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Assessing cardiotonic steroids involvement in hypertensive rat models with *Helicobacter pylori* infections

by

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Dissertation submitted in partial fulfilment of the requirements for the degree

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In

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Co-supervisor: Mrs M. Leuschner (University of Pretoria)

Executive summary

Introduction: Hypertension is an important public health challenge worldwide, being the leading cause of cardiovascular disease, morbidity and mortality. It is particularly prevalent in people in sub-Saharan Africa, especially in urban areas. There is an urgent need to develop strategies to prevent, detect, treat, and control hypertension effectively in the African region. *Helicobacter pylori,* a gram-negative bacterium responsible for many gastric disorders worldwide, has been associated with hypertension in some previous studies; where blood pressure of patients with *Helicobacter pylori* infection did not subside after hypertensive treatment, when compared to patients without *Helicobacter pylori* infections. This effect was suggested to be due to *Helicobacter pylori* produced and modified cardiotonic steroids that are found in elevated concentrations in hypertensive patients. Cardiotonic steroids are positive inotropic agents which are known to increase blood pressure. A sensitive analytical method is needed to detect and quantify the low concentrations of cardiotonic steroids in biological samples.

Materials and Methods: An extraction method was optimised using reversed phase Solid Phase Extraction. A targeted liquid chromatography tandem mass spectrometry method using an Agilent binary series 1100/1200 LC system with a Kinetex C18 RP column (100 x 2.1 mm, 2.6 µm) coupled to a Sciex 4000QTRAP tandem mass spectrometer was developed and validated for the detection and quantitation of 9 different cardiotonic steroids in both solvent and whole blood. The method was validated according to the International Conference on Harmonization guidelines with regards to precision, accuracy, sensitivity, selectivity, linearity, range, limit of detection, limit of quantification, reproducibility, recovery, carry-over and stability. Media from *Helicobacter pylori* cultures and faecal samples from human and different normo- and hypertensive rat strains were analysed. Data analysis was performed with Analyst® Software (version 1.5.2) and multiple t-test and Kruskal Wallis test using GraphPad Prism 8 software.

Results and Discussion: The calibration curves of tested cardiotonic steroids were linear over a concentration range of 0.1-40 ng/mL with coefficients of determination greater than 0.990 except for telocinobufagin. The analytical method was selective with an estimated limit of detection and limit of quantification between 0.02-0.5 ng/mL and 0.1-2 ng/mL respectively. All tested cardiotonic steroids showed good recovery of over 70%. Accuracy and precision were found to be within acceptable limits of 15% and 20% at lowest limit of quantification for almost all the analytes and their stability in blood and solvent at room temperature, 4°C, -20°C and -80°C was tested for a month. Cardiotonic steroids were detected in *Helicobacter pylori* cultures and faecal samples with the exception of ouabain and proscillaridin A which were not detected at all. Although *Helicobacter pylori* were shown to produce cardiotonic steroids *in vitro*, no evidence of the effect of *Helicobacter pylori* on cardiotonic steroids production was detected in different normo- and hypertensive rat groups.

Conclusion: The quantitative analytical method was successfully validated, over expected *in vivo* concentration ranges for 8 different cardiotonic steroids. The extraction and analytical methods were both successfully applied to *Helicobacter pylori* cultures and faecal rat samples where cardiotonic steroids were detected.

Keywords:

Cardiotonic steroids, Derivatisation, *Helicobacter pylori*, Hypertension, Liquid chromatography, Mass spectrometry.

Declaration of originality

Department of Pharmacology
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Project title: Assessing cardiotonic steroids involvement in hypertensive rat models with *Helicobacter pylori* infections

Declaration

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this dissertation is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
3. I have not used work previously produced by another student or any other person to hand in as my own.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

SIGNATURE OF STUDENT: 

Date: 20/02/2020

Dedication

To my parents

Francis and Colbertine Mbengama

For being my support since day one. For your prayers, unconditional love, encouragement, finances, and for helping me to become the woman I am today. You are my heroes.

Acknowledgements and funding

I would not have accomplished this without the grace of God almighty. For I can do all things through him who strengthens me.

To my dear husband Gaettan Katamba, thank you for all your love, patience, amazing support, and encouragement, for listening to my complaints and excitements even when you did not understand what I was talking about and for always pushing me even when I felt like I could not make it. To my little angel Athalia Katamba, who had to suffer my absence after only six weeks in this world, you are the reason I leave the bed every morning and tell myself I can do it.

To my family, thank you for your prayers, unconditional love and support, and for always making me feel like I am not far from home with our long chats and video calls. Lastly for always reminding me that I needed to finish my MSc.

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To my friends Blanche Holel, Bongekile Rabede, Keith Ncube and Shamiso Mlambo, thank you for making this journey less stressful than it is. Thank you for your morale, spiritual, and physical support. I love and appreciate you all.

To my dear friend Vuyelwa Buque, who departed this world before seeing the results of our early mornings, late and sleepless nights, thank you for being my shoulder to cry on for the past three years. You will be forever remembered.

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Ethics

Ethics approval, reference number 292/2017, was obtained from the University of Pretoria's Research Ethics Committee for the drawing blood from healthy volunteers. Approval was also obtained from the Master of Science Committee of the Faculty of Health Sciences from the University of Pretoria to conduct this research project.

Ethical approval was obtained from the Human Research Ethics Committee and Animal Ethics Screening Committee of the University of the Witwatersrand (M170582) for to the collection and growth of the *Helicobacter pylori* from hypertensive rat models who were tested positive for *H. pylori* and (2018-0942/c) for the use of rats.

Study outputs

Podium presentations

ZF. Masso, M. Leuschner, G. Candy, AD. Cromarty. Detection and quantification of different endogenous cardiotonic steroids by liquid chromatography tandem mass spectrometry. **Health Sciences Faculty Day, University of Pretoria, South Africa, 21 August 2019.**

ZF. Masso, M. Leuschner, G. Candy, AD. Cromarty. Detection and quantification of different endogenous cardiotonic steroids by liquid chromatography tandem mass spectrometry. **South African Society for Basic and Clinical Pharmacology conference**. (**Young Scientist Oral Presentation Competition in Basic Pharmacology). 5 October 2019.**

Poster presentations

ZF. Masso, M. Leuschner, G. Candy, AD. Cromarty. Detection and quantification of different endogenous cardiotonic steroids by liquid chromatography tandem mass spectrometry. **School of Clinical Medicine, Biennial Research Day, University of Witwatersrand. 23 October 2019.**

GP. Candy, A. Thiba, A. Idowu, H. Mullah, S. Dinat, MJ. Nel, K. Rumbold, ZF. Masso, AD. Cromarty. Evidence for association of *Helicobacter pylori* and hypertension. **School of Clinical Medicine, Biennial Research Day, University of Witwatersrand. 23 October 2019**.

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List of abbreviations

°C	Degree Celsius
%	Percentage
µL	Microlitre
µL/min	Microlitre per minute
µM	Micromolar
µm	Micrometre
3BHSD	3beta-hydroxysteroid dehydrogenase
ACTH	Adrenocorticotropic hormone
AGEs	Advanced glycation end products
APCI	Atmospheric pressure chemical ionisation
APPI	Atmospheric pressure photoionization ionisation
ATP	Adenosine Triphosphate
BHI	Brain Heart Infusion
Ca2+	Calcium
CHF	Congestive heart failure
Cm	Centimetre
Cps	Counts per second
CTS	Cardiotonic steroids
CV	Coefficient of variation
CYP450	Cytochrome 450
DSS	Dahl salt sensitive rats
DNH	Dansyl hydrazine
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
ESI	Electrospray ionisation
FA	Formic acid
FBS	Faecal Bovine Serum
FDA	Food and Drug Administration
GIT	Gastrointestinal tract

H. pylori	*Helicobacter pylori*
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High pressure liquid chromatography
Hrs	Hours
HSD3B7	Hydroxy-delta-5-steroid dehydrogenase
ICAM	Intercellular adhesion molecule
ICH	International Council for Harmonisation
IEC	Ion-exchange chromatography
IL	Interleukin
IS	Internal standard
Jnk	c-Jun N-terminal kinase
K^+	Potassium
KDa	Kilodalton
Kpa	Kilopascal
KV	Kilovolt
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LLOQ	Lowest limit of quantitation
LOD	Limit of detection
LV	Left ventricular
MAPK	Microtubule associated protein kinase
MBG	Marinobufagin
MEK	Mitogen-activated protein kinase
Min	Minutes
mL	Millilitre
mM	Millimolar
mm	Millimetre
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MTBE	Methyl tert-butyl ether

Na^+	Sodium
Na^+/K^+-ATPase	Sodium/potassium pump
NaCl	Sodium chloride
ng/mL	Nanogram per millilitre
nM	Nanomolar
nm	Nanometre
NO	Nitric oxide
NPLC	Normal-phase liquid chromatography
PE	Preeclampsia
pH	Potential of Hydrogen
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
Psi	Pounds per square inch
Q	Quadrupole
QC	Quality control
RAS	Renin-angiotensin system
ROS	Reactive oxygen species
RPLC	Reverse-phase liquid chromatography
RSD	Relative standard deviation
S/N	Signal to noise
SEC	Size-exclusion chromatography
SHR	Spontaneously hypertensive rats
SPE	Solid phase extraction
TCB	Telocinobufagin
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TOF	Time of Flight
UV	Ultraviolet
WHO	World Health Organization

Chapter 1. Literature review

1.1 Introduction, Definitions and Classifications

Cardiotonic steroids (CTS) are a class of lipophilic steroid-like hormones found in plants and mammals. They are synthesized by some plants and animals as a strategy to reduce or prevent damage inflicted by herbivores or predators.[1,2,3] They are classified as sodium/potassium adenosine triphosphatase (Na^+/K^+-ATPase) inhibitors and are divided into two structurally distinct classes based on the aglycone steroid-like core structure: cardenolides have a characteristic 5-membered unsaturated lactone (butyrolactone) ring attached to the steroid nucleus at carbon position 17 and a hydroxyl at position 14; while bufadienolides with a double unsaturated 6-membered lactone (α-pyrone) ring at the carbon 17 position of the steroid nucleus (Figure 1-1).[1,4,5,6,7]

Further division is made depending on the presence or absence of attached sugar residues bound to the aglycone moiety. The cardiac glycosides have one to four sugar molecules attached to the aglycone (either cardenolide or bufadienolide) steroid core.[6,8] The CTS are composed of three major structural components: First, a steroid core, in which rings AB and CD are *cis*-fused, whereas rings BC are *trans*-fused. Hydroxyls occur at carbon positions 3- and 14- with the glycoside sugar chain attached at the 3-OH group (Figure 1-1). Many cardiac aglycones also have OH groups at 12- and 16- positions. These additional hydroxyl groups influence the partitioning of the cardiac glycosides into the aqueous media and greatly affect their duration of action.

Secondly, the 5- or 6-membered lactone ring at position 17 are used to clarify the molecule as cardenolides and bufadienolides respectively. The third component is a variable number of sugar residues attached to the 3β-OH group. [4,6,8,9]

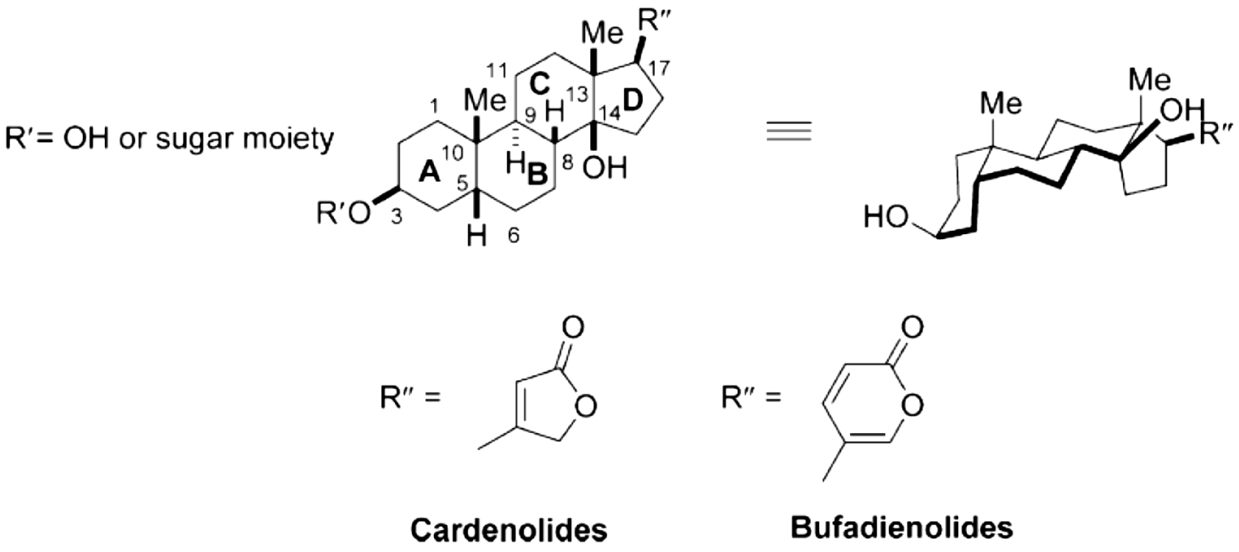


Figure 1-1. General structure of CTS. Reproduced with permission (License number: 4646230063160).

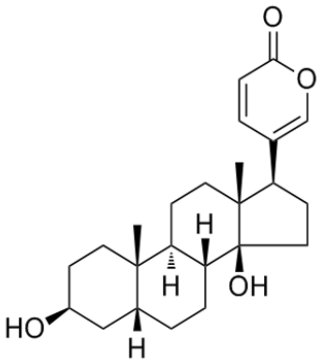
The sugars most commonly bound to the aglycone core include L-rhamnose (ouabain), D-glucose, D-digitoxose (digitoxin and digoxin), D-digitalose, D-digginose, D-sarmentose, L-vallarose, and D-fructose. The presence of a sugar moiety in CTS increases the inhibitory potency by stabilising the Na^+/K^+-ATPase enzyme·CTS complex through interactions of the 3'-α-hydroxyls with both proton donating and proton-accepting groups on the Na^+/K^+-ATPase enzyme, but the effect varies considerably in relation to the number of hydroxyl group substitutions on the steroid core. The presence of acetyl groups on the sugar chain affects the lipophilic character and the kinetics of the CTS. The rate of reactivation of CTS-bound Na^+/K^+-ATPase, is proportional to the rate of CTS dissociation, and is strongly dependent on the presence of the sugars.[8,9]

Endogenous CTS in blood circulate bound to transport proteins, and their levels in plasma vary from picomolar to nanomolar concentrations at different physiological conditions.[1,10] Nanomolar (nM) concentrations of CTS (in the range of 0.5–20 nM) such as ouabain-like factors, digoxin, marinobufagin, bufalin and telocinobufagin, have been detected in serum of experimental animals and humans.[1,5] Some CTS have been used for more than 200 years for the treatment of congestive heart failure and some other cardiac diseases,

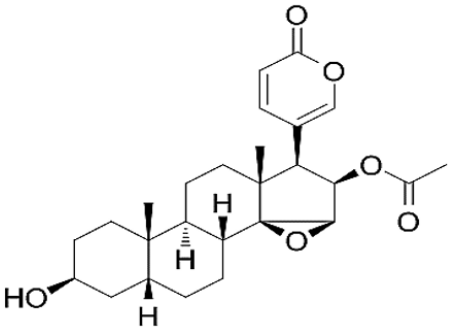
notably atrial arrhythmias. They have a narrow therapeutic index making them toxic, even at low concentrations.[1,6,11]

1.1.1 Endogenous bufadienolides

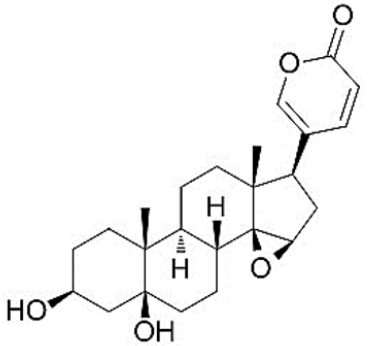
Amphibians are known to produce bufadienolides with the highest concentration being in their skin; and many of these CTS have also been identified in human blood and urine. Bufadienolide-containing preparations from frog and toad skin have been used for the treatment of congestive heart failure (CHF) in traditional medicine of the Far East [3,10,12], and are reported to be involved in many biological activities, such as regulation of cardiac tone, anaesthesia, blood pressure regulation, respiratory stimulation and anti-neoplastic activity.[13,14] Figure 1-2 shows figures of different bufadienolides.



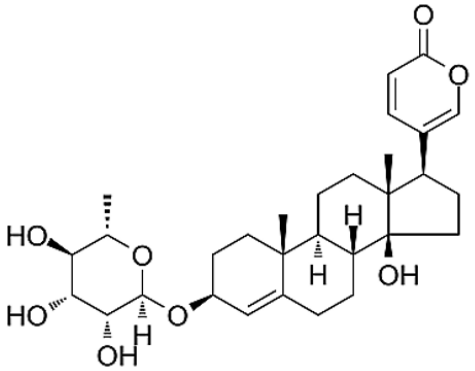
Bufalin



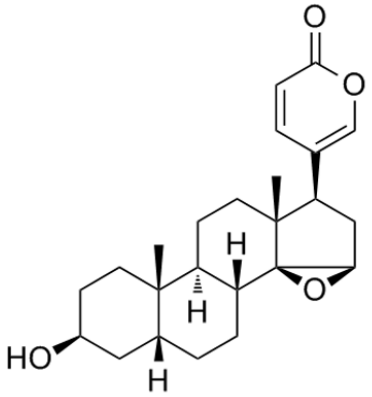
Cinobufagin



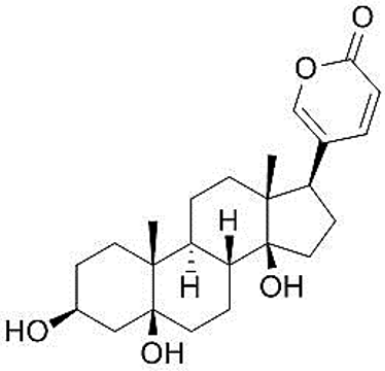
Marinobufagin



Proscillaridin A



Resibufogenin



Telocinobufagin

Figure 1-2. Bufadienolides structures. Drawn using ACD/ChemSketch Freeware 12

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Bufalin

From *Bufo gargarizans*, bufalin is the active but toxic component in traditional Chinese medicine used to rescue patients with heart failure. Its chemical structure has a 6-membered lactone ring attached to the hydrophobic steroidal core, and possesses strong inhibitory potency on Na^+/K^+-ATPase. Endogenously, it has been extracted from human and rat bile and plasma.[15,16]

Bufalin is known to block vasodilation and increase vasoconstriction, vascular resistance, and blood pressure by inhibiting the Na^+/K^+-ATPase.[17] It is a very potent Na^+/K^+-ATPase inhibitor, and its derivatives were the first bufadienolides positively identified in mammals.[10]

Marinobufagin

Marinobufagin (MBG) has been isolated and identified endogenously from human and bovine urine, serum and plasma. It acts as a vasoconstrictor, and its concentration is elevated following myocardial infarction, volume expansion, sodium-induced hypertension, primary aldosteronism, chronic renal failure, congestive heart failure and preeclampsia (PE) in humans. Previous studies have shown that, the increase in plasma levels of MBG promotes natriuresis and compensates for the genetically impaired pressor natriuretic mechanism in both humans and rats. It shows natriuretic properties through selective inhibition with a greater affinity for the ouabain resistant α1 isoform of Na^+/K^+-ATPase, the main sodium (Na^+) pump isoform of the kidney. [4,12,3,7,18]

Besides its natriuretic effect, increases in MBG production inhibit erythrocyte and smooth muscle Na^+/K^+-ATPase activity to cause vasoconstriction. Long-term infusion of nanomolar concentrations of MBG in animals raises arterial blood pressure and has been shown to lead to cardiomyopathy and increased collagen production in the heart, and may cause fibrosis as in uraemic cardiomyopathy.[15,19]

Telocinobufagin

Telocinobufagin (TCB) is the reduced form of MBG; it differs from MBG by having a hydroxyl at the 14-position versus an epoxy group in the 14,15-position seen in MBG. It was identified as a constituent of human serum and plasma. Its plasma concentration is higher than that of MBG and it increases in patients with end stage renal failure. TCB was reported as the most potent CTS, with the greatest suppressive effect on the Na^+/K^+ ATPase. [3,4,7,10,20]

Proscillaridin A

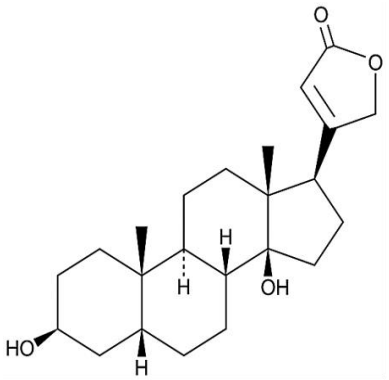
Proscillaridin A was reported in human plasma and bovine adrenal glands using cross-reactivity with an antibody.[3,12,15]

Others

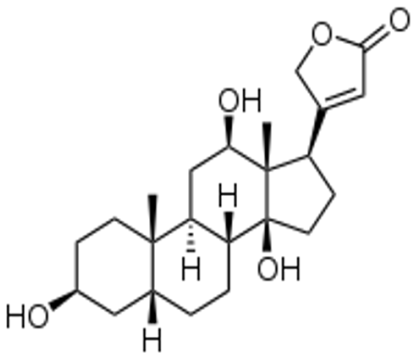
There are many other bufadienolides including; 19-norbufalin isolated from human cataractous lenses.[19] Resibufogenin, and cinobufagin are structurally similar; with cinobufagin having almost the same pharmacological activity as bufalin, but it is less potent; while resibufogenin has the weakest pharmacological effect of these three. It differs from MBG only in the absence of a hydroxyl group in the β-5 position and was proved to antagonise the effect of MBG in many studies.[17,21,22]

1.1.2 Endogenous cardenolides

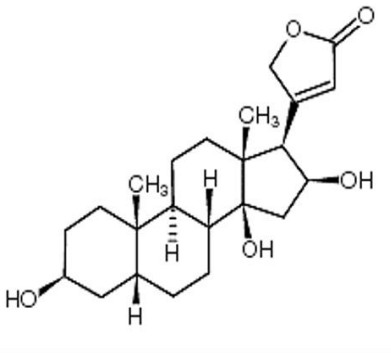
The cardenolides have been isolated from plants, insects and animals, and are serving as important clinical agents especially in heart conditions.[1] Figures 1-3 and 1-4 show some important endogenous cardenolide aglycones (genin) and glycosides respectively.



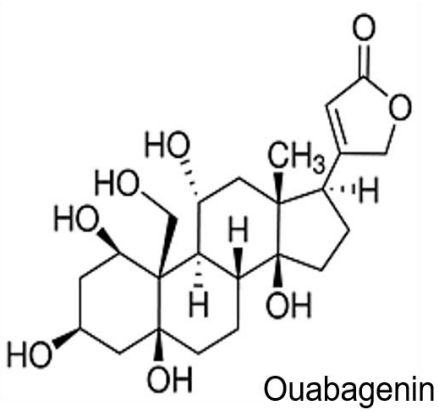
Digitoxigenin



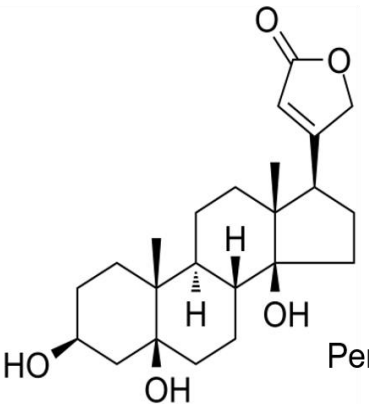
Digoxigenin



Gitoxigenin

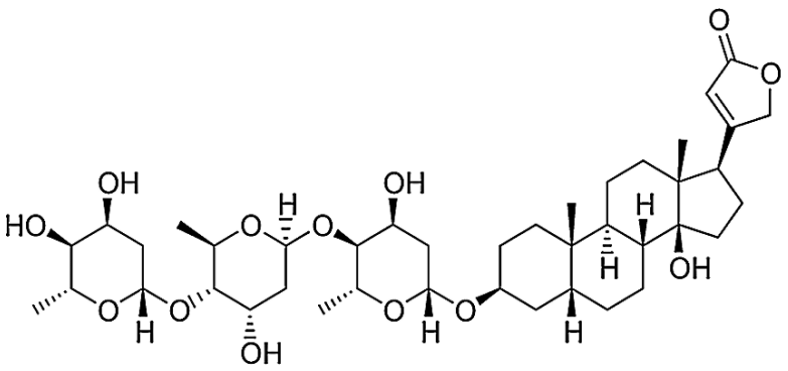


Ouabagenin

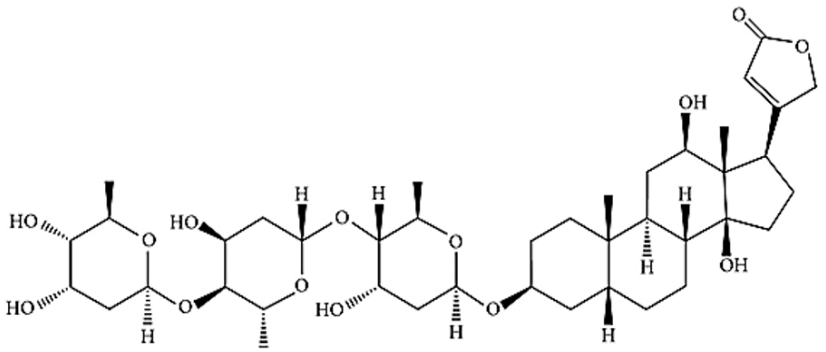


Periplogenin

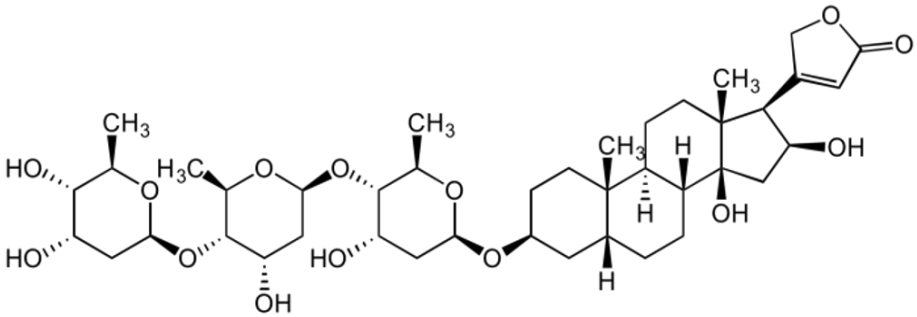
Figure 1-3. Aglycone cardenolide structures. Drawn using ACD/ChemSketch Freeware 12



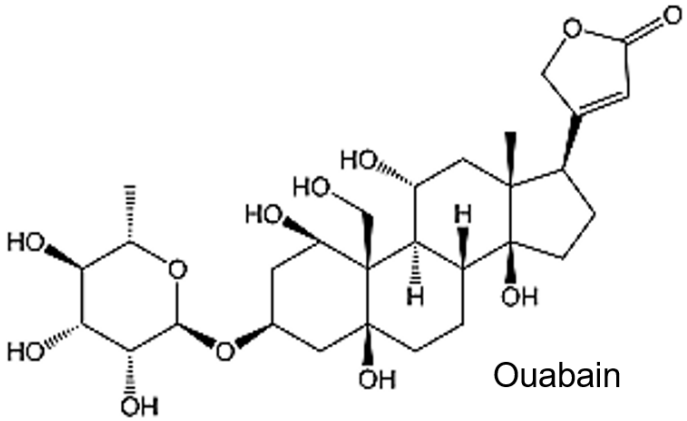
Digitoxin



Digoxin



Gitoxin



Ouabain

Figure 1-4. Glycoside cardenolide structures. Drawn using ACD/ChemSketch Freeware 12

Ouabain

Ouabain was discovered in human plasma for the first time in 1991 by Hamlyn's and Haupert's groups[23], it was the first CTS shown to be a specific inhibitor of the Na^+/K^+-ATPase and has been most frequently associated with disease states. It binds to the extracellular region and mainly to the phosphorylated E2-P conformation or more specifically to the E2-P ground state of Na^+/K^+-ATPase and blocks the E2–E1 transition of the enzyme, inhibiting its activity. Increased concentrations of endogenous ouabain were reported in conditions like sodium imbalance, hypertension, chronic renal failure, hyperaldosteronism, congestive heart failure and preeclampsia, and can modulate both apoptosis and inflammation at concentrations below those that inhibit ion transport.[1,4,6,7,18]

Recent research has suggested that endogenous ouabain was not a natriuretic hormone but, was involved in the adaptation of humans to sodium depletion as no direct correlation with sodium uptake existed but rather correlated with the urinary loss of potassium (K^+). However, ouabain was shown to cause the release of MBG which is a natriuretic hormone. Moreover, while it has high affinity for the α2/α3 isoforms of Na+/K+-ATPase, the target for a natriuretic hormone, the tubular cells of the rat kidney, express mainly the α1 isoform, which does not show sensitivity to ouabain. Ouabain was then found to be kaliuretic. This has long-term consequences for protein synthesis and affects other hormonal involved in the control of the circulatory system, including catecholamine release and synthesis from atrial and chromaffin tissue cells, acetylcholine release, secretion of the atrial natriuretic hormone, endothelin 1, nitric oxide (NO), alteration of aldosterone synthesis and the renin-angiotensin system (RAS).[4,10,12,19,23]

Previous studies have shown that, in salt-hypertensive patients, approximately 50% of Caucasians with uncomplicated essential hypertension and patients with hyperaldosteronism exhibit elevated concentrations of endogenous ouabain. This finding implies that high sodium concentrations in plasma and cerebrospinal fluid stimulate the release of endogenous ouabain via a Na^+ sensor or endothelial Na^+ channels in the brain. Also, its concentration rises in blood upon increased Na^+ uptake, hypoxia, and physical exercise.[4,12,18,23]

Digoxin

Digoxin is by far the most commonly prescribed cardiac glycoside across the world, although the longer-acting digitoxin is popular in Europe. It has been identified endogenously in human urine, blood plasma, adrenal gland and breast cyst fluid. It is a prominent CTS used in therapy for heart failure and arrhythmias, acting indirectly on the heart by depressing the activated neuroendocrine system and the adrenergic and renin-angiotensin system; with recommended therapeutic plasma concentrations of 0.8–2.0 ng/mL. Slightly higher plasma concentrations are toxic or lethal at 3 ng/mL and 5 ng/mL respectively. Increased arrhythmia has been reported from patients with digoxin serum level of 1.7 ng/mL and 2.5 ng/mL.[4,6,11,24] An endogenous increased concentration of digoxin is seen in patients with renal failure, liver dysfunction, preeclampsia, during prolonged and strenuous exercise and in new-born infants.[4,11,12]

It has been speculated that digoxin treatment leads to an internalisation of CTS receptors in brain, and hence, to the inability of the sodium-induced ouabain to activate the release of hypertensinogenic hormones.[4,19] Thus, in contrast to ouabain, digoxin does not induce hypertension but, rather, reverses ouabain-induced hypertension. The digoxin-induced arterial baroreflex opposes the sympathetic excitatory pressor responses of ouabain in both the periphery and the brain and no longer activates the chemoreflex in patients with chronic heart failure.[4,12,18] This may also be due to a decrease in plasma renin activity and angiotensin II, aldosterone, and epinephrine levels, and a significant increase in the levels of atrial and brain natriuretic peptides. Furthermore, the higher hydrophobicity of digoxin as compared to ouabain may contribute and result in altered tissue distribution.[18]

Digitoxin

Digitoxin has a well-established clinical profile and is also used for treatment of heart failure. Its toxic concentration is 30 ng/mL in plasma and above this concentration becomes lethal. Its aglycone digitoxigenin, is a natural CTS commonly found in many herbal plants and in humans, it is also used for heart failure treatment and it is less toxic than digitoxin.[11,25,26]

Periplogenin

Periplogenin plays a protective role in regulating hyperthyroidism and associated cardiovascular problems. It was also reported to have an inhibitory effect on histamine release of mast cells either cultured in vitro or in antigen-pulsed rats; and it possesses anti-cancer properties.[27,28]

Other cardenolides include ouabagenin, gitoxin and gitoxigenin.

1.2 Synthesis and metabolism

The biosynthesis of endogenous CTS has been proved to occur in the hypothalamus and adrenal cortex (the zona fasciculata and glomerulosa). Adrenalectomies in rats resulted in the reduction of plasma digoxin-like immunoreactivity and MBG levels, which indicated that adrenals were the major source of CTS in mammals. The brain might also be a source of CTS as their concentration was shown to be higher in the midbrain than in blood plasma.[1,11,18,19,23,29] Both bufadienolide and cardenolide CTS are derived from cholesterol; but only cardenolide synthesis requires cholesterol side chain cleavage, with hydroxycholesterol shown to be one of the CTS precursors. The release of endogenous CTS is controlled by various humoral stimuli including adrenocorticotropic hormone (ACTH), interleukin 11 (IL-II), vasopressin, and catecholamines.[10,18,30]

Ouabain was proved to be synthesised in mammalian adrenal gland and hypothalamus, and present in the pituitary and medullary neurons. Pregnenolone and progesterone are biosynthetic precursors of endogenous ouabain and digoxin. Ouabain release is regulated by different molecules depending on the organism. Bovine adrenocortical cells show sensitivity to ACTH, stimulation of adrenergic receptors agonists, and IL-II by acting on angiotension 2 receptors. In human adrenocortical cells, its release is insensitive to ACTH or IL-II, whereas phenylephrine and vasopressin stimulated it. In rat adrenocortical cells, ouabain secretion increases on nicotinic cholinoceptors stimulation. Cholesterol side chain cleavage by cytochrome P450 (CYP450) is essential for ouabain synthesis. The pharmacological inhibition of the conversion of pregnenolone to progesterone was seen to reduce the synthesis of ouabain *in vitro*.[1,10,18,19,29]

Fedorova *et. al.* in 2015, demonstrated that MBG is not synthesised endogenously by the traditional steroidogenesis pathway like other steroids; but, rather by the “acidic” bile acid pathway (Figure 1-5). The traditional pathway includes cholesterol cleavage by CYP7A1, an enzyme expressed only in the liver. Moreover, MBG is synthesised in non-hepatic tissues. The side chain cleavage of MBG was done by CYP27A1, an enzyme expressed in the extra-hepatic tissues, including adrenal glands; and oxidises cholesterol into bile acids, which appeared to be a precursor of MBG.[29,31]

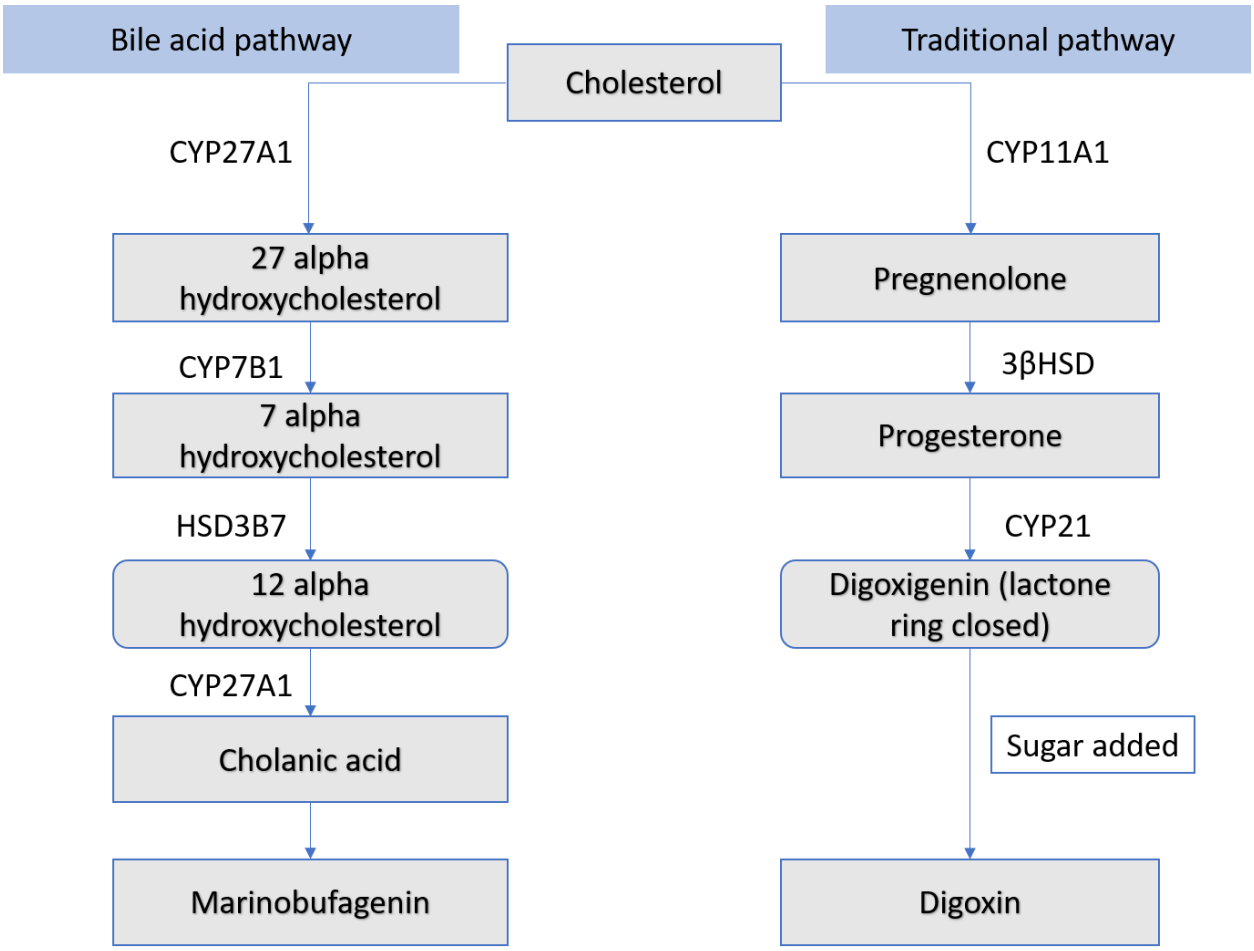


Figure 1-5. Scheme of biosynthesis of MBG from bile acids pathway (acidic pathway) and digoxin from the traditional steroidogenesis.

Digitoxigenin was reported to be metabolised by rat liver homogenates, with only 26.6% parent drug left after 2 hours of incubation.[25,32] Digitoxin was shown to be metabolised mainly in the liver in a stepwise manner, by hydrolysis of the trisaccharide portion to bis- and mono-digitoxosides, and then digitoxigenin. It may also be hydroxylated at the 12 8-position, resulting in the formation of digoxin. The inactive 3-a-hydroxy epimer of digitoxigenin is formed in the liver. The a-hydroxylated compounds may be conjugated with glucuronic or sulfuric acid. It is then excreted mostly as metabolites and partly unaltered.[32,33,34,35]

Digoxin is also metabolised mainly in the liver, by removal of the sugar moieties to form digoxigenin, which is further metabolised to inactive metabolites which is then excreted in free or conjugated forms. It also is reduced to dihydrodigoxin, a relatively inactive form, which was proved to happen in the gastrointestinal tract by gut microbiota. However, only a small percentage of digoxin is metabolised in human liver cells, it is excreted in the urine, with up to 80% of the dose unaltered. It can also be found as a metabolite of deslanoside, digitoxin, lanatoside C and metildigoxin.[32,33,35,36,37,38,39]

Bufadienolides are metabolised by two major metabolic pathways: hydroxylation and dehydrogenation mediated by CYP3A in human and some animal species' liver microsomes. Furthermore, the metabolites of bufalin were demonstrated to exhibit a strong selective inhibition effect on the CYP3A4 metabolic enzyme, which could induce the function loss of CYP3A4 and result in severe drug–drug interactions.[13,14,16,22] CTS are excreted in urine and bile.[16,22,32,35,40]

1.3 Mechanism of actions

More than 50 years ago, CTS were hypothesized to represent the exogenous counterparts of endogenous factors involved in the regulation of cardiovascular system. Within the past few years, many studies demonstrated that they were also produced endogenously in mammals and were implicated in many physiological processes.[10] They were involved in processes such as, cardiac and kidney function, salt metabolism, hypertension, cell proliferation, and cellular half-life.[4,5,7,12]

1.3.1 The sodium/potassium pump (Na^+/K^+-ATPase)

The sodium/potassium pump is an active transport system that is highly conserved in all eukaryotic cells. It is a member of the P-type ATPase family of membrane-incorporated proteins, and it was initially discovered by Jens Skou as an ion pump in 1957. It is directly responsible for the maintenance of the low intracellular sodium/potassium ratio by the active transport of these ions across the plasma membrane (moving $3Na^+$ out of the cell in exchange for $2K^+$) using the hydrolysis of adenosine tri-phosphate (ATP) to provide the necessary energy. The Na^+/K^+-ATPase transport pump controls multiple essential cellular functions including glucose and amino acid transport. It maintains the electrical membrane potential, which is necessary for nerve transmission and muscle contraction, excitability and many other cellular functions, which depend on the necessary sodium-potassium gradients. The Na^+/K^+-ATPase can also drive secondary active co- and counter- transporters, which are coupled to the gradient of extracellular to intracellular sodium, such as passive ion transport via the sodium/ calcium (Na^+/Ca^{2+}-) exchanger. It also acts via ATP hydrolysis, determining a very substantial fraction of the cellular metabolic rate of most tissues.[1,6,7,10,41]

The Na^+/K^+-ATPase (Figure 1-6) consists of two polypeptides in equimolar ratios. The catalytic subunit α has a molecular mass of approximately 100-113 kDa and holds the binding sites for Na^+, K^+, ATP, and CTS. It has four isomers (α1-α4) and ten transmembrane segments (M1-M10). The glycosylated glycoprotein subunit β has a molecular mass of 35-60 kDa depending on the glycosylation pattern in each tissue. It has only one transmembrane domain and three isomers (β1-β3). It has as roles the maintenance of the holoenzyme functionality, as well as the transport and assembly of the enzyme to plasma membrane. The third subunit is the γ subunit of 8–14 kDa, which was first identified as a component involved in the binding of ouabain. It possesses a unique signature sequence from FXYD family; which role seems to modulate kidney Na^+/K^+-ATPase kinetic properties, by decreasing Na^+ or k^+ affinity depending on the Na^+/K^+-ATPase isozymes. The FXYD proteins, including the Ƴ subunit, are not an integral

part of Na^+/K^+-ATPase, but are associated with specific domains of αβ-subunit complex and modulate catalytic properties of the Na^+/K^+-ATPase.[6,7,10,41]

The Na^+/K^+-ATPase contains the binding sites for sodium and CTS on the extracellular segments and the binding sites for K^+ and ATP on the intracellular loops. There are four isoforms of Na^+/K^+-ATPase, which allow numerous combinations of complexes among the tissues with different characteristics including differential sensitivities to different CTS.[6,7,10] The Na^+/K^+-ATPase subunit α isoforms are expressed in a tissue-specific fashion. The distribution of the α1 isoform is ubiquitous; it is also the main isozyme expressed in the kidney. This isoform can form a signalling complex with signalling proteins including Src, which regulates many essential cellular functions such as sodium homeostasis, protein trafficking, gene expression and cell growth. The α2 isoform is expressed in adult heart, vascular smooth muscle, skeletal muscle, brain (preferentially the glial cells), adipocytes, cartilage, placenta and bone. The expression of the α2 isoform is insulin-dependent. The α3 isoform is expressed mostly in excitable tissues, being most abundant in the central and peripheral nervous tissues and in the conductive system of the heart. It is found in muscles, placenta, ovaries, erythrocytes, heart, and neurons. Finally, the α4 isoform, which seems to be testis-specific.[6,7,10] Regarding the Na^+/K^+-ATPase β subunit isoforms, β1 is also expressed ubiquitously, while β2 is found in cartilages, nervous system, heart, erythrocytes, and muscles. β 3 is expressed in cartilages, brain, erythrocytes, liver, lungs, retina, and testis.[6]

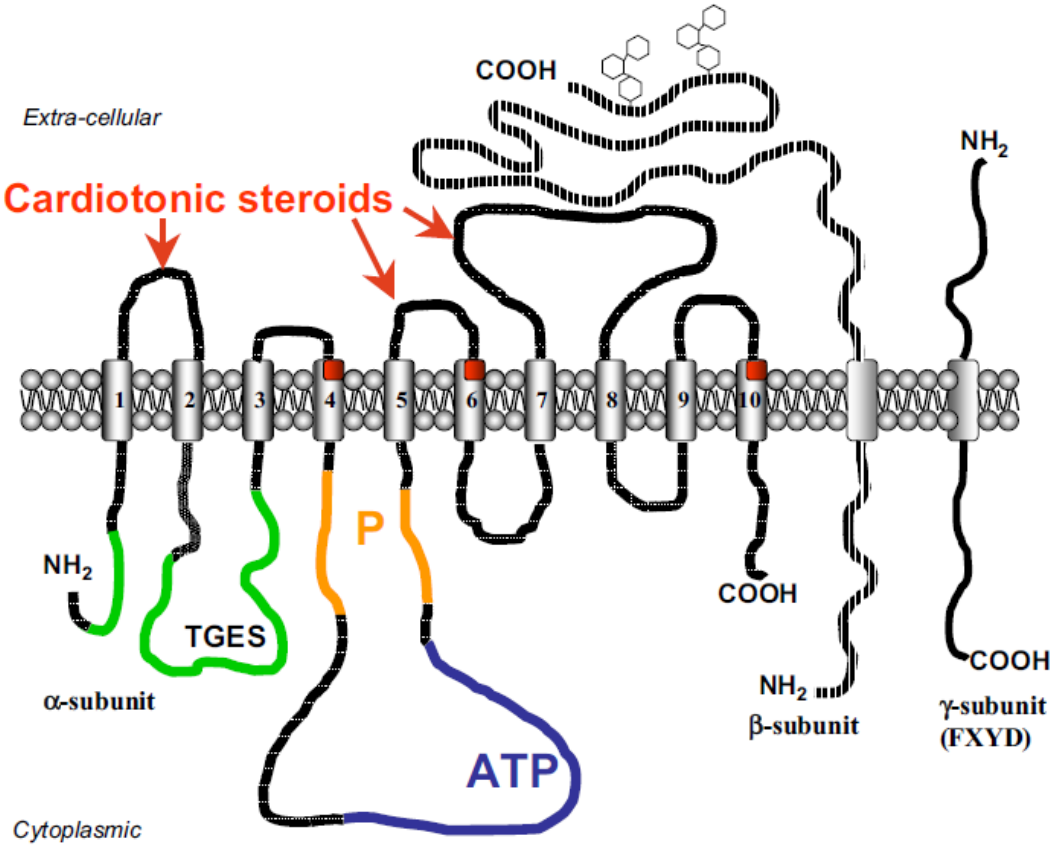


Figure 1-6. Structure of Na^+/K^+-ATPase.[10] (with permission)

Figure 1-6 shows the αβ heterodimer composition of the Na^+/K^+ ATPase, which associates with the subunit γ. The α subunit extracellular segments form a binding site for CTS shown in red, which include TM1–TM2, TM5–TM6, and TM7–TM8 loops and several amino acids from the transmembrane regions M4, M6, and M10. Blue shows the binding site for ATP which is located on the intracellular loop TM4–TM5, and forms the "pocket" for this nucleotide. P shown in orange on TM4-TM5, is the phosphorylation domain. Green shows the actuator domain, specifically its TGES motif, which is responsible for the dephosphorylation step, and is constituted by the cytoplasmic NH2-terminal and TM2–TM3 intracellular loop. The regulatory β glycoprotein subunit extracellular part interacts with a conserved motif SYGQ on the extracellular loop TM7–TM8 of α subunit.[6,10]

1.3.2 Na^+/K^+-ATPase as a specific receptor for cardiotonic steroids

The main mechanism of action of CTS is their ability to bind and inhibit the ubiquitous Na^+/K^+-ATPase pump. The core structure of cardiac glycosides consists of a tetracyclic steroidal framework, which is considered the pharmacophoric moiety responsible for their inhibition on Na^+/K^+-ATPase.[4,5,42]

CTS have a specific binding site on the extracellular loops of the subunit of the Na^+/K^+-ATPase. Some amino acids from the transmembrane regions also interact with ouabain; this suggests that the hydrophobic regions of CTS may actually be inserted in the membrane inside where they interact with the subunit of Na^+/K^+-ATPase. According to Bagrov *et. al.* (2009), amino acids from residues 111 to 122 form the most important part of the putative CTS binding site. They also mentioned that the dissimilarities in the amino acid sequence between different species and different isoforms are responsible for the fundamental differences in the sensitivity of the Na^+/K^+-ATPase isoforms to a variety of CTS.[6,10]

Pessôa *et. al.* (2018) explained the importance of each part of the CTS in the inhibitory activities on Na^+/K^+-ATPase. The presence of an unsaturated lactone ring (five-membered for cardenolides and six-membered for bufadienolides), attached in a correct configuration at C17, the steroid nucleus in a chair configuration, with the AB and CD ring bonds in a *cis* configuration and the BC bond in a *trans* configuration, a hydroxyl group at C14, and an appropriate sugar at C3 are all required for activity.[6]

The steroid nucleus of a CTS interacts with transmembrane domains TM4–TM6. Three phenylalanine residues (Phe323, Phe790, and Phe793) were reported to be a key factor for a correct chair and a cis configuration of the AB and CD ring junctions. The close hydrophobic pocket formed by these residues, was displaced by the CTS steroid nucleus, thus allowing for a better accommodation of this group. The hydroxyl group at C14b was able to form a hydrogen bond with Thr804, which is a key residue in ouabain binding. Contrary to the hydroxyl group at C11a like in digoxin, was not involved with the

interactions, and its presence can reduce the CTS affinity due to a sterical hindrance when compared to CTS without hydroxylation at the same position as in digitoxin.[6]

The number and type of sugar units that are linked to the C3 position, can drive changes in the relative binding affinity of CTS. A rhamnose residue that is present in ouabain was demonstrated to interact with Glu319 and Arg887 by hydrogen bonds, hence, ouabain was shown to have a higher affinity in comparison to ouabagenin, which does not present a sugar moiety. Rhamnose's importance in increasing the binding affinity was also observed when adding it to digitoxigenin, yielding evomonoside, which showed an increase in binding affinity of over 18-fold when compared to digitoxigenin. Moreover, the reduction of digitoxose units for digoxin and digitoxin showed an increase in the binding affinity, but the removal of all sugar units caused a decrease in the CTS affinity. The gitoxin-based compound helveticoside, presenting a digitoxose, showed a decreased binding affinity in comparison to cymarin, which contains a cymarose.[6]

Cornelius *et. al.* (2013) explained the interaction between ouabain and Na^+/K^+-ATPase. They found that ouabain bound deeply into the transmembrane domain with the lactone ring placed near the K-binding sites and the sugar exposed to the solvent. The α-face of ouabain which is hydrophobic interacted with three Phe residues, whereas the hydrophilic β-face showed little interaction with protein residues, and this was reported as the reason for the low affinity to ouabain. However, in the homology model of ouabain-bound Na+/K+-ATPase in the high affinity state and in the high affinity E2P·ouabain crystal structure M1/M2 interacted with the ouabain β-face, allowing residues such as, Gln-118 and Asn-129 to interact with hydroxyl groups on the CTS steroid core.[8]

To reach the binding site, ouabain causes rearrangements of the M1–M4 transmembrane helices to create a cavity. Thus, association and dissociation of ouabain are relatively slow processes. High concentrations of sodium chloride (NaCl) reactivate enzyme activity after inhibition by ouabagenin, but not by ouabain as the presence of its rhamnose residue prevented the reactivation; demonstrating the pivotal role of the sugar moiety for closing the extracellular cation pathway.[6,8]

The therapeutic effect of CTS lies in their reversible inhibition on the membrane-bound Na^+/K^+-ATPase located in the human myocardium. Inhibition of Na^+/K^+-ATPase leads to the elevation of intracellular Na^+ concentration, due to a delay in the restoration of the Na^+/K^+ balance before the next cell's depolarization. As an osmotically active ion, high intracellular Na^+ leads to an excessive water uptake, which would cause cell death, therefore, Na^+ needs to be carried out of the cell through another transport system; which in turn activates the passive Na^+/Ca^{2+} exchanger resulting in an increase of the intracellular Ca^{2+} concentration, as the gradient will be disrupted and the exchanger may work in a reverse direction carrying Na^+ out of the cell and importing Ca^{2+} inside. The elevated intracellular Ca^{2+} concentration binds to the troponin C complex, which then lead to an increase in myocardial contractility, by increasing the velocity and extent of sarcomere shortening, thus translating into an increased stroke work for a given filling volume of pressure.[6,23,26,42]

CTS have been used for the treatment of cardiac diseases for decades. In recent years, researchers found that Na^+/K^+-ATPase could also act as a receptor through its interaction with Src kinase, converting the ligand-binding signalling to the activation of protein kinase cascades. Other investigations have indicated that, a signal cascade involving epidermal growth factor receptor (EGFR) and phospholipase C (PLC) are initiated by CTS via their binding to Na^+/K^+-ATPase resulting in an increase in collagen expression. These findings suggest that CTS like digitoxigenin may be potential wound healing therapeutics via stimulating collagen synthesis, one of the most important mechanisms in wound healing; which is also beneficial in skin repair.[25]

Figure 1-7 shows a schematic representation of CTS secretion control and functions in mammalian cardiovascular system and volume control.

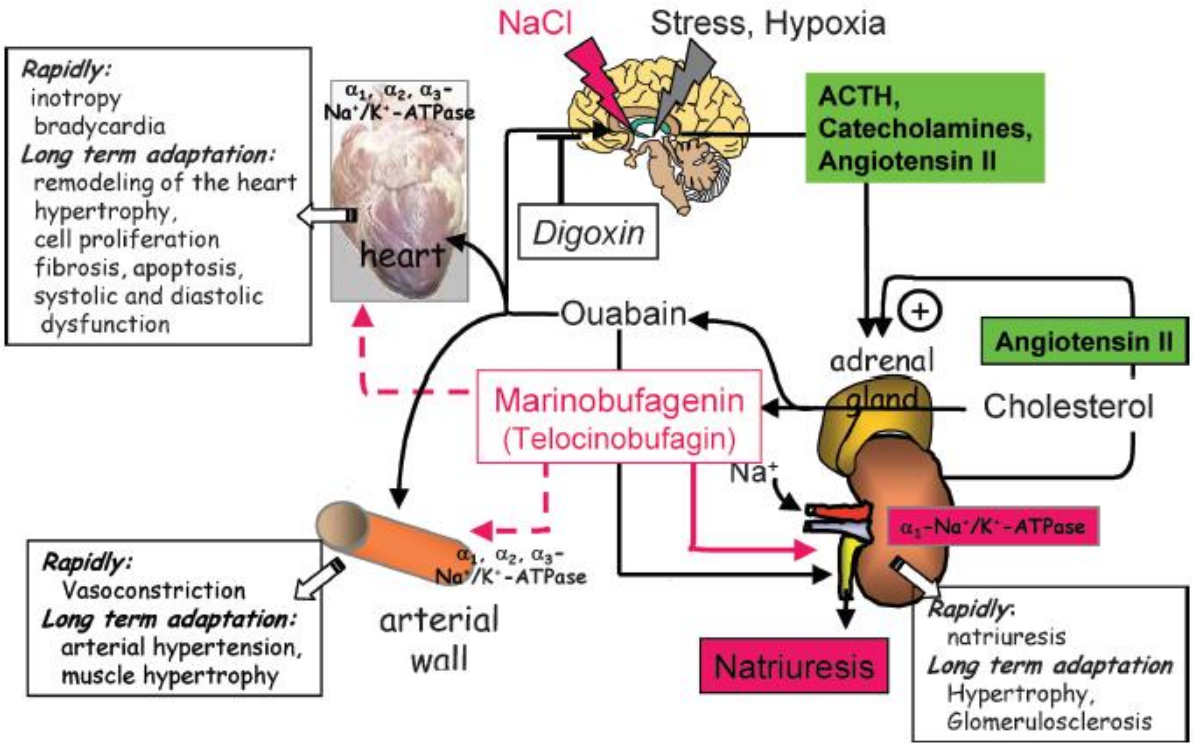


Figure 1-7. A schematic representation of the probable secretory control and function of endogenous CTS in the mammalian cardiovascular system and volume control. Synthesis of endogenous CTS in the adrenal cortex from cholesterol is under the control of catecholamines, ACTH and angiotensin II. Increased NaCl in the midbrain cells may lead to its secretion. The rapid effects on the heart, arterial wall and kidney are followed by a remodelling of the organs after prolonged exposition to endogenous CTS. Reproduced with permission (License number: 4666591240055).

1.4 Functions *in vivo*

Although all CTS inhibit the Na^+/K^+-ATPase, different CTS have different affinities for various isoforms and at different sites in the body, hence different physiological effects. At a molecular level, these differences may be caused by altered interaction times of the cardiac glycoside receptor site with hydrophobic versus hydrophilic CTS, by a slightly different conformation of the subunit upon CTS binding due to an induced-fit mechanism and also, by variations of the gene expression of the various intracellular signalling and proteins in the target cells.[4]

1.4.1 Cardiotonic steroids and cell signalling

Many studies done indicate that CTS binding to Na^+/K^+-ATPase elicit cell signalling events leading to oxidative stress, hypertrophic growth and fibrosis (MBG, ouabain and digoxin), inflammation and apoptosis (bufadienolides and ouabain), and cell differentiation (bufadienolides). Plasmalemmal Na^+/K^+-ATPases that reside in the caveolae of cells, do not seem to actively pump sodium, and are closely associated with other key signalling proteins such as, Src and EGFR. Binding of CTS to the plasmalemmal Na^+/K^+-ATPase induces the endocytosis of the CTS-Na^+/K^+-ATPase-Src-EGFR complex in a manner analogous to that for classic receptor tyrosine kinases.[6,7,10,17,23]

Exposure of several different cell types in culture to several CTS induced rapid phosphorylation of the EGFR in a Src-dependent manner. Ouabain was shown to trigger a tyrosine-kinase protein starting an intracellular-signalling cascade that constitutes a stimulus for EGFR. This pathway cascade then leads to the transcription of genes encoding for pro-fibrotic factors, promoting cardiac hypertrophy, and finally lead to heart failure. The phosphorylation pattern for the EGFR is distinctly different from that observed when EGFR induces autophosphorylation. In addition to the EGFR, other signalling proteins seem to be recruited including PLC, transient receptor potential proteins, phosphoinositide 3-kinase, and several isoforms of protein kinase C.[10,12,23]

Fluorescence transfer methods using intact cells suggested that under basal circumstances, the caveolar Na^+/K^+-ATPase binds with Src and maintains Src in an inactive form. The binding of CTS to the Na^+/K^+-ATPase induces a conformation change that allows activation of Src. The active Src can then phosphorylate other proteins. Na^+/K^+-ATPase and Src together seem to form an effective receptor tyrosine kinase.[7,10,12]

The binding of CTS to Na^+/K^+-ATPase increased cellular reactive oxygen species (ROS); which can consequently increase cytosolic Ca^{2+} concentrations and activate extracellular signal-regulated Kinase (ERK). ROS increases depend on the function of Ras, and mitochondrial K^+ channels.[7,10,12] Figure 1-8 shows different pathways affected by CTS binding to the Na^+/K^+-ATPase.

The relationship between signalling through the Na^+/K^+-ATPase and cell death. It was demonstrated that high concentrations of ouabain seems to induce a death signal in endothelial cells that can be readily dissociated from the effects of Na^+/K^+-ATPase inhibition by extracellular potassium depletion. Digoxin, digitoxin, oleandrin, bufalin and ouabain were shown to induce immunogenic and autophagy cell death in a panel of human cancer cell lines through a regulation of specific signalling pathways. The interactions between signalling through the Na^+/K^+-ATPase and cell death seem to be both complex and variable, depending on cell type and on treatment conditions.[6,7,10]

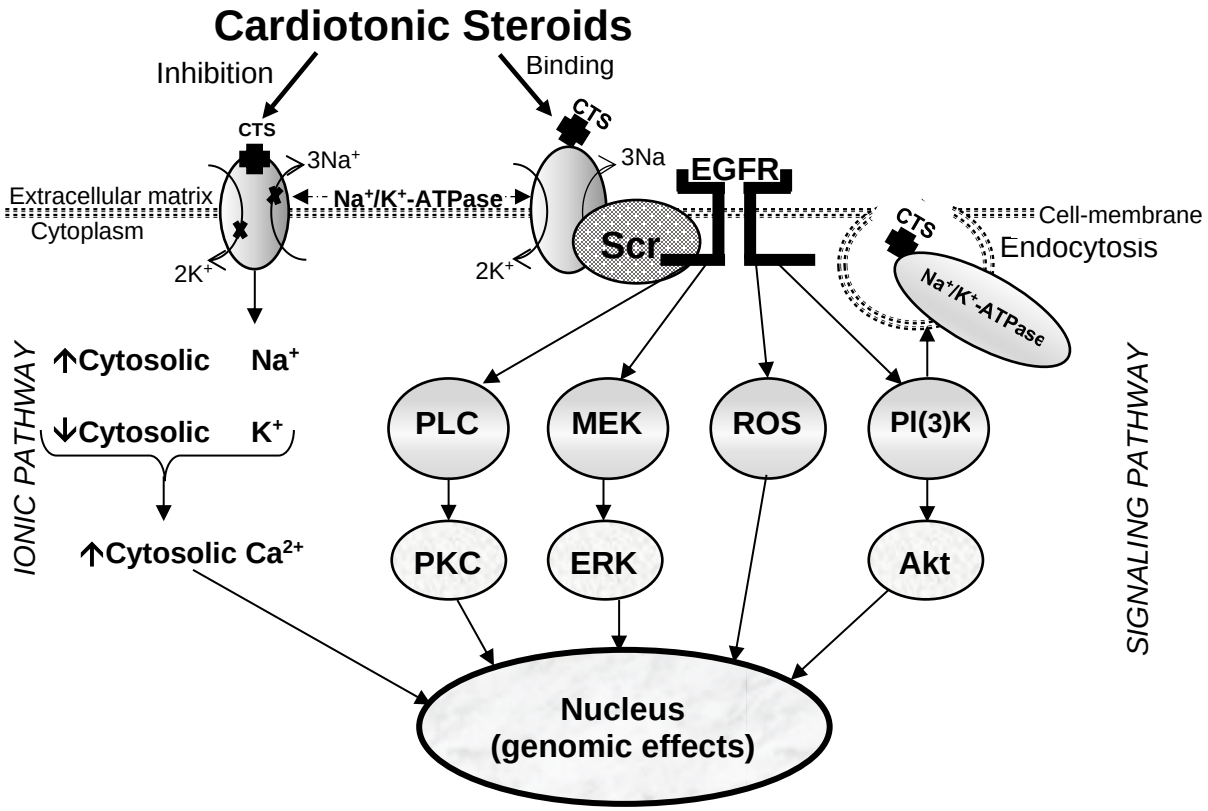


Figure 1-8. Schematic diagram of pathways for CTS effects. The ionic pathway on the left side shows the inhibition Na+ and K+ exchange through the Na^+/K^+-ATPase. On the right side, the binding of the CTS to the Na^+/K^+-ATPase activates Src, which, in turn, transactivates the EGFR and PLC pathways. This will then lead to a cascade that involves generation of ROS, activation of mitogen-activated protein kinase (MAPK) ERK through activation of its (MAPK) MEK, activation of Phosphoinositide 3-kinase (PI3K), stimulation of endocytosis and activation of Akt, as well as activation of protein kinase C (PKC).[10]

1.4.2 Effects of cardiotonic steroids on kidney and sodium metabolism

Increases in dietary sodium shown to cause CTS induced endocytosis, causing altered sodium reabsorption. Specifically, high dietary sodium increased urine excretion of MBG along with decreased proximal tubular Na^+/K^+-ATPase expression, and increased presence of the Na^+/K^+-ATPase in both early and late endosomes.[10,12] Many CTS have been reported in elevated concentrations in patients with chronic renal failure.[4,23]

Experiments done on both animals and humans demonstrated that, increases in MBG accompany decreases in renal function. More importantly, MBG shown to induce endocytosis of the proximal tubular Na^+/K^+-ATPase and decreases renal sodium reabsorption. Increases in the circulating levels of MBG accompany salt loading, which may in turn induce decreases in both basolateral and apical sodium transport in the proximal tubule through both the classic or signalling mechanism and also the Na^+/K^+-ATPase-Src-EGFR pathway. This decrease in renal sodium reabsorption would then result in increases in urinary sodium excretion. Renal 1 Na^+/K^+-ATPase isoform is responsible for MBG regulation of renal sodium excretion.[4,10,12]

Digoxin however, is not natriuretic when administered in typical clinical doses *in vivo* to experimental animals or normal human subjects, but supra-pharmacological doses of digoxin do alter sodium transport in the isolated kidney and impair transcellular transport of virtually all renal epithelial cells. Therefore, through direct effects on renal epithelium, some CTS (MBG specifically) function as natriuretic substances and have renal effects that are important physiologically.[10]

1.5 Cardiotonic steroids and hypertension

The World Health Organization (WHO) has defined hypertension as systolic blood pressure ≥160 mm Hg and diastolic blood pressure >95 mm Hg. Hypertension is a leading cause of morbidity and mortality worldwide affecting over 600 million people; and has been reported as the 4th highest contributor to premature death in developed countries

and 7th in the developing countries. Recent reports indicate that nearly 1 billion adults (nearly a quarter of the world's population) had hypertension in 2000, and this is predicted to increase to 1.56 billion (29%) by 2025. According to the WHO, hypertension is estimated to cause 7.5 million deaths, about 12.8% of the total of all worldwide deaths annually. This accounts for 57 million disability adjusted life years.[43,44,45]

More than 95% of individuals with hypertension are classified as having essential hypertension, implying that the exact cause is unknown. Hypertension prevalence studies done in sub-Saharan Africa suggested that the prevalence of cardiovascular disease and hypertension is increasing rapidly, with average blood pressures now higher in Africa than in Europe and USA; resulting in high morbidity and mortality from potentially preventable complications such as stroke, myocardial infarction, and renal failure.[45,46,47,48,49] The results of that study showed that prevalence of hypertension was higher in urban than rural areas in all studies that covered both areas, and showed an increase with increasing age in most studies. Hypertension is of public health importance in sub-Saharan Africa, particularly in urban areas, with evidence of considerable under-diagnosis, treatment, and control. There is an urgent need to develop strategies to prevent, detect, treat, and control hypertension effectively in the African region.[45,47]

Many factors contribute to the aetiology of hypertension. These include genetic and lifestyle factors. However, in Africa, it is mainly associated with environmental and lifestyle factors and has a stronger association and causal link with tobacco use, excessive use of alcohol, physical inactivity, unhealthy diet (high salt intake and, insufficient fruit and vegetable consumption) and obesity. Particularly, dietary salt has been acknowledged as a major contributing factor to increased blood pressure in salt-sensitive individuals.[19,45,50]

Under normal physiological conditions, the NO pathway, RAS and insulin are major factors involved in a complex biochemical network that maintains vascular homeostasis. Alteration in any one of these factors potentially affects all others, leading to impaired blood pressure regulation and hypertension. Insulin resistance and its downstream effects, including altered glucose metabolism and advanced glycation end product (AGEs) formation, as well as loss of bioavailable NO, angiotensin II-mediated alterations

and oxidative stress have all been implicated in the development of hypertension.[46] Proposed mechanism of salt sensitive or essential hypertension are shown in Figure 1-9.

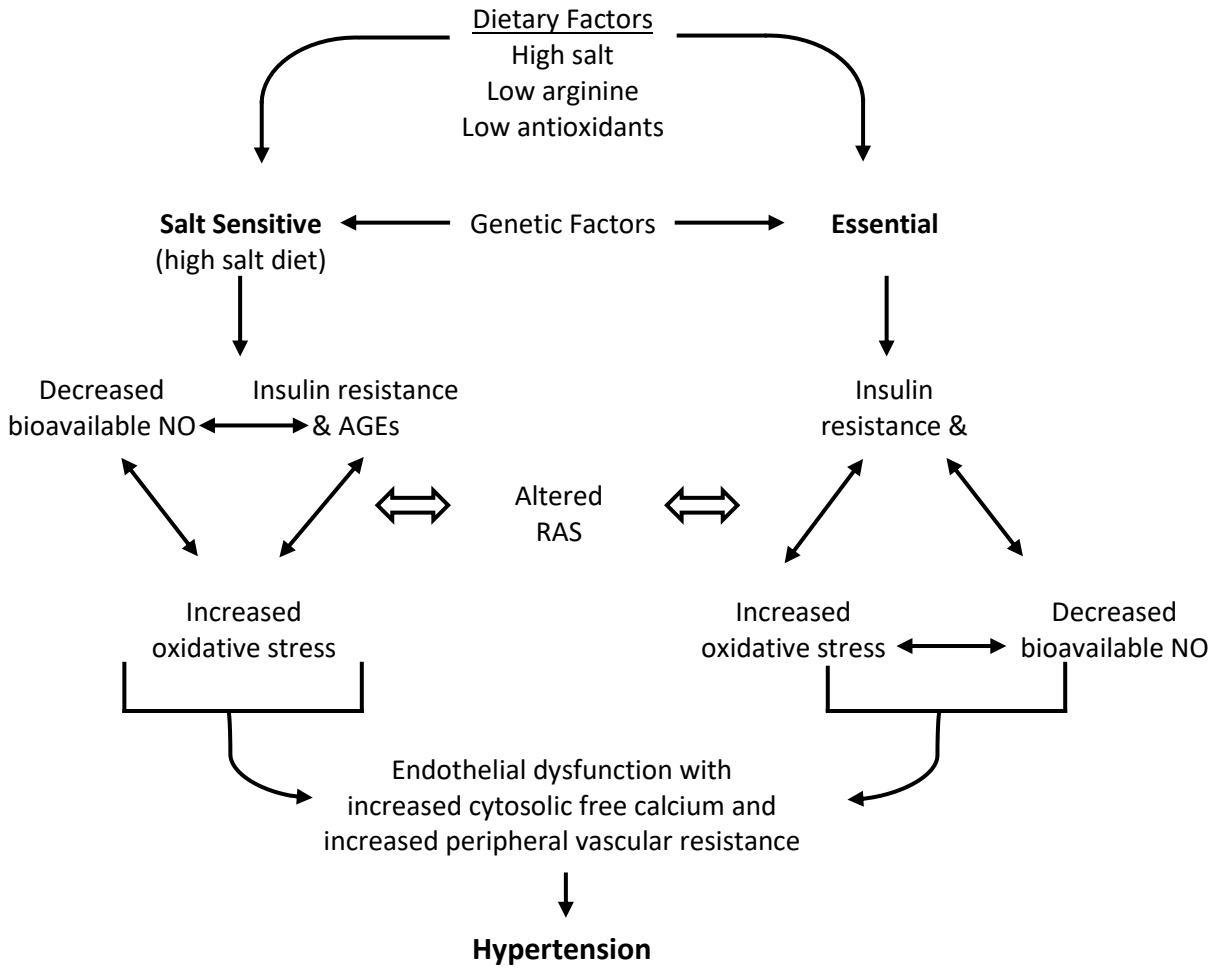


Figure 1-9. Proposed mechanisms of salt-sensitive and essential hypertension. Reproduced with permission (License number: 4647071276870).

The role of the different endogenous CTS in cardiac and kidney function and the regulation of salt and mineral metabolism unlocks the concept that endogenous CTS play an essential role in the physiology and pathophysiology of blood pressure regulation and therefore development of arterial hypertension (Salt-induced). This is through the inhibition of plasma Na^+/K^+-ATPase activity which is positively associated with elevated

blood pressure in hypertensive patients; where peripheral resistance increases due to increased myogenic tone. Increased sympathetic nerve activity are also implicated.[4,12,51]

According to the concept of natriuretic hormone, CTS are produced with the adaptive aim to regulate sodium excretion via inhibition of the Na^+/K^+-ATPase in the renal tubules. However, in patients with salt-sensitive hypertension, exaggerated CTS response produces inhibition of the Na^+/K^+-ATPase in the vasculature leading to vasoconstriction; also, their action on blood pressure was shown to be dependent on the activation of renin–angiotensin system in rats by ouabain.[3,12,15,23] Figure 1-10 shows the Interactions between brain endogenous ouabain, RAS, and MBG in pathogenesis of NaCl-sensitive hypertension.

Physiological effects of endogenous ouabain on blood pressure and cardiac activity are consistent with the “Na-lag” hypothesis. This hypothesis assumes that, in cardiac and arterial myocytes, a CTS-induced local increase of Na^+ concentration, initiated by inhibition of Na^+/K^+-ATPase, leads to an increase of intracellular Ca^{2+} concentrations, via a backward-running Na^+/Ca^{2+} passive exchanger. Increased calcium then activates cardiac and smooth muscle contraction. These positive inotropic actions of CTS forms the basis for the use of digoxin and digitoxin in heart failure.[4,6,10,11] Digoxin and digitoxin on the other hand, do not raise blood pressure, but lower the hypertensive effect of ouabain.[18,4,12]

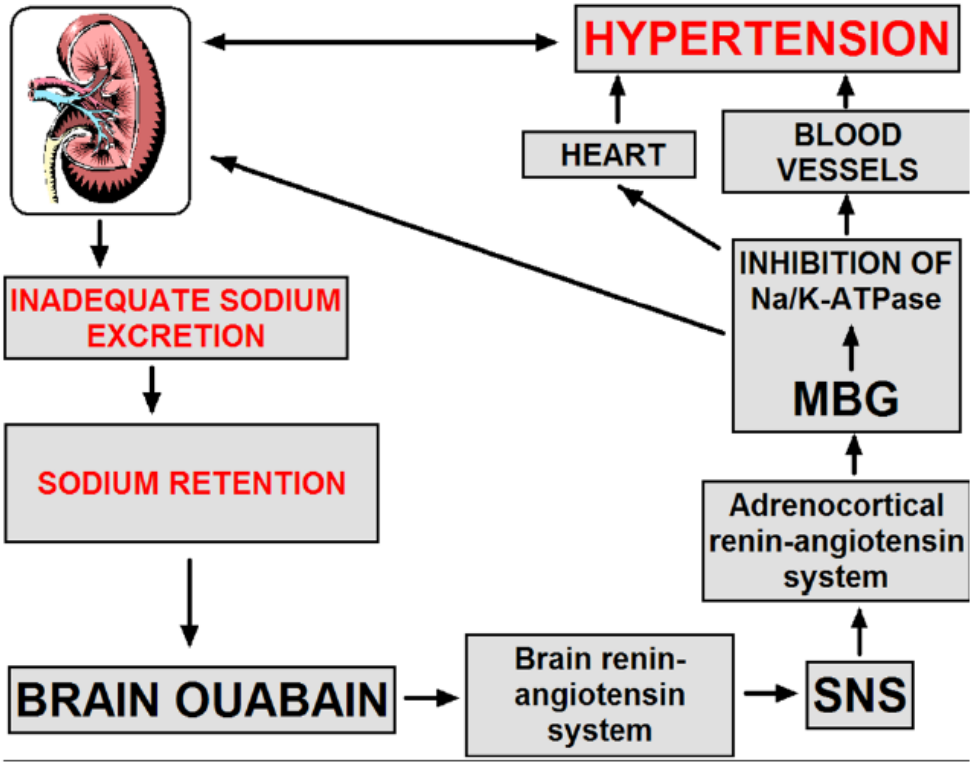


Figure 1-10. Interactions between brain endogenous ouabain, RAS, and marinobufagenin in pathogenesis of NaCl-sensitive hypertension. Reproduced with permission (License number: 4647070513483).

1.6 Hypertension and *Helicobacter pylori*

Helicobacter pylori (*H. pylori*) is one of the most common pathogens affecting humankind, infecting approximately 50% of the world's population. This pathogen is a gram-negative spiral shaped bacterium that has the unique ability to colonise the human gastric mucosa. The infection is usually acquired early in life and may persist a lifetime, unless treated.[52,53,54,55,56,57] The outcome of the infection may involve a combination of the bacterial factors, host factors, as well as environmental factors.[53,57]

*Helicobacte*r species colonise the gastric or intestinal mucosa of many mammalian and avian hosts and induce histologic inflammation. The association of *H. pylori* with gastritis, peptic ulcer disease, and gastric cancers makes it a significant human pathogen.[56,58] Infection with *H. pylori* cagA-positive strains is responsible for gastritis and peptic ulcers and further contributes to the development of gastric cancer and marginal zone B-cell

lymphoma.[52,59] *H. pylori* assimilates host cholesterol, modifies it by glycosylation, and incorporates the glycosylated cholesterol onto its surface via a cholesterol glucosyltransferase, encoded by cgt.[60,61]

Cellular cholesterol is a vital component of the membrane, which contributes to membrane dynamics and functions. It is important in VacA intoxication and phagocyte evasion during *H. pylori* infection. The glycosylation of free cholesterol (FC) by *H. pylori* has various biological significances for the survival of the bacterium, making the bacterium capable of evading host immune system, such as phagocytosis by macrophages and activation of antigen-specific T cells, and surviving in the gastric mucosal tissues for long periods.[52,60,61]

A hallmark feature of infection with *H. pylori* is a pronounced inflammatory response and the inability of the host to clear the infection, which results in a persistent infection, increased acid production followed by tissue damage.[60]

Many studies have assessed the associations between hypertension and *H. pylori* infection. Some data showed a positive association with significant elevated blood pressures in patients with *H. pylori* infection compared to patients without the infection. This association was observed only in patients with *H. pylori* virulence factor CagA-positive. After treatment for the eradication of the infection, the blood pressures dropped significantly in those patients.[54,56,62,63,64,65]

One of the hypotheses published on the relationship between hypertension and *H. pylori* was that, *H. pylori* may cause the activation of the cytokine cascade with the release of vasoactive substances, such as nitric oxide by vascular endothelium which will interfere with fibrinogen level and cause the reduction of the normal capacity of muscular relaxation, leading to vasoconstriction and adverse hemodynamic balance.[54,56,62,64] However, there is no evidence of the mechanism through which *H. pylori* affects blood pressure. Further studies are needed to investigate the mechanism through which potential role of *H. pylori* in elevated blood pressure in hypertensive patients.

As mentioned above, *H. pylori* uses cholesterol or bile acids to adapt to the harsh conditions in the stomach including low pH.[60] The cholesterol may be metabolised to produce compounds that affect the blood pressure. Alternatively, CTS are synthesised from cholesterol and these affect cardiac output and blood pressure.[4] Beevers *et. al*. (2004) suggested that high salt intake may in some way facilitate *H. pylori* infection. Another study has reported an association between the intake of salty food and the risk of *H. pylori* infection, which might explain the association of *H. pylori* with hypertension as there is a known relation between high salt intake and hypertension.[56,63,66]

H. pylori infects the stomach and the first part of the small intestine (duodenum). All the secondary products or molecules produced from the bacterial cholesterol break down or any other chemical reaction by *H. pylori*, might be detected at the site of infection. CTS produced from cholesterol can be absorbed from the gastrointestinal tract (GIT); with glycosidic cardenolides being absorbed more easily than the bufadienolides.[67,33,40,68] They are reported to pass through an entero-hepatic circulation, after which they are excreted via either urine or together with bile, implying that CTS or their precursors can then be detected in faeces.[32,35,68,69]

1.7 High Performance Liquid Chromatography tandem Mass Spectrometry (HPLC-MS/MS)

Other techniques besides MS such as; nuclear magnetic resonance and antibody based assays have been used for identification of different CTS in biological samples.[4,10,20] However, liquid chromatography tandem mass spectrometry (LC-MS/MS) provides an excellent approach that can offer a combined sensitivity and very high selectivity assay for small molecules at very low endogenous concentrations. Moreover, different LC-MS/MS approaches, such as different ionisation techniques and mass analyser technology, can be used to detect multiple compounds simultaneously.[70] Figure 1-11 shows the typical HPLC-MS instrumentation

1.7.1 Principles

LC-MS/MS is an analytical technique that combines the physical separation capabilities of liquid chromatography with the mass analysis capabilities of mass spectrometry; making it a powerful and selective technique with very high sensitivity and accuracy in separation, detection and potential identification of trace level compounds in biological samples.[71]

High Performance Liquid Chromatography

HPLC is a separation technique. It separates the sample components according to their molecular weight and polarity. This separation is based on the differences in the affinity or retention strength of the analytes between the stationary phase and the mobile phase.[70]

Mass Spectrometry

MS is a highly sensitive detection technique that operates by converting the analyte molecules to a charged (ionised) state, then separates the resulting ions based of their mass-to-charge ratios (m/z), and measures the intensity of each ion. This information indicates the concentration of each ion (ion intensities) plotted against the m/z values on a mass spectrum, which is used to both identify and quantitate specific sample components.[20,72]

Instrumentation

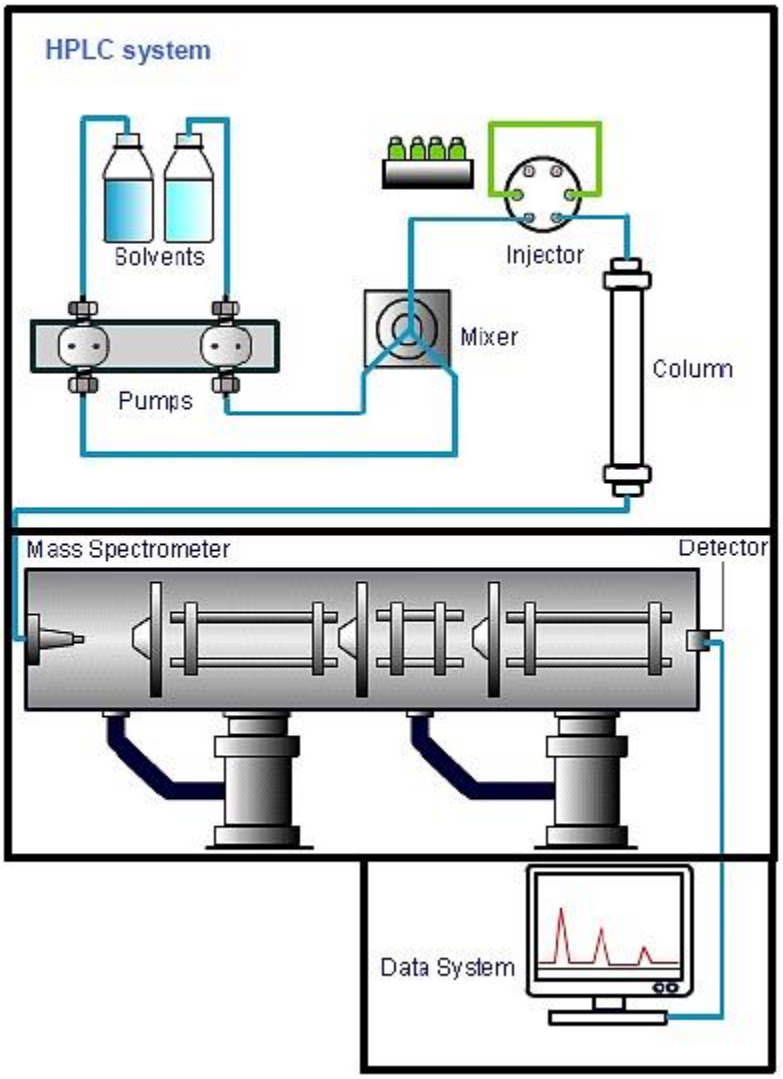


Figure 1-11. HPLC-MS instrumentation. Image from *https://www.chromacademy.com*. With permission

HPLC unit

Figure 1-12 illustrates how HPLC works and Table 1-1 explains the different techniques used. An HPLC system consist of a solvent reservoir, a binary mixing pump; sample injector and column oven with the following functions:

Solvent Reservoir: Mobile phases (Solvents) are contained in glass reservoirs. In HPLC, it is usually a mixture of polar and non-polar liquid components of which respective concentrations are varied depending on the complexity of the sample.[73]

Pump: A pump uses the mobile phase from the solvent reservoir and forces it through the system's column and detector. Depending on a number of factors including column dimensions, particle size of the stationary phase, the flow rate and composition of the mobile phase, operating pressures of up to 42000 kPa (about 6000 psi) can be generated.[73]

Sample injector: The injector can be a manual injection or an automated injection system. An injector for an HPLC system should provide injection of the liquid sample within the range of 0.001-100 mL of volume with high reproducibility and under high pressure.[73]

Column: Columns: An applicable column is chosen for the separation and attached to the HPLC system. These columns are usually made of polished stainless steel, between 50 and 300 mm long and have an internal diameter of between 2 and 5 mm. They are commonly filled with a stationary phase with a particle size of 1.7–10 µm. Columns with internal diameters of less than 2 mm are often referred to as microbore columns.[73]

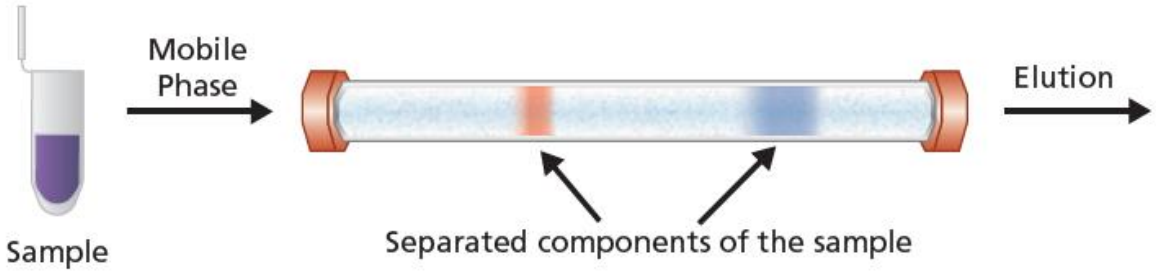


Figure 1-12. An illustration of how HPLC works. The sample is injected into the column and get separated into 2 analytes bands, which get eluted from the column. With permission from Shimadzu Corporation.

The Mass Spectrometer

The mass spectrometer has three major components: the ionisation source, analyte mass analyser and an ion detector:

Ion source: It is where eluting analytes from the HPLC are ionized by either removal or addition of electrons. Ions are produced by the use of strong electric fields in the vapour or condensed phase. There are many different ionization methods, which include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo ionisation (APPI).[72,74]

ESI is considered a soft ionisation technique, which means little energy is imparted to the analyte, which causes minimal fragmentation. ESI works well with polar, least volatile, or thermally unstable molecules and is thus well suited to the analysis of many metabolites, xenobiotics and peptides. Liquid samples are pumped through a metal capillary maintained at 3 to 5 kV and nebulised at the tip of the capillary to form a fine spray of charged droplets. The capillary is usually orthogonal to, or off-axis from, the entrance to the mass spectrometer in order to minimise contamination. The droplets are rapidly evaporated by the application of heat and dry nitrogen, and the residual electrical charge on the droplets is transferred to the analytes. The ionised analytes are then transferred into the high vacuum of the mass spectrometer via a series of small apertures and focusing voltages.[70,72] Figure 1-13 shows ESI ionisation in positive mode.

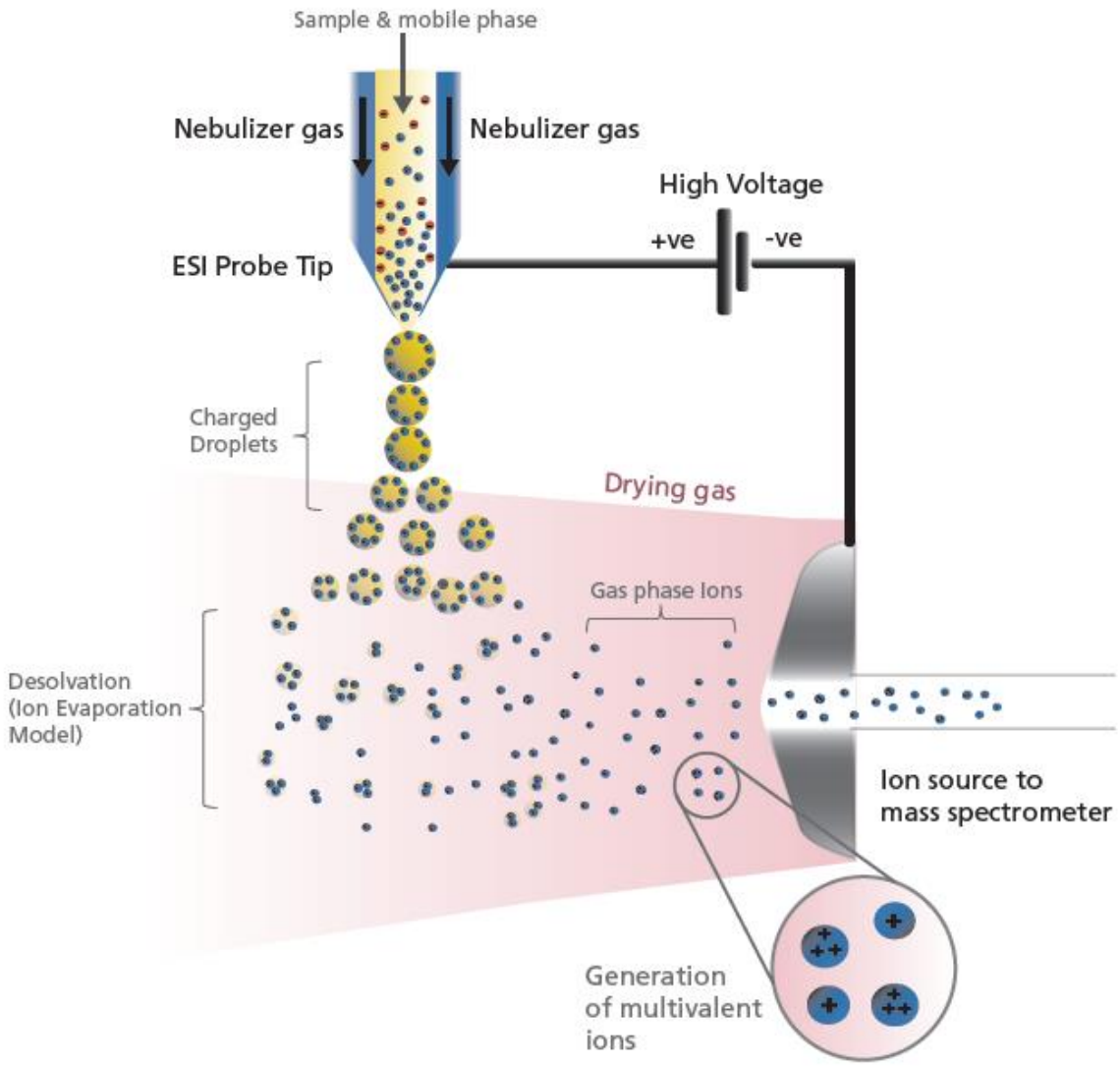


Figure 1-13. Schematic of the ionization and desolvation processes in ESI positive mode. With permission from Shimadzu Corporation.

Ion guide: An electrostatic lens that efficiently introduces the generated ions in the MS.[74]

Skimmer cone: It has a sampling orifice of reduced diameter that sample gas phase ions and reduce the gas load entering the vacuum system of the mass analyser device.[74]

Mass analyzer: The mass analyser separates analyte ions or their fragments based on their mass-to-charge ratio. Different mass analysers include, quadrupole, ion trap, time of flight (TOF), and magnetic sector.[70,72,74]

Quadrupole analyser: This device (figure 1-14) uses electric fields in order to separate ions according to their mass to charge ratio as they pass along the central axis of four parallel equidistant rods. As long as the position of an ion from the centre of the rods, remains less than r0, indicated in figure 11 by the x and y-axis, the ion will be able to pass through the quadrupole without touching the rods. This is called a non-collisional or stable trajectory.[70,74]

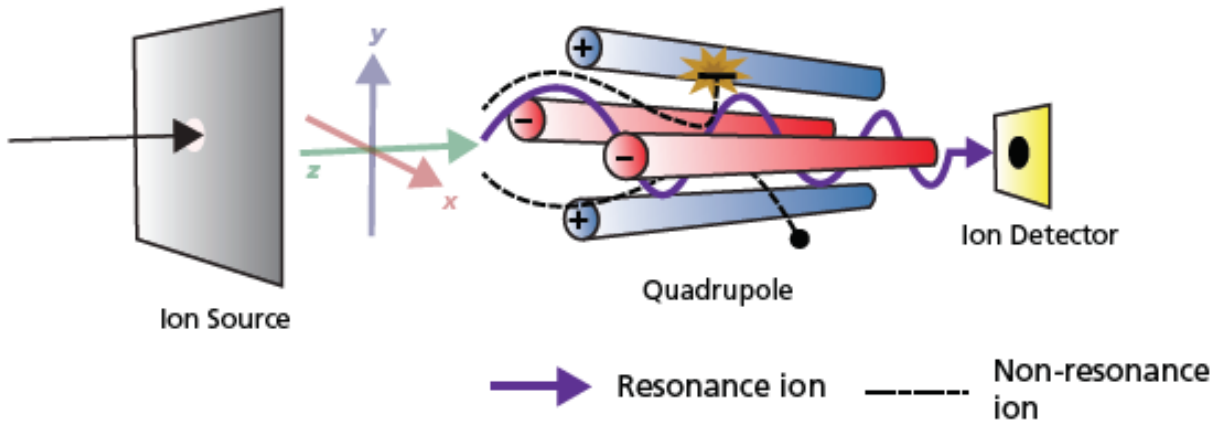


Figure 1-14. Quadrupole analyser. With permission from Shimadzu Corporation.

Ions can be induced to undergo fragmentation by collisions with a neutral gas such as hydrogen, nitrogen or argon, a process called collision induced dissociation. A quadrupole is a type of collision cell that has been designed to maintain the low pressure of the collision gas required for dissociation and transmit most of the fragment ions that are produced. A particularly useful mass spectrometer configuration is obtained by placing two quadrupole mass analysers (MS1 and MS3) connected in series with a collision cell in between. This combination is called a triple quadrupole mass spectrometer and is an example of tandem MS in which two or more stages of mass analysis are independently

applied. The advantage of tandem MS is the greatly increased specificity of the analysis over single stage mass analysis.[70,72]

Targeted multiple reaction monitoring (MRM) (Figure 1-15) are used to simultaneously filter and detect multiple molecular ion/daughter ion pairs according to their m/z values.[70,72]

Detector: A unit that detects the separated ions. It generates a signal from incident ions either by generating secondary electrons, which are further amplified, or by inducing a current generated by moving charges.[72]

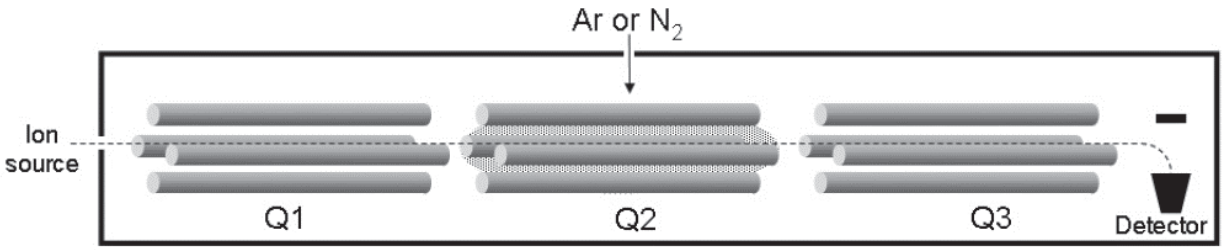


Figure 1-15. A triple quadrupole mass spectrometer. Q1 and Q3 act as mass filters and can be independently fixed, scanned or stepped. Q2 is a collision cell that contains a low pressure inert gas.[72]

1.7.2 Advantages of using HPLC-MS/MS in the quantitative analysis

There are a number of advantages in using LC-MS/MS for quantitative analysis of molecules in very low concentrations *in vivo*, such as:

- The development of sensitive and selective determination methods for trace level compounds is essential to elucidate their biological roles and functions in living systems. LC-MS/MS is frequently utilised for this purpose.
- Structural isomers that are difficult to separate using HPLC can also be difficult to .

detect with MS. The ions generated for use in MS from structural isomers can be too similar to show differences in m/z ratios. One way of overcoming this problem is to use LC-MS/MS, where after ionization for MS, some of the ions are further fragmented. This allows the detection of structurally related isomers

- In particular, with ESI ion source it is the most common method, since it requires lower temperature for ionization compared to other ionization methods, and thus it can be used for thermally unstable compounds.
- Furthermore, the decrease in the noise level by tandem MS analysis improves the signal-to-noise ratios and hence enables sensitive detection of the targeted compounds.[75]

1.7.3 Derivatisation

This process consists of transforming a chemical compound into another similar compound called the derivative or derivate, and it is done by altering one or more of the functional groups contained within the analyte. In LC-MS/MS it is done to enhance the detection sensitivity by introducing an appropriate ionisable moiety that increases the hydrophilic nature and provides suitable structures to enhance chromatographic separation.[71]

Not all compounds can be favourably analysed by LC-ESI-MS/MS. The ionisation efficiencies of some compounds can be extremely low and such compounds cannot be detected at trace concentrations. Ketones, aldehydes and lactones are neutral functional groups. The ionisation efficiencies in ESI of these compounds are usually low; and CTS all contain lactones in their structures. Furthermore, their biological concentrations are extremely low which will make them hard to be detected. To increase sensitivity, a chargeable moiety may be introduced into these compounds to enhance the ionisation efficiency.[71,75]

Dansyl hydrazine (DNH) has been used as a pre-separation derivatisation agent for the fluorometric detection of sugars, plant growth regulators, ketosteroids, and other biological carbonyl compounds in both thin layer chromatography (TLC) and HPLC

separations. By using DNH in the presence of a strong acid, it is possible to introduce a fluorescent label onto a ketosteroid, and form dansylhydrazones.[76,77] Figure 1-16 shows the derivatisation mechanism of DNH

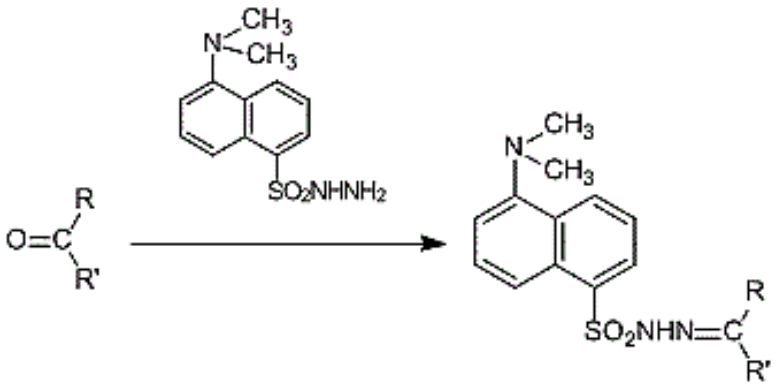


Figure 1-16. A mechanism of derivatisation of Dansyl hydrazine. Drawn using ACD/ChemSketch Freeware 12

1.8 Hypothesis

The hypothesis of this study was:

Cholesterol derived *Helicobacter pylori* produced CTS or CTS precursors can be detected in faeces of a hypertensive rat model using sensitive liquid chromatography tandem mass spectrometry methodology.

1.9 Aim and Objectives

The aim of the study was to develop a targeted LC-MS/MS technique to quantify cardiotonic steroid compounds in biological samples of hypertensive rat models and to determine if *Helicobacter pylori* produce cardiotonic steroid compounds when grown in culture with high salt and/or cholesterol.

With the initial objectives:

- To optimize sample preparation with and without a selective derivatisation procedure for identification and quantitation of cardiotonic steroid compounds by liquid chromatography tandem mass spectrometry using cardiotonic steroid standards.

- To develop and validate a liquid chromatography tandem mass spectrometry technique to analyse *Helicobacter pylori* culture supernatant and biological samples for cardiotonic steroids.

- To assay conditioned *ex vivo* culture media from *Helicobacter pylori* cultures, where the bacteria were originally isolated from the stomach and duodenal microbiome of hypertensive patients, and try determine the identity and amount of cardiotonic steroid compounds formed.

- To analyse biological samples from hypertensive rats and patients with or without *Helicobacter pylori* infections using the validated quantitative LC-MS/MS method to assess cardiotonic steroid concentrations in faeces.

Chapter 2. Materials and Methods

2.1 Chemical reagents

Analytical Standards bufalin, digitoxigenin, digitoxin, digoxin, ouabain, proscillaridin a, and dansyl hydrazine for derivatisation were purchased from Sigma Aldrich (Pty) Ltd. (St Louis, USA); marinobufagin, periplogenin, and telocinobufagin were purchased from Leapchem (Zhejiang, China) supplied by DLD Scientific (Durban, RSA). Isotope labelled surrogate standards D3-digoxin was purchased from Santa Cruz Biotechnology (Texas, USA).

MS grade (purity > 99.9%) acetonitrile, ethanol, methanol, analytical grade (≥98%) formic acid; reagents and solvents trifluoroacetic acid, acetic acid, chloroform, butanol, ethyl acetate and hexane were purchased from Merck (Pty) Ltd. (Darmstadt, Germany). Ammonium formate was purchased from Sigma Aldrich (St Louis, USA). MS grade isopropanol was purchased from Fluka Analytical. Double-deionized pyrogen-free water of >18 MΩ was produced in-house using an ELGA Genetics water purification system.

2.2 Instrumentation

An Agilent 1100/1200 combined series HPLC system (Agilent Technologies, Waldbronn, Germany) coupled to a Sciex 4000 QTRAP triple quadruple tandem-mass spectrometer, equipped with a Turbo V™ ESI source (Sciex, Toronto, Canada) was used. The LC system consisted of a degasser, binary mixing pump, column oven and autosampler. Analyst® Software version 1.5.2 (Sciex, Toronto, Canada) was used to manage the system and for optimisation of analyte detection parameters, data acquisition and data analysis. A Labconco Vacutec Centrivap™ Concentrator (Missouri, USA) was used to dry the samples. A Phenomenex™ Gemini C18 column, 100 mm × 2.1 mm (Separations, Johannesburg, RSA) was used for analytes separation. The LC-MS instrumentation is located at the University of Pretoria in the Department of Pharmacology.

2.3 Standard stock solutions

Stock solutions of the different CTS standard were made up at a concentration of 0.1 mg/mL, and DNH at a concentration of 1 mg/mL in methanol. Stock solutions were stored at -20°C until used. From CTS stock solutions, working standard solutions were made up at a concentration of 500 ng/mL by diluting 5 µL of the stock in 995 µL methanol. Further dilutions were made to 10 ng/mL by diluting in methanol. The two working standard solutions were used to spike whole blood and solvent at different concentrations to make up the calibration curves for method validation and quantitative sample analysis.

2.4 Derivatisation

CTS were derivatised individually using DNH by adding 50 µL of CTS to 50 µL of dansyl hydrazine stock solution and an acid (Hydrochloric acid (HCl), sulfuric acid (H_2SO_4) or trifluoroacetic acid (TFA) and mixed well. The reaction conditions were optimised with respect to temperature, pH, molar ratio and time (see Table 2-1) in order to yield the highest ratio of derivatised product to underivatised starting CTS.

Table 2-1. Different parameters tested to optimise CTS derivatisation using dansyl hydrazine. The molar ratio is given as CTS: dansyl hydrazine: acid.

Molar ratio	Temperature (°C)	Acid	Time
1:1:5	23	TFA	10 min
1:2:5	40	H_2SO_4	30 min
1:5:5.5	50	HCl	45 min
1:5:10	60		60 min
1:5:20	80		12 hrs
			18 hrs
			24 hrs
			48 hrs

The optimised conditions with the highest yield of derivatives were: CTS, DNH and HCL in a molar ratio of 1:2:5, at either room temperature (23°C) or 60°C for 60 min. After the mixture was transferred into an Eppendorf vial and covered with an aluminium foil, it was vortex mixed for 10 min using Vortex Genie2 at room temperature, and it was either left shaking for 50 min at room temperature or incubated at 60°C for 1 h. After the reaction

was completed at these two conditions, an aliquot of the solution was spotted on a TLC plate and the rest transferred into amber glass autosampler vials for LC-MS/MS analysis.

2.5 Thin layer chromatography

TLC is a widely used laboratory chromatographic technique, which involves a stationary phase of a thin layer of adsorbent like silica gel, aluminium or cellulose on a flat, inert substrate. It has the advantage of faster runs, sufficient separation and a wide choice of different adsorbents. It is used for following the progress of organic chemical reactions and for assaying the purity of organic compounds in phytochemistry and biotechnology. It takes advantage of the different affinity of the analyte with the mobile and stationary phases to achieve the separation of a complex mixture of organic molecules.[78,79]

TLC was used to determine whether CTS were effectively derivatised are being or not. After derivatisation, 2 µL of each standard solution derivatised and underivatised was spotted onto a 10 cm x 10 cm aluminium baked silica plate (Merck, Darmstadt, Germany) and allowed to dry for 1 min. The plate was then placed in a saturated glass tank containing the mobile phase. Following development, plates were allowed to dry and visualised under a UV lamp at 366 or 254 nm in order to detect fluorescent compounds. The presence and colour of the spots under UV light was recorded. The plates were then sprayed with anisaldehyde stain and incubated at 110°C for 10 min.

Anisaldehyde stain was made up by mixing 1 mL of anisaldehyde and 1 mL of H_2SO_4 with 18 mL of pure ethanol. Different mixtures of solvents were used as mobile phases including methylene chloride, methanol, formamide, cyclohexane, acetone, acetic acid, chloroform, acetonitrile, ammonium solution, hexane, benzene, butanol, and water. The final optimised mobile phase consisted of 49% cyclohexane, 49% acetone, and 2% acetic acid. Acetaldehyde derivatized with DNH was used as the positive control.

2.6 Sample collection

All the patient samples were collected at the Charlotte Maxeke Johannesburg Academic Hospital.

2.6.1 Blood samples

Blood was collected from healthy volunteers in 8 mL sodium-citrate BD Vacutainer tube. Aliquots of 500 µL of whole blood were spiked with different CTS and the surrogate, vortex mixed, added with 2 mL of 25% methanol in water, vortex mixed, put on ice for 10 min, centrifuged at 3200 x*g* for 10 min, after which the supernatant was collected.

2.6.2 Faecal samples

Human faecal samples were collected at the Charlotte Maxeke Johannesburg Academic Hospital Hypertensive Clinic and rat faeces from experimental rat models (Normotensive Wistar rats, spontaneously hypertensive rats (SHR), and Dahl salt sensitive rats (DSS), infected with 3 strains of cagA+ *H. pylori* for SHR and 2 strains of cagA+ *H. pylori* in each of DSS and Wistar rats) housed at the Central Animal Services of the University of Witwatersrand, where the animal study was approved and performed. Briefly the faecal material was freeze dried, finely ground then 1.0 g suspended in 10 mL of solvent consisting of either methyl tert-butyl ether (MTBE), methanol, methanol:chloroform (1:2), or isopropanol:chloroform for human samples and butanol or methanol for rat faecal samples. The mixtures were ultrasonicated for 15 min, vortex mixed for 1 h, clarified by centrifugation for 10 min and the organic phase removed and dried under a stream of nitrogen at ambient temperature. The samples were resolubilised in 5 ml of the same extraction solvent and stored at -20°C until used.

2.6.3 *H. pylori* cultures

Gastroscopic biopsies were done by a Gastroenterologist at the Charlotte Maxeke Johannesburg Academic Hospital for stomach/duodenal microbiome collection. This was collected from consenting adult patients with *H. pylori* infection and mild hypertension. The study was approved by the University of Witwatersrand ethics (M170582). Patient records were used for screening of hypertensive patients and ^{14}C-urea breath tests were conducted to prove the presence of *H. pylori* infections.

After growing bacteria isolated from the recovered stomach/duodenal microbiome of these patients, each strain of *H. pylori* was streaked onto prepared Columbia Blood Agar

(19 g/L) supplemented with 7% Foetal Bovine Serum (FBS) and Skirrows Campylobacter supplement (antibiotic mix – 5 mg vancomycin and 2 mg trimethoprim). The plates were incubated under microaerobic conditions for 72 hrs at 37°C; using an anaerobic tank and a gas pack (Micro-Aeropack NGC).

Isolated colonies from each of the strains were then used to inoculate cagA+ strain *H. pylori* in 10 mL of 37 g/L Brain Heart Infusion (BHI), supplemented with Skirrows Campylobacter supplement and 7% FBS. The liquid cultures were again incubated under microaerobic conditions, with 100 rpm shaking for 24 hours at 37°C. OD readings were taken at 600 nm every 3 hours to monitor the phase of growth.

BHI was further supplemented with either cholesterol, salt or both. Cholesterol was solubilised in ethanol (5 mg/ml), and it was added to prepared BHI to a final concentration of 50 µg/ml. BHI cholesterol supplemented (10 mL) was then inoculated with 1 mL *H. pylori* liquid culture, incubated under microaerobic conditions for 24 hours at 37°C with shaking at 100 rpm with a 2 cm throw. The same procedure was done for salt supplemented BHI at a final concentration of 15 mg/mL. Bacterial growth was assessed and harvest at stationary phase.

2.7 Solid phase extraction

Solid phase extraction (SPE) was used to extract, concentrate and clean up the samples before LC-MS/MS analysis. This sample preparation technique uses a solid stationary phase and a liquid mobile phase to isolate the analytes from complex mixtures before the LC-MS/MS analysis to detect and quantify analytes in the sample.[80] For this study, C18 Strata reverse phase SPE cartridges (Separations, South Africa) were used.

A three-step sample preparation method (Pre-SPE, SPE and Pre-analysis) was used for sample preparation of all the samples.

2.7.1 Spiked blood and solvent

The calibration curves for method validation were prepared by spiking 500 µL of either blood or solvent at different concentrations of the prepared standards. The chosen

concentrations were within the concentration range of CTS expected to be found in biological samples. The surrogate standard D3-digoxin was added at a constant concentration of 0.5 ng/mL. The calibration curve concentrations are given in Table 2-2.

Table 2-2. Calibration curve dilutions. Working solution 1 (500 ng/mL) and 2 (10 ng/mL).

Concentrations (ng/mL)	Working Solution 1 (µL)	Working Solution 2 (µL)
0.02	-	1
0.05	-	2.5
0.1	-	5
0.2	-	10
0.5	-	25
1	-	50
2	2	-
5	5	-
10	10	-
20	20	-
30	30	-
40	40	-

Pre-SPE: The individual standards were added to glass tubes, after which they were dried, and 500 µL of either blood or solvent were added. The tubes were vortex mixed, then 2 mL of 25% methanol was added, put on ice for 10 min, centrifuged at room temperature at a speed of 3200 *xg* for 10 min. The supernatant was loaded onto the preconditioned SPE cartridges. To the remaining pellets, 500 µL of 25% methanol was added, followed by centrifugation at 3200 *xg*, and the supernatant was loaded into the SPE cartridges.

SPE: Cartridges were preconditioned with 1 mL of methanol and 1 mL of water, equilibrated with 1 mL of a 10 mM ammonium acetate solution at pH 6.4. After loading, they were washed with 1 mL of 10 mM ammonium acetate in 30% methanol, and eluted with 1 mL of chloroform: isopropanol (9:1) into glass tubes. Both washing and elution fractions were collected separately and dried.

Pre-analysis: The eluted fractions were reconstituted with 50 µL of acetonitrile:water (6:4), and vortex mixed, while the wash fractions were reconstituted with 500 µL of methanol,

vortex mixed, sonicated, and centrifuged at 16000 x*g* for 5 min, the supernatants were collected, dried and reconstituted with 100 µL of acetonitrile:water solution (3:7) containing 0.1% FA. The final supernatants were transferred into amber glass autosampler vials for LC-MS/MS analysis. An injection volume of 5 µL was used.

2.7.2 Faecal samples

Pre-SPE: Faecal samples were dried and reconstituted with 1 mL of 25% methanol in water, containing 0.5 ng/mL of surrogate standard. The solution was vortex mixed for 10 min, sonicated for 10 min, and centrifuged at room temperature at 3200 x*g* for 10 min. The supernatant was then loaded onto the SPE cartridges.

SPE: Cartridges were preconditioned with 1 mL of methanol and 1 mL of water and then equilibrated with 1 mL of a 10 mM ammonium acetate solution at pH 6.4. After loading, the cartridges were washed with 1 mL of the 10 mM ammonium acetate in 30% methanol. The CTS analytes were eluted with a 1 mL chloroform:isopropanol in a ratio of 9:1 into 2 mL Eppendorf tubes. Both the washing and eluted fractions were dried in a vacuum centrifuge and stored at -80°C until use.

Pre-analysis: The eluted fractions were reconstituted with 500 µL of methanol, vortex mixed for 5 min, sonicated for 10 min, and centrifuged at 16 000 x*g* for 5 min. The supernatant was transferred to glass tubes, dried, reconstituted with 200 µL of acetonitrile:water mixture (6:4), vortex mixed, sonicated for 5 min, and centrifuged at 3200 x*g* for 10 min; while the wash fractions were reconstituted with 100 µL of acetonitrile:water (3:7) containing 0.1% formic acid (FA). Samples were then vortex mixed for 5 min, sonicated for 10 min, and centrifuged at 16000 x*g* for 5 min. Supernatants were transferred into amber glass autosampler vials for LC-MS/MS analysis. An injection volume of 5 µL was used.

2.7.3 *H. pylori* cultures

Pre-SPE: The cultures were dried, and reconstituted with 1 mL of MTBE, containing 0.5 ng/mL of the surrogate standard. The tubes were vortex mixed, then sonicated for 40 min,

centrifuged at room temperature at a speed of 3200 *xg* for 10 min, and the supernatant was loaded into the SPE cartridges.

SPE: Cartridges were preconditioned with 1 mL of methanol and 1 mL of water, equilibrated with 1 mL of a 10 mM ammonium acetate solution at pH 6.4. After loading, they were washed with 1 mL of 10 mM ammonium acetate in 30% methanol, and eluted with 1 mL of chloroform:isopropanol (9:1) into glass tubes. Both washing and elution fractions were dried using a vacuum centrifuge.

Pre-analysis: The elution fractions were reconstituted with 100 µL of acetonitrile:water (6:4), vortex mixed, sonicated for 10 min, centrifuged at room temperature at 3200 x*g* for 10 min; while the wash fractions were reconstituted with 100 µL of acetonitrile/ water solution (3:7) containing 0.1% FA, vortex mixed, sonicated for 10 min, and centrifuged at 16000 x*g* for 5 min, The final supernatants were transferred into amber glass autosampler vials for LC-MS/MS analysis. An injection volume of 20 µL was used.

2.8 LC-MS/MS method optimisation

For analysis, the prepared samples were injected into the LC-MS/MS system and run following the optimised MS parameters The MS conditions were optimised according to ionisation observed using both derivatised and underivatised CTS analytes. Derivatised standards were diluted in methanol and underivatised standards in acetonitrile containing 5 mM ammonium formate. These were infused directly into the ESI source using a 1 mL Harvard syringe pump at a flow rate of 20 µL/ min.

The masses of the analytes of interest were determined using a Q1+ scan. The declustering potential and collision energies were optimised using the parameter-ramp functions, in order to find more intense precursor ion pairs for all analytes. The gradient profiles, reconstitution solvents, pH and column temperature were changed to assess the effects on the separation of the analytes, their retention times, as well as peak symmetry, analyte peak area (in counts per second, (cps)), analyte peak height (cps) and peak shape.

2.9 LC-MS/MS method validation

Method validation is the formal procedure of establishing and documenting that a method is capable of producing results that are fit for the intended purpose. The method used in this study was fully validated in whole blood to demonstrate reliability and reproducibility for its intended use. Method validation for quantitative assay was performed according to the ICH-GLP guidelines. The following parameters were assessed: Linearity, selectivity, accuracy, precision, absolute and relative recoveries, limit of detection, limit of quantitation, reproducibility, matrix effect, and stability.

2.9.1 Linearity and Sensitivity

The linearity of an analytical procedure is its ability to obtain test results which are directly proportional to the concentration of analyte in the sample, within a given range. The linearity of the method was assessed by analysing a minimum of five points calibration curves of each analyte standard in triplicate at each concentration level on three separate days. Peak area ratio of the analyte to surrogate standard versus the nominal concentration were fitted by least-squares linear regression using 1/X as a weighting factor. The concentration range used for each analyte are detailed in Table 2-3 below.

Table 2-3. Concentration ranges used for each analyte to make calibration curves.

Analytes	Concentrations (ng/mL)
Bufalin	0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10
Digitoxigenin	0.5, 1, 2, 5, 10, 20, 30, 40
Digitoxin	0.2, 0.5, 1, 2, 5, 10, 20, 30
Digoxin	0.2, 0.5, 1, 2, 5, 10, 20, 30
Marinobufagin	0.5, 1, 2, 5, 10, 20, 30, 40
Ouabain	2, 5, 10, 20, 30, 40
Periplogenin	0.1, 0.2, 0.5, 1, 2, 5, 10, 20
Telocinobufagin	2, 5, 10, 20, 25, 30, 40

2.9.2 Selectivity

Selectivity is the ability of bioanalytical method to differentiate and quantify the analyte in the presence of other components in the sample. The absence of interfering components is accepted where the response is less than 20% of the lower limit of quantification for the analyte and 5% for the internal standard (IS).

The selectivity of the method was evaluated by analysing different double blank samples (no analyte or surrogate) of whole blood to check for interfering peaks of analytes of interest. Also, blank samples (blank whole blood samples + surrogate) were analysed, to verify the absence of native analytes in the IS solution.

2.9.3 Accuracy and Precision

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted as either a conventional true value or an accepted reference value, and the value found. The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the sample under the prescribed conditions.

The intra- and inter- day precision and the accuracy of the method were calculated from quality control samples (QC). Three runs included a full calibration curve and QC standards at three different concentrations (QC low, QC medium, and QC high) of individual compounds in nine replicates. A QC sample at the LLOQ for all analytes were analysed to confirm that the LLOQ were within an acceptable limit of variance of 15% for both precision and accuracy assessments. The accuracy was calculated as the percentage deviation of the measured concentration from the nominal concentration. Intra- and inter-batch precisions were indicated by the coefficient of variation (CV).

2.9.4 Recovery and Matrix effect

The recovery assesses the extraction efficiency of the analytes from the samples using the developed method. Matrix effects refer to complications encountered during analysis of complex biological samples due to multiple components, endogenous or exogenous,

present in the sample matrix which can cause ionisation suppression or enhancement of the analytes of interest.

The extraction recovery was determined by comparing the mean peak areas of three different concentrations (QC low, QC medium, and QC high) of the individual compounds from a whole blood matrix, spiked with standards before the complete SPE extraction, with those obtained from a whole blood matrix spiked after the extraction. Matrix effects were determined by comparing the mean peak areas from matrix spiked after the extraction and the individual standards spiked into solvent.

2.9.5 Limit of detection and quantitation

The limit if detection of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected. The lowest amount, which can be quantitatively, determined with suitable precision and accuracy, is the limit of quantitation. The limit of detection was defined as the analyte concentration that produced a S/N of at least > 3. The limit of quantification was defined where the S/N were > 10.

2.9.6 Stability

The stability of each analyte in both stock solution and matrix were investigated. The stability was evaluated by infusing freshly prepared samples, which were then aliquoted into 4 samples. The first was stored at 4°C, the second at -20°C, the third at -80°C and the last one was kept in the autosampler (19°C). The stability was checked at week 1, 2, 3 and 4. The peak areas obtained by triplicate injections inter and intra-day were compared to those of a freshly prepared solution.

2.9.7 Reproducibility

The reproducibility of the method was assessed by injecting freshly prepared solutions of each analyte in triplicate over a period of seven consecutive days for day-to-day variation. The percentage relative standard deviation (%RSD) between peak areas from each injection was then calculated.

2.9.8 Carry over

Carry over was assessed by two consecutive injections of a blank solvent after the injection of the highest concentration of each calibration curve for the individual analytes.

2.10 Statistics

Analyst software version 1.5.2 was used to evaluate the analytical data using descriptive statistics, recovery, precision, linearity, linear regression and Pearson correlation. Quantitative data was calculated using calibration curves created in matrix matched calibrations using the built in quantitation algorithms.

The statistical differences were determined using multiple t-test, Holm-Sidak method and comparisons of different rat groups were performed using the Kruskal Wallis assay of GraphPad Prism 8. The P-value of less than 0.05 was considered to be statistically significant between groups for all tests.

Chapter 3. Results and Discussion

3.1 Derivatisation and LC-MS/MS method development

There are various studies in literature reporting the development of methods for the determination of some of the CTS in human plasma, serum, and urine by LC-MS, and some described their determination in rat urine or liver. However, only a few studies have been published on validated methods for the analysis of more than five different CTS, including both polar and less polar CTS in whole blood. Furthermore, to the best of the authors' knowledge, the present study is the first to report a LC-MS optimised method for the analysis of CTS in *H. pylori* cultures and faecal materials. Thus, optimised bioanalytical methods need to be developed to detect very low quantities of the CTS in cultures media.

An optimised derivatisation method for CTS with DNH was developed. A molar ratio 1:2:5 of CTS, DNH and HCL respectively, was found to work better either heated at 60°C, or kept at room temperature for an hour. Five CTS (Bufalin, digitoxigenin, digitoxin, digoxin and ouabain) were derivatised individually and the spectra for four of them are shown in Figure 3-1. Optimised mass spectrometric detection parameters (declusting potential (DP), and collision energy (CE)) for the derivatives with their respective product ion pairs, are shown in Table 3-1

A method for underivatised CTS was also developed and optimised. This was to compare the underivatised CTS to the derivatised ones in order to determine whether the derivatisation assay sufficiently increased the MS sensitivity of the analytes. Optimised mass spectrometric detection parameters (DP, and CE) for precursor ions and product ions (monoprotonated and ammonium adducts), are shown in Table 3-2. For both derivatised and underivatised CTS a multiple reaction monitoring (MRM) algorithm was used for optimised selectivity and sensitivity during mass spectrometric analysis using the optimised parameters.

As indicated in Figure 3-1, digitoxigenin (A) gave the same derivative (641.0) irrespective of the temperature used (A1 at room temperature or A2 at 60°C). Contrary to digitoxin (B), ouabain (C) and digoxin (D) which gave different derivatives at room temperature (23°C) (B1-D1: 1031.9, 851.8, 1048.0) and at 60°C (B2-D2: 641.6, 685.3, 657.6). Furthermore, digitoxigenin derivate's intensity was higher at room temperature compared to that of the 60°C product; while all the other derivatives had higher intensities at 60°C. Another difference observed between the two temperatures used was that, at room temperature they all showed only one derivative peak (Figure 3-1 (A1, B1, C1, D1)), but when heated to 60°C, many peaks were formed from the derivatisation molecule, which supposedly reacted with the analytes to give different derivate peaks (Figure 3-1 (A2, B2, C2, D2)).

Table 3-1. Optimised MS-MS parameters for derivatised CTS standards.

Analyte	Monoisotopic mass (g/mol)	Derivative (m/z)	Optimised DP (V)	Product ions (m/z)	CE (V)
Bufalin	386.532	653.4	46	236.4 266.4	53 22
Dansyl hydrazine	265.300	n/a	50	115.2 156.4	73 48
Digitoxigenin	374.521	641.6	46	236.4 266.4	53 20
Digitoxin	764.900	641.6	42	236.4 266.4	61 30
Digoxin	780.949	657.5	66	236.4 171.4	48 87
Ouabain	584.652	851.5	75	236.4 266.4	81 28

Table 3-2. Optimised MS detection parameters for precursor ions of the underivatised CTS standards.

Analyte	Chemical Formula	Monoisotopic Mass (g/mol)	$[M+H]^+$	$[M+NH_4]^+$	DP (V)
Bufalin	$C_{24}H_{34}O_4$	386.532	387.3		98
D3-digoxin	$C_{41}H_{64}O_{14}$	783.967		801.6	61
Digitoxigenin	$C_{23}H_{34}O_4$	374.521		392.5	83
Digitoxin	$C_{41}H_{64}O_{13}$	764.900		782.6	54
Digoxin	$C_{41}H_{64}O_{14}$	780.949		798.5	57
Marinobufagin	$C_{24}H_{32}O_5$	400.225	401.6		78
Ouabain	$C_{29}H_{44}O_{12}$	584.652	585.7		56
Periplogenin	$C_{23}H_{34}O_5$	390.24		408.5	45
Proscillaridin A	$C_{30}H_{42}O_8$	530.650	531.5		126
Telocinobufagin	$C_{24}H_{34}O_5$	402.24	403.6		78

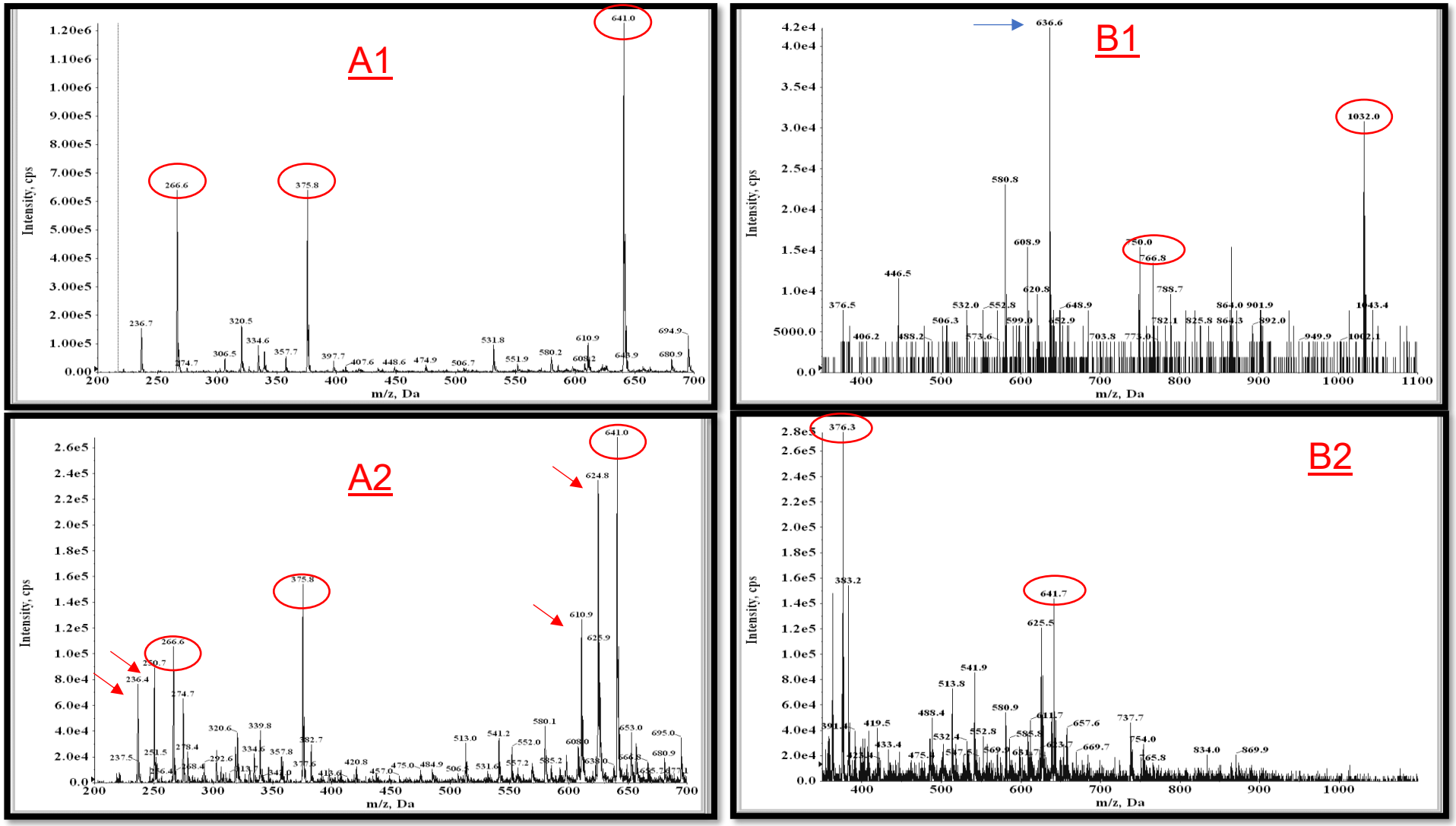


Figure 3-1. Q1 scan of derivatised CTS standards at room temperature (1) and 60°C (2). Digitoxigenin (A) and, Digitoxin (B).

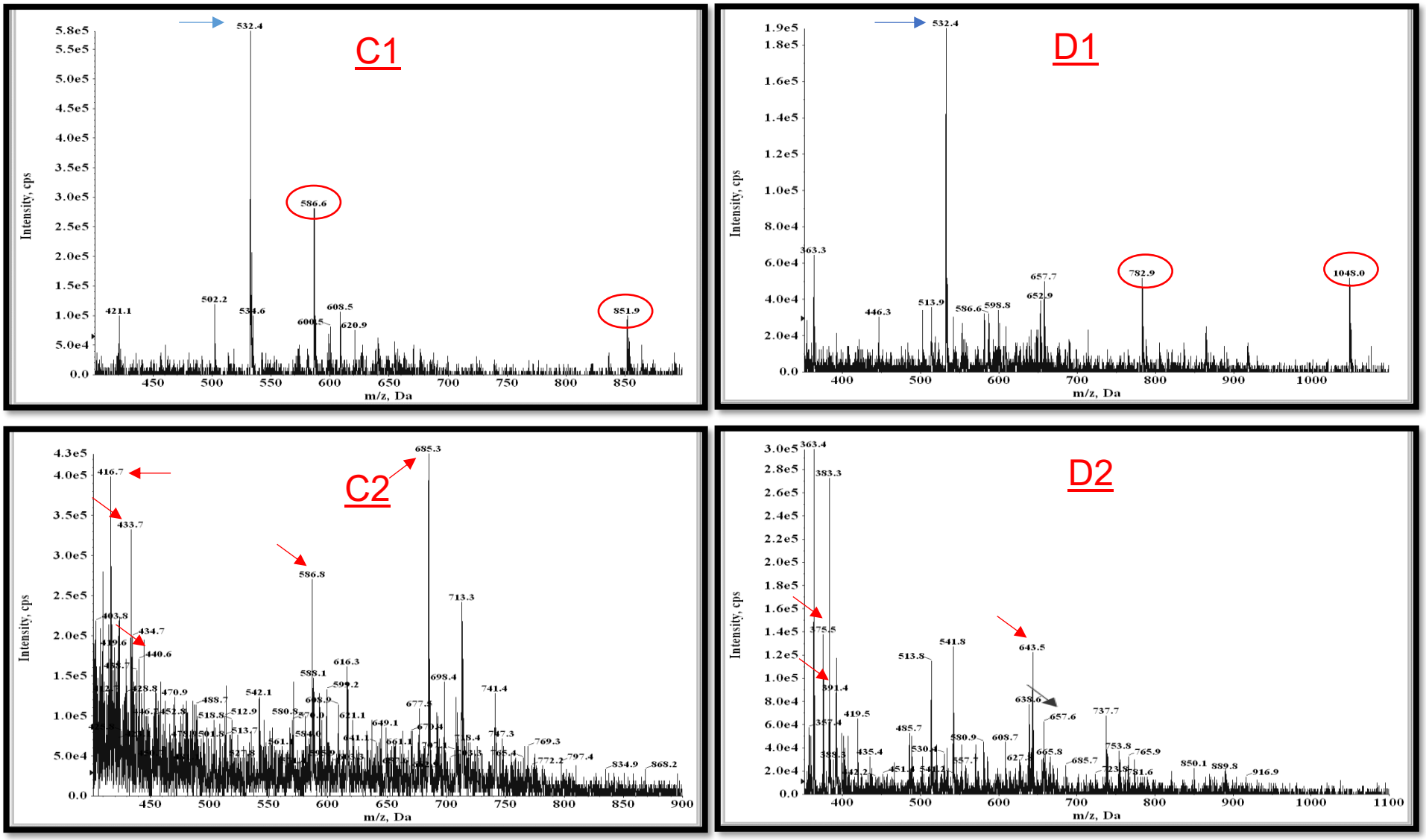


Figure 3-1 (continued). Q1 scan of derivatised CTS standards at room temperature (1) and 60°C (2). Ouabain (C) and Digoxin (D).

CTS that yielded different derivatives at different temperatures had one thing in common; they were all glycosides. This means that the sugar moieties attached to them were being affected differently at different derivatisation temperatures. A Q1 scan of the derivatised mixtures at room temperature, seen in Figure 3-1 (B1, C1, & D1) revealed a peak higher in intensity (blue arrows) than the precursor ion and the derivative as indicated by the red blocks. The Q1 ion of 636.6 Da, observed in Figure 3-1 B1, is a fragment from digitoxin obtained after removal of one sugar (β-D Digitoxose, 148.158 g/mol). The 532.4 Da ion observed in both Figure 1 C1 and D1 is possibly a dimer of DNH. It stands to reason that at room temperature the derivatisation reactions were not completed for most of the analytes and that the excess amount of DNH in the solution formed homodimers, which were not observed at 60°C.[81]

Many peaks were observed when the CTS were derivatised at 60°C (Figure 3-1 (B2, C2, & D2)) which were not detected at room temperature, as indicated by the red arrows. B2 for instance, shows a Q1 ion of 376.3 Da, which is the aglycone ion for Digitoxin, after removal of its sugar moieties (3 β-D digitoxoses) which happens through acidic hydrolysis.[82] The aglycone ion then reacted with DNH to give 641.7 Da peak as the derivative. Similarly, the aglycone ion peaks 391.4 and 440.6 for Digoxin and Ouabain, respectively were observed on the spectrum and many other fragments which reacted with DNH to give different derivatives.

These results show that the glycosides' sugar moieties are cleaved from the aglycones at 60°C. It has been stated that, similar to sugar molecules, the glycosidic bonds in the molecular structure of the cardiac glycosides break through acid hydrolysis.[82] The present study indicated that the acid hydrolysis reaction is also temperature related as the same acidic conditions were used for derivatisation at room temperature which did not show any breakdown of cardiac glycosides except for the one sugar breaking from Digitoxin. This could be attributed to the stability of digitoxin at room temperature. It has been confirmed by literature that higher temperatures increase the hydrolysis process.[83]

The derivatisation reaction with DNH takes place through a dehydration reaction, where hydrazine reacts with ketone or lactone groups to form the corresponding hydrazones by

the release of water molecule (Figure 1-16).[75] This implies that the expected derivative masses should follow the equation:

$$\boldsymbol{Derivative} \rightleftharpoons [(\boldsymbol{AB} - \boldsymbol{H2O}) + \boldsymbol{H}^{+}]$$

Where: A is the CTS molecule and
B is DNH

However, all the derivatives obtained during the current study at room temperature and at 60°C followed the equation:

$$\boldsymbol{A} + \boldsymbol{B} \rightleftharpoons [\boldsymbol{AB} + \boldsymbol{H}^{+}]$$

Furthermore, the MRM product ions of all the derivates were the same (Figure 3-2); and the highest intensities were the same as DNH precursor ion 266.4 Da and its most abundant product ions 236.4 Da. Thus, TLC was performed to confirm the derivatisation reaction. The only compound that was observed under UV light was DNH as a green fluorescent spot. After staining the plate with Anisaldehyde stain, all the CTS were observed as blue spots. The derivatised samples did not show any spot different form the underivatised samples. The spot colours and retention values were still the same for both samples. This indicated that, either the derivatisation reaction was not taking place at all or it was happening with only a small concentration of the analytes that could not be observed on the plate.

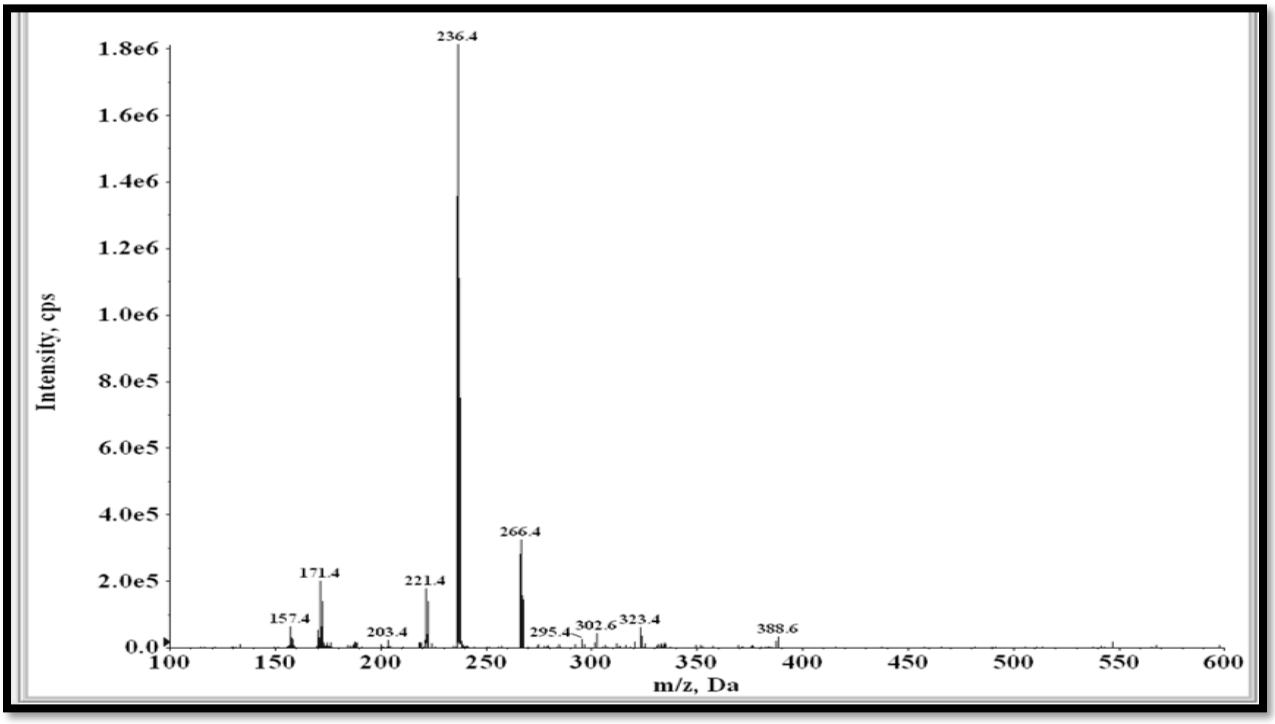


Figure 3-2. Product ions obtained from all CTS standards derivatives.

This brought up a possibility that, all the peaks observed on the spectrums after derivatisation might be the molecules combined through a very weak bond. Many studies confirm the common formation of dimers, in MS with ESI and it could be concentration dependent. As this was done at µM concentrations, this might also be the case here. Moreover, when the same procedure was performed at nM concentrations the derivative peaks were almost absent. This would explain the fact that the same product ions were observed for all the CTS after derivatisation. During fragmentation, the combined molecules break into two original compounds which are DNH and CTS, and because DNH is easily ionisable, thus it has the highest intensity and it is shown as the most abundant product ion in the solution.[81,84] However this would need to be confirmed.

“For a derivatisation reagent to be suitable for MS, it should produce more than 95% derivatives, it should not cause any rearrangements or other structural alterations of compounds during formation of the derivative, it should not contribute to loss of the sample during the reaction, it should produce a derivative that will not interact with the

column and finally, it should produce a derivative that is stable with respect to time." [85] According to all the results discussed above, the optimised derivatisation method did not fulfil all the requirements. Firstly, the derivatisation reaction could not be confirmed; secondly, the reaction observed was incomplete as the precursor ions of underivatized CTS were still observed at high intensities; thirdly, the derivatives were not stable with respect to time; and lastly, the formation of different derivatives made it difficult to choose which one to use as the method was not reproducible. Therefore, due to all these uncertainties, the derivatisation method could not be used for the study purpose.

3.2 LC-MS/MS method optimisation for underivatised CTS

Optimisation of spectrometric ionisation and MS/MS detection of the compounds were done by direct infusion via a Harvard syringe pump into the ESI source, whilst running in both positive and negative ionization modes. The positive mode provided systematically higher sensitivity for each of the compounds. The transitions of all the compounds were chosen according to their specificity and their maximum intensity according to the CE applied, and all fragment ions were confirmed by literature.[13,25,36,86,87,88,89] Optimised MS/MS detection parameters are given in Table 3-3.

The protonated molecules $[M + H]^+$ and the sodium adducts ions $[M + Na]^+$ were observed when the analytes were dissolved in 0.1% FA methanol, but although their intensities were very high and stable, there were no stable fragment ions for the sodium adducts $[M + Na]^+$. The formic acid in methanol was then replaced with 5 mM ammonium formate in acetonitrile as the solvent. With this solvent, the presence of the ammonium adduct ions $[M + NH_4]^+$ and the protonated molecules were observed for all the analyte standards. Figure 3-3 shows the precursor ions of each analyte standard at the optimised DP.

Ammonium adducts were the predominant ions for all the glycosides (Digitoxin, digoxin, D3-digoxin, and proscillaridin A (Figure 3-3 (C, D & I)) except for ouabain (Figure 3-3 (F)) which had the protonated molecule at the highest intensity and stable precursor ion. However, proscillaridin A ammonium adduct did not produce stable fragments; $[M + H]^+$ was then chosen as its precursor ion. The same applies to digitoxigenin and periplogenin (Figure 3-3 (B & G)), which had $[M + H]^+$ as the predominant ions but the ammonium

adducts generated the highest intensity and most stable fragment ions and were therefore used as precursor ions. $[M + H]^+$ were also chosen as precursors for bufalin, MBG and TCB (Figure 3-3 (A, E & H)).

Ammonium and sodium adducts, $[M + NH_4]^+$ or $[M + Na]^+$, are always present with CTS due to the strong affinity of their heterosides for ammonium and sodium ions.[90] $[M + Na]^+$ adducts presence was likely due to trace levels of sodium present from glassware, bottles or impurities in the solvents. Table 3-2 and 3-3 show the MS/MS optimised parameters for the underivatised CTS standards.

Table 3-3. Optimised MS/MS detection parameters for the product ions of the underivatised CTS standards.

Analyte	Monoisotopic Mass (g/mol)	Product ions (m/z)	Fragmentation	CE (V)
Bufalin	386.532	351.4 145.2	$[M+H-2H_2O]^+$	31 51
D3-digoxin	783.967	654.3 97.4	[a]$[M+H-D]^+$ [a]$[D+H-H_2O-OH]^+$	15 75
Digitoxigenin	374.521	339.2 90.9	$[M+H-2H_2O]^+$	25 96
Digitoxin	764.900	635.2 113.3	[a]$[M+H-D]^+$ [a]$[D+H-H_2O]^+$	16 65
Digoxin	780.949	651.7 97.4	[a]$[M+H-D]^+$ [a]$[D+H-H_2O-OH]^+$	19 68
Marinobufagin	400.225	365.2 159.5	$[M+H-2H_2O]^+$	26 43
Ouabain	584.652	385.0 403.4	[b]$[M+H-R-2H_2O]^+$ $[M+H-R-H_2O]^+$	26 19
Periplogenin	390.240	337.3 124.9	$[M+H-3H_2O]^+$	26 50
Proscillaridin A	530.650	84.0 513.4	 $[M+H-H_2O]^+$	65 27
Telocinobufagin	402.240	384.9 171.3	$[M+H-2H_2O]^+$	31 51

[a]D=digitoxose (130 g/mol); [b]R=Rhamnose (164.16 g/mol)

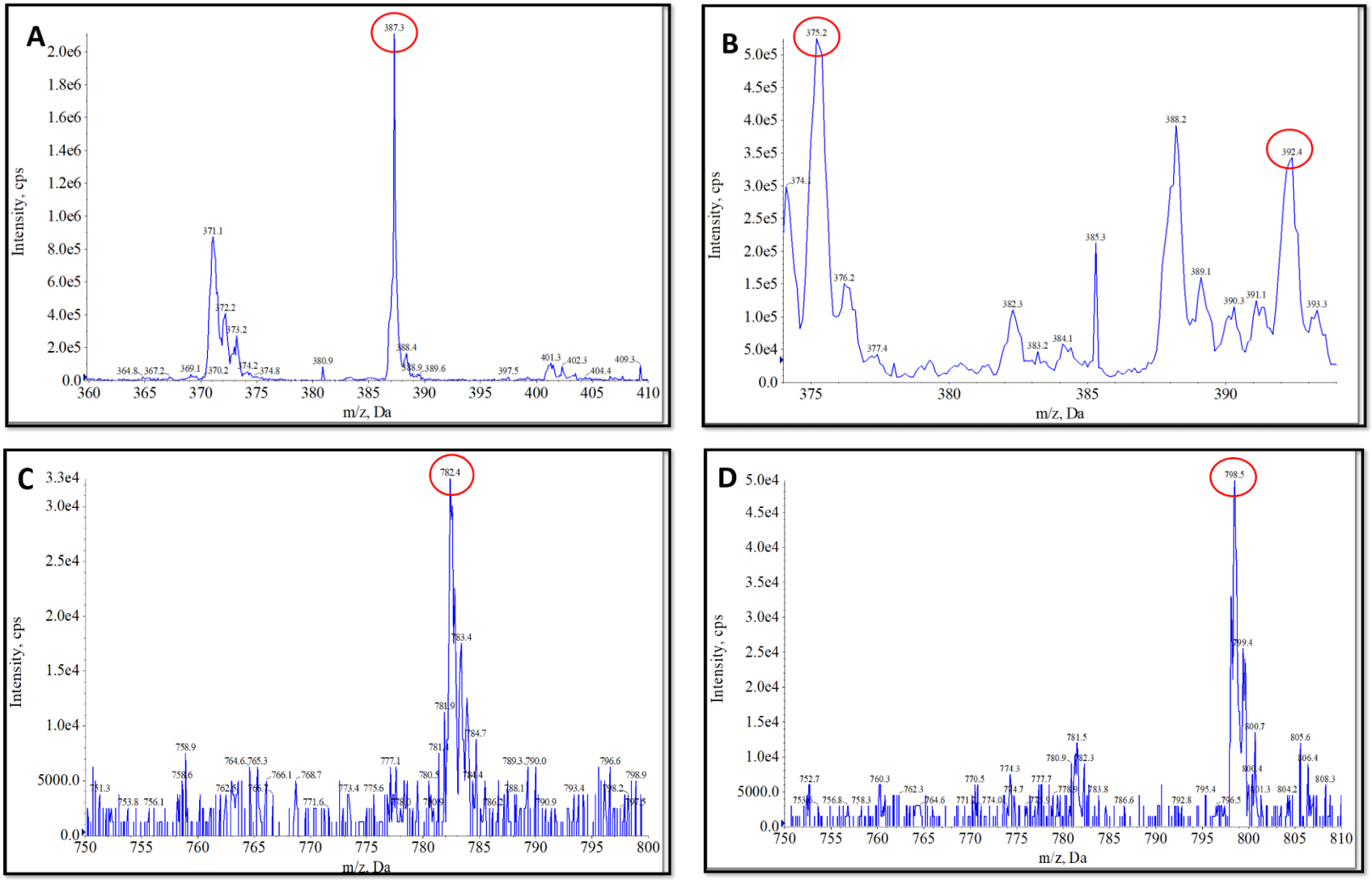


Figure 3-3. scan of underivatized CTS standards in 5 mM ammonium formate in acetonitrile. Bufalin (A), Digitoxigenin (B), Digitoxin (C), Digoxin (D).

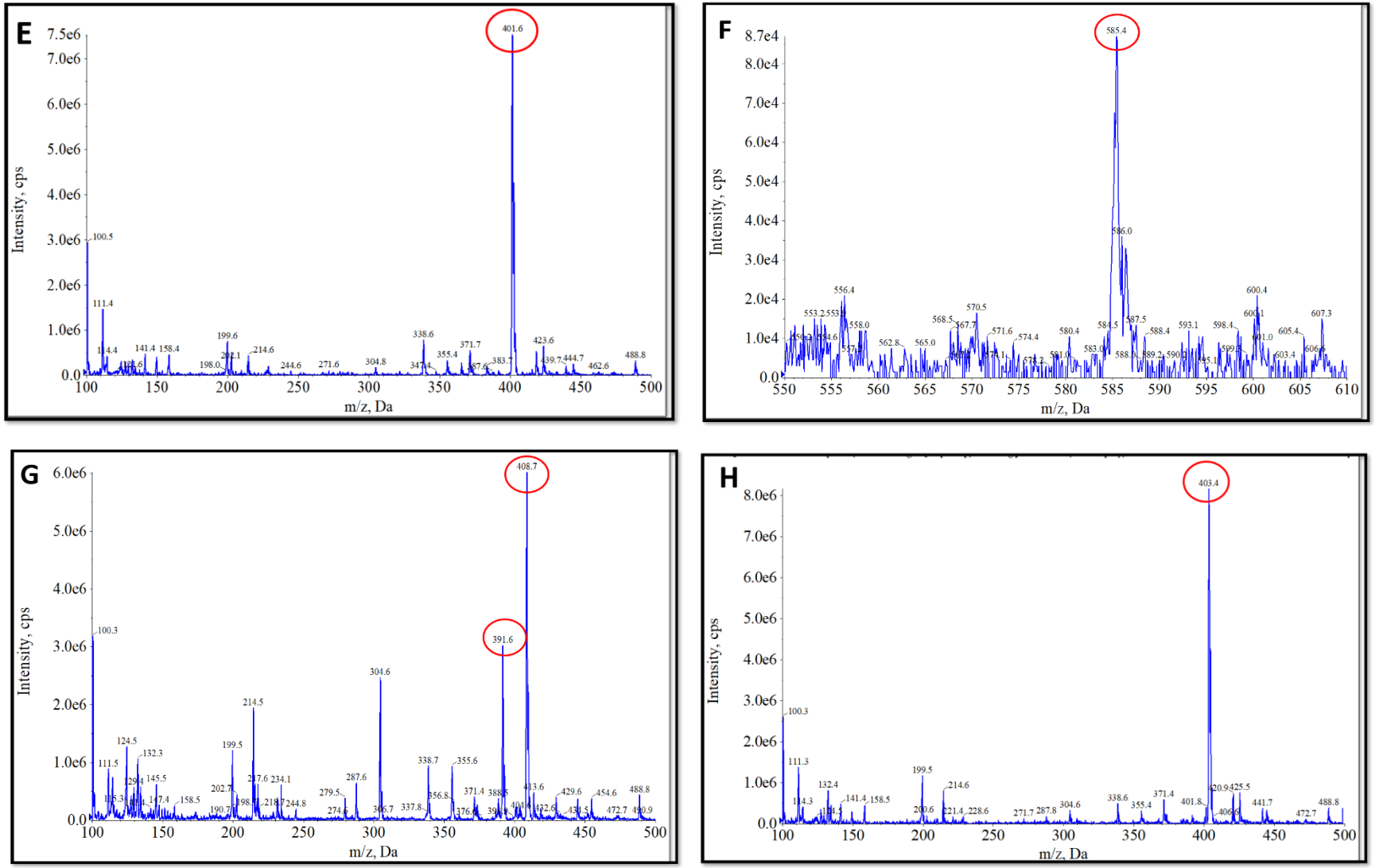


Figure 3-3. (continued). Q1 scan of underivatized CTS standards in 5 mM ammonium formate in acetonitrile. Marinobufagin (E), Ouabain (F), Periplogenin (G), Telocinobufagin (H).

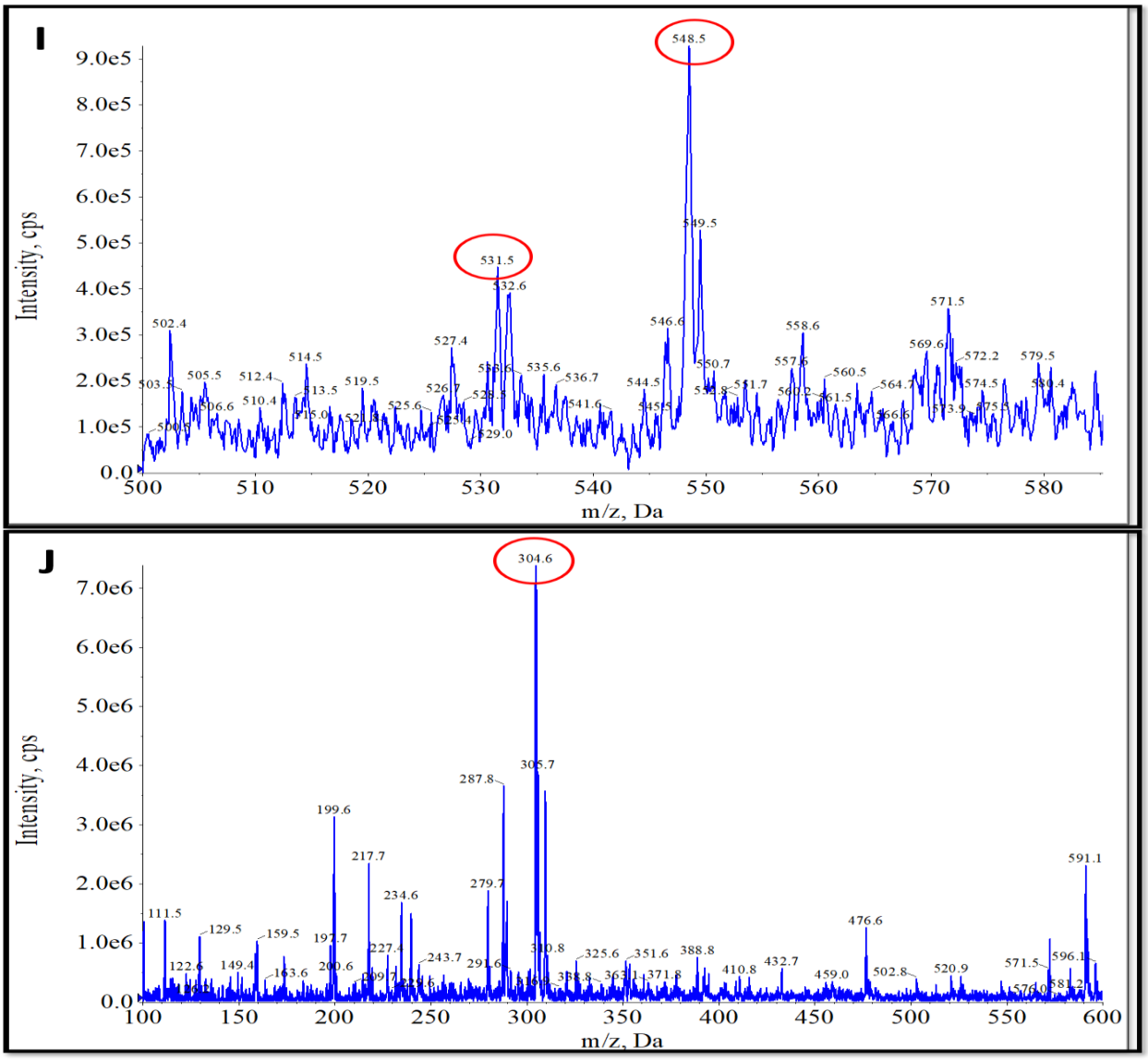


Figure 3-3. (continued). Q1 scan of underivatized CTS standards in 5 mM ammonium formate in acetonitrile. Proscillaridin A before degradation (I), and Proscillaridin A after degradation (J).

Several conditions were tested in order to optimise the LC-MS/MS method. Reverse phase chromatography was used on two columns, Kinetex C18, 100 x 4.6 mm, 2.6 µm column and Kinetex Biphenyl , 100 x 4.6 mm, 2.6 µm column, with mobile phases; A: 0.1% FA in water, B: 0.1% FA in acetonitrile or A: 5 mM ammonium formate in water, B: 5 mM ammonium formate in 90% acetonitrile. The separation was better achieved with the C18 column and mobile phases buffered with ammonium formate.

The final chromatographic method was developed and optimised using a Kinetex C18, 100 x 4.6 mm, 2.6 µm column for the separation of the analytes which were quantified employing the optimised MS/MS detection parameters and MRM transitions. Figure 3-4 illustrates the chromatographic separation of nine different CTS used in this study and the IS (D3-digoxin) which was used to compensate for possible interfering matrix effect or instrument instability. Tables 3-4 and 3-5 show the final optimised parameters and mobile phases gradient for LC-MS/MS.

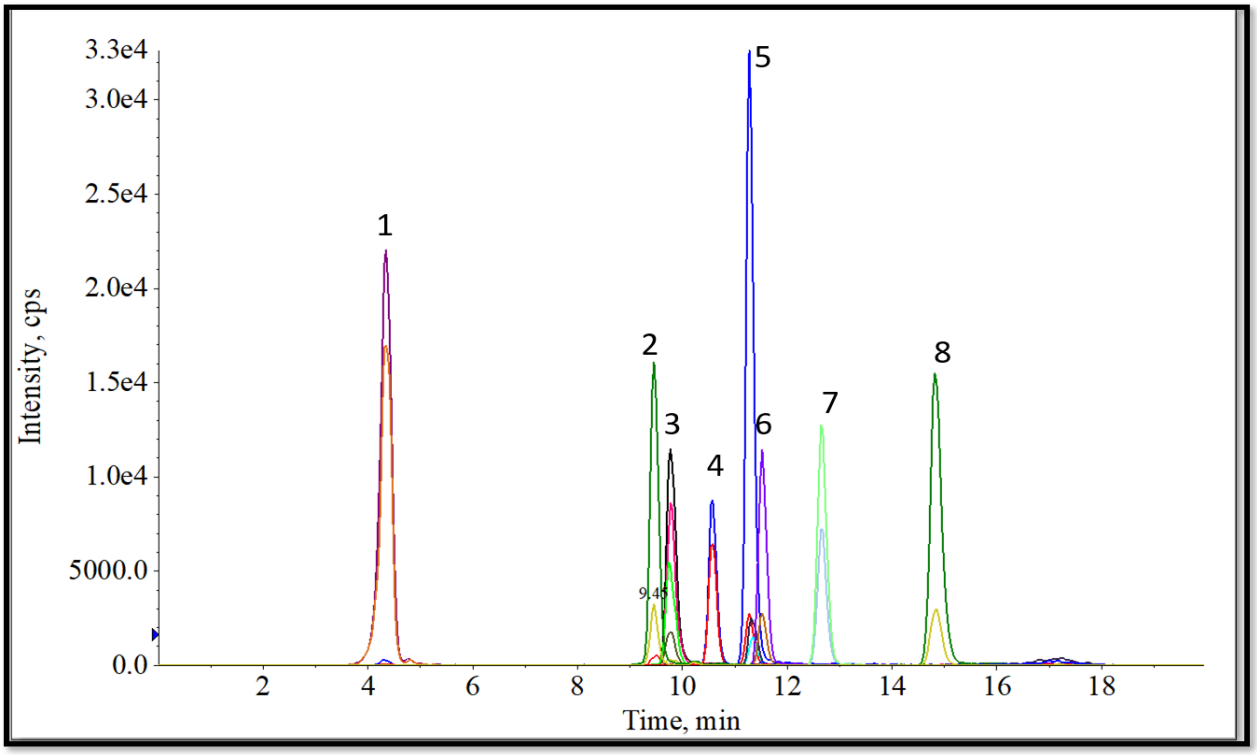


Figure 3-4: Chromatogram obtained from the underivatised CTS standard mix with elution order and concentration: 1 – Ouabain (4.3 min; 40 ng/mL), 2 – Periplogenin (9.4 min; 5 ng/mL), 3 – Digoxin/D3-digoxin (9.9 min; 5/0.5 ng/mL), 4 – Telocinobufagin (10.6 min; 20 ng/mL), 5 – Digitoxigenin/Proscillaridin A (11.3/11.4 min; 20/40 ng/mL), 6 – Marinobufagin (11.6 min; 10 ng/mL), 7 – Bufalin (12.7 min; 2 ng/mL), 8 – Digitoxin (14.9 min; 5 ng/mL).

Table 3-4. Final optimised LC-MS/MS parameters for underivatised CTS standards.

Parameters	Value
Curtain gas	23
Ionspray voltage	4500 V
Source temperature	450°C
Ion source 1	35
Ion source 2	30
Mode	Positive
Mobiles phases	A: 5 mM Ammonium Formate, Water. pH 6.5 B: 5 mM Ammonium Formate, 90% Acetonitrile.
Flow rate	300 µL/min
Run time	23 min
Injection volume	5 µL
Column temperature	40°C

Table 3-5. Final time program for the LC pumps for the optimised chromatographic separation.

Total time (min)	A (%)	B (%)
0.00	90.0	10.0
0.50	90.0	10.0
3.00	75.0	25.0
5.00	70.0	30.0
7.00	65.0	35.0
8.00	60.0	40.0
12.00	55.0	45.0
13.00	45.0	55.0
14.00	40.0	60.0
15.00	15.0	85.0
16.00	10.0	90.0
18.50	10.0	90.0
18.60	90.00	10.00
23.00	90.00	10.00

Different dilution solvents were also tested by reconstituting the dried CTS standard mix in different solvent compositions before LC-MS/MS analysis to determine optimised peak shape, peak symmetry and ESI efficiency. The following solvent mixtures were used; 30% 5 mM ammonium formate: 70% acetonitrile, 5 mM ammonium formate in 60% acetonitrile: 40% water, 0.1% FA in 60% acetonitrile: 40% water, and 60% acetonitrile: 40%water. Both the peak intensities and shapes were optimal using a combination of 60%

acetonitrile: 40% water, except for Ouabain, which gave a better peak shape at acidified conditions. Therefore a 0.1% FA in 30% acetonitrile: 70% water diluent was used for Ouabain only.

Figure 3-4 shows that Proscillaridin A was co-eluting with digitoxigenin (Peak 5) and Figure 3-5 shows the two peaks extracted from the total ion chromatogram. This could have been resolved with further chromatographic optimisation, but the analyte standard was shown to degrade. Figure 3-3 (I and J) shows degradation from 531.5 m/z to 304.6 m/z and due to limited availability of the stock standard, this was not included in method validation. However, because its elution time was already known, its MRM transition parameters were included in the final qualitative study of biological samples.

Periplogenin and digoxin (Figure 3-4; Peak 2 & 3), digitoxigenin and MBG (Figure 3-4; Peak 5 & 6) showed an acceptable separation but eluted very close to each other compared to others. This might be due to their chemical similarities. For instance, digoxin fragments are from its aglycone moiety which is digoxigenin with a chemical formula $C_{23}H_{34}O_5$ and molar mass 390,51 g/mol, and this is an isomer of periplogenin (see Figure 1-3).

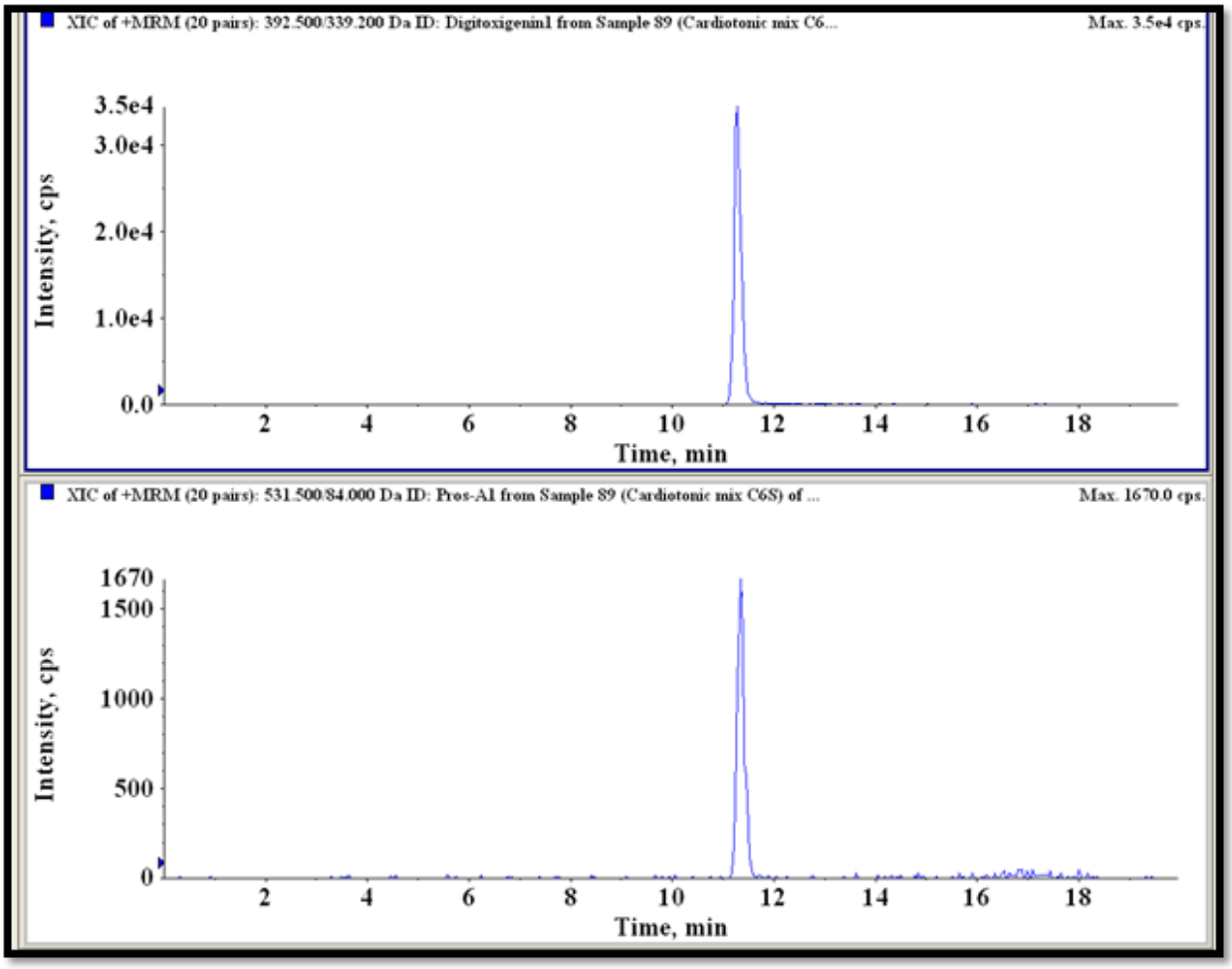


Figure 3-5. Co-elution of digitoxigenin (top) and proscillaridin A (bottom)

The run time for the validated method was 18 min; however, after running faecal samples, the runtime was extended to 23 min (running at 90% organic eluent) due to the persistence of late – eluting lipophilic residues present in the faecal samples, that caused significant carry-over. The extended run-time ensured complete elution of the interfering matrix components.

3.3 Extraction method optimisation

CTS compounds are very similar in structure, but with different chemical properties and exhibit diverse biological responses. They range from polar-hydrophilic compounds such as ouabain, to less-polar-hydrophobic compounds like bufalin, digoxin and MBG. To optimise CTS extraction from biological samples, two methods from previous studies

were compared using six CTS standards. One was used for hydrophobic CTS (Table 3-6, Method 1)[87] and the other for Ouabain, which is hydrophilic (Table 3-6, Method 4)[30]. Methods 2 and 3 are modified Methods of 1 and 4 in which buffers were not used before extraction. The two methods from literature differ in the pH used before and during extraction. Buffer with pH 9 (basic) was used for hydrophobic CTS (Method 1) and pH 2 (acidic) was used for hydrophilic CTS Ouabain (Method 4).

Table 3-6. Different SPE methods used to optimise the extraction of the CTS analyte mix.

SPE steps	Method 1	Method 2	Method 3	Method 4
Dilution solvent before SPE	100 mM CH3COONH4, 10% MeOH in H_2O	25% MeOH in H_2O	25% MeOH in H_2O	0.1% TFA, 10% MeOH in H_2O
Condition	Methanol Water	Methanol Water	Methanol Water	Methanol Water
Equilibrium	10 mM CH3COONH4 in H_2O	10 mM CH3COONH4 in H_2O	0.1% TFA in H_2O	0.1% TFA in H_2O
Wash	10 mM CH3COONH4, 10% MeOH in H_2O	10 mM CH3COONH4, 10% MeOH in H_2O	0.1% TFA, 30% MeOH in H_2O	0.1% TFA, 30% MeOH in H_2O
Elution	Chloroform/IPA 19:1	Chloroform/IPA 19:1	Chloroform/IPA 19:1	Chloroform/IPA 19:1

Buffering samples before SPE could enhance extraction of either hydrophobic or hydrophilic analytes as expected from their chemical properties, however, an unbiased extraction (Methods 2 and 3) during this study found enhanced peak intensities when compared to the original Methods (Figure 3-6). A 30–50 fold increase in signal intensity were observed between Method 1 and the modified Method 2. Similarly, an increase of peak area of between 10–20 fold were found when using Method 3 vs Method 4.

The results showed that all the Methods worked for all the analytes except for ouabain which is more hydrophilic. A small fraction of ouabain was found to elute with the other analytes with the remainder of the ouabain found in the SPE wash fractions (Figure 3-7). Using Method 1, more ouabain eluted in the wash fraction than in the eluent, whereas approximately equal amounts were collected between the wash and the eluent fractions

during Methods 2 and 3. On the other hand using Method 4, more of the ouabain eluted in the eluent SPE fraction, which explains why FA was used in this method for ouabain extraction. Regardless of this finding, a small amount was still lost in the SPE wash fraction. This might be due to the poor solubility of ouabain in chloroform.[11]

Due to the differences in polarity and hydrophobicity of the analytes, Method 2 was chosen since it allowed for complete elution of ouabain in the wash (30% methanol) whilst retaining all other analytes that were collected in the SPE elution fraction resulting in the highest observed analytes intensities compared to the other methods. Figure 3-8 shows the results after final method optimisation.

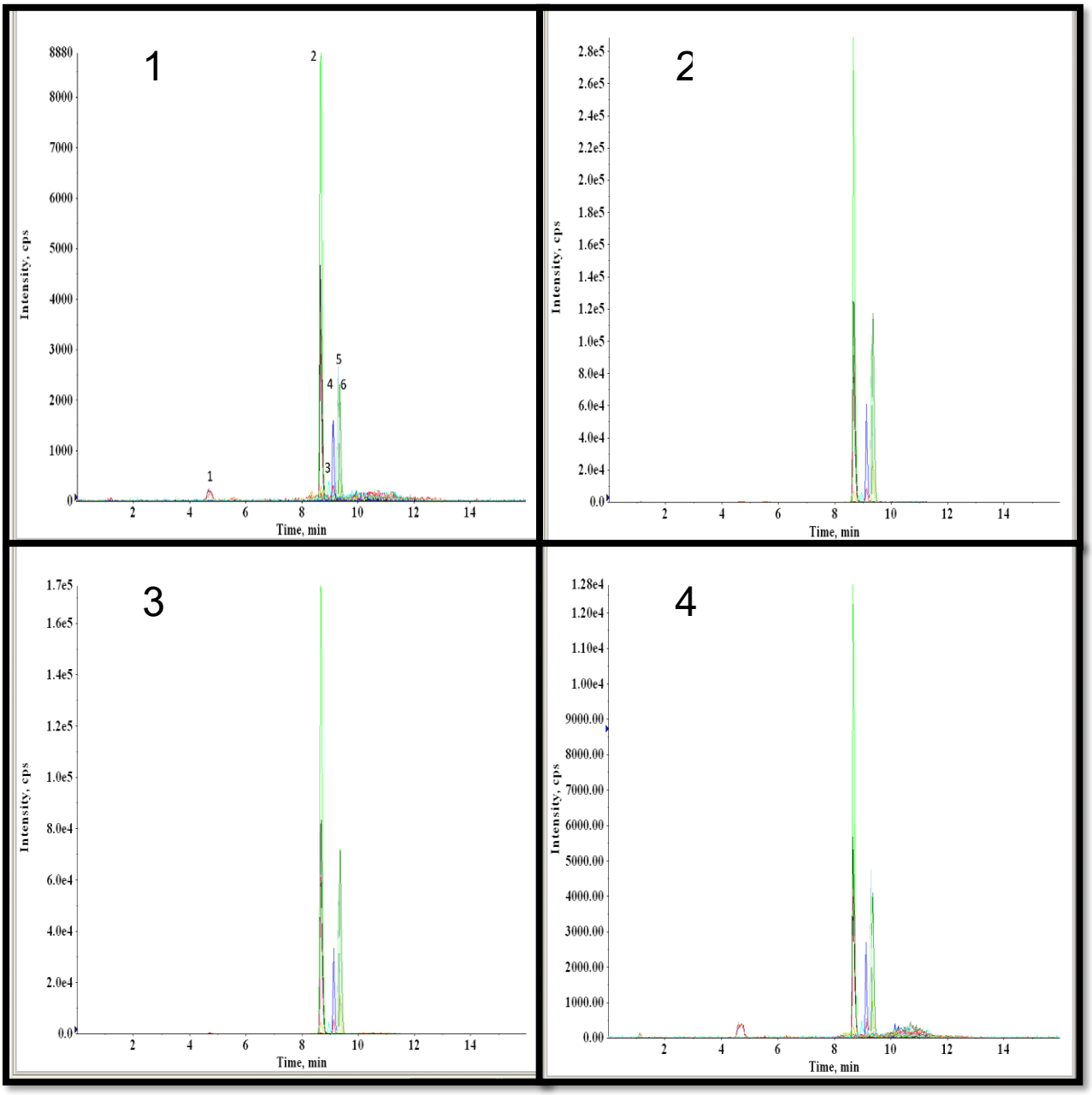


Figure 3-6. The four different SPE methods used for extraction optimisation of the CTS standards. The elution order are as follows: Ouabain (1), Digoxin (2), Proscillaridin A (3), Digitoxigenin (4), Bufalin (5), and Digitoxin (6).

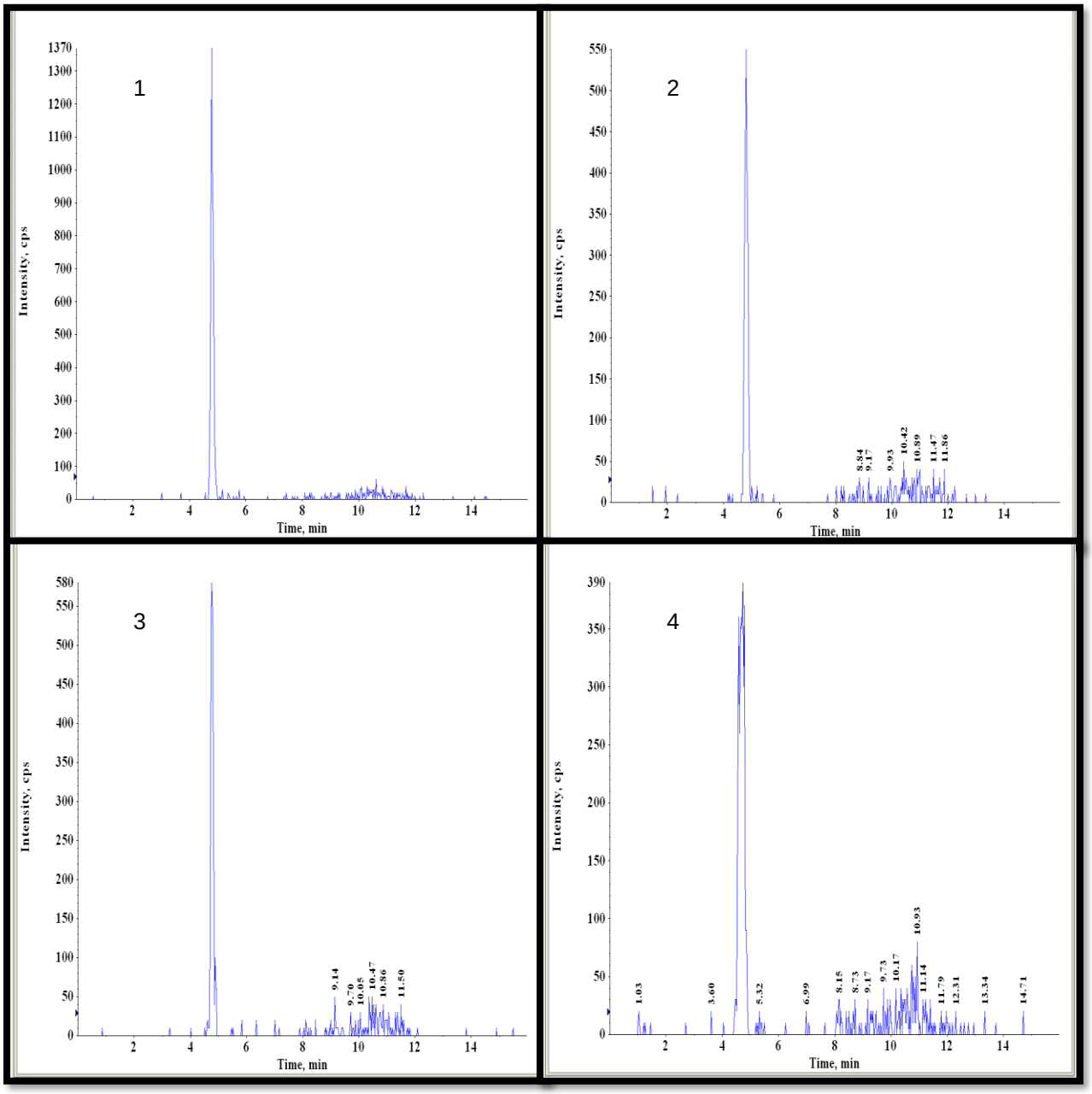


Figure 3-7. Presence of ouabain in the SPE wash fractions between the four different SPE methods used.

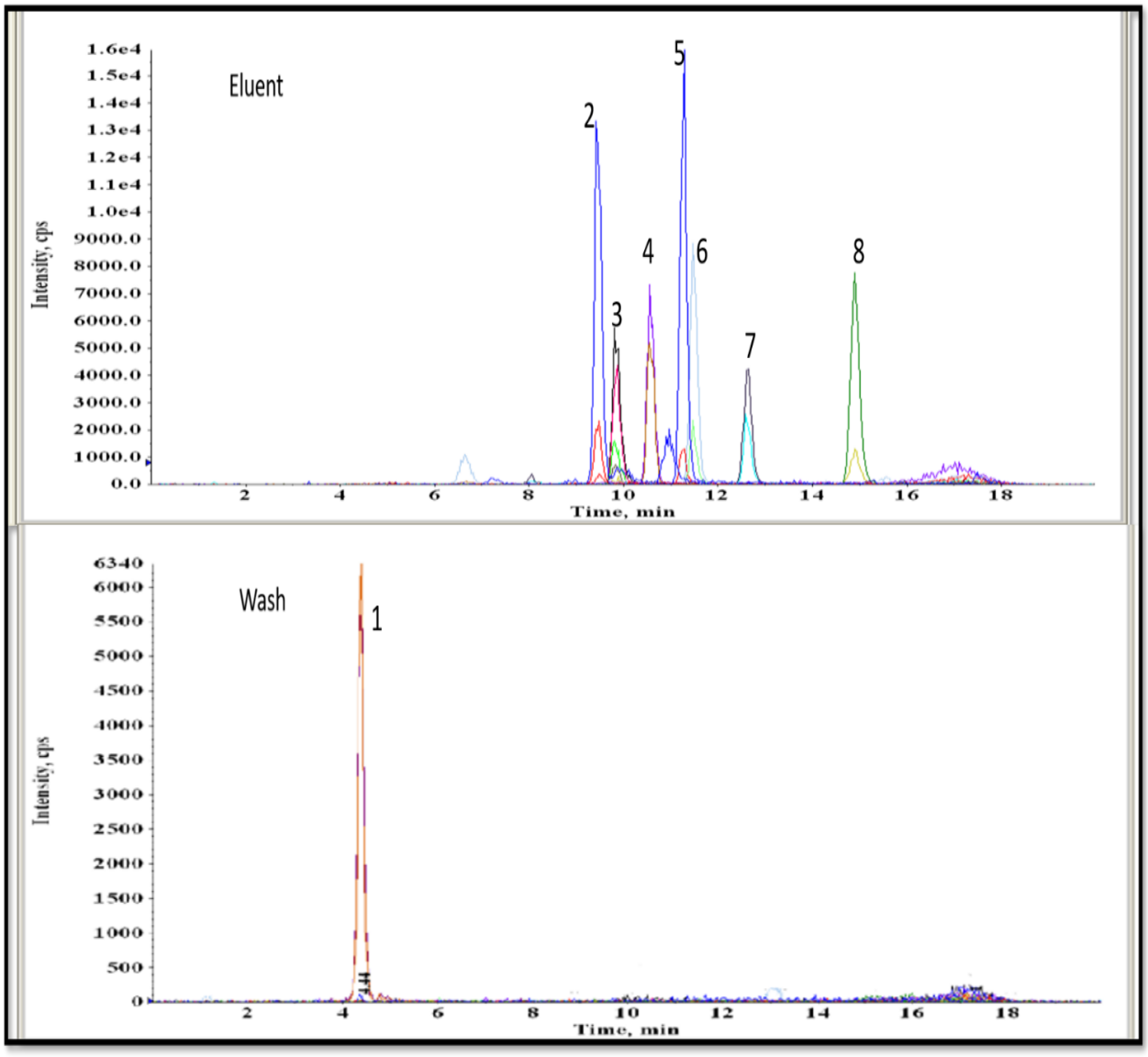


Figure 3-8. Chromatograms obtained after final SPE optimisation. Ouabain (1), Periplogenin (2), Digoxin/D3-digoxin (3), Telocinobufagin (4), Digitoxigenin (5), Marinobufagin (6), Bufalin (7), Digitoxin (8).

Faecal samples were first extracted using liquid/liquid extraction before SPE, because of their solid states. Recovery of this extraction was also tested but only for digoxin and digitoxin which were spiked into the faecal matrix before extraction at different concentrations of 2, 10, and 50 µg/mL and diluted further to 10, 50, and 250 ng/mL before running them on the LC-MS/MS to avoid system saturation. The results are shown in Table 3-7. Digitoxin showed very high recoveries which were out of the normal range.

This is explained by ion enhancement from the matrix effect, as faeces contain many molecules which can interfere with the analytes of interest.[91]

Table 3-7. Digitoxin and Digoxin liquid/liquid extraction recoveries from faeces. Mean ± SD (n=6).

Concentrations (µg/mL)	Digoxin (%)	Digitoxin (%)
2	101.7±4.3	164±10
10	94.75±0.2	155±01
50	83.65±1.8	121±16

3.4 Analytical method validation

Nine CTS were used for this study and eight of them were validated in whole blood and solvent for quantification. The developed method was validated according to criteria set by the ICH. Matrix effects and selectivity of whole blood samples were assessed first, due to the complex matrix as human blood consists of numerous proteins, lipoproteins and many small molecules. Linearity, LOD, LLOQ, recovery, accuracy and intra- and interday precision were assessed using at least 5 points calibration curves for each analyte, on three different days. Reproducibility was assessed on seven consecutive days. Analyte stabilities were also assessed at different temperatures during one month. The concentration ranges used were within the expected biological concentrations of CTS.

3.4.1 Selectivity

In order to evaluate the method selectivity, blank blood samples (with no analyte or surrogate standard added) from five healthy volunteers were analysed to check for peaks that might interfere with the detection of the CTS. Also, negative samples (blank blood samples including surrogate standard) were analysed, to verify the absence of native interfering compounds in the surrogate solution. There were no significant interfering peaks at the retention times of bufalin, digitoxigenin, digitoxin, digoxin, ouabain and TCB. A compound with the same mass and fragment as MBG was found to elute 4.5 min earlier, which means it was more polar than MBG and it did not seem to interfere with the detection of the analyte of interest (Figure 3-9 top). Three peaks with the same mass and fragment as periplogenin were observed; one at the exact same retention time which

caused the analyte's ion enhancement, and two others eluted 1 and 2 minutes later, they might be impurities of the same mass and fragments as periplogenin (Figure 3-9 bottom). This indicates that the method was selective for most the analytes.

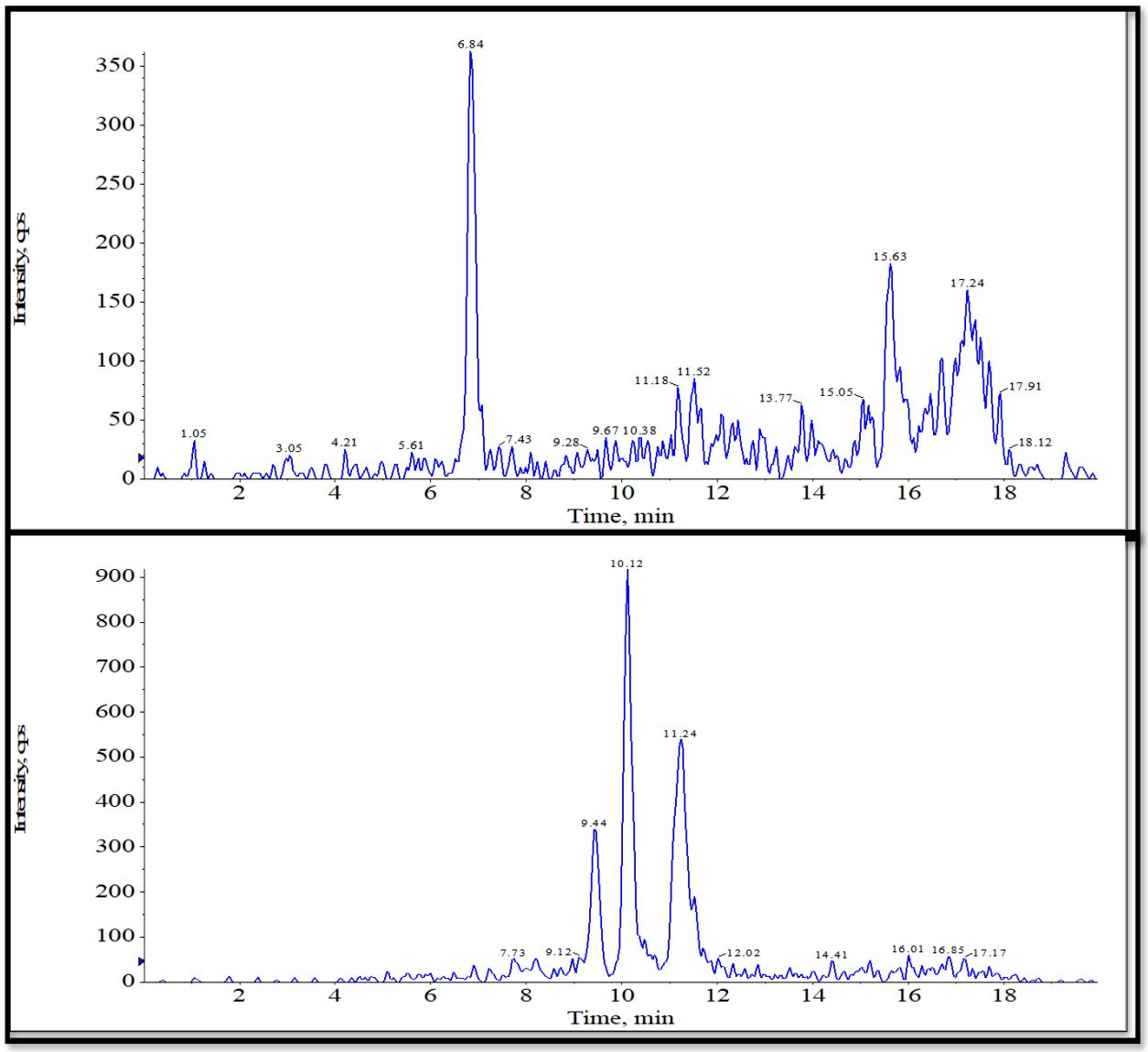


Figure 3-9. Whole blood blank showing a peak (6.8 min) with the same mass and fragment as marinobufagin (expected time 11.5 min) (top figure), and the three peaks (bottom figure) with the same mass and fragment as periplogenin (expected time 9.4 min).

3.4.2 Recovery

The extraction recoveries were very good and ranged from 70% to 102% for all nine analytes. Extraction yields in this study were higher or the same as those suggested in the screening methods already published.[11,24,25,36,87] Matrix effects for all the analytes studied ranged from −35.3% to 9.6%. Digitoxin and ouabain had matrix effects of 4.7 and 9.6 respectively, which means they showed ion suppression due to other molecules in the matrix. Ion suppression for ouabain is explained by the fact that it's being eluted in the SPE wash fraction with all the other unwanted molecules. However, together with digoxin and MBG which had matrix effects of -8 and -9.8 respectively, indicate no significant matrix effect was observed. Contrary for bufalin, digitoxigenin, periplogenin, and TCB, which had significant matrix effects as shown in Table 3-8, causing ion enhancements.

These results suggested that the extraction method employed in this study produced reproducible and very good recoveries for all the analytes and although matrix effects caused ion enhancement for some of the compounds, the quantitative study was not unduly affected.

Table 3-8. Method validation values.

Analyte	Extraction Recovery (%±SD)	CV (%)	Accuracy (%)	Matrix effect (%)	Concentration Range (ng/mL)	LOD (ng/mL)	LLOQ (ng/mL)
Bufalin	102	1.7-17.9	84-120	-35.3	0.1-10	0.02	0.1
Digitoxigenin	77.9±7.9	5.3-19.2	82-113	-18.5	0.5-30	0.2	0.5
Digitoxin	84.8±7.2	0.8-18.1	75-115	4.7	0.2-20	0.05	0.1
Digoxin	83±9.1	2.8-26.3	86-122	-8	0.2-10	0.05	0.1
Marinobufagin	88.5±8.3	2.1-26.7	85-118	-9.8	0.5-20	0.2	0.5
Ouabain	89.2±9.2	2.5-14.7	88-114	9.6	2-40	0.5	2
Periplogenin	84.7±8.6	4.1-17.9	88-120	-31.4	0.1-10	0.01	0.05
Telocinobufagin	81.1±11	0.5-13.9	89-129	-30.7	2-80	0.5	2

3.4.3 Carry over

Carryover was assessed by injecting two consecutive blank solvent samples after each calibration curve's highest concentration. There were no peaks observed in the blanks, indicating that the carryover from residues on the autosampler needle or switching valves were negligible. Also, no carryover from late-eluting residues on the column was observed.

3.4.4 Stability

The percentage peak areas derived from the stored standard solutions in comparison with those of freshly prepared solutions the first day of stability testing, were considered stable if the %RE was no greater than ±15% considering the daily instrument signal variations. Results of the stability testing are summarised in Table 3-9. These results indicate that CTS were stable in solvent for less than a week at room temperature and 4°C except for digitoxin, ouabain and periplogenin which were stable for more than a week. Digitoxin and digoxin were stable for at least a month at both -20°C and -80°C. Ouabain was stable for the same duration but only at -80°C. Periplogenin and TCB were stable for less than a month at either -20°C and -80°C. Bufalin, digitoxigenin and MBG were not stable at all the tested temperatures.

CTS were not stable in whole blood at any of the tested temperatures. Except for digitoxigenin which showed stability at -20°C and -80°C for over a week, and telocinobufagin was also stable for more than a week at all the tested temperatures and for a month at -80°C. They both were more stable in blood than in solvent. According to literature, CTS are stable for at least a month when stored at -20°C which was not the case with the results found in this study.[25,88] This might be because of the different matrix and solvent used from previous studies and more tests are recommended with different donors to confirm this.

Table 3-9. Stability test in reconstituted solvent. Spiked solvent (n=6) and spiked whole blood (n=3) at week 1, 2, 3 and 4.

Analytes	Solvent				Blood			
	Room t°	4°C	-20°C	-80°C	Room t°	4°C	-20°C	-80°C
Bufalin	<1	<1	1	1	<1	<1	<1	<1
Digitoxigenin	<1	<1	1	1	<1	<1	<2	<3
Digitoxin	<3	<3	4	4	<1	<1	<1	<1
Digoxin	<1	<1	4	4	<1	<1	<1	<1
Marinobufagin	<1	<1	1	1	<1	<1	<1	<1
Ouabain	<1	<2	2	4	<1	<1	<1	<1
Periplogenin	<2	<2	3	3	<1	<1	<1	<1
Telocinobufagin	<1	<1	2	2	<2	<2	<2	4

3.4.5 Linearity

Acceptable linearity was obtained within the tested concentration working ranges for all the CTS standards in both solvent and matrix. Figure 3-10 shows calibration curves of the CTS in matrix with the ratio of analyte peak area and surrogate standard plotted against analyte concentration. The weighted (1/x) linear calibration equations are summarised in Table 3-10 and the quantification ranges are listed in Table 3-8. The coefficients of determination (r^2) of the calibration equations were greater than or equal to 0.9900. TCB showed a lower average coefficient of determination (less than 0.9900), which could be explained by the instability of the analyte at room temperature (discussed in Section 3.4.4). Moreover, TCB showed the biggest variation in intra-day precision results compared to the other CTS, which might contribute to its low coefficient of determination.

Table 3-10. Calibration curve equations in matrix. Mean values ± SD.

y=mx+b			
Analyte	**M**	**b**	**r^2**
Bufalin	0.782±1.49	0.308±0.26	0.9900
Digitoxigenin	0.158±0.26	0.187±0.17	0.9917
Digitoxin	0.248±0.25	0.858±0.65	0.9933
Digoxin	0.179±0.19	0.307±0.19	0.9911
Marinobufagin	0.103±0.15	0.219±0.30	0.9945
Ouabain	0.033±0.02	0.039±0.03	0.9946
Periplogenin	0.282±0.15	0.361±0.28	0.9910
Telocinobufagin	0.029±0.14	0.06±0.07	0.9874

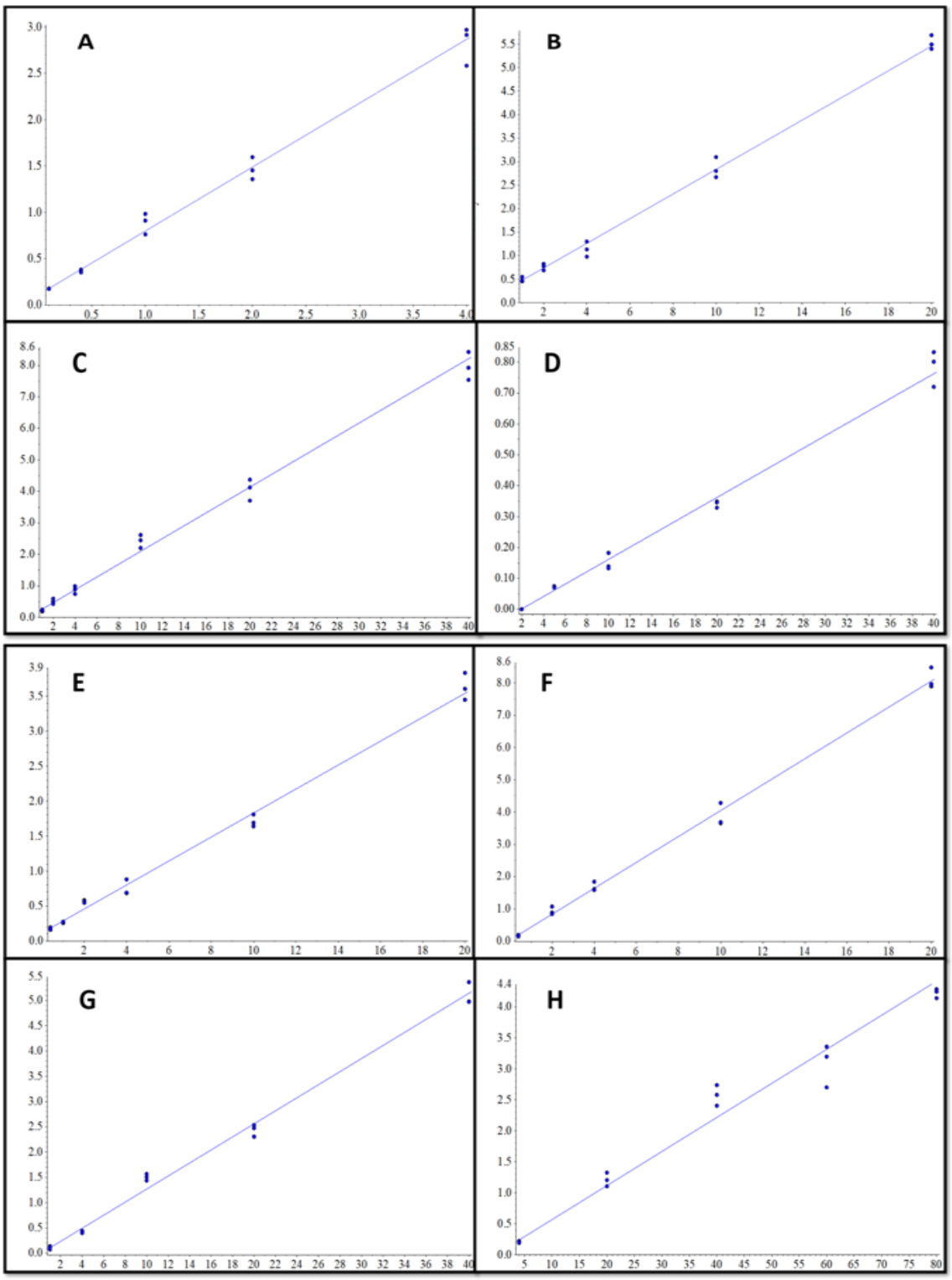


Figure 3-10. CTS standards calibration curves. The ratio of analyte peak area and surrogate standard (Y) plotted against analyte concentration (X). Bufalin, r^2=0.9943 (A), Digitoxin, r^2=0.9951 (B), Digitoxigenin, r^2=0.9939 (C), Ouabain, r^2=0.9955 (D), Digoxin, r^2=0.9923 (E), Periplogenin, r^2=0.9968 (F), Marinobufagin, r^2=0.9947 (G), Telocinobufagin, r^2=0.9909 (H).

3.4.6 Accuracy and Precision

The accuracy and precision values for the majority of the analytes in whole blood were within the acceptable ranges of 85% to 115% (80% to 120% at LLOQ) across the linear analyte ranges and within 20% at the LLOQ concentration for accuracy and with precision values of up to 15% (20% LLOQ), as shown in Table 3-8. Digoxin and digitoxin had an accuracy of 125% and 75% respectively at their LLOQ. Digoxin and MBG had a precision of over 20% at their LLOQ as well, which were out of the ICH guidelines acceptance criteria, but since very low concentrations were used in this study, these data points were included. The 129% accuracy value at LLOQ for TCB on the other hand, was due to its instability. This indicates that the method is precise and accurate for the determination of CTS in whole blood.

3.4.7 Limit of detection and quantitation

LOD and LOQ were estimated from extracted samples spiked with decreasing concentrations of the studied compounds. LOD as low as a few picograms of CTS could be detected with a signal to noise ratio greater than 3. With whole blood samples, the LOD were achieved from 0.02 to 0.5 ng/mL; and LLOQ with a signal to noise ratio of at least 10, from 0.05-2 ng/mL as summarised in Table 3-8. These results were the same as found in previous studies and even better for some compounds, which could be due to the better recovery achieved by the optimised SPE method. [11,36,87]

3.4.8 Reproducibility

The deviations of the peak areas of analytes freshly made at seven consecutive days were between 0.24 and 3.58. The CV values ranged from 8.7% to 18% with the exception of TCB which had a CV of 24.1 (Table 3-11); and as mentioned above, its stability problem is the cause of this high variations. These results indicated that the method was reproducible for all the analytes at seven different days.

Table 3-11. Method reproducibility test standard deviations and coefficient of variation.

Analyte	SD (%)	CV (%)
Bufalin	1.63	14.5
Digitoxigenin	0.32	16.6
Digitoxin	3.58	18.0
Digoxin	1.28	8.7
Marinobufagin	0.84	15.4
Ouabain	0.29	14.0
Periplogenin	3.05	17.2
Telocinobufagin	0.24	24.1

The assay was thoroughly assessed and validated to establish that its performance was fit-for-purpose. It showed enough sensitivity to all the analytes for very low biological concentrations of CTS. It was also selective for most of the analytes for quantification purposes without any interferences. The assay was also accurate and precise for all the analytes. Extraction recoveries were better than most of the extraction used in previous studies. The method was then fit for the analysis of biological samples for detection and quantification of CTS.

3.5 Application of the developed method to cultures and faecal samples

3.5.1 *H. pylori* cultures

The *H. pylori* cultures (n=11) were analysed with the developed LC-MS/MS method. Five CTS (digitoxigenin, digitoxin, digoxin, marinobufagin and periplogenin) were detected in cultures. Digitoxin, digoxin and marinobufagin were detected consistently in all the cultures whether supplemented with cholesterol, salt or both; while digitoxigenin and periplogenin were detected in only some of the samples. MBG showed three different peaks at 9.8, 10.3, and 10.8 min (Figure 3-11), while the standard peak was detected at 11.2 min (discussed in Section 3.5.3). The detected peaks had very low intensities below the LOQ.

This result shows that, both the optimised extraction method and developed analytical method were successfully applied to the analysis of bacterial cultures and showed that *H. pylori* does produce CTS when grown in cultures.

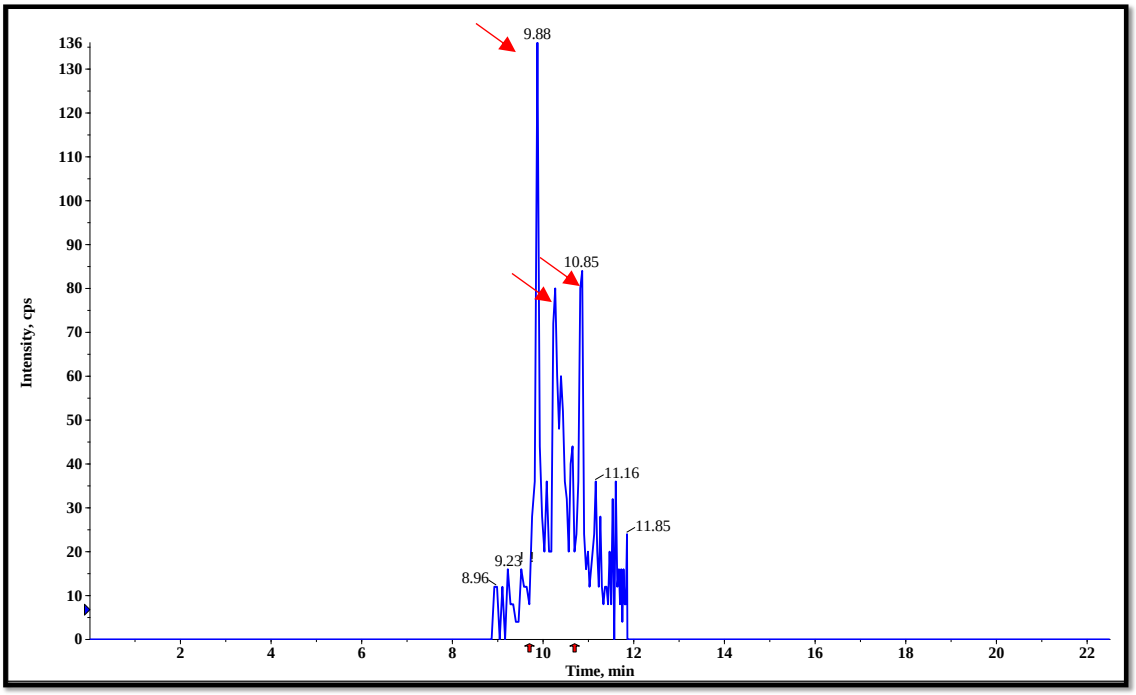


Figure 3-11. MBG detected in *H. pylori* cultures shows three different peaks.

3.5.2 Human faecal samples

a. Liquid/liquid extraction

Faecal samples from a volunteer were subjected to liquid/liquid extraction to extract the more lipophilic CTS using different solvents (MTBE, methanol, IPA/chloroform, and methanol/chloroform (1:2)) prior to SPE. Five endogenous CTS were detected in all the faecal extracts (Figure 3-12). Table 3-12 shows elution times of the endogenous CTS detected in human faecal samples in comparison to different CTS standards made up in solvent confirming the identity of CTS in faecal samples. The developed LC-MS/MS method was then successfully used to human faecal extracts.

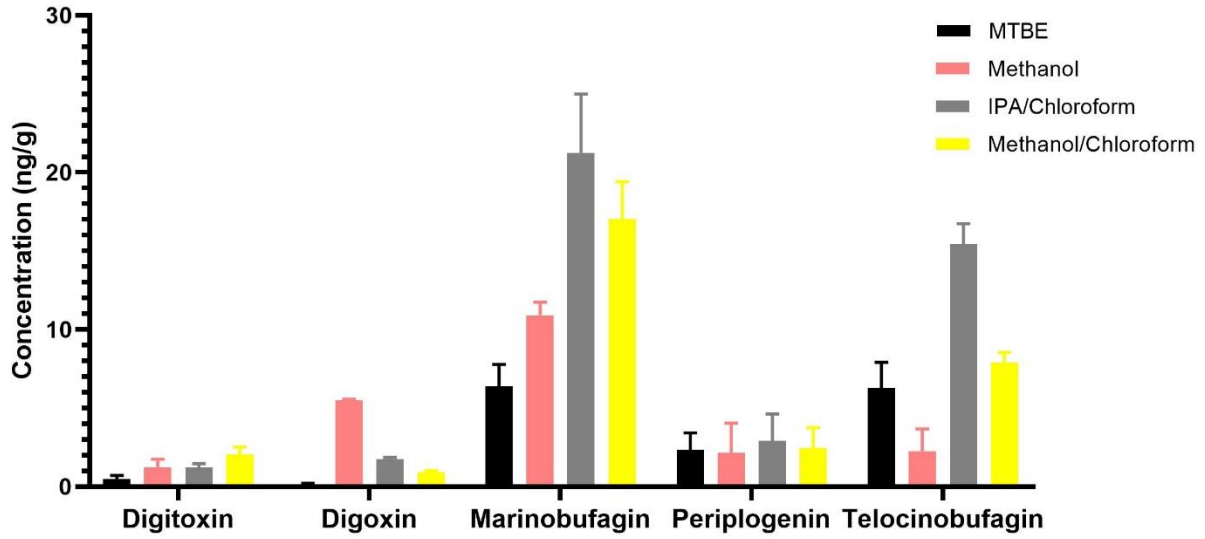


Figure 3-12. CTS extractions from different liquid/liquid extraction solvents. MTBE (n=8); Methanol, IPA/Chloroform & Methanol/Chloroform (n=6).

However, periplogenin and TCB chromatograms showed more than one peak at different elution times (Figures 3-13, red arrows). Periplogenin showed two peaks in all the faecal samples; peak one at 9.1 min and peak two at 9.7 min. The expected time according to the standard was 9.2 min, hence, peak one was considered to be periplogenin. Surprisingly, the first peak had the highest intensity in three out of the four solvents used during LLE, whereas the second peak had the highest intensity in samples extracted with MTBE. Moreover, even the standards in solvent showed a second peak at 9.8 min, 0.6 min after the "correct" peak.

This is consistent with results from the LC-MS/MS method validation done in a whole blood matrix, where two to three peaks were detected for periplogenin in blank blood samples and in blood spiked at very low concentrations (Figure 3-9). Also, periplogenin detected in *H. pylori* cultures showed two peaks. Hence, standard in solvent, in blood, and the analyte detected in cultures and faecal samples showed a second peak eluting 0.6-0.7 min after the expected peak. This peak seemed to have higher intensity than the expected peak in faecal samples extracted with MTBE. Based on these findings, the two peaks are possibly just periplogenin isomers, which is common with CTS.[92,93,94]

TCB also showed three peaks in some of the samples. The expected peak at 10.2 min and the other ones at 9.5 min and 10.7 min. Which might also be isomers, or just impurities with the same mass and fragments as TCB. Hence, only the peak at the expected retention time from the analyte standard made up in solvent was considered.

The difference between concentrations of the CTS analytes detected from different extraction mixtures were statistically significant for digoxin, digitoxin, and TCB. With MTBE extraction having the lowest concentrations. Digoxin and digitoxin (Glycosides) had the highest concentrations when methanol was used as the extraction solvent, and TCB (aglycone) had the highest concentrations with solvent mixtures which contain chloroform. Results from the statistical analysis are shown in Table 3-13.

Table 3-12. CTS elution times (minutes) in spiked solvent and human faecal samples (mean ± SD).

	Digitoxin	Digoxin	Marinobufagin	Periplogenin	Telocinobufagin
Analyte standard	14.8	9.5	11.1	9.2	10.2
Sample	14.8 ± 0.04	9.5 ± 0.02	11.1 ± 0.05	9.1 ± 0.04	10.2 ± 0.14

Table 3-13. Statistical differences of CTS concentrations from different extraction solvents. Statistical significance (**p≤0.01, ***P≤0.001)

	MeOH vs MTBE	IPA/CHCl3 vs MTBE	MeOH/CHCl3 vs MTBE	MeOH vs IPA/CHCl3	MeOH vs MeOH/CHCl3	IPA/CHCl3 vs MeOH/CHCl3
Digitoxin	**	***	***	no	no	no
Digoxin	***	**	**	***	***	no
Marinobufagin	no	No	**	no	no	no
Periplogenin	no	No	no	no	no	no
Telocinobufagin	no	**	***	no	***	***

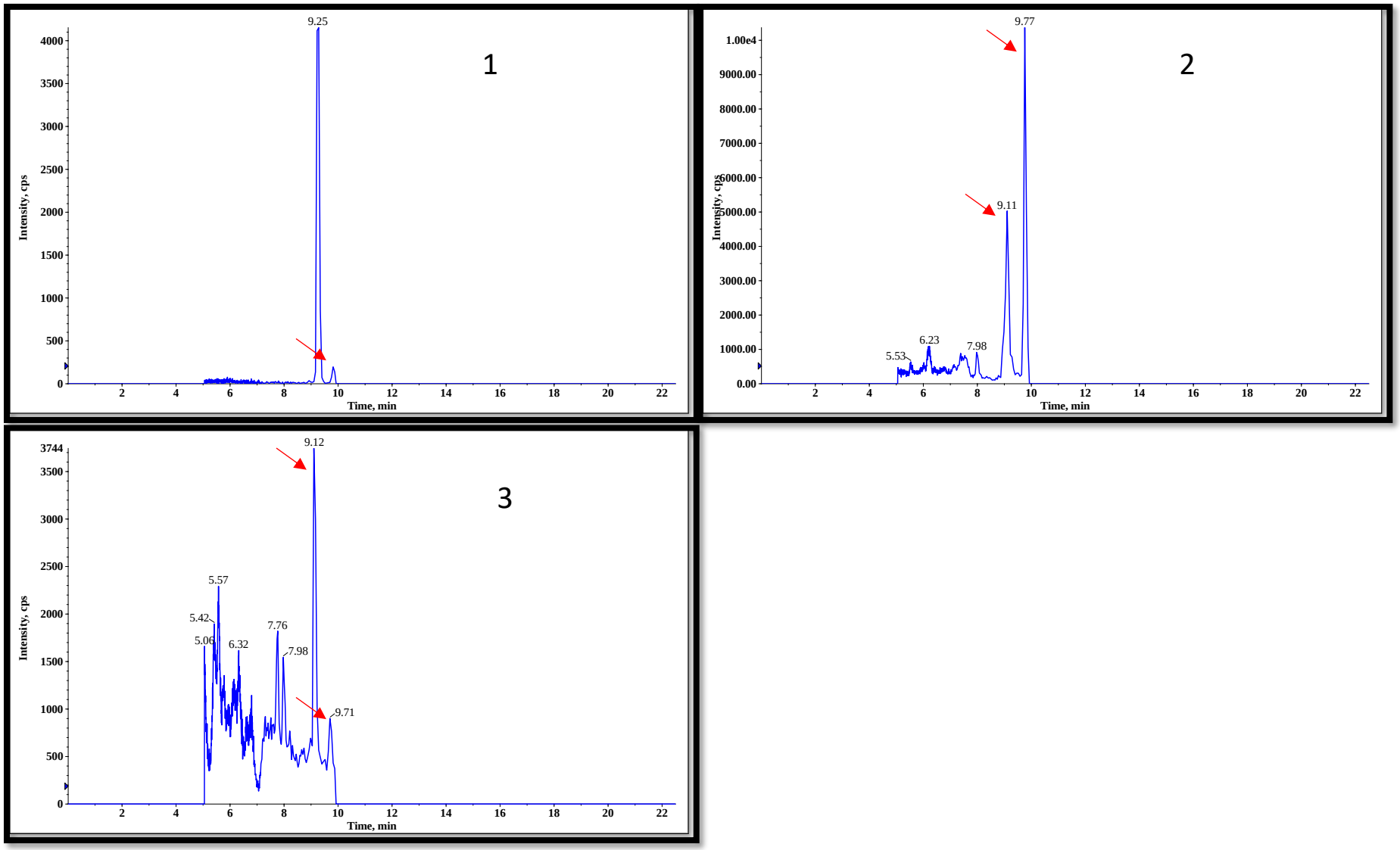


Figure 3-13. Periplogenin chromatograms. Standard in solvent (1), MTBE faecal extraction (2), and methanol faecal extraction (3).

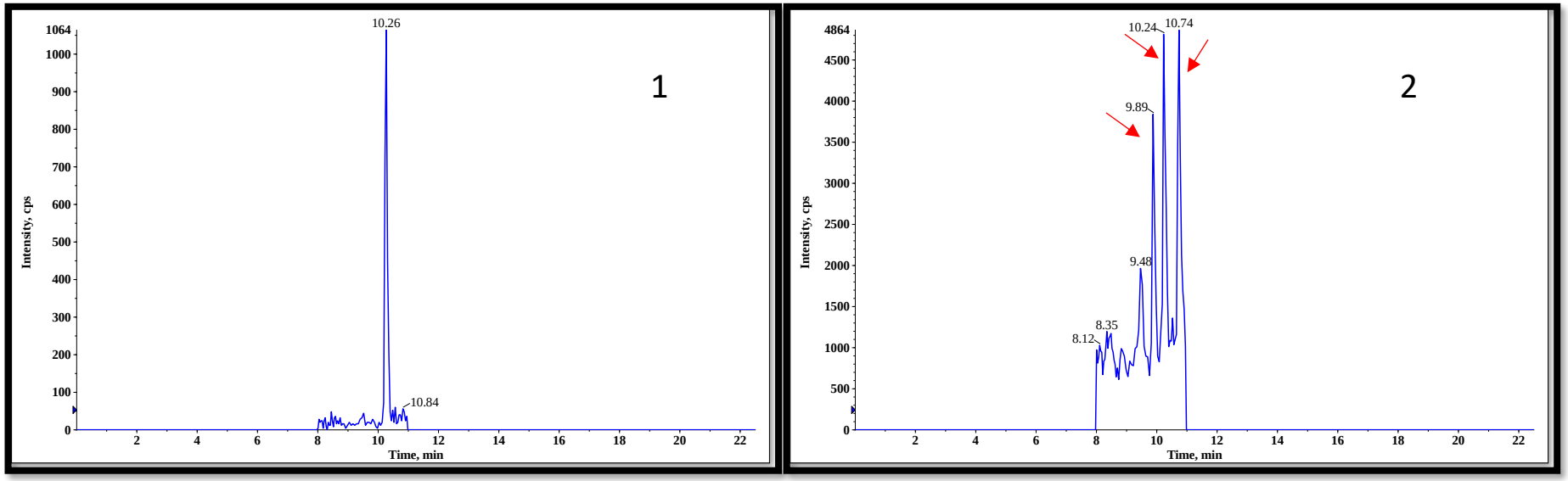


Figure 3-13 (continued). Telocinobufagin chromatograms. Standard in solvent (1) and faecal sample methanol extraction (2).

b. Samples from hypertensive patients

Human faecal samples from hypertensive volunteers (n=8) with unknown *H. pylori* status were analysed for the detection and quantitation of different CTS using the developed LC-MS/MS method after LLE and SPE sample clean up. The results are shown in Table 3.14 below. Only 3 CTS out of 9 were detected in hypertensive patients' samples.

Table 3-14. Detected CTS (ng/g) in faecal samples from hypertensive patients.

Subject number	Digitoxigenin	Marinobufagenin	Periplogenin
1			
2			0.9
3		6.6	7.8
4			15.7
5		2.3	
6		3.5	
7		3.0	7.9
8	1.4		

Total of three CTS were detected in hypertensive faecal samples, with glycosides (digitoxin and digoxin) having lower concentrations than the aglycones (MBG, periplogenin and TCB). Many reasons can contribute to this absence to very low concentrations of the glycosides.

According to literature, cardenolides are absorbed easily in the GIT, with ±70% absorption rate for the glycosides from the duodenum into the portal vein. Bufadienolides on the other hand have lower absorption rates and all CTS are absorbed better from the duodenum comparing to the stomach. They go through entero-hepatic circulation and after their metabolism which happens mainly in the liver, they are excreted through biliary and urinary excretions as either metabolites or unaltered.[32,33,35,40,68]

Digitoxin, digoxin and their aglycones are excreted mostly in the bile (10%-30% of the total enterally absorbed concentrations), ouabain's excretion is done mostly in the urine with only ±5% in the bile.[32,35,40] However, cardenolides are excreted mostly in the bile as metabolites rather than unchanged. Of the total CTS concentrations excreted in both bile

and urine, unchanged digoxin in bile accounts for only 5-8% and digitoxin 4-13%. Digitoxigenin is excreted only as metabolites.[34,35]

The absence or very low concentrations of digitoxin, digoxin, and digitoxigenin might be due to the fact that they are mostly excreted as metabolites in bile. Moreover, although they are very well absorbed in the GIT, they go through metabolism, first in the stomach by acid hydrolysis, and then hepatic first pass effect.[34] In addition to that, digoxin was shown to be metabolised in the GIT by gut microbiota specifically *eubacterium lentum*..[37,38,39] All these might then cause a decrease in concentrations of the unchanged molecules absorbed, and excreted.

Bufadienolides MBG and TCB were detected in higher concentrations compared to the others, despite going through stomach metabolism and first pass effect.[67] They also go through biotransformation by gut microbiota including *Comamonas testosterone, Bacillus sp, Mucor polymorphosporus, Pseudomonas aeruginosa* etc.[3,95,96] They are shown to be metabolised in the liver and their metabolites are excreted in urine and bile.

Because they are reported to be poorly absorbed in the GIT, higher bufadienolide concentrations could imply that they are produced in the gut, and the non-absorbed fraction excreted in faeces, or they might be excreted into the gut via the bile as MBG and its reduced form TCB, which are present at higher concentrations in hypertensive patients,[4,7] but these concepts would need to be investigated further.

3.5.3 Rats faecal samples

Rat faecal samples were analysed for detection and quantitation of CTS. Table 3-15 shows elution times of different CTS standards in solvent, and CTS detected from rats' faecal samples extracted with methanol, and then re-extracted with butanol by liquid/liquid extraction before SPE.

Table 3-15. CTS elution times (minutes) in spiked solvent and rats' faecal samples (mean ± SD). Butanol and methanol liquid/liquid extraction.

	Analyte standard	Butanol extract	Methanol extract
Bufalin	12.5	12.6 ± 0.08	12.9 ± 0.08
Digitoxigenin	11.1	10.9 ± 0.05	11.1 ± 0.05
Digitoxin	14.7	14.7 ± 0.04	14.7 ± 0.04
Digoxin	9.5	9.5 ± 0.04	9.5 ± 0.04
Marinobufagin	11.2	9.7 ± 0.05	10.0 ± 0.11
Telocinobufagin	10.2	10.1 ± 0.16	10.1 ± 0.16

CTS aglycones (except TCB) extracted with methanol eluted 0.2-0.3 min later comparing to those re-extracted with butanol. This might be due to matrix effect as the two solvents can also extract many other different lipophilic compounds from the same sample. The elution times for glycosides from samples match the standards times, with slight difference for aglycones. MBG, contrary to human faecal samples, eluted 1.2-1.5 min earlier than the expected time (Figure 3.14), just like in cultures where it eluted 1.4 earlier and with three different peaks (Figure 3-11). This makes it almost impossible to confirm whether it's MBG which had a time shifting due to matrix effect, or they are just its isomers.

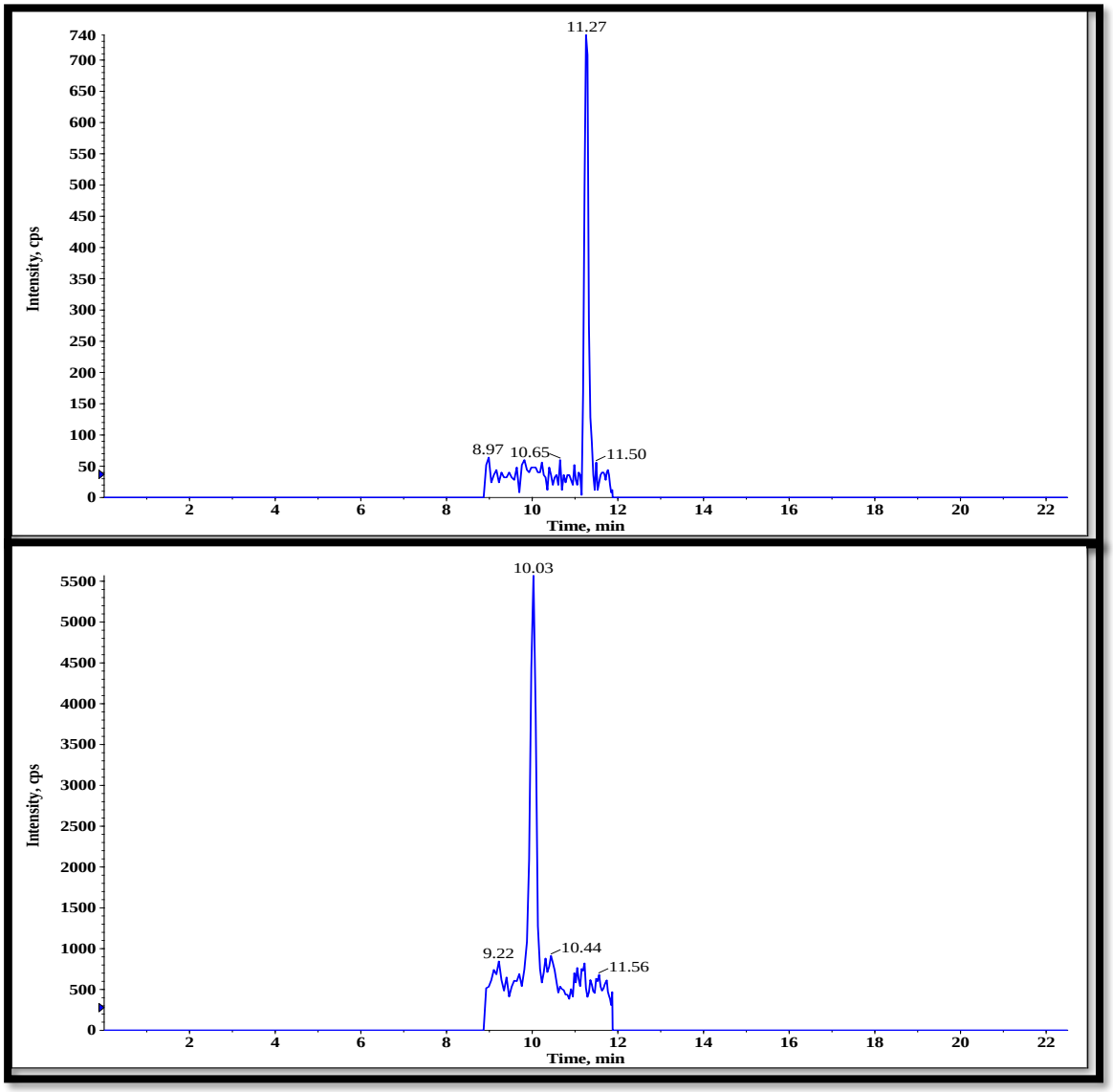


Figure 3-14. Marinobufagin chromatograms. Standard in solvent (top) and rat's faecal sample methanol extraction (bottom).

Figure 3-15 shows different CTS detected in faecal samples of different rat strains. The concentrations were below the LOQ except for digitoxigenin. Therefore, peak areas normalised to the surrogate standard were used to compare different rat strains CTS levels. Table 3-16 shows the differences in intensities of different CTS detected in each rat strain.

Glycosides (Digitoxin and digoxin) just as in human's faecal samples had the lowest intensities. The fact that they are excreted mostly as metabolites, might be one reason.[34,37] Digoxin showed a significant difference between DSS group treated with salt and the two other DSS groups, while the exercise seemed to lower the CTS intensities in SHR rats. Digitoxin showed the same pattern as digoxin, but the differences between groups were not statistically significant.

Digitoxigenin showed almost the same pattern as the other cardenolides (Digoxin and digitoxin), as its SHR group treated with exercise which, contrary to the others had higher intensity than its control group. As discussed in Section 3.1.2.b, digitoxigenin was shown to be excreted in bile as metabolites only.[32] However, it is detected in both human and rat faecal samples at high concentrations. It might be the aglycone metabolite of digitoxin or, it is the unchanged parent product which could now be detected because of the sensitive MS/MS method developed which has many advantages such as high sensitivity and specificity, elimination of interferences, targeted detection, high throughput, comparing to colorimetric methods used in previous studies.

TCB was not detected in Wistar or the SHR control group, and its DSS control group had the highest intensity for bufalin instead of the DSS treated with salt group, like seen with all the other CTS. MBG, on the other hand showed almost the same trend as digitoxin but with significant differences between different groups, and with the exception of the DSS group treated with green diet which had a higher intensity than its control group.

These results support the known facts about CTS and hypertension, where the DSS rat strains which represent the salt sensitive hypertensive subjects showed an increase in intensities of CTS in the salt treated group. This group showed the highest intensity of all the CTS detected except for bufalin. A salt free diet was shown to decrease CTS

concentrations in previous studies, which was well represented here by the green diet group, which showed lower level of CTS comparing to those treated with salt, and the control group with the exception of MBG.[19,50]

Exercise proved to decrease blood pressure in human and rats.[97,98] SHR group treated with exercise had no significant difference with the control group, but had lower intensities of digitoxin, digoxin and MBG. The Wistar group, a normotensive rat strain without *H. pylori* infections, also had the same CTS detected except TCB; and even showed higher intensities of some of CTS than SHR treated with exercise and DSS treated with green diet.

The current LC-MS/MS developed method was used successfully to detect and quantify CTS in different physiological conditions in rats. Although these results reflect the relationship between CTS and hypertension, where DSS with the highest systolic blood pressure (Table 3-16) also showed higher CTS levels, there was no conclusive evidence that *H. pylori* increases specific CTS levels sufficiently to promote hypertension in different rat strains. SHR with more *Helicobacter species* (3 vs 2 in DSS and Wistar) a higher abundance (15% vs 5% in DSS and Wistar) thereof, higher concentrations of CTS would have been expected, which was not shown in this study. These data are unable to provide an explanation for *Helicobacter spp*. in hypertension.

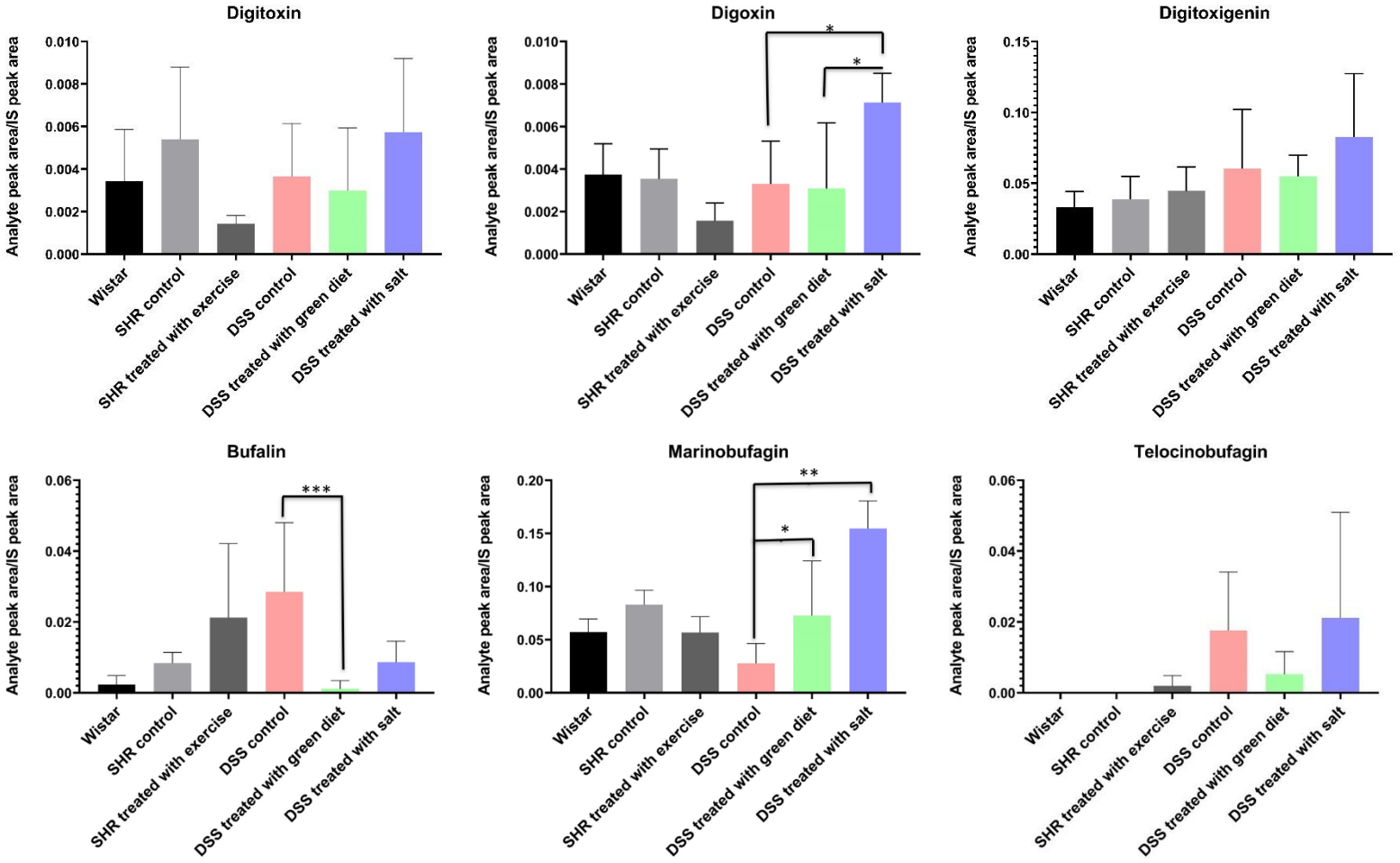


Figure 3-15. Comparative CTS detected in different rats' faecal samples. Note that Y axes have different scales depending on the intensities of each CTS detected. Wistar & SHR control (n=9); SHR treated with exercise & DSS treated with salt (n=6); DSS control (n=18); DSS treated with green diet (n=15). Statistical significance (*p≤0.05, **p≤0.01, ***p≤0.001).

Table 3-16. Difference in intensities of different CTS detected in each rat strain. Systolic blood pressure ranges (mm Hg). (n/d= not detected. += the lowest intensity detected, ++++++= the highest intensity detected)

	Wistar (141-167 mm Hg)	SHR control (197-220 mm Hg)	SHR treated with exercise (197-220 mm Hg)	DSS control (226-250 mm Hg)	DSS treated with green diet (226-250 mm Hg)	DSS treated with salt (226-250 mm Hg)
Bufalin	+	+++	++	+++	+	+++
Digitoxigenin	+++	++++	+++	++++	++++	+++++
Digitoxin	++	++	+	+	++	+
Digoxin	++	+	+	+	++	++
Marinobufagin	++++	+++++	++++	+++	+++++	++++++
Ouabain	n/d	n/d	n/d	n/d	n/d	n/d
Periplogenin	n/d	n/d	n/d	n/d	n/d	n/d
Proscillaridin A	n/d	n/d	n/d	n/d	n/d	n/d
Telocinobufagin	n/d	n/d	+	++	+++	++++

Chapter 4. Conclusion and recommendations

4.1 Conclusion

The purpose of this study was to develop a sensitive method for the detection and quantification of the very low concentrations of CTS in biological samples. Derivatisation was initially used in order to increase the sensitivity to quantitate the very low CTS concentrations. Due to problems encountered such as, multiple derivatives from single compounds, incomplete derivatisation, the instability of the derivatives, and the formation of alternative derivatives affecting the reproducibility of the method; the derivatisation method could not be used for the study purpose. An alternative LC-MS/MS method was developed and validated for the underivatised CTS with sufficient sensitivity to detect expected concentrations of these CTS in biological samples. Due to this, blood samples from hypertensive patients could not be analysed as the expected concentrations in blood are lower than expected and could not be detected with an underivatised cardiotonic steroid LC-MS/MS method, probably due to CTS binding to red blood cells and to certain proteins because of their high lipophilicity.

The LC-MS/MS method was validated in positive ESI mode, which showed higher sensitivity and stability for each of the compounds than negative ionisation. The monoprotonated ions $[M+H]^+$ were most abundant for the bufadienolides and the cardenolide ouabain, while other cardenolides produced stable ammonium adduct ions $[M+NH_4]^+$. Chromatographic separation was achieved on a Kinetex C18, 100 x 4.6 mm, 2.6 µm column, with a 23 min run. The final mobile phase conditions were; A: 5 mM ammonium formate in water and B: 5 mM ammonium formate in 90% acetonitrile. Sufficient resolution for nine tested CTS was achieved with an optimised gradient elution method.

The difference in polarity and hydrophobicity of different CTS made it challenging to extract all the CTS using a single generic sample preparation method. Thus, a reversed phase SPE method was optimised where almost all the CTS were eluted with chloroform/IPA 19:1, ouabain was an exception which eluted in the wash fraction (30%

methanol) due to its higher hydrophilicity. Moreover, ouabain was reconstituted with acetonitrile:water (30:70) buffered with 0.1% FA in which it showed optimal peak shape, peak symmetry and ESI efficiency. The eluted cardiotonic fraction on the other hand was reconstituted with acetonitrile:water (60:40).

The optimised extraction method in this study allowed recoveries of between 70% and 100% for all the analytes; with no significant matrix effects for digitoxin, digoxin, MBG and ouabain. Bufalin, digitoxigenin, periplogenin, and TCB showed significant ion enhancement in whole blood samples but this did not seem to affect the quantification of these analytes. Matrix effects are common with ESI sources; however, this can be better avoided with effective extraction or analytical method optimisation.

The complete assay was validated in whole blood because, the primary biological sample which will be used to compare CTS concentrations in hypertensive patients with and without *H. pylori* infections, is blood. The method showed very limited carry over, good selectivity and high sensitivity with minimal interferences for most of the analytes during quantification. LOD from extracted spiked blood samples was estimated to be between 0.02-0.5 ng/mL; and the LLOQ between 0.05-2 ng/mL, which is within the expected biological blood concentrations, and it shows that the developed method was sensitive enough for detection and quantification of CTS in blood samples.

All CTS standards showed acceptable linearity within the tested working concentration ranges which were within the *in vivo* concentrations. The accuracy and precision values for the majority of the analytes were also within the acceptable ranges; with the exception of digoxin, digitoxin, MBG and TCB, which had some values out of the normal ranges, but this was explained by the very low concentrations used in this study. The stability test showed that CTS standards digitoxin, digoxin and ouabain were stable in solvent for a whole month at both -20°C and -80°C. Periplogenin and TCB were stable for less than a month, and bufalin, digitoxigenin and MBG were not stable under the same storage conditions. In contrast, in whole blood only digitoxigenin and TCB were stable in whole blood for over a week at -20°C and -80°C contrary to the results in solvent. Finally, the method was reproducible for all the analytes over seven consecutive days.

TCB was the most challenging standard to achieve the validation. It had a CV value of 24.1% for reproducibility, 129% of accuracy at its LLOQ, its average coefficient of determination was less than 0.9900, and showed the biggest variation in intra-day precision results compared to the other CTS standards. The instability of this compound at room temperature potentially contributed to the large variability during longer batch run times where triplicate injections were performed. The extended time that samples needed to stay in the autosampler at room temperature could have contributed to this instability. With a low temperature controlled autosampler this could be improved. The developed method was successfully applied to *in vitro H. pylori* cultures and faecal samples from hypertensive humans and rats. Although CTS were detected, they were at very low concentrations. CTS cardenolides were detected in the *H. pylori* cultures where salt stress or excessive cholesterol were present, showing that *H. pylori* do produce CTS. CTS were also detected in faeces unchanged with exception of ouabain and proscillaridin A which were not detected at all. This confirms that CTS can be detected unchanged in faeces.

The low concentrations of CTS were attributed to the fact that they are not only excreted in faeces, but also excreted as metabolites when excreted in faeces. Moreover, the CTS undergo metabolism in the stomach and GIT by microorganisms and acid hydrolysis, and also demonstrate hepatic first pass effects which decrease their parent compound concentrations before distribution and excretion. Periplogenin and TCB show other peaks which were hypothesised to be their isomers as literature also confirms the existence of CTS isomers.

To conclude, the extraction and analytical methods developed in this study were able to extract, detect and quantify nine different CTS in a single LC-MS/MS run. Eight CTS were successfully validated in whole blood at the low concentrations expected *in vivo* but could not quantitate human plasma samples. *H. pylori* culture conditioned media samples were analysed and affirmed the hypothesis of the study by showing that *H. pylori* can produce CTS in *vitro*. Faecal samples also showed CTS production *in vivo*, but there was no evidence on the effect of *H. pylori* on CTS plasma levels in different conditions.

4.2 Recommendations

For the continuation of this project, there are a few considerations to take into account. Now that it has been confirmed that *H. pylori* produce CTS, blood and faecal samples from hypertensive patients both with and without *H. pylori* infections should be analysed and compared to confirm whether *H. pylori* affect blood pressure through CTS production or not. For faecal samples, it would be helpful to also add potential CTS metabolites to the analytical method, as most of the excreted CTS in faeces were found to be metabolites. It would yield higher concentrations than the precursor alone and would clarify if the detected CTS in faeces are excreted products or non-absorbed CTS.

Derivatisation, as stated before would help to increase the detection sensitivity of the analytes with the extreme low concentrations in blood. Selection of a derivatisation reagent such as methylhydroxylamine hydrochloride and further optimisation will also be of importance as dansyl hydrazine did not provide efficient derivatisation of the samples in this study. This would increase sensitivity and improve the overall parameters (LOD, LOQ, selectivity, chromatographic separation efficiency); enabling comparison of blood samples from hypertensive patients with and without *H. pylori* infections.

To confirm the identity of CTS isomers detected and also identify and confirm the derivative formation and structures, NMR could be used as it is important to prove if the derivatisation is really taking place under the selected conditions and which reaction is the dominant reaction under the conditions used.

Sample preparation is a very important part of the study, as it helps with concentrating the analyte and removing interfering matrix components and reduction of background noise signals. Although the extraction method in this study worked for most of the CTS, ouabain eluted in the wash fraction which is not ideal as the wash fraction includes undesirable molecules which can interfere with analyte analysis. Further optimisation or the use of other extraction techniques such as supercritical fluid extraction which is promising for an efficient analyte extraction can be considered. It can also help to resolve matrix effects seen with some of the CTS.

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Appendixes

Informed consent form

Principal Investigator: Zelie Flavienne Masso
Organization: Pharmacology Department, University of Pretoria
Contact number: 0607538895
Email: Flaviennemasso@gmail.com

Study title: Assessing cardiotonic steroids involvement in hypertensive patients with *Helicobacter pylori* infections

Introduction

You are being invited to take part in this study; the researchers are collecting blood samples to develop an analytical method for different cardiotonic steroids identification in plasma. This part of the study requires at least 5 healthy participants. This form explains the reason and all the procedures involved in this study. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the investigator. You should not agree to take part unless you are completely happy about all the procedures and data collection involved. This information will help you decide whether you wish to be part of the study and a copy of a signed form will be given to you.

Purpose of the study

The purpose of this study is to determine if *Helicobacter pylori* does produce cardiotonic steroid compounds when grown in culture and to use a developed targeted technique with an instrument called liquid chromatography tandem mass spectrometry (LC-MS/MS) to identify and quantitate the cardiotonic steroid compounds in those cultures and faecal samples of hypertensive subjects with/without *Helicobacter pylori* infections.

Criteria to participate in the study

To take part in this part of the study you must be a healthy adult aged 21 years of age or older. You cannot participate in this study if you are pregnant.

Study procedures

If you agree to participate in the study, you will be requested to provide 15 ml of blood sample to be collected in blood collecting tubes. Samples will be spiked with cardiotonic steroids and analysed using LC-MS/MS methodology to identify and quantitate cardiotonic steroids. There will be no intervention in this study, meaning that you will not be asked to take any pharmaceutical drug or any other substance. The results collected from the LC-MS/MS system will be used to develop a sensitive analytical method for the detection and quantification of different cardiotonic steroids concentrations between healthy and hypertensive participants (with/without *Helicobacter pylori* infections).

Possible risks and discomforts

If you take part of this study, to the best of my knowledge, the only discomfort involved will be the needle stick that may hurt. There is a small risk of bruising and fainting, and a rare risk of infection.

Withdrawal

Your participation in the study is completely voluntary, you have the right to withdraw from the project at any time without adverse consequences or giving reasons and without any prejudice to your privacy.

Possible benefits

There are no benefits or incentives for your participation in this study.

Ethical approval

This Protocol will be submitted to the Faculty of Health Sciences Research Ethics Committee, University of Pretoria and written approval will be granted by that committee. The study has been structured in accordance with the Declaration of Helsinki (last update: 2013), which deals with the recommendations guiding doctors in Biomedical Research involving human/subjects. A copy of the Declaration may be obtained from the investigator should you wish to review it.

Questions

In case you have any questions about the study or your rights, please contact the investigator or Prof Duncan Cromarty cell: 0733064220 or at duncan.cromarty@up.ac.za

Confidentiality

All samples collected will be collected with only a code number for identity. Any data and information provided in this study will be treated with utmost confidentiality, and your anonymity is fully granted. This means that your name will not be used in any reports or scholarly publications based on this research. Results will be published in peer reviewed journals or presented at a conference in such a fashion that participants remain unidentifiable, meaning that the results obtained in this study will be used for academic purposes only.

Subject information and consent form signature page

I have read and understood the above information before signing this consent form. The contents and meaning of this information have been explained to me clearly. I have been given the opportunity to ask questions and I am satisfied that they have been answered satisfactorily.

I hereby volunteer to take part in this study.

Name of the subject…….

Signature of subject……..

Date……..

Signature of individual conducting informed consent discussion……..

Date……

Name of individual conducting informed consent discussion……

Signature of witness…….

Date……..

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 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.

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Exoires 03/14/2020.

12 September 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 292-2017
Title: Assessing cardiotonic steroids involvement in hypertensive patients with Helicobacter pylori infections

Dear Ms ZF Masso

The **Annual Renewal** as supported by documents received between 2019-08-21 and 2019-09-11 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-09-11.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-09-12.
- Please remember to use your protocol number (292-2017) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health)

Research Ethics Committee
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Arcadia 0007, South Africa
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Fakulteit Gesondheidswetenskappe
Lefapha la Disaense tša Maphelo

R14/49 Prof Geoffrey Candy

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M170582

NAME: (Principal Investigator)	Prof Geoffrey Candy
DEPARTMENT:	Surgery
PROJECT TITLE:	Effect of Helicobacter Pylori Eradication on Blood Pressure in Patients with Hypertension and without Type 2 Diabetes (NIDDM)
DATE CONSIDERED:	26/05/2017
DECISION:	Approved unconditionally
CONDITIONS:	Renewal for the period 26/05/2017 - 26/02/2022 Previously M120481
SUPERVISOR:	
APPROVED BY:	Prof P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	02/06/2017

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 301, Third floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in May and will therefore be due in the month of May each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date 08/06/2017

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES