The effect of sulforaphane on oxidative stress and biotransformation in HepaRG cells

A Crous

20574711

Dissertation submitted in the fulfillment of the requirements for the degree *Magister Scientiae* in Biochemistry at the Potchefstroom Campus of the North-West University

Supervisor: Dr R Louw

Co-supervisor: Mr E Erasmus

September 2013

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

ABSTRACT

Sulforaphane is an isothiocyanate found in high concentrations in cruciferous vegetables like broccoli. Sulforaphane has received much attention due to the evidence that it inhibits phase I carcinogen-bioactivating enzymes and/or induces phase II antioxidant enzymes as well as metallothioneins (MTs) (Perocco *et al.,* 2006; Clarke *et al.,* 2008; Yeh & Yen, 2009). Since MTs and antioxidant enzymes are involved in the scavenging of reactive oxygen species (ROS), the question was raised whether sulforaphane can provide protection against increased oxidative stress and if sulforaphane exposure of a human hepatocellular carcinoma cell line, like HepaRG cells, will have a negative impact on phase I and II biotransformation in these cells. Oxidative stress was exogenously induced in HepaRG cells with *tert*-Butyl hydroperoxide (t-BHP). Phase I and phase II biotransformation pathways were assessed with caffeine, paracetamol, aspirin, sodium benzoate, and para-aminobenzoic acid, respectively, as probe substances. Through the use of a liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) assay, the biotransformation of caffeine in phase I and the formation of paracetamol, aspirin, sodium benzoate and para-aminobenzoic acid conjugates in phase II were investigated. This involved elucidating the time it took for the whole probe to be completely biotransformed during phase I biotransformation and the unique conjugates formed during phase II biotransformation in HepaRG cells.

The optimal t-BHP concentration and exposure time in HepaRG cells were standardized with a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. LC-ESI-MS/MS assays to monitor phase I and phase II biotransformation were optimized and validated. The optimal sulforaphane concentration and exposure time in HepaRG cells were standardized with a MTT assay. To evaluate the possible protective effect of sulforaphane against oxidative stress, HepaRG cells were pre-incubated with sulforaphane followed by the induction of oxidative stress with t-BHP and the quantification of the amount of viable cells with a MTT assay. To investigate the effect of sulforaphane on phase I and phase II

biotransformation pathways, HepaRG cells were first pre-incubated with sulforaphane followed by the addition of a specific probe substance and the assessment of the biotransformation of the probe with a LC-ESI-MS/MS assay.

The results partially supported the hypothesis of the study that sulforaphane will protect HepaRG cells against oxidative stress without negatively influencing phase I and phase II biotransformation. The results indicated that sulforaphane provided partial protection against t-BHP induced oxidative stress and had no effect on phase II paracetamol biotransformation in HepaRG cells.

Key terms: Sulforaphane, oxidative stress, t-BHP, MTT, phase I and phase II biotransformation, LC-ESI-MS/MS, HepaRG cells.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

LIST OF SYMBOLS

γ	Gamma
β	Beta
µ	Micro
n	Nano
º C	Degree Celsius
%	Percentage
>	Greater than
<	Less than
TM	Trademark
±	Plus minus

LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
CGase	Cysteinglycinase
CO_2	Carbon dioxide
-COOH	Carboxyl group
CYP450	Cytochrome P450
ddH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EC	Enzyme commission
EDTA	Ethylenediaminetetraacetic acid
EPHX-1	Epoxide hydrolase
ESI	Electron spray ionization
ETC	Electron transport chain
FBS	Foetal bovine serum
GSH	Glutathione
GST	Glutathione-S-transferase
GTP	γ-glutamyl transpeptidase
H_2O	Water
H_2O_2	Hydrogen peroxide
HepG2/HepaRG cells	Human liver carcinoma cells
HMOX-1	Heme oxygenase-1

HPLC	High pressure liquid chromatography
IS	Internal standard
kb	Kilobase
kDa	Kilodalton
LC	Liquid chromatography
LC-ESI-MS/MS	Liquid chromatography-electrospray ionization-mass spectrometry
L-Glut	L-Glutamine
MRM	Multi reaction monitoring
mRNA	Messenger ribonucleic acid
MS	Mass spectrometer
MS/MS	Triple quadrupole mass analyser
MT	Metallothionein
MTS	Metallothioneins
mtDNA	Mitochondrial deoxyribonucleic acid
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPQI	*N*-acetyl-p-benzoquinoneimine
NAT	*N*-acetyl-transferase
NAT-1	*N*-acetyl-transferase-1

NEAA	Non-essential amino acids
-NH_2	Amino group
O_2	Oxygen
O_2-	Superoxide anion
-OH	Hydroxyl group
OH^-	Hydroxyl radical
OXPHOS	Oxidative phosphorylation
PAABA	Para-acetamidobenzoic acid
PAAHA	Para-acetamidohippuric acid
PABA	Para-aminobenzoic acid
PAHA	Para-aminohippuric acid
PBS	Phosphate buffered saline
Pen/Strep	Penicillin/streptomycin antibiotic solution
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rt	Retention time
SH	Thiol
SOD	Superoxide dismutase
SULT	Sulfotransferases
t-BHP	*tert*-Butyl hydroperoxide
UGT	UDP-glucuronosyltransferases
WME	Williams medium E
γ-GCS	Gamma-glutamyl cysteine ligase

LIST OF FIGURES

Figure:

LIST OF TABLES

Table:

LIST OF EQUATIONS

Equation:

ACKNOWLEDGEMENTS

I would to express my sincere gratitude to the following persons for their contributions made to this study:

Firstly, to God I give all the honour.

Dr. R. Louw, my supervisor, for his guidance, patience, and support.

Mr. E. Erasmus, my co-supervisor, for his guidance with the chromatography analysis.

Mr. Peet Jansen van Rensburg, for his guidance and help with the LC-ESI-MS/MS.

Mrs Barbara Bradley, for checking and editing the language of this dissertation.

The National Research Foundation (NRF) for financial assistance throughout this study.

My family, for their support and motivation throughout this study.

Finally, and most important, I would like to thank my husband, Wessel, for his patience, support and kind words of love.

CHAPTER 1

Introduction

The process of adenosine-5'-triphosphate (ATP) synthesis in the mitochondrion is dependent on the presence of oxygen (O_2) and at risk to the formation of free radicals and oxidative stress. Oxidative stress is a situation created by an imbalance between free radical formation and antioxidant systems leading to cell injury and death. Antioxidants protect the body against free radicals, and the damaging effects caused by free radicals, by preventing the formation of reactive oxygen species (ROS) through enzyme-catalyzed removal by antioxidant enzymes or through metal chelation to metallothioneins (MTS). Biotransformation systems in hepatic cells consist of two phases, known as phase I and phase II. Phase I enzymes function by biotransforming a variety of endogenous and exogenous chemicals through oxidation, reduction, and/or hydrolysis reactions to either expose or add a functional group. Phase II enzymes function by catalyzing the conjugation of phase I metabolites to various water soluble molecules and accelerate the rate of metabolite excretion. Sulforaphane are found in high concentrations in cruciferous vegetables like broccoli. Sulforaphane has received much attention due to the evidence that it function as an indirect antioxidant through the inhibition of phase I enzymes, preventing the formation of more reactive molecules, and the activation of phase II antioxidant enzymes, as well as MTS.

Hypothesis

Since MTS and antioxidant enzymes are involved in ROS scavenging, the question was raised whether sulforaphane can provide protection against increased oxidative stress. Another question that was raised was if sulforaphane treatment of a human hepatocellular carcinoma cell line, like HepaRG cells, will have a negative impact on phase I and II biotransformation in these cells. **The hypothesis of this study was: "Sulforaphane will protect HepaRG cells against induced oxidative stress without negatively influencing phase I and II biotransformation".**

Aim and objectives

The aim of this study was to determine the effect of sulforaphane on induced oxidative stress and phase I and phase II biotransformation in HepaRG cells.

The following objectives were formulated:

1) The standardization of the optimal *t*-BHP concentration and incubation time to induce oxidative stress in HepaRG cells

2) The optimization of LC-ESI-MS/MS assays to monitor phase I and phase II biotransformation in HepaRG cells

3) The assessment of the effect of sulforaphane on *t*-BHP-induced oxidative stress in HepaRG cells

4) The assessment of the effect of sulforaphane on phase I and phase II biotransformation in HepaRG cells

Chapter 2 contains a comprehensive literature review on oxidative stress, phase I and phase II biotransformation, sulforaphane, and HepaRG cells. The chapter concludes with the experimental approach of this study. Chapter 3 describes the tissue culture techniques used to culture HepaRG cells for experimental use, followed by the methods used to induce and evaluate oxidative stress in these cells. Finally, the methods used to prepare HepaRG cell samples for analysis by a liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS) assay are discussed. In Chapter 4 the optimization and the validation of LC-ESI-MS/MS assays used to quantify the biotransformation of each probe substance to selected phase I and phase II metabolites are discussed. Chapter 5 follows, where the optimization of phase I and phase II biotransformation assays in HepaRG cells are discussed. Chapter 6 includes the investigation of the effects of sulforaphane on oxidative stress and biotransformation in HepaRG cells. Finally Chapter 7 concludes the study and gives future prospects.

CHAPTER 2

Literature review

2.1 OXIDATIVE STRESS

Oxygen is needed by most organisms to sustain life. Despite the life-giving advantages of O_2, it can be disadvantageous as well. O_2 may lead to the formation of excess ROS and a situation known as oxidative stress. ROS are formed when O_2 is reduced (Migliore & Coppedè, 2008) to form the superoxide anion (O_2-), hydroxyl radicals (OH-), and hydrogen peroxide (H_2O_2). Thus ROS are radical species of O_2 which are in a more reactive state compared to molecular O_2. Oxidative stress is a situation created by an imbalance between free radical formation and antioxidant systems leading to cell injury and death (Migliore & Coppedè, 2008).

2.1.1 Sources of ROS

Endogenous sources of ROS are by-products of the metabolism. ROS formation begins with the leaking of an electron mainly from complex I and complex III, part of the electron transport chain (ETC), which leads to the reduction of O_2 to O_2- (Ishii *et al.*, 2006). The production of O_2- by the ETC thus occurs continuously during normal aerobic metabolism (Tiano *et al.*, 1983). This is followed by the reduction of O_2- to H_2O_2 due to the dismutation of O_2- which can occur spontaneously, especially at low pH, or can be catalyzed by superoxide dismutase (SOD). A further reaction may lead to the formation of OH^-. These OH^- are extremely reactive and will most likely react with the first molecule they encounter (Hancock *et al*., 2001). Some ROS, especially H_2O_2 are key signalling molecules, while others appear to be extremely dangerous to biological systems. The amount of damage is determined by the level of ROS produced in cells and the effectiveness of antioxidant systems within cells (Turrens, 2003). Exogenous sources of ROS can be the result of an external exposure to compounds normally not found in an organism's metabolism (xenobiotics), which

causes oxidative stress through various mechanisms, such as uncoupling of the ETC, and depletion of antioxidant systems (Rau *et al.*, 2004).

2.1.2 Effect of ROS in cells

At the correct levels, ROS molecules have a role in the regulation of many important cellular events, including transcription factor activation, gene expression, differentiation, and cell proliferation (Martín *et al.,* 2001). However, high levels of ROS can cause injury to cells through damage to membrane lipids, proteins, and nucleic acids (Beckman & Ames, 1998; Ishii *et al*., 2006). ROS causes the oxidation of membrane lipids, especially polyunsaturated fatty acids found in high amounts in the mitochondrial membrane, leading to membrane dysfunction, changes in the structural and functional integrity of mitochondria, and cell death (Beckman & Ames, 1998; Chaudhuri *et al.,* 2007). Oxidation of proteins cause the cross linking of structural proteins and changes in the conformation of receptors, membrane pumps, enzymes carrier proteins, or peptide hormones. Oxidation of nucleic acids causes damage to base and sugar groups, single- and double-strand breaks and cross linking to other molecules (Beckman & Ames, 1998; Liska *et al.,* 2006). Despite a smaller size (± 16.6 kilobase), relative to the nuclear genome, the mitochondrial genome is very important for normal cellular function, encoding thirteen respiratory chain subunits of the oxidative phosphorylation (OXPHOS) system (Holmuhamedov *et al.,* 2003). Cleavage of the mtDNA thus causes impairment in ATP formation. The result is irreversible damage to mitochondria and cell death (Ishii *et al*., 2006).

2.1.3 Antioxidants

Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation of oxidizing chain reactions. Antioxidants protect the body against free radicals and the damaging effects caused by free radicals, by preventing the formation of ROS through enzyme-catalyzed removal by antioxidant enzymes or through metal chelation to metallothioneins (MTS). Together these antioxidant enzymes and MTS form the antioxidant defence system that protects at different

sites in the body against different types of ROS. However, cellular damage is not only caused by free radicals but also by non-radical mechanisms. An antioxidant system with various functions is thus needed. There are two types of antioxidants known as direct and indirect antioxidants. Direct antioxidants, like MTS and antioxidant defence enzymes, are able to take part in physiological, biochemical or cellular processes to inactivate free radicals or prevent chemical reactions that generate free radicals. Indirect antioxidants, like sulforaphane, are not able to take part in radical or redox reactions but they function by boosting the antioxidant capacity of cells through various mechanisms, and thus help to protect against oxidative stress (Fahey & Talalay, 1999).

2.1.4 Induction of oxidative stress in *in vitro* models

Depending on the method used to induce oxidative stress, *in vitro* studies of oxidative stress can be divided into exogenous and endogenous. A number of factors need to be considered before deciding between an endogenous and exogenous method to induce oxidative stress. First, the site where oxidative stress is generated must be considered, along with type and the amounts of ROS produced. This is because each ROS has different characteristics when it comes to chemical reactivity and stability. Thus it is expected that cell injury caused by endogenously induced oxidative stress would be different from cell injury caused by exogenously induced oxidative stress. Secondly, the type and level of oxidative stress must also be taken into consideration. Two methods of cell death exist, known as apoptosis and necrosis. The method is determined by the type and level of oxidative stress. Lower concentrations of oxidants cause apoptosis, and higher concentrations necrosis (Shiba & Shimamoto, 1999).

2.1.4.1 *tert*-Butyl hydroperoxide

tert-Butyl hydroperoxide (*t*-BHP) is an organic hydroperoxide and is routinely used to induce oxidative stress in *in vitro* models (Lapshina *et al.,* 2005; Lima *et al.,* 2006). After *t*-BHP crosses the cellular membrane it causes damage such as peroxidation

of membrane lipids, DNA damage, cellular ATP depletion, and a loss in mitochondrial membrane potential, which all eventually lead to cell death (Lapshina *et al.,* 2005; Lima *et al.,* 2006). *t*-BHP also causes the depletion of the most abundantly produced endogenous antioxidant, glutathione (GSH), due to the oxidation of GSH. GSH thus has an important part in *t*-BHP-induced liver cell death. This has been proven in hepatocytes where it was observed that the depletion of GSH levels before the administration of *t*-BHP lead to much higher cell death than in cells with normal GSH levels before *t*-BHP was added (Nishida *et al.,* 1997).

2.2 BIOTRANSFORMATION

On a daily basis humans are exposed to toxins in the form of exogenous substances, not usually found in the metabolism (xenobiotics), and endogenous substances which are products of the metabolism. The first line of defence against these toxins is a biotransformation system which consists of all mechanisms used to convert these toxins into more water soluble metabolites for excretion through the urine or the stool. Biotransformation mechanisms are action and target specific. Some function only in the bowl, others in the liver, blood, kidney or the skin (Liska *et al.*, 2006). The liver is the most important organ in the body where toxins are biotransformed. As illustrated in Figure 2.1, the role of the liver in the biotransformation of toxins is accomplished by two groups of enzymatic modifications known as phase I transformation reactions (hydrolysis, oxidation, and reduction) and phase II conjugation reactions (sulfation, glucuronidation, GSH conjugation, and amino acid conjugation) (Liska *et al.*, 2006; Zamek-Gliszczynski *et al.,* 2005). Together, phase I and phase II provide a variety of metabolic transformation reactions making the elimination of almost any toxin possible (Iyer *et al.,* 2010).

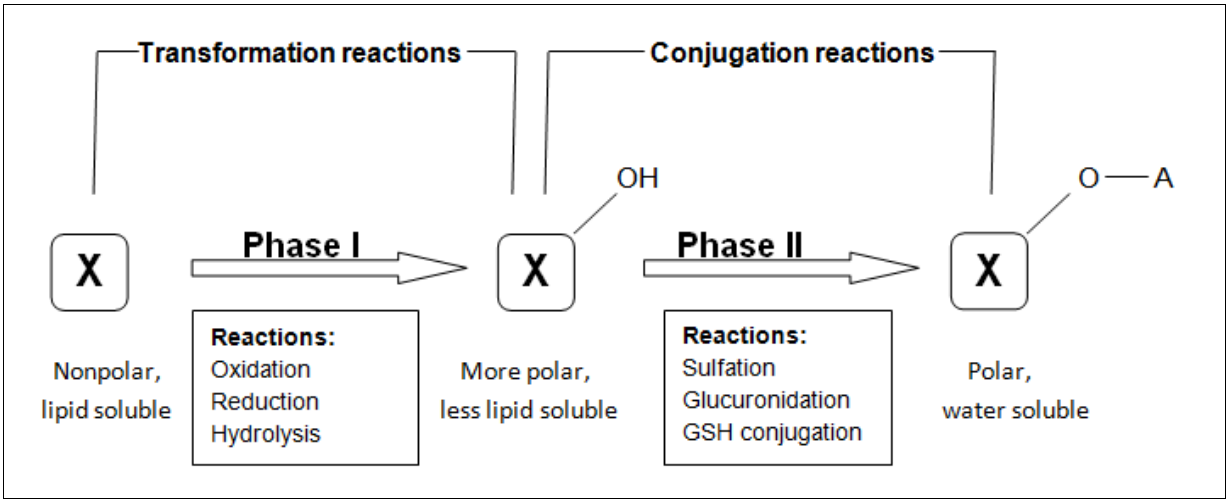

Figure 2.1: The liver biotransformation pathways. The liver functions as the main biotransformation organ in the body. Through a range of transformation (phase I) and conjugation (phase II) reactions, using nutrient cofactors, lipid-soluble exogenous and endogenous toxins (X) are transformed into more water soluble metabolites that can be excreted through the urine or the stool. Phase I enzymes function by biotransforming endogenous and exogenous toxins through oxidation, reduction, or hydrolysis reactions to either expose or add a functional group (OH). Phase II enzymes function by catalyzing the conjugation of phase I metabolites through sulfation, glucuronidation, GSH conjugation, and amino acid conjugation (O-A) to various water soluble molecules and accelerate the rate of metabolite excretion through the urine or the stool. An imbalance in the system leads to the formation of more polar intermediary metabolites which are not further transformed in phase II, leading to secondary tissue damage and the formation of free radicals (Adapted from: Liska *et al*., 2006).

2.2.1 Phase I

Phase I enzymes function by biotransforming toxins through oxidation, reduction, and/or hydrolysis reactions which expose functional groups to form reactive sites. A hydroxyl (-OH), a carboxyl (-COOH), or an amino ($-NH_2$) group are usually added depending on the structure of the molecule being biotransformed. This can improve the water solubility and allow direct elimination from phase I or allow phase II conjugation of the compound (Liska *et al*., 2006). The cytochrome P450 enzyme family has a very important role in phase I biotransformation reactions (Brandon *et al.,* 2003).

2.2.1.1 Cytochrome P450 enzymes

Although many types of phase I biotransformation enzymes exist, cytochrome P450 enzymes (CYP450) are the most common (Liska *et al.,* 2006). CYP450 enzymes

belong to a family of genes that encode for an array of enzymes catalyzing the oxidation of a wide variety of exogenous and endogenous toxins. They are responsible for the transformation of most toxins, as they have a wide range of substrate specificities in order to handle a broad spectrum of different compounds. In humans these enzymes are mostly found in the liver. CYP1A2, CYP2A6, CYP2B1/2, CYP2B6, CYP2C8, CYP2C9, CYP2C18/2C19, CYP2D6, CYP2E1, CYP3A4 and CYP4A11 are all the CYP450 enzymes families found in the liver (Brandon *et al.,* 2003). CYP450 enzymes function by using O_2 and the co-factor reduced nicotinamide adenine dinucleotide (NADH) to either add an -OH, an -COOH, or an $-NH_2$ group. During this step fat-soluble toxins are sometimes transformed into more polar toxins which can cause damage to proteins, ribonucleic acid (RNA), and DNA if not inactivated in phase II (Liska *et al*., 2006; Zamek-Gliszczynski *et al.,* 2005).

2.2.2 Phase II

Phase II reactions follow phase I reactions or can function independently. Phase II enzymes function by catalyzing the conjugation of phase I polar metabolites to various water soluble molecules for excretion through the urine or the stool. This involves sulfate conjugation, glucuronic acid conjugation, glutathione conjugation, and amino acid conjugation (Zamek-Gliszczynski *et al.,* 2005; Chang *et al*., 2010). All these conjugation reactions are catalyzed by various conjugation enzymes (Brandon *et al.,* 2003).

2.2.2.1 Conjugation enzymes

Phase II conjugation enzymes include UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), glutathione-S-transferase (GST), *N*-acetyl-transferase-1 (NAT-1), and epoxide hydrolase (EPHX1) (Liska *et al*., 2006; Westerink & Schoonen, 2007). Conjugation reactions can occur with a variety of substances, and involve cofactors derived from the metabolism (eg. glucuronic acid, sulfate, glycine, or glutathione). During a conjugation reaction, one of these water soluble cofactors is attached to a toxic molecule. These conjugation reactions cause an increase in the

molecular weight and water solubility of compounds (Liska *et al*., 2006; Zamek-Gliszczynski *et al.,* 2005).

Sulfate conjugation

Sulfate conjugation (sulfation) is the most common phase II liver biotransformation mechanism. Sulfation of a toxin increases the water solubility of the compound to prepare them for excretion from the body through urine or the stool. Although sulfation has a high affinity for toxins, they are quickly saturated. Sulfation thus functions together with glucuronic acid conjugation (glucuronidation) on overlapping substrates. Sulfation is the most common at low substrate concentrations and glucuronidation at high substrate concentrations, when sulfation has been saturated. Sulfation reactions of different xenobiotics occur at different sites in the cell. Conjugation of hydroxyl, amino, N-oxide, and sulfhydryl groups on xenobiotics occurs in the cytosol. Sulfation of carbohydrates attached to peptides or lipids, as well as proteins, occur in the Golgi network (Zamek-Gliszczynski *et al.,* 2005).

Glucuronic acid conjugation

As mentioned, conjugation of a toxin with glucuronic acid (glucuronidation) occurs on many of the same substrates as sulfation. Glucuronidation is most common at high substrate concentrations when sulfation has become saturated, because of co-substrate depletion or enzyme saturation. Like sulfation, glucuronidation also aims to biotransform toxins into more water soluble compounds for excretion through urine or stool. Unlike sulfation, glucuronidation of toxins occurs only inside microsomal membranes (Zamek-Gliszczynski *et al.,* 2005).

Glutathione conjugation

Substrates for the GSH conjugation reaction are often strong electrophiles, making GSH conjugation very important. Substrates for GSH conjugation include parent

compound electrophiles, electrophilic phase I metabolites, and certain phase II conjugates. GSH conjugation can occur spontaneously due to the high intracellular GSH concentrations (~ 10 mM) in the liver, but this reaction is much more effective when catalyzed by the glutathione-S-transferase (GST) antioxidant enzyme. GST facilitated metabolism mainly occurs in the cytosol but GST also functions in the endoplasmic reticulum. High intracellular GSH levels in the liver is difficult to deplete, thus GSH is the highest co-factor used in the four conjugation reactions. If GSH should be depleted it would result in liver damage (Zamek-Gliszczynski *et al.,* 2005).

Amino acid conjugation

Amino acid conjugation, an important biotransformation route, functions independent of phase I enzyme reactions (Kasuya *et al.,* 2000). Aromatic acids are mainly biotransformed through amino acid conjugation. Glycine, taurine, arginine and ornithine are used in the phase II amino acid conjugation reactions. The conjugation of a specific amino acid is determined by the type of aromatic acid being biotransformed. In humans, the glycine conjugation pathway is mostly involved in phase II reactions (Liska *et al*., 2006; Beyoğlu *et al.,* 2012). Aromatic acids such as benzoic acids are biotransformed through the conjugation of glycine, producing a more water soluble hippuric acid molecule (Kasuya *et al.,* 2000; Beyoğlu *et al.,* 2012).

2.2.3 Function of phase I and phase II biotransformation

Exposure to compounds normally not found in an organism's metabolism (xenobiotics), contributes towards endogenous and exogenous formation of ROS which may cause oxidative stress (Rau *et al.*, 2004). Most xenobiotics are also highly fat-soluble and can thus accumulate in fat, if not biotransformed. The existence and proper functioning of all enzymes involved in the biotransformation system, used to convert these xenobiotics into more water soluble metabolites for excretion through the urine or the stool, are thus very important. For the successful biotransformation of xenobiotics the functioning of both phase I and phase II must be in balance.

Biotransformation enzymes normally function sufficiently enough to decrease the potential damage from xenobiotics. However, dysfunction of these systems can arise when they are overloaded or imbalanced. In phase I, inhibition of the CYP450 enzymes can be due to competitive inhibition when two or more compounds compete for the same enzyme, leading to the overloading of the system. Phase II enzymes are inhibited by the depletion of co-factor levels like sulfate, glucuronic acid or GSH. The biotransformation of xenobiotics in phase I can transform a xenobiotic into a more reactive molecule. Depending on the structure of the molecule being biotransformed, during phase I biotransformation reactions, CYP450 enzymes use O_2 and the co-factor NADH to add a -OH to prepare the molecule for phase II biotransformation. This may lead to the formation of a more reactive molecule. If this molecule is not further biotransformed in phase II, like in the case of an imbalance between phase I and phase II, damage to lipids, proteins and nucleic acids may follow (Liska *et al.,* 2006).

2.2.4 Assessment of biotransformation

The use of animal models to assess biotransformation is increasingly being replaced with *in vitro* cellular models. Cellular models provide the use of human cell cultures. This eliminates any biotransformation differences between animal and human and provides a less complex study system. Since the liver is the most important organ in the body where toxins are biotransformed, it is frequently used in tests where the toxicity and biotransformation of compounds is investigated (Jover *et al.,* 1992). Several *in vitro* liver models have since been used and developed (Brandon *et al.,* 2003; Anthérieu *et al.,* 2012).

In the study of specific drug-metabolizing enzymes, hepatocytes and other liver cell lines provide all enzymes and are thus considered as a representative model of liver specific metabolism (Brandon *et al.,* 2003; Aninat *et al*., 2006; Guillouzo *et al.,* 2007; Lübberstedt *et al*., 2011). However, primary hepatocytes are not easy to obtain, do not survive very long and can undergo early and irregular physical changes (Aninat *et al*., 2006; Guillouzo *et al*., 2007; Lübberstedt *et al*., 2011). Their liver specific

functions, particularly CYP450 enzymes and their response to inducers can also show major variation between donors (Aninat *et al*., 2006; Guillouzo *et al.,* 2007; Pernelle *et al.,* 2011). Human liver cell lines derived from tumor cells are thus mostly used in studies of biotransformation, as they have the advantage of continuous growth and reproducible metabolism. These cells express phase I and phase II enzymes at relatively stable concentrations and are easier to obtain and culture (Brandon *et al.,* 2003). The liver carcinoma cell line known as HepG2 has been used extensively as an alternative cellular model. However, the use of this cell line in toxicology studies is limited by the fact that they lack several liver specific functions. The HepaRG cell line expresses most of the liver specific genes at higher levels compared to other cell lines, especially in biotransformation enzyme activity (Lambert *et al.,* 2009; Pernelle *et al.,* 2011; Anthérieu *et al.,* 2012). The assessment of the biotransformation metabolic activity can be done by challenging phase I and phase II reactions with specific probe substances whose biotransformation pathways are well known. The individual introduction of probes into cells provides the possibility to investigate the role of either phase I or phase II in the complete biotransformation of the probe (Schrader *et al.,* 1999; Liska *et al*., 2006).

2.2.4.1 Assessment of phase I biotransformation with caffeine as probe substance

Caffeine (1, 3, 7-trimethylxanthine) is the xenobiotic to which humans are mostly exposed. Caffeine is mainly biotransformed in the liver with only 5% being directly eliminated through the urine (Miners & Birkett, 1996). Caffeine is a useful probe for assessing the activity of CYP1A1 isoforms in humans and can thus be used as a probe for the investigation of phase I biotransformation. As a probe it has the advantage that it is inexpensive and, at the correct concentration, does not have any harmful effects. Variations in caffeine concentration and the tempo of formation of specific caffeine metabolites can be used to assess phase I biotransformation activity (Schrader *et al.,* 1999; Liska *et al*., 2006). Caffeine N-demethylation activity, indicated by the formation of the specific caffeine metabolites: theophylline, paraxanthine, and theobromine, can then be used as an indication of the human *in vitro* liver CYP1A2 activity (Miners & Birkett, 1996; Hickman *et al*., 1998).

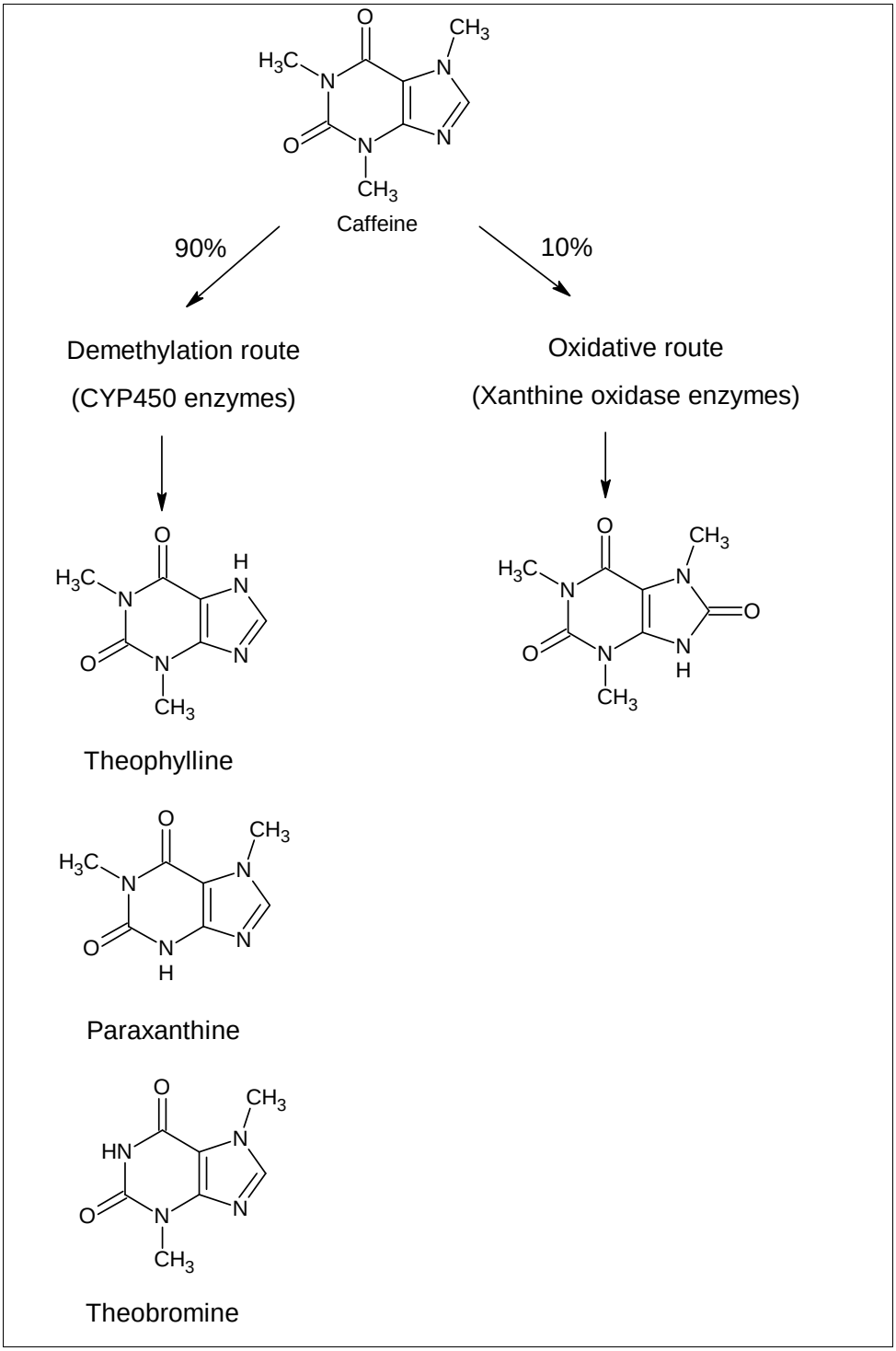

Figure 2.2: *In vitro* biotransformation of caffeine. The figure illustrates the *in vitro* caffeine biotransformation pathway to the primary metabolites theophylline, paraxanthine, theobromine, and 1-3-7-trimethyluric acid. These N-demethylation reactions are catalyzed by CYP1A2 and account for approximately 90% of caffeine elimination in humans. The other 10% are eliminated through an oxidative route by the enzyme xanthine oxidase (Miners & Birkett, 1996) (Adapted from Gokulakrishnan et al., 2005).

Caffeine is metabolized in humans by the CYP450 enzymes CYP1A2, CYP3A4, CYP2E1, xanthine oxidase and N-acetyl transferase to various methylxanthines, methylutates, and urasil derivatives (Gokulakrishnan *et al.,* 2005). Although various caffeine metabolites can be detected *in vivo* in urine, *in vitro* caffeine is only biotransformed to the primary metabolites paraxanthine (84%), theophylline (4%), and theobromine (12%). These metabolites are formed when caffeine undergoes demethylation of the nitrogen (N-demethylation) sites one, three and seven (Cazeneuve *et al*., 1994). As illustrated in Figure 2.2, these N-demethylation reactions are catalyzed by CYP1A2 and account for approximately 90% of caffeine elimination in humans. The other 10% are eliminated through an oxidative route by the enzyme xanthine oxidase (Miners & Birkett, 1996).

2.2.4.2 Assessment of phase II sulfate, glucuronic acid, and glutathione conjugation with paracetamol as probe substance

Paracetamol (4-acetamidophenol) is used as a probe substance to investigate phase II sulfate conjugation (sulfation), glucuronic acid conjugation (glucuronidation), and glutathione (GSH) conjugation reactions (Liska *et al.,* 2006). Most toxins must undergo both phase I and phase II biotransformation to be successfully excreted from the body, but molecules like paracetamol, with sites already open to conjugation can directly undergo phase II biotransformation. As indicated in Figure 2.3, paracetamol is mainly biotransformed by phase II sulfation and glucuronidation pathways to form paracetamol sulfate and paracetamol glucuronide. These metabolites are more water soluble than their parent compound and are thus excreted through the urine. If these pathways are inhibited or compromised due to depleted co-factor status, paracetamol is biotransformed through an alternate pathway requiring a phase I biotransformation (CYP2E1 mediated N-hydroxylation) to form N-acetyl-p-benzoquinoneimine (NAPQI), a highly neurotoxic substance. NAPQI can cause severe damage if not cleared by GSH conjugation to form paracetamol mercapturate (Liska *et al.,* 2006; Zamek-Gliszczynski *et al.,* 2005; Lohmann & Karst, 2006).

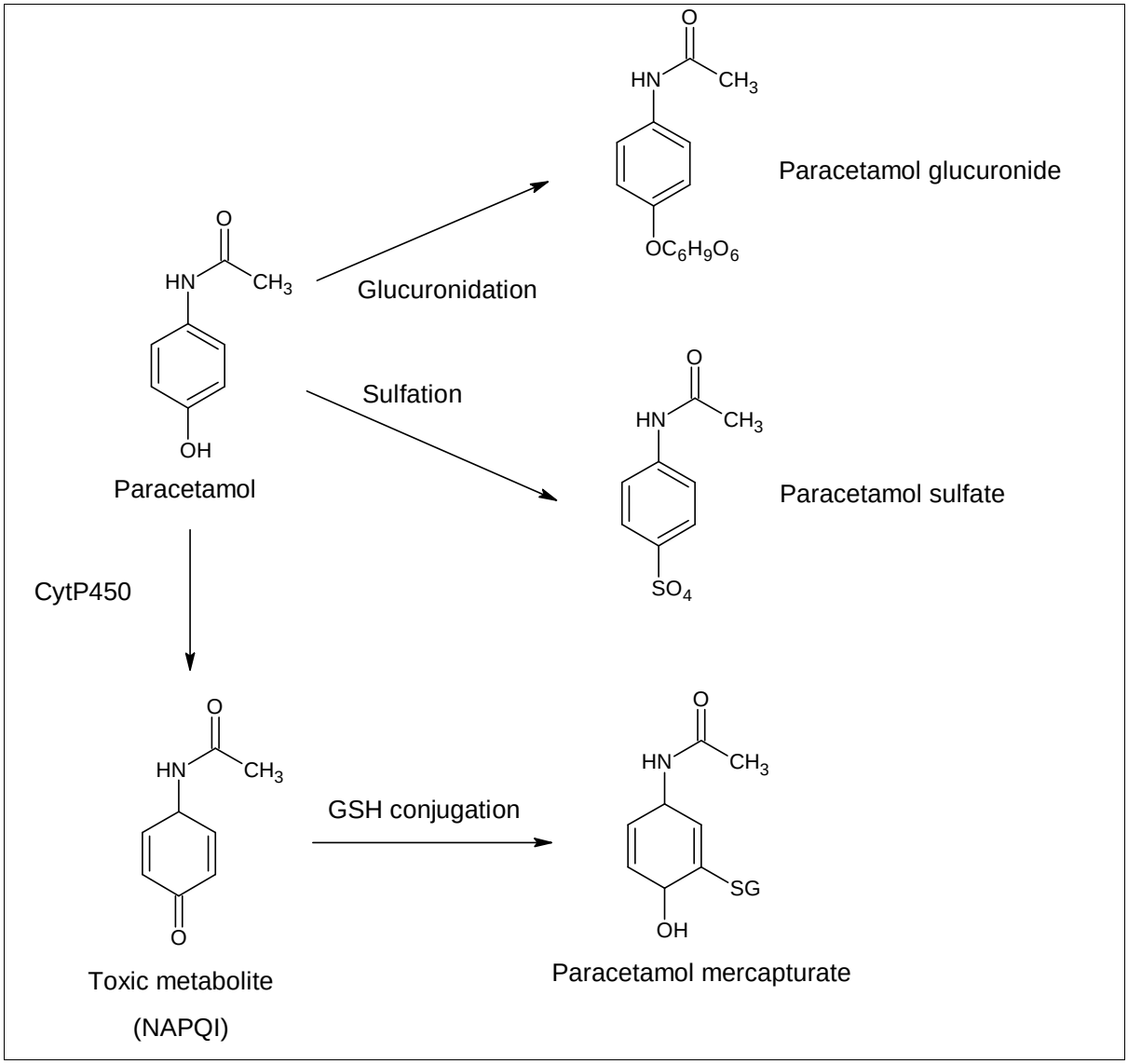

Figure 2.3: Biotransformation of paracetamol in humans. The figure illustrates the phase II biotransformation pathway of paracetamol. Paracetamol is biotransformed to the major metabolites paracetamol glucuronide and paracetamol sulfate through sulfation and glucuronidation reactions. If these pathways are inhibited or compromised due to depleted co-factor status, paracetamol is biotransformed through an alternate pathway requiring a phase I biotransformation (CYP2E1 mediated N-hydroxylation) to form the biotransformed intermediate, N-acetyl-p-benzoquinoneimine (NAPQI), a highly neurotoxic substance. NAPQI can cause severe damage if not cleared by glutathione (GSH) conjugation to form the metabolite paracetamol mercapturate (Adapted from Liska *et al.,* 2006).

In humans, the biggest part of paracetamol is metabolized through the glucuronidation (50-60%) and sulfation (25-35%) pathways. Only a small amount (2-10%) of paracetamol is converted to the toxic metabolite NAPQI and eventually paracetamol mercapturate under normal circumstances (Lohmann & Karst, 2006). As mentioned previously, although sulfation displays a higher affinity for

paracetamol, they are quickly saturated, leaving glucuronidation which has a higher capacity for paracetamol conjugate formation. CYP450 enzymes have a very low affinity for paracetamol. NAPQI is only formed at higher paracetamol concentrations when sulfation and glucuronidation pathways are over saturated. Thus, only a small percentage of paracetamol is converted to NAPQI (Slikker *et al.,* 2004; Zamek-Gliszczynski *et al.,* 2005). GSH in the liver has an important role in protecting cells against NAPQI through conjugation or its antioxidant function or both. Depletion of liver GSH levels causes NAPQI to bind covalently with the thiol groups in cellular macromolecules. The loss of thiol groups leads to liver cell necrosis. Thus the levels of GSH will determine the toxicity of paracetamol to cells (Slikker *et al.,* 2004; Lohmann & Karst, 2006). Despite the conversion of paracetamol into these conjugates, some paracetamol is still excreted directly through the urine (Lohmann & Karst, 2006).

2.2.4.3 Assessment of phase II amino acid conjugation with aspirin, sodium benzoate and para-aminobenzoic acid as probe substances

In humans, glycine conjugation is the main amino acid conjugation pathway and can be used to evaluate phase II amino acid conjugation activity (Liska *et al.,* 2006; Beyoğlu *et al.,* 2012). Aromatic acids such as benzoic acids are biotransformed by glycine conjugation into more water soluble molecules. Specific aromatic acids can thus be used as probes to assess the activity of phase II glycine conjugation. Aspirin, the food preservative sodium benzoate and para-aminobenzoic acid have all been used in studies as probes to investigate the activity of the phase II glycine conjugation pathway (Kasuya *et al.,* 2000; Beyoğlu *et al.,* 2012).

Aspirin

As indicated in Figure 2.4, aspirin (acetylsalicylic acid) is firstly degraded into salicylic acid, which is eventually biotransformed through glycine conjugation and glucuronic acid conjugation to form the major metabolite salicyluric acid and the minor metabolites salicyl glucuronides (Liska *et al*., 2006). The quantification of the

formation of the major metabolite salicyluric acid, through glycine conjugation of salicylic acid in cells, can be used as an indication of phase II glycine conjugation activity.

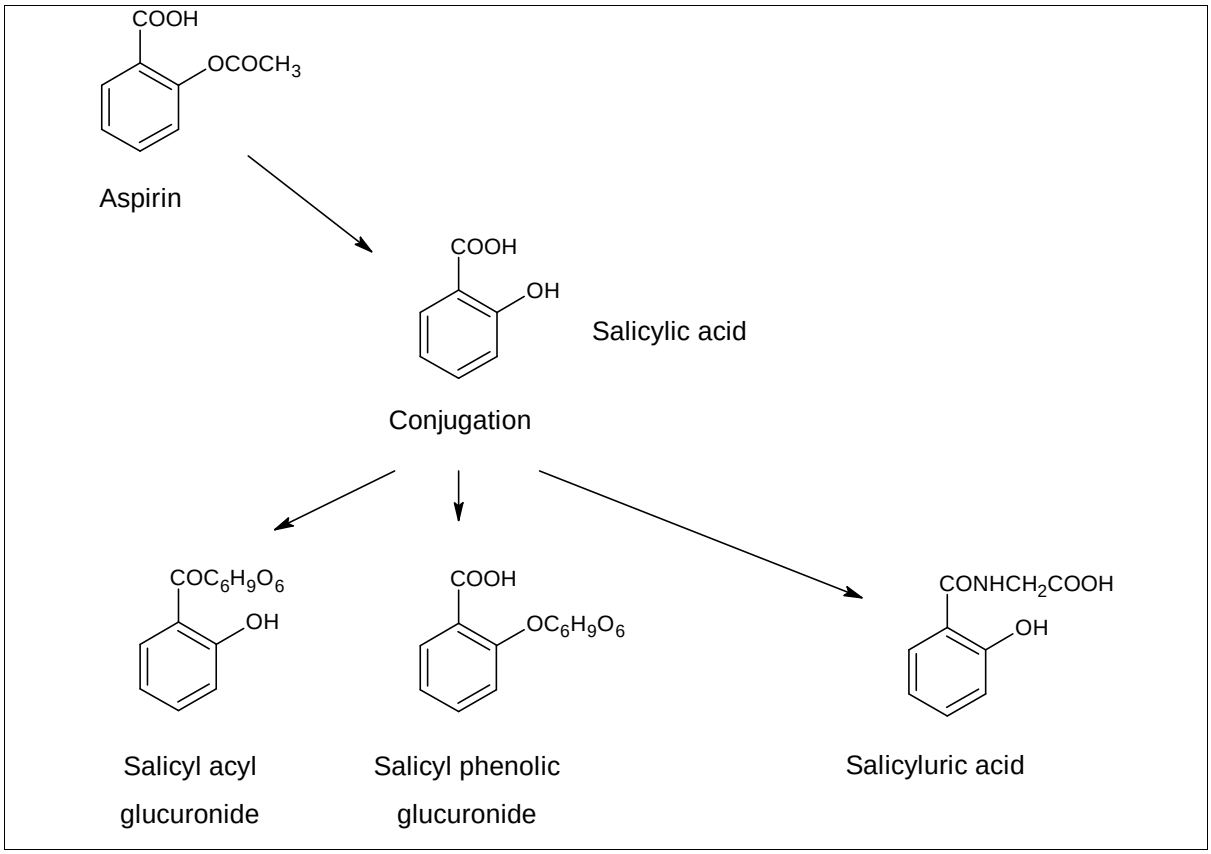

Figure 2.4: Biotransformation of aspirin in humans. The figure illustrates the phase II biotransformation pathway of aspirin. Aspirin is firstly degraded into salicylic acid, which is eventually biotransformed through glycine conjugation and glucuronic acid conjugation to form the major metabolite salicyluric acid and the minor metabolites salicyl glucuronides (Adapted from Liska *et al.,* 2006).

Sodium benzoate

As indicated in Figure 2.5, during glycine conjugation, sodium benzoate is biotransformed to hippuric acid (Beyoğlu *et al.,* 2012). The quantification of the formation of hippuric acid in cells can be used as an indication of phase II glycine conjugation activity.

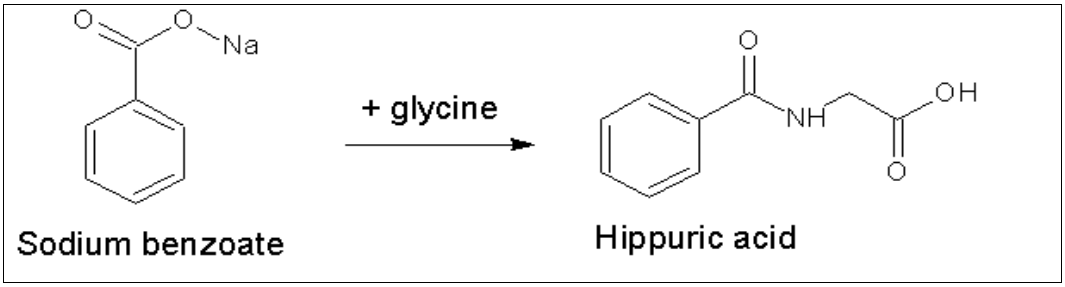

Figure 2.5: Biotransformation of sodium benzoate. The figure illustrates the phase II liver amino acid conjugation pathway in which sodium benzoate is biotransformed to hippuric acid through glycine conjugation (Beyoğlu *et al.,* 2012).

Para-aminobenzoic acid

During phase II glycine conjugation reactions, para-aminobenzoic acid (PABA) is biotransformed to three metabolites, as indicated in Figure 2.6. Glycine conjugation of PABA leads to the formation of para-aminohippuric acid (PAHA). Conjugation with glycine and acetyl-CoA lead to the formation of para-acetamidobenzoic acid (PAABA). Conjugation with glycine and acetyl-CoA produce para-acetamidohippuric acid (PAAHA) (Lebel *et al.,* 2003). Quantification of para-aminohippuric acid formation in cells can be used as an indication of phase II glycine conjugation activity.

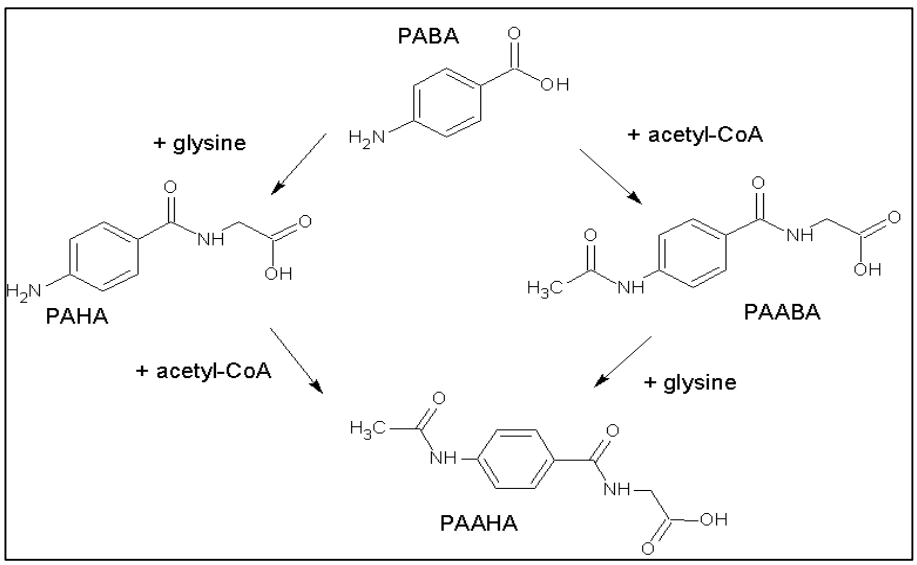

Figure 2.6: Biotransformation of para-aminobenzoic acid (PABA). The figure illustrates the phase II amino acid conjugation pathway of para-aminobenzoic acid (PABA).PABA is biotransformed into either para-aminohippuric acid (PAHA), para-acetamidobenzoic acid (PAABA) or para-acetamidohippuric acid (PAAHA) by combining with glycine (+glycine) or (+acetyl-CoA), respectively (Adapted from Lebel *et al.,* 2003).

2.3 SULFORAPHANE

In humans, research has showed that the high intake of cruciferous vegetables, part of the *Cruciferae* plant family, like broccoli, cabbage and cauliflower can be the cause of a lower risk of cancer. This is believed to be due to the high content of specific biological active compounds found in these vegetables, known as glucosinolates. Glucoraphanin is the glucosinolate found in the highest amounts in these vegetables. Glucoraphanin has obtained much attention as a possible agent that can be used to prevent the development of cancer (chemopreventive agent). It is assumed that the protective effect is because of the inhibition of phase I carcinogen-bioactivating enzymes and/or induction of phase II antioxidant enzymes by isothiocyanates (Perocco *et al.,* 2006; Anwar-Mohamed & El-Kadi, 2008; Yeh & Yen, 2009).

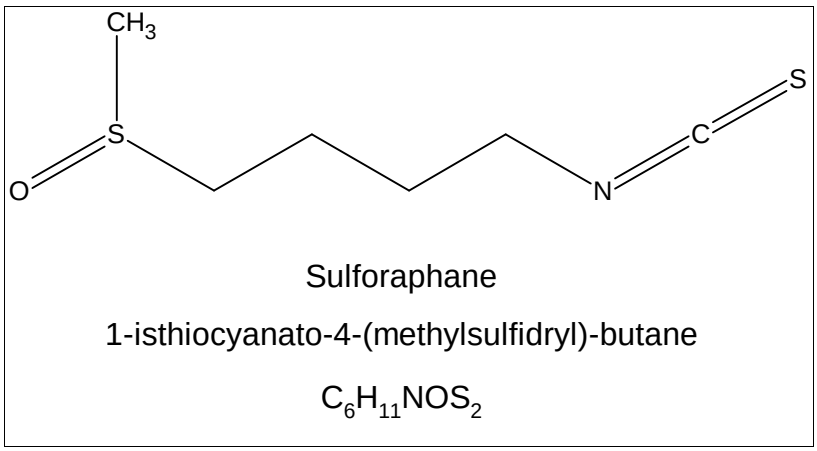

Figure 2.7: The structure of sulforaphane.

Sulforaphane, also known as 1-isothiocyanato-4-(methylsulfinyl)-butane, is an isothiocyanate found in high concentrations in cruciferous vegetables like broccoli. Isothiocyanates are derived from glucosinolates. Sulforaphane has received much attention due to the evidence that sulforaphane inhibits phase I enzymes and activates phase II antioxidant enzymes, as well as MTS (Perocco *et al.,* 2006; Clarke *et al.,* 2008; Yeh &Yen, 2009). The inhibition of phase I prevents the conversion of possible procarcinogens into carcinogens. The activation of phase II enzymes is

important in the prevention of cancer as they biotransform carcinogens into inactive metabolites, which are excreted from the body, thus preventing any cellular damage. If not inactivated, carcinogens can cause DNA damage which leads to genomic instability and possible cancer development. DNA damage is also caused by oxidative stress. ROS is thought to play multiple roles in tumor initiation, progression and maintenance. To prevent this, free radicals are scavenged by MTS, also induced by sulforaphane (Yeh & Yen, 2005; Clarke *et al.,* 2008; Yeh &Yen, 2009). Sulforaphane has been proven to be a potent protector against carcinogens and oxidative damage in cell culture as well as in carcinogen-induced and genetic animal cancer models. Sulforaphane has also been included in human clinical trials with a focus on the chemistry, metabolism, absorption and factors influencing the availability of sulforaphane to specific organs after intake (Clarke *et al.,* 2008; Elbarbry & Elrody, 2011).

2.3.1 Sulforaphane metabolism

As mentioned, the most abundant glucosinolate in cruciferous vegetables is glucoraphanin. The first reaction in the metabolism of sulforaphane involves the transformation of glucoraphanin into sulforaphane (Figure 2.8 A). This reaction is catalyzed by the enzyme myrosinase (β-thioglucoside glucohydrolase; EC. 3.2.3.1), which cleaves the glycine from the glucosinolate to form glucose. Myrosinase are released from the plant cell upon damage to the plant, such as chewing of the raw vegetable (Fimognari & Hrelia, 2006; Elbarbry & Elrody, 2011).

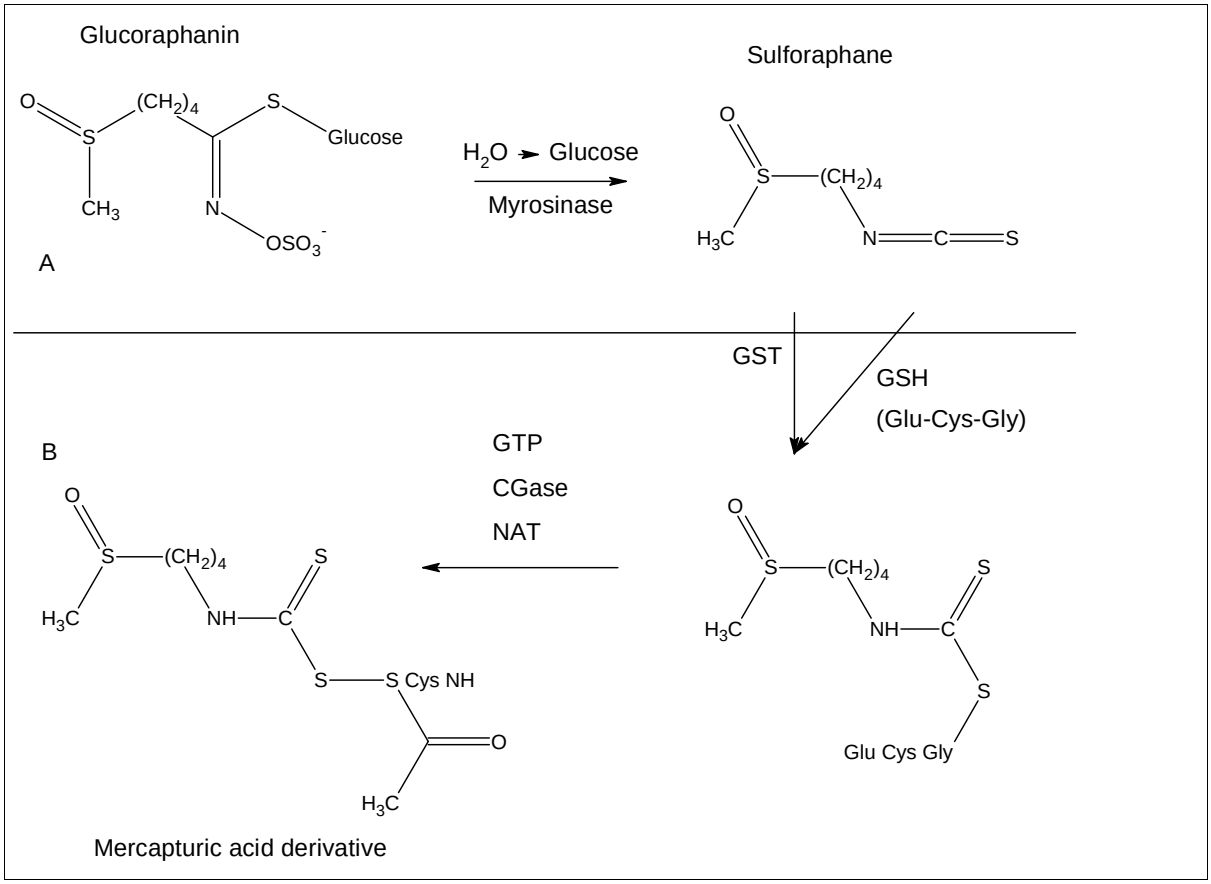

Figure 2.8: Sulforaphane metabolism. The figure illustrates (A) the hydrolysis of glucoraphanin to sulforaphane by myrosinase, and (B) the metabolism of sulforaphane to a mercapturic acid derivative (Adapted from: Elbarbry & Elrody., 2011).

After absorption, sulforaphane is metabolized into a mercapturic acid derivative (Figure 2.8 B). The first step in this enzymatic transformation of sulforaphane involves a reaction catalyzed by glutathione-S-transferase (GST) that causes sulforaphane to undergo conjugation to GSH. The cleavage of glutamine and glycine by the enzymes γ-glutamyl transpeptidase (GTP) and cysteinglycinase (CGase) produces an L-cysteine conjugate. This conjugate is then acetylated by the enzyme *N*-acetyltransferase (NAT) to produce an *N*-acetyl-L-cysteine conjugate, also known as a mercapturic acid derivative, which is excreted into the urine (Elbarbry & Elrody, 2011).

2.3.2 Sulforaphane as an antioxidant

Sulforaphane functions as an indirect antioxidant, providing protection against oxidative stress by boosting the antioxidant capacity of cells through various mechanisms (Fahey & Talalay, 1999). This was experimentally proven in previously done studies by Zhang *et al* (1992); Yeh & Yen (2005); Anwar-Mohamed & El-Kadi (2008); Yeh and Yen (2009) and Sestili *et al* (2010), where the effect of sulforaphane on MTS and biotransformation was investigated. These studies indicated that sulforaphane can effectively induce MT genes. MTS are a family of low molecular mass (6–7 killodalton), cysteine (Cys)-rich, inducible, intracellular proteins that bind heavy metals with high affinity. MTS thus maintain the homeostasis of essential metals, detoxify heavy metals, and protect against oxidative stress (Yeh and Yen, 2009). Yeh and Yen (2005) found that the levels of both MT-I and MT-II messenger RNA (mRNA) increase in a concentration-dependant manner upon treatment of cells with sulforaphane. Sulforaphane has also been shown to be a potent inducer of phase II antioxidant enzymes (Zhang *et al*., 1992; Yeh and Yen., 2009; Sestili *et al*., 2010). By exposing HepG2 cells to sulforaphane, an increase in the mRNA activity of the antioxidant enzymes Heme oxygenase-1 (HMOX-1), NAD(P)H: quinone oxidoreductase (QR), glutathione-S-transferase (GST), gamma-glutamyl cysteine ligase (γ-GCS), and glutathione reductase (GR) was observed. The induction of phase II enzymes by sulforaphane also leads to an increase in the activity of g-glutamylcysteine synthetase. This enzyme is the rate-limiting enzyme of GSH synthesis and an increase in enzyme activity will lead to an increase in GSH levels. As GSH is already present in millimolar concentrations in all cells, such increases in GSH will most probably intensify cellular antioxidant defences (Fahey & Talalay, 1999).

2.4 HUMAN HEPATOCELLULAR CARCINOMA CELLS (HEPARG CELLS) AS A MODEL TO INVESTIGATE BIOTRANSFORMATION

It was recently found that the liver cell line, derived from a human hepatocellular carcinoma, known as HepaRG cells can be used as a valuable *in vitro* model for the investigation of cytochrome P450 (CYP450) induction by drug compounds in

humans. HepaRG cells were shown to maintain liver functions and to express genes for various liver specific proteins, including CYP450 enzymes and transporters of the phase II system (Guillouzo *et al.,* 2007; Kanebratt & Andersson*,* 2008; Lambert *et al.,* 2009; Lübberstedt *et al*., 2011). Studies done by Guillouzo *et al* (2007) and Kanebratt & Andersson (2008) showed that HepaRG cells expressed CYP2B6, CYP2C9, CYP2E1 and CYP3A4, which is in contrast to other hepatocellular carcinoma cell lines like HepG2 cells. CYP3A4 has an important role in the biotransformation of about 50% of drugs in humans (Pernelle *et al.,* 2011). However, the level of expression is depended on the period of confluency of the cells. When HepaRG cells were most differentiated, they expressed CYP450 mRNA at levels comparable to primary human hepatocytes. Stable gene expression for up to thirty days was also reported (Guillouzo *et al.,* 2007; Jossé *et al.,* 2008; Kanebratt & Andersson*,* 2008; Lübberstedt *et al*., 2011). The activity and responsiveness to the inducers CYP3A4 and CYP1A2 were also found to remain relatively stable (Jossé *et al.,* 2008).This proved that HepaRG cells could be a useful model for *in vitro* studies of drug metabolism and toxicity and act as a suitable substitute for primary hepatocytes (Guillouzo *et al.,* 2007; Lübberstedt *et al*., 2011).

2.5 EXPERIMENTAL APPROACH

The experimental approach of this study was as follows:

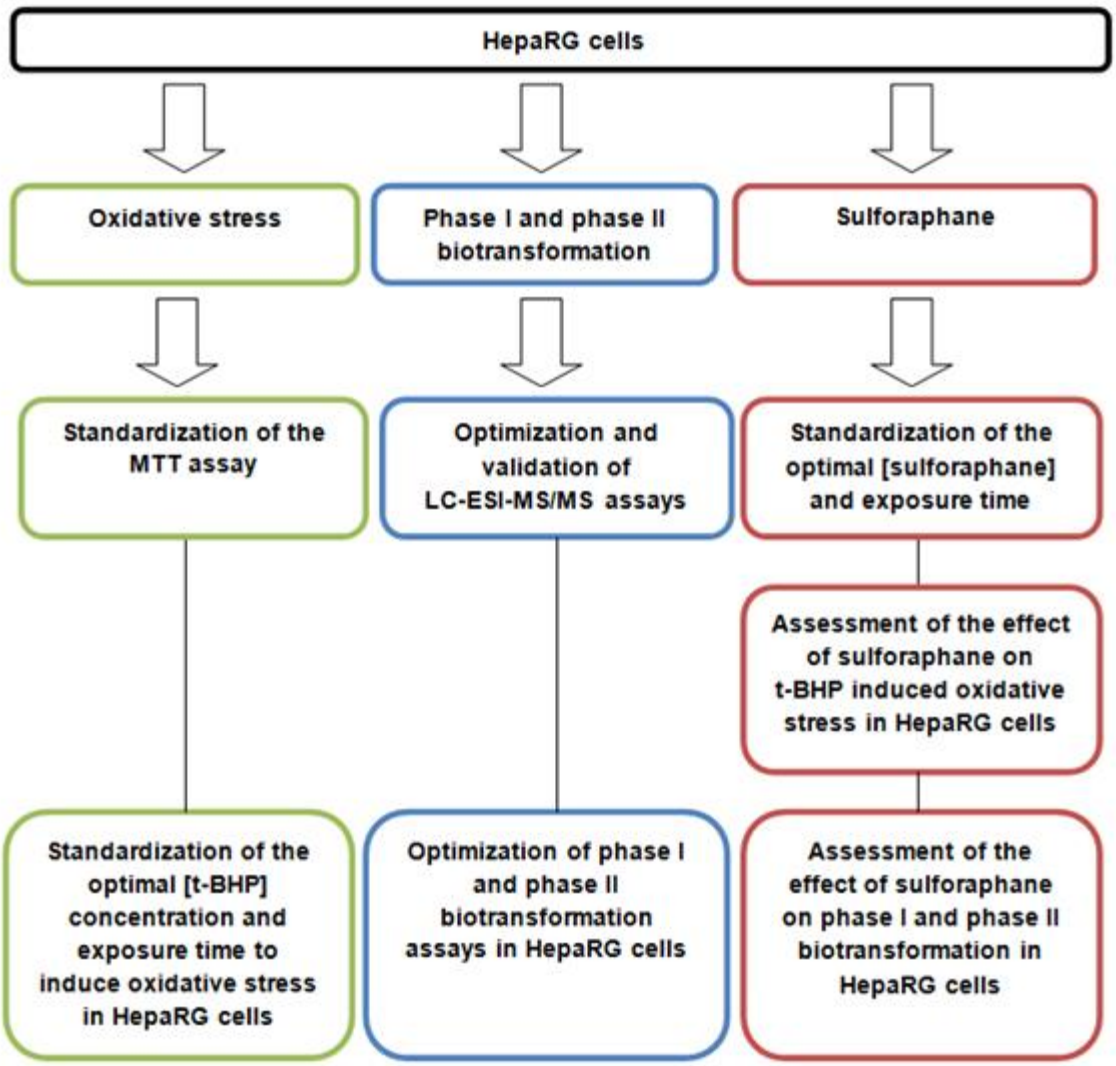

Figure 2.9: Visual representation of the experimental approach of this study.

CHAPTER 3

Tissue cultures

3.1 Introduction

The HepaRG cell line was established from a liver tumor in a female patient suffering from hepatitis C. It is likely that HepaRG cells were developed from tubular structures in the liver (billary ducts) and not from primary hepatocytes, due to long term hepatitis C infection (Guillouzo *et al*., 2007; Parent *et al.*, 2004). HepaRG cells are progenitor cells because they are able to differentiate into two different cell lines when seeded at a low density (2.6 x 10^4 cells/cm^2) (Guillouzo *et al.,* 2007; Kanebratt & Andersson*,* 2008; Lübberstedt *et al*., 2011; Pernelle *et al.,* 2011). These HepaRG cell cultures then contain hepatocyte-like and biliary-like epithelial cells with a hepatocyte population of approximately 50-55% (Kanebratt & Andersson*,* 2008; Lübberstedt *et al*., 2011). Hepatocyte-like cells express various phase I and phase II biotransformation enzymes at levels close to those in hepatocytes (Anthérieu *et al.,* 2012). Guillouzo *et al* (2007) and Kanebratt & Andersson (2008) showed that by adding 2% dimethyl sulphoxide (DMSO) to the cells, the hepatocyte-like cells were able to differentiate into more granular cells that closely resembled adult primary hepatocytes. When HepaRG cells are seeded at high density (0.45 x 10^6 cells/cm^2) they have a restricted proliferation activity and keep their hepatocyte-like features (Guillouzo *et al.,* 2007). Cryopreserved HepaRG cells (Lot: N1956555) were obtained from Biopredic International (Rennes, France) in a 1ml cryopreserved vial containing > 8 x 10^6 cells. From these, more cultures were established to obtain enough cells for experimental use.

3.2 Materials and culturing methods

3.2.1 Chemicals and reagents

The purest available reagents were purchased. These reagents included: 95% ethanol (C_2H_5OH, Rochelle Chemicals), trypsin-EDTA (10 x dilution of 0.5%

trypsin, 5.3 mM EDTA.4Na, BioWhittikar™), WME (Williams medium E, Invitrogen™), with 10% (v/v) FBS (foetal bovine serum, E.U approved origin, BioWhittikar™), 1% (v/v) L-Glut (L-Glutamine, BioWhittikar™), 1% (v/v) NEAA (non-essential amino acids, BioWhittikar™), and 1% (v/v) Pen/Strep (penicillin/streptomycin antibiotic solution, 5 000 units/ml penicillin G sodium and 5 000 µg/ml streptomycin sulfate in 0.85% saline, BioWhittikar™), HepaRG maintenance/metabolism medium supplement (Invitrogen™), thiazol blue tetrazolium bromide (trypan blue solution, 97.5% (TLC), suitable for cell culture, Sigma-Aldrich), PBS (phosphate buffered saline without calcium or magnesium, Sigma-Aldrich), DMSO (dimethyl sulphoxide, Sigma-Aldrich), *t*-BHP (*tert*-Butyl hydroperoxide, Sigma-Aldrich), MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, 97.5% (TLC), suitable for cell culture, Sigma-Aldrich) and acetic acid (CH3COOH, Sigma-Aldrich).

3.2.2 HepaRG cellular growth conditions

HepaRG cells were cultured at 37 ºC in 5% medical CO_2 in a HERA cell incubator (Kendro Laboratory Products). The base WME growth medium consisted of 500 ml high glucose WME to which 50 ml FBS, 5 ml L-Glut, 5 ml NEAA and 5 ml Pen/Strep were added, respectively. The working growth medium consisted of the complete base WME growth medium, to which 15 ml HepaRG maintenance/metabolism supplement was added, to form a supplement working growth medium.

HepaRG cells were cultured in a high glucose growth medium. To enable survival in conditions of low O_2, cancer cell lines mainly use glycolysis to produce energy, while suppressing oxidative phosphorylation (OXPHOS) (Diaz-Ruiz *et al.,* 2010). Theoretically, the functions of the different components of the media are as follows: FBS improve cell attachment and supply extra nutrients and hormone-like growth factors that increase the growth of healthy cells (Wilson & Walker, 2005). Cancer cells also use glutaminolysis as another source of energy. The amino acid, glutamine is degraded in this pathway to produce extra energy needed for cell proliferation and survival (Diaz-Ruiz *et al.,* 2010). NEAA provide a source of amino acids used by

cells to biosynthesize proteins and also provide amino acid cofactors used in phase II conjugation reactions. Although NEAA can be synthesized by cells, adding extra NEAA decrease the metabolic burden on cells and allow for faster proliferation. Pen/Strep antibiotic solution prevents the growth of a wide range of bacteria. Penicillin inhibits the last step in bacterial cell wall synthesis and streptomycin blocks protein synthesis (Wilson & Walker, 2005). HepaRG maintenance/metabolism medium supplement is a specialised medium supplement for HepaRG cell cultures intended to be used in metabolism studies. It is used to supplement the daily growth and metabolism of HepaRG cell cultures.

3.2.3 HepaRG standard culturing procedures

The following procedures were carried out in a sterilised Clear Flow ^{TM} laminar flow chamber. All objects were sterilised externally with 70% ethanol solution before they were placed inside the laminar flow chamber. All of the necessary steps were followed to ensure a sterile working environment.

3.2.3.1 Start-up of HepaRG cultures and change of growth medium

HepaRG cells were cultured in 25 cm^2 Nunclon EasYFlask (Angled necks and vented caps, AEC-Amersham) cell culture flasks containing either 5 ml base- or working WME growth medium. A frozen vial of HepaRG cells was thawed (not completely, crystals still remained in the vial). The contents of the vial were then transferred to a centrifuge tube with the 2 ml growth medium. After centrifugation at 1 200 *g* and 22 ºC for 3 minutes, the supernatant was discarded. The pellet was then resuspended in 1 ml growth medium. The total volume cell suspension was added to a 25 cm^2 cell culture flask containing 4 ml growth medium to prepare a total of 5 ml of cell suspension. The cell suspension was evenly spread over the bottom surface of the cell culture flask to ensure even cell proliferation over the whole surface of the cell culture flask. The flask was then sterilised externally and incubated. On the next day the growth medium was removed and replaced with 5 ml fresh growth medium. After this initial change, cell growth was inspected daily and the growth medium

changed every second day to ensure HepaRG cells had enough supplements and growth factors to proliferate successfully.

To replace the growth medium, the cell culture flask was firstly removed from the incubator, sterilised, and placed in the laminar flow chamber. The growth medium was decanted in a large falcon tube and 5 ml fresh growth medium was added. The cell culture flask was then sterilised again and placed back in the incubator. The growth medium was aspirated into a large vacuum container for disposal at a later stage. This was done until the cell culture flask reached a total confluence of about 80%-90%. The cells were then trypsinized (Section 3.2.3.2) and either transferred to a new cell culture flask for culturing purposes, harvested (Section 3.2.3.3) and seeded into cell culture plates (Section 3.2.3.4 and 3.2.3.5) for experimental purposes, or cryopreserved (Section 3.2.3.6) in vials for later use.

3.2.3.2 Trypsinization of HepaRG cells

This procedure started with the removal of growth media by transferring it to a waste container. As cellular growth medium deactivates trypsin-EDTA, the previous step was followed by washing the cells twice with PBS to remove all growth medium. This was followed by adding 0.5 ml trypsin-EDTA to a 25 cm^2 cell culture flask after which the cell culture flask was sterilised and incubated for 2-3 minutes at 37 ºC. After the removal of the cell culture flask from the incubator, the cells were shaken loose by tapping the cell culture flask on the hand palm to form a trypsin-EDTA cell suspension. The cell suspension in the flask was then transferred to a 10 ml conical tube. To remove the trypsin-EDTA present in the growth medium and avoid any further cellular damage, the tube was centrifuged at 1 200 *g* and 22 ºC for 3 minutes. The supernatant was discarded and the pellet suspended in 2 ml fresh growth medium. This solution was the master solution used for either experimental or culturing purposes.

3.2.3.3 Harvesting of HepaRG cells

To harvest HepaRG cells for experimental purposes, an 80%-90% confluent cell culture flask was trypsinized as described in Section 3.2.3.2. Depending on the size of the cell culture plate, a pre-determined amount of the master solution (calculated in Section 3.2.3.4) was then transferred to a pre-determined amount of wells, present in a TPP (Techno Plastics Product, AEC-Amersham) 96-well flat bottom cell culture plate. The cell culture plate was then sterilised and placed in the incubator to allow for proliferation of the cells.

To harvest HepaRG cells for culturing purposes, an 80%-90% confluent cell culture flask was trypsinized as described in Section 3.2.3.2. To split one 25 cm^2 cell culture flask in two 25 cm^2 cell culture flask (1:2), the 2 ml master solution was divided in two portions of 1 ml each. Each 1 ml portion was transferred to a new 25 cm^2 cell culture flask to which 4 ml of fresh growth medium was added to make up a total of 5 ml of cell suspension (this formula can be adjusted to split the cells into more than two fractions). The cell culture flasks were then sterilised and placed in the incubator.

3.2.3.4 Counting of HepaRG cells

This method was used to determine the amount of HepaRG cells in 1 ml of master solution. This was necessary to ensure cells were seeded at the correct density. One 80-90 % confluent 25 cm^2 cell culture flask was used to make up a master solution of cells using the trypsinization method described in Section 3.2.3.2. 10 µl of the master solution was then added to a mixture of 15 µl PBS and 25 µl trypan blue solution in a 1 ml Eppendorf ^{TM} tube (Merck) to give a total volume of 50 µl. In order to count cells using a microscope, 20 µl of the 50 µl solution was added to a heamocytometer. As illustrated in Figure 3.1, 10 µl was added on the X part and 10 µl on the Y part of the haemocytometer. The haemocytometer was then positioned on a light microscope to view the trypan blue coloured cells. Five squares, of X and Y on the haemocytometer, were counted and the total was used to calculate the total number of cells present per ml in the master solution (Equation 3.1).

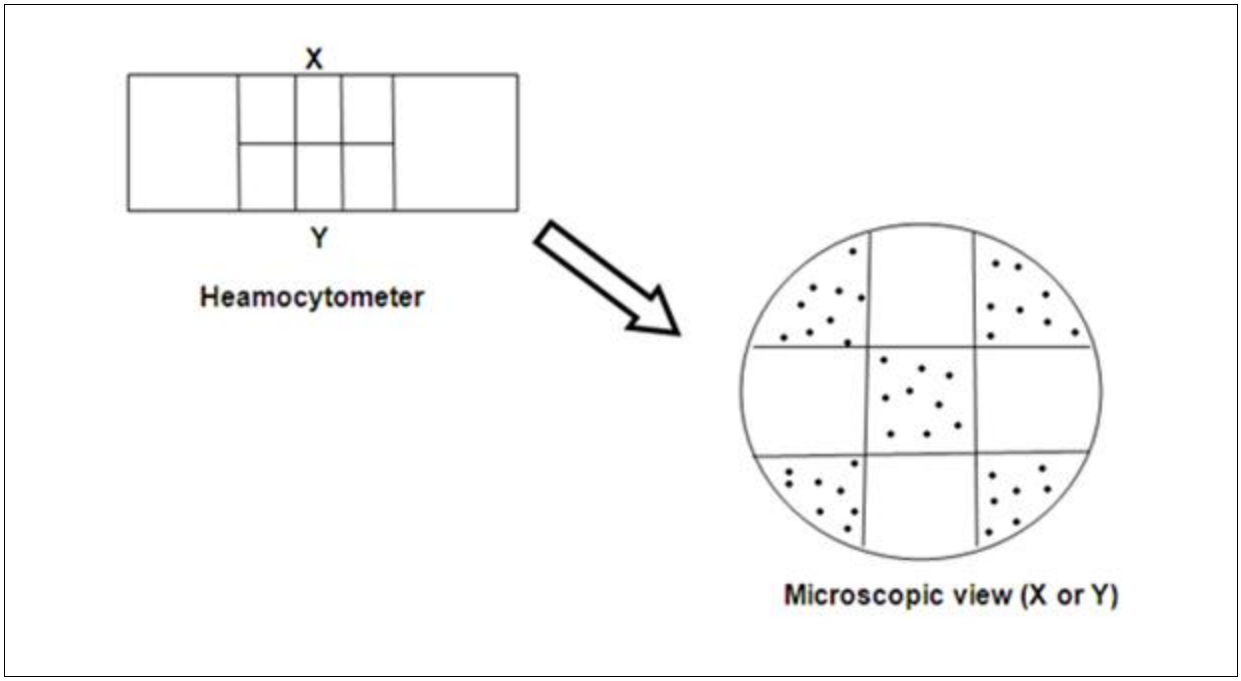

Figure 3.1: An illustration of the haemocytometer method used to count HepaRG cells. To count the amount of coloured cells, 10 µl of the cell suspension was withdrawn from the master solution in the flask and added to 15 µl PBS and 25 µl trypan blue dye in an Eppendorf tube to give a total volume of 50 µl. 20 µl of this solution was transferred to a heamocytometer to count the cells using a light microscope (10 µl on X and 10 µl on Y). The haemocytometer was then placed underneath a microscope to view the trypan blue coloured cells. Five squares of the heamocytometer were counted and the total used to calculate the total amount of cells present per ml master solution using Equation 3.1.

Equation 3.1: Equations used for the calculation of the amount of HepaRG cells per millilitre of master solution.

Total amount of cells counted (X + Y) / 2 = average amount present on heamocytometer
Average amount present on heamocytometer / 5 blocks = amount of cells per block
Amount of cells per block x (5 x 10^5) dilution factor = amount of cells per ml

3.2.3.5 Seeding of HepaRG cells into wells

TPP (Techno Plastics Product, AEC-Amersham) 96-well flat bottom cell culture plates were used. HepaRG cells were seeded at a density of 1 x 10^5 cells per well. The amount of master solution in µl to be added to each well were calculated from the total amount of cells per ml, obtained from Equation 3.1, using Equation 3.2.

Equation 3.2: Equations used for the calculation of the amount of HepaRG cells to be seeded per well of a Techno Plastic Product 96-well flat bottom cell culture plate.

From Equation 3.1 the amount of cells present per ml in the master solution was calculated.

Amount of cells needed per well:

For a 96-well cell culture plate: 1×10^5 cells per well

Thus to calculate the amount of master solution to be added per well to obtain 0.10×10^6 cells per well:

For a 96-well cell culture plate = 1×10^5 / amount of cells per ml
= answer x 1000 (convert to µl)
= amount of cell suspension in µl to be added to each well for 1×10^5 cells per well

Table 3.1: Seeding of HepaRG cells into Techno Plastic Product 96-well flat bottom cell culture plates.

Plate format	Number of viable cells per well ($\times 10^5$)	Volume per well (ml)	Cell concentration ($\times 10^5$/ml)
96-well cell culture plate	1	0.10	1

In the case of a 96-well plate, after the cell suspension was added to the well, the well was topped up to a final volume of 200 µl using cell culture media.

Even though great care was taken to ensure HepaRG cells were seeded at the same density, the amount of cells present in each well, after confluence is reached, differed from each other. This can be due to different rates of cell proliferation and death. Thus, for all experiments in this study, cells were seeded in a TPP 96-well cell culture plate in triplicate. This ensured that, like in the case of the MTT assay, an average absorption value could be determined and any possible human experimental errors could be minimalized. After the cells were seeded, the covered cell culture plate was sterilised and incubated for one day after which the growth medium was replaced with 200 µl fresh growth medium. The growth medium was replaced every second day and the growth monitored using a light microscope until adhesion and proliferation of the HepaRG cells on the bottom of each of the seeded wells was 80%-90% confluent.

3.2.3.6 Cryofreezing of HepaRG cells

An 80%-90% confluent 25 cm^2 cell culture flask was trypsinized according to methods described in Section 3.2.3.2. After the supernatant was discarded, the pellet was suspended in 1 ml freezing media which consisted of 6.3 ml FBS and 0.7 ml DMSO. The FBS was added to improve the cell attachment and supply extra nutrients and hormone-like growth factors that elevate the growth of healthy cells (Wilson & Walker, 2005) once the cells were thawed and cultured. The DMSO prevented the formation of ice crystals and the lysis of cells during thawing. The volume was then transferred to a 1 ml cryovial. After each cryovial was correctly marked, they were frozen in liquid nitrogen or stored at -80 ºC.

3.3 Induction of oxidative stress in HepaRG cells with *tert*-Butyl hydroperoxide

3.3.1 Introduction

In order to investigate the effect of sulforaphane on oxidative stress in HepaRG cells, oxidative stress was exogenously induced with *t*-BHP. As discussed in Section 2.1.4.1, *t*-BHP is an organic hydroperoxide and is routinely used to induce oxidative stress in *in vitro* models (Lapshina *et al.,* 2005; Lima *et al.,* 2006). After *t*-BHP crosses the cellular membrane, it causes damage such as peroxidation of membrane lipids, DNA damage, cellular ATP depletion, and a loss in mitochondrial membrane potential, which all eventually lead to cell death (Lapshina *et al.,* 2005; Lima *et al.,* 2006). *t*-BHP also causes depletion in the levels of the most abundantly produced endogenous antioxidant in eukaryotic cells, GSH. The liver is one of the organs in the body with the highest content of GSH (Nishida *et al.,* 1997). *t*-BHP could thus be used to exogenously induce oxidative stress in HepaRG cells.

The toxicity of *t*-BHP towards HepaRG cells were indicated by the amount of viable cells, quantified by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. In Section 3.3.2.2, the standardization of the MTT assay in HepaRG cells is discussed, followed by the standardization of the optimal *t*-BHP concentration and time of exposure to induce oxidative stress in HepaRG cells in Section 3.3.2.3.

3.3.2 The cell viability test

The use of tetrazolium salt in a quantifiable colorimetric assay for cell survival and proliferation was first described by Mosmann (1983). The MTT assay is a colorimetric assay, used in this study to determine the toxicity of substances towards HepaRG cells.

3.3.2.1 Principle of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

As illustrated in Figure 3.2, the principle of the MTT assay is based on the conversion of the yellow, water soluble substrate, MTT, by the metabolically active mitochondrial dehydrogenase enzymes to form deep purple coloured formazan salts, which are insoluble in water. After the insoluble crystals have been dissolved, the concentration of the formazan product can be colorimetrically quantified (Freimoser *et al*. 1999).

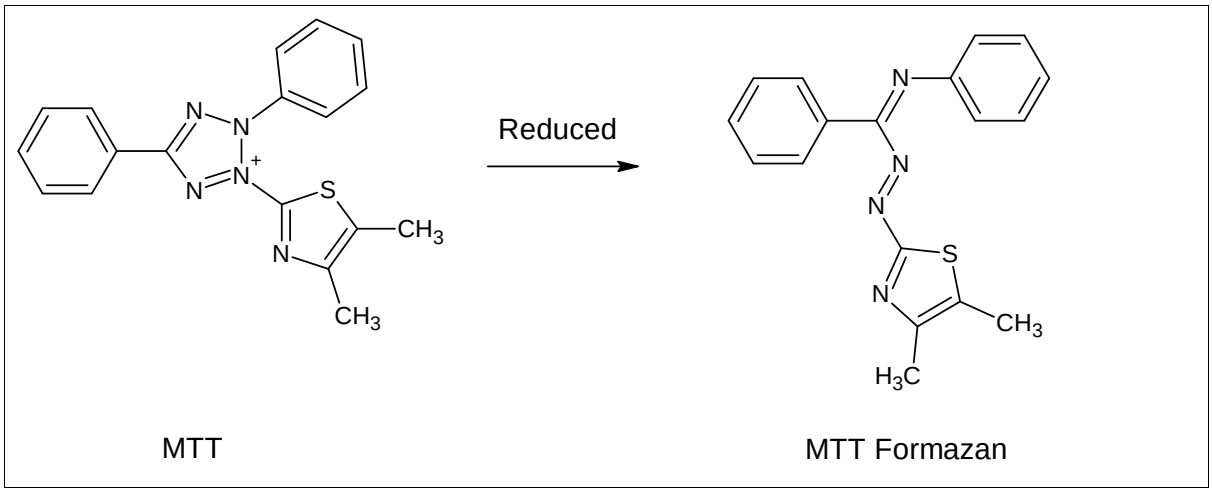

Figure 3.2: An illustration of the principle of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. In live cells the metabolically active mitochondrial dehydrogenase enzymes will reduce the yellow, water soluble substrate, MTT, to form deep purple coloured MTT formazan salts, which are insoluble in water (Adapted from Mosmann, 1983).

3.3.2.2 Standardization of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

Standardization of the MTT assay was done to test the principle of the MTT assay and to ensure the consistent functioning of the colour reaction. Positive and negative controls were also included to serve as baselines to which MTT results could be compared. As a positive control, acetic acid was used to cause almost complete cell death. This control acted as an indication of a colour reaction obtained from a MTT assay at low cell viability. To the negative control, no chemicals were added. This control acted as an indication of a colour reaction obtained from a MTT assay at almost 100% cell viability. However, the volume of acetic acid needed per well to act as a positive control had to be optimized.

Pre-incubation of cells with acetic acid

TPP 96-well cell culture plates (80%-90% confluent) were used for the MTT assay and all analyses were performed in triplicate. Negative control cells, which contained a final volume of 200 µl growth media, were not treated at all, while positive control wells were treated with increasing volumes of acetic acid (10 µl-100 µl topped up to a final volume of 200 µl using growth media). Blank wells received 200 µl PBS. These wells acted as the blanks for the MTT assay and did not contain any cells at all. The covered cell culture plate was then sterilised and incubated for 30 minutes in the incubator.

The conditions of the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

After the 30 minute incubation period was over, the cell culture plate was removed from the incubator, sterilised, and placed in the laminar flow chamber. Cells were washed twice with PBS to remove any residual acetic acid and dead cells. This was followed by adding 20 µl of the MTT solution (5 mg/ml) to each well and 180 µl of base growth media for survival of the live cells. The plate was sterilised and incubated for 5 hours to allow the metabolically active mitochondrial dehydrogenase

enzymes, present in the viable cells, to reduce the yellow, water soluble substrate, MTT, to form the deep purple coloured MTT formazan salts. Following the incubation period, 200 µl DMSO was added to each well to dissolve the crystals. The plate was shaken lightly to assist with the process. Optical density was measured in a BIO-TEC FL600 fluorescence plate reader (Analytical & Diagnostics Products) at a wavelength of 560 nm. The background absorption was measured at 630 nm and automatically subtracted by the plate reader software to provide the delta absorption. The percentage of viable cells was calculated by Equation 3.3.

Equation 3.3: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay: Calculation of the percentage of viable cells.

% cellular viability = (Δ sample – Δ blank) / (Δ control – Δ blank) x 100

Where: ***Δ sample =*** *absorbance of the sample at 560 nm – absorbance of the sample at 630 nm* ***Δ blank =*** *absorbance of the blank at 560 nm – absorbance of the blank at 630 nm* ***Δ control =*** *absorbance of the cell control at 560 nm – absorbance of the cell control at 630 nm*

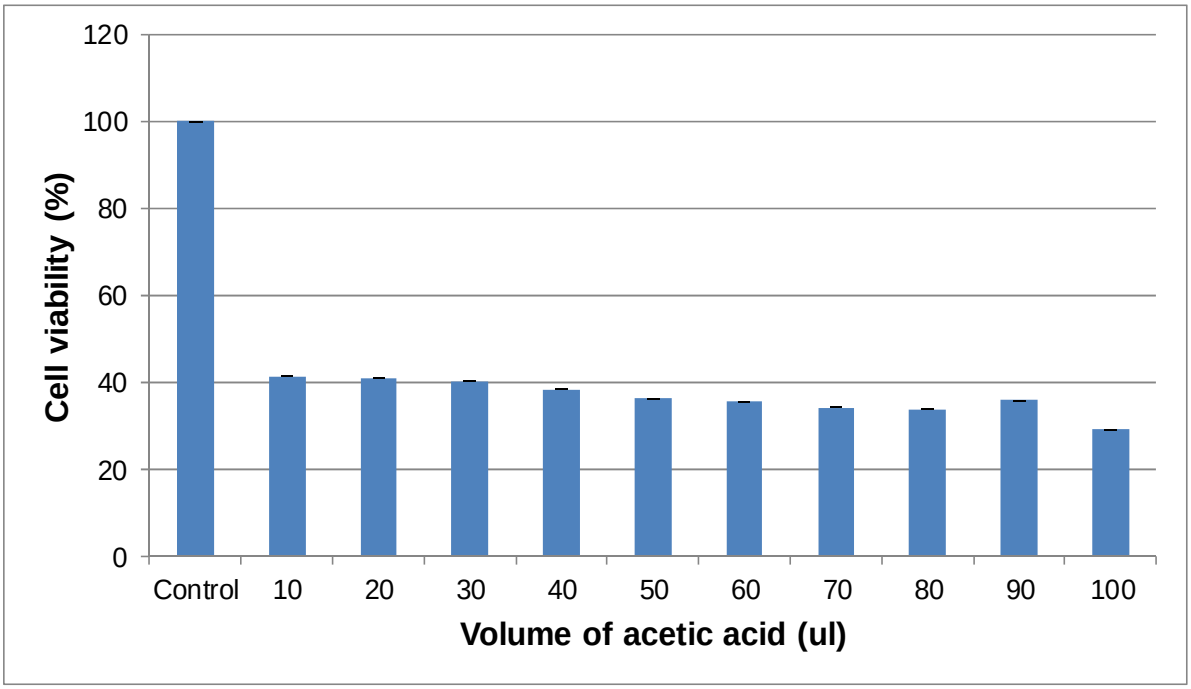

Figure 3.3: The effect of acetic acid on the cell viability in HepaRG cells The Y-axis represents the cell viability (%) and the X-axis the volume of acetic acid in microliters (µl) added to the HepaRG cells. 80%-90% confluent cells were treated with increasing volumes of acetic acid (10 µl-100 µl topped up to a final volume of 200 µl using growth media). The cells were then incubated for 30 minutes. To the control wells no chemicals were added. The cell viability was determined with the MTT assay. Each data point in the figure represents the mean ± SD values of three separate wells.

From Figure 3.3 it can be observed that the cell viability was set at 100% and the positive controls (acetic acid wells) had a cell viability that ranged between 30%-40%. This was a clear indication that the acetic acid caused a high amount of cell death when added to HepaRG cells. The results also indicated that the MTT assay was successful. This was supported by the difference in colour observed between the control wells (with high cell viability) and acetic acid wells (with low cell viability). In the control wells, a higher amount of cells were viable and thus higher amounts of the metabolically active mitochondrial dehydrogenase enzyme was present to convert the yellow MTT to form the deep purple coloured formazan salts. These wells displayed a dark purple colour after the formazan salts were dissolved in DMSO. The colour in the positive control wells (acetic acid), where less mitochondrial dehydrogenase enzymes were present, varied between light purple to yellow. From Figure 3.3 it was also observed that 10 µl of acetic acid already caused a high percentage of cell death, indicated by the low % of cell viability observed in the figure. It was thus decided that 10 µl of acetic acid added to HepaRG cells for 30 minutes would act as the positive control in the MTT assay for the rest of this study.

3.3.2.3 Standardization of the optimal *tert*-Butyl hydroperoxide concentration and time of exposure to induce oxidative stress in HepaRG cells

t-BHP affects each cell type differently (Lapshina *et al.,* 2005; Lima *et al.,* 2006). It was thus necessary to first examine the effect of different *t*-BHP concentrations on HepaRG cells to determine which concentration of *t*-BHP will consistently cause maximum *in vitro* oxidation without causing any major cell death. This was done by adding different concentrations of *t*-BHP to HepaRG cells for different periods of time, followed by the MTT assay to determine the cell viability of the cells after exposure to *t*-BHP.

Pre-treatment of cells

After inspecting the confluence of the cells the next step was to add different concentrations of *t*-BHP to the cells. Based on a study done by Lima (2006), where

the human hepatocellular carcinoma cell line, HepG2 cells, were exposed to 1 mM and 2 mM *t*-BHP for different time periods between 2 to 8 hours, it was decided to test a *t*-BHP concentration range from 0 mM to 10 mM at the time intervals of 1 hour, 3 hours and 6 hours. A broad *t*-BHP concentration range was used to determine which concentration of *t*-BHP caused minimal cell death and which concentration caused major cell death. As mentioned, the aim was to only induce oxidative stress in HepaRG cells without causing major cell death. Thus it was decided to use 0.25 mM, 0.5 mM, 0.75 mM, 1 mM, 5 mM and 10 mM *t*-BHP, respectively to investigate the effect of increasing concentrations of *t*-BHP on the HepaRG cells. Each well contained 190 µl fresh base WME growth medium and 10 µl of a specific *t*-BHP stock solution (dissolved in PBS) which added to give a final volume of 200 µl in each well. Three identical plates were prepared and incubated for 1 hour, 3 hours and 6 hours, respectively. After this incubation, the MTT assay was performed on all wells, as described in Section 3.3.2. The results obtained from the completed MTT assay are displayed in Figure 3.4.

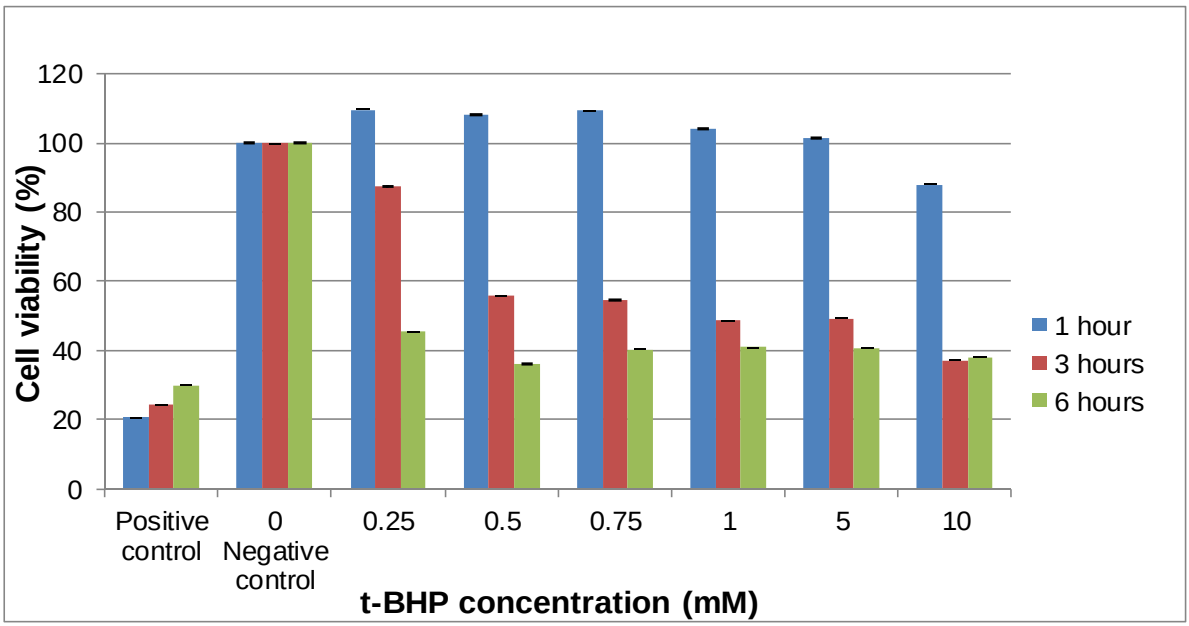

Figure 3.4: The effect of different *tert*-Butyl hydroperoxide concentrations and times of exposure on the cell viability of HepaRG cells. The Y-axis represents the cell viability (%) and the X-axis the different *t*-BHP concentrations in millimolar (mM) added to the HepaRG cells. After inspecting the confluence of the cells, different concentrations of *t*-BHP (0.25 mM-10 mM) was added to the cells. To the positive control acetic acid was added and to the negative control, no chemicals. Three identical plates were prepared and incubated for 1 hour, 3 hours and 6 hours, respectively. After this incubation, the MTT assay was performed on all wells. Each data point in the figure represents the mean ± SD values of three separate wells.

Figure 3.4 represents the effect of different *t*-BHP concentrations (0.25 mM-10 mM) and exposure times (1, 3 and 6 hours) on the viability of HepaRG cells. From Figure 3.4 it can be observed that at 1 hour HepaRG cell viability ranged between 100%-110% when exposed to 0.25 mM-5 mM of *t*-BHP. Compared to the negative control, an increase in the % cell viability was observed which indicated an increase in HepaRG cell growth over the period of *t*-BHP incubation. This was a clear indication that 1 hour of 0.25 mM-5 mM *t*-BHP exposure was not sufficient enough to cause oxidative stress in the HepaRG cells. The figure also indicates that when HepaRG cells were incubated for 6 hours with 0.25 mM-10 mM of *t*-BHP it caused major cell death. This was supported by the low cell viability (between 30%-43%) and proves that 6 hours of exposure to 0.25 mM-10 mM of *t*-BHP was long enough for *t*-BHP to not only cause oxidative stress but also major cellular damage. From the figure it can be observed that after HepaRG cells were exposed for three hours to 0.25 mM *t*-BHP 85% of cells were still viable and to 0.50 mM *t*-BHP for three hours 58%. As it was not certain which level of cell viabillity was sensitive enough to induce oxidative stress and indicate the possible protective effect of sulforaphane, it was decided to use both 0.25 mM and 0.50 mM *t*-BHP concentrations, added to the cells for three hours, in all further experiments where oxidative stress is induced in HepaRG cells.

3.4 Biotransformation in HepaRG cells

3.4.1 Introduction

As discussed in Section 2.2.4, the use of animal models to assess biotransformation is increasingly replaced with *in vitro* cellular models. Since the liver is the most important organ in the body where toxins are biotransformed, it is frequently used in tests where the toxicity and biotransformation of compounds is investigated (Jover *et al.,* 1992). Human liver cell lines derived from tumor cells are thus mostly used in studies of biotransformation as they have the advantage of continuous growth and reproducible metabolism (Brandon *et al*., 2003). The HepaRG cell line expresses most of the liver specific genes at higher levels compared to other cell lines, especially in biotransformation enzyme activity (Lambert *et al.,* 2009; Pernelle *et al.,* 2011; Anthérieu *et al.,* 2012).

The assessment of the biotransformation metabolic activity can be done by challenging phase I and phase II reactions with specific probe substances whose biotransformation pathways are well known. The individual introduction of probes into cells provides the possibility to investigate the role of either phase I or phase II in the complete biotransformation of the probe (Schrader *et al.,* 1999; Liska *et al*., 2006). To investigate the effect of sulforaphane on biotransformation in HepaRG cells, phase I and phase II biotransformation pathways were assessed with caffeine, paracetamol, aspirin, sodium benzoate, and para-aminobenzoic acid, respectively, as probe substances. The identification and quantification of the selected phase I and phase II metabolites of each probe substance required a specific analytical method, and sample preparation was an essential part of the analytical procedure (Kostiainen *et al.,* 2003). HepaRG cells were incubated with these probe substances. This was followed by the preparation of the samples for analysis by liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS/MS). The methods used to incubate HepaRG cells with the specific probes, as well as the sample preparation method are discussed in Chapter 3. The LC-ESI-MS/MS assays used to analyse the samples are discussed in Chapter 4.

3.4.1.1 Pre-treatment of cells

Cells where seeded in a 96-well cell culture plate and used when the confluence was 80%-90%. First the cell media was removed and 190 µl fresh base WME growth medium was added to each well, followed by 10 µl of a pre-determined concentration probe substance, which added up to a total volume of 200 µl in each well. The covered plate was then sterilised and incubated for the pre-determined period of time needed for the biotransformation of the specific probe.

3.4.1.2 Cell lysis

In order to quantify the formed metabolites of each probe substance, the cell content needed to be released from the cell into the surrounding media. After the incubation time with the probe substance, the plate was removed from the incubator. The

200 µl base WME growth medium, present in each well, was transferred to a 1.5 ml microcentrifuge tube (Merck). This was followed by adding 50 µl distilled water (ddH_2O) to each well of the plate. This caused endosmosis in the cells which led to cell lysis. To ensure complete cell lysis a 200 µl pipette point was used to scrape the bottom of the wells. The 50 µl water volume was then added to the 200 µl base WME growth medium, in the microcentrifuge tube, to form a 250 µl solution.

3.4.1.3 Acetonitrile deproteinisation of samples

Compounds which may interfere in the LC-ESI-MS/MS spectrum, like proteins and salts, were removed during sample preparation. This was necessary to prevent damage of the chromatographic column and increase the selectivity and sensitivity of the analysis (Kostiainen *et al.,* 2003). WME growth medium contains a high protein and salt concentration. Thus to prevent damage to the column used in the LC-ESI-MS/MS assay, these proteins needed to be removed from the 250 µl solution through an acetonitrile deproteinisation method. To avoid the hydrolysis of conjugates like glucuronides and sulfates, the organic solvent acetonitrile was used in the precipitation of proteins (Kostiainen *et al.,* 2003).

First, 250 µl acetonitrile was added to the existing 250 µl tissue extract, present in each microcentrifuge tube, to form a 500 µl solution. The addition of the ddH_2O and acetonitrile diluted the original 200 µl base WME growth medium with a factor of 2.5 (x 2.5). The tubes were placed on ice for 10 minutes to aid in the precipitation of proteins. After 10 minutes the tubes were removed from the ice and centrifuged at 25 000 x *g* at 4 °C for 30 minutes. A protein pellet could be seen at the bottom of each tube after they were centrifuged. The entire volume of supernatant was transferred to a new microscentrifuge tube. The tubes with the supernatants were again centrifuged at 25 000 *g* at 4 °C for 30 minutes to ensure all protein was removed from the solution. The entire volume of supernatant was again transferred to a new microcentrifuge tube. The 500 µl supernatant was then diluted (x 1.3) by adding 385 µl of the supernatant to 615 µl ddH_2O to form a 1000 µl solution. These

tubes were either frozen at -80 °C for later use or used immediately for analysis using a LC-ESI-MS/MS assay.

3.4.1.4 Freeze-drying of samples

Before the samples could be analyzed by the LC-ESI-MS/MS, each sample was freeze-dried. The 1000 µl solution was diluted further (x 2.6) by adding 400 µl of the solution and 200 µl internal standard (IS) together in a test tube (Plastpro Scientific) to form a 600 µl solution. Each tube was then stored in a -80 °C freezer for ± 1 hour or until all fluids were frozen. This was followed by placing the tubes in a freeze-dryer for ± 5 hours, or until all the samples were dried. The pellets were dissolved in 400 µl aqueous mobile phase which consisted of 1 ml formic acid in 1000 ml MilliQ water (pH 3.5). Finally, 100 µl of this solution was added to an insert in a vial for analysis by a LC-ESI-MS/MS. A final dilution factor of 8.45 (2.5 x 1.3 x 2.6) was calculated. The final concentration (mg/L) of phase I or phase II metabolites, present in the LC-ESI-MS/MS analyzed samples, was calculated by multiplying the calculated concentration value (Equation 4.2) of each metabolite with an 8.45 dilution factor.

CHAPTER 4

Optimization of LC-ESI-MS/MS assays to monitor phase I and phase II biotransformation

4.1 Introduction

The assessment of the biotransformation metabolic activity can be done by challenging phase I and phase II reactions with specific probe substances whose biotransformation pathways are well known. The individual introduction of probes into cells provides the possibility to investigate the role of either phase I or phase II in the complete biotransformation of the probe (Schrader *et al.,* 1999; Liska *et al*., 2006). To investigate the effect of sulforaphane on biotransformation in HepaRG cells, phase I and phase II biotransformation pathways was investigated with caffeine, paracetamol, aspirin, sodium benzoate, and para-aminobenzoic acid, respectively, as probe substances. After the incubation of HepaRG cells with a specific probe substance, the cells were prepared for LC-ESI-MS/MS analyses as described in Sections 3.4.1.1 to 3.4.1.4. However, an LC-ESI-MS/MS assay for the metabolites of each probe substance first had to be established.

4.2 Materials, standards, and solutions

4.2.1 Chemicals and reagents

The purest available reagents were purchased. These reagents included: caffeine ($C_8H_{10}N_4O_2$, Reagentplus^{TM}, powder, Sigma-Aldrich), paracetamol (4-acetamidophenol, $CH_3CONHC_6H_4OH$, 98%-101%, powder, Sigma-Aldrich), 2-acetamidophenol ($CH_3CONHC_6H_4OH$, 97%, powder, Sigma-Aldrich), paracetamol mercapturate ($C_{13}H_{15}N_2NaO_5S$, solid, Toronto Research Chemicals), paracetamol sulfate (C8H9NO5S, solid, ≥ 97%, HPLC, Sigma-Aldrich), paracetamol glucuronide (C14H17NO8, Sigma-Aldrich), aspirin ($C_9H_8O_4$, Sigma-Aldrich), salicyl glycine (C9H9NO4, US Biologicals), L-sulforaphane ($C_6H_{11}NOS_2$, ≥ 97%, HPLC, oil, Sigma-

Aldrich), acetonitrile UV (C_2H_3N for HPLC, Honeywell^{TM}) and formic acid (HCOOH, 98%, Sigma-Aldrich).

4.2.2 Solutions

For the optimization of the MS and the chromatographic conditions, a 1 mg/L and a 10 mg/L standard solution of each metabolite and internal standard (IS) was prepared in MilliQ water.

For the validation of the LC-ESI-MS/MS assay a standard calibration range was prepared, consisting of 0.01 mg/L, 0.1 mg/L, 0.5 mg/L, 1 mg/L, and 10 mg/L samples of each metabolite. The IS concentration remained the same in all of the samples at 0.5 mg/L. All of the samples were prepared in the aqueous mobile phase (mobile phase A).

4.2.3 Internal standard

A 20 mg/L IS stock solution was prepared by diluting 0.002 g of 2-acetamidophenol in 100 ml MilliQ water. From this stock solution further dilutions were made to create a 0.5 mg/L and 1 mg/L internal standard solution, used in the optimization and validation of the LC-ESI-MS/MS assay.

4.2.4 Buffers and mobile phases

A gradient system with two mobile phases was used for chromatographic separation of the metabolites. The aqueous phase consisted of 1 ml formic acid in 1000 ml MilliQ water (pH 3.5) (phase A) and the organic phase consisted of acetonitrile (phase B).

4.3 Assessment of phase I biotransformation using caffeine as probe substance

As discussed in Section 2.2.4.1, caffeine is a useful enzyme probe for assessing the activity of CYP1A1 isoforms in humans and can thus be used as a probe to assess phase I biotransformation reactions. When using caffeine as a probe substance, the rate of specific caffeine metabolite formation can be used to assess phase I biotransformation activity (Schrader *et al.,* 1999; Liska *et al*., 2006). *In vitro*, caffeine is biotransformed into the primary metabolites known as theobromine (12%), theophylline (4%) and paraxanthine (84%). These metabolites are formed when caffeine undergoes N-demethylation (Cazeneuve *et al*., 1994). N-demethylation reactions are catalyzed by CYP1A2 and account for approximately 90% of caffeine elimination in humans (Miners & Birkett, 1996).

4.3.1 Optimization and validation of the LC-ESI-MS/MS assay to quantify selected caffeine metabolites

Caffeine biotransformation pathways have been analyzed in studies using the physical separation capabilities of a high pressure liquid chromatography (HPLC) assay (Iyer *et al.,* 2010; Lakshmi & Nilanjana*,* 2011). Initially, an HPLC assay was used to quantify caffeine concentration. Although a small decrease in the caffeine concentration was observed, after HepaRG cells were incubated with caffeine as a probe substance, this decrease was very small compared to the variation of the assay (data not shown). Thus, it was decided to rather assess phase I biotransformation by following the formation of specific caffeine metabolites (theobromine, theophylline and paraxanthine) instead of trying to monitor the decrease in the concentration of caffeine itself. In order to quantify the caffeine metabolites, a more specific and selective assay was needed.

When it comes to the identification, structure characterisation and quantitative analysis of drug metabolites, LC-ESI-MS/MS methodology is often used, due to its

sensitivity and specificity (Kostiainen *et al.,* 2003; Ho *et al.,* 2003; Chen *et al.,* 2007). The high selectivity and sensitivity of the LC-ESI-MS/MS allows for the quantitative analysis of drugs and their metabolites at very low concentrations in complex biological samples (Kostiainen *et al.,* 2003). The LC-ESI-MS/MS thus combined the physical separation capabilities of the HPLC with the mass analysing capabilities of the MS (Kostiainen *et al.,* 2003; Ho et al., 2003; Chen *et al.,* 2007).

4.3.1.1 Optimization of the MS conditions for the quantification of selected caffeine metabolites

The first step in the standardization of the LC-ESI-MS/MS assay for the quantification of the selected phase I and phase II metabolites was to optimize the MS conditions. Agilent MassHunter optimizer software was used for this optimization. MassHunter works on chromatographic and electrophoretic mass spectral data to extract information, reduce data complexity, eliminate potential interferences and generate a list of molecular features (Anon, 2005).

Specifications of the LC-ESI-MS/MS

An Agilent 1200 series LC (Santa Clara, CA, USA) was used for sample handling and mobile phase delivery. The analytes of interest were chromatographically separated with a reversed phase Luna 5µ C18 (2) 100A Phenomenex column of 250 mm x 2.0 mm, 5 µm particle size, and 100A pore size with a 4 mm x 3.0 mm Security Guard C18 guard column (Agilent Technologies). Samples were injected at a volume of 4 µl per sample and a constant flow rate of 0.2 ml/min was maintained throughout the run. Quantification was performed on an Agilent 6410 Triple Quadrupole mass spectrometer (Santa Clara, CA, USA) operated in positive ionization mode.

Optimizing MS/MS conditions

Analytical standards of each metabolite and IS were prepared as described in Section 4.2.2. These standards (1 mg/L) were used to optimize the MS conditions for

the quantification of each metabolite, respectively. The prepared samples were each directly infused into the MS/MS without the use of a column for chromatographic separation. The MassHunter optimizer was used to automatically optimize the settings on the MS/MS for optimal detection and quantification of each metabolite. The information obtained from the MassHunter optimizer software was used to prepare the following multi reaction monitoring (MRM) conditions for the selective detection and quantification of theobromine, theophylline, paraxanthine and the internal standard (Table 4.1).

Table 4.1: Multi reaction monitoring conditions for the quantification of theobromine, theophylline, paraxanthine and internal standard.

Compound name	Precursor ion (m/z)	Product ion (m/z)	Fragmentor	Collision energy
Theobromine	181.17	138.1	92	16
Theophylline	181.17	124	86	16
Paraxanthine	181.17	124	106	20
Internal standard	152.1	110.1	68	8

* (m/z) = mass-to-charge ratio.

MassHunter software was used to optimize the conditions for the detection of the selected caffeine metabolites (theobromine, theophylline, and paraxanthine) and internal standard with the LC-ESI-MS/MS.

4.3.1.2 Chromatographic separation of theobromine, theophylline, paraxanthine and internal standard

A gradient system with two mobile phases was used for chromatographic separation of the metabolites (Table 4.2). The aqueous phase consisted of 1 ml formic acid in 1000 ml MilliQ water (pH 3.5) (phase A) and the organic phase consisted of acetonitrile (phase B). The formic acid was added to the aqueous phase to improve chromatographic peak shape and to provide a source of protons in the reverse phase LC-ESI-MS/MS. The organic solvent lowered the polarity of the mobile phase, causing the molecules to elute from the column.

Table 4.2: Mobile phase gradient used for the chromatographic separation of the selected caffeine metabolites (theobromine, theophylline and paraxanthine).

Time (min)	% Mobile phase B	Flow rate (ml/min)
0	5	0.2
4	5	0.2
14	30	0.2
17	100	0.2
21	100	0.2
25	5	0.2

*min = minutes, % = percentage, (ml/min) = millilitres per minute

The mobile phases used for chromatographic separation were a aqueous phase of 1 ml formic acid in 1000 ml MilliQ water (pH 3.5) (mobile phase A) and an organic phase which consisted out of acetonitrile (mobile phase B).

The conditons described in Table 4.2 were used to analyse a mixture containing theobromine, theophylline, paraxanthine and internal standard (each at a concentration of 1 mg/L). The results are depicted in Figure 4.1.

Figure 4.1: Chromatographic separation of theobromine, theophylline, paraxanthine and internal standard. The Y-axis represents the abundance of each identified peak and the X- axis the retention time (minutes) of each specific peak. (A) Represent the total ion chromatogram (TIC), (B) MRM of theobromine, (C) MRM of theophylline and paraxanthine, and (D) MRM of 2-acetamidophenol.

MRM was used to monitor and quantify theobromine, theophylline, paraxanthine and the IS. The total ion chromatogram (TIC), as indicated in Figure 4.1, is the sum of all of the MRM's used for the detection of theobromine, theophylline, paraxanthine and the IS. When the gradient for the separation of theobromine, theophylline and paraxanthine (Table 4.3) was used, theobromine (181.2 m/z-138.1 m/z) had a retention time of ± 8.8 minutes, theophylline and paraxanthine (181.2 m/z-124 m/z) had a retention time of ± 10.2 minutes and the IS (152.1 m/z-110.1 m/z) had a retention time of ± 14.8 minutes. Also indicated in Figure 4.1, theophylline and paraxanthine were detected in one peak instead of two separate peaks. The chromatographic conditions used could not separate the two compounds and

therefore they had the same retention time. The two compounds also displayed the same precursor (181.2 m/z) and product ions (124 m/z). Separate MRM analyses of 1 mg/L theophylline and 1 mg/L paraxanthine confirmed that the two compounds could not be distinguished using the given MRM parameters (data not shown). Literature also indicated that theobromine and theophylline cannot be separated into two separate peaks with reversed phase chromatography, but only through an ion paring technique (O'Broin *et al.,* 1980). Since the quantification of theobromine, theophylline and paraxanthine was to demonstrate the biotransformation rate of caffeine under a given set of experimental conditions, the sum of the concentrations of the three metabolites is proportional to the decrease of caffeine. It was thus decided that the separate quantification of theophylline and paraxanthine were unnecessary. Theobromine and paraxanthine were quantified as one peak in all further experiments where phase I biotransformation was assessed using caffeine as probe substance.

4.3.1.3 Validation of the LC-ESI-MS/MS assay used for the quantification of theobromine, theophylline and paraxanthine

Method validation has an important part in the development of an assay as it affects the quality of data the assay produces (Peters *et al.,* 2007). The standardized LC-ESI-MS/MS assay for the quantification of each selected phase I and phase II metabolite thus had to be validated before it could be used. A calibration curve of metabolite against internal standard was constructed to determine the linearity of the assay. The results are depicted in Figure 4.2 – Figure 4.5.

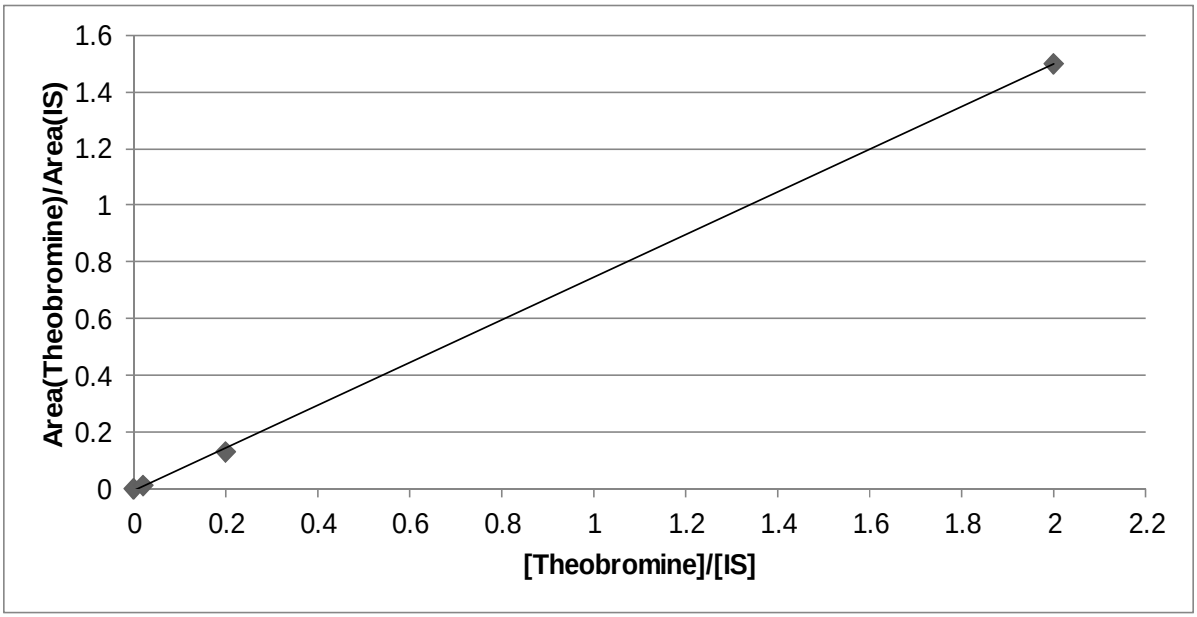

Figure 4.2: Calibration curve of theobromine. The Y-axis represent the values obtained when the response of theobromine was divided by the response of the internal standard (IS) and the X-axis the values of the concentration of theobromine was divided by the concentration of the IS. The theobromine concentration ranged between 0.01- 1 mg/L while the IS concentration was 0.5 mg/L in each sample. R^2 = 0.99.

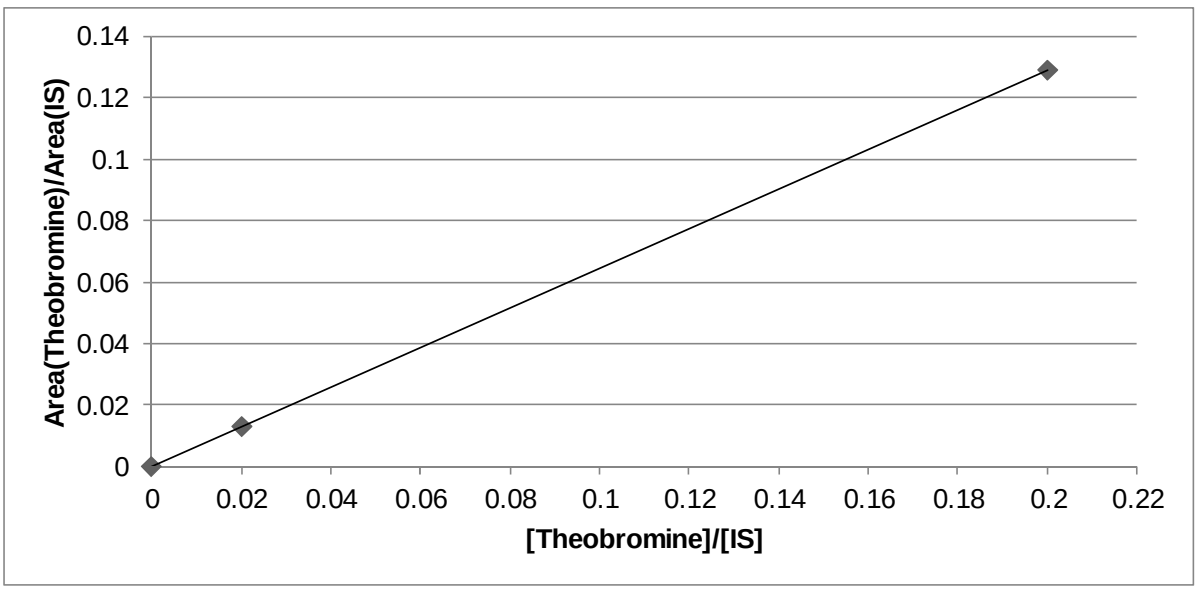

Figure 4.3: Calibration curve of theobromine in the lower concentration range (R^2 > 0.99)

In Figures 4.2 and 4.3, the standardized LC-ESI-MS/MS assay for theobromine was linear over the concentration range of 0.01 to 1 mg/L (R^2 = 0.99). Thus the

standardized MRM assay could be used to quantify theobromine (in this concentration range) in HepaRG cells (Chapters 5 and 6).

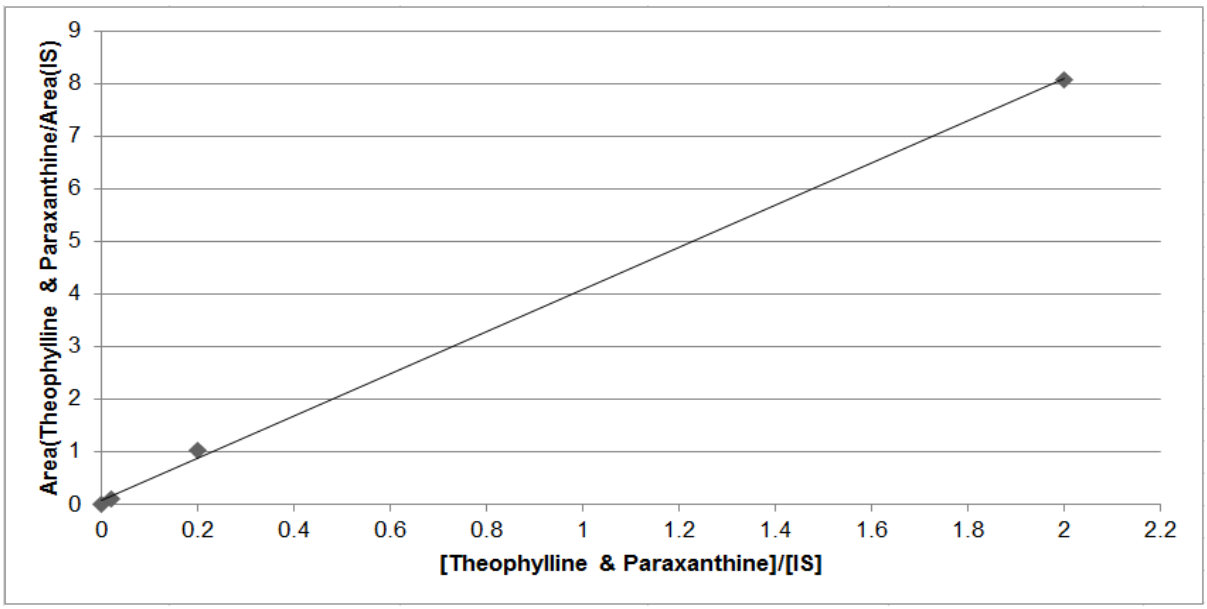

Figure 4.4: Calibration curve of theophylline and paraxanthine The Y-axis represent the values obtained when the response of theophylline and paraxanthine was divided by the response of the internal standard (IS) and the X-axis the values of the concentration of theophylline and paraxanthine was divided by the concentration of the IS. The theophylline and paraxanthine concentration ranged between 0.01- 1 mg/L while the IS concentration was 0.5 mg/L in each sample. R^2 = 0.99.

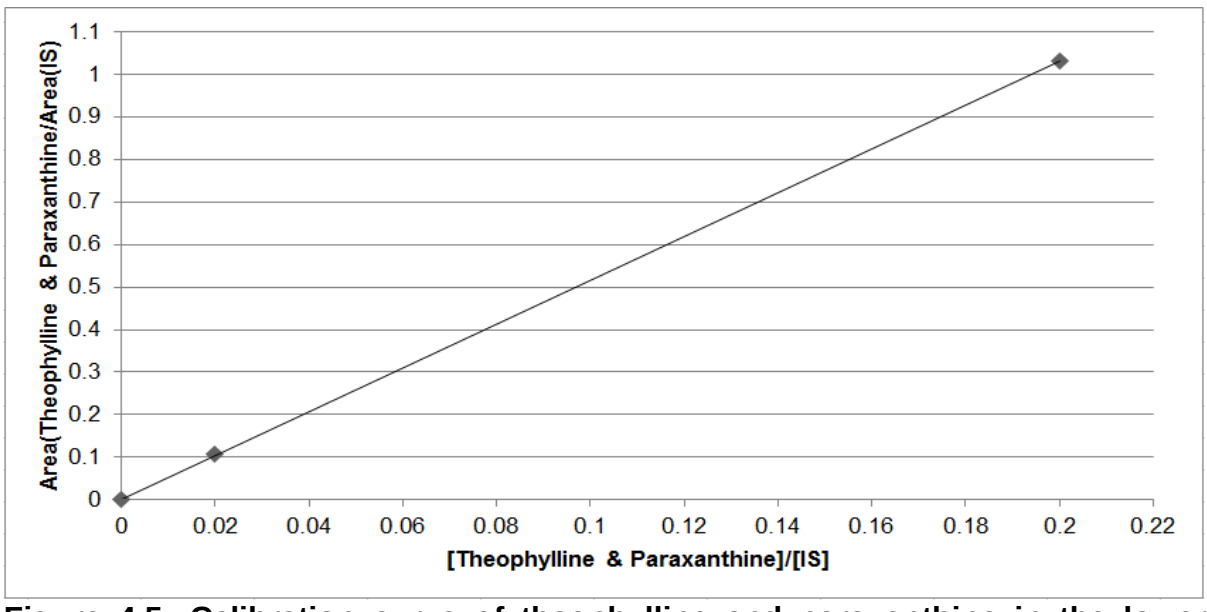

Figure 4.5: Calibration curve of theophylline and paraxanthine in the lower concentration range (R^2 > 0.99).

In Figures 4.4 and 4.5, the standardized LC-ESI-MS/MS assay for theophylline and paraxanthine was linear over the concentration range of 0.01 to 1 mg/L (R^2 = 0.99). Thus the standardized MRM assay could be used to quantify theophylline and paraxanthine (in this concentration range) in HepaRG cells (Chapters 5 and 6).

Equation 4.1: Calculation of the response factors of the selected phase I and phase II metabolites relative to the internal standard.

Response factor = Response (IS) x [Analyte] / Response (analyte) x [IS]

The data used to demonstrate the linearity for the quantification theobromine (Figure 4.1) could be used to calculate the response factor (RF) of theobromine relative to the internal standard (Equation 4.1). However, in the case of theophylline and paraxanthine the calibration curve could not be used to calculate the RF as the calibration curve was constructed from a mixture of these metabolites which consisted of 50% theophylline and 50% paraxanthine. Although theophylline and paraxanthine were quantified as one peak, the percentage of theophylline and paraxanthine present in each peak varied. Phase I and phase II biotransformation pathways were compared and it was not necessary for the absolute quantification of the concentration of each formed metabolite. It was thus decided to use a RF value of 1 to only relatively quantify the theobromine, theophylline and paraxanthine concentration, using Equation 4.2.

Equation 4.2: Calculation of the concentration of the selected phase I and phase II metabolites.

[Analyte] = Response (analyte) x [IS] / Response (IS)

Where:

Response IS = the response of the internal standard (2-acetamidophenol)

[Analyte] = the concentration of the analyte

Response (analyte) = the response of the analyte

[IS] = the concentration of the internal standard (2-acetamidophenol)

4.4 Assessment of phase II biotransformation using paracetamol as probe substance

As discussed in section 2.2.4.2, paracetamol is used as a probe substance to assess phase II sulfate conjugation, glucuronic acid conjugation, and glutathione conjugation pathways (Liska *et al.,* 2006). In humans, the biggest part of paracetamol is metabolized through the glucuronidation (50%-60%) and sulfation (25%-35%). Only a small amount of paracetamol is converted to the toxic metabolite NAPQI and paracetamol mercapturate (2%-10%) (Lohmann & Karst, 2006). The formation of these selected paracetamol metabolites can be used to assess phase II biotransformation activity.

4.4.1 Optimization and validation of the LC-ESI-MS/MS assay to quantify selected paracetamol metabolites

Paracetamol biotransformation pathways have been analyzed in studies using the physical separation capabilities of a high pressure liquid chromatography (HPLC) assay (Neyrinck *et al.,* 1999). An HPLC assay was used to quantify paracetamol concentration and the formation of the selected phase II paracetamol metabolites (paracetamol glucuronide, paracetamol sulfate and paracetamol mercapturate). However, it was decided to rather assess phase II biotransformation by following the formation of specific paracetamol metabolites (paracetamol glucuronide, paracetamol sulfate and paracetamol mercapturate) instead of trying to monitor the decrease in the concentration of paracetamol itself. In order to quantify the selected phase II paracetamol metabolites, a more specific and selective LC-ESI-MS/MS assay was needed.

4.4.1.1 Optimization of the MS conditions for the quantification of selected paracetamol metabolites

The information obtained from the MassHunter optimizer software was used to prepare the following MRM conditions for the selective detection and quantification of paracetamol glucuronide, paracetamol sulfate, paracetamol mercapturate and the IS (Table 4.3).

Table 4.3: Multi reaction monitoring conditions for the quantification of paracetamol glucuronide, paracetamol sulfate, paracetamol mercapturate and the internal standard.

Compound name	Precursor ion (m/z)	Product ion (m/z)	Fragmentor	Collision energy
Paracetamol glucuronide	328.1	152.1	80	4
Paracetamol sulfate	232	152.1	96	12
Paracetamol mercapturate	271.1	140	140	20
Internal standard	152.1	110.1	68	8

* (m/z) = mass-to-charge ratio

MassHunter software was used to optimize the conditions for the detection of the selected paracetamol metabolites (paracetamol glucuronide, paracetamol sulfate and paracetamol mercapturate) and internal standard with the LC-ESI-MS/MS.

4.4.1.2 Chromatographic separation of paracetamol glucuronide, paracetamol sulfate, paracetamol mercapturate and internal standard

Table 4.4: Mobile phase gradient used for the chromatographic separation of the selected paracetamol metabolites (paracetamol glucuronide, paracetamol sulfate and paracetamol mercapturate).

Time (min)	% Mobile phase B	Flow rate (ml/min)
0	5	0.2
9	5	0.2
11	25	0.2
17	85	0.2
19	100	0.2
24	100	0.2
28	5	0.2

*min = minutes, % = percentage, (ml/min) = millilitres per minute

The mobile phases used for chromatographic separation were a aqueous phase of 1 ml formic acid in 1000 ml MilliQ water (pH 3.5) (mobile phase A) and an organic phase which consisted out of acetonitrile (mobile phase B).

The conditons described in Table 4.4 were used to analyse a mixture contianing paracetamol glucuronide, paracetamol sulfate, paracetamol mercapturate and internal standard (each at a concentration of 1 mg/L). The results are depicted in Figure 4.6.

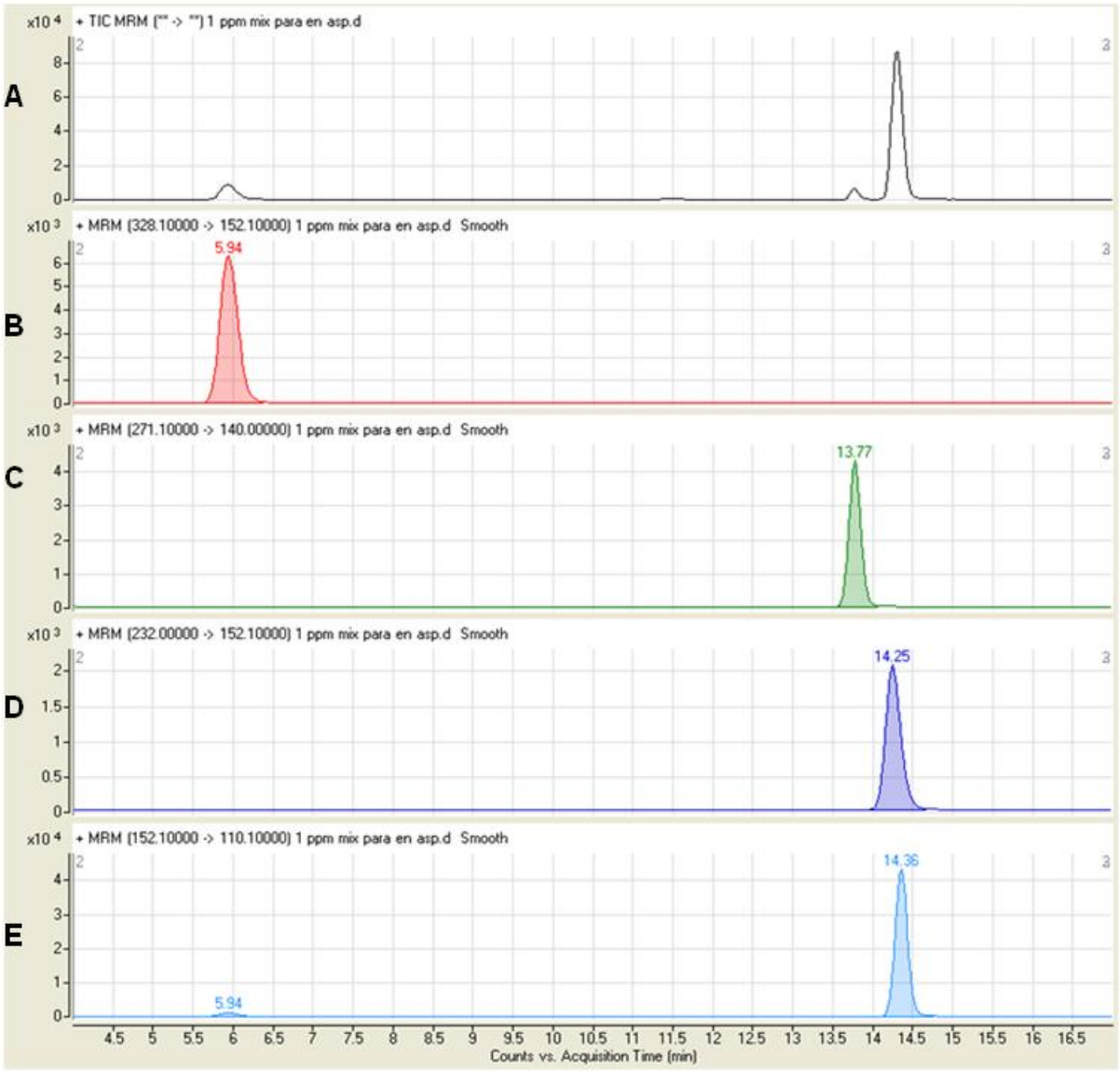

Figure 4.6: Chromatographic separation of paracetamol glucuronide, paracetamol sulfate, paracetamol mercapturate and internal standard. The Y-axis represents the abundance of each identified peak and the X- axis the retention time (minutes) of each specific peak. (A) Represent the total ion chromatogram (TIC), (B) MRM of paracetamol sulfate, (C) MRM of paracetamol mercapturate, (D) MRM of paracetamol sulfate, and (E) MRM of 2-acetamidophenol (IS).

MRM was used to monitor and quantify paracetamol glucuronide, paracetamol sulfate, paracetamol mercapturate and IS. The TIC, as shown in Figure 4.6, is the sum of all of the MRM's used for the detection of paracetamol glucuronide, paracetamol sulfate, paracetamol mercapturate and IS. When the gradient for the chromatographic separation of paracetamol glucuronide, paracetamol sulfate, paracetamol mercapturate (Table 4.4) was used, paracetamol glucuronide (328.1-152.1 m/z) had a retention time of ± 5.9 minutes, paracetamol sulfate (232-152.1

m/z) had a retention time of ± 14.3 minutes, paracetamol mercapturate (271.1-140 m/z) had a retention time of ± 13.7 minutes, and the IS (152.1-110.1 m/z) had a retention time of ± 14.4 minutes.

4.4.1.3 Validation of the LC-ESI-MS/MS assay used for the quantification of paracetamol glucuronide, paracetamol sulfate and paracetamol mercapturate

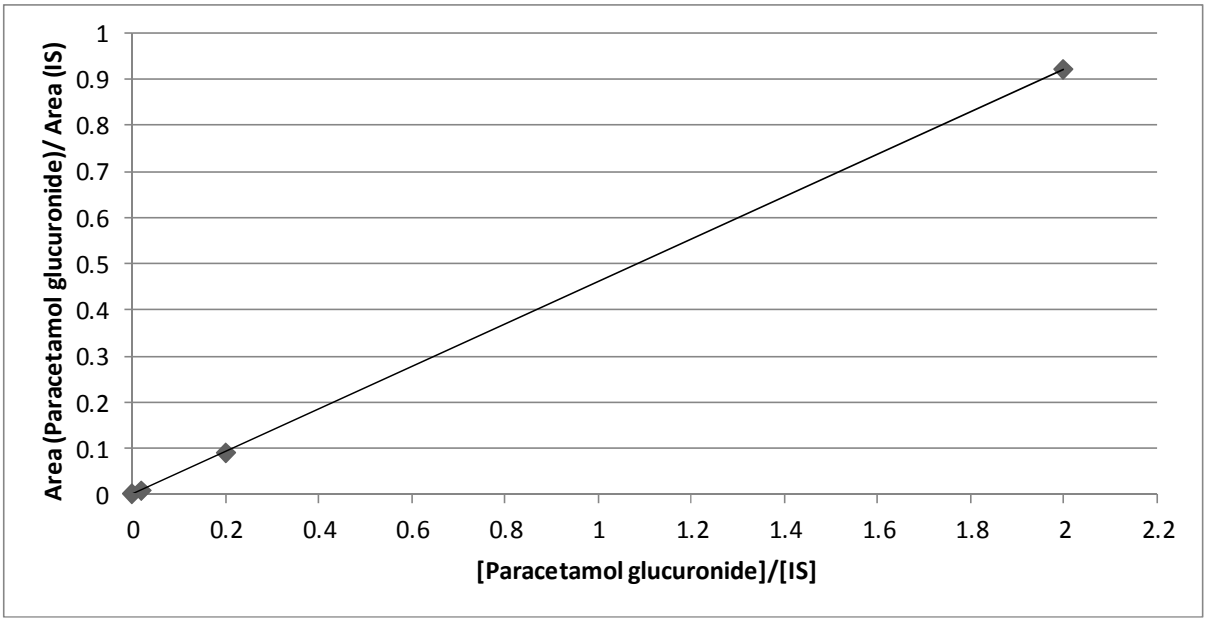

Figure 4.7: Calibration curve of paracetamol glucuronide. The Y-axis represent the values obtained when the response of paracetamol glucuronide was divided by the response of the internal standard (IS) and the X-axis the values of the concentration of paracetamol glucuronide was divided by the concentration of the IS. The paracetamol glucuronide concentration ranged between 0.01 mg/L-1 mg/L while the IS concentration was 0.5 mg/L in each sample. $R^2 > 0.99$.

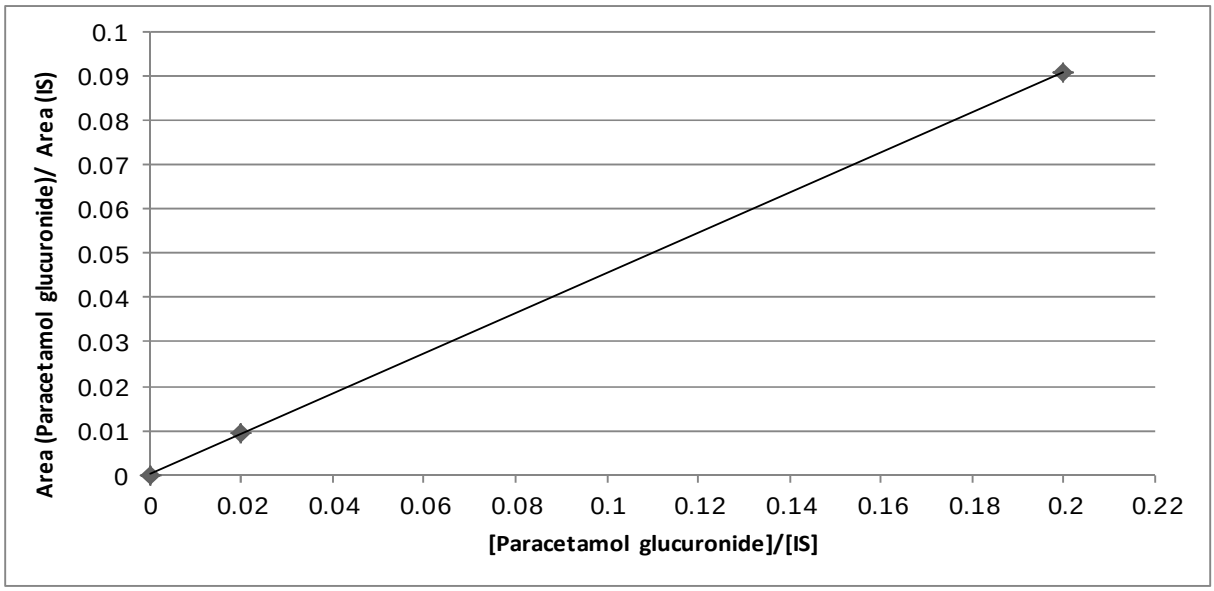

Figure 4.8: Calibration curve of paracetamol glucuronide in the lower concentration range (R^2 > 0.99).

In Figures 4.7 and 4.8, the standardized LC-ESI-MS/MS assay for paracetamol glucuronide was linear over the concentration range of 0.01 to 1 mg/L (R^2 > 0.99). Thus the standardized MRM assay could be used to quantify paracetamol glucuronide (in this concentration range) in HepaRG cells (Chapters 5 and 6).

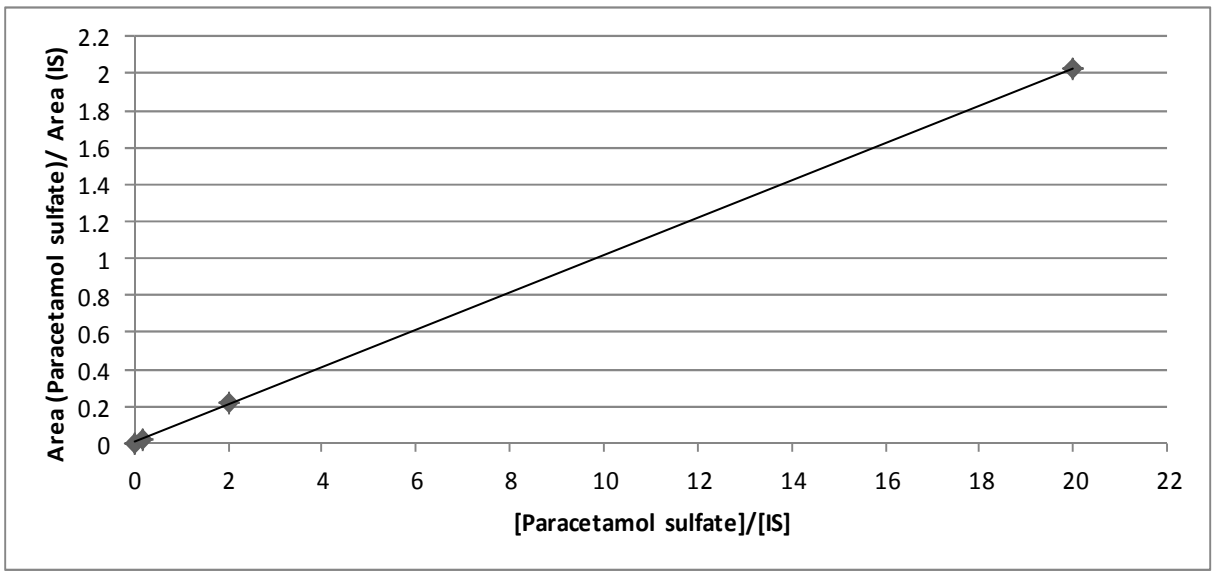

Figure 4.9: Calibration curve of paracetamol sulfate. The Y-axis represent the values obtained when the response of paracetamol sulfate was divided by the response of the internal standard (IS) and the X-axis the values of the concentration of paracetamol sulfate was divided by the concentration of the IS. The paracetamol sulfate concentration ranged between 0.1 mg/L-10 mg/L while the IS concentration was 0.5 mg/L in each sample. (R^2 = 0.99).

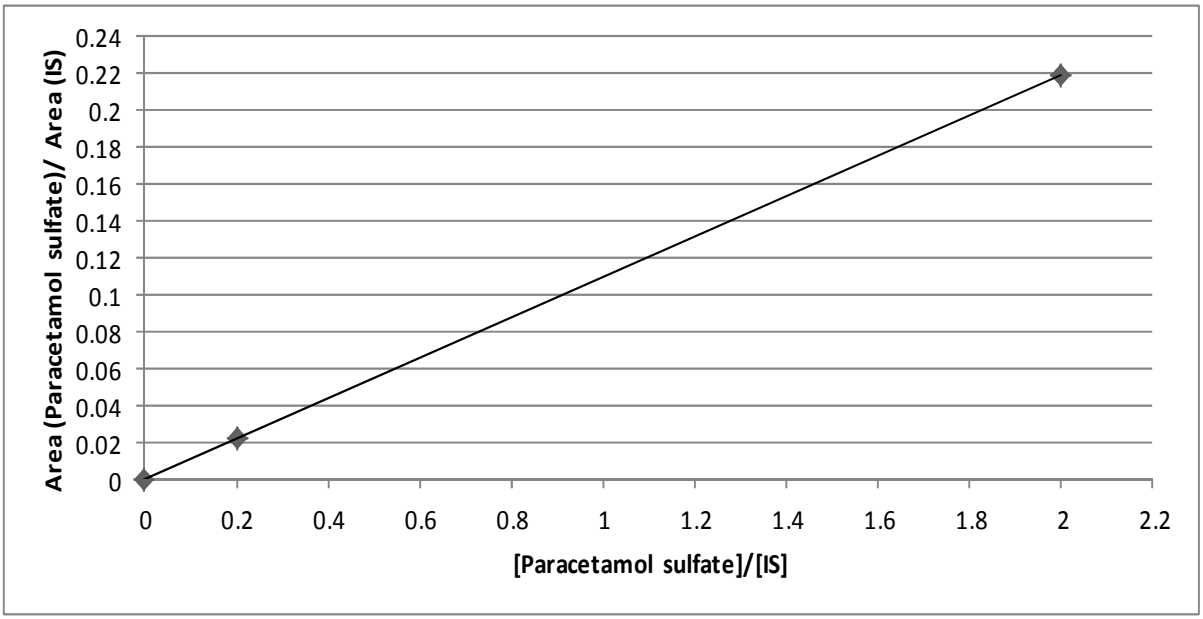

Figure 4.10: Calibration curve of paracetamol sulfate in the lower concentration range (R^2 > 0.99).

In Figures 4.9 and 4.10, the standardized LC-ESI-MS/MS assay for paracetamol sulfate was linear over the concentration range of 0.1 to 10 mg/L (R^2 = 0.99). Thus the standardized MRM assay could be used to quantify paracetamol sulfate (in this concentration range) in HepaRG cells (Chapters 5 and 6).

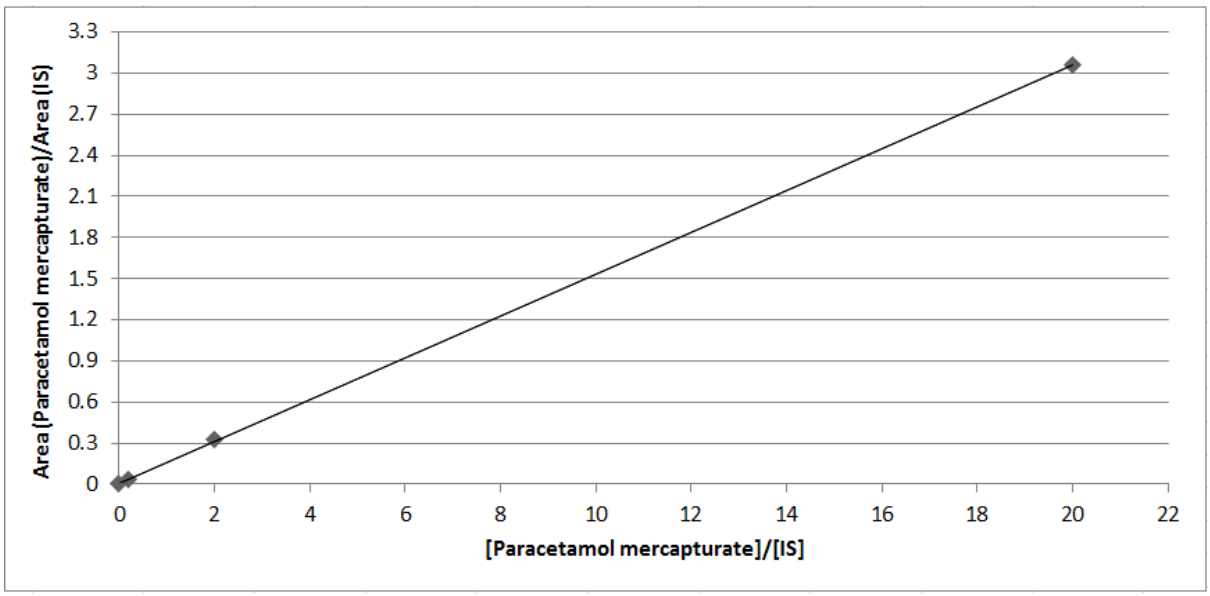

Figure 4.11: Calibration curve of paracetamol mercapturate. The Y-axis represent the values obtained when the response of paracetamol mercapturate was divided by the response of the internal standard (IS) and the X-axis the values of the concentration of paracetamol mercapturate was divided by the concentration of the IS. The paracetamol mercapturate concentration ranged between 0.1 - 10 mg/L while the IS concentration was 0.5 mg/L in each sample. (R^2 = 0.99).

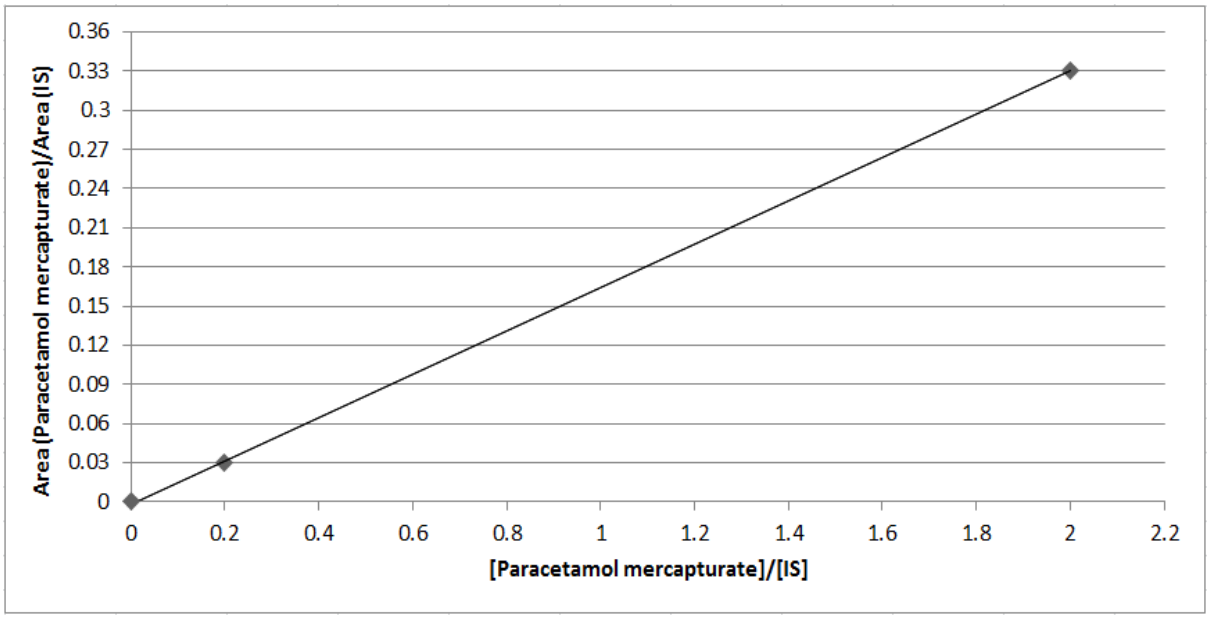

Figure 4.12: Calibration curve of paracetamol mercapturate in the lower concentration range (R^2 = 0.99).

In Figures 4.11 and 4.12, the standardized LC-ESI-MS/MS assay for paracetamol mercapturate was linear over the concentration range of 0.1 to 10 mg/L (R^2 = 0.99). Thus the standardized MRM assay could be used to quantify paracetamol mercapturate (in this concentration range) in HepaRG cells (Chapters 5 and 6). The data used to demonstrate the linearity for the quantification of paracetamol sulfate, paracetamol glucuronide and paracetamol mercapturate (Figure 4.7-Figure 4.12) could be used to calculate the response factor (RF) of these metabolites relative to the internal standard (Equation 4.1). As discussed in Section 4.3.1.3, because phase I and phase II biotransformation pathways were compared, it was not necessary for the absolute quantification of the concentration of each formed metabolite. It was thus decided to use a RF value of 1 to only relatively quantify the paracetamol sulfate, paracetamol glucuronide and paracetamol mercapturate concentration, using Equation 4.2.

4.5 Assessment of phase II biotransformation using aspirin, salicylic acid, sodium benzoate and 4-aminobenzoic acid as probe substances

As discussed in Section 2.2.4.3, in humans, glycine conjugation is the main amino acid conjugation pathway and can thus be used to evaluate the phase II amino acid conjugation pathway (Liska *et al*., 2006; Beyoğlu *et al.,* 2012). Specific aromatic acids such as aspirin, the food preservative sodium benzoate, and para-aminobenzoic acid have all been used in studies as probes to investigate the activity of the phase II glycine conjugation pathway (Kasuya *et al.,* 2000; Beyoğlu *et al.,* 2012). The formation of the selected glycine conjugation metabolites: salicyluric acid, hippuric acid and para-aminobenzoic acid can be used to assess phase II glycine conjugation activity.

4.5.1 Optimization and validation of the LC-ESI-MS/MS assay to quantify selected glycine conjugation metabolites

The phase II glycine conjugation pathways of benzoic acids have been analyzed studies using the physical separation capabilities of a high pressure liquid chromatography (HPLC) assay (Kasuya *et al.,* 2000). An HPLC assay was used to quantify aspirin concentration and the formation of the selected phase II glycine conjugation metabolite (salicyluric acid). As no salicyluric acid formation was quantified (results not shown), it was decided to rather assess phase II glycine conjugation by following the formation of the selected phase II glycine conjugation metabolites (salicyluric acid, hippuric acid and para-aminohippuric acid), using salicylic acid, sodium benzoate, and para-aminobenzoic acid as probe substances instead. In order to quantify the selected phase II glycine conjugation metabolites, a more specific and selective LC-ESI-MS/MS assay was needed.

4.5.1.1 Optimization of the MS conditions for the quantification of selected glycine conjugation metabolites

The information obtained from the MassHunter optimizer software was used to prepare the following MRM conditions for the selective detection and quantification of salicyluric acid, hippuric acid, para-aminohippuric acid and the internal standard (Table 4.5).

Table 4.5: Multi reaction monitoring conditions for the quantification of salicyluric acid, hippuric acid, para-aminohippuric acid and the internal standard.

Compound name	Precursor ion (m/z)	Product ion (m/z)	Fragmentor	Collision energy
Salicyluric acid	196.1	121.1	70	12
Hippuric acid	180.1	105	70	12
Para-aminohippuric acid	195.1	120	70	12
Internal standard	152.1	110.1	68	8

* (m/z) = mass-to-charge ratio

MassHunter software was used to optimize the conditions for the detection of the selected glycine conjugation metabolites (salicyluric acid, hippuric acid and para-aminohippuric acid) and internal standard with the LC-ESI-MS/MS.

4.5.1.2 Chromatographic separation of salicyluric acid, hippuric acid, para-aminohippuric acid and internal standard

Table 4.6: Mobile phase gradient used for the chromatographic separation of the selected phase I glycine conjugation metabolites (salicyluric acid, hippuric acid and para-aminohippuric acid).

Time (min)	% Mobile phase B	Flow rate (ml/min)
0	5	0.2
9	5	0.2
11	25	0.2
17	85	0.2
19	100	0.2
24	100	0.2
28	5	0.2

*min = minutes, % = percentage, (ml/min) = millilitres per minute

The mobile phases used for chromatographic separation were a aqueous phase of 1 ml formic acid in 1000 ml MilliQ water (pH 3.5) (mobile phase A) and an organic phase which consisted out of acetonitrile (mobile phase B).

The conditons described in Table 4.6 was used to analyse a mixture containing salicyluric acid, hippuric acid, para-aminohippuric acid and internal standard (each at a concentration of 1 mg/L). The results are depicted in Figure 4.13.

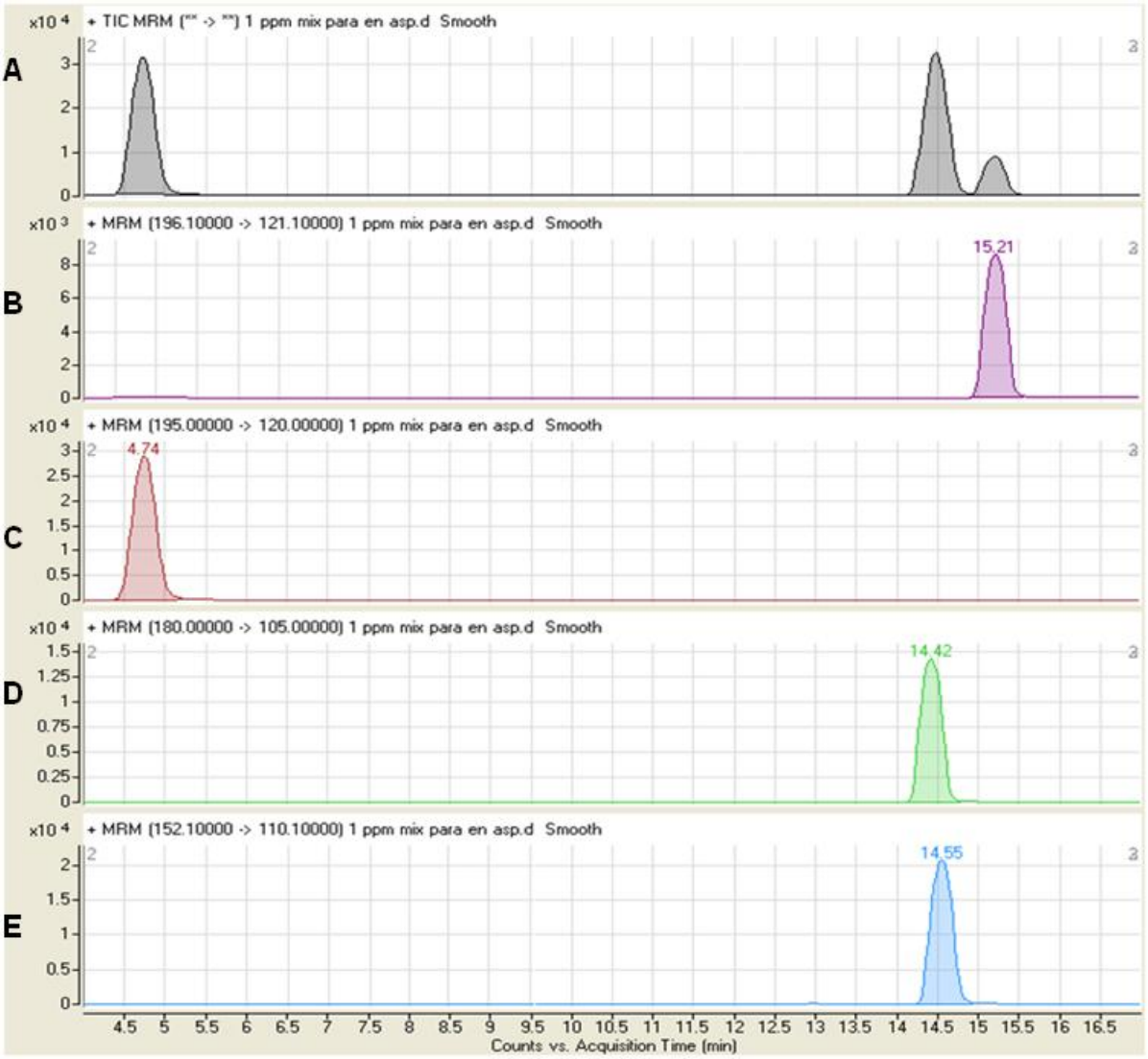

Figure 4.13: Chromatographic separation of salicyluric acid, hippuric acid, para-aminohippuric acid and internal standard. The Y-axis represents the abundance of each identified peak and the X- axis the retention time (minutes) of each specific peak. (A) Represent the total ion chromatogram (TIC), (B) MRM of salicyluric acid, (C) MRM of para-aminohippuric acid, (D) MRM of hippuric acid, and (E) MRM of 2-acetamidophenol (IS).

MRM was used to monitor and quantify salicyluric acid, hippuric acid, para-aminohippuric acid and IS. The TIC, as indicated in Figure 4.13, is the sum of all of the MRM's used for the detection of salicyluric acid, hippuric acid, para-aminohippuric acid and IS. When the gradient for the separation of salicyluric acid, hippuric acid and para-aminohippuric acid (Table 4.6) was used, salicyluric acid (196-121 m/z) had a retention time of ± 15.2 minutes, para-aminobenzoic acid (195 m/z-120 m/z) had a retention time of ± 4.7 minutes, hippuric acid (180 m/z-105

m/z) had a retention time of ± 14.4 minutes, and the IS (152.1 m/z-110.1 m/z) had a retention time of ± 14.6 minutes.

4.5.1.3 Validation of the LC-ESI-MS/MS assay for the quantification of salicyluric acid, hippuric acid and para-aminohippuric acid

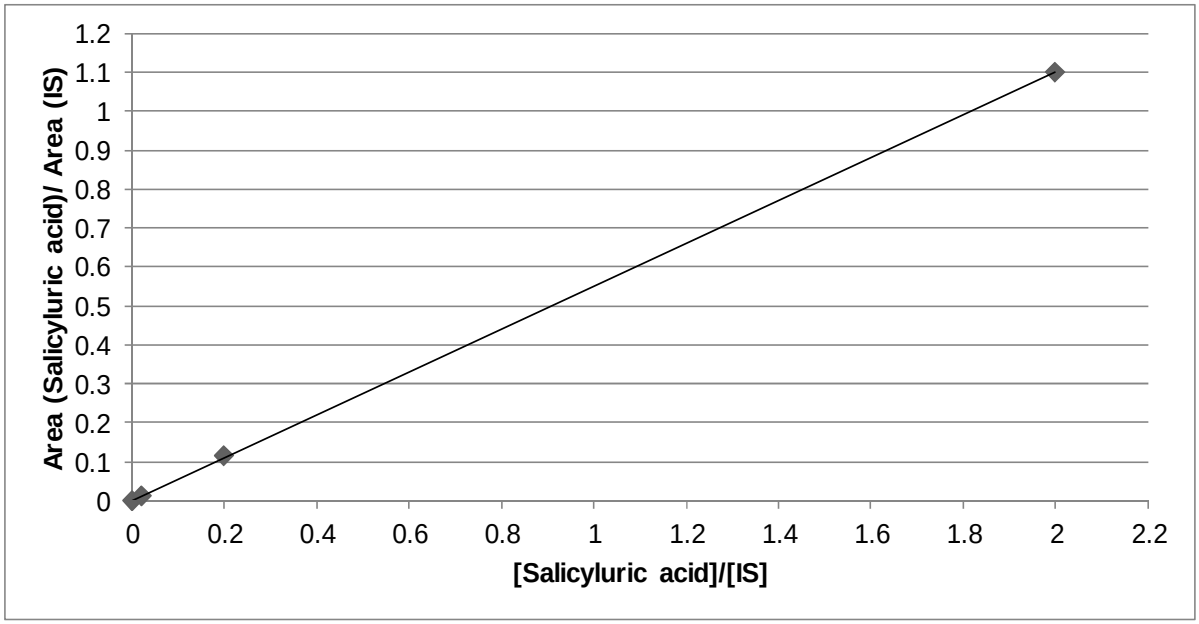

Figure 4.14: Calibration curve of salicyluric acid. The Y-axis represent the values obtained when the response of salicyluric acid was divided by the response of the internal standard (IS) and the X-axis the values of the concentration of salicyluric acid was divided by the concentration of the IS. The salicyluric acid concentration ranged between 0.01 mg/L-1 mg/L while the IS concentration was 0.5 mg/L in each sample. $R^2 > 0.99$.

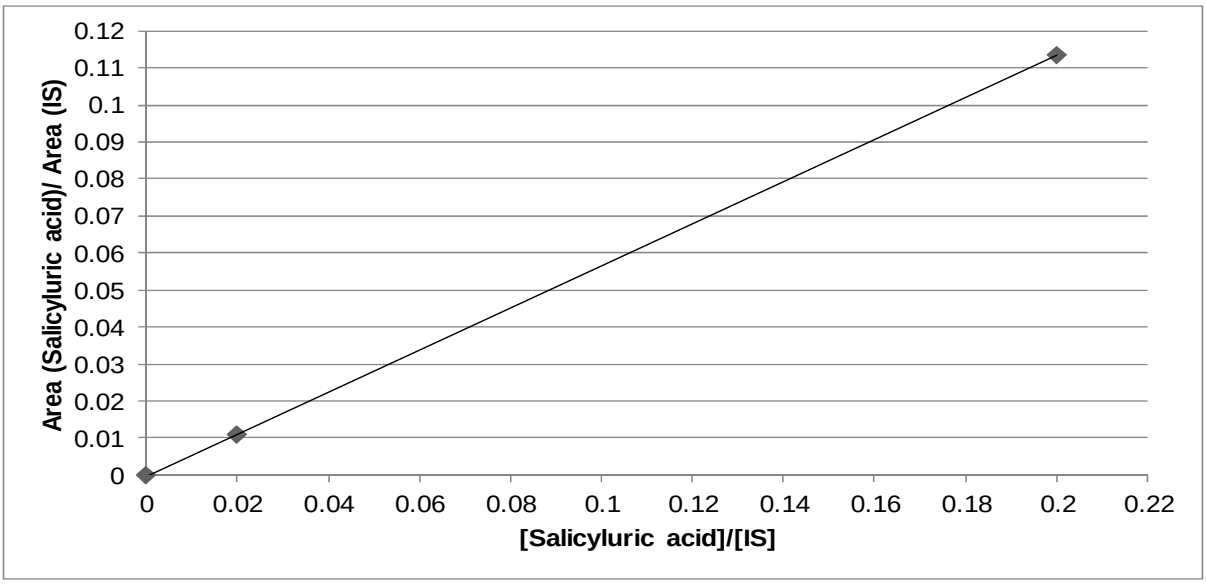

Figure 4.15: Calibration curve of salicyluric acid in the lower concentration range (R^2 > 0.99).

In Figures 4.14 and 4.15, the standardized LC-ESI-MS/MS assay for salicyluric acid was linear over the concentration range of 0.01 to 1 mg/L (R^2 > 0.99). Thus the standardized MRM assay could be used to quantify salicyluric acid (in this concentration range) in HepaRG cells (Chapters 5 and 6).

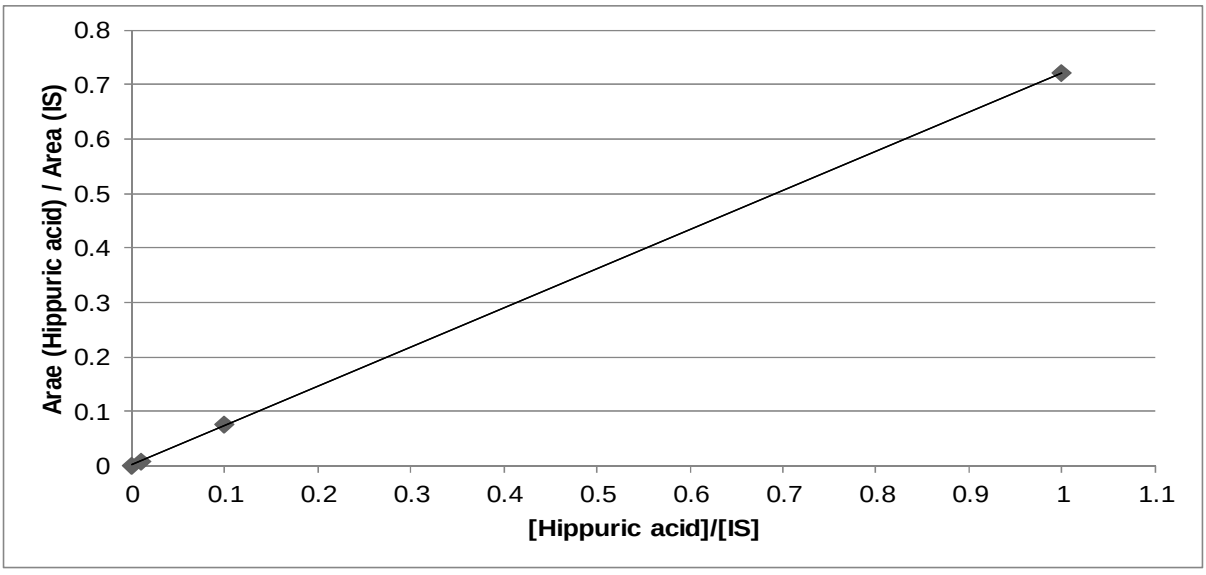

Figure 4.16: Calibration curve of hippuric acid. The Y-axis represent the values obtained when the response of hippuric acid was divided by the response of the internal standard (IS) and the X-axis the values of the concentration of hippuric acid was divided by the concentration of the IS. The hippuric acid concentration ranged between 0.01 mg/L-1 mg/L while the IS concentration was 1 mg/L in each sample. R^2 > 0.99.

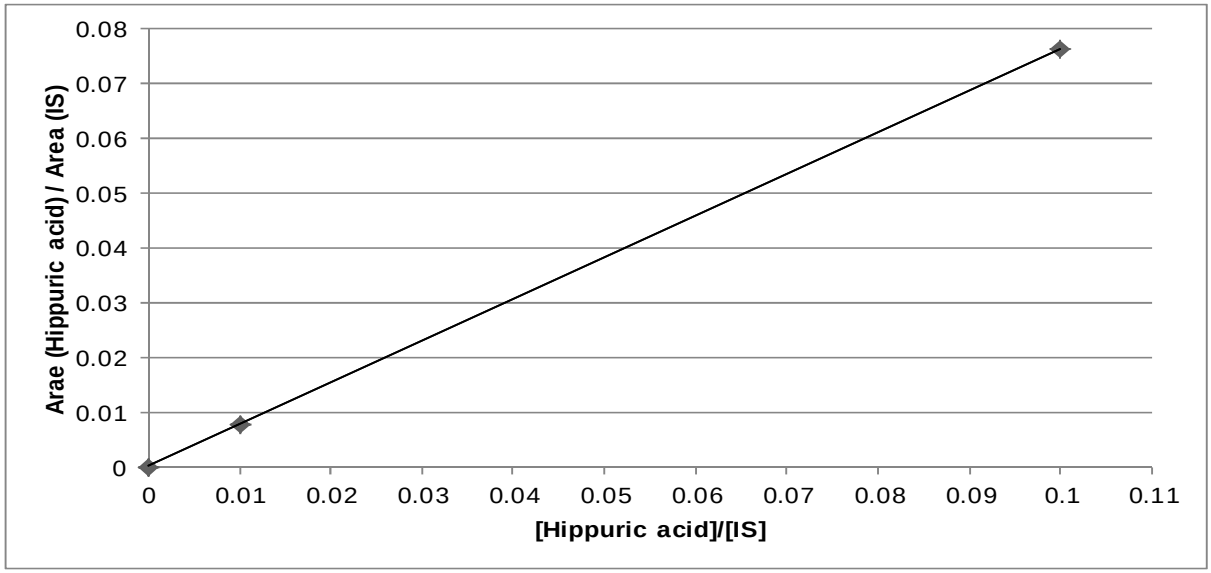

Figure 4.17: Calibration curve of hippuric acid in the lower concentration range (R^2 > 0.99).

In Figures 4.16 and 4.17, the standardized LC-ESI-MS/MS assay for hippuric acid was linear over the concentration range of 0.01 to 1 mg/L (R^2 > 0.99). Thus the standardized MRM assay could be used to quantify hippuric acid (in this concentration range) in HepaRG cells (Chapters 5 and 6).

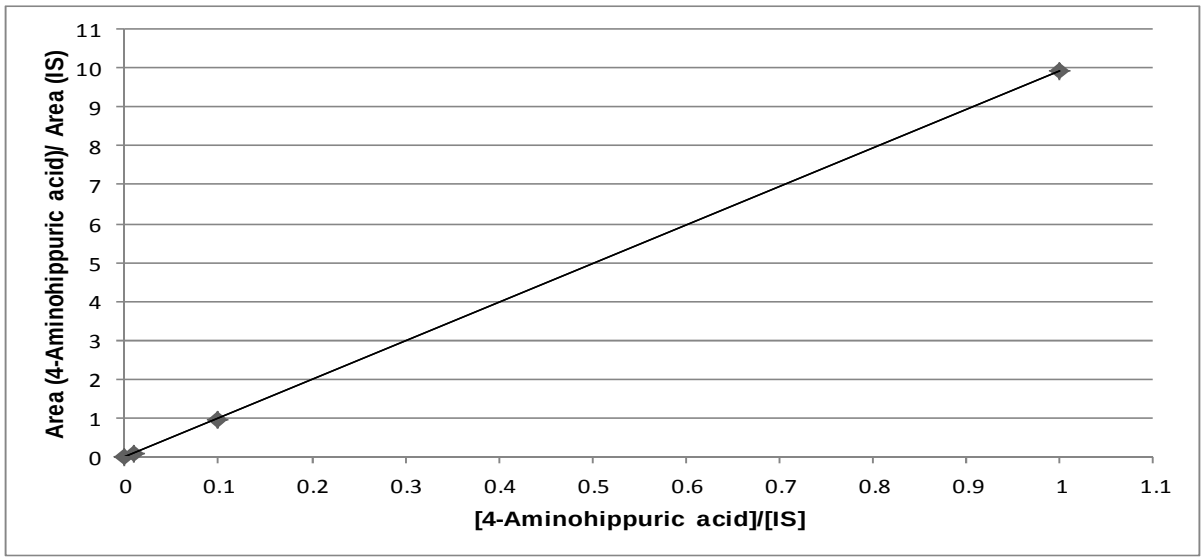

Figure 4.18: Calibration curve of 4-aminohippuric acid. The Y-axis represent the values obtained when the response of 4-aminohippuric acid was divided by the response of the internal standard (IS) and the X-axis the values of the concentration of 4-aminohippuric acid was divided by the concentration of the IS. The 4-aminohippuric acid concentration ranged between 0.01 mg/L-1 mg/L while the IS concentration was 1 mg/L in each sample. R^2 > 0.99.

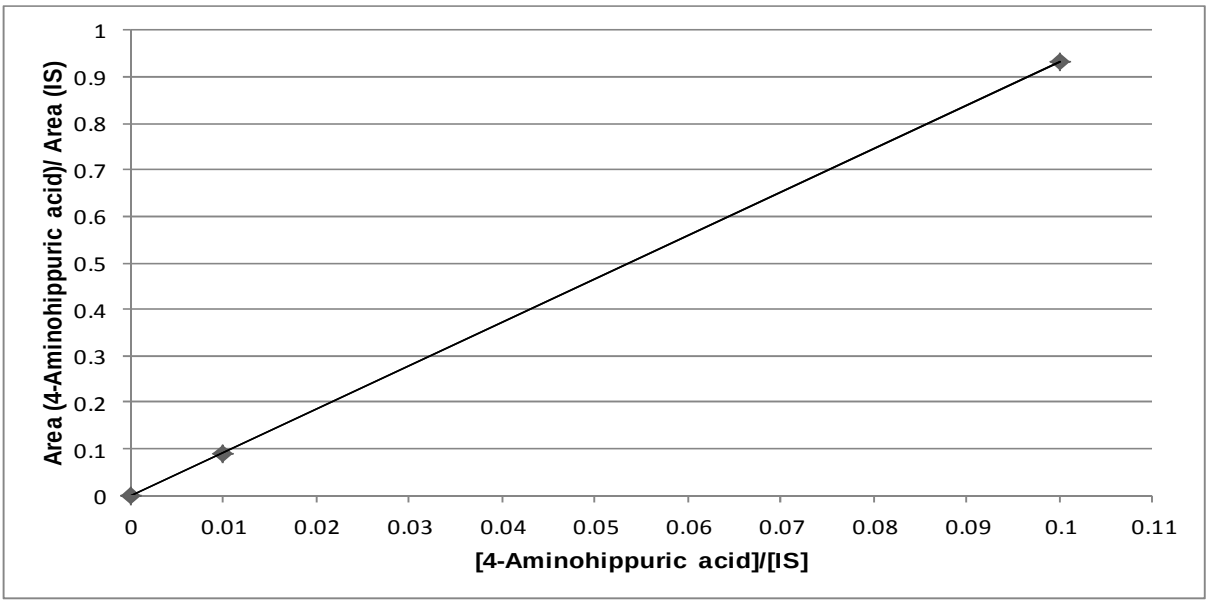

Figure 4.19: Calibration curve of 4-aminohippuric acid in the lower concentration range (R^2 > 0.99).

In Figures 4.18 and 4.19, the standardized LC-ESI-MS/MS assay for 4-aminohippuric acid was linear over the concentration range of 0.01 to 1 mg/L (R^2 > 0.99). Thus the standardized MRM assay could be used to quantify 4-aminohippuric acid (in this concentration range) in HepaRG cells (Chapters 5 and 6).The data used to demonstrate the linearity for the quantification of salicyluric acid, hippuric acid and para-aminohippuric acid (Figure 4.14-Figure 4.19) could be used to calculate the RF of these metabolites relative to the internal standard (Equation 4.1). As discussed in Section 4.3.1.3 and 4.4.1.3, because phase I and phase II biotransformation pathways were compared, it was not necessary for the absolute quantification of the concentration of each formed metabolite. It was thus decided to use a RF value of 1 to only relatively quantify the salicyluric acid, hippuric acid and para-aminohippuric acid concentration, using Equation 4.2.

4.6 Conclusion

The LC-ESI-MS/MS assays to monitor phase I and phase II biotransformation were optimized and validated. The assessment of phase I and phase II biotransformation pathways with specific probe substances could thus be done through the identification and quantification of selected phase I and phase II metabolites with LC-ESI-MS/MS assays.

CHAPTER 5

Optimization of phase I and phase II biotransformation assays in HepaRG cells

5.1 Introduction

As discussed in Section 2.4, HepaRG cells can be used as a valuable *in vitro* model for the investigation of cytochrome P450 (CYP450) induction by drug compounds in humans. HepaRG cells were shown to maintain liver functions and to express genes for various liver specific proteins, including CYP450 enzymes and transporters of the phase II system (Guillouzo *et al.,* 2007; Kanebratt & Andersson*,* 2008; Lambert *et al.,* 2009; Lübberstedt *et al*., 2011). However, HepaRG cells are able to differentiate into two different cell lines when seeded at a low density (2.6 x 10^4 cells/cm^2) (Guillouzo *et al.,* 2007; Kanebratt & Andersson*,* 2008; Lübberstedt *et al*., 2011; Pernelle *et al.,* 2011). These HepaRG cell cultures then contain hepatocyte-like cells and biliary-like epithelial cells with a hepatocyte population of approximately 50%-55% (Kanebratt & Andersson*,* 2008; Lübberstedt *et al*., 2011). Hepatocyte-like cells express various phase I and phase II biotransformation enzymes at levels close to those in hepatocytes (Anthérieu *et al.,* 2012). Guillouzo *et al* (2007) and Kanebratt & Andersson (2008) showed that by adding 2% DMSO to the cells, the hepatocyte-like cells were able to differentiate into more granular cells that closely resembled adult primary hepatocytes. Additionally, the suppliers also recommend the use of HepaRG metabolism medium supplement. This is a specialised medium supplement for HepaRG cell cultures intended to be used in metabolism studies. It is used to supplement the growth and metabolism of HepaRG cells before a probe substance is added.

To optimize phase I and phase II biotransformation assays in HepaRG cells, the effect of DMSO and HepaRG metabolism supplement first had to be investigated. To achieve this, the effect of DMSO working WME growth medium (DMSO media), HepaRG metabolism supplement working WME growth medium (supplement

media), and base WME growth medium on phase I and phase II biotransformation of the selected probe substances were investigated in HepaRG cells. As no experiments were done during this study to indicate possible changes in phase I and phase II enzyme activity after the DMSO media and supplement media were added, only variations in the concentration of the probe substance after incubation as well as the formation of selected metabolites could be used as an indication of the effect of DMSO and supplement on phase I and phase II biotransformation activity in HepaRG cells.

5.2 The effect of dimethyl sulphoxide on phase I and phase II biotransformation assays in HepaRG cells

In experiments done by Aninat *et al* (2006), Kanebratt & Andersson (2008), and Lübberstedt *et al* (2011), after the initial seeding and the attachment of HepaRG cells to the bottom of the cell culture flask, cells were treated for ± 2 weeks with 2% DMSO to obtain maximum differentiation. To investigate the effect of DMSO working WME growth medium (DMSO media prepared as described in Section 3.2.2) on phase I and phase II biotransformation in HepaRG cells, it was decided to expose cells to DMSO media for different time periods (5 days and 10 days). As control, cells were used that were not exposed to any DMSO media at all (no DMSO media). After the incubation period with DMSO media was over, the media was removed and the cells washed with PBS. This was followed by incubating the cells for 72 hours with supplement (HepaRG metabolism supplement working WME growth medium; prepared as described in Section 3.2.2). After the supplement incubation period, the media was removed and replaced with 190 µl fresh base WME growth medium and 10 µl of a selected probe solution (dissolved in PBS).

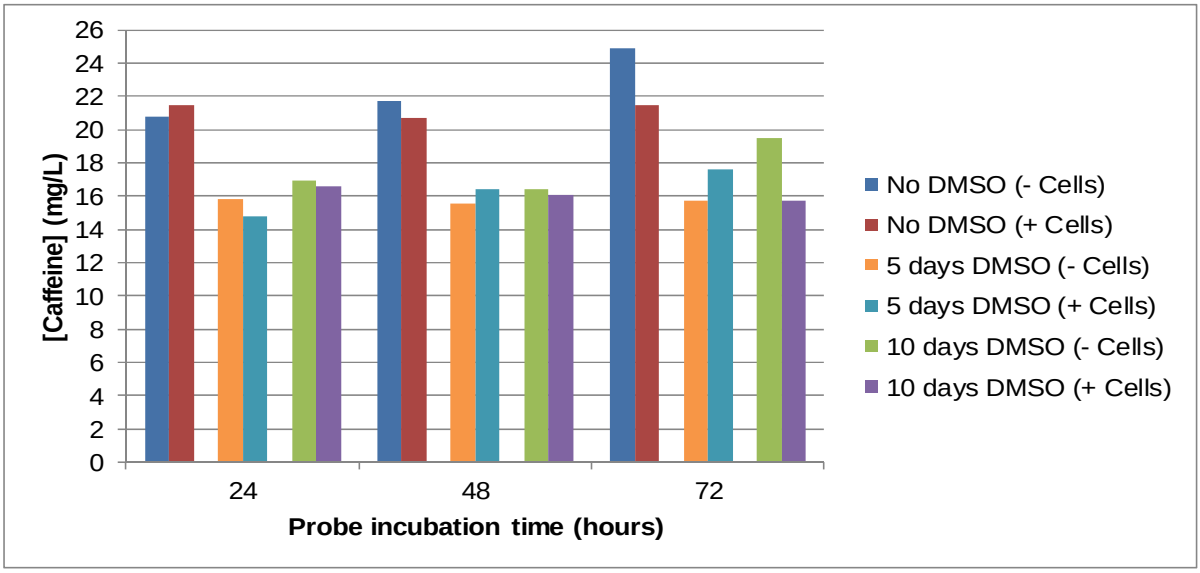

Figure 5.1: The effect of dimethyl sulphoxide on phase I biotransformation in HepaRG cells. The Y-axis represents the caffeine concentration (mg/L) and the X-axis the time (in hours) HepaRG cells were incubated with the probe. Cells were incubated with DMSO media for 0 (no DMSO), 5 and 10 days. After this, all cells were incubated for 72 hours with supplement media. Phase I biotransformation was assessed by incubating the cells for different time periods (24, 48 and 72 hours) with caffeine. Control wells were also treated with DMSO, supplement, and caffeine but the wells contained no cells (- Cells).

Figure 5.1 represents the effect of DMSO and different incubation times with the probe substance (caffeine) on phase I biotransformation in HepaRG cells. The figure compares the caffeine concentration in no DMSO, 5 day DMSO, 10 day DMSO samples without cells (- cells) and with cells (+ cells). From Figure 5.1 it can be observed that there was no visible difference in caffeine concentration between the samples without cells and those with cells, where biotransformation of caffeine was expected. This was observed for the no DMSO, 5 day DMSO and 10 day DMSO samples. However, as illustrated in Figure 5.1, a difference in caffeine concentration was observed between the samples incubated with no DMSO and those which were incubated with DMSO. A possible reason for these results could be that the long period of DMSO incubation had a negative influence on HepaRG cells and thus phase I caffeine biotransformation activity in the cells. It became evident that the HepaRG cells, used in this study, were highly sensitive to DMSO. Indicated by the high amount of floating cells observed in the WME growth medium, cell death occurred from the first day of DMSO incubation. After a 5 to 10 day DMSO incubation period, it was possible that the damage to the HepaRG cells was

irreversible and the amount of observed viable cells present too low for the successful biotransformation of caffeine.

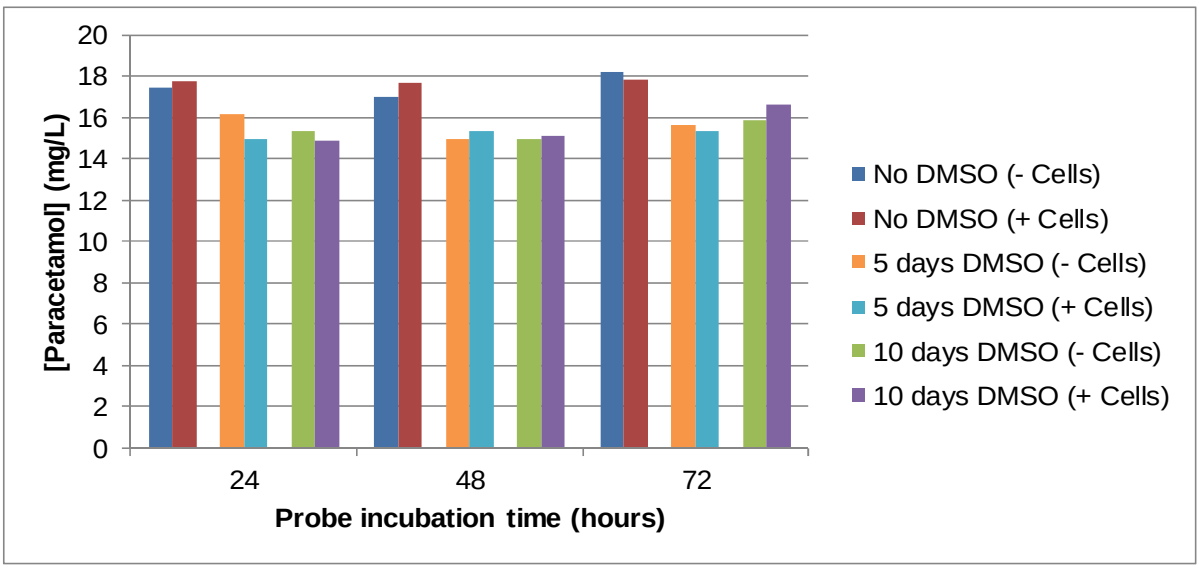

Figure 5.2: The effect of dimethyl sulphoxide on phase II biotransformation in HepaRG cells. The Y-axis represents the paracetamol concentration (mg/L) and the X-axis the time (in hours) HepaRG cells were incubated with the probe. Cells were incubated with DMSO media for 0 (no DMSO), 5 and 10 days. After this, all cells were incubated for 72 hours with supplement media. Phase II biotransformation was assessed by incubating the cells for different time periods (24, 48 and 72 hours) with paracetamol. Control wells were also treated with DSMO, supplement, and paracetamol but the wells contained no cells (- Cells).

Figure 5.2 represent the effect of DMSO and different incubation times with the probe substance (paracetamol) on phase II biotransformation in HepaRG cells. The figure compares the paracetamol concentration in no DMSO, 5 day DMSO, 10 day DMSO samples without cells (- cells) and with cells (+ cells). From Figure 5.2 it can be observed that there was no visible difference in paracetamol concentration between the samples without cells and those with cells, where biotransformation of paracetamol was expected. This was observed for the no DMSO, 5 day DMSO and 10 day DMSO samples. As in the case of phase I caffeine biotransformation, the high amount of floating cells observed in the WME growth medium, indicated that this could be due to the long period of DMSO incubation which caused irreversible damage to the HepaRG cells and the unsuccessful biotransformation of paracetamol.

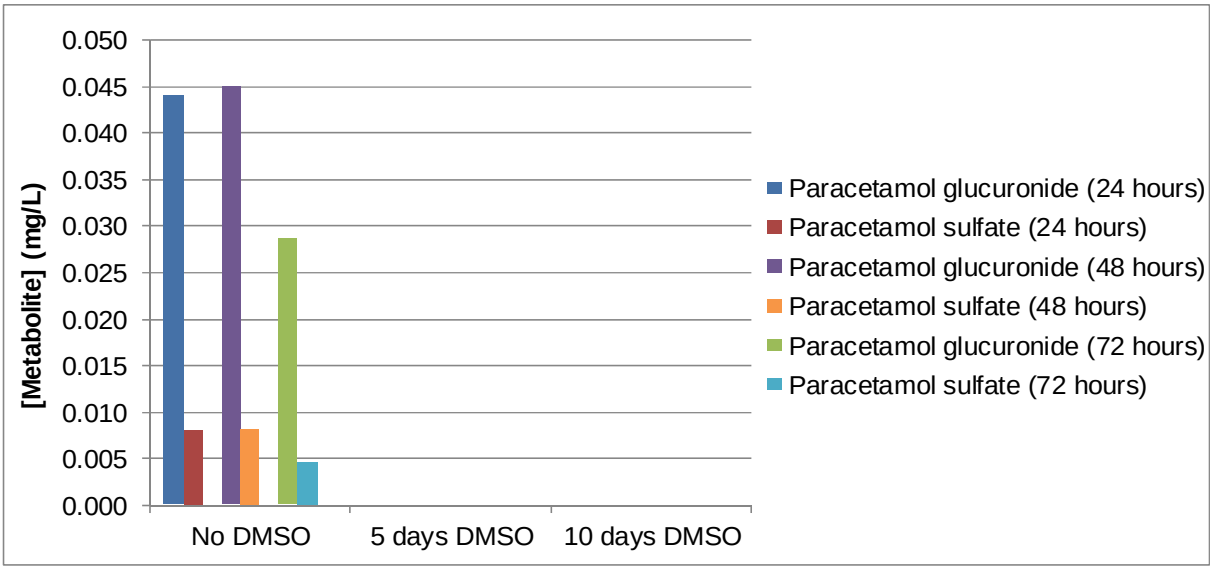

Figure 5.3: The effect of dimethyl sulphoxide on the formation of selected phase II paracetamol metabolites in HepaRG cells. The Y-axis represents the metabolite concentration (mg/L) and the X-axis the different DMSO media incubation periods. Cells were incubated with DMSO media for 0 (no DMSO), 5 and 10 days. After this, all cells were incubated for 72 hours with supplement media. Phase II biotransformation was assessed by incubating the cells for different time periods (24, 48 and 72 hours) with paracetamol.

Figure 5.3 represent the effect of DMSO and different incubation times with the probe substance (paracetamol) on the formation of the selected phase II paracetamol metabolites (paracetamol glucuronide, paracetamol sulfate and paracetamol mercapturate) in HepaRG cells. The figure compares the paracetamol glucuronide and paracetamol sulfate concentration quantified in the no DMSO, 5 day DMSO, 10 day DMSO samples with cells (+ cells) after a 24 hour, 48 hour and 72 hour paracetamol incubation period. No paracetamol mercapturate formation was quantified. As mentioned in Section 2.5.4.2, in humans the biggest part of paracetamol is biotransformed through glucuronic acid (50%-60%) and sulfate conjugation (25%-35%). Only a small amount of paracetamol is converted to the toxic metabolite NAPQI and paracetamol mercapturate (2%-10%) (Lohmann & Karst, 2006). CYP450 has a lower affinity for paracetamol. This causes NAPQI formation to be greater at higher paracetamol concentrations (Slikker et al., 2004; Zamek-Gliszczynski *et al.,* 2005). It was thus possible that the concentration of paracetamol added to the HepaRG cells were high enough for sulfotransferase to be saturated, as a higher amount of paracetamol glucuronide formation was observed than paracetamol sulfate, and that the concentration was too low for NAPQI and paracetamol mercapturate formation. In all further experiments where phase II

biotransformation was assessed using paracetamol as probe substance, the formation of only paracetamol glucuronide and paracetamol sulfate were expected. From Figure 5.3 it can also be observed that, although it was at a very low concentration, paracetamol was only biotransformed in the samples to which no DMSO was added. No metabolite formation was observed in the 5 day DMSO and 10 day DMSO samples. This was observed for a 24, 48 and 72 hour paracetamol incubation period. As with the phase I caffeine results in Figure 5.1, the results in Figure 5.3 again supported the possibility that DMSO had a negative influence on the viability of HepaRG cells which caused the unsuccessful biotransformation of paracetamol.

Based on the results in Section 5.2, obtained from the investigation of the effect of DMSO on phase I and phase II biotransformation in HepaRG cells (Figure 5.1-Figure 5.3) and the major variation observed in the HPLC data, it was decided to not analyse the rest of the DMSO samples. Although a small decrease in the caffeine and paracetamol concentration was observed, after HepaRG cells were incubated with the probe substances, this decrease was very small compared to the variation of the assay (data not shown). It was decided to rather assess phase I and phase II biotransformation by following the formation of specific metabolites instead of trying to monitor the decrease in the concentration of the probe itself (assays described in Chapter 4). Due to the effect of DMSO on the viability of the HepaRG cells and thus phase I and phase II biotransformation, the effect of DMSO on phase II glycine conjugation of aspirin was not investigated. Phase I and phase II biotransformation assays could thus not be optimized in HepaRG cells pre-treated with DMSO. Therefore all future assays were done without DMSO pre-incubation.

5.3 The effect of HepaRG metabolism supplement on phase I and phase II biotransformation assays in HepaRG cells

To investigate the effect of HepaRG metabolism supplement working WME growth medium (supplement media prepared as described in Section 3.2.2) on phase I and phase II biotransformation in HepaRG cells, four different experimental groups were

used: the first group contained HepaRG cells, supplement, and probe; the second group contained supplement and probe; the third group contained only supplement and the forth group contained HepaRG cells and supplement. The experimental design using the four groups was to assess a number of things in a single experiment: firstly if the probe substance is indeed formed; secondly if the formation of the metabolite/s is enzyme dependent (biotransformation reaction) or a spontaneous reaction; and lastly if probe substance is indeed needed for the formation of the selected metabolite/s (or can the metabolite/s be formed from the tissue culture media or the supplement). HepaRG cells were incubated for 72 hours with supplement in 96-well cell culture plates before the biotransformation was assessed by using different probe substances.

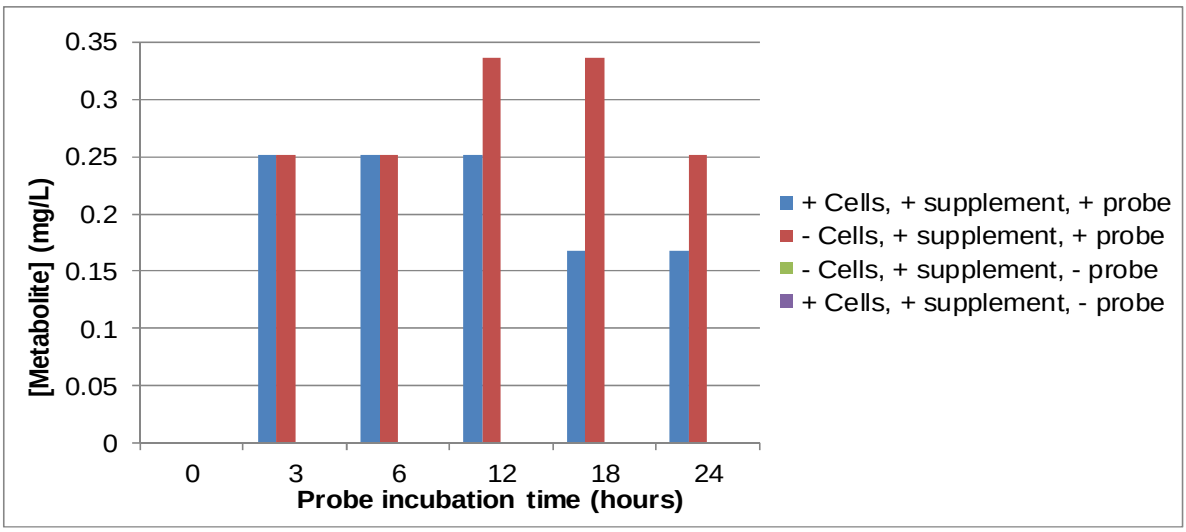

Figure 5.4: The effect of HepaRG metabolism supplement on the formation of the selected phase I caffeine metabolite (theobromine) in HepaRG cells. The Y-axis represents the theobromine concentration (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Four different experimental groups were prepared: the first group contained HepaRG cells, supplement, and probe; the second group contained supplement and probe; the third group contained only supplement and the forth group contained HepaRG cells and supplement. Cells were incubated for 72 hours with supplement media. Phase I biotransformation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with caffeine.

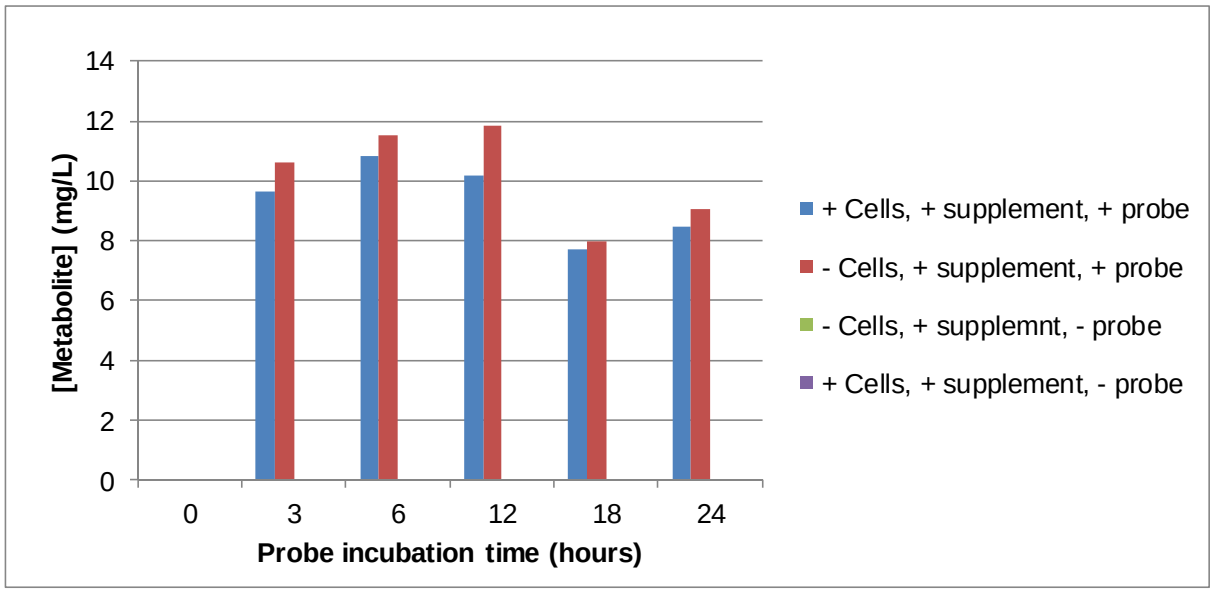

Figure 5.5: The effect of HepaRG metabolism supplement on the formation of the selected phase I caffeine metabolite (theophylline and paraxanthine) in HepaRG cells. The Y-axis represents the theophylline and paraxanthine concentration (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Four different experimental groups were prepared: the first group contained HepaRG cells, supplement, and probe; the second group contained supplement and probe; the third group contained only supplement and the forth group contained HepaRG cells and supplement. Cells were incubated for 72 hours with supplement media. Phase I biotransformation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with caffeine.

Figure 5.4 and 5.5 represent the effect of HepaRG metabolism supplement on the formation of the selected phase I caffeine metabolites (theobromine, theophylline and paraxanthine) in HepaRG cells. The biotransformation of caffeine at shorter incubation times (3, 6, 12, 18, and 24 hours) was also assessed. Figure 5.4 compares the theobromine concentration and Figure 5.5 the theophylline and paraxanthine concentration quantified in the samples with different combinations of cells (+ cells), supplement (+ supplement) and caffeine (+ probe). From Figure 5.4 and 5.5 it can be observed that no theobromine, theophylline and paraxanthine formation was observed in the samples where no probe substance was added (-probe). This indicated that the formation of these metabolites was only possible if caffeine was added to the cells. From Figure 5.4 and 5.5 it can also be observed that theobromine, theophylline and paraxanthine formation was already detected after 3 hours of incubation, indicating that caffeine biotransformation is indeed rapid in HepaRG cells. Theobromine, theophylline and paraxanthine formation was observed in the samples containing cells, supplements) and probe (where caffeine was

expected to be biotransformed), as well as in the samples without cells (- cells), containing only supplements and probe. This indicated that the formation of selected caffeine metabolites was independent on the presence of HepaRG cells.

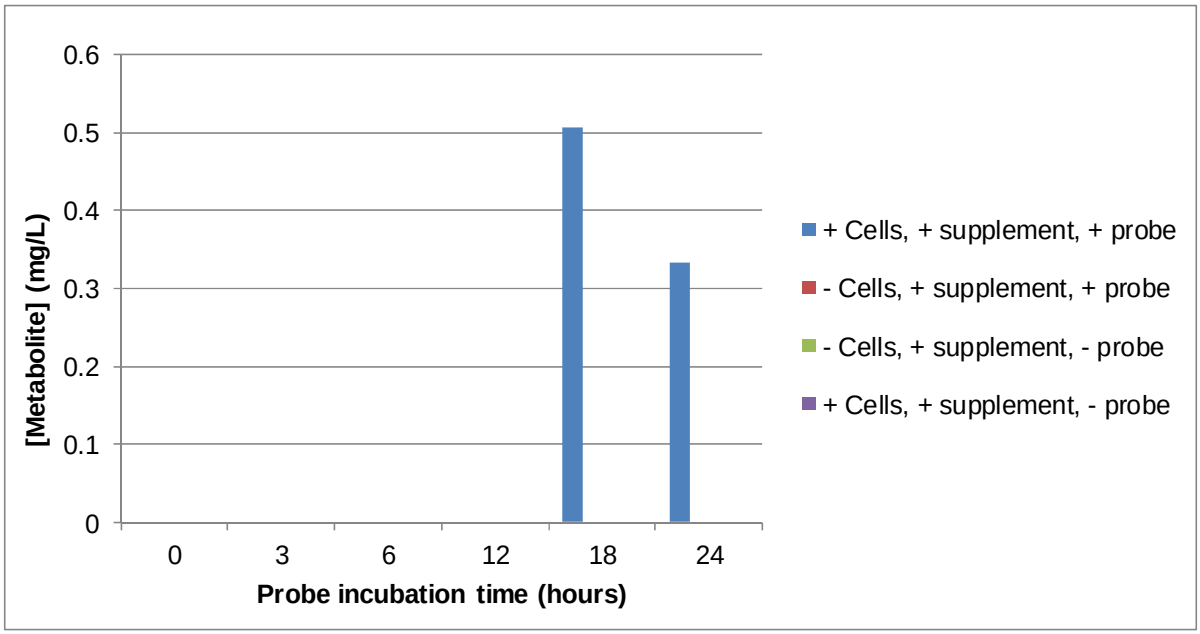

Figure 5.6: The effect of HepaRG metabolism supplement on the formation of the selected phase II paracetamol metabolite (paracetamol glucuronide) in HepaRG cells. The Y-axis represents the paracetamol glucuronide concentration (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Four different experimental groups were prepared: the first group contained HepaRG cells, supplement, and probe; the second group contained supplement and probe; the third group contained only supplement and the forth group contained HepaRG cells and supplement. Cells were incubated for 72 hours with supplement media. Phase II biotransformation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with paracetamol.

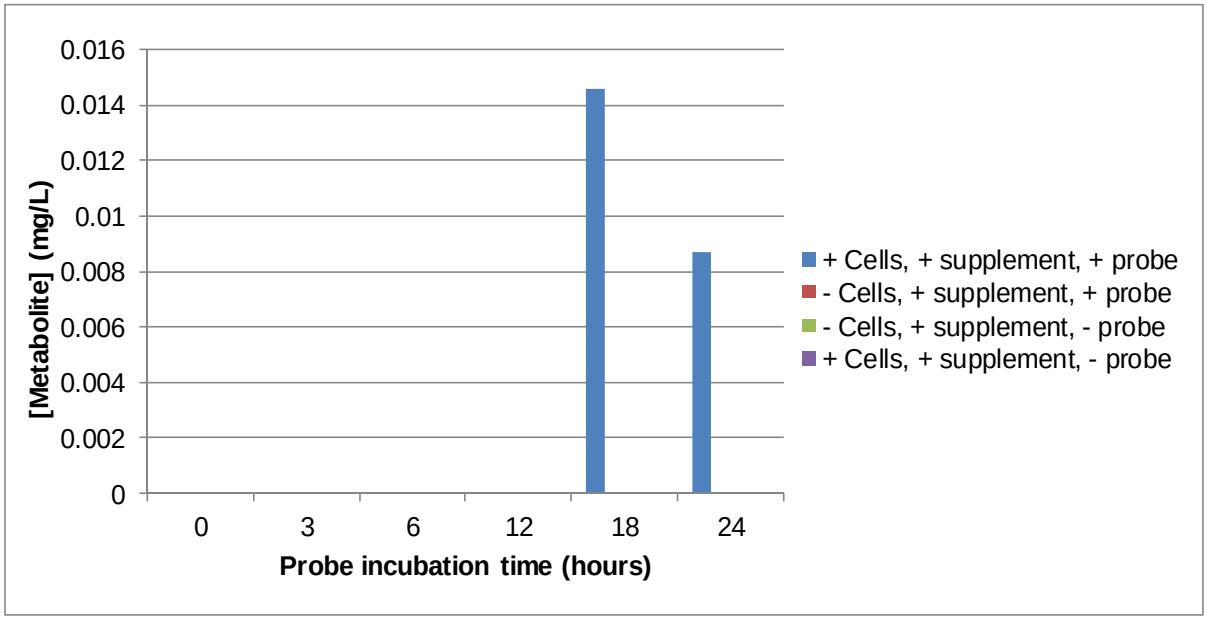

Figure 5.7: The effect of HepaRG metabolism supplement on the formation of the selected phase II paracetamol metabolite (paracetamol sulfate) in HepaRG cells. The Y-axis represents the paracetamol sulfate concentration (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Four different experimental groups were prepared: the first group contained HepaRG cells, supplement, and probe; the second group contained supplement and probe; the third group contained only supplement and the forth group contained HepaRG cells and supplement. Cells were incubated for 72 hours with supplement media. Phase II biotransformation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with paracetamol.

Figure 5.6 and 5.7 represent the effect of HepaRG metabolism supplement on the formation of the selected phase II paracetamol metabolites (paracetamol glucuronide and paracetamol sulfate) in HepaRG cells. The biotransformation of paracetamol was assessed at shorter incubation times (3, 6, 12, 18, and 24 hours). Figure 5.6 represent the paracetamol glucuronide concentration and Figure 5.7 the paracetamol sulfate concentration quantified in the samples with different combinations of cells (+ cells), supplement (+ supplement) and caffeine (+ probe) after a 3, 6, 12, 18, and 24 hour paracetamol incubation period. From Figure 5.6 and 5.7 it can be observed that no paracetamol glucuronide and paracetamol sulfate formation was observed if cells and probe were not present. This indicated that phase II paracetamol biotransformation to the selected metabolites (paracetamol glucuronide and paracetamol sulfate) was only due to phase II biotransformation enzyme activity in HepaRG cells and not due to enzyme activity in the supplement. From Figure 5.6 and 5.7 it can also be observed that the formation of both paracetamol glucuronide and paracetamol sulfate was only quantifiable in the 18 and 24 hour samples. No

paracetamol metabolite formation could be quantified in the 3, 6 and 12 hour samples. These results indicated that it was possible that *in vitro* phase II paracetamol biotransformation were more successful at longer periods of incubation with paracetamol and that a 3 – 12 hour incubation period was a too short for sulfate and glucuronic acid conjugation to occur. No paracetamol mercapturate could be detected in any of the samples analyzed. As mentioned in Section 2.5.4.2, in humans the biggest part of paracetamol is biotransformed through glucuronic acid (50-60%) and sulfate conjugation (25-35%). Only a small amount of paracetamol is converted to the toxic metabolite NAPQI and paracetamol mercapturate (2-10%) (Lohmann & Karst, 2006). CYP450 has a lower affinity for paracetamol. This causes NAPQI formation to be greater at higher paracetamol concentrations (Slikker et al., 2004; Zamek-Gliszczynski *et al.,* 2005). It was thus possible that the concentration of paracetamol added to the HepaRG cells were high enough for sulfotransferase to be saturated, as a higher amount of paracetamol glucuronide formation was observed than paracetamol sulfate, and that the concentration was too low for NAPQI and paracetamol mercapturate formation. In all further experiments where phase II biotransformation was assessed using paracetamol as probe substance, the formation of only paracetamol glucuronide and paracetamol sulfate was expected.

As discussed in Section 5.1, aspirin, sodium benzoate and para-aminobenzoic acid were used to assess phase II glycine conjugation activity in HepaRG cells. After aspirin was added to HepaRG cells, no salicyluric acid formation was observed (results not shown). As mentioned in Section 2.5.4.3, during aspirin biotransformation, aspirin is firstly degraded into salicylic acid, which is eventually biotransformed through glycine conjugation and glucuronic acid conjugation, to form the major metabolite salicyluric acid (Liska *et al*., 2006). A possible reason for no salicyluric acid formation could be that the aspirin incubation period in the HepaRG cells were too short for aspirin to be biotransformed from aspirin to salicylic acid and then to salicyluric acid. It was thus decided to use salicylic acid as a probe substance instead of aspirin to assess the phase II glycine conjugation pathway. By directly adding salicylic acid to the HepaRG cells, the first step in the aspirin biotransformation pathway was eliminated. This increased the chances of salicyluric acid formation and decreased the probe incubation period. In all further experiments

salicylic acid, sodium benzoate and para-aminobenzoic acid were used as probe substances to assess phase II glycine conjugation in HepaRG cells. By comparing the biotransformation pathways of the probes, the best probe to assess phase II glycine conjugation could be identified.

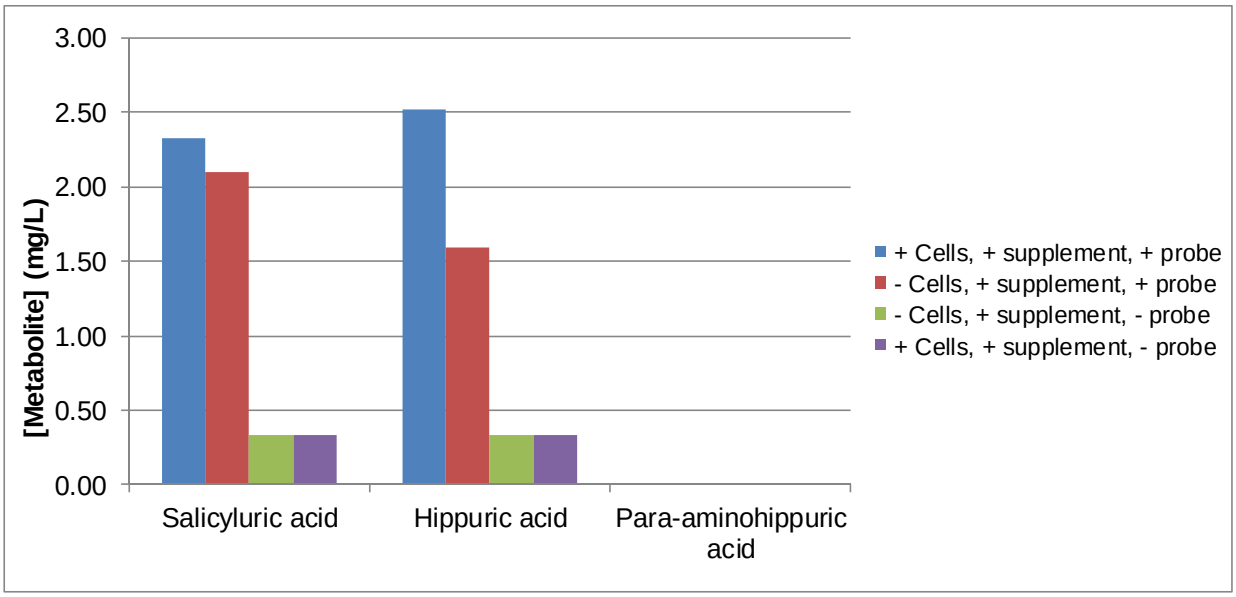

Figure 5.8: The effect of HepaRG metabolism supplement on the formation of the selected phase II glycine conjugation metabolites (salicyluric acid, hippuric acid and para-aminohippuric acid) in HepaRG cells. The Y-axis represents the metabolite concentration (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Four different experimental groups were prepared: the first group contained HepaRG cells, supplement, and probe; the second group contained supplement and probe; the third group contained only supplement and the forth group contained HepaRG cells and supplement. Cells were incubated for 72 hours with supplement media. Phase II glycine conjugation was assessed by incubating the cells for 24 hours with salicylic acid, sodium benzoate or para-aminobenzoic acid.

Figure 5.8 represent the effect of HepaRG metabolism supplement on the formation of the selected phase II glycine conjugation metabolites (salicyluric acid, hippuric acid and para-aminohippuric acid) in HepaRG cells. No para-aminohippuric acid could be quantified with the LC-ESI-MS/MS assay. From Figure 5.8 it can be observed that salicyluric acid and hippuric acid formation was quantified in all of the samples. Relative to the rest of the samples, a small amount of salicyluric acid and hippuric acid was quantified in the samples with only supplement (- cells, + supplement, - probe) and in the samples with supplement and cells (+ cells, + supplement, - probe). This indicated that these metabolites could be formed even if the probe was not added to the sample. From Figure 5.8 it can also be observed that

when the salicylic acid and sodium benzoate probes were added to only supplement (- cells, + supplement, + probe), a high amount of salicyluric acid and hippuric acid was formed. This again indicated, as in the case of phase I caffeine biotransformation, that the phase II glycine conjugation of salicylic acid and hippuric acid was independent of the presence of HepaRG cells. However, illustrated in Figure 5.8, higher salicyluric acid and hippuric acid concentrations were quantified in the samples with cells (+ cells, + supplement, + probe) compared to the samples without cells (- cells, + supplement, + probe). This indicated that a certain percentage of the formed metabolites were due to phase II glycine conjugation activity in HepaRG cells. The figure clearly indicates that, compared to the salicyluric acid concentration, a higher concentration of hippuric acid formation was possibly due to phase II glycine conjugation activity in HepaRG cells. Based on these results it was decided to use sodium benzoate as probe substance to assess phase II glycine conjugation in all further experiments.

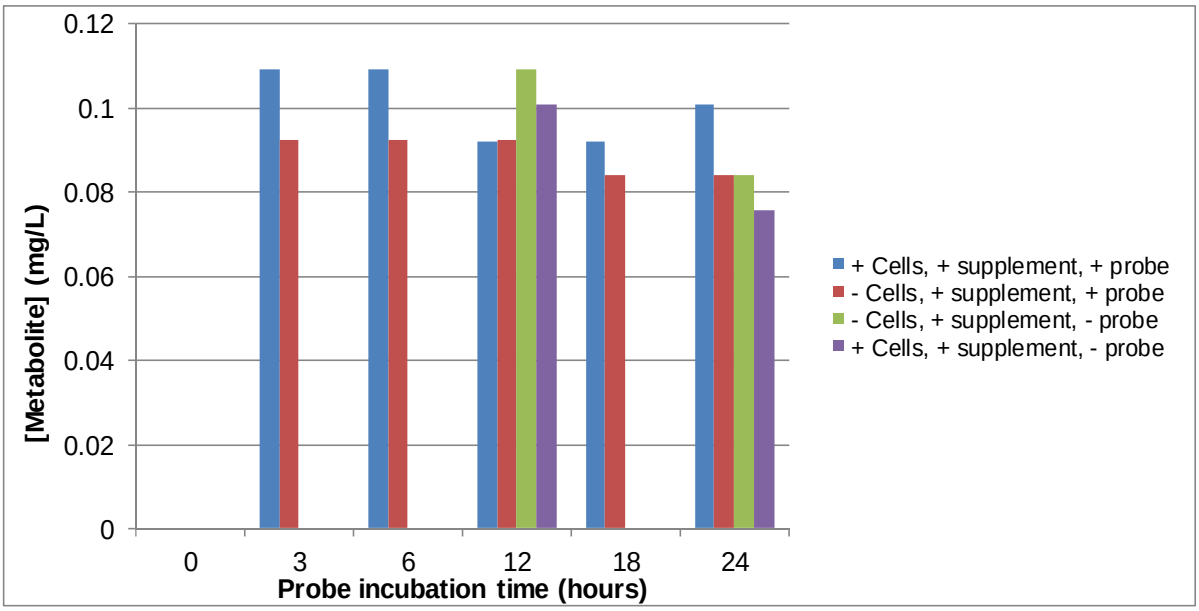

Figure 5.9: The effect of HepaRG metabolism supplement on the formation of the selected phase II glycine conjugation metabolite (hippuric acid) in HepaRG cells. The Y-axis represents the hippuric acid concentration (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Four different experimental groups were prepared: the first group contained HepaRG cells, supplement, and probe; the second group contained supplement and probe; the third group contained only supplement and the forth group contained HepaRG cells and supplement. Cells were incubated for 72 hours with supplement media. Phase II glycine conjugation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with sodium benzoate.

Figure 5.9 represents the effect of HepaRG metabolism supplement on the formation of the selected phase II glycine conjugation metabolite (hippuric acid) in HepaRG cells. From Figure 5.9 it can be observed that after a 12 and 24 hour sodium benzoate incubation period, hippuric acid was formed in all of the samples, and after 3, 6, and 18 hours only in the samples with HepaRG cells to which supplement and sodium benzoate was added (+ cells, + supplement, + probe) and in the samples which contained only supplement and sodium benzoate (- cells, + supplement, + probe). This indicated that it was possible that phase II glycine conjugation of sodium benzoate occurred as early as 3 hours in HepaRG cells. Based on these results, it was decided to focus on the 3 hour results, because a high amount of hippuric acid was already present in these samples and hippuric acid was only quantified in two of the prepared samples. From the 3 hour sample in Figure 5.9 it can be observed that hippuric acid was again quantified in the samples with cells and those without. Although a higher amount of hippuric acid was quantified in the samples with cells (+ cells, + supplement, + probe) than in those without cells, the difference was small. From this result it seems that only a small fraction of the formed hippuric acid is due to phase II glycine conjugation by the HepaRG cells.

Based on the results in Section 5.3, obtained from the investigation of the effect of HepaRG metabolism supplement on phase I and phase II biotransformation in HepaRG cells (Figure 5.4-Figure 5.9), it was decided not to analyse the rest of the supplement media samples. As the composition of the HepaRG metabolism supplement was unknown and no samples were prepared without supplements it was not certain if the metabolite formation, observed in the samples with cells, were due to phase II glycine conjugation activity in the cells or due to enzyme activity in the HepaRG metabolism supplement or FBS added to the WME growth medium. Phase I and phase II biotransformation assays could thus not be optimized in HepaRG cells pre-treated with HepaRG metabolism supplement. Therefore all future assays were done without a supplement media pre-incubation.

5.4 The optimized phase I and phase II biotransformation assays in HepaRG cells

To optimize phase I and phase II biotransformation assays in HepaRG cells, samples with cells (+ cells), where phase I and phase II biotransformation activity was expected, and samples without cells (- cells) were prepared in triplicate. HepaRG cells were cultured in only base WME growth medium until they were confluent. After confirming the confluence of the cells, 190 µl fresh base WME growth medium was added to the wells followed by 10 µl of the specific probe solution (dissolved in PBS) and the plates incubated for different time periods (3, 6, 12, 18, and 24 hours). After each specific probe incubation period was completed, the plate was removed from the incubator and the samples prepared as described in Section 3.4.1.2-Section 3.4.1.4, and analyzed with the optimized LC-ESI-MS/MS assays (Chapter 4). The results are displayed in Figure 5.10-Figure 5.14.

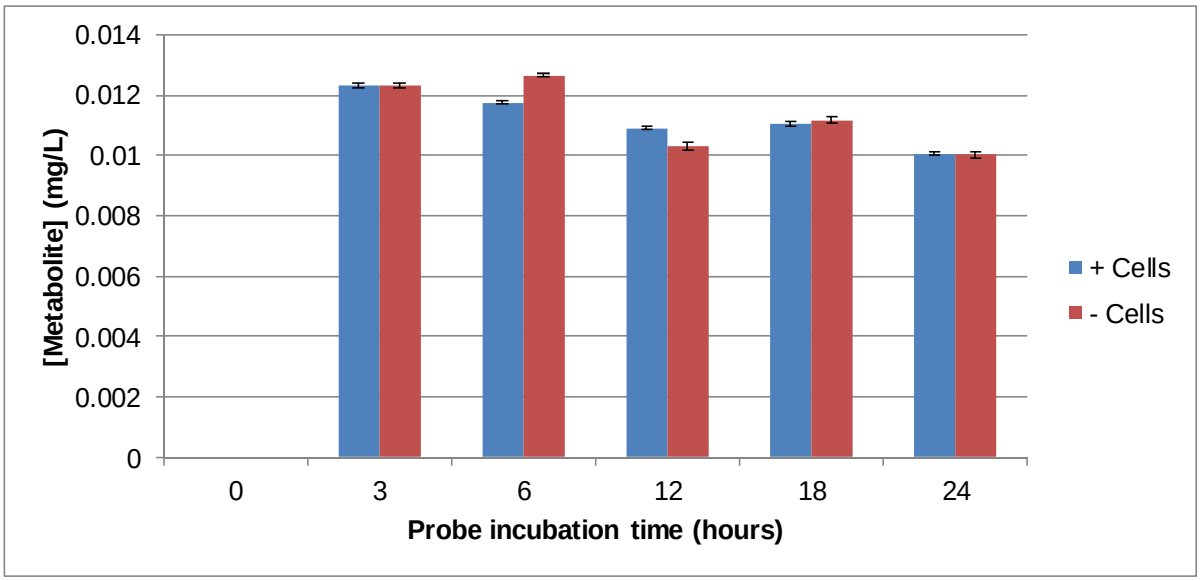

Figure 5.10: Phase I caffeine biotransformation to theobromine in HepaRG cells. The Y-axis represents the theobromine concentration in milligrams per litre (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Samples with (+ cells) and without cells (- cells) were prepared in triplicate. Phase I biotransformation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with caffeine. Each data point in the figure represents the mean ± SD values of three separate samples. Two way ANOVA: $p < 0.01$ (effect of time) and $p = 0.63$ (+/- cells).

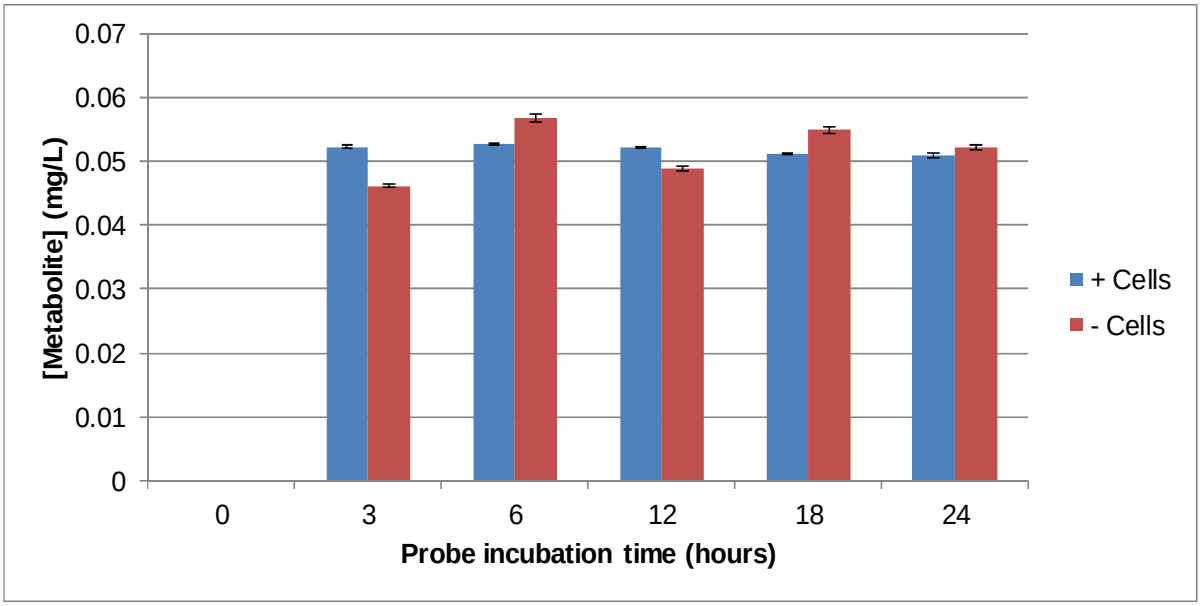

Figure 5.11: Phase I caffeine biotransformation to theophylline and paraxanthine in HepaRG cells. The Y-axis represents the theophylline and paraxanthine concentration in milligrams per litre (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Samples with (+ cells) and without cells (- cells) were prepared in triplicate. Phase I biotransformation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with caffeine. Each data point in the figure represents the mean ± SD values of three separate samples. Two way ANOVA: $p < 0.01$ (effect of time) and $p = 0.97$ (+/- cells).

Figure 5.10 and 5.11 represent phase I caffeine biotransformation to the selected metabolites (theobromine, theophylline and paraxanthine) in HepaRG cells. Figure 5.10 compares the theobromine concentration and Figure 5.11 the theophylline and paraxanthine concentration quantified in the samples with cells (+ cells) to the samples without cells (- cells) after a 3, 6, 12, 18, and 24 hour caffeine incubation time. From Figure 5.10 and 5.11 it can be observed that theobromine, theophylline and paraxanthine were quantified in the samples with cells and in the samples without cells. From the figures it can also be observed that there was no significant difference in the theobromine, theophylline and paraxanthine concentrations quantified in the samples with cells, where phase I caffeine biotransformation activity was expected, and the samples without cells ($p = 0.97$) after a 3, 6, 12, 18, and 24 hour caffeine incubation time ($p < 0.01$). There was thus no certainty whether the metabolite formation was due to phase I caffeine biotransformation activity in the cells or due to other reactions. As no DMSO or supplement was added to the cells, these results indicated that phase I caffeine biotransformation in HepaRG cells to the selected metabolites was most likely due to caffeine being biotransformed by

enzymes already present in the base WME medium. To supplement the growth of HepaRG cells, 10 % FBS was added to the base WME growth medium. The serum contains enzymes (Lee et al., 2001; Salvaggio et al., 1991) which could have influenced the phase I cytochrome P450 (CYP450) enzyme activity in the HepaRG cells. Nakama *et al* (1995) compared the activity of CYP450-dependant monooxygenase (MFO), involved in catalyzing phase I biotransformation of endogenous and exogenous xenobiotics, in HepG2 cells cultured in growth medium with serum with HepG2 cells cultured in serum-free growth medium. The results indicated MFO activity were twice as high in the cells cultured in serum-free medium compared to the MFO activity in the HepaRG cells cultured in medium with serum. This illustrated that enzymes present in the serum influenced the expression and activity of phase I enzymes. When evaluating the activity of specific enzymes involved in biotransformation reactions, the use of serum-free medium minimizes the amount of variables and allows the culture of cells within a defined set of conditions (Nakama *et al.,* 1995). The phase I caffeine biotransformation assay could thus not be optimized in HepaRG cells.

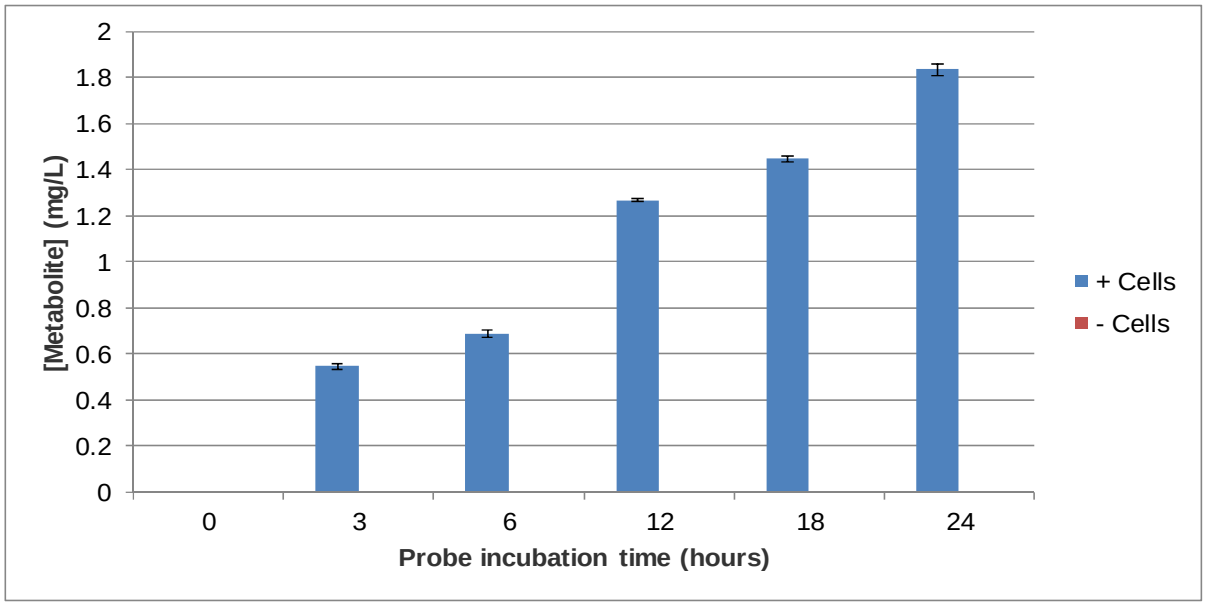

Figure 5.12: Phase II paracetamol biotransformation to paracetamol glucuronide in HepaRG cells. The Y-axis represents the paracetamol glucuronide concentration in milligrams per litre (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Samples with (+ cells) and without cells (- cells) were prepared in triplicate. Phase II biotransformation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with paracetamol. Each data point in the figure represents the mean ± SD values of three separate samples. Two way ANOVA: $p < 0.01$ (effect of time) and $p < 0.01$ (+/- cells).

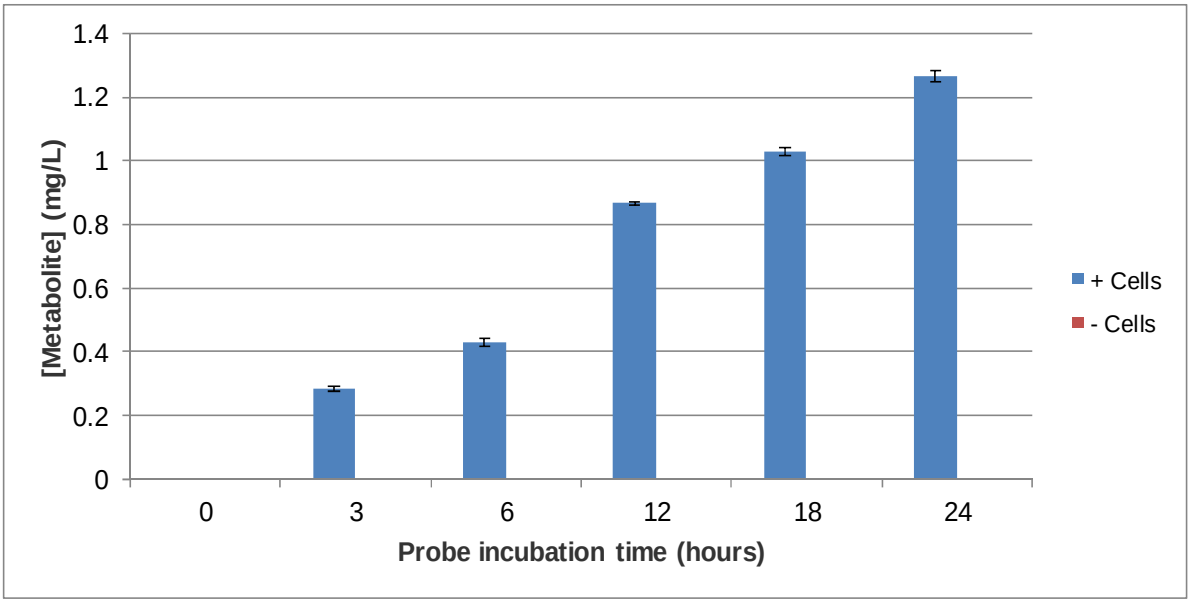

Figure 5.13: Phase II paracetamol biotransformation to paracetamol sulfate in HepaRG cells. The Y-axis represents the paracetamol sulfate concentration in milligrams per litre (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Samples with (+ cells) and without cells (- cells) were prepared in triplicate. Phase II biotransformation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with paracetamol. Each data point in the figure represents the mean ± SD values of three separate samples. Two way ANOVA: $p < 0.01$ (effect of time) and $p < 0.01$ (+/- cells).

Figure 5.12 and 5.13 represent phase II paracetamol biotransformation to the selected metabolites (paracetamol glucuronide and paracetamol sulfate) in HepaRG cells. Figure 5.12 compares the paracetamol glucuronide concentration and Figure 5.13 the paracetamol sulfate concentration quantified in the samples with cells (+ cells) to the samples without cells (- cells) after a 3, 6, 12, 18, and 24 hour paracetamol incubation time. From Figure 5.12 and 5.13 it can be observed that the formation of paracetamol glucuronide and paracetamol sulfate was only quantified in the samples with cells ($p < 0.01$). The figures also indicate an increase in the formation of the metabolites over time with the highest quantified concentration at 24 hours ($p < 0.01$). Figure 5.12 and 5.13 also illustrate paracetamol metabolite formation as early as 3 hours. This supported the theory that supplement had a negative impact on HepaRG cells (Figure 5.6 and 5.7). These results indicated that phase II paracetamol biotransformation to the selected metabolites (paracetamol glucuronide and paracetamol sulfate) was due to phase II biotransformation activity in HepaRG cells and not due to other reactions in the base WME growth medium. The phase II paracetamol biotransformation assay was thus optimized in HepaRG cells.

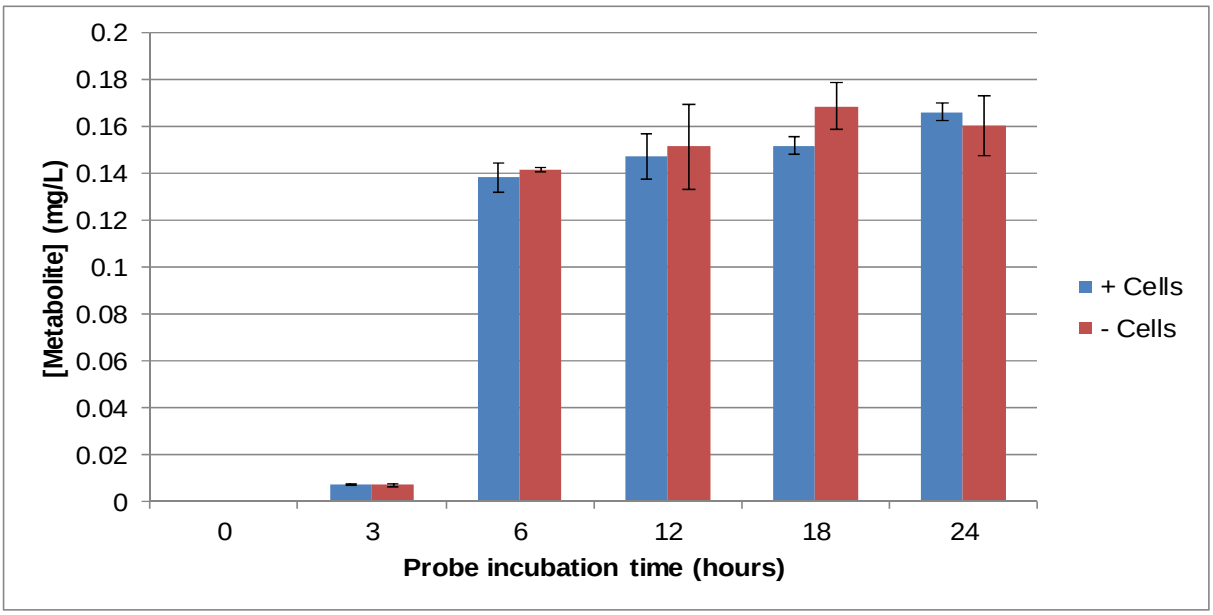

Figure 5.14: Phase II glycine conjugation of sodium benzoate to hippuric acid in HepaRG cells. The Y-axis represents the hippuric acid concentration in milligrams per litre (mg/L) and the X-axis the period of time (in hours) HepaRG cells were incubated with the probe. Samples with (+ cells) and without cells (- cells) were prepared in triplicate. Phase II glycine conjugation was assessed by incubating the cells for different time periods (3, 6, 12, 18, and 24 hours) with sodium benzoate. Each data point in the figure represents the mean ± SD values of three separate samples. Two way ANOVA: $p < 0.01$ (effect of time) and $p = 0.27$ (+/- cells).

Figure 5.14 represent phase II glycine conjugation of sodium benzoate to the selected metabolite (hippuric acid) in HepaRG cells. Figure 5.14 compares the hippuric acid concentration quantified in the samples with cells (+ cells) to the samples without cells (- cells) after a 3, 6, 12, 18, and 24 hour sodium benzoate incubation time. From the figure it can be observed that hippuric acid was quantified in the samples with cells and in the samples without cells. Compared to the results in Figure 5.8 and 5.9 where hippuric acid formation was quantified, in the samples with and in the samples without cells, after cells were pre-incubated with supplement media, from the Figure 5.14, it can be observed that there was no significant difference in the hippuric acid concentrations quantified in the samples with cells, where phase I caffeine biotransformation activity was expected, and the samples without cells ($p = 0.27$). As no DMSO or supplement were added to the cells, the results in Figure 5.14 indicated that phase II glycine conjugation of sodium benzoate in HepaRG cells to hippuric acid was most likely due to sodium benzoate being biotransformed by enzymes already present in the base WME growth medium. The phase II glycine conjugation assay could thus not be optimized in HepaRG cells.

5.5 Conclusion

To optimize phase I and phase II biotransformation assays in HepaRG cells, the effect of DMSO and HepaRG metabolism supplement was investigated. Due to the effect of DMSO on the viability and thus, the biotransformation activity of HepaRG cells which lead to the unsuccessful biotransformation of the probe substances, phase I and phase II biotransformation assays could not be optimized in HepaRG cells, pre-treated with DMSO media. As the composition of the HepaRG metabolism supplement was unknown and no samples were prepared without supplements, it was not certain if the metabolite formation, observed in the samples with cells, were due to phase I or phase II biotransformation activity in HepaRG cells or due to enzyme activity in the HepaRG metabolism supplement or WME growth medium. Phase I and phase II biotransformation assays could thus not be optimized in HepaRG cells pre-treated with HepaRG metabolism supplement media. Therefore all further assays were done without DMSO and supplement.

To optimize phase I and phase II biotransformation assays, HepaRG cells were thus cultured in only base WME growth medium. As no DMSO or supplement were added to the cells, the results obtained indicated that phase I caffeine biotransformation and phase II glycine conjugation in HepaRG cells to the selected metabolites was most likely due to the probes being biotransformed by enzymes already present in the FBS added to the base WME medium, since the rate of biotransformation was the same with and without cells. Phase I caffeine biotransformation and phase II glycine conjugation assays could not be optimized in HepaRG cells. Only the phase II paracetamol biotransformation assay could be optimized in HepaRG cells, illustrated by the time-dependent formation of paracetamol glucuronide and paracetamol sulfate.

CHAPTER 6

Sulforaphane, oxidative stress and biotransformation in HepaRG cells

6.1 Introduction

As discussed in Section 2.3, sulforaphane is an isothiocyanate found in high concentrations in cruciferous vegetables like broccoli. Isothiocyanates are derived from specific biological active compounds found in these vegetables known as glucosinolates. Sulforaphane has received much attention due to the evidence that sulforaphane inhibits phase I carcinogen-bioactivating enzymes and/or induces phase II antioxidant enzymes as well as metallothioneins (MTS) (Perocco *et al.,* 2006; Clarke *et al.,* 2008; Yeh & Yen, 2009). The inhibition of phase I prevents the conversion of possible procarcinogens into carcinogens. The activation of phase II enzymes is important in the prevention of cancer as they biotransform carcinogens to inactive metabolites that are excreted from the body, thus preventing any cellular damage. If not inactivated, carcinogens can cause DNA damage which leads to genomic instability and possible cancer development. DNA damage is also caused by oxidative stress. Reactive oxygen species (ROS) is thought to play multiple roles in tumor initiation, progression and maintenance. To prevent this, free radicals are scavenged by MTS, also induced by sulforaphane (Yeh & Yen., 2005; Clarke *et al.,* 2008; Yeh &Yen, 2009).

6.2 Sulforaphane

6.2.1 The effect of sulforaphane on induced oxidative stress in HepaRG cells

6.2.1.1 Standardization of the sulforaphane concentration and incubation time in HepaRG cells

The first step in the assessment of the effect of sulforaphane on induced oxidative stress and biotransformation in HepaRG cells was to standardize the sulforaphane

concentration and time of exposure in HepaRG cells. This was done to determine which sulforaphane concentration and what period of time HepaRG cells could be exposed to sulforaphane without causing major cell death. As no experiments were done during this study to physically measure the phase I and phase II enzyme activity and MT expression in HepaRG cells after sulforaphane was added, sulforaphane concentrations proven in the literature to have an effect on phase I and phase II biotransformation and MT expression in cells were used. A study done by Yeh & Yen (2005), where the human hepatocellular carcinoma cell line, HepG2 cells, were incubated for 24 hours with increasing concentrations (20 μM-100 μM) of sulforaphane, indicated when cells were exposed to 1 μM-20 μM of sulforaphane for 24 hours the cell viability ranged between 90%-100% after which it decreased. Yeh & Yen (2005) also found increasing concentrations of sulforaphane caused increasing MT-I and MT-II mRNA expression in HepG2 cells. Anwar-Mohamed & El-Kadi (2009) investigated the effect of sulforaphane on the expression of CYP1A1 mRNA. After HepG2 cells were exposed to increasing concentrations of sulforaphane (1 μM-10 μM) for 6 hours, they found the maximum expression of CYP1A1 mRNA was at 5 μM sulforaphane. In order to investigate the optimal incubation time where sulforaphane will cause maximum induction of CYP1A1 mRNA, cells were exposed to 5 μM sulforaphane for different periods of time between 1 - 24 hours. The results indicated maximum induction of CYP1A1 mRNA expression occurred at 6 hours after which it decreased. Based on the results of these studies it was decided to expose HepaRG cells to 1 μM, 2.5 μM, 5 μM, 10 μM and 20 μM sulforaphane, respectively for 1, 2, 3 and 6 hours. Sulforaphane stock solutions of 1 μM, 2.5 μM, 5 μM, 10 μM, and 20 μM were prepared using DMSO as solvent according to the method published by Anwar-Mohamed & El-Kadi (2009).

Pre-treatment of cells

After inspecting the confluence of the cells, the different sulforaphane concentrations were added to cells in triplicate. To each well 190 μl fresh base WME growth medium was added followed by 10 μl of a specific sulforaphane concentration, which added up to a total volume of 200 μl in each well. Identical plates were prepared for a 1, 2, 3 and 6 hour incubation times and the plates incubated. After each incubation

time, the MTT assay was performed on all wells, as described in Section 3.3.2. The results obtained from the completed MTT assay are displayed in Figure 6.1.

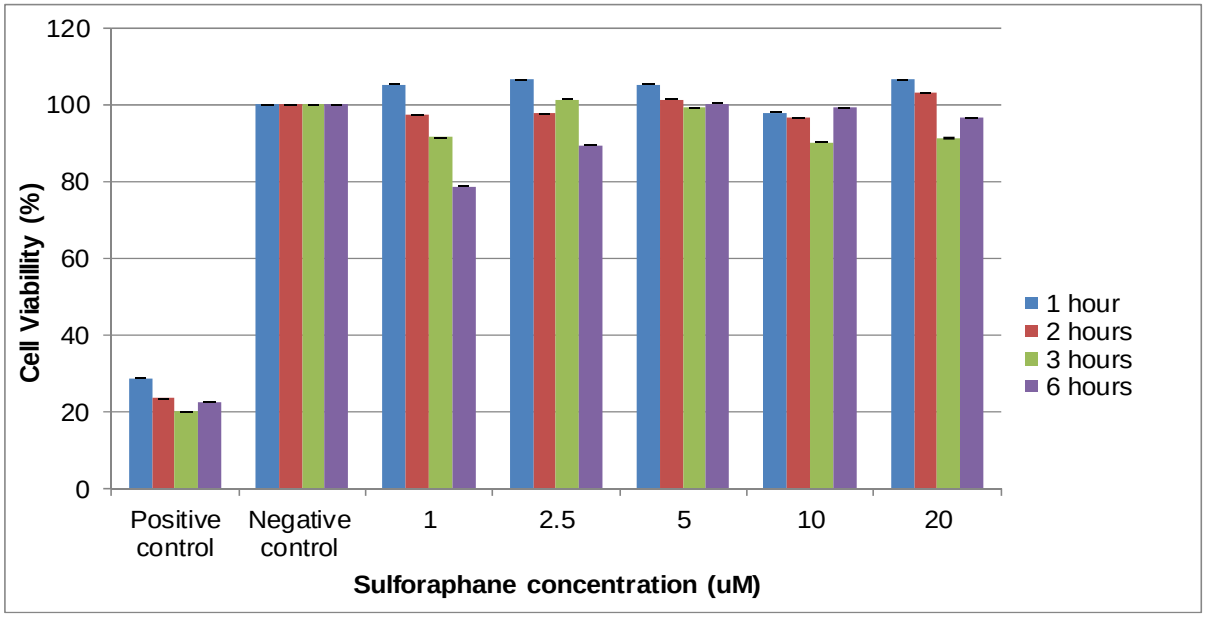

Figure 6.1: The effect of different sulforaphane concentrations and incubation times on the cell viability of HepaRG cells. The Y-axis represents the percentage (%) cell viability and the X-axis the sulforaphane concentration in micromolar (µM). After checking the confluence of the cells, different doses of sulforaphane (1 µM-20 µM) was added to the cells and the cell culture plates incubated for different time periods (1, 2, 3 and 6 hours). To the positive control acetic acid was added and to the negative control no chemicals. The cell viability was determined with a MTT assay. Each data point in the figure represents the mean ± SD values of three separate samples. Two way ANOVA: p = 0.02 (effect of time) and p = 0.39 (sulforaphane concentration).

Figure 6.1 represent the effect of different sulforaphane concentrations and incubation times on the cell viability of HepaRG cells. From Figure 6.1 it can be observed that there was no significant difference in the percentage of viable cells after HepaRG cells were exposed to the different sulforaphane concentrations (p = 0.39) and incubation times (p = 0.02), indicating no major cell death. All of the tested sulforaphane concentrations and incubation times could thus be used to assess the effect of sulforaphane on oxidative stress in HepaRG cells. However, studies done by Anwar-Mohamed & El-Kadi (2009), Zhang *et al* (1992), Yeh and Yen (2009), and Sestili (2010), indicated the maximum increase in the mRNA activity of CYP1A1 and phase II antioxidant enzymes was at a 6 hour incubation period with 5 µM sulforaphane. Compared to the negative control with 100% cell viability and the positive control with a cell viability of 22%-30%, from Figure 6.1 it can be observed,

after HepaRG cells were incubated for 6 hours with 5 µM sulforaphane, the average cell viability was 100% indicating exposure to 5 µM sulforaphane for 6 hours had no effect on the viability of the HepaRG cells. A 6 hour sulforaphane incubation time was thus used in all further experiments.

6.2.1.2 Standardization of the *tert*-Butyl hydroperoxide concentration to illustrate the protective effect of sulforaphane against oxidative stress in HepaRG cells

During this part of the study the *t*-BHP concentration and time of exposure, which would induce enough oxidative stress in the cells to activate the cells antioxidant defence system and illustrate the protective effect of sulforaphane, was standardized (Figure 6.2 and 6.3). As no experiments were done during this study to physically measure the difference in oxidative stress in the cells after *t*-BHP and sulforaphane was added, a difference in cell viability acted as the only indication of the effect of *t*-BHP and sulforaphane on the HepaRG cells. As discussed in Section 6.2.1, based on the studies done by Anwar-Mohamed & El-Kadi (2009), Zhang *et al* (1992), Yeh and Yen (2009), and Sestili (2010), and the results in Figure 6.1, it was decided to incubate HepaRG cells for 6 hours with 1 µM, 2.5 µM, 5 µM, 10 µM, and 20 µM to first activate the cells antioxidant defence system, before *t*-BHP was added. Indicated in the MTT assay results obtained from the standardization of the *t*-BHP concentration in HepaRG cells, displayed in Figure 3.4 in Section 3.3.2.3, the cells had a cell viability of 80% after being exposed to 0.25 mM *t*-BHP for 3 hours and 58% after being exposed to 0.50 mM *t*-BHP for 3 hours. Based on these results, it was decided to use both concentrations to induce oxidative stress in HepaRG cells. Sulforaphane stock solutions of 1 µM, 2.5 µM, 5 µM, 10 µM, and 20 µM were prepared as described in Section 6.2.1. *t*-BHP stock solutions of 0.25 mM and 0.50 mM were prepared as described in Section 3.3.2.3.

Pre-treatment of cells

After inspecting the confluence of the cells, the first step was to add different sulforaphane concentrations to the cells in triplicate, as described in Section 6.2.1.1. The only difference was the cells were incubated with the sulforaphane

concentrations for only 6 hours. To assess the effect of sulforaphane on HepaRG cells, the sulforaphane control samples were incubated with 5 µM sulforaphane for 6 hours. Identical plates were prepared for 0.25 mM and 0.50 mM *t*-BHP. After the 6 hour incubation period, the base WME growth medium in each well was removed and replaced with 190 µl fresh base WME growth medium and 10 µl 0.25 mM *t*-BHP or 10 µl 0.50 mM *t*-BHP to exogenously induce oxidative stress in the HepaRG cells. This was followed by incubating the plates for 3 hours. To assess the effect of *t*-BHP on HepaRG cells, the *t*-BHP control samples were incubated with 0.25 µM or 0.50 µM *t*-BHP for 3 hours. Identical plates were prepared for 0.25 mM and 0.50 mM *t*-BHP. After the incubation period, the MTT assay was performed on all wells. The results obtained from the completed MTT assay are displayed in Figure 6.2.

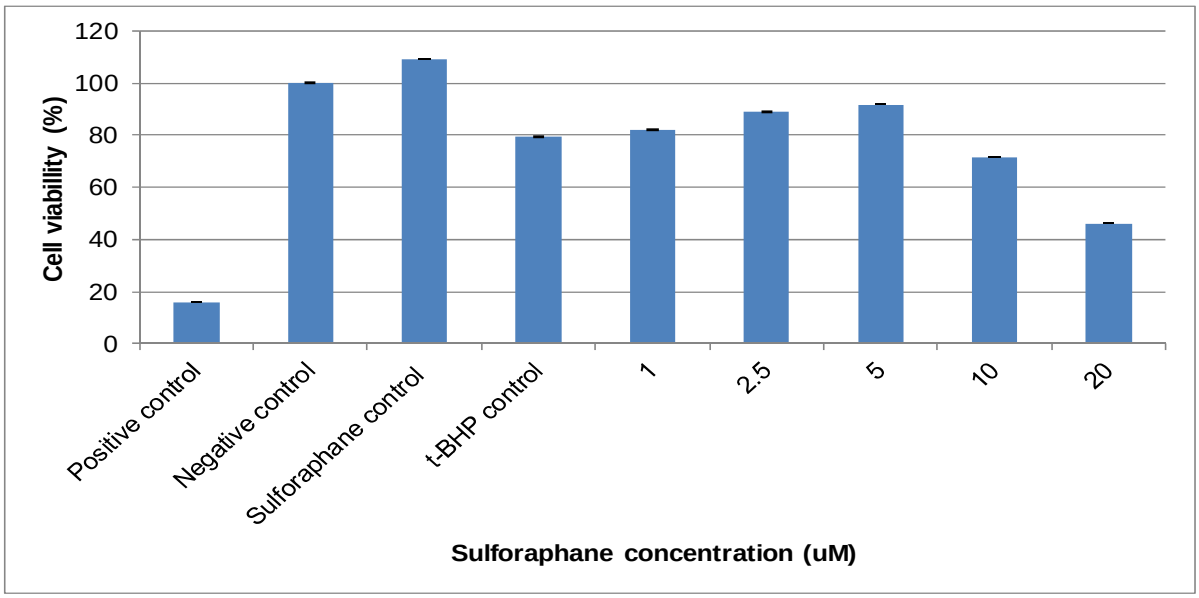

Figure 6.2: The effect of sulforaphane on 0.25 mM *tert*-Butyl hydroperoxide-induced oxidative stress in HepaRG cells. The Y-axis represents the cell viability (%) and the X-axis the positive control, negative control, sulforaphane control, *t*-BHP control and the different sulforaphane concentrations in micromolar (µM). After checking the confluence of the cells, different doses of sulforaphane (1 µM-20 µM) was added to the cells for 6 hours. To the positive control acetic acid was added, to the negative control no chemicals, to the sulforaphane control 5 µM sulforaphane was added, and to the *t*-BHP control 0.25 µM. This was followed by adding 0.25 mM *t*-BHP to the cells for 3 hours. The cell viability was determined with the MTT assay. Each data point in the figure represents the mean ± SD values of three separate samples.

Figure 6.2 represents the effect of different sulforaphane concentrations on 0.25 µM *t*-BHP-induced oxidative stress in HepaRG cells. Compared to the *t*-BHP control, to which 0.25 mM *t*-BHP was added, from Figure 6.2 it can be observed, that there was

an increase in the amount of viable cells in the samples pre-incubated with 1 µM, 2.5 µM, and 5 µM sulforaphane. The 5 µM sulforaphane samples showed the highest increase in cell viability of 11%. This illustrated the possible protective effect of sulforaphane against oxidative stress in HepaRG cells. At higher sulforaphane concentrations (10 µM and 20 µM) a decrease in the cell viability was observed. As mentioned, Anwar-Mohamed & El-Kadi (2009) investigated the effect of sulforaphane on the expression of CYP1A1 mRNA. After HepG2 cells were exposed to increasing concentrations of sulforaphane (1 µM-10 µM) for 6 hours, they found the maximum expression of CYP1A1 mRNA was at 5 µM sulforaphane. They also found that a further increase in sulforaphane concentration caused a decrease in the induction of CYP1A1. This could cause a decrease in protection against the *t*-BHP-induced oxidative stress, which will lead to a higher amount of cell death after exposure to *t*-BHP, thus resulting in the lower cell viability observed at a 10 µM and 20 µM sulforaphane concentration.

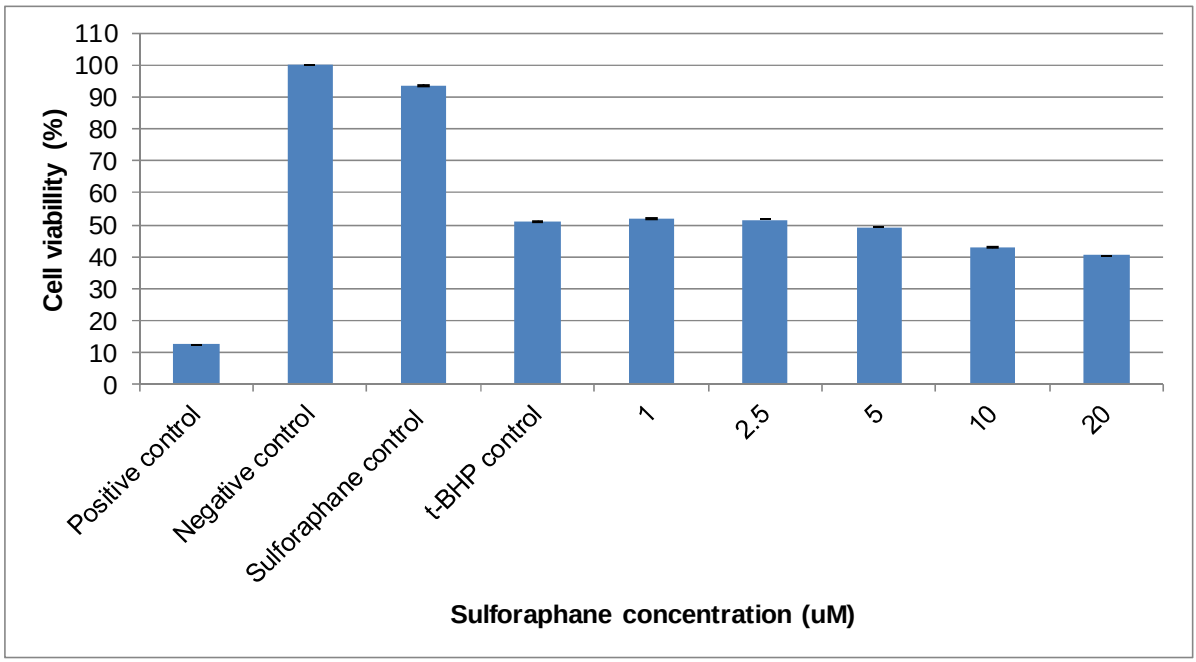

Figure 6.3: The effect of sulforaphane on 0.50 mM *tert*-Butyl hydroperoxide-induced oxidative stress in HepaRG cells. The Y-axis represents the cell viability (%) and the X-axis the positive control, negative control, sulforaphane control, *t*-BHP control and the different sulforaphane concentrations in micromolar (µM). After checking the confluence of the cells, different doses of sulforaphane (1 µM-20 µM) was added to the cells for 6 hours. To the positive control acetic acid was added, to the negative control no chemicals, to the sulforaphane control 5 µM sulforaphane was added, and to the *t*-BHP control 0.50 µM. This was followed by adding 0.50 mM *t*-BHP to the cells for 3 hours. The cell viability was determined with the MTT assay. Each data point in the figure represents the mean ± SD values of three separate samples.

Figure 6.3 represents the effect of different sulforaphane concentrations on 0.50 µM *t*-BHP-induced oxidative stress in HepaRG cells. Compared to the *t*-BHP control, to which only 0.50 mM *t*-BHP was added, from Figure 6.3 it can be observed that there was almost no increase in cell viability in the cells pre-treated with 1 µM, 2.5 µM and 5 µM sulforaphane. These results indicated that there was no protective effect from the tested sulforaphane concentrations against the 0.50 mM *t*-BHP-induced oxidative stress in HepaRG cells. This could be due to the high level of oxidative stress and cellular damage caused by the higher *t*-BHP concentration. As discussed previously at Figure 6.2, at higher sulforaphane concentrations (10 µM and 20 µM) the HepaRG cell viability decreased. Although the tested sulforaphane concentrations did not provide any protective effect against 0.50 mM *t*-BHP-induced oxidative stress, compared to the results in Figure 6.2, it was decided to test the effect of higher sulforaphane concentrations against 0.50 mM *t*-BHP-induced oxidative stress.

Pre-treatment of cells

After inspecting the confluence of the cells the first step was to the 1 µM, 5 µM, 10 µM, 50 µM, 100 µM, 500 µM, and 1000 µM sulforaphane concentrations to the cells as described in Section 6.2.1.1, and the plate incubated for 6 hours. To assess the effect of DMSO on HepaRG cells, the DMSO control samples were incubated with 10 µl of the 0.05% DMSO solution for 6 hours. After the incubation period was over, 0.50 mM *t*-BHP was added to the cells for 3 hours, as described in Section 6.2.2.1. After the *t*-BHP incubation time was over, the MTT assay was performed on all wells. The results obtained from the completed MTT assay are displayed in Figure 6.4.

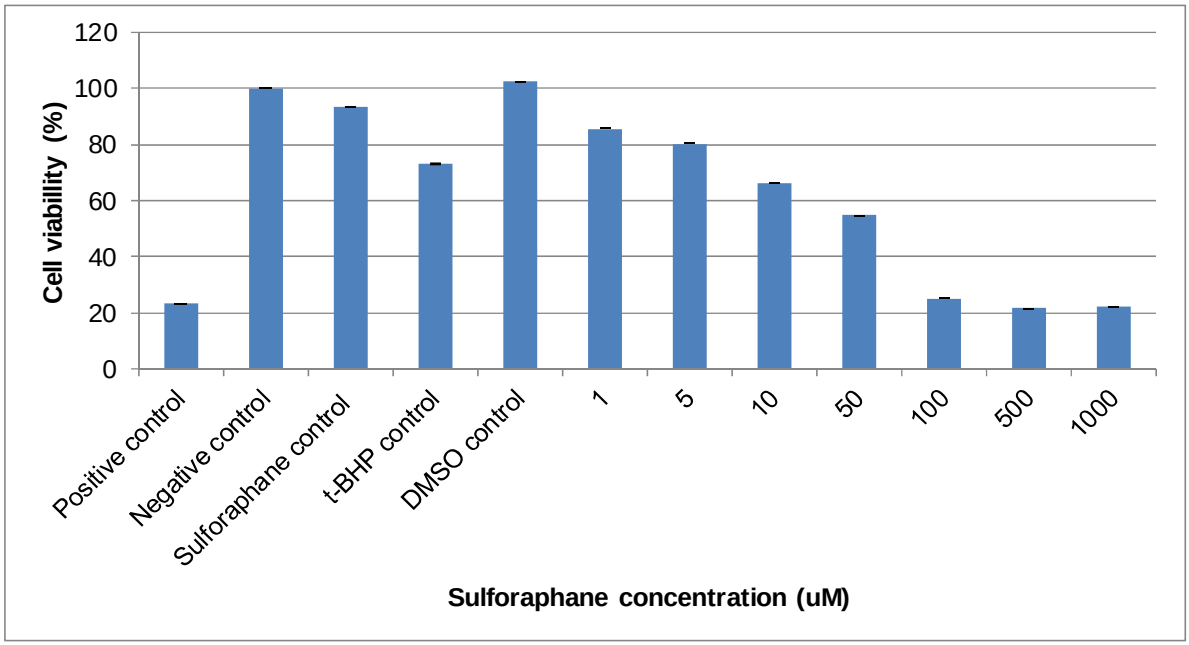

Figure 6.4: The effect of higher sulforaphane concentrations on 0.50 µM *tert*-Butyl hydroperoxide-induced oxidative stress in HepaRG cells. The Y-axis represents the cell viability (%) and the X-axis the positive control, negative control, sulforaphane control, *t*-BHP control, DMSO control, and the different sulforaphane concentrations in micromolar (µM). After checking the confluence of the cells, different doses of sulforaphane (1 µM-1000 µM) was added to the cells for 6 hours. To the positive control acetic acid was added, to the negative control no chemicals, to the sulforaphane control 5 µM sulforaphane was added, and to the *t*-BHP control 0.50 µM. This was followed by adding 0.50 mM *t*-BHP to the cells for 3 hours. The cell viability was determined with the MTT assay. Each data point in the figure represents the mean ± SD values of three separate samples.

Figure 6.4 represents the effect of higher sulforaphane concentrations on 0.50 µM *t*-BHP-induced oxidative stress in HepaRG cells. Compared to the *t*-BHP control, to which only 0.50 mM *t*-BHP was added, from Figure 6.4 it can be observed that there was an increase in the amount of viable HepaRG cells in the samples pre-treated with 1 µM and 5 µM sulforaphane. At higher sulforaphane concentrations (10-1000 µM) a decrease in the cell viability was observed. As also illustrated in Figure 6.2 and Figure 6.3, this could be due to the high sulforaphane concentrations causing cell death before *t*-BHP was added to the cells. From Figure 6.4 it could also be observed, the samples to which only DMSO was added had an average cell viability of 102%, proving that the DMSO in the sulforaphane solution had no effect on the viability of the HepaRG cells before *t*-BHP was added. Figure 6.4 also illustrates a 1 µM sulforaphane concentration was the most effective in providing a possible protective effect against 0.50 mM *t*-BHP-induced oxidative stress. The average cell viability of the *t*-BHP control was 65% and the 1 µM sulforaphane sample 86%. A

21% increase in cell viability over the 3 hour *t*-BHP incubation period was thus observed after 0.50 mM *t*-BHP was added. However, as mentioned previously, studies done by Anwar-Mohamed & El-Kadi (2009), Zhang *et al* (1992), Yeh and Yen (2009), and Sestili (2010), where human HepG2 cells were treated with various concentrations of sulforaphane at different periods of time, the maximum increase in the mRNA activity of CYP1A1 and phase II antioxidant enzymes was indicated at a 6 hour incubation period with 5 μM sulforaphane. From Figure 6.4 it can be observed, compared to the *t*-BHP control with an average cell viability of 65%, a 15% increase in cell viability of the 5 μM sulforaphane sample over the 3 hour *t*-BHP incubation period was observed after 0.50 mM *t*-BHP was added to the HepaRG cells. Based on these results, it is clear that pre-incubation of HepaRG cells with 5 μM sulforaphane for 6 hours provides only partial protection against 0.50 mM *t*-BHP exogenously-induced oxidative stress (as measured by % cell viability) in HepaRG cells.

6.2.2 The effect of sulforaphane on phase II paracetamol biotransformation in HepaRG cells

The question remains if the incubation of HepaRG cells for 6 hours with 5 μM sulforaphane has a negative effect on phase II paracetamol biotransformation activity in HepaRG cells. As discussed in Section 5.4, compared to the phase I caffeine biotransformation and phase II glycine conjugation results, paracetamol was the only probe substance which could be successfully used to evaluate the phase II sulfate and glucuronic acid conjugation pathways in HepaRG cells. Based on these results, it was decided to incubate the cells for 3, 6, 12, 18 and 24 hours with 1 mM paracetamol after a 6 hour pre-incubation period with 5 μM sulforaphane. A LC-ESI-MS/MS assay (Section 4.4) was used to quantify the formation of the selected phase II paracetamol metabolites: paracetamol sulfate and paracetamol glucuronide.

Pre-treatment of cells

Two different samples were prepared in triplicate for each period of incubation: the cells in the first samples were incubated with only 1 mM paracetamol for 3, 6, 12, 18

and 24 hours. The cells in the second samples were first incubated with 5 µM sulforaphane for 6 hours after which 1 mM paracetamol was added and the cells for 3, 6, 12, 18 and 24 hours. If sulforaphane had an effect on phase II paracetamol biotransformation activity, there would be a significant difference in the concentration of the selected phase II paracetamol metabolites: paracetamol sulfate and paracetamol glucuronide, quantified in the samples to which only paracetamol and the samples to which sulforaphane and paracetamol were added. After each incubation period was over, the plates were removed from the incubator and the samples prepared (Section 3.4.1.3-Section 3.4.1.7). Samples were analyzed in triplicate with a LC-ESI-MS/MS assay, used to quantify paracetamol sulfate and paracetamol glucuronide in each sample. The results are displayed in Figure 6.5 and Figure 6.6.

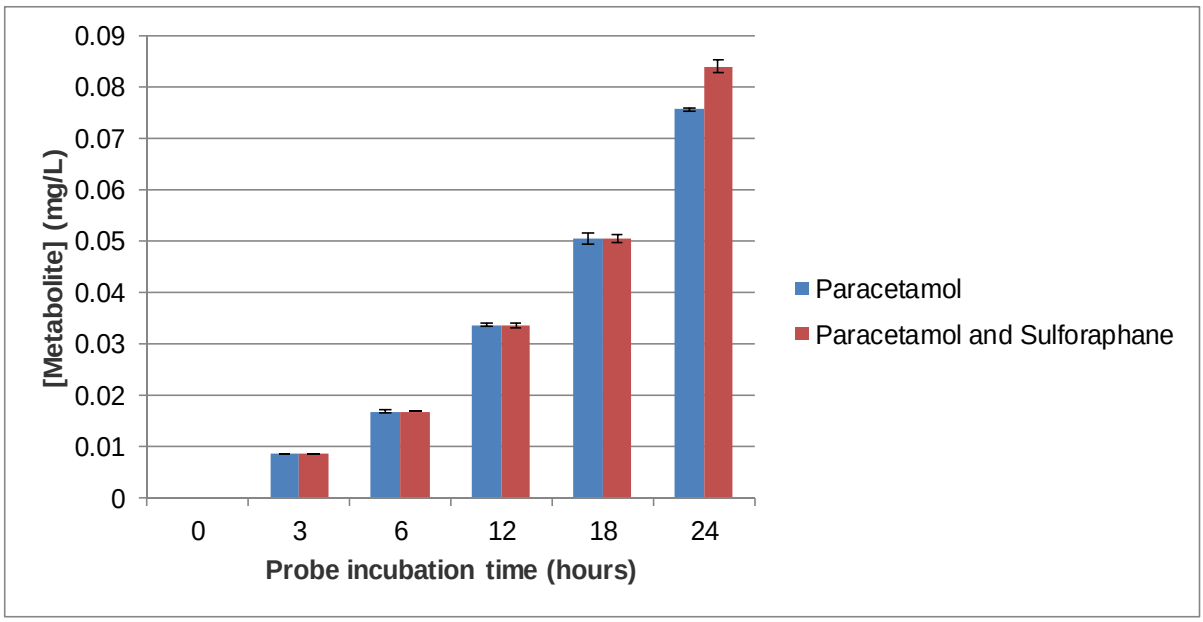

Figure 6.5: The effect of sulforaphane on the formation of the selected phase II paracetamol metabolite (paracetamol glucuronide) in HepaRG cells. The Y-axis represents the paracetamol glucuronide concentration in milligrams per litre (mg/L) and the X-axis the different periods of time in hours HepaRG cells were incubated with the paracetamol probe. Two different samples were prepared in triplicate for each period of incubation: the cells in the first samples were incubated with only 1 mM paracetamol for 3, 6, 12, 18 and 24 hours. The cells in the second samples were first incubated with 5 µM sulforaphane for 6 hours after which 1 mM paracetamol was added and the cells for 3, 6, 12, 18 and 24 hours. After each incubation time, the paracetamol glucuronide concentration present in each sample was quantified with a LC-ESI-MS/MS assay. Each data point in the figure represents the mean ± SD values of three separate samples. Two way ANOVA: $p < 0.01$ (effect of time) and $p = 0.28$ (sulforaphane concentration).

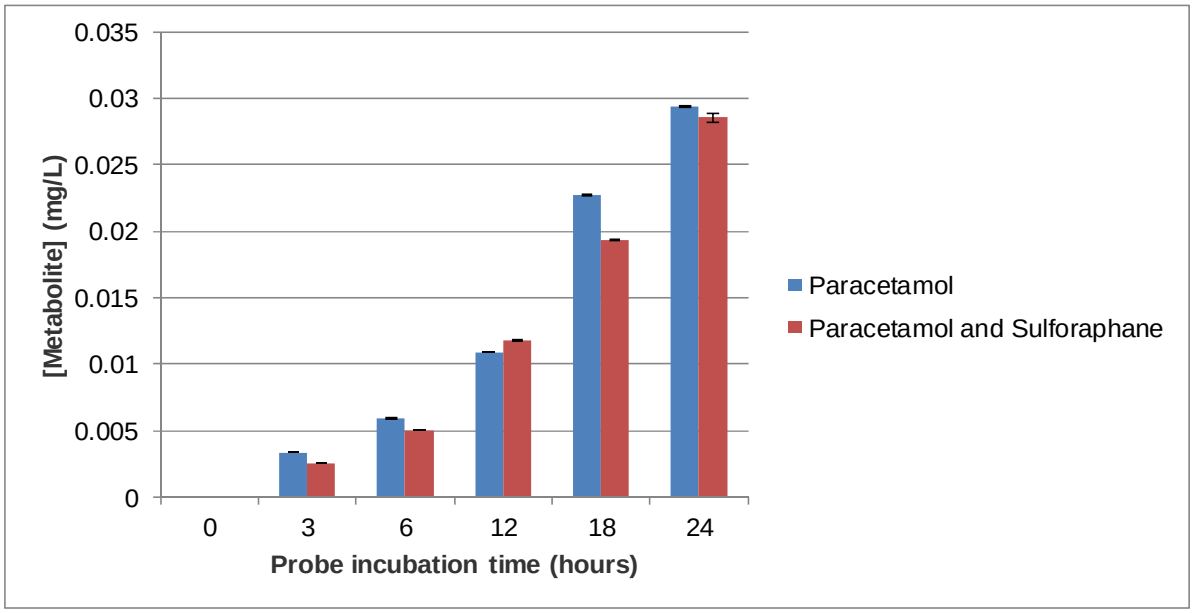

Figure 6.6: The effect of sulforaphane on the formation of the selected phase II paracetamol metabolite (paracetamol sulfate) in HepaRG cells. The Y-axis represents the paracetamol sulfate concentration in milligrams per litre (mg/L) and the X-axis the different periods of time in hours HepaRG cells were incubated with the paracetamol probe. Two different samples were prepared in triplicate for each period of incubation: the cells in the first samples were incubated with only 1 mM paracetamol for 3, 6, 12, 18 and 24 hours. The cells in the second samples were first incubated with 5 µM sulforaphane for 6 hours after which 1 mM paracetamol was added and the cells for 3, 6, 12, 18 and 24 hours. After each incubation time, the paracetamol sulfate concentration present in each sample was quantified with a LC-ESI-MS/MS assay. Each data point in the figure represents the mean ± SD values of three separate samples. Two way ANOVA: $p < 0.01$ (effect of time) and $p = 0.06$ (sulforaphane concentration).

Figure 6.5 and 6.6 represent the effect of the sulforaphane period on phase II paracetamol biotransformation in HepaRG cells. Figure 6.5 compares the paracetamol glucuronide and Figure 6.6, the paracetamol sulfate concentration quantified in the paracetamol samples, with the paracetamol glucuronide and paracetamol sulfate concentration quantified in the paracetamol and sulforaphane samples. From Figure 6.5 and 6.6 it can be observed that there were no significant differences, in the quantified paracetamol glucuronide (Figure 6.5) and paracetamol sulfate (Figure 6.6) concentrations, when comparing the paracetamol samples and the paracetamol and sulforaphane samples at a 3, 6, 12, 18 and 24 hour paracetamol incubation period ($p < 0.01$). This indicated that the phase II paracetamol biotransformation activity in HepaRG cells thus remained the same after cells were pre-incubated with sulforaphane ($p = 0.06$). Sulforaphane thus had no visible negative effect on the phase II paracetamol biotransformation pathway in HepaRG cells.

6.3 Conclusion

The optimal sulforaphane concentration and incubation time in HepaRG was standardized with a MTT assay. The results indicated that exposure of HepaRG cells to the different concentrations for up to 6 hours caused no major cell death. All of the tested sulforaphane concentrations and incubation times could thus be used to assess the effect of sulforaphane on induced oxidative stress in HepaRG cells. However, studies done by Anwar-Mohamed & El-Kadi (2009), Zhang *et al* (1992), Yeh and Yen (2009), and Sestili (2010), indicated the maximum increase in the mRNA activity of CYP1A1 was at a 6 hour incubation period with 5 µM sulforaphane. The results indicated exposure of HepaRG cells to 5 µM sulforaphane for 6 hours had no effect on the viability of the HepaRG cells. A 6 hour sulforaphane incubation time was thus used in all further experiments. The assessment of the effect of sulforaphane on oxidative stress in HepaRG cells indicated that the pre-incubation of HepaRG cells with 5 µM sulforaphane for 6 hours provide only partial protection against 0.50 mM *t*-BHP-induced oxidative stress (as measured by an increase in the % cell viability) in HepaRG cells. Compared to the phase I caffeine biotransformation and phase II glycine conjugation results, displayed in Section 5.4, the phase II paracetamol biotransformation assay was the only assay which could be experimentally proven to be active in HepaRG cells. The effect of sulforaphane on phase I and phase II biotransformation could thus only be assessed on the phase II paracetamol biotransformation pathway. Based on these results, it was concluded that sulforaphane provided partial protection against oxidative stress in HepaRG cells. Sulforaphane also had no visible negative effect on phase II biotransformation in HepaRG cells.

CHAPTER 7

Discussion, conclusions and future recommendations

7.1 Introduction

Sulforaphane has received much attention due to the evidence that sulforaphane functions as an indirect antioxidant, by inhibiting phase I carcinogen-bioactivating enzymes and/or inducing phase II antioxidant enzymes, as well as MTS (Perocco *et al.,* 2006; Clarke *et al.,* 2008; Yeh & Yen, 2009). Since MTS and antioxidant enzymes are involved in the scavenging of ROS, the question was raised whether sulforaphane can provide protection against increased oxidative stress. Another question that was raised was if sulforaphane treatment of a human hepatocellular carcinoma cell line, like HepaRG cells, will have a negative impact on phase I and II biotransformation in these cells.

The aim of this study was to determine the effect of sulforaphane on oxidative stress and on phase I and phase II biotransformation in HepaRG cells. The objectives of this study included the standardization of the optimal *t*-BHP concentration and incubation time to induce oxidative stress in HepaRG cells. The toxicity of *t*-BHP towards HepaRG cells was indicated by the amount of viable cells, quantified by a MTT assay. LC-ESI-MS/MS assays, which were more selective and specific, compared to HPLC assays, were used to quantify the formation of selected phase I and phase II metabolites. The next objective was the optimization and validation of LC-ESI-MS/MS assays in HepaRG cells and the optimization of phase I and phase II biotransformation assays in HepaRG cells. This was followed by the standardization of the optimal sulforaphane concentration and incubation time in HepaRG cells with the MTT assay. The study concluded with the assessment of the effect of sulforaphane on oxidative stress (MTT assay) and biotransformation (LC-ESI-MS/MS assay) in HepaRG cells.

7.2 Discussion

7.2.1 Standardization of the optimal *tert*-Butyl hydroperoxide concentration and incubation time to induce oxidative stress in HepaRG cells

The optimal *t*-BHP concentration and incubation time which would induce oxidative stress in HepaRG cells without causing major cell death was standardized with a MTT assay. The results indicated a 3 hour exposure of the HepaRG cells to 0.25 mM *t*-BHP, which resulted in a high cell viability of 85%, was sufficient enough to cause oxidative stress in HepaRG cells without causing any major cell death. These conditions were used in all further experiments where oxidative stress was induced in HepaRG cells.

7.2.2 Optimization of the LC-ESI-MS/MS assays to monitor phase I and phase II biotransformation in HepaRG cells

Due to the need for a selective and specific assay, which specifically focussed on the formation and quantification of the selected phase I and phase II metabolites, it was decided to use LC-ESI-MS/MS assays to monitor phase I and phase II biotransformation pathways in HepaRG cells. LC-ESI-MS/MS assays were developed for the quantification of the selected phase I caffeine metabolites (theobromine, theophylline and paraxanthine), the selected phase II paracetamol metabolites (paracetamol glucuronide, paracetamol sulfate and paracetamol mercapturate), and the selected phase II glycine conjugation metabolites (salicyluric acid, hippuric acid and para-aminohippuric acid). The optimized LC-ESI-MS/MS assays for each metabolite were validated with acceptable linearity ($R^2 \geq 0.99$). The assessment of phase I and phase II biotransformation pathways with specific probe substances could thus be done through the identification and quantification of selected phase I and phase II metabolites with LC-ESI-MS/MS assays.

7.2.3 Optimization of phase I and phase II biotransformation assays in HepaRG cells

The optimization of phase I and phase II biotransformation assays in HepaRG cells involved the assessment of the effect of DMSO (DMSO media), HepaRG

metabolism supplement (supplement media), and base WME growth medium on phase I and phase II biotransformation activity in HepaRG cells. Guillouzo *et al* (2007) and Kanebratt & Andersson (2008) indicated that the addition of 2% DMSO to the HepaRG cells aided in the formation of more hepatocyte-like cells and, with the supplement, lead to an increase in phase I and phase II biotransformation enzyme activity. As no experiments were done during this study to indicate possible changes in phase I and phase II enzyme activity after DMSO media and supplement media was added, only the formation of the selected metabolites of each probe substance, quantified with LC-ESI-MS/MS assays, could be used as an indication of phase I and phase II activity in HepaRG cells.

Due to the effect of DMSO on the viability and thus, the biotransformation activity of HepaRG cells which lead to the unsuccessful biotransformation of the probe substances, phase I and phase II biotransformation assays could not be optimized in HepaRG cells, pre-treated with DMSO media. As the composition of the HepaRG metabolism supplement was unknown and no samples were prepared without supplements, it was not certain if the metabolite formation, observed in the samples with cells, were due to phase I or phase II biotransformation activity in HepaRG cells or due to enzyme activity in the HepaRG metabolism supplement or FBS, added to the WME growth medium. Phase I and phase II biotransformation assays could thus not be optimized in HepaRG cells pre-treated with HepaRG metabolism supplement. After cells were incubated with only base WME growth medium and no DMSO or supplement, again only the phase II paracetamol biotransformation assay was experimentally proven to be active in HepaRG cells. As no DMSO or supplement were added to the cells, these results indicated that phase I caffeine biotransformation and phase II glycine conjugation of sodium benzoate to the selected metabolites in HepaRG cells were most likely due to the probes being biotransformed by enzymes already present in the serum added to the base WME medium. Only the phase II paracetamol biotransformation assay could be optimized in HepaRG cells. The effect of sulforaphane on biotransformation could thus only be assessed through the phase II paracetamol biotransformation pathway in HepaRG cells.

7.2.4 Sulforaphane, oxidative stress and biotransformation in HepaRG cells

The optimal sulforaphane concentration and incubation time in HepaRG was standardized with a MTT assay. The results indicated that exposure of HepaRG cells to the different concentrations for up to 6 hours caused no major cell death. All of the tested sulforaphane concentrations and incubation times could thus be used to assess the effect of sulforaphane on oxidative stress in HepaRG cells. However, studies done by Anwar-Mohamed & El-Kadi (2009), Zhang *et al* (1992), Yeh and Yen (2009), and Sestili (2010), indicated the maximum increase in the mRNA activity of CYP1A1 was at a 6 hour incubation period with 5 µM sulforaphane. Compared to the negative control with 100% cell viability and the positive control with a cell viability of 22 - 30%, from Figure 6.1 it can be observed, after HepaRG cells were incubated for 6 hours with 5 µM sulforaphane, the average cell viability was 100% indicating exposure to 5 µM sulforaphane for 6 hours had no effect on the viability of the HepaRG cells. A 6 hour sulforaphane incubation time was thus used in all further experiments. The assessment of the effect of sulforaphane on oxidative stress in HepaRG cells indicated that the pre-incubation of HepaRG cells with 5 µM sulforaphane for 6 hours provide only partial protection against 0.50 mM *t*-BHP-induced oxidative stress in HepaRG cells. Although the possible protective effect of sulforaphane against the 0.25 mM *t*-BHP-induced oxidative stress was observed, the small difference in cell viability between the *t*-BHP control samples and the sulforaphane pre-treated samples, lead to the decision that the *t*-BHP concentration did not cause enough oxidative stress in HepaRG cells to be certain the increase in cell viability was due to the protective effect of sulforaphane. The assessment of the effect of sulforaphane on phase II paracetamol biotransformation in HepaRG cells indicated that the incubation of HepaRG cells for 6 hours with 5 µM sulforaphane had no visible negative impact on phase II paracetamol biotransformation activity in HepaRG cells.

7.3 Conclusions

The hypothesis of this study was: “Sulforaphane will protect HepaRG cells against oxidative stress without negatively influencing phase I and II biotransformation”. Based on the results of the study, it could be concluded that sulforaphane provided

partial protection against oxidative stress in HepaRG cells. Sulforaphane also had no visible influence on phase II paracetamol biotransformation in HepaRG cells.

7.4 Recommendations for further studies

7.4.1 Quantification of the amount of oxidative stress

During this study, the optimal *t*-BHP concentration and time of exposure which would induce sufficient oxidative stress in HepaRG cells without causing major cell death, was standardized with a MTT assay. A possible recommendation for future studies would be to physically assess the amount of oxidative stress induced by *t*-BHP in HepaRG cells. A ROS detection kit can be used to detect the amount of ROS produced in the cells with the oxidation-sensitive non-fluorescent probe 20, 70-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA diffuses into the cells where it is oxidized in the presence of peroxides to the fluorescent 20, 70-dichlorofluorescin. The intensity of the fluorescence indicates the amount of oxidative stress (Li *et al.,* 2010). As discussed in Section 2.1.4.1, *t*-BHP also causes the depletion of GSH, the most abundantly produced endogenous antioxidant. GSH levels in cells are depleted due to the oxidation of GSH in the presence of high amounts of *t*-BHP (Nishida *et al.,* 1997). The amount of oxidative stress in the cells can also be determined by assessing the GSH content in the cells after incubation with *t*-BHP. Using a method introduced by Tietzke (1969) the total GSH, and the oxidized GSH (GSSG) content in the cells can be determined. The use of a GSH scavenger, 1-methyl-2-vinylpyridinium triflate (M2VP), allows the measurement of GSSG, thus allowing the measurement of GSH and GSSG.

7.4.2 Assessment of phase I and phase II biotransformation enzyme activity

As no experiments were done during this study to indicate possible changes in phase I and phase II enzyme activity after probe substances or sulforaphane was added to the cells, only variations in the formation of the selected phase I and phase II metabolites, quantified with LC-ESI-MS/MS assays, could be used as an indication of the effect on the biotransformation activity in HepaRG cells. Possible

recommendations for future studies include the assessment of the effect of probes and sulforaphane on phase I and phase II biotransformation through the evaluation of the activity of specific phase I and phase II enzymes in HepaRG cells after the incubation of the cells with a probe substance or sulforaphane. On a transcriptional level the mRNA of specific CYP450 enzymes and MTS, extracted from the cells, can be quantified with real-time polymerase chain reaction (RT-PCR). On a protein level a protein extraction and Western Blot analysis can be used to quantify proteins specific to the expression of each CYP450 enzyme and MTS (Anwar-Mohamed & El-Kadi, 2009) (Yeh & Yen, 2005).

7.4.3 The use of serum-free growth medium

To assess phase I and phase II enzyme activity, after probe substances or sulforaphane is added to the cells, the use of serum-free growth medium is recommended. This will eliminate any possible enzyme activity present in the serum, minimize the amount of variables and allow the culture of the cells a defined set of conditions (Nakama *et al.,* 1995).

References

- ANINAT, C., PITON, A., GLAISE, D., LE CHARPENTIER, T., LANGOUËT, S., MOREL, F., GUGUEN-GUILLOUZO, C. & GUILLOUZO, A. 2006. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metabolism and Disposition,* 34: 75-83 p.
- ANON. 2005. Agilent mass hunter software. Quick start guide. *Agilent Technologies, Inc.* USA, September.
- ANON. 2009. Agilent mass hunter software. Quick start guide. *Agilent Technologies, Inc.* USA, December.
- ANTHÉRIEU, S., CHESNÉ, C., LI, R., GUGUEN-GUILLOUZO, C. & GUILLOUZO, A. 2012. Optimization of the HepaRG cell model for drug metabolism and toxicity studies. *Toxicology in Vitro,* 26: 1278-1285 p.
- ANWAR-MOHAMMED, A. & EL-KADI, A.O.S. 2008. Sulforaphane induces CYP1A1 mRNA, protein, and catalytic activity levels via an AhR-dependant pathway in murine hepatoma Hepa1c1c7 and human HepG2 cells. *Cancer Letters,* 275: 93-101 p.
- BECKMAN, K.B. & AMES, B.N. 1998. The free radical theory of aging matures. *Physiological Reviews,* 78: 547-581 p.
- BEYOĞLU, D., SMITH, R.L. & IDLE, J.R. 2012. Dog bites man or man bites dog? The enigma of amino acid conjugations. *Biochemical Pharmacology,* 83: 1331-1339 p.
- BRANDON, F.A., RAAP, C.D., MEIJERMAN, I., BEIJNEN, J.H. & SCHELLENS, J.H.M. 2003. An update on in vitro test methods in human hepatic drug biotransformation research: pros and cons. *Toxicology and Applied Pharmacology*, 189: 233-246 p.
- CAZENEUVE, C., PONS, G., REY, E., TRELUYER, J-M., CRESTEIL, T., THIROUX, G., D'ATHIS, P. & OLIVE, G. 1994. Biotransformation of caffeine in human liver microsomes from foetuses, neonates, infants and adults. *Br J Clin Pharmac*, 37: 405-412 p.
- CHANG, H-S., KO, M., ISHIZUKA, M., FUJITA, S., YABUKI, A., HOSSAIN, M.A. & YAMATO, O. 2010. Sodium 2-propenyl thiosulfate derived from garlic induces

phase II detoxification enzymes in rat hepatoma H4IIE cells. *Nutrition Research,* 30: 435-440 p.

- CHAUDHURI, S., BANERJEE, A., KAUSHIK, B., SENGUPTA, B. & SENGUPTE, P.K. 2007. Interaction of flavonoids with red blood cell membrane lipids and proteins: Antioxidant and antihemolytic effects. *International Journal of Biological Macromolecules,* 41: 42-48 p.
- CHEN, Y., MONSHOUWER, M. & FITCH, W. 2007. Analytical tools and approaches for metabolite identification in early drug discovery. *Pharmaceutical Research*, 24: 248-257 p.
- CLARKE, J.D., DASHWOOD, R.H. & HO, E. 2008. Multi-targeted prevention of cancer by sulforaphane. *Cancer Letters,* 269: 291-304 p.
- DIAZ-RUIZ, R., RIGOULET, M. & DEVIN, A. 2010. The Warburg and Crabtree effects: On the origin of cancer cell energy metabolism and of yeast glucose repression*. Biomedica et Biophysica Acta,* 1807: 568-576 p.
- ELBARBRY, F. & ELRODY, N. 2011. Potential health benefits of sulforaphane: A review of the experimental, clinical and epidemiological evidences and underlying mechanisms. *Journal of Medicinal Plants Research*, 5: 473-484 p.
- FAHEY, J.W. & TALALAY, P. 1999. Antioxidant functions of sulforaphane: A potent inducer of phase II detoxification enzymes. *Food and Chemical Toxicology,* 37: 973-979 p.
- FERNÁNDEZ-VIZARRA, E., FERRÍN, G., PÉREZ-MARTOS, A., FERNÁNDEZ-SILVA, P., ZEVIANI, M. & ENRÍQUEZ, J.A. 2010. Isolation of mitochondria for biogenetical studies: An update. *Mitochondrion,* 10: 253-262 p.
- FIMOGNARI, C. & HRELIA, P. 2006. Sulforaphane as a promising molecule for fighting cancer. *Mutation Research,* 635: 90-104 p.
- FREIMOSER, F.M. JAKOB, C.A., AEBI, M. & TUOR, U. 1999. The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities. *Appl. Environ. Microbiology,* 65: 3727-3729 p.
- GOKULAKRISHNAN, S., CHANDRARAJ, K. & GUMMADI, N. 2005. Microbial and enzymatic methods for the removal of caffeine. *Enzyme and Microbial Technology,* 37: 225-232 p.

- GUILLOUZO, A., CORLU, A., ANINAT, C., GLAISE, D., MOREL, F. & GUGUEN-GUILLOUZO, C. 2007. The human hepatoma HepaRG cells: A highly differentiated model for studies of liver metabolism and toxicity of xenobiotics. *Chemico-Biological Interactions,* 168: 66-73 p.
- HANCOCK, J.T., DESIKAN, R. & NEILL, S.J. 2001. Role of reactive oxygen species in cell signalling pathways. *Biochemical Society Transactions*, 29: 345-350 p.
- HICKMAN, D., WANG, J-P, WANG, Y. & UNADKAT, J.D. 1998. Evaluation of the selectivity of *in vitro* probes and suitability of organic solvents for the measurement of human cytochrome P450 monooxygenase activities. *Drug Metabolism and Disposition*, 26: 207-215 p.
- HO, C.S., LAM, C.W.K., CHAN, M.H.M., CHEUNG, R.C.K., LAW, L.K., LIT, L.C.W., NG, K.F., SUEN, M.W.M. & TAI, H.L. 2003. Electrospray Ionisation Mass Spectrometry: Principles and Clinical Applications. *Clin Biochem Rev*., 24: 3-12 p.
- HOLMUHAMEDOV, E., JAHANGIR, A., BIENENGRAEBER, M., LEWIS, L.D. & TEIZIC, A. 2003. Deletion of mtDNA disrupts mitochondrial function and structure, but not biogenesis*. Mitochondrion,* 3: 13-19 p.
- ISHII, N., ISHII, T. & HARTHMAN, P.S. 2006. The role of the electron transport SDHC gene on lifespan and cancer. *Mitochondrion,* 7: 24-28 p.
- IYER, V.V., YANG, H., IERAPETRITOU, M.G. & ROTH, C.M. 2010. Effects of Glucose and Insulin on HepG2-C3A Cell Metabolism. *Biotechnology and Bioengineering*, 107: 347-356 p.
- JOSSÉ, R., ANINAT, C., GLAISE, D., DUMONT, J., FESSARD, V., MOREL, F., POUL, J-M., GUGUEN-GUILLOUZO, C. & GUILLOUZO, A. 2008. Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. *Drug Metabolism and Disposition,* 36: 1111-1118 p.
- JOVER, R., PONSODA, X., CASTELL, J.V. & GÓMEZ-LECHÓN, M.J. 1992. Evaluation of the cytotoxicity of ten chemicals on human cultured hepatocytes: predictability of human toxicity and comparison with rodent cell culture systems. *Toxic in Vitro,* 6: 47-52 p.

- KANEBRATT, K. P. & ANDERSSON, T.B. 2008. Evolution of HepaRG cells as an in vitro model for human drug metabolism studies. *Drug Metabolism and Disposition,* 36: 1444-1452 p.
- KASUYA, F., YAMAOKA, Y., OSAWA, K.I. & FUKUI, M. 2000. Difference of the liver and kidney in glycine conjugation of ortho-substituted benzoic acids. *Chemico-Biological Interactions*, 125: 39-50 p.
- KOSTIAINEN, R., KOTIAHO, T., KUURANNE, T. & AURIOLA, S. 2003. Liquid chromatography/atmospheric pressure ionisation-mass spectrometry in drug metabolism studies. *Journal of Mass Spectrometry,* 38: 357-372 p.
- LAKSHMI, V. & NILANJANA, D.A.S. 2011. Biodegradation of caffeine by *Trichosporon asahii* isolated from caffeine contaminated soil. *International Journal of Engineering Science and Technology*, 3: 7988-7997 p.
- LAMBERT, C.B., SPIRE, C., RENAUD, M-P., CLAUDE, N. & GUILLOUZO, A. 2009. Reproducible chemical-induced changes in gene expression profiles in human hepatoma HepaRG cells under various experimental conditions. *Toxicology in vitro,* 23: 466-475 p.
- LAPSHINA, E.A., ZAVODNIK, I.B., LABIENIEC, M., REKAWIECKA, K.R. & BRYSZEWSKA, M. 2005. Cytotoxic and genotoxic effects of *tert-Butyl hydroperoxide* on Chinese hamster B14 cells. *Mutation Research,* 583: 189-197 p.
- LEBEL, S., NAKAMACHI, Y., HEMMING, A., VERJEE, Z., PHILLIPS, J.M. & FURUYA, K.N. 2003. Glycine conjugation of para-aminobenzoic acid (PABA): A pilot study of a novel prognostic test in acute liver failure in children. *Journal of Pediatric Gastroenterology and Nutrition,* 36: 62-71 p.
- LEE, D-H., HA, M-H. & CHRISTIANI, D.C. 2001. Body weight, alcohol consumption and liver enzyme activity–a 4-year follow-up study. *International Journal of Epidemiology*, 30: 766-770 p.
- LIMA, C.F., FERNÁNDEZ-FERREIRA, M. & PEREIRA-WILSON, C. 2006. Phenolic compounds protect HepG2 cells from oxidative damage: Relevance of glutathione levels. *Life sciences,* 79: 2056-2068 p.
- LISKA, D., LYON, M. & JONES, D.S. 2006. Detoxification and Biotransformational Imbalances. *EXPLORE*, 2: 122-140.

- LOHMANN, W. & KARST, U. 2006. Simulation of the detoxification of paracetamol using on-line electrochemistry/liquid chromatography/mass spectrometry. *Analytical and Bioanalytical Chemistry*, 386: 1701-1708 p.
- LÜBBERSTEDT, M., MÜLLER-VIEIRA, U., MAYER, M., BIEMEL, K.M., KNÖSPEL, F., KNOBELOCH, D., NÜSSLER, K.A., GERLACH, J.C. & ZEILINGER, K. 2011. HepaRG human hepatic cell line utility as a surrogate for primary human hepatocytes in drug metabolism assessment in vitro. *Journal of Pharmacological and Toxicological Methods,* 63: 59-68 p.
- MARTÍN, C., MARTÍNEZ, R., NAVARRO, R., RUIZ-SANZ, J.I., LACORT, M. & RUIZ-LARREA. 2001. tert-Butyl hydroperoxide-induced lipid signalling in hepatocytes: involvement of glutathione and free radicals. *Biochemical Pharmacology,* 62: 705-712 p.
- MIGLIORE, L. & COPPEDÈ, F. 2008. Environmental-induced oxidative stress in neurodegenerative disorders and aging. *Mutation Research,* 674: 73-84 p.
- MINERS, J. O. & BIRKETT, D.J. 1996. The use of caffeine as a metabolic probe for human drug metabolizing enzymes. *Gen. Pharmacol.*, 2: 245-249 p.
- MOSMANN, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods,* 65: 55-63 p.
- NAKAMA, A., KURODA, K. & YAMADA, A. 1995. Induction of cytochrome P450-dependant monooxygenase in serum-free cultured Hep G2 cells. *Biochemical Pharmacology,* 50: 1407-1412 p.
- NEYRINCK, A., EECKHOUDT, S.L., MEUNIER, C.J., PAMPFER, S., TAPER, H.S., VERBEECK, R.K. & DELZENNE, N. 1999. Modulation of paracetamol metabolism by Kupffer cells: a study on rat liver slices. *Life Sciences,* 65: 2851-2859 p.
- NISHIDA, K., OHTA, Y. & ISHIGURO, I. 1997. Modulating role of endogenous reduced glutathione in tert-Butyl hydroperoxide-induced cell injury in isolated rat hepatocytes. *Free Radical Biology & Medicine*, 23: 453-462 p.
- O'BROIN, J.D., TEMPERLEY, I.J. & SCOTT, J.M. 1980. Separation and quantification of theophylline and paraxanthine by reverse phase liquid chromatography. *Clinical chemistry,* 26.

- PARENT, R., MARION, M-J., FURIO, L., TRÉPO, C. & PETIT, M-A. 2004. Origin and characterization of a human bipotent liver progenitor cell line. *Gastroenterology,* 126: 1147-1156 p.
- PERNELLE, K., LE GUEVEL, R., GLAISE, D., GAUCHER-DI STASIO, C., LE CHARPENTIER, T., BOUAITA, B., CORLU, A. & GUGUEN-GUILLOUZO, C. 2011. Automated detection of hepatotoxic compounds in human *hepatocytes* using HepaRG cells and image-based analysis of mitochondrial dysfunction with JC-1 dye. *Toxicology and Applied Pharmacology,* 254: 256-266 p.
- PEROCCO, P., BRONZETTI, G., CANISTRO, D., VALGIMIGLI, L., SAPONE, A., AFFATATO, A., PEDULLI, G.F., POZETTI, L., BROCCOLI, M., IORI, R., BARILLARI, J., SBLENDORIO, V., LEGATOR, M.S., PAOLINI, M. & ABDEL-RAHNAN, S.Z. 2006. Glucoraphanin, the bioprecursor of the widely extolled chemopreventive agent sulforaphane found in broccoli, induces Phase I xenobiotic metabolizing enzymes and increases free radical generation in rat liver. *Mutation Research,* 595: 125-136 p.
- PETERS, F.T., DRUMMER, O.H., & MUSSHOFF, F. 2007. Validation of new methods. *Forensic Science International*, 165: 216-224 p.
- RAU, M.A., WHITAKER, J., FREEDMAN, J.H. & DI GIULIO. 2004. Differential susceptibility of fish and rat liver cells to oxidative stress and cytotoxicity upon exposure to prooxidants. *Comparative Biochemistry and Physiology, Part C,* 137: 335-342 p.
- SALVAGGIO, A., PERITI, M., MIANO, L., TAVANELLI, M. & MARZORATI, D. 1991. Body mass index and liver enzyme activity in serum. *Clin. Chem.,* 37: 720-723 p.
- SCHRADER, E., KLAUNICK, G., JORRITSMA, U., NEURATH, H., HIRSCH-ERNST, K., KAHL, G.F. & FOTH, H. 1999. High-performance liquid chromatography method for simultaneous determination of [1-methyl-^{14}C] caffeine and its eight major metabolites in rat urine. *Journal of Chromatography B,* 726: 195-201 p.
- SESTILI P., PAOLILLO, M., LENZI, M., COLOMBO, E., VALLORANI, L., CASADEI, L., MARTINELLI, C. & FIMOGNARI, C. 2010. Sulforaphane induces DNA single strand breaks in cultured human cells. *Mutation Research,* 689: 65-73 p.

- SHIBA, D. & SHIMAMOTO, N. 1999. Attenuation of endogenous oxidative stress–induced cell death by cytochrome P450 inhibitors in primary cultures of rat hepatocytes. *Free Radical Biology & Medicine,* 27: 1019-1026 p.
- SLIKKER, W., ANDERSON, M.E., BOGDANFFY, M.S., BUS, J.S., COHEN, S.D., CONOLLY, R.B., DAVID, R.M., DOERRER, N.G., DORMAN, D.C., GAYLOR, D.W., HATTIS, D., ROGERS, J.M., SETZER, R.W., SWENBERG, J.A. & WALLACE, K. 2004. Dose-dependent transitions in mechanisms of toxicity: case studies. *Toxicology and Applied Pharmacology,* 201: 226-294 p.
- TURRENS, J.F. 2003. Mitochondrial formation of Reactive Oxygen Species. *The Journal of Physiology*, 552: 335-344 p.
- WESTERINK, W.M.A. & SCHOONEN, W.G.E.J. 2007. Phase II enzyme levels in HepaG2 cells and cryopreserved primary human hepatocytes and their induction in HepaG2 cells. *Toxicology in Vitro,* 21: 1592-1602 p.
- WILSON, K. & WALKER. J. 2005. Principles and techniques of biochemistry and molecular biology, 6th edition: 82-84 p.
- YEH, C-T. & YEN, G-C. 2005. Effect of sulforaphane on metallothionein expression and induction of apoptosis in human hepatoma HepG2 cells. *Carcinogenesis*, 26: 2138-2148 p.
- YEH, C-T. & YEN, G-C. 2009. Chemopreventive functions of sulforaphane: A potent inducer of antioxidant enzymes and apoptosis. *Journal of Functional Foods,* 1: 23-32 p.
- ZAMEK-GLISZCZYNSKI, M.J., HOFFMASTER, K.A., NEZASA, K., TALLMAN, M.N. & BROUWER, K.L.R. 2005. Integration of hepatic drug transporters and phase II metabolizing enzymes: Mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. *European Journal of Pharmaceutical Sciences*, 27: 447-486 p.
- ZHANG, Y., TALALAY, P., CHO, C-G., & POSNER, G.H. 1992. A major inducer of anticarcinogenic protective enzymes from broccoli: Isolation and elucidation of structure. *Proceedings of the National Academy of Sciences of the United States of America*, 89: 2399-2403 p.