00	3	.lcd	Rat 6 002	Rat 6 002	9.692	50943	6721	0.00
Rat 6 003	Bile Acids 00	4	.lcd	Rat 6 003	Rat 6 003	9.678	129957	17521	0.00
Rat 6 004	Bile Acids 00	5	.lcd	Rat 6 004	Rat 6 004	9.684	100133	13157	0.00
Rat 6 005	Bile Acids 00	1	.lcd	Rat 6 005	Rat 6 005	9.671	85728	12561	0.00
Rat 6 006	Bile Acids 00	2	.lcd	Rat 6 006	Rat 6 006	9.672	92408	12112	0.00
Rat 6 007	Bile Acids 00	3	.lcd	Rat 6 007	Rat 6 007	9.671	106737	14492	0.00
Rat 6 008	Bile Acids 00	4	.lcd	Rat 6 008	Rat 6 008	9.737	377	100	0.00
Rat 6 009	Bile Acids 00	5	.lcd	Rat 6 009	Rat 6 009	9.683	13014	1967	0.00
Blank 002	Bile Acids 00	6	.lcd	Blank 002	Blank 002	9.675	452	120	0.00
Rat 24 001	Bile Acids 00	7	.lcd	Rat 24 001	Rat 24 001	9.686	91931	12890	0.00
Rat 24 002	Bile Acids 00	8	.lcd	Rat 24 002	Rat 24 002	9.679	7839	1171	0.00
Rat 24 003	Bile Acids 00	9	.lcd	Rat 24 003	Rat 24 003	9.671	124228	15591	0.00
Rat 24 004	Bile Acids 00	10	.lcd	Rat 24 004	Rat 24 004	9.674	117711	14699	0.00
Rat 24 005	Bile Acids 00	11	.lcd	Rat 24 005	Rat 24 005	9.677	117369	15852	0.00
Rat 24 006	Bile Acids 00	12	.lcd	Rat 24 006	Rat 24 006	9.664	135269	190047	0.00
Rat 24 007	Bile Acids 00	13	.lcd	Rat 24 007	Rat 24 007	9.661	163502	21177	0.00
Rat 24 008	Bile Acids 00	14	.lcd	Rat 24 008	Rat 24 008	9.844	355	94	0.00
Rat 24 009	Bile Acids 00	15	.lcd	Rat 24 009	Rat 24 009	9.674	29107	4336	0.00
Blank 003	Bile Acids 00	16	.lcd	Blank 003	Blank 003	10.105	324	86	0.00
Average						9.715	72266	9694	0.00
% RSD						1.061	80.122	78.677	0.00
Maximum						10.105	163502	21177	0.00
Minimum						9.661	324	86	0.00
Standard deviation						0.103	57894	7627	0.00
ID#1 Compound Name: Deoxycholic acid m/z: 391.2845									
	Title			**Sample Name**	**Sample ID**	**Ret Time**	**Area**	**Height**	**Conc.**
Blank 001	Bile Acids 00	1	.lcd	Blank 001	Blank 001	6.86	2041	361	0.00
Rat 6 001	Bile Acids 00	2	.lcd	Rat 6 001	Rat 6 001	7.018	2387830	322839	0.00
Rat 6 002	Bile Acids 00	3	.lcd	Rat 6 002	Rat 6 002	7.019	184553	25538	0.00
Rat 6 003	Bile Acids 00	4	.lcd	Rat 6 003	Rat 6 003	7.015	549923	74999	0.00
Rat 6 004	Bile Acids 00	5	.lcd	Rat 6 004	Rat 6 004	7.013	305063	42021	0.00
Rat 6 005	Bile Acids 00	1	.lcd	Rat 6 005	Rat 6 005	7.009	158939	18990	0.00
Rat 6 006	Bile Acids 00	2	.lcd	Rat 6 006	Rat 6 006	7.01	472701	62556	0.00
Rat 6 007	Bile Acids 00	3	.lcd	Rat 6 007	Rat 6 007	7.004	338980	45303	0.00
Rat 6 008	Bile Acids 00	4	.lcd	Rat 6 008	Rat 6 008	--	--		N.D. (W/B)
Rat 6 009	Bile Acids 00	5	.lcd	Rat 6 009	Rat 6 009	7.023	64460	9495	0.00

Blank 002	Bile Acids 00	6	.lcd	Blank 002	Blank 002	7.237	880	232	0.00
Rat 24 001	Bile Acids 00	7	.lcd	Rat 24 001	Rat 24 001	7.018	204091	29902	0.00
Rat 24 002	Bile Acids 00	8	.lcd	Rat 24 002	Rat 24 002	7.023	50929	7117	0.00
Rat 24 003	Bile Acids 00	9	.lcd	Rat 24 003	Rat 24 003	7.019	646921	87731	0.00
Rat 24 004	Bile Acids 00	10	.lcd	Rat 24 004	Rat 24 004	7.012	172582	21315	0.00
Rat 24 005	Bile Acids 00	11	.lcd	Rat 24 005	Rat 24 005	7.017	226335	30614	0.00
Rat 24 006	Bile Acids 00	12	.lcd	Rat 24 006	Rat 24 006	7.007	208591	26551	0.00
Rat 24 007	Bile Acids 00	13	.lcd	Rat 24 007	Rat 24 007	7.007	108218	13661	0.00
Rat 24 008	Bile Acids 00	14	.lcd	Rat 24 008	Rat 24 008	--	--		N.D. (W/B)
Rat 24 009	Bile Acids 00	15	.lcd	Rat 24 009	Rat 24 009	7.006	73941	9974	0.00
Blank 003	Bile Acids 00	16	.lcd	Blank 003	Blank 003	--	--		N.D. (W/B)
Average						7.018	396054	52899	0.00
% RSD						0.939	142.767	143.1	0.00
Maximum						7.237	2387830	322839	0.00
Minimum						6.86	880	232	0.00
Standard deviation						0.066	56436	75858	0.00
ID#1 Compound Name: Deoxycholic acid m/z: 409.3676									
	Title			**Sample Name**	**Sample ID**	**Ret Time**	**Area**	**Height**	**Conc.**
Blank 001	Bile Acids 00	1	.lcd	Blank 001	Blank 001	9.703	1110	252	0.00
Rat 6 001	Bile Acids 00	2	.lcd	Rat 6 001	Rat 6 001	9.707	412852	50618	0.00
Rat 6 002	Bile Acids 00	3	.lcd	Rat 6 002	Rat 6 002	9.711	52701	7158	0.00
Rat 6 003	Bile Acids 00	4	.lcd	Rat 6 003	Rat 6 003	9.703	122676	17675	0.00
Rat 6 004	Bile Acids 00	5	.lcd	Rat 6 004	Rat 6 004	9.706	53543	7040	0.00
Rat 6 005	Bile Acids 00	1	.lcd	Rat 6 005	Rat 6 005	9.695	136413	16883	0.00
Rat 6 006	Bile Acids 00	2	.lcd	Rat 6 006	Rat 6 006	9.693	141448	19698	0.00
Rat 6 007	Bile Acids 00	3	.lcd	Rat 6 007	Rat 6 007	9.693	122109	15132	0.00
Rat 6 008	Bile Acids 00	4	.lcd	Rat 6 008	Rat 6 008	9.859	657	168	0.00
Rat 6 009	Bile Acids 00	5	.lcd	Rat 6 009	Rat 6 009	9.723	12162	1688	0.00
Blank 002	Bile Acids 00	6	.lcd	Blank 002	Blank 002	9.741	712	144	0.00
Rat 24 001	Bile Acids 00	7	.lcd	Rat 24 001	Rat 24 001	9.706	202744	25756	0.00
Rat 24 002	Bile Acids 00	8	.lcd	Rat 24 002	Rat 24 002	9.719	11569	1471	0.00
Rat 24 003	Bile Acids 00	9	.lcd	Rat 24 003	Rat 24 003	9.703	202936	21929	0.00
Rat 24 004	Bile Acids 00	10	.lcd	Rat 24 004	Rat 24 004	9.701	170362	20090	0.00
Rat 24 005	Bile Acids 00	11	.lcd	Rat 24 005	Rat 24 005	9.761	218185	25852	0.00
Rat 24 006	Bile Acids 00	12	.lcd	Rat 24 006	Rat 24 006	9.684	401524	40805	0.00
Rat 24 007	Bile Acids 00	13	.lcd	Rat 24 007	Rat 24 007	9.677	328179	35515	0.00
Rat 24 008	Bile Acids 00	14	.lcd	Rat 24 008	Rat 24 008	9.747	817	202	0.00
Rat 24 009	Bile Acids 00	15	.lcd	Rat 24 009	Rat 24 009	9.695	42550	4862	0.00
Blank 003	Bile Acids 00	16	.lcd	Blank 003	Blank 003	9.677	1106	278	0.00
Average						9.698	125541	14915	0.00
% RSD						0.664	104.411	99.189	0.00
Maximum						9.859	412852	50618	0.00

Appendix B: Ethics

AESC 2012 M&E

Please note that only typewritten applications will be accepted.

UNIVERSITY OF THE WITWATERSRAND
ANIMAL ETHICS SCREENING COMMITTEE
MODIFICATIONS AND EXTENSIONS TO EXPERIMENTS

a. Name: Geoffrey P Candy

b. Department: School of Animal, Plant and Environmental Science

c. Experiment to be modified / extended **AESC NO**

Original AESC number	**2018**	**09**	**42 /C**
Other M&Es :			0

d. Project Title: **Synthesis of cardiotonic steroids from ^{14}C-(4C)-labelled cholesterol in rat models of hypertension**

	No.	**Species**
e. Number and species of animals originally approved:	12 12 12	SHR rats Dahl Salt-sensitive rats WKY rats
f. Number of additional animals previously allocated on M&Es:	0	0
g. Total number of animals allocated to the experiment to date:	2	
h. Number of animals used to date:	2	1 SHR and 1 Dahl salt sensitive rats

i. Specific modification / extension requested:

a) Additional 2 SHR and 2 Dahl salt sensitive rats;
b) Of these 1 SHR rat and 1 Dahl rat will be gavaged with 14C-C-cholesterol as per protocol to optimise experimental conditions ;
c) The other 1 SHR rat and 1 Dahl rat will be fed gelatin cubes for 3 days and thereafter gelatin cubes with 1% cholesterol and 4% sodium chloride only, for 21 days. The rats will be transferred to metabolic cages for the collection of faecal samples each 3rd day;
d) Addition of co-workers –
- Hannah Mullah – will be undertaking the experimental work and using this toward an MSc;
- Sarhana Dinat – is running a related study using antibiotics in the same rat models and will be assisting Ms Mullah;
- Anza Thibia – upon euthanasia, Ms Thibia will be harvesting the stomach and gastrointestinal tract to determine associations of gut bacteria (microbiome) and the conversion of14C-cholesterol to bile acids, and, if the hypothesis is correct, to the cardiotonic steroids
- Dr. Ayodeji James Idowu – post –doctoral fellow – culture of Helicobacter spp. from the rats.

j. Motivation for modification / extension:

The 2 rats allocated for the gavage are being used to test the dosing of 14C-cholesterol and develop/optimise techniques – radiolabel dose, extraction methods, use of metabolic cages and GC/MS conditions;
We are uncertain under what conditions cardiotonic steroids may/may not form. We

AESC 2012 M&E

hypothesize that the *Helicobacter spp.* modifies cholesterol and upregulates genes in the presence of salt (NaCl). With the rats fed high cholesterol/salt, we should determine if these are required in the diet to produces cardiotonic steroids. The doses have been determined from the literature. The gelatin cubes are used to "solubilize" the cholesterol (it will float on water as a solid) and is given as 1% (w/w) of the animals weight. The NaCl could be mixed in the same cubes (to 4% of body weight) as the cholesterol.

Signature: Geoff Candy

Date: 29/05/2019

RECOMMENDATIONS

Additional 2 SHR and 2 Dahl salt sensitive rats including 1 SHR rat + 1 Dahl rat gavaged with 14C-C-cholesterol as per protocol and 1 SHR rat + 1 Dahl rat fed gelatin cubes with 1% bw cholesterol and 4% bw sodium chloride.

Addition of co-workers Hannah Mullah, Sarhana, Anza Thibia and Dr. Ayodeji James Idowu to the protocol – condition: to assist CAS orientation course.

Date: 30/05/2019

Signature:

Chairman, AESC pp.

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2018/09/42/C

APPLICANT: Professor G Candy

SCHOOL: Surgery
DEPARTMENT:
LOCATION:

PROJECT TITLE: Synthesis of steroids from 14C-(4C)-labeled cholesterol in rat models of hypertension

Number and Species

12X 8-10 weeks male WKY rats, 12X8-10 weeks male Dahl salt resistant rats, 12X 8-10 weeks male Dahl salt sensitive rats and 12X8-10 weeks male Spontaneously hypertensive rats

Approval was given for the use of animals for the project described above at an AESC meeting held on 2018/09/25. This approval remains valid until 2020/11/26.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

The Biosafety clearance needs to be obtained before the start of the experiments

Signed: _____ (Chairperson, AESC) Date: 27/11/2018

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 24/11/18

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

Research Office

INSTITUTIONAL BIOSAFETY COMMITTEE (IBC)
(R 14/16)

CLEARANCE CERTIFICATE **PROTOCOL NUMBER: 20190401**

BRIEF DESCRIPTION OF APPLICATION:

Synthesis of steroids from 14C-(4C)-labeled cholesterol in rat models of hypertension

APPLICANT: Prof GP Candy

SUPERVISOR: N/A

SCHOOL/DEPARTMENT : Surgery

DATE CONSIDERED: 03 June 2019

EXPIRY DATE: 03 June 2024

DECISION OF COMMITTEE: Approved unconditionally

1. This clearance certificate expires on 03 June 2024 and may be renewed on application
2. An annual report must be provided on the anniversary date of this certificate, for as long as the project continues
3. Notification of any proposed modifications must be submitted on the attached form
4. Unreported changes to the application may invalidate the clearance given by the IBC

DATE: 03 June 2019 **CHAIRPERSON:** _____
(Professor J Larkin)

DECLARATION OF APPLICANT:

To be completed in duplicate and **one copy** returned to the Research Office, Phillip Tobias Building, Room 301, 3rd Floor, 29 Princess of Wales Terrace, Parktown, Faculty of Health Sciences, University.

1. I have read, understood and accepted the approval conditions above.
2. I agree to submit a yearly progress report to the Committee and to submit an interim report on the form provided, in the event of any significant unforeseen event, e.g. suspension of a drug trial, temporary closure or relocation of my laboratory, etc.
3. I note that the University Safety Officer, or his/her representative, may at any reasonable time inspect my laboratory or trial site to ensure compliance with current Health and Safety legislation. I undertake to offer my full co-operation in any such inspection.
4. I have read, understood and will comply with the *recommended standard operating procedures for the handling of biohazardous materials* posted at http://web.wits.ac.za/Academic/Research/Biosafety.htm
5. I declare (delete as appropriate) that:
   a. I have all the approvals required by statute or regulation and by the funding agencies supporting this work, or
   b. that I will not begin work until such approvals are obtained

Signed: _____ **Date:** 19/6/2019

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C: Plagiarism report

Final dissertation.docx

ORIGINALITY REPORT

15%	9%	13%	2%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ncbi.nlm.nih.gov Internet Source	1%
2	pharmrev.aspetjournals.org Internet Source	1%
3	Buckalew, Vardaman M.. "Endogenous Digitalis-Like Factors: An Overview of the History", Frontiers in Endocrinology, 2015. Publication	<1%
4	Yanan Zhang, Xue Li, Di Xu, Mengyun Wu, Yinghui Dai, Mingyu Xia, Dong Wang. "Cloning and characterization of steroid 5β-reductase from the venom gland of Bufo bufo gargarizans", International Journal of Biological Macromolecules, 2021 Publication	<1%
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7	Submitted to Ain Shams University	