EVALUATION OF GRAPEFRUIT EXTRACT FOR THE PREVENTION OF PARACETAMOL-INDUCED HEPATOTOXICITY AFTER OVERDOSE IN RATS

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ABSTRACT

Paracetamol is a widely used analgesic and antipyretic agent. While it is generally safe for use at recommended doses, acute overdose of paracetamol can cause potentially fatal liver damage. Despite the understanding that some cytochrome P450 isoforms are responsible for activation of paracetamol to the hepatotoxic metabolite, *N*-acetyl-*p*-benzoquinone-imine (NAPQI), the use of enzyme inhibitors of therapeutic value for prevention and/or treatment of paracetamol hepatotoxicity is still not well researched. Therefore, grapefruit juice, a well known enzyme inhibitor, was investigated for the prevention of hepatotoxicity after paracetamol overdose in rats.

A high performance liquid chromatography (HPLC) method for the determination of paracetamol in plasma was developed. It involved protein precipitation of 50 µl of paracetamol spiked plasma with zinc sulphate followed by centrifugation. The supernatant was directly injected into the HPLC. The sample was eluted with a mobile phase of 0.01% trifluoroacetic acid in distilled water: acetonitrile (75: 25, v/v) over a Phenomenex C_{18} (4.60 x 250 mm) 5 µ analytical column at 1 ml/min. 4-Aminoacetophenone was used as the internal standard. Under these conditions, paracetamol and 4-aminoacetophenone eluted at retention times of 4.2 minutes and 6.2 minutes, respectively. The average calibration curve (0 - 20 µg/ml) was linear with a regression equation of $y = 0.0603x + 0.089$, and a regression coefficient of $r^2 = 0.9957$. The method was used to measure paracetamol concentrations in rat plasma.

Grapefruit juice was evaluated for prevention of paracetamol-induced hepatotoxicity. Sprague Dawley rats were used and approval from the animal ethics committee was obtained. Rats were treated with a once-off oral dose of saline, paracetamol only, paracetamol + grapefruit juice low dose and paracetamol + grapefruit juice high dose.

A commercially available grapefruit derivative, bergamottin, was also evaluated. Thereafter, 5 rats from each group were sacrificed after 24, 48 and 72 hours. Blood samples were collected for liver function tests, full blood count and paracetamol concentration. A piece of liver was sent for histopathology.

Hepatotoxicity was induced with a single oral dose of paracetamol 1725 mg/kg. The liver enzymes were significantly elevated [ALT 1359 (1073 - 1645); AST 837 (647 - 1026)] when paracetamol was administered alone. The full blood count indicated a very low platelet count [311 (95 - 526)] at 48 hours. Upon co-administration of paracetamol with grapefruit juice, the hepatotoxicity caused by a toxic dose of paracetamol was antagonised. The liver enzymes were lowered [ALT 11 (1 - 90); AST 131 (92 - 492)] and similar results were obtained when paracetamol was co-administered with bergamottin [ALT 164 (121 - 220); AST 14 (14 - 38)].

In conclusion, grapefruit juice prevented the hepatotoxicity caused by paracetamol in a rat model. Its enzyme inhibition ability could be responsible for its hepatoprotective activity. Hence, grapefruit juice could be a more therapeutic and economical alternative to *N*-acetylcysteine in the treatment of paracetamol-induced hepatotoxicity. Further investigation to determine the exact mechanism that is responsible for its hepatoprotective effect is recommended for further studies.

DECLARATION OF INDEPENDENT WORK

I, Refuoe Baleni, hereby declare that the dissertation hereby submitted by me for M.Med.Sc degree in Pharmacology at the University of the Free State is my own independent work and has not previously been submitted by me at another university or faculty for admission to a degree or diploma or any other qualification. I furthermore cede copyright of the dissertation to the University of the Free State.

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Signature **Date**

SUPERVISOR'S DECLARATION

I, Professor A. Walubo, the supervisor of the dissertation entitled: Evaluation of grapefruit extract for the prevention of paracetamol-induced hepatotoxicity in rats, hereby certify that the work in this project was done by Refuoe Baleni at the Department of Pharmacology, University of the Free State.

I hereby approve submission of this dissertation and also affirm that this has not been submitted previously to this or any other institution for admission to a degree or any other qualification.

_____ _____

Signature **Date**

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TABLE OF CONTENTS

ABBREVIATIONS

ALP	alkaline phosphatase
ALT	alanine amnotransferase
AST	aspartate aminotransferase
BGT	bergamottin
Cal	calibration
COX	cyclooxygenase
CV	coefficient of variation
CYP1A2	cytochrome P450 enzyme 1A2
CYP2E1	cytochrome P450 enzyme 2E1
CYP3A4	cytochrome P450 enzyme 3A4
CYP450	cytochrome P450
CZN	chlorzoxazone
EDTA	ethylamine-diamine-tetraacetic acid
GFJ	grapefruit juice
Hb	hemoglobin
Hct	haematocrit
HPLC	high performance liquid chromatography
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration

MCV	mean corpuscular volume
NAC	*N*-acetylcysteine
NAPQI	*N*-acetyl-*p*-benzoquinone-imine
PARA	paracetamol
Plt	platelets
RBC	red blood cell
RDA	recommended daily allowance
ROS	reactive oxygen species
RPM	revolution per minute
Rx	treatment
SD	Sprague-Dawley
SD	standard deviation
TEAH	tetraethylammoniumhydroxide
TEAP	tetraethylammoniumphosphate
UV	ultraviolet
WBC	white blood cell

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

GENERAL INTRODUCTION

Paracetamol (acetaminophen) is a widely used over-the-counter analgesic and antipyretic agent. It is commonly used for the relief of headaches and other minor aches and pains, and it is a major ingredient in numerous cold and flu remedies. In combination with opiod analgesics, paracetamol can also be used in the management of more severe pain such as post surgical pain and providing palliative care in advanced cancer patients. Acute overdose of paracetamol can cause potentially fatal liver damage. The risk of overdosing is heightened by chronic alcohol consumption (Refat *et al*., 2013).

Damage to the liver results not from paracetamol itself, but from one of its metabolites, *N*-acetyl-*p*-benzoquinone-imine (NAPQI). NAPQI depletes the liver's natural antioxidant, glutathione and directly damages cells in the liver, leading to liver failure.

In a previous report by Walubo and co-workers (2004), it was demonstrated that administration of a hepatotoxic dose of paracetamol in combination with known cytochrome P450 enzyme inhibitors, i.e., ketoconazole, isoniazid and caffeine, prevented the development of paracetamol-induced hepatotoxicity. Unfortunately, because of their side effects and therapeutic use, these drugs could not be investigated further for treatment or prevention of paracetamol-induced hepatotoxicity.

Thus, the aim of the study was to evaluate grapefruit extract for the prevention of paracetamol-induced hepatotoxicity after overdose in rats.

PART I: AN OVERVIEW OF PARACETAMOL

2.1 BACKGROUND

2.1.1 Physical and chemical properties

Paracetamol ($C_8H_9NO_2$) is a white crystalline substance with a molecular weight of 151.2 g/mol and a melting point of 169 - 171°C. It is soluble in water and ethanol (Frank Ellis, 2002).

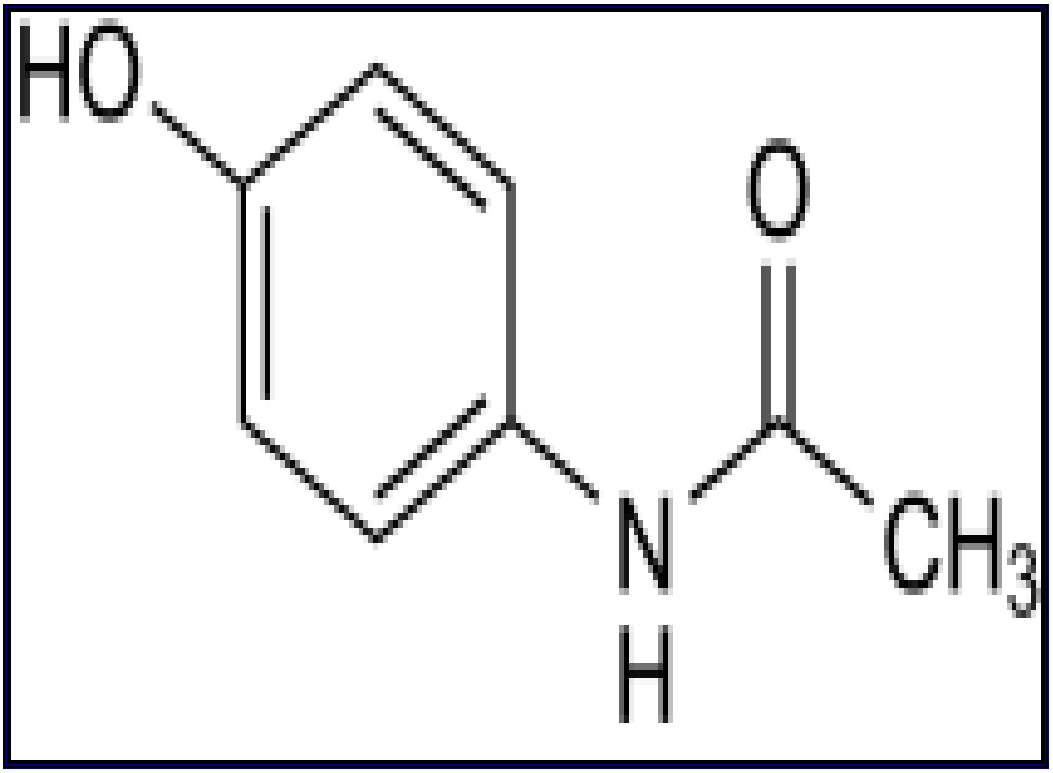

Figure 2.1: The chemical structure of paracetamol (From: Frank Ellis, 2002)

2.1.2 Mechanism of action

Paracetamol is used worldwide as an anti-pyretic and analgesic drug. The therapeutic dose of paracetamol, 500 mg, is generally safe and free from adverse effects, but an acute overdose (15 to 20 tablets) can bring about centrilobular hepatic necrosis (Fazlul Hug, 2007).

The complete mechanism of action of paracetamol is still not well understood. It is believed that paracetamol inhibits the synthesis of prostaglandins in the central nervous system (CNS), which accounts for its anti-pyretic and analgesic properties. However, paracetamol has shown to have less effect on cyclooxygenase (COX) in the peripheral tissues, which explains its weak anti-inflammatory properties (Harvey and Champe, 2009).

2.1.3 Pharmacokinetics

2.1.3.1 Absorption and distribution

After oral administration, paracetamol exhibits bioavailability of 88%, total body clearance of 5ml/min/kg and volume of distribution of 0.8 L/kg. Paracetamol is not highly bound to plasma proteins, approximately 3% of it is excreted unchanged in the urine. Furthermore, paracetamol crosses the blood brain barrier (Toussaint *et al.,* 2010).

Despite differences in individual plasma paracetamol concentrations quantified 60 minutes after oral administration, the time to peak concentration is almost 45-60 minutes for normal release tablets. The absorption of paracetamol is minimised when the drug is taken with food. Therefore, in order to achieve quick pain relief, paracetamol should not be taken after a carbohydrate rich meal (Bertolini *et al.,* 2006).

2.1.3.2 Metabolism

Paracetamol is metabolised by three main pathways in the liver after oral administration, namely, glucuronidation, sulphation and oxidation (Figure 2.2). It is converted to reactive metabolites by human cytochrome P450 isoforms CYP2E1, CYP1A2 and CYP3A4, and as a result, causes toxicity. The hepatotoxicity ensues due to death of hepatocytes by *N*-acetyl-*p*-benzoquinone-imine (NAPQI). NAPQI is a highly reactive metabolite which can bind to glutathione. In small quantities, NAPQI is immediately detoxified by conjugation with glutathione, but when high doses of paracetamol (>10g) are ingested, NAPQI is produced in excess and causes glutathione stores to deplete quickly. Subsequently, the overpowering unconjugated NAPQI binds to hepatocytes, and this leads to liver injury (Tanaka *et al.,* 2000).

2.1.3.3 Elimination

Paracetamol is eliminated through the kidneys by glomerular filtration with successive tubular reabsorption, during which the highly polar glucuronide and sulphate conjugates are actively secreted by the tubules. In healthy individuals, the elimination half-life is 2 to 4 hours, while in older patients the average half-life increases significantly due to a reduction in paracetamol clearance. On the other hand, in premature infants the average half-life is 11 hours, while it is 4 to 5 hours in newborns (Bertolini *et al.,* 2006).

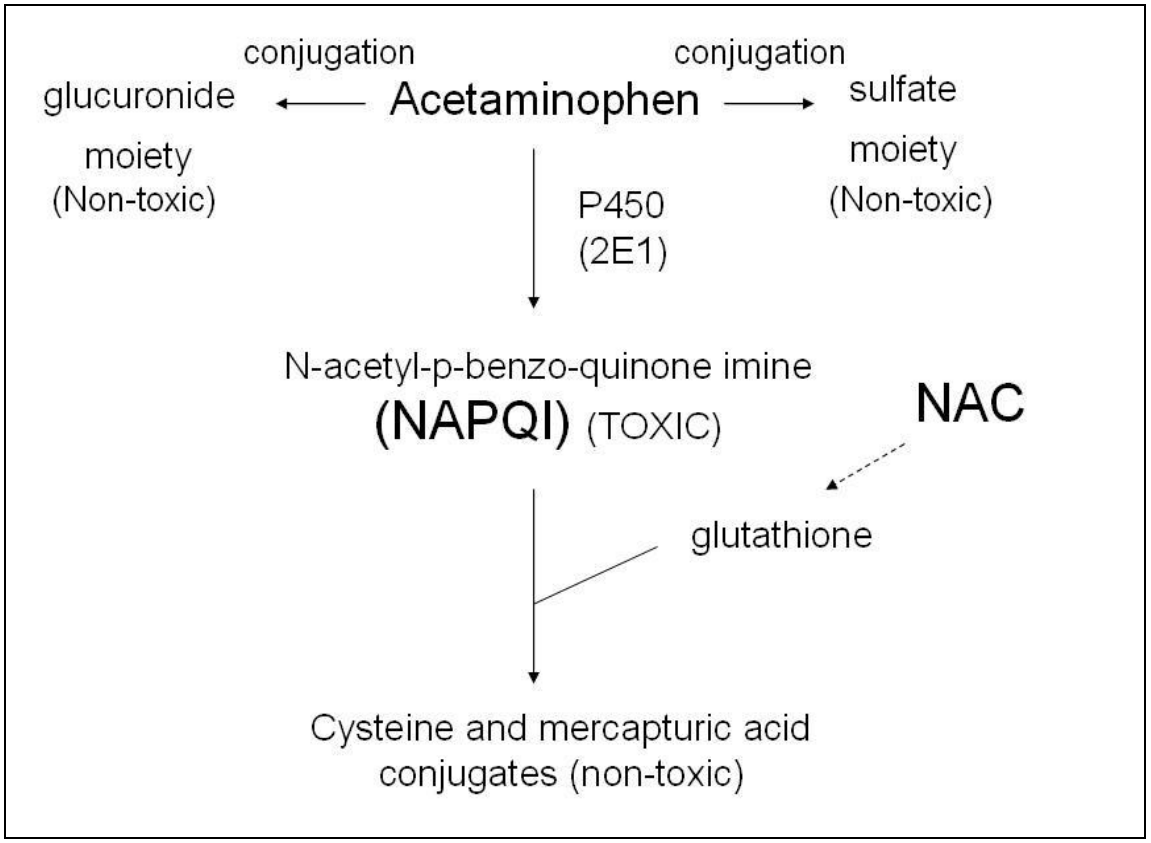

Figure 2.2: The metabolic pathways of paracetamol, and the mechanism of, and protection against, paracetamol-induced hepatotoxicity

(From: Anker *et al.,* 1994)

2.1.4 Adverse effects

When taken in therapeutic doses, paracetamol has shown to be safe. However, when the usual therapeutic range is exceeded, paracetamol is able to induce serious and fatal hepatotoxicity (Toussaint *et al.,* 2010). The drug has been affliated with agranulocytosis, neutropenia, thrombocytopenia and pancytopenia, while in dogs, pigs and cats, oxidative hemolysis and methaemoglobinemia have been observed. Barr (2008), reported that paracetamol, in contrast to other opiod analgesics, does not result in euphoria and other mood disorders.

2.1.5 Drug interactions

Paracetamol has been found to increase the anti-coagulant effects of warfarin by inhibition of the metabolism of oral formulations of the drug, or interference with hepatic synthesis of factors II, VII, IX and X. Phenytoin and fosphenytoin have shown to lower the bioavailability of paracetamol in patients receiving anti-convulsants (Bertolini *et al.,* 2006).

Alcohol-paracetamol syndrome is defined as the development of acute toxic hepatic symptoms in long-term alcoholics who take paracetamol in doses which are generally considered to be safe. Concomitant use of alcohol and paracetamol may potentiate the CYP2E1-mediated metabolism of paracetamol to the hepatotoxic metabolite *N*-acetyl-*p*-benzoquinone-imine (NAPQI). However, in non-alcoholic patients, NAPQI is usually rapidly detoxified by conjugation with glutathione. In alcoholic patients the accumulation of NAPQI results from the induction of CYP2E1 and the depletion of glutathione (Bertolini *et al.,* 2006).

PART II: AN OVERVIEW OF CYTOCHROME P450

2.2 THE ROLE OF CYTOCHROME P450 IN DRUG METABOLISM

2.2.1 Drug metabolism

Drug metabolism is the enzymatic conversion of a drug into a metabolite. The metabolism of drugs takes place primarily in the liver in two phases: in phase I the drug is oxidised in the liver microsomes, and in phase II is where the metabolite from phase I is conjugated in the liver cells. The cytochrome P450 enzymes play an important role in drug metabolism because they catalyse the phase I reactions in the microsomes (Gunaratna, 2000).

2.2.2 Factors affecting drug metabolism

2.2.2.1 Physical and chemical properties of the drug

The molecular size, shape, lipophilicity, acidity/basicity, electronic characteristics and pKa influence the interaction of a drug with the metabolising enzymes (Taxak and Bharatam, 2014).

2.2.2.2 Biochemical factors

Drug-drug interactions are the results of the impact of one drug on the metabolism of another drug (Taxak and Bharatam, 2014).

i. Enzyme induction

Enzyme induction is a process that increases the rate of metabolism of a drug which affects the duration and intensity of the drug action. For instance, barbiturates induce the metabolism of coumarins and phenytoin, while the metabolism of pentobarbitals and coumarins are potentiated by the use of alcohol (Taxak and Bharatam, 2014).

ii. Enzyme inhibition

Enzyme inhibition results from a process of blocking the catalytic site of cytochrome P450 enzymes and thus decreasing the conversion of drugs to metabolites. Subsequently, the duration which the drug remains in the body is increased, causing the drug to accumulate and give rise to toxicity (Taxak and Bharatam, 2014).

2.2.2.3 Biological factors

Diet, smoking, alcohol consumption and concomitant drug therapy may affect the outcome of drug metabolism. Polycyclic aromatic hydrocarbons (PAH) produced by cigarette smoking induce CYP1A2, which is responsible for the metabolism of PAH to carcinogens and results in lung and colon cancer. Grapefruit is a known dietary constituent that inhibits CYP3A4, while herbal medicines like St. John's Wort increases the possibility of the occurrence of drug interactions to occur (Gunaratna, 2000).

2.3 CYTOCHROME P450 ISOFORMS

The cytochrome P450 enzymes are found predominately in the liver, however, some are present in the intestine. These enzymes play an important role in the metabolism of the majority of medications and are involved in the mechanism by which most pharmacokinetic drug interactions occur (Horn and Hansten, 2014).

2.3.1 CYP1A2

The importance of CYP1A2 in drug interactions has escalated due to the fact that it metabolises a large number of drugs, which makes it a source of drug interactions (Horn and Hansten, 2014). However, CYP1A2 can be inhibited by some drugs, natural substances and other compounds (Zhou, 2010).

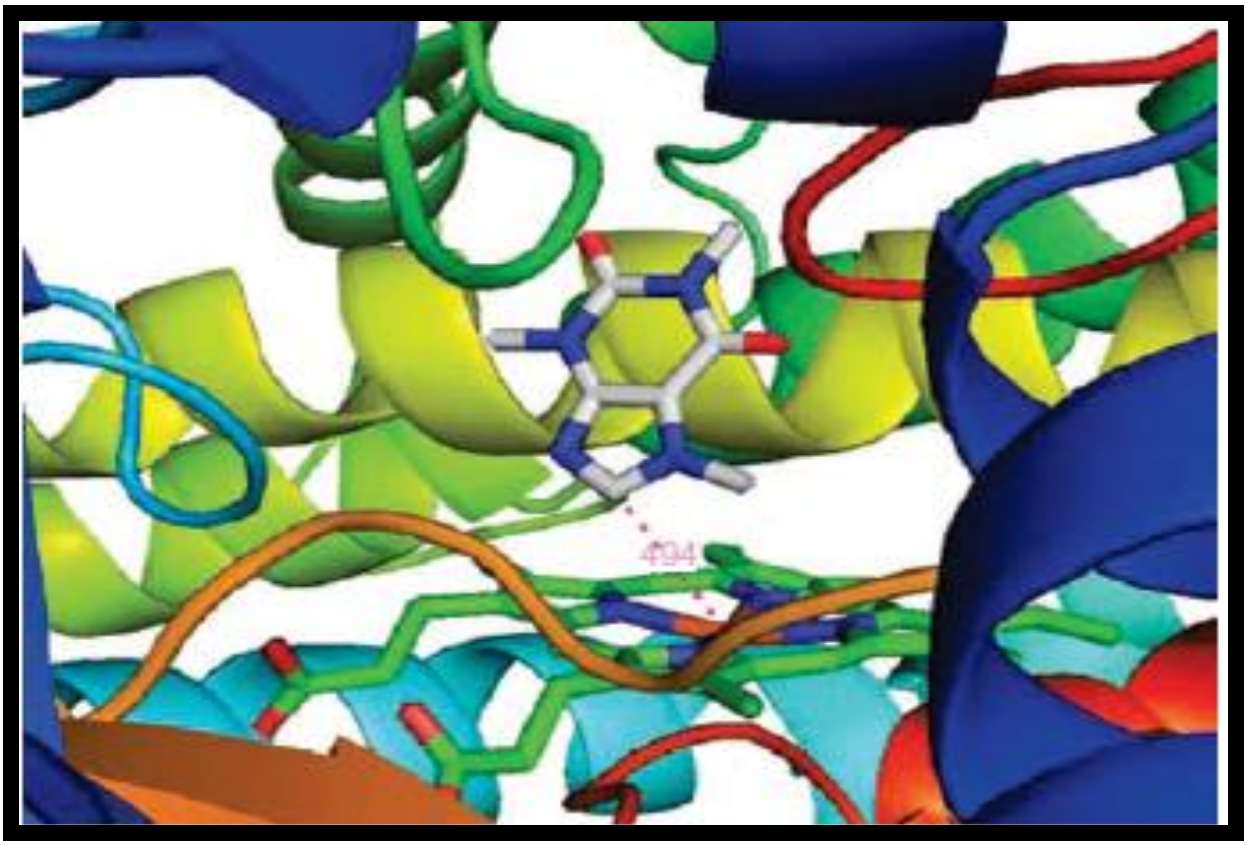

Figure 2.3: Structure of cytochrome P450 1A2, (From: Shu-Feng Zhou *et al.,* 2010).

Table 2.1: Substrates, inhibitors and inducers of CYP1A2

Substrates	**Inhibitors**	**Inducers**
Paracetamol	Grapefruit juice	Tobacco
Caffeine	Ciprofloxacin	Carbamazepine
Clozapine	Cimitidine	Phenobarbital
Theophylline	Fluvoxamine	
	Omeprazole	

2.3.2 CYP2E1

CYP2E1 is responsible for the metabolism of ethanol, paracetamol and pro-carcinogens like nitrosamines. Excessive amounts of reactive oxygen species (ROS) and toxic intermediates are produced by the CYP2E1-mediated metabolism of compounds such as ethanol.

The various liver diseases that are associated with chronic alcohol consumption are mainly caused by the increased CYP2E1 protein levels and induced enzymatic activity (Leung *et al*., 2013).

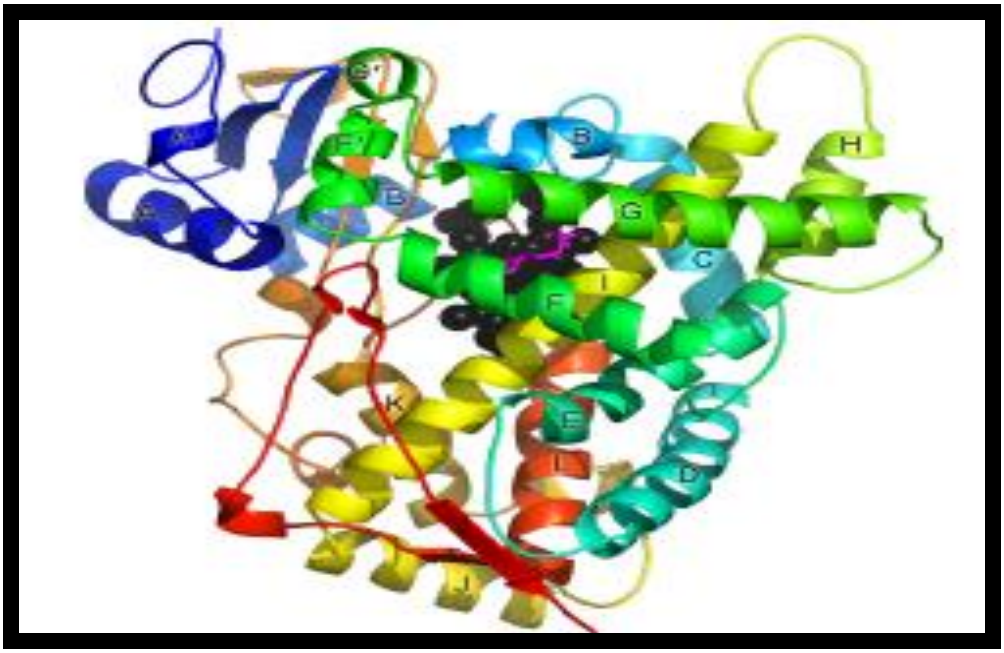

Figure 2.4: Structure of cytochrome P450 2E1 with omega- imidazolyl decanoic fatty acid, (From: Porubsky *et al*., 2010).

Table 2.2: Substrates, inhibitors and inducers of CYP2E1

Substrates	**Inhibitors**	**Inducers**
Halothane	Diethyldithiocarbamate	Ethanol
Paracetamol	Disulfiram	Isoniazid
Ethanol		
Theophylline		
Chlorzoxazone		

2.3.3 CYP3A4

CYP3A4 is responsible for the metabolism of approximately half of the drugs available on the market. Most of the drugs are used regularly and known to be inhibitors of CYP3A4, which consequently gives rise to drug toxicity. It has, however, been shown that CYP3A4 can be induced as well. The decreased efficacy of the substrate is due to the lowering of its plasma concentration by CYP3A4 inducers. Reduced drug efficacy is often thought to be due to a lack of patient compliance, however, this could be a result of drug interactions (Horn and Harnsten, 2014).

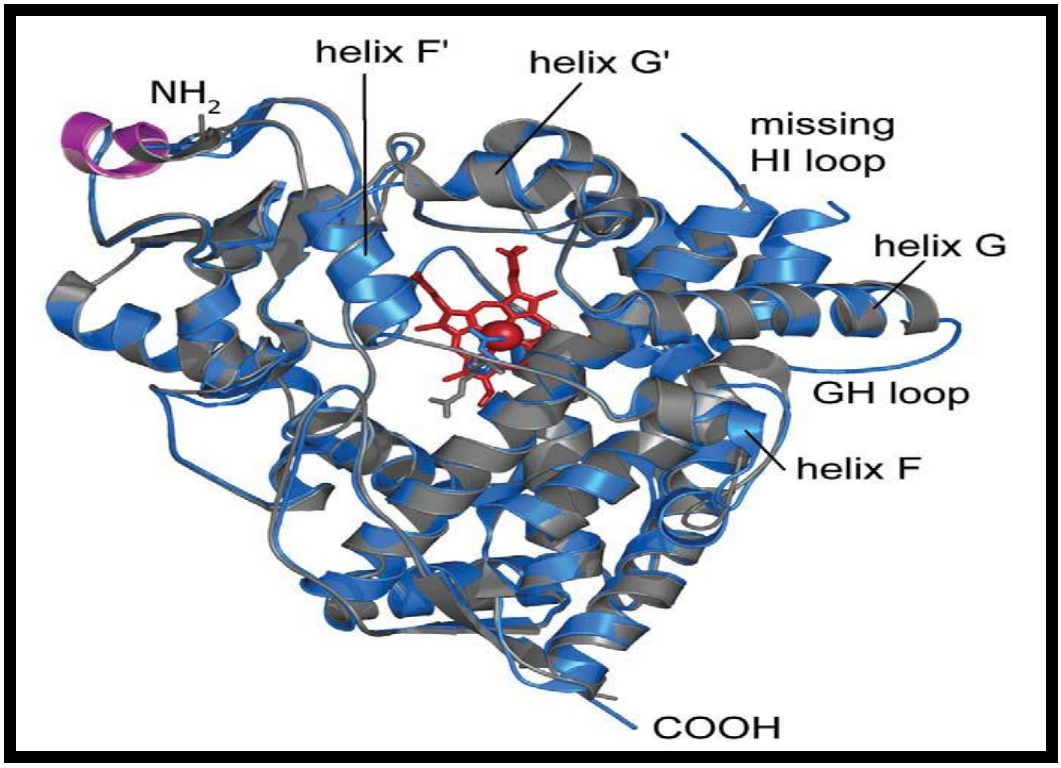

Figure 2.5: Structure of cytochrome P450 3A4, (From: Scott and Halpert, 2005).

Table 2.3: Substrates, inhibitors and inducers of CYP3A4

Substrates	**Inhibitors**	**Inducers**
Amitriptylline	Grapefruit juice	Carbomazepine
Benzodiazepines	Nefazodone	Phenytoin
Calcium channel blockers	Venlafaxine	Refampin
Erythromycin	Protease inhibitors	Dexamethasone
Ketoconazole	Cyclosporine	Theophylline
Amiodarone	Erythromycin	Nevirapine
Atazanavir	Flvoxamine	St. John’s wort
Bupropion	Fluconazole	Phenobarbital
Budesonide	Fluoxetine	Modafinil
Clarythromycin	Ritonavir	Rifambutin
Cisapride	Verapamil	Griseofulvin

PART III: AN OVERVIEW OF GRAPEFRUIT AS AN ENZYME INHIBITOR

2.4 GRAPEFRUIT

2.4.1 Background

Grapefruit was first developed in the West Indies in the early 1700s and is thought to be a hybrid of orange and shaddock. Today, there are three major types of grapefruit, namely, white, pink/red and ruby red. Up to 69% of the recommended daily allowance (RDA) for vitamin C can be provided by grapefruit juice (Kiani and Imam, 2007).

Grapefruit juice is also known to be rich in vitamin A, fibre and potassium. Furthermore, research has indicated that grapefruit juice may contain compounds which are responsible for boosting heart health and reducing the risk of heart disease (Barett, 2013).

2.4.2 Pharmacologically active compounds

Several pharmacologically active compounds, including the primary flavonoids, naringin and hesperidin, and furanocoumarins, bergamottin and 6,'7'-dihydroxybergamottin, are found in grapefruit juice. Factors such as type, origin and quantity of the grapefruit may affect the concentration of flavonoids and furanocoumarins in grapefruit juice (Papandreou and Phily, 2014).

Flavonoids are present in grapefruit juice in the form of glycosides. Here, naringin is the predominant and most abundant flavonoid. After ingestion, glycosides are converted to aglycones and sugars by intestinal flora, and these compounds have the ability to inhibit the CYP450 enzymes. Flavonoids continue to be a subject of interest, especially naringenin, since grapefruit juice contains high quantities of the compound, which cannot be found in other citrus juices (Kiani and Imam, 2007).

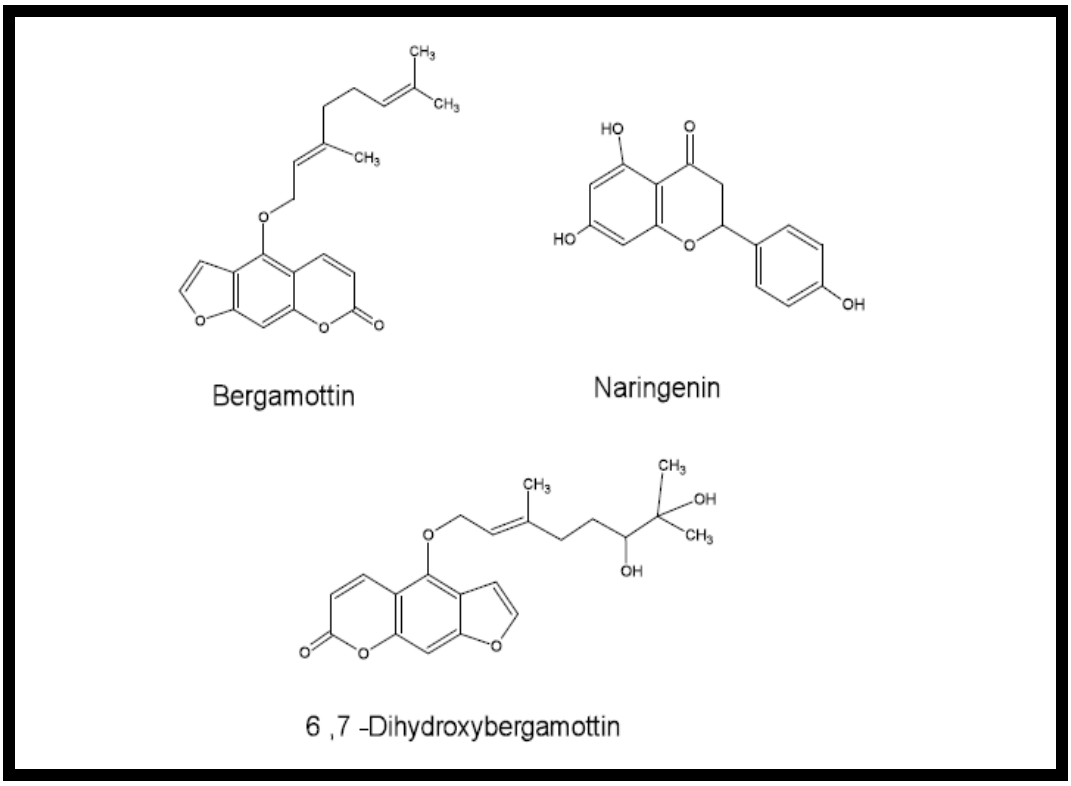

Figure 2.6: Active chemicals in grapefruit juice (From: Xu *et al.,* 2013)

2.4.3 Drug interaction with grapefruit juice

Grapefruit juice mainly exerts its effects on CYP3A4 by inhibiting the activity of the enzyme. Within 4 hours of ingestion, grapefruit juice can reduce the cellular levels of CYP3A4. Subsequently, the bioavailability is increased for as long as 24 hours, with 30% of its effects still detectable (Kiani and Imam, 2007).

Grapefruit juice has shown to affect individuals differently due to the variation in enteric CYP3A4 protein expression. Most drugs that interact with grapefruit juice undergo primary metabolism in the intestine. Although grapefruit juice leads to an increase in drug plasma concentrations, it has not shown a significant effect on the half-life of drugs (Kiani and Imam, 2007).

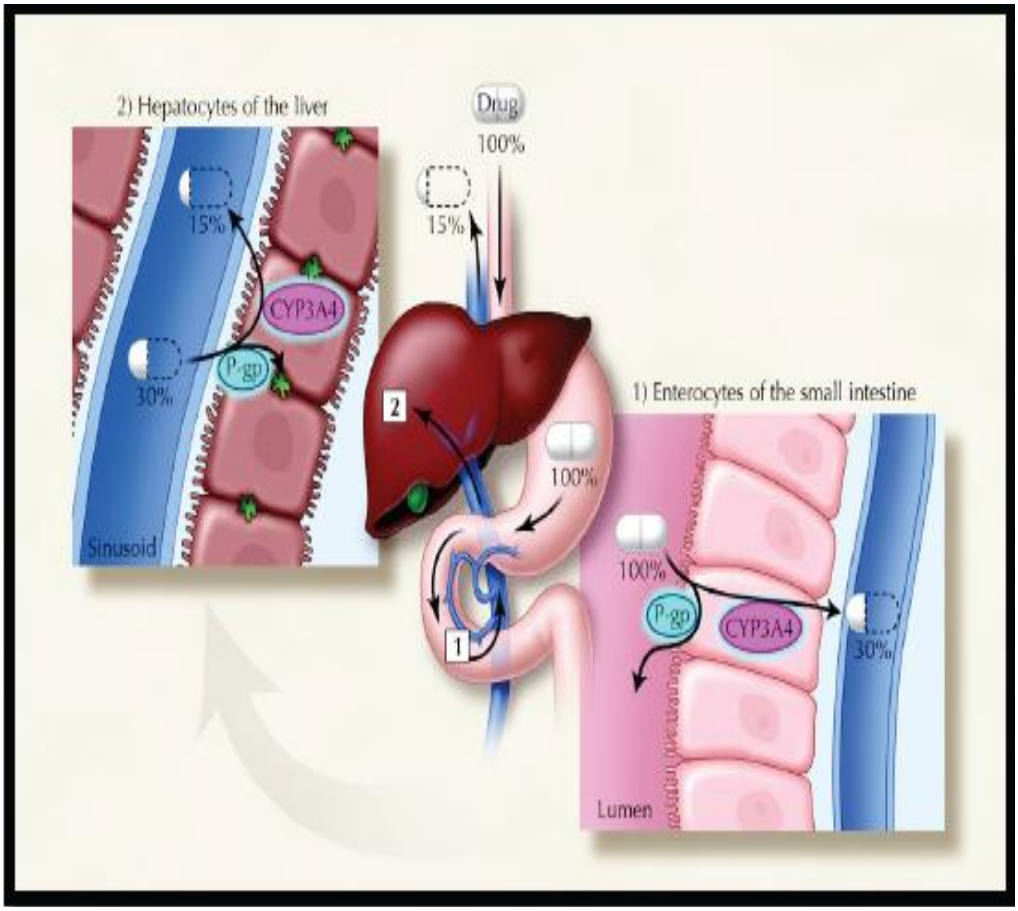

Figure 2.7: Diagram showing the pathway of a drug being metabolised by CYP3A4 in the liver and small intestine (From: Bailey *et al.,* 2003).

2.4.4 Clinical significance of drug interactions with grapefruit juice

2.4.4.1 The clinical significance of drug interactions relies on the following:

i. Change in drug pharmacokinetics

If the plasma drug concentration increases due to grapefruit juice, it might result in adverse drug effects (Bailey *et al.*, 1998).

ii. Patient susceptibility

Patients that rely on intestinal CYP3A4 activity for drug elimination are particularly susceptible for interaction with grapefruit juice (Bailey *et al*., 1998).

iii. Grapefruit juice type and amount

The level of interaction may vary due to different brands, different batches of juice, as well as the quantity of active ingredients in the fruit (Bailey *et al.,* 1998).

2.4.4.2 Content and role of furanocoumarins in grapefruit juice-drug interaction

Bergamottin, a furanocoumarin present in grapefruit juice, was originally thought to be responsible for the inhibition of CYP450 enzymes. Unfortunately, the relevance of bergamottin in the clinical interaction is uncertain. However, administration of bergamottin in its pure form enhances the oral bioavailability of some drugs, but this effect is not as potent as that of grapefruit juice (Muntingh, 2011). 6,'7'-Dehydroxybergamottin (DHB) is the most abundant and important furanocoumarin and is known to be an inhibitor of CYP3A4, hence a likely culprit of the interaction. Studies have shown that the use of fresh grapefruit juice is mostly favoured, however, it has been proposed that preparations of the pulp, peel and core of the fruit might also contain compounds which could participate in the interaction (Muntingh, 2011). Furanocoumarins from grapefruit juice are said to induce food-drug interaction with many CYP3A4 substrates. This is due to the reversible inhibition and irreversible mechanism-based metabolism of CYP3A4 (Bouwer *et al.,* 2006).

PART IV: AN OVERVIEW OF PARACETAMOL-INDUCED HEPATOTOXICITY

2.5 PARACETAMOL HEPATOTOXICITY

The hepatotoxicity occurs due to injury of the liver by the toxic metabolite of paracetamol. When taken in therapeutic doses, paracetamol is quickly metabolised by glucuronidation and sulphation in the liver (Figure 2.8 - page 18). Approximately 2% is excreted in the urine, while 5-10% is metabolised by cytochrome P450 to *N*-acetyl-*p*-benzoquinone-imine (NAPQI; Chun *et al.,* 2009). In normal doses, NAPQI is quickly detoxified by conjugation with glutathione and it is excreted through the kidneys (Walubo *et al.,* 2004).

As the dose of paracetamol increases, glutathione becomes depleted and NAPQI accumulates and leads to hepatic and centrilobular necrosis (Figure 2.9 - page 18). To reduce absorption of paracetamol, gastric lavage, activated charcoal ingestion and induction of emesis by ipecacuanha can be performed.

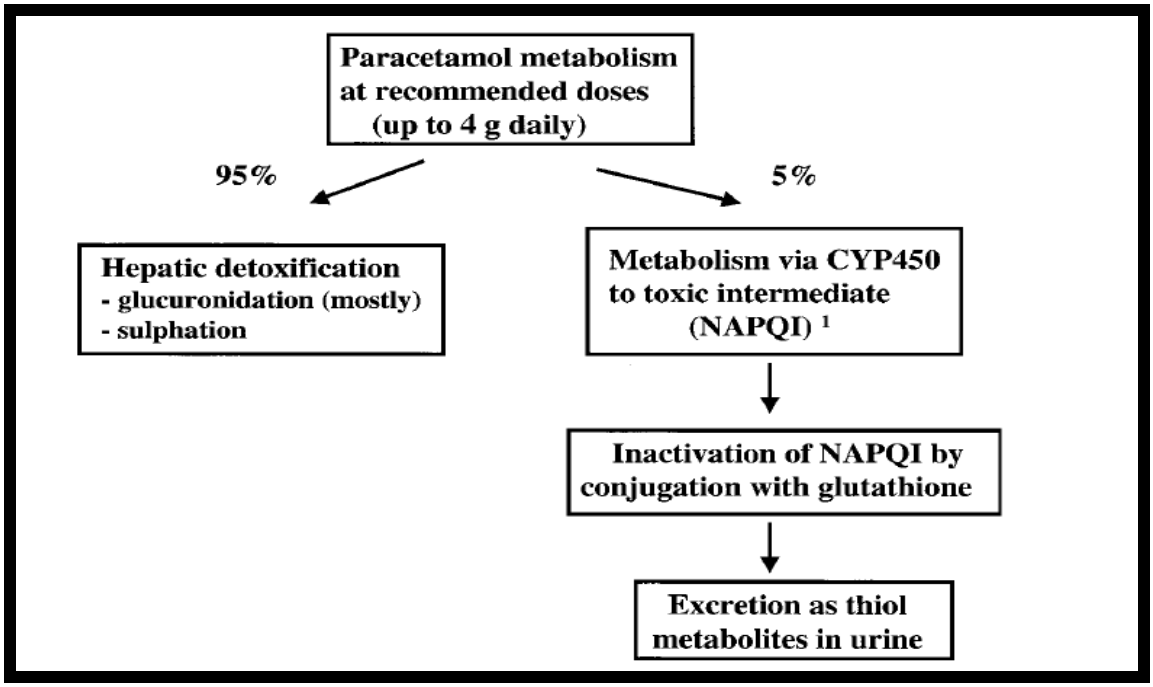

Figure 2.8: Schematic representation of paracetamol metabolism at recommended dose (From: Riordan and Williams, 2002)

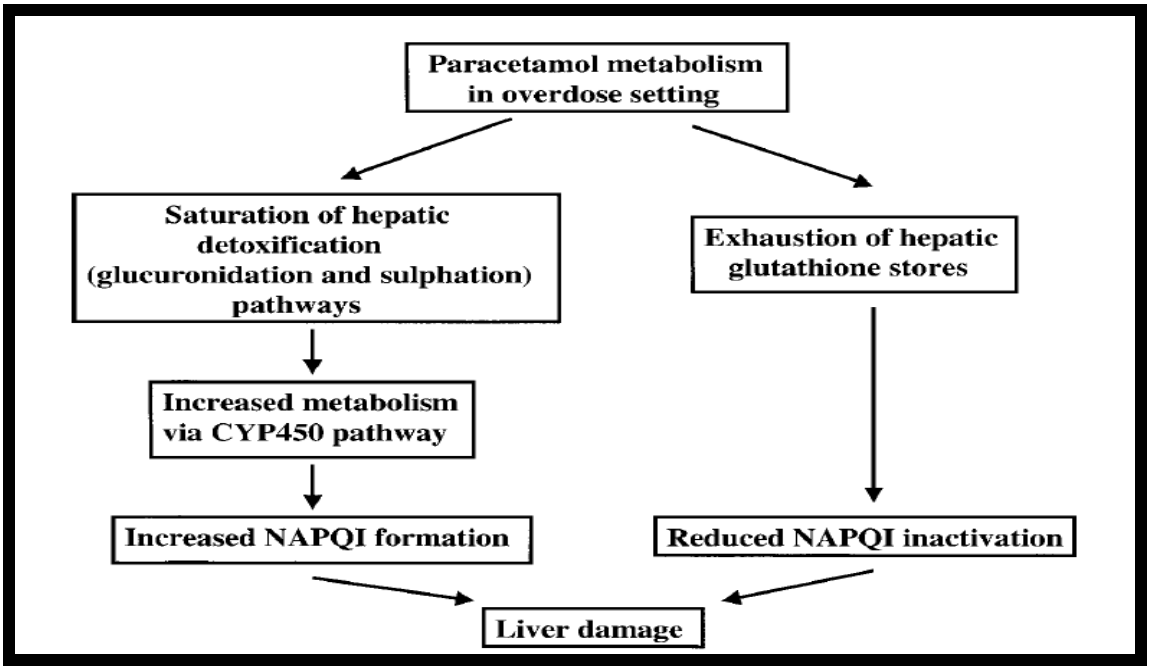

Figure 2.9: Key changes in paracetamol metabolism during overdose (From: Riordan and Williams, 2002)

Currently, *N*-acetylcysteine (NAC) is an accepted antidote that is able to reduce the risk of hepatotoxicity and mortality in patients with acute liver failure, if administered early enough. If given in the first 8-10 hours after ingestion, NAC can be highly effective in protecting against severe liver damage, renal failure and death. The recommended dose of NAC is 140 mg/kg, followed by 70 mg/kg every 4 hours for 17 doses (Chun *et al.,* 2009).

NAC acts by replenishing the glutathione stores, thereby enhancing NAPQI detoxification. Unfortunately, NAC does not stop the production of NAPQI and it can cause mild to moderate side effects, which include nausea, vomiting, abdominal pain, diarrhea and rash (hypersensitivity) when administered orally, whereas when administered intravenously, NAC can cause anaphylactic reactions (Walubo *et al.,* 2004: Chun *et al.,* 2009).

In a study by Walubo *et al*. (2004), it was demonstrated that administration of a hepatotoxic dose of paracetamol with a combination of known enzyme inhibitors, ketoconazole, isoniazid and caffeine, prevented the development of paracetamol-induced hepatotoxicity. Unfortunately, because of their side effects and therapeutic use, these drugs could not be investigated further for the treatment or prevention of paracetamol-induced hepatotoxicity.

Grapefruit juice on the other hand, is known to inhibit a wide range of CYP450 enzymes such as CYP1A2, CYP3A4 and CYP2E1 (Guo and Yamazoe, 2004).

Evaluation of grapefruit will help determine whether it can be used to prevent paracetamol-induced hepatotoxicity after overdose by inhibiting cytochrome P450 enzymes, CYP3A4, CYP1A2 and CYP2E1.

CHAPTER 3

REVIEW OF ANALYTICAL METHODS FOR DETERMINATION OF PARACETAMOL IN PLASMA

3.0 SUMMARY

The assays that are used to measure the concentration of paracetamol in plasma are not readily available, regardless of the increasing number of overdose cases (Shihana *et al.,* 2010). Methods that are available are either expensive or time consuming, such as colorimetric, spectrophotometric and high performance liquid chromatography (Chun *et al*., 2008).

3.1 Colorimetric and spectrophotometric methods

Colorimetric and spectrophotometric methods may give false results of paracetamol levels, because they are based on unspecific acid hydrolysis of the drug without prior solvent extraction (Shihana *et al.,* 2010). Acetaminophen metabolites, acid-labile acetaminophen conjugates, are also hydrolysed to *4*-aminophenol in acidic conditions giving gross overestimates of the true free acetaminophen concentration.

3.2 High performance liquid chromatographic essay

The preferred analytical method for emergency estimation of the plasma paracetamol concentration is high performance liquid chromatography (Campanero *et al*., 1999).

In their method, Campanero *et* al. (1999), used *p*-Propionamidophenol as an internal standard and extraction was performed by a simple liquid-liquid extraction with ethyl acetate. The internal standard, reversed phase analytical column and the diode array detector used in this method are not available in our laboratory.

Soysa and Kolambage (2010), phased out the extraction of paracetamol with ethyl acetate which is ideal during method development because the preparation is simpler and quick.

The method is quite rapid and has shown to be sensitive and accurate. Even so, the method requires the use of a column size that is currently not available in our department.

The high performance liquid chromatography method used by Vertzoni *et al*. (2003) was reviewed. This method showed to be very sensitive and accurate. Also, small plasma volumes were used, which is one of the major objectives of our method development. However, the instrumentation used is very expensive and not available in our laboratory.

Brunner *et al.* (1999) reported a method with simple sample preparation and a short run time, which is appealing when developing a method, especially when large numbers of samples have to be analysed. In spite of this, the pH of a mobile phase was too low, which might put the column life-time at stake.

A rapid, simple and sensitive high performance liquid chromatography method for detection of paracetamol in human plasma was described by Arayne and colleagues (2009). In this method, paracetamol was isolated from plasma by addition of acetonitrile and zinc sulphate, which is an ideal objective for our method development. However, this method required the use of instrumentation that is not available in our laboratory.

All the above-mentioned methods could not be adopted due to solvents and instrumentation used. Nevertheless, protein precipitation by zinc sulphate, as described by Arayne and colleagues (2009), was considered as this has been used successfully in our laboratory.

CHAPTER 4

OBSERVATIONS FROM THE REVIEW

4.1 OBSERVATIONS FROM THE REVIEW

In summary it was observed that:

- Paracetamol is commonly used for the relief of headaches and other minor aches and pains.
- Hepatotoxicity results not from paracetamol itself, but from one of its metabolites, NAPQI, which depletes the liver's natural antioxidant, glutathione, and directly damages cells in the liver, leading to liver failure.
- Cytochrome P450 enzymes, CYP2E1, CYP1A2 and CYP3A4 convert approximately 5% of paracetamol to NAPQI.
- A number of factors can potentially increase the risk of developing paracetamol toxicity:
    1. Chronic excessive alcohol consumption can induce CYP2E1, and thus increase the potential toxicity of paracetamol.
    2. Concomitant use of other drugs that induce CYP enzymes, such as antiepileptics including carbamazepines, phenytoin and barbiturates.
- Understanding the mechanism of paracetamol-induced hepatotoxicity will assist in finding a suitable method for the prevention thereof.
- Co-administration of paracetamol with inhibitors of cytochrome P450 (ketoconazole, isoniazid and caffeine) prevented the development of paracetamol-induced hepatotoxicity in rats.
- Unfortunately, these enzyme inhibitors may not be favourable due to their side effects and therapeutic use. Hence, they could not be investigated any further.

- Thus, there is a need to evaluate naturally occurring enzyme inhibitors such as grapefruit for the prevention of paracetamol-induced hepatotoxicity.
- It is envisaged that inclusion of a small amount of enzyme inhibitor in each paracetamol tablet will lead to enzyme inhibition when many tablets are taken, (e.g. fewer than 10 tablets), which will then prevent activation of paracetamol to NAPQI.

4.2 CHARACTERISATION OF GRAPEFRUIT

- A study cannot be considered scientifically valid if the natural product is not characterised.
- Quality control of raw materials and finished products from medicinal plants is done by chromatographic fingerprinting for identification purposes.

4.3 AIM

The aim of the study is to evaluate grapefruit extract and commercially isolated grapefruit derivative, bergamottin, for prevention of paracetamol-induced hepatotoxicity after overdose.

4.4 OBJECTIVES

i. To characterise grapefruit using HPLC for identification purposes

a) A characterised product ensures reproducibility and consistency in results.
b) Fingerprinting acts as a reference for subsequent products
c) It may be helpful in explaining variation in results amongst other researchers
d) The current study will ensure that claims regarding the enzyme inhibition of grapefruit are made on a characterised product, and
e) Lastly, when the product is released, the fingerprint can be used for quality control purposes.

ii. To develop a method of analysis of paracetamol in plasma

a) This would help to determine the concentration of paracetamol before and after grapefruit has been given.

iii. To evaluate grapefruit juice for the prevention of paracetamol-induced hepatotoxicity

a) Knowledge as to whether enzyme inhibitors present in grapefruit can be used for prevention of paracetamol-induced hepatotoxicity is essential.
b) The results will contribute to the development of effective guidelines for the prevention of paracetamol-induced hepatotoxicity.

CHAPTER 5

DIRECT INTERACTION OF GRAPEFRUIT WITH PARACETAMOL *IN VITRO*

5.0 SUMMARY

This study was divided into two parts, namely: characterisation of grapefruit by high performance liquid chromatography, and testing the mixture of paracetamol and grapefruit juice for enzyme inhibitor properties.

In the first part of the study, characterisation of grapefruit was performed by analysis of two grapefruit samples, namely, grapefruit peel extract and un-extracted grapefruit juice. The peel extract was prepared by homogenizing the peels using a food blender, sieving the pulp formed and centrifuging the homogenate for 15 min at 11963 *g* (10 000 r.p.m). Extraction was done with ethyl acetate and the supernatant was centrifuged for 15 min. A rotary evaporator was used to evaporate the organic layer with the temperature set at 45°C. When almost dry, the crude extract was weighed, reconstituted with water. The un-extracted juice sample was prepared from a freshly squeezed grapefruit.

After preparation of the two samples, they were analysed on the HPLC with the following conditions: the mobile phase of 10% acetonitrile added to TEAP buffer: 100% acetonitrile with 100 µl H_3P0_4 (sulphuric acid) over a Phenomenex® C18 column (150 x 4.60 mm, 3 µ) analytical column at 1 ml/min. Under these conditions, the peaks in the peel extract did not match with any of the furanocoumarins UV spectra, especially bergamottin. The unextracted juice on the other hand, had a peak that was identified as bergamottin and verified with a UV spectrum. Therefore, it was concluded that unextracted grapefruit juice be used for subsequent experiments.

In the second part of the study, a mixture of paracetamol and grapefruit juice was tested for enzyme inhibitor properties. Three pure cytochrome P450 enzymes, CYP1A2, CYPE1 and CYP3A4, were used as controls.

To analyse CYP1A2, the assay involved addition of CYP1A2 pure enzyme, 0.6 mM EDTA, 30 mM magnesium sulphate and 25 nM ethoxyresorufin added to 0.1 M HEPES buffer.

Analysis of CYP2E1 comprised of the addition of CYP2E1 pure enzyme, 0.848 mg/ml chlorzoxazone to a 0.1 M sodium phosphate buffer.

Finally, to analyse CYP3A4, pure CYP3A4 enzyme, 100 mM magnesium chloride and 0.5 mg/ml midazolam were added to 0.1 M sodium phosphate buffer. In all samples, 10 mg/ml of paracetamol was added.

For all the enzyme assays performed, a mixture of grapefruit juice and paracetamol was added. This kind of interaction has not been previously reported. Because grapefruit juice is a well known enzyme inhibitor, it was contemplated that this could potentially decrease the liver toxicity when a small amount of an enzyme inhibitor is added to a paracetamol tablet.

The results show that CYP3A4 was the most inhibited enzyme followed by CYP2E1 and CYP1A2. This kind of inhibition was expected as it is well known that grapefruit juice is a potent inhibitor of CYP3A4 and many other enzymes including CYP2E1 and CYP1A2. Direct interaction of paracetamol and grapefruit juice have proved that this mixture could play an important role in the prevention of paracetamol-induced hepatotoxicity by inhibiting the enzymes which are responsible for the production of NAPQI when induced and consequently lead to hepatic failure.

5.1 MATERIALS AND REAGENTS

5.1.1 Apparatus

A 5810 R centrifuge (Eppendorf; Germany) and an Automatic Refrigerated Low Centrifuge (Sorvall; USA) were used for separating samples. Food blender (Philips) was used to homogenate the grapefruit peels and the pulp was removed with a sieve (Checkers Hyper, South Africa). A rotary evaporator and aspirator (Buchi) were used to concentrate the extract and make it a crude extract. Precision balance (SPB 52 and SPB 31 Scaltec Instruments, Goettingen Germany) was used to weigh centrifuge tubes and analytical bergamottin. The HPLC system (Hewlett Packard model 1100) for grapefruit extract analyses was equipped with an autosampler (Waldbronn, Germany), and UV detector (Tokyo, Japan). Compounds were separated using a Phenomenex C_{18} (4.60 x 150 mm) 3 µ analytical column coupled to a SecurityGuard™ C_{18} (4 x 3 mm) guard column (Phenomenex®, Torrance, CA, USA). A Labcon (Maraisburg, SA) shaking water bath was used for incubation of pure enzymes during *in vitro* studies. Absorbance of resorufin was measured using a Varian Cary Eclipse Fluorescence spectrophotometer.

5.1.1 Reagents

Sigma-Aldrich™ (St.Louis,MO, USA) provided the following drug standards and chemicals: acetaminophen, bergamottin, D-glucose-6-phosphate monosodium salt, β-nicotinamide adenine dinucleoyide phosphate sodium salt, glucose-6-phosphate dehydrogenase, magnesium sulphate, ethoxyresorufin, resorufin, chlorpropamide, carbamazepine, midazolam, hydoxymidazolam, magnesium chloride, HEPES, chorzoxazone, 6-hydrochlorzoxazone, 4-aminoacetophenone and sodium hydroxide. Sulphuric acid (H_3PO_4), tetraethylammoniumhydroxide (TEAH), sodium phosphate monobasic, sodium phosphate dibasic powder and perchloric acid and ethyl acetate, were purchased from Merck (Darmstadt, Germany).

HPLC grade methanol and acetonitrile were purchased from Honeywell Burdick and Jackson International Inc. (Muskegon, MI, USA).The Star Ruby Red Grapefruits were bought from a local supermarket.

PART I: CHARACTERISATION OF GRAPEFRUIT EXTRACT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

5.2 INTRODUCTION

This part describes the characterisation of grapefruit extract using a high performance liquid chromatography method. The aim is to verify the presence of bergamottin, a major component of grapefruit juice and also a well known enzyme inhibitor. Verification was done by matching the UV-spectrum of bergamottin pure standard with bergamottin found in grapefruit extract and un-extracted grapefruit.

5.3 PREPARATION OF GRAPEFRUIT EXTRACT AND JUICE SAMPLES

Fifty (50) Star Ruby grapefruits were peeled and the peels and juice were separated and prepared differently for subsequent sample analysis.

5.3.1 Preparation of grapefruit extract from peels.

Grapefruit peels were homogenised for 2 minutes using a food blender, and 1000 ml of distilled water was added whilst blending, to form a homogenate. The homogenate was sieved to remove the pulp, after which it was centrifuged with a low centrifuge for 15 minutes at 11963 *g* (10000 r.p.m). Thereafter, the supernatant was extracted with ethyl acetate, shaken vigorously and centrifuged for 15 minutes. The aqueous phase was removed and the organic layer evaporated to nearly dry using a rotary evaporator at 45^0 C. Finally, the crude extract was weighed, reconstituted with water, and stored at -20^0 C until analysis (Figure 5.1 – page 31).

5.3.2 Preparation of grapefruit juice

The juice was squeezed from the fruit by hand (Figure 5.2 – page 31). Thereafter, it was filtered and centrifuged for 5 minutes at 7026 *g* (13400 r.p.m). The sample was stored at 4^0 C until analysis.

5.4 SAMPLE PREPARATION FOR ANALYSIS

5.4.1 Bergamottin standard

A stock solution was prepared by dissolving 1 mg of bergamottin in 1 ml of acetonitrile. The stock solution was further diluted to 100 µg/ml with the mobile phase (as described in Section 5.5).

5.4.2 Grapefruit extract from peels

100 µl of the grapefruit extract was further centrifuged at 7026 *g* (13400 r.p.m) for 5 minutes and 20 µl of the supernatant was directly injected into the HPLC.

5.4.3 Grapefruit juice (unextracted)

The squeezed juice was centrifuged at 7026 *g* (13400 r.p.m) for 5 minutes and the supernatant was directly injected into the HPLC.

5.5 PREPARATION OF MOBILE PHASE

The mobile phase comprised Solvent A and B. Solvent A consisted of 10% acetonitrile added to TEAP buffer, whereas Solvent B contained 100% acetonitrile and 100 µl of (sulphuric acid) H_3PO_4. TEAP buffer was prepared by weighing 2.9 g of H_3PO_4 filled to 400 ml with distilled water and 15.54 g of TEAH, and then filled to 500 ml. An isocratic mixture was prepared by mixing 30% of Solvent A with 70% of Solvent B.

5.6 CHROMATOGRAPHIC CONDITIONS

A Phenomenex® C_{18} column (150 x 4.60 mm, 3 µm particle size) was used for separation of compounds. The flow rate was set at 1.0 ml/min and the wavelength of the UV detector was set at 210 nm and injected a volume of 100 µl.

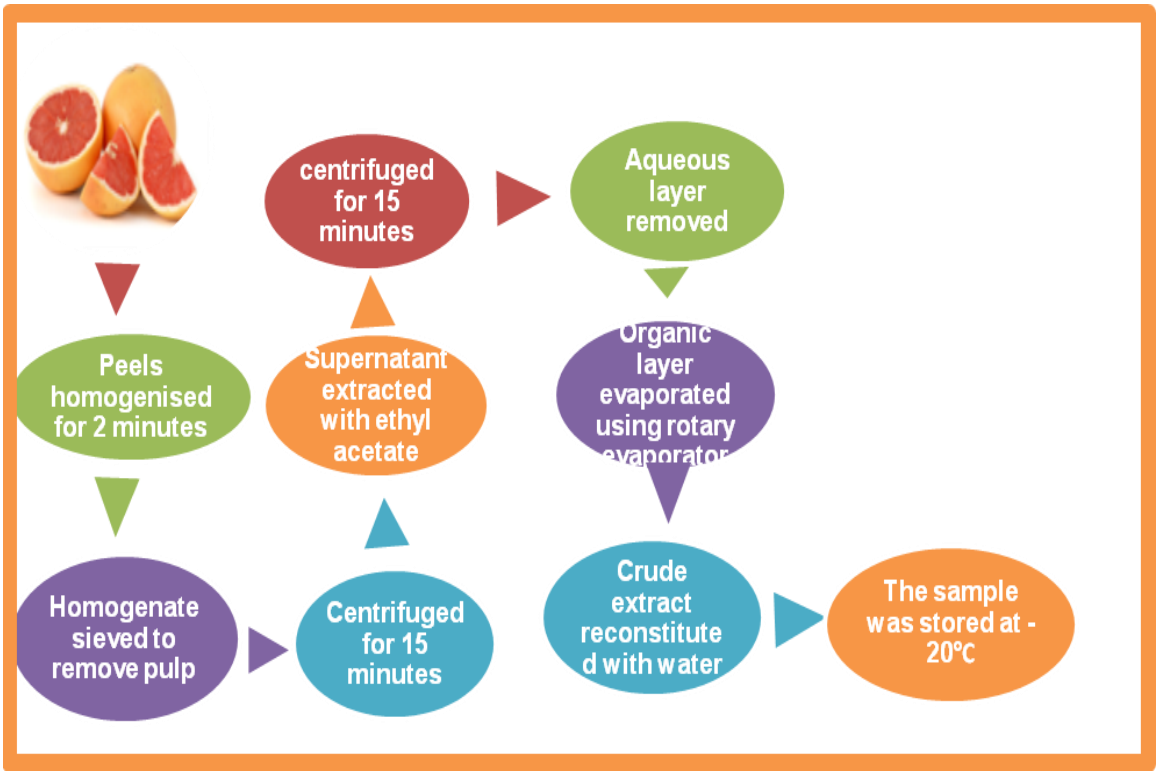

Figure 5.1: Flow chart indicating the preparation of grapefruit extracts from the grapefruit peels

Figure 5.2: Freshly manually squeezed grapefruit juice

5.7 RESULTS

Figure (5.3 a – 5.3 b) – page 33), indicates the chromatograms of ethyl acetate and grapefruit extract. The peaks on the extract did not match with the UV-spectrum of bergamottin. Figure (5.4 a - 5.4 b) on page 34, shows chromatograms of grapefruit juice and a UV-spectrum of peak with retention time of 16.983 minutes. The UV-spectrum matched the UV-spectrum of bergamottin standard (Figure 5.5 b – page 35).

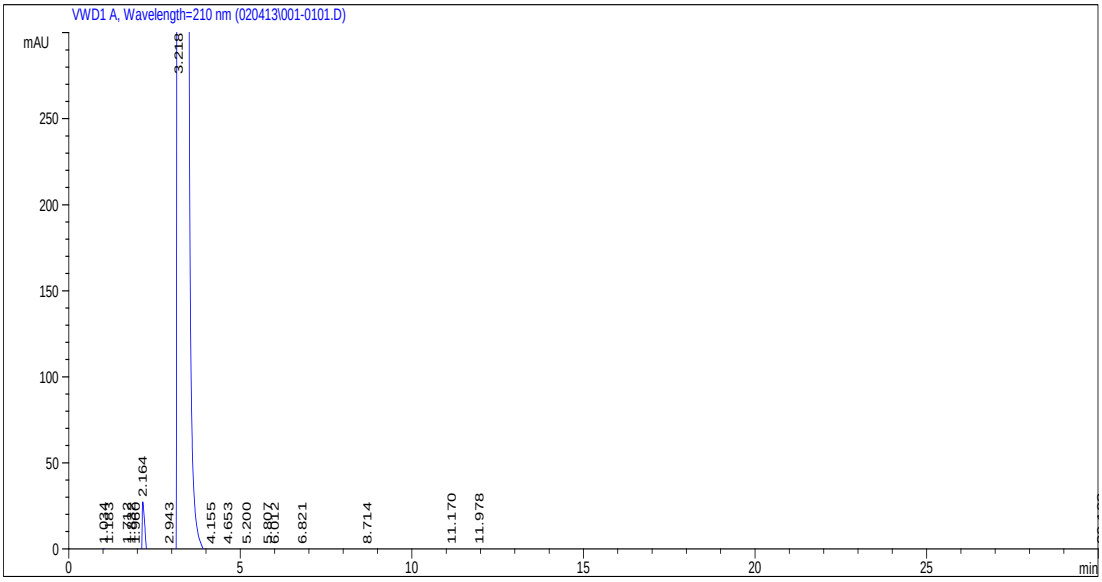

Figure 5.3 a): A chromatogram of ethyl acetate

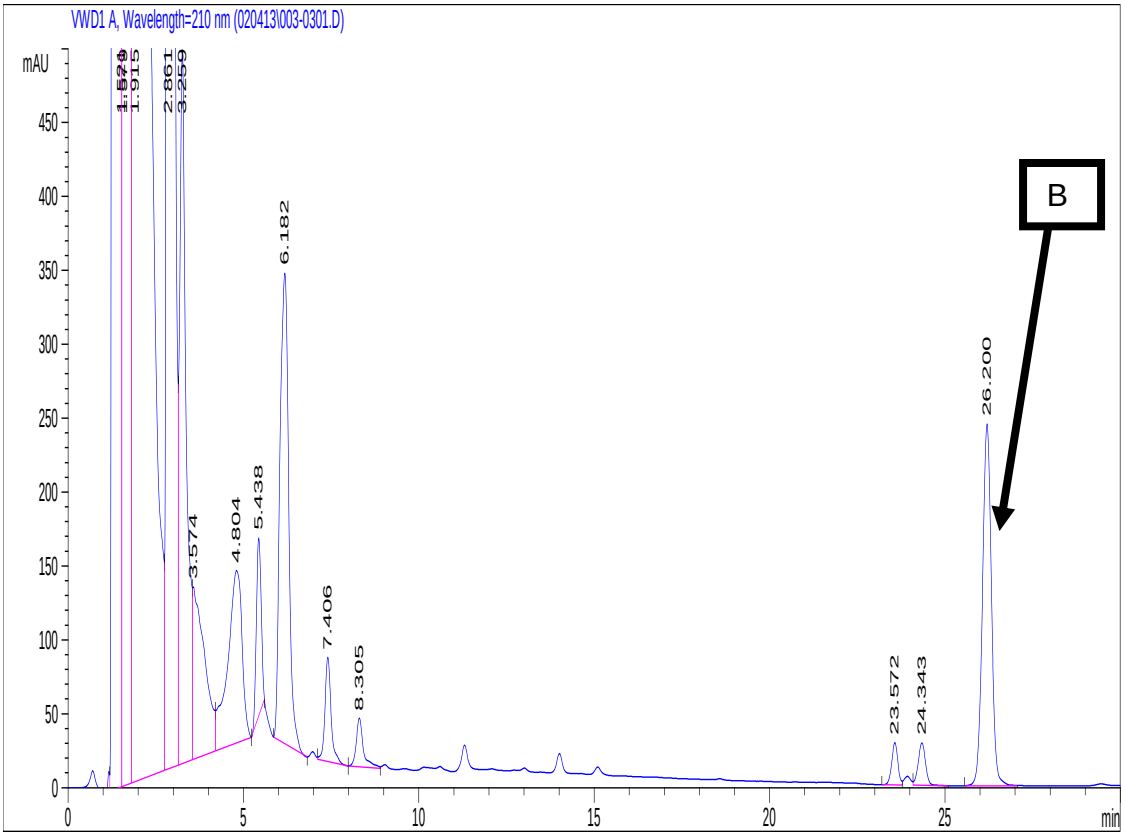

Figure 5.3 b): A chromatogram of grapefruit extract sample (B = assumed bergamottin peak)

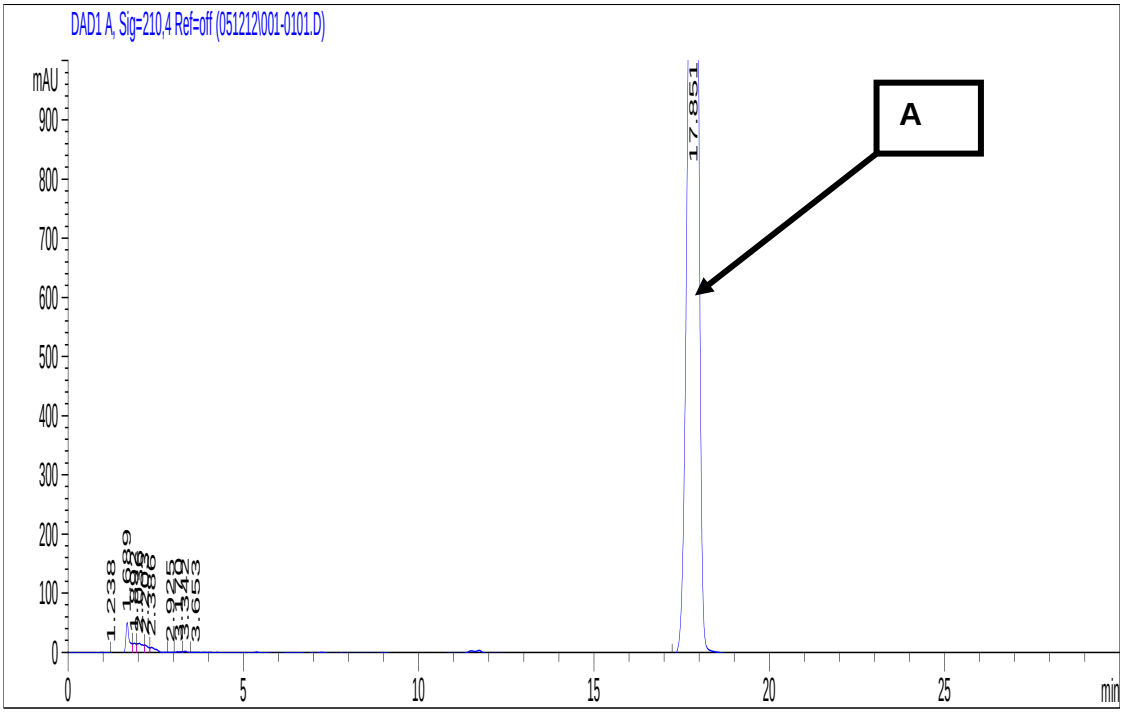

Figure 5.4 a): A chromatogram of bergamottin standard (Peak A= Bergamottin)

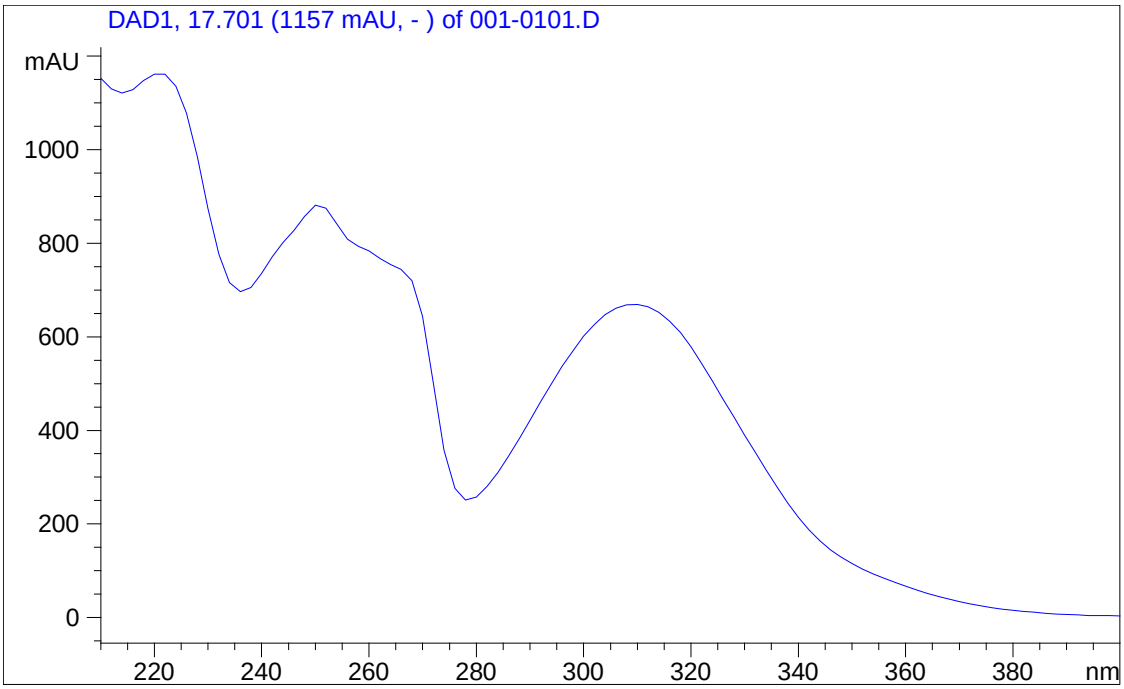

Figure 5.4 b): A UV spectrum of bergamottin standard (Peak A)

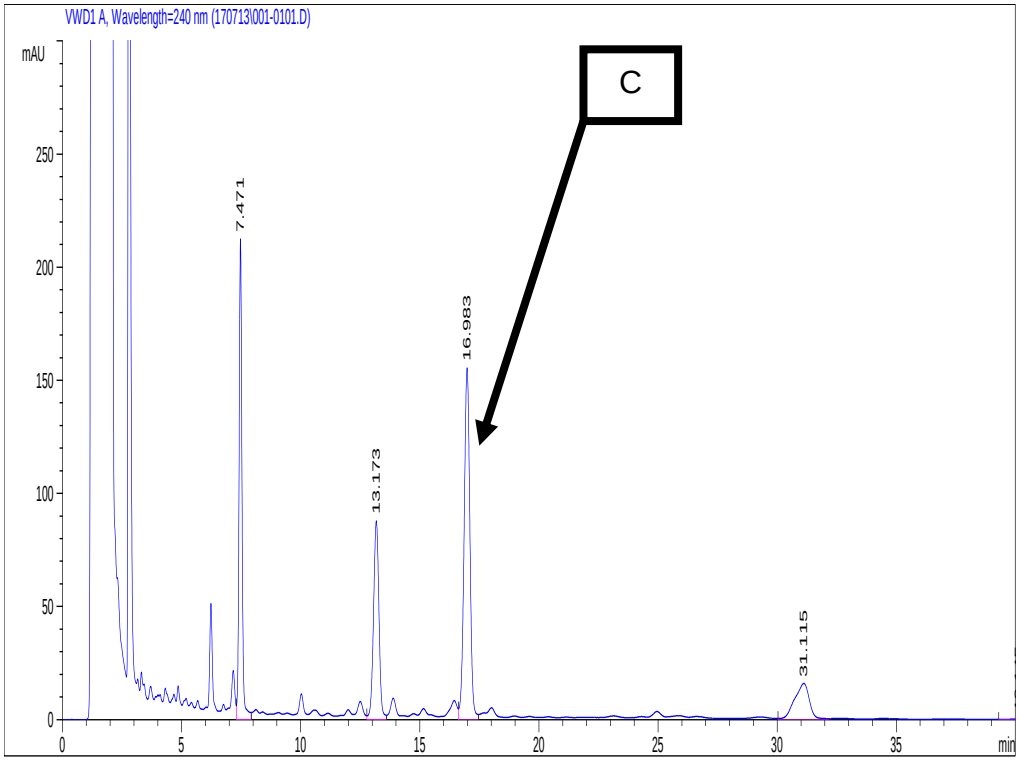

Figure 5.5 a): A chromatogram of unextracted grapefruit juice sample (C = bergamottin in grapefruit juice)

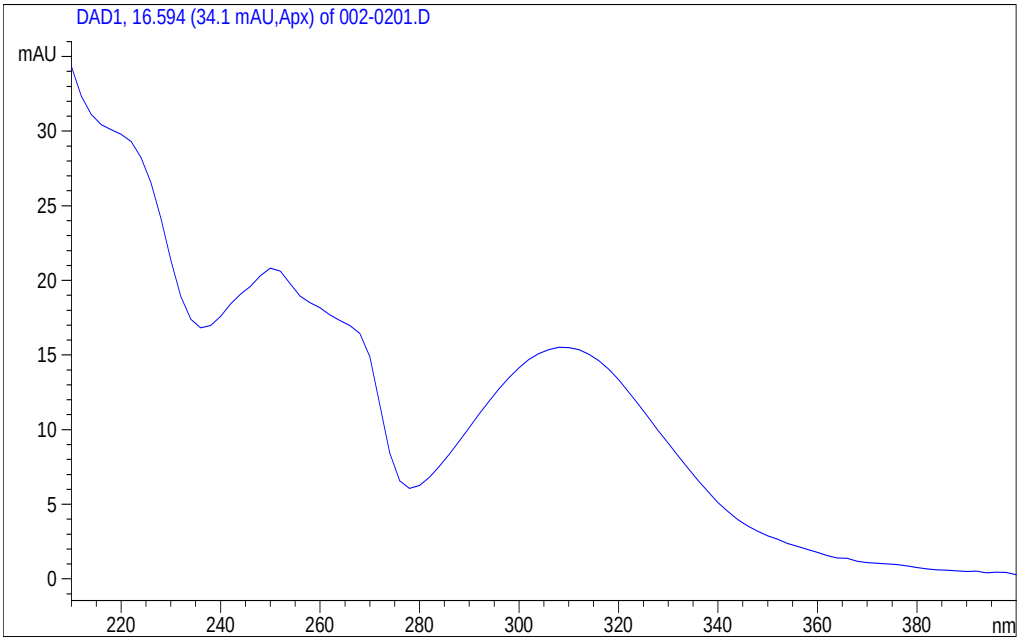

Figure 5.5 b): A chromatogram of the UV spectra of bergamottin in grapefruit juice.

5.8 COMMENT

Characterisation of grapefruit juice was successfully accomplished using high performance liquid chromatography to identify and verify the presence of bergamottin in the extract. Verification was done by using UV-spectrum and retention time.

The result after attaining the UV-spectrum verified that peak (B) with retention time of 26.200 minutes (Fig 5.3 b – page 33) in the peel extract was not bergamottin. It is believed that the extraction and rigorous evaporation process could have caused the degradation of furanocoumarins in the peel extract and hence, none of them could be identified. Therefore, evaluation of peel extract was stopped.

The unextracted grapefruit juice showed a peak at 16.983 minutes (Fig 5.5 a – page 35) and was verified by UV-spectrum as bergamottin (Figure 5.5 b – page 35).

PART II: TESTING THE MIXTURE OF PARACETAMOL AND GRAPEFRUIT JUICE FOR ENZYME INHIBITOR PROPERTIES

5.9 INTRODUCTION

The effect of the mixture of paracetamol and grapefruit juice on the activity of CYP1A2, CYP2E1 and CYP3A4 was tested *in vitro*. To each enzyme assay (sections 5.9.1 - 5.9.3), a mixture of grapefruit juice and paracetamol was added and tested in different volumes. The pure cytochrome P450 enzymes, CYP1A2, CYP2E1 and CYP3A4 were used as controls.

5.9.1 CYP1A2 assay

(a) Sample preparation

To 62.5 µl of 0.1 M HEPES potassium salt buffer (pH 7.4) was added final concentration of: 10 µl pure CYP1A2 enzymes, 0.6 mM EDTA, 30 mM $MgSO_4$ and 25 nM ethoxyresorufin. Six calibration samples were prepared by adding different volumes of resorufin to achieve the following concentration range: 0, 50, 100, 150, 200 and 250 pmol/ml. Samples were pre-incubated for 5 minutes at 37 °C, while the reaction was started by the addition of the NADP regenerating system. The total reaction volume of the sample was 250 µl. Ultimate incubation was continued for 10 minutes at 37 °C and stopped by the addition of 2.5 ml cold acetonitrile (Appendix A). All samples were prepared in duplicate.

(b) Spectrophotometric conditions

The sample was transferred to a quarts cuvette and resorufin absorption read at wavelengths of excitation of 560 nm and emission 585 nm, respectively.

5.9.2 CYP2E1 assay

(a) Sample preparation

To 120 µl of 0.1 M sodium phosphate buffer (pH 7.4) was added, final concentration of: 10 µl pure CYP2E1 enzymes and 0.848 mg/ml chlorzoxazone. Six calibration samples were prepared by adding different volumes of 6-hydroxychlorzoxazone to achieve the following concentration range: 0, 0.760, 2.155, 4.310, 6.466, and 8.621 nmol/ml. Samples were pre-incubated for 5 minutes at 37 °C, while the reaction was started by addition of the NADP regenerating system. The total reaction volume of the sample was 250 µl. Ultimate incubation was continued for 10 minutes at 37 °C and stopped with 40 µl of 0.1 M hydrochloric acid and 10 µl 4-aminoacetophenone (internal standard, Appendix A). All samples were prepared in duplicate.

(b) Sample extraction

A C_{18} solid phase extraction cartridge (1 ml) was conditioned with 1 ml HPLC grade methanol and 1 ml deionised water. The enzyme mixture was placed on the column and allowed to elute. Thereafter, the column was washed with 500 µl of deionised water. Finally, put into a fresh test tube, the compounds were eluted with 200 µl sodium phosphate buffer (pH 4.5): acetonitrile (55:45). Of the collected eluent, 50 µl was injected into the HPLC for analysis.

(c) Chromatographic conditions

An HPLC system, as described in 6.2.3, was used for analysis. Chromatographic separation of chlorzoxazone, 6-hydrochlorzoxazone and 4-aminoacetophenone was achieved by running the mobile phase at flow rate of 1 ml/min. The mobile phase consisted of solvent A, sodium phosphate buffer, pH 4.5, and solvent B, HPLC grade acetonitrile. For gradient separation, the proportion of solvent A and B was initially 70:30 for 3 minutes. This was changed to 60:40 over 1 minute, after which it was changed to 50:50 over 1 minute, and finally maintained for 5 minutes.

For re-equilibrium purposes, a post-run of 2 minutes was performed at the initial ratio of 70:30. Compounds were detected by UV at a wavelength of 280 nm.

5.9.3 CYP3A4 assay

a) Sample preparation

To 120 µl of 0.1 M sodium phosphate buffer (pH 7.4) was added, final concentration of: 10 µl pure CYP3A4 enzyme, 100 mM magnesium chloride and 0.5 mg/ml midazolam. Six calibration samples were prepared by adding different volumes of 1-hydroxymidazolam to achieve the following concentration range: 0, 1.25, 2.50, 5.00, 7.50 and 10.00 nmol/ml. Samples were pre-incubated for 5 minutes at 37 °C, while the reaction was started by the addition of the NADP regenerating system. The total reaction volume of the sample was 250 µl. Ultimate incubation was continued for 20 minutes at 37 °C and stopped with 250 µl of cold HPLC grade acetonitrile and 50 µl carbamazepine (internal standard, Appendix A). All samples were prepared in duplicate.

b) Sample extraction

The sample was alkalinised with sodium hydroxide and extracted with diethyl ether by liquid-liquid extraction. After extraction, the supernatant was removed and evaporated to dryness under a stream of nitrogen, reconstituted with 150 µl of mobile phase, and 100 µl was injected into the HPLC for analysis.

c) Chromatographic conditions

An HPLC system, as described in section 6.2.3, was used for analysis. Chromatographic separation of midazolam, 1-hydroxymidazolam and carbamazepine (internal standard) was achieved by running the mobile phase at a flow rate of 1 ml/min. The mobile phase consisted of solvent A, sodium acetate buffer, pH 4.0, and solvent B, HPLC grade acetonitrile. An isocratic mixture was prepared by mixing solvent A and B in the ratio of 55:45. Compounds were detected by UV at a wavelength of 220 nm.

5.10 RESULTS

5.10.1 Calibrations

The calibration curves for the tested enzymes are shown on the next page. For CYP1A2, the calibration curve was linear with a correlation coefficient of 0.99 and a linear regression equation of y = 0.3425x + 1.5435. The calibration curve of CYP2E1 was linear with a correlation coefficient of 0.99 and a linear regression equation of y = 0.3607x + 0.0093. Lastly, CYP3A4 calibration curve was linear with a correlation coefficient of 0.99 and a linear regression equation of y = 0.2493x + 0.011.

a) CYP1A2

Table 5.1: Resorufin calibration data

Metabolite resorufin (pmol/ml)	Absorbance
0	0
50	21.22
100	31.91
150	56.49
200	72.09
250	84.44

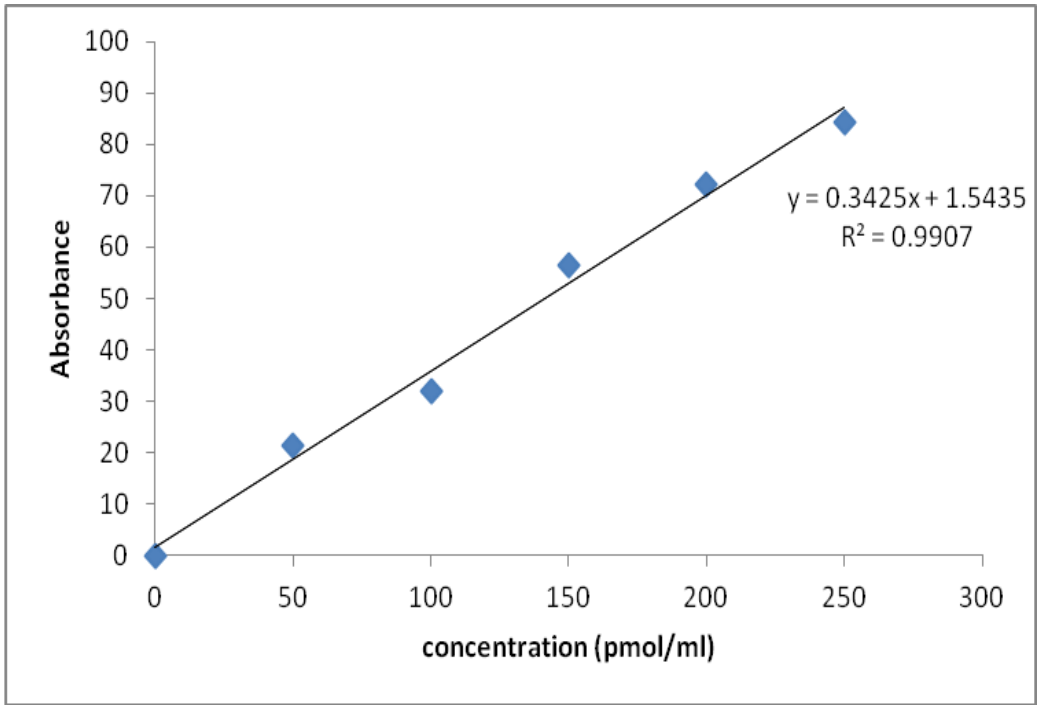

Figure 5.6: Calibration curve of resorufin concentration versus absorption

b) CYP2E1

Table 5.2: 6-hydroxychlorzoxazone calibration data

Metabolite 6-hydrochorzoxazone (nmol/ml)	ratio
0	0
0.1	0.058
0.4	0.138
0.8	0.315
1.2	0.445
1.6	0.579

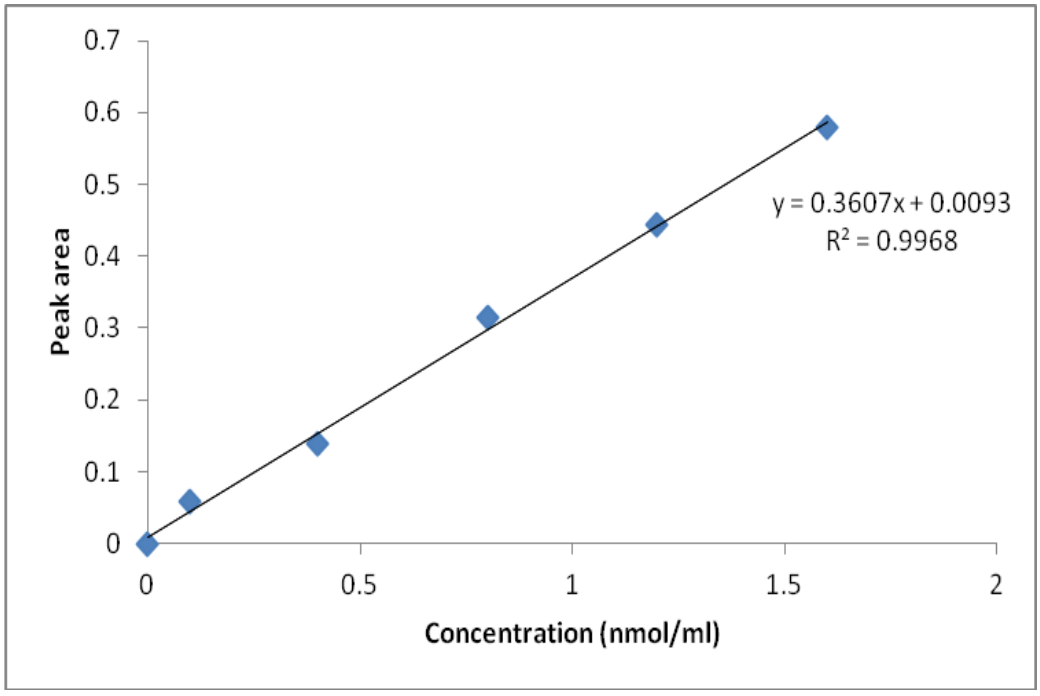

Figure 5.7: Calibration curve of 6-hydroxychlorzoxazone versus peak area

c) CYP3A4

Table 5.3: 1-hydoxymidazolam calibration data

Metabolite 1-hydroxymidazolam (pmol/ml)	ratio
0.000	0.000
0.313	0.086
0.625	0.175
1.250	0.354
1.875	0.445
2.500	0.642

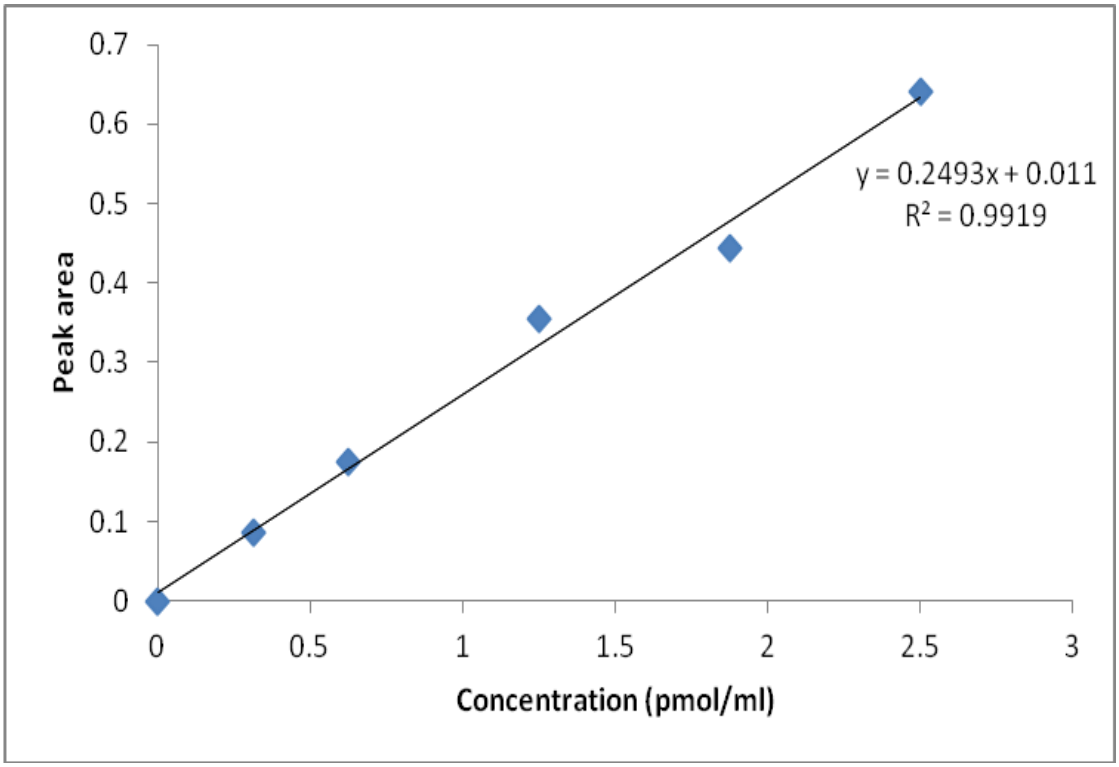

Figure 5.8: Calibration curve of 1-hydroxymidazolam versus peak area

5.10.2 Cytochrome P450 enzyme activity *in vitro*

a) CYP1A2

Table 5.4 and Figure 5.9 – page 44 show the summary of the results of the effect of paracetamol and grapefruit juice on the activity of CYP1A2 *in vitro*. The activity of CYP1A2 was inhibited as the amount of the mixture of paracetamol + grapefruit juice increased. The inhibition of CYP1A2 may be attributed to the drug-drug interaction with grapefruit juice.

Table 5.4: Summary of the effect of the mixture of paracetamol and grapefruit juice on the activity of CYP1A2 *in vitro*

PAR+GFJ (µl)	Reaction rate (nmol/min*mg protein)	SD	% Control
Control	**82.45**	**1.25**	**100**
PAR+GFJ (5 µl)	85.13	0.74	**103**
PAR+GFJ (10 µl)	30.35	0.17	**64**
PAR+GFJ (15 µl)	47.56	1.45	**56**
PAR+GFJ (20 µl)	7.638	0.15	**25**

PAR = Paracetamol; **GFJ** = Grapefruit juice; **SD** = Standard deviation

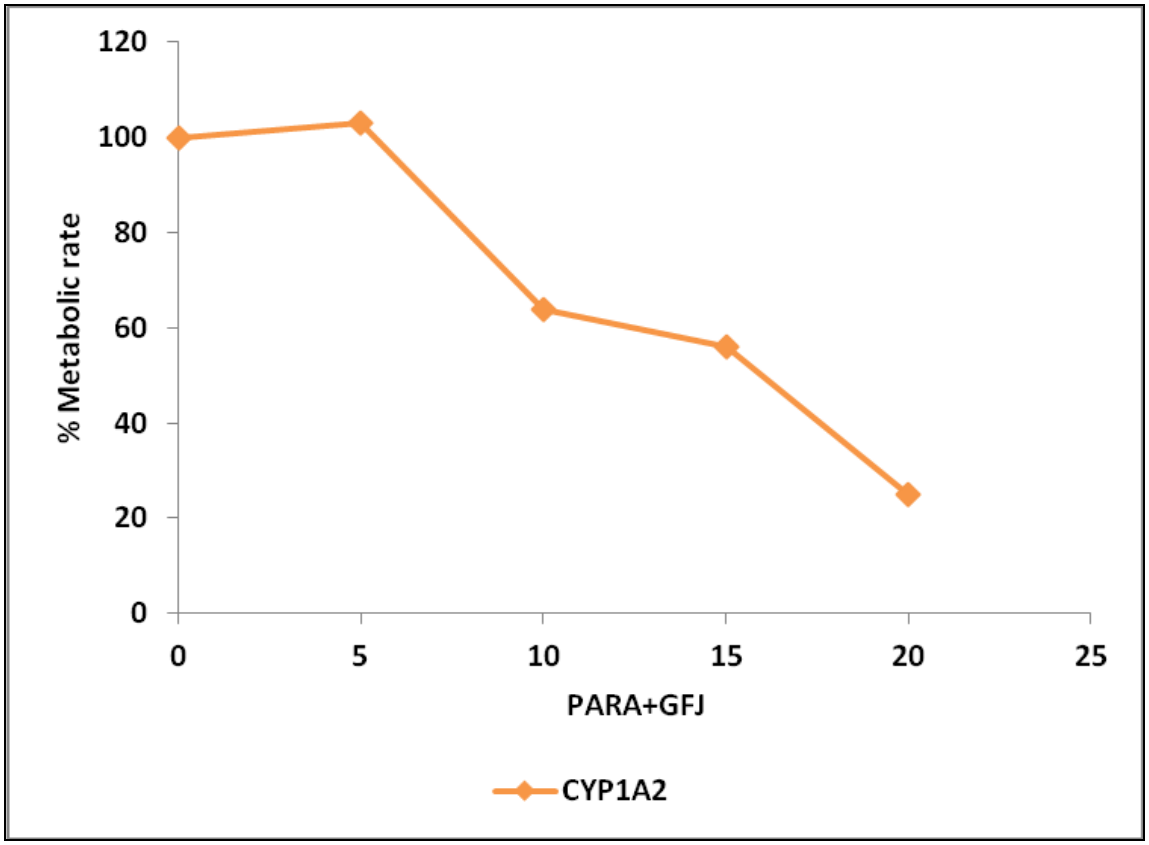

Figure 5.9: CYP1A2 activity after of PAR+GFJ *in vitro*

b) CYP2E1

Table 5.5 and Figure 5.10 – page 46, show the summary of the results of the effect of paracetamol and grapefruit juice on the activity of CYP2E1 *in vitro*. The activity of CYP2E1 was gradually inhibited as the amount of the mixture of paracetamol + grapefruit juice increased. The inhibition of CYP2E1 may be attributed to the drug-drug interaction with grapefruit juice.

Table 5.5: Summary of the effect of the mixture paracetamol and grapefruit juice on the activity of CYP2E1 *in vitro*

PAR+GFJ (µl)	Reaction rate (nmol/min*mg protein)	SD	% Control
Control	0.24	0.06	**100**
PAR+GFJ (5 µl)	0.83	0.19	**43**
PAR+GFJ (10 µl)	0.65	0.09	**34**
PAR+GFJ (15 µl)	0.56	0.11	**29**
PAR+GFJ (20 µl)	0.31	0.27	**16**

PAR = Paracetamol; **GFJ** = Grapefruit juice; **SD** = Standard deviation

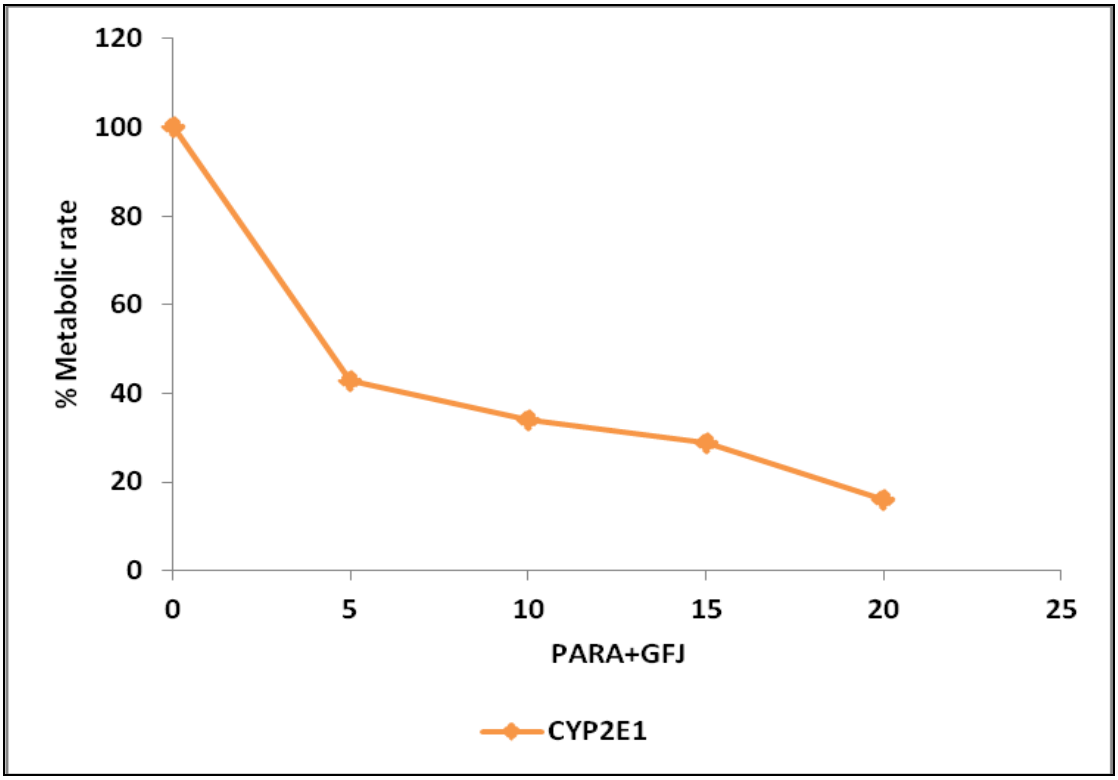

Figure 5.10: CYP 2E1 activity after of PAR+GFJ *in vitro*

c) CYP3A4

Table 5.6 and Figure 5.11 – page 47, show the summary of the results of the effect of paracetamol and grapefruit juice on the activity of CYP3A4 *in vitro*. The activity of CYP3A4 was significantly inhibited as the amount of the mixture of paracetamol + grapefruit juice increased. The inhibition of CYP3A4 may be attributed to the drug-drug interaction with grapefruit juice.

Table 5.6: Summary of the effect of the mixture of paracetamol + grapefruit juice on the activity of CYP3A4 *in vitro*

PAR+GFJ (µl)	Reaction rate (nmol/min*mg protein)	SD	% Control
Control	**88.50**	**0.00**	**100**
PAR+GFJ (5 µl)	46.83	10.61	**56**
PAR+GFJ (10 µl)	19.17	6.36	**19**
PAR+GFJ (15 µl)	16.42	4.36	**19**
PAR+GFJ (20 µl)	7.42	1.77	**9**

PAR = Paracetamol; **GFJ** = Grapefruit juice; **SD** = Standard deviation

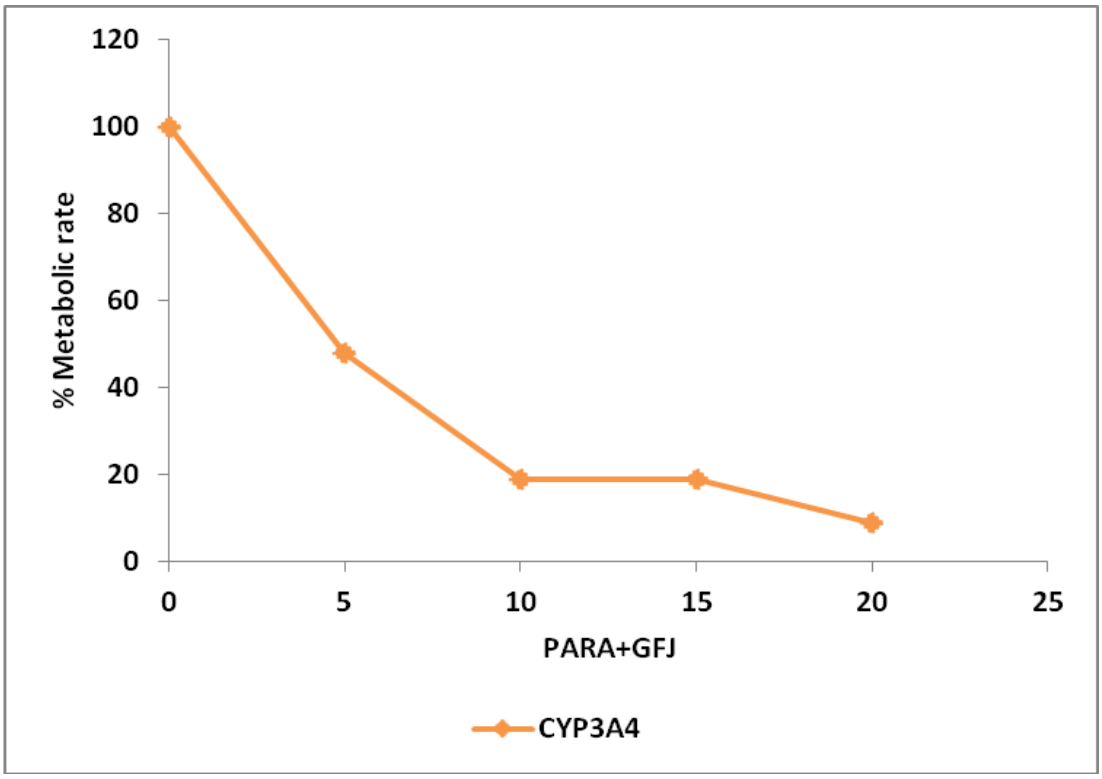

Figure 5.11: CYP3A4 activity after of PAR+GFJ *in vitro*

Table 5.7: Summary of the effect of the mixture of paracetamol + grapefruit juice on the activity of CYP1A2, CYP2E1 and CYP3A4 (Percentage control)

PAR+GFJ (µl)	CYP1A2	CYP2E1	CYP3A4
0	100	100	100
5	103	43	56
10	56	34	19
15	64	29	19
20	25	16	9

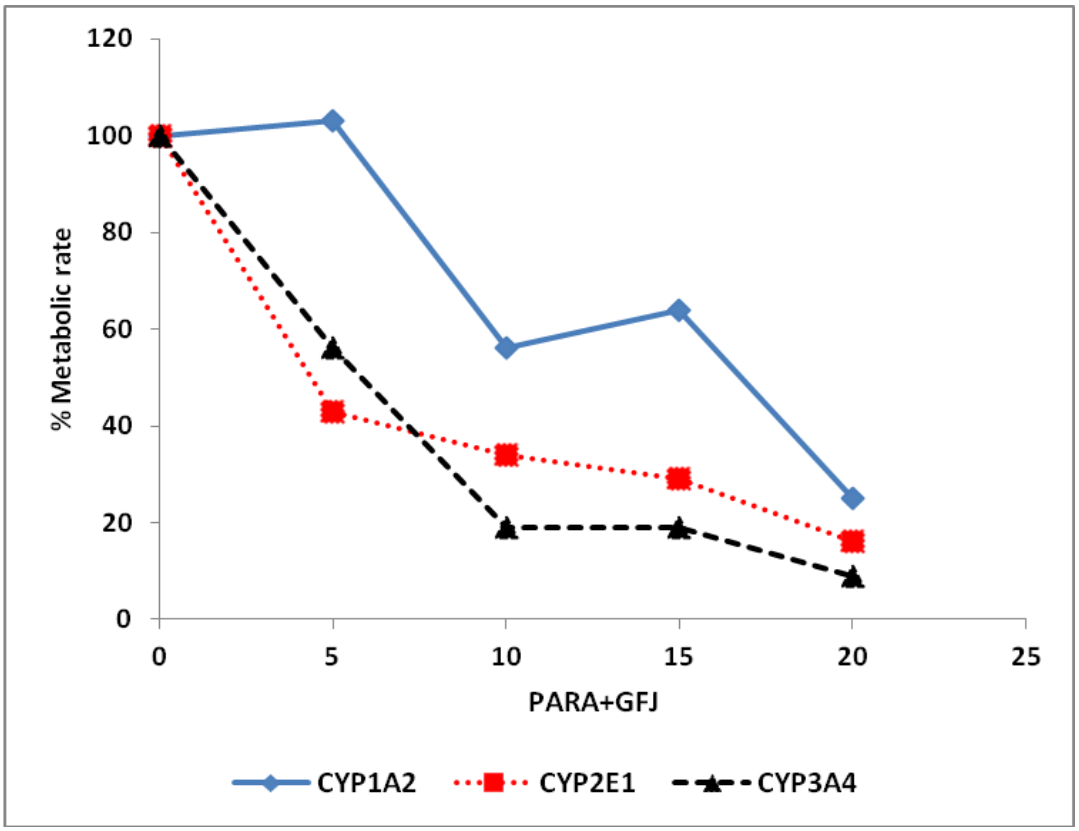

Figure 5.12: Summary of the activity of all enzymes

5.11 COMMENT

When a mixture of paracetamol and grapefruit juice was added to the cytochrome P450 assays respectively, a significant enzyme inhibition was demonstrated with CYP3A4 as the most inhibited, followed by CYP2E1 and CYP1A2. This type of enzyme inhibition is very important due to the fact that it interferes with the primary mechanism of paracetamol hepatotoxicity.

Therefore, grapefruit juice could be used for prevention of paracetamol hepatotoxicity by combining it with the paracetamol preparations such that an ingestion of a potentially hepatotoxic dose of paracetamol with appropriate enzyme inhibitors would provide protection against hepatotoxicity by inhibiting the activation of a toxic metabolite. This will be further confirmed in the following animal study where a hepatotoxic dose of paracetamol, followed by grapefruit juice, will be given to rats (Chapter 7).

CHAPTER 6

A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR THE DETERMINATION OF PARACETAMOL IN PLASMA.

6.0 SUMMARY

A high performance liquid chromatography (HPLC) method for the determination of paracetamol in plasma was developed. It involved protein precipitation of 50 µl of paracetamol spiked plasma with zinc sulphate, followed by centrifugation. The supernatant was directly injected into the HPLC. Elution of the sample was done with a mobile phase of 0.01% trifluoroacetic acid in distilled water: acetonitrile (75: 25, v/v) over a Phenomenex C_{18} (4.60 x 250 mm) 5 µ analytical column at 1 ml/min. 4-Aminoacetophenone was used as the internal standard. Under these conditions paracetamol and 4-aminoacetophenone eluted at retention times of 4.2 minutes and 6.2 minutes, respectively. The average calibration curve (0 - 20 µg/ml) was linear with a regression equation of y = 0.0603x + 0.089, and regression coefficient of r^2 = 0.9957. This method was used to measure paracetamol concentrations in rat plasma.

6.1 INTRODUCTION

In this chapter, a high performance liquid chromatography method for the determination of paracetamol in plasma is described.

6.2 MATERIALS AND METHODS

6.2.1 Apparatus

Precision and analytical balances (SPB 52 and SPB 31, Scaltec Instruments, Goettingen, Germany) were used to weigh gram and milligram amounts of reagents and drug standards, respectively. A vortex mixer (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY, USA) and a micro centrifuge (Minispin, Epperndorf, Hamburg, Germany) were used for mixing and quick spinning of the samples.

6.2.2 Reagents and chemicals

The analytical standards of paracetamol (acetaminophen), 4-aminoacetophenone, caffeine and propionamedophenol were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). HPLC grade acetonitrile, methanol and ethyl acetate were purchased from Honeywell Burdick and Jackson (Muskegon, MI, USA). Merck Laboratories (Darmstadt, Germany) supplied trifluoroacetic acid, acetic acid, potassium hydroxide and potassium dihydrogen phosphate. Zinc sulphate was purchased from BDH Laboratory, England.

6.2.3 Chromatographic system

The HPLC system was an Agilent, Hewlett Packard 1100 series, equipped with a 1260 Infinity quaternary pump (Waldbronn, Germany) with a 1260 Infinity degasser attached to a G1313A autosampler (Waldbronn, Germany), and a G1314A UV wavelength detector (Tokyo, Japan). Data were collected using Chemstation software.

6.3 PRELIMINARY EXPERIMENTS

6.3.1 Selection of a mobile phase

A mobile phase of 1% acetic acid added to potassium dihydrogen phosphate (KH_2PO_4) buffer (solvent A) and methanol (solvent B) (95:5, v/v) was tried. The internal standard did not elute and paracetamol eluted very late. The same mobile phase was used with different gradient conditions, but with no success as paracetamol did not elute. It was decided to move on to another method.

A method which was developed and used in the similar study at the Department of Pharmacology was tried. A mobile phase of 0.01% trifluoroacetic acid added to distilled water (solvent A) and 100% acetonitrile (solvent B) was tried out initially using gradient conditions, but the peaks were poorly resolved. Isocratic conditions for the above mobile phase were tried out (80:20, v/v). Finally, paracetamol and 4-aminoacetophenone were separated and all peaks were sharp and resolved. Therefore, this mobile phase was selected for subsequent experiments.

6.3.2 Preparation of standard solutions

A stock solution of paracetamol (1 mg/ml) was prepared by dissolving 1 mg of paracetamol in 1 ml of distilled water. From the stock, the following standard solutions were prepared by further dilution with distilled water: 2, 10, 30 and 30 and 40 µg/ml.

For the internal standard, 4-aminoacetoaminophenone, a stock solution (1 mg/ml) was prepared in distilled water by dissolving 1 mg of 4-aminoacetophenone in 1 ml distilled water, and this was further diluted with distilled water to prepare a working solution (50 µg/ml) Thereafter, calibration samples were prepared by spiking 50 µl of plasma with appropriate volumes of the standard solutions of paracetamol to obtain final concentrations of 1, 5, 10, 15 and 20 µg/ml.

6.3.3 Selection of internal standard

The following drugs were tested as a possible internal standard: caffeine, theophylline, propionamidophenol and 4-aminoacetophenone. Eventually, 4-aminoacetophenone was selected as the appropriate internal standard, as it showed no interference with paracetamol and it eluted very well (Figure 6.1 b – page 56).

6.3.4 Sample Preparation

6.3.4.1 Liquid-liquid extraction

A liquid-liquid extraction using ethyl acetate (Campanero *et al.,* 1999) was tried. The results were poor as the plasma peaks interfered with peaks of interest. This liquid-liquid extraction method was therefore abandoned.

6.3.4.2 Extraction by centrifugation with zinc sulphate

Based on the method which was developed and used for similar studies at the Department of Pharmacology, zinc sulphate was used to precipitate proteins.

To plasma spiked with paracetamol, 30 µl of 2.5% zinc sulphate was added, the sample was vortexed for 15 seconds and centrifuged for 10 minutes at 7026 *g* (13400 r.p.m). The supernatant was directly injected into the HPLC. Paracetamol was well extracted.

6.4 FINAL CONDITIONS

6.4.1 Sample preparation

To 50 µl of plasma spiked with paracetamol, 50 µl of internal standard was added, after which the sample was vortexed for 10 seconds. Then, 30 µl of 2.5 % zinc sulphate was added to the sample in order to precipitate the proteins in the solution. The sample was vortexed for 15 seconds and centrifuged at 7026 *g* (13400 r.p.m) for 10 minutes. The supernatant was directly injected into the HPLC.

6.4.2 Chromatographic conditions

Separation of paracetamol and 4-aminoacetophenone was achieved by running the mobile phase at a flow rate of 1 ml/min. over a Phenomenex® C_{18} (4.60 x 250 mm) 5 µ analytical column, coupled to a Phenomenex® SecurityGuard™ C_{18} (4 x 3 mm) guard column (Torrance, CA, USA). Compounds were detected by UV at a wavelength of 240 nm. The mobile phase consisted of 0.01% trifluoroacetic acid in distilled water (solvent A) and 100% HPLC grade acetonitrile (solvent B) run isocratically (80:20 v/v). The run time for the method was 10 minutes.

6.5 METHOD VALIDATION

6.5.1 Linearity/ Calibration

The linearity of an analytical method indicates its ability to obtain the response directly proportional to the concentration of the analyte in the sample within a definite range. Here, calibration was performed by analysing plasma samples spiked with paracetamol at a concentration range of 1, 5, 10, 15 and 20 µg/ml, on different days for 5 days. Calibration curves were created by plotting the peak area ration of paracetamol to 4-aminoacetophenone, versus the concentration of paracetamol. The curves were analysed by linear regression using the GraphPad Instat program.

6.5.2 Accuracy of the assay

The accuracy of a bioanalytical method is a measure of the systematic error or bias and is defined as the agreement between the measured value and the true value (Tesfu, 2004). For this study, accuracy was tested at 1, 10 and 20 µg/ml, and the test was repeated five times. Thereafter, accuracy values were derived from a calibration curve. The results obtained were used to calculate the coefficient of variation using the following formula: (standard deviation / mean) x 100.

6.5.3 Stability

The stability of an analyte is often critical in biological samples even over a short period of time. Degradation is not unusual even when all precautions are taken to avoid specifically known stability challenges of the analyte (e.g. light sensitivity). It is therefore important to verify that there is no sample degradation between the time of collection of the sample and analysis that would compromise the result of the study. Stability evaluation is done to show that the concentration of the analyte at the time of analysis corresponds to the concentration of an analyte at the time of sampling (Hartmann *et al.* (1998). The stability of paracetamol in plasma was tested at three concentrations, namely, 1, 10 and 20 µg/ml. The samples were stored at room temperature, 4°C and -20°C and analysed after 0, 8, 12 and 24 hours (short-term stability), and 14, 30 and 60 days (long-term stability).

6.5.4 APPLICATION OF THE METHOD

The method was tested by analysing the plasma of rats, after oral administration of paracetamol.

6.6 RESULTS

6.6.1 Chromatographic performance

Figures 6.1.a) - 6.1 d – page 56-57) are the representative chromatograms for the standard solutions, blank plasma and spiked plasma. From the standard solutions it was observed that the peaks were well resolved, with paracetamol eluting at 3.755 minutes and the internal standard at 6.303 minutes. The blank plasma showed no interference from plasma. This observation was also clear in the spiked plasma samples. Retention times for plasma spiked with paracetamol and 4'aminoacetophenone were 4.247 minutes and 6.246 minutes respectively. The total run time was 10 minutes.

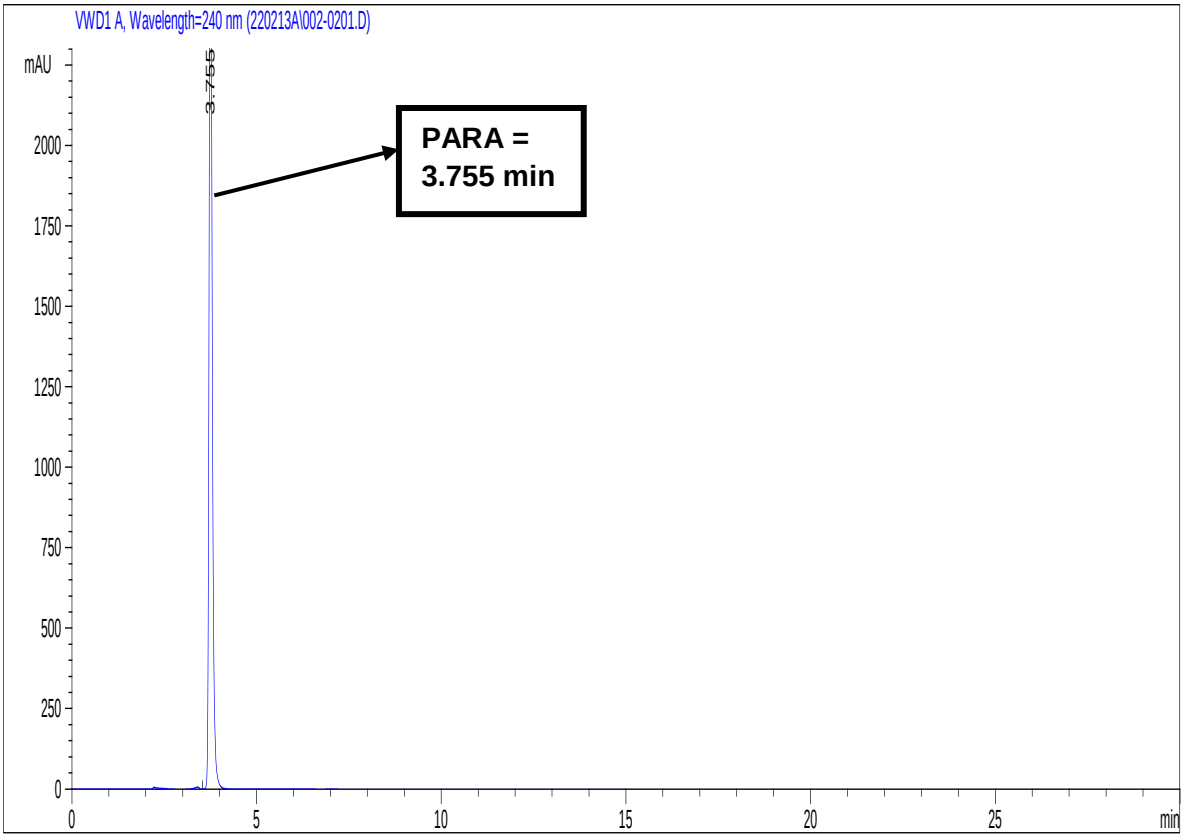

Figure 6.1 a): Chromatogram of paracetamol in water

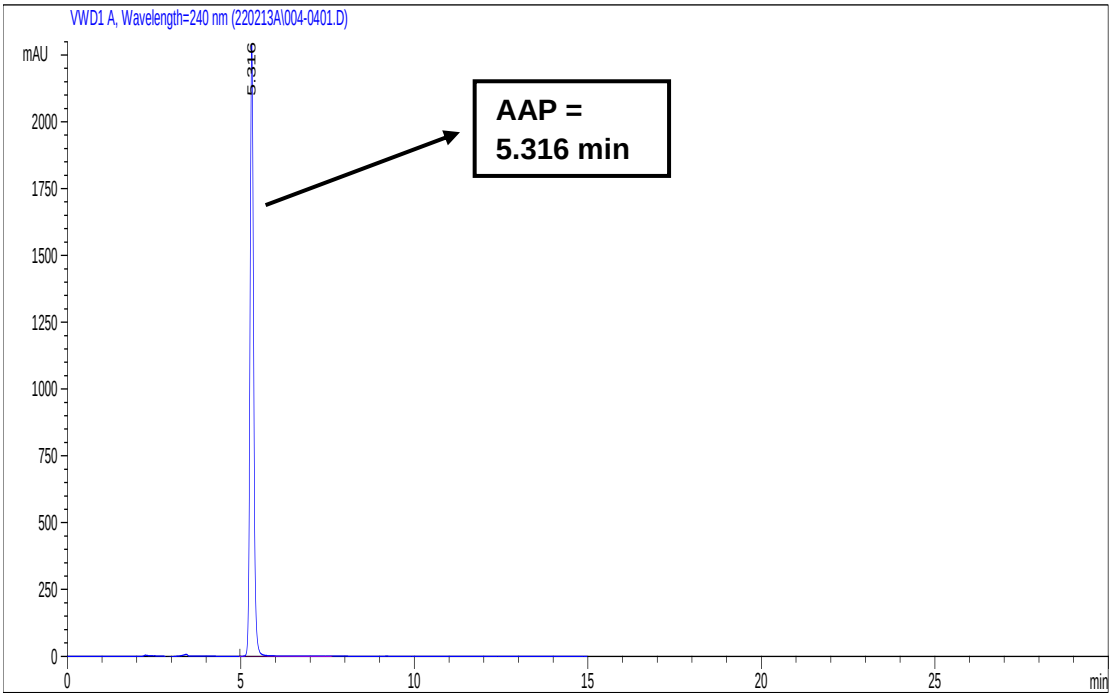

Figure 6.1 b): Chromatogram of 4-aminoacetophenone in water

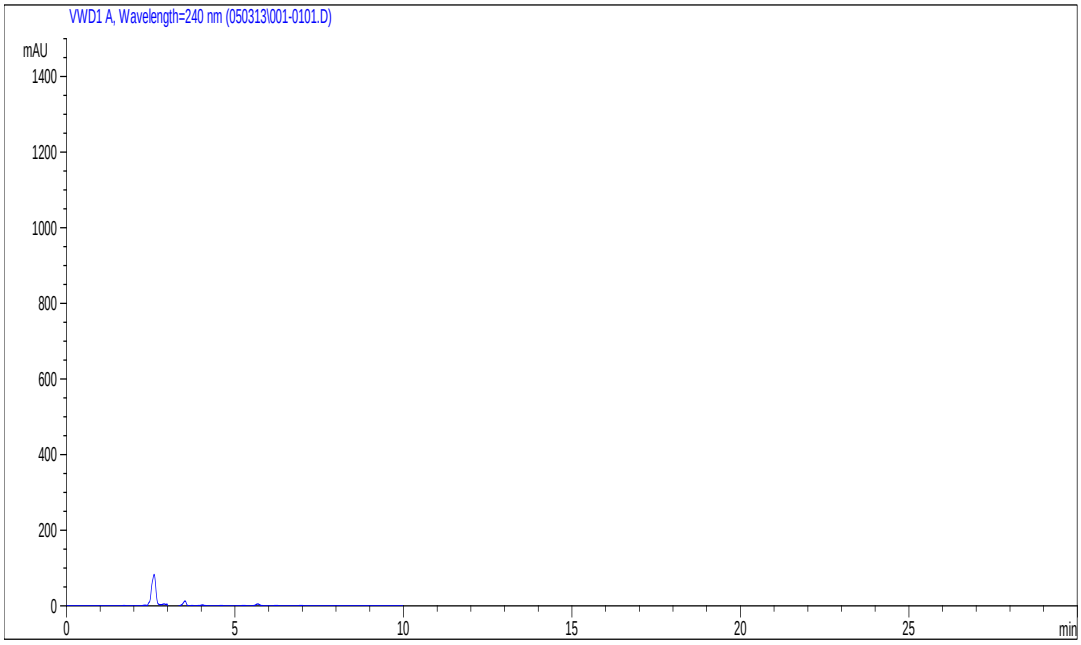

Figure 6.1 c): Chromatogram of a blank plasma sample

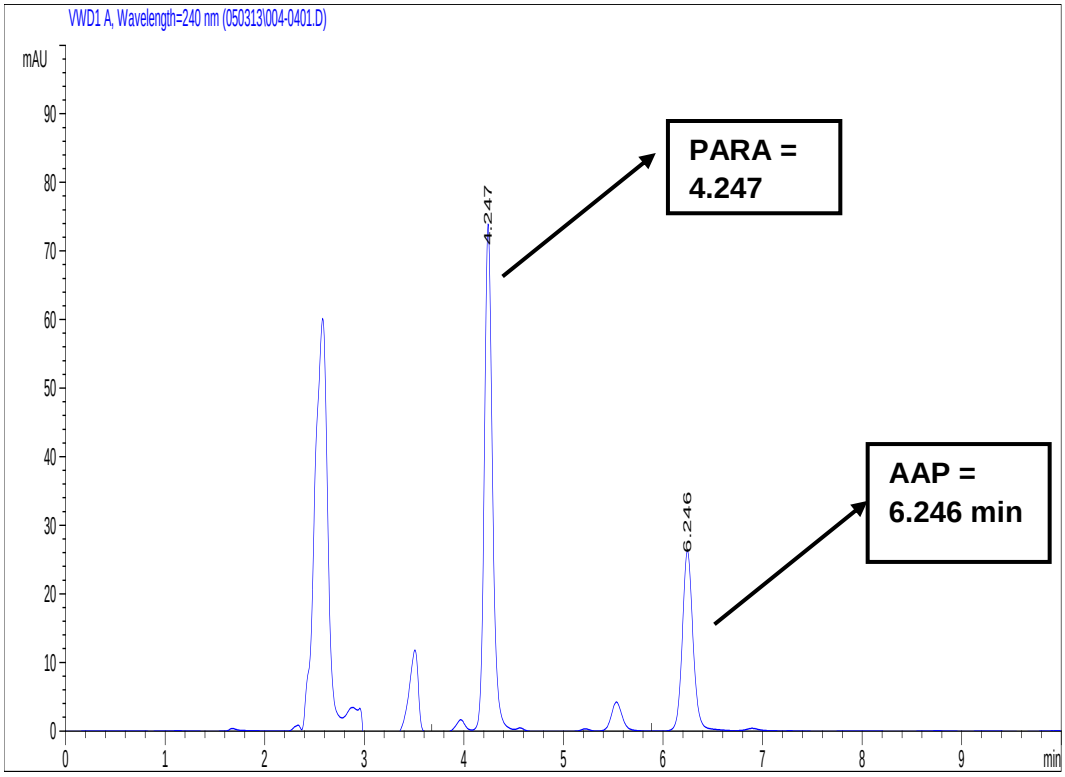

Figure 6.1.d): Chromatogram of a plasma sample spiked with internal standard and 10 ug/ml paracetamol

6.6.2 Calibration curve

The summary data for the calibration over five days is shown in Table 6.1, while the average calibration curve is shown in Figure 6.2 (page 59) (see Appendix B for individual calibrations). The calibration curve was linear with a regression equation of y = 0.0603x + 0.089 and a correlation coefficient of r^2 = 0.9957. The coefficient of variation (CV %) was less than 15%.

Table 6.1: HPLC calibration for paracetamol over 5 days using ratios of area paracetamol/area internal standard

	PARA Concentration				
Day	**1 µg/ml**	**5 µg/ml**	**10 µg/ml**	**15 µg/ml**	**20 µg/ml**
1	0.11	0.44	0.78	1.06	1.38
2	0.13	0.42	0.76	0.91	1.07
3	0.14	0.39	0.74	1.06	1.34
4	0.13	0.35	0.74	0.99	1.32
5	0.11	0.41	0.71	0.90	1.30
Mean	**0.12**	**0.40**	**0.74**	**0.98**	**1.28**
SD	0.02	0.03	0.03	0.07	0.12
% CV	12	8	3	8	10

SD = standard deviation; **CV %** = coefficient of variation

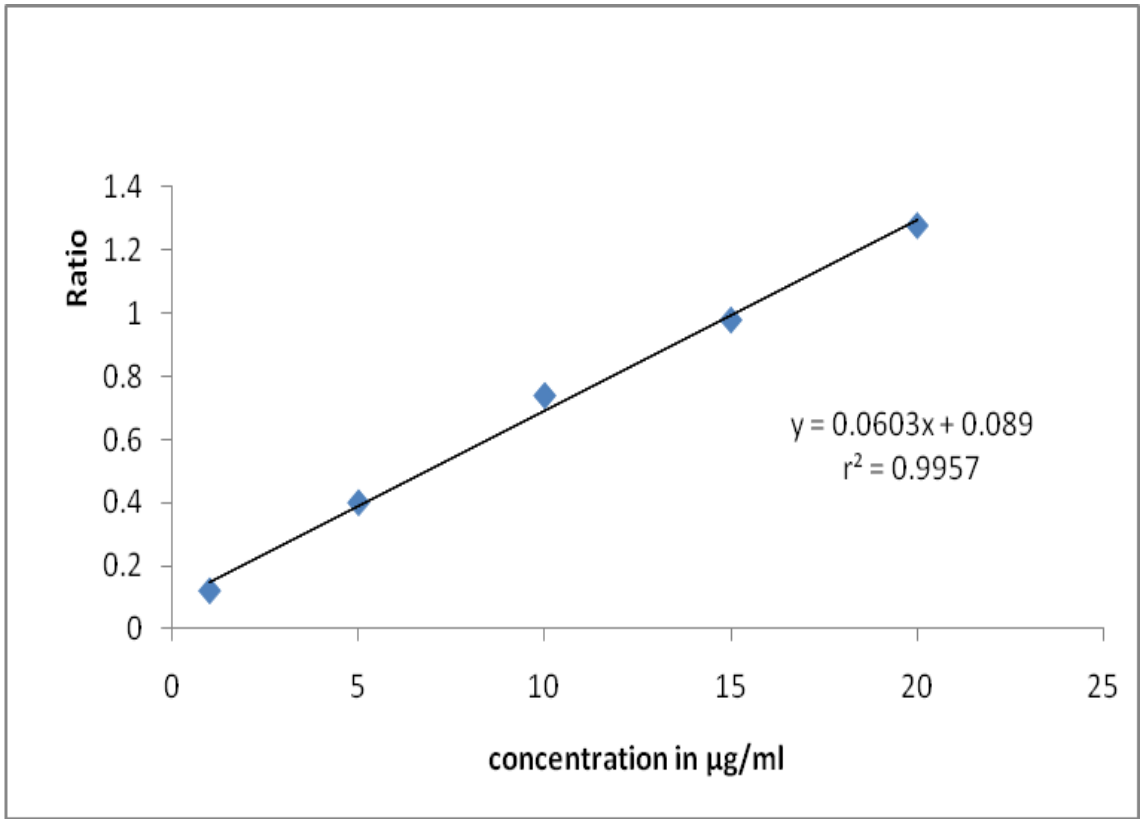

Figure 6.2: 5 Day calibration curve of paracetamol

6.6.3 Accuracy

According to the data in Table 6.2 – page 60, accuracy was 103%, 105% and 96% at 1, 10 and 20 µg/ml, respectively (view Appendix C for fully detailed accuracy data tables). The CV % was <10 for all the samples.

6.6.4 Stability

From the stability results (Tables 6.3 a – page 60) and 6.3 b – page 61), paracetamol proved to be very stable at all concentrations, temperatures and over time.

Table 6.2: Summary of accuracy data of paracetamol in plasma at 1, 10 and 20 µg/ml

Conc. prepared (n = 5) (µg/ml)	Conc. measured (µg/ml)	Mean accuracy (%)	SD	CV%
1	1.03	**103**	0.1	9.0
10	10.51	**105**	0.3	2.8
20	19.10	**96**	0.2	1.0

SD = standard deviation; **CV %** = coefficient of variation

Table 6.3 a): Summary of long-term stability data of paracetamol in plasma at -20°C

Temp	Conc. Prep (µg/ml)	14 days		30 days		60 days	
		Conc. Measd.	% Stab.	Conc. Measd.	% Stab	Conc. Measd.	%Stab
-20°C	1 µg/ml	0.93 ± 0.01	**93**	0.54 ± 0.0	**54**	0.57± 0.02	**57**
	10 µg/ml	10.92 ± 0.06	**109**	10.66 ± 0.02	**107**	8.42 ± 0.38	**84**
	20 µg/ml	18.14 ± 0.3	**92**	19.38 ± 0.5	**97**	18.84 ± 1.11	**94**

Table 6.3 b): Summary of short-term stability data of paracetamol in plasma 1, 10 and 20 µg/ml at room temperature, 4ºC, -20ºC

Temp	Conc prep (µg/ml)	0 hours		8 hours		12 hours		24 hours	
		conc. measd.	% Stab.	conc. measd.	% Stab.	conc. measd.	% Stab.	conc. measd.	%Stab.
Room Temp	1 µg/ml	0.98±0.04	**98**	0.95±0.01	**95**	0.96±0.05	**96**	0.96±0.03	**96**
	10 µg/ml	10.18±0.3	**102**	9.60±0.21	**96**	9.81±0.26	**98**	10.00±0.05	**100**
	20 µg/ml	18.54±0.8	**93**	18.89±0.52	**94**	18.62±0.51	**93**	19.09±1.14	**95**
4^0C	1 µg/ml	_____		0.92±0.01	**92**	1.04±0.2	**104**	0.94±0.02	**94**
	10 µg/ml	_____		11.28±0.5	**113**	11.84±0.2	**118**	11.22±0.06	**112**
	20 µg/ml	_____		20.01±1.03	**100**	20.72±0.7	**104**	18.51±0.4	**93**
-20^0C	1 µg/ml	_____		0.94±0.01	**94**	0.93±0.2	**93**	0.91±0.01	**91**
	10 µg/ml	_____		11.21±0.2	**112**	11.48±0.3	**115**	10.98±0.3	**110**
	20 µg/ml	_____		19.51±0.6	**98**	20.72±1.2	**99**	19.00±0.8	**95**

6.6.5 Application of the method

Figures 6.3 a) and 6.3 b – page 66) are the representative chromatograms for the blank rat plasma and paracetamol in rat plasma at 24 hours after 1725 mg/kg administration, respectively. Paracetamol concentration was calculated as 12.75 µg/ml.

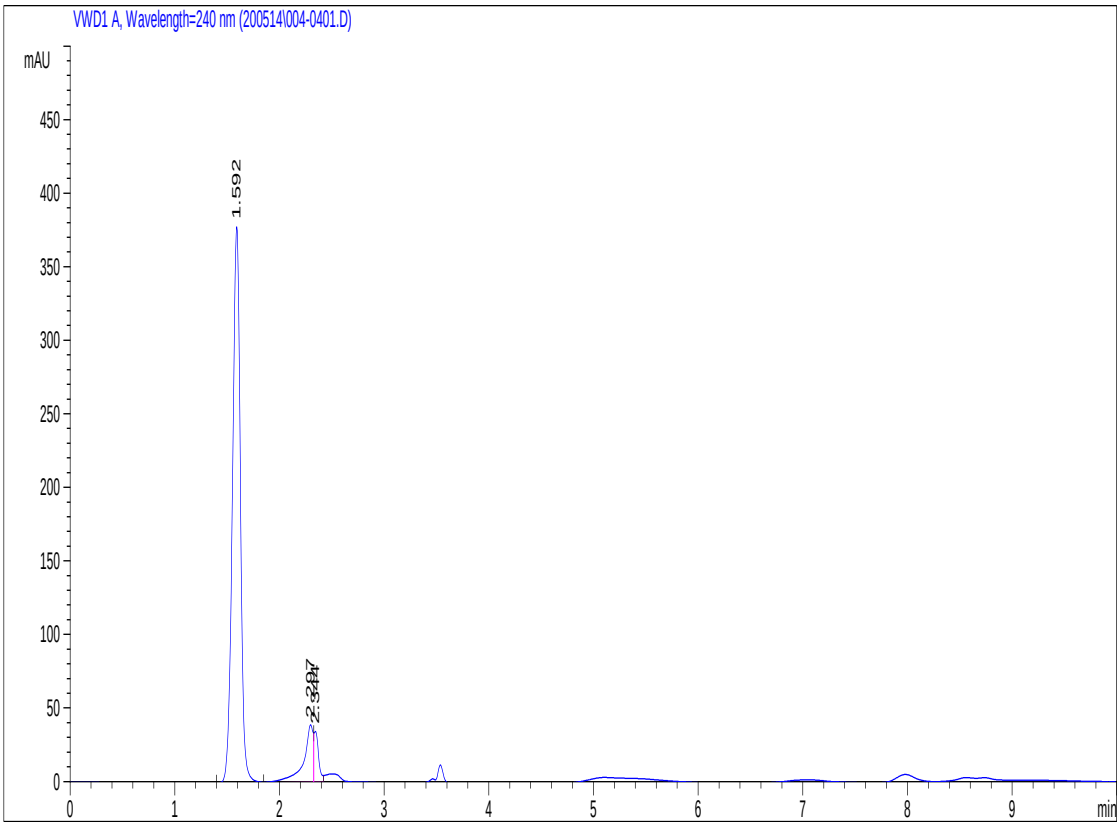

Figure 6.3 a): Chromatogram of blank rat plasma

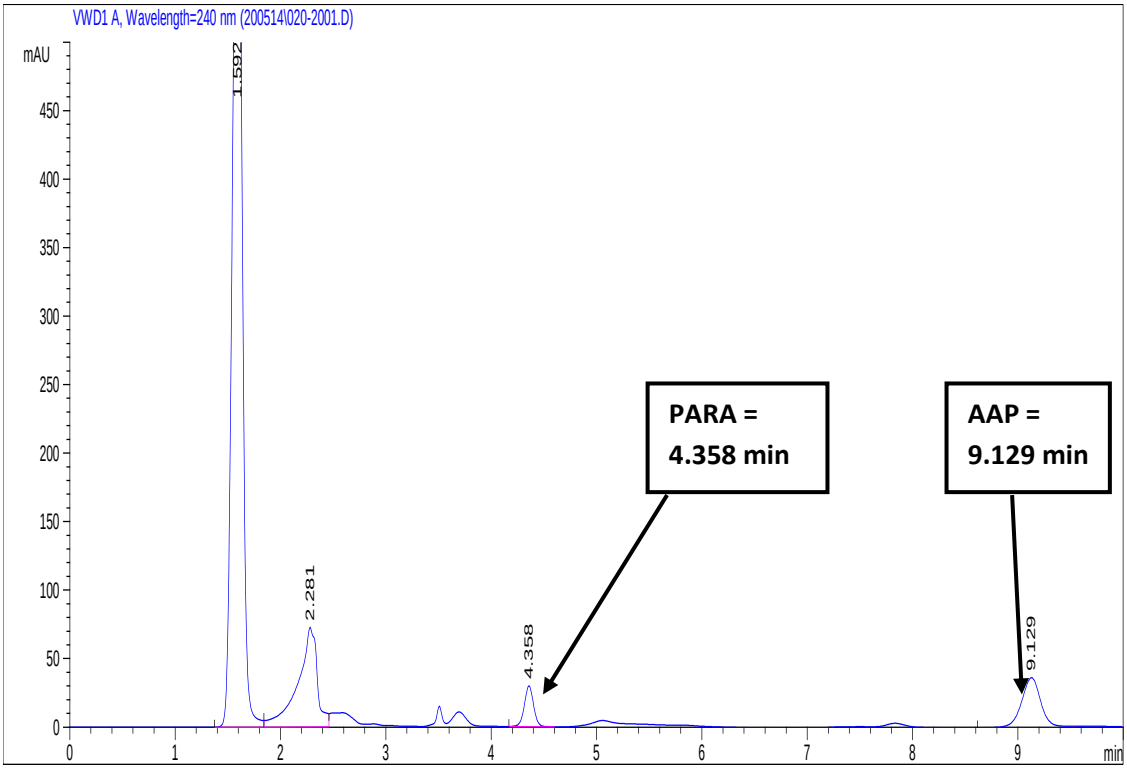

Figure 6.3 b): Chromatogram of paracetamol in rat plasma at 24 hours after 1725 mg/kg treatment.

6.7 COMMENT

An accurate and effective HPLC method for measurement of paracetamol in plasma was developed. Sharp, symmetrical peaks of paracetamol and the internal standard were observed in the chromatograms produced (Figure 6.1 d – page 57). The average calibration curve (Figure 6.2 - page 59) was linear with a regression equation of y = 0.0603x + 0.089 and a correlation coefficient of r^2 = 0.9957; in addition the CV% was ± 8.2% (Table 6.1 – page 58). Accuracy at 1, 10 and 20 µg/ml was 103%, 105% and 96%, respectively (Table 6.2 – page 60). The method was used to measure paracetamol concentration in rats (Figure 6.3 b - page 63).

Paracetamol is a readily available drug and the source of many overdose cases worldwide. This method can be used for measuring the concentration of paracetamol in patients who have or are suspected to have overdosed with paracetamol.

CHAPTER 7

EVALUATION OF GRAPEFRUIT JUICE FOR THE PREVENTION OF PARACETAMOL-INDUCED HEPATOTOXICITY IN A RAT MODEL

7.0 SUMMARY

Here, grapefruit juice was evaluated for prevention of paracetamol-induced hepatotoxicity. Ethical approval was obtained, and Sprague Dawley rats were used. The rats were divided into 30 groups. The groups were treated with saline, paracetamol, bergamottin, grapefruit juice, paracetamol+ bergamottin (low and high doses) and paracetamol+ grapefruit juice (low and high doses), respectively with a one-off oral dose. The rats were then sacrificed after 24, 48 and 72 hours. Haematology tests, liver function tests, histopathology and paracetamol level analysis were done.

Administration of a toxic dose of paracetamol (1725 mg/kg) elevated the liver enzymes (ALT and AST) significantly when compared to the control group. Upon physical observation of the liver during surgery, the livers of the rats at 48 hours exhibited moderate to severe hepatic injury. The haematology results, especially platelet count, revealed a very low platelet count at 48 hours which is indicative of thrombocytopenia. Paracetamol plasma concentrations in rats treated with paracetamol only were higher at 24 hours and there was a slight decrease at 48 and 72 hours due to drug metabolism.

However, the hepatic injuries of the rats treated with paracetamol only were antagonised by co-administration of paracetamol with grapefruit juice and also co-administration with bergamottin. The significant decrease in liver enzymes (ALT and AST) and the slight increase in platelet count at 72 hours revealed that grapefruit juice and bergamottin may have hepatoprotective effects against paracetamol-induced hepatotoxicity.

Similarly, the concentration of paracetamol in the plasma of rats treated with paracetamol + grapefruit juice/bergamottin were lowered as opposed to when paracetamol was administered alone.

The results of this study indicate that grapefruit juice can be used as an effective hepatoprotective agent against paracetamol-induced hepatotoxicity.

7.1 INTRODUCTION

This chapter describes the investigations performed in order to establish whether grapefruit juice can be used to prevent paracetamol-induced hepatotoxicity by observing the changes in the full blood count, liver function and plasma concentrations.

It is envisioned that addition of a small yet significant amount of an enzyme inhibitor will help with prevention of paracetamol hepatotoxicity during an overdose.

7.2 MATERIALS AND METHODS

7.2.1 Apparatus

Precision and analytical balances (Scaltec Instruments, Goettingen, Germany) were used to weigh rats and gram and milligram amounts of reagents and drug standards, respectively. Feeding needles (16 G-3", curved 3 mm ball; Poppers and sons, Inc., NY, USA) were used for oral gavage, and a dissection kit (Lasec S.A., Bloemfontein, South Africa) was used to perform rat surgeries. A vortex mixer (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY, USA) and a micro centrifuge (Minispin, Epperndorf, Hamburg, Germany) were used for mixing and quick spinning of the rat plasma samples. An Agilent high performance liquid chromatography (HPLC, Hewlett Packard model 1100) equipped with an autosampler (Waldbronn, Germany) and UV detector (Tokyo,Japan) was used to analyse paracetamol. Compounds were separated using a Phenomenex® C_{18} (4.60 x 250 mm) 5 µ analytical column, coupled to a SecurityGuard^{TM} C_{18} (4 x 3 mm) guard column (Phenomenex®, Torrance, CA, USA).

7.2.2 Materials

Paracetamol as syrup and tablets were purchased from a local pharmacy while the juice was squeezed from Star Ruby grapefruits bought from a local supermarket.Bergamottin pure compound was obtained from Sigma Aldrich Inc. (St Louis, MO, USA). Saline solution (Euro-Med Laboratories Inc., Cavite, Phillipines) was kindly sponsored by the Toxicology Laboratory, University of the Free State. HPLC grade methanol and acetonitrile were purchased from Honeywell Burdick and Jackson International Inc. (Muskegon, MI, USA). Liquid nitrogen was obtained from Parexel International (Bloemfontein, South Africa).

7.3 ANIMAL CARE

Ethical approval (ETOVS 10/12) was obtained from the Animal Ethic Committee of the University of the Free State before the animal experiments were started. Sprague-Dawley (SD) rats weighing between 200-250 g were used. Animals were kept and treated at the Animal House of the University of the Free State. Here they were fed and looked after by qualified staff and their cages cleaned once a week. Standard rat chow and water was available to the animals *ad libitum.* All animals were inspected for visible adverse events every day. During oral administration, the respective drug was drawn into a syringe and administered to the rats with a feeding needle by gavage.

7.4 EXPERIMENTAL DESIGN

7.4.1 Preliminary experiment: Determination of a hepatotoxic dose of paracetamol

A preliminary experiment to determine a dose of paracetamol that will be able to induce hepatotoxicity was conducted. Here, the paracetamol dosage was tested at; 1000, 1725 and 1822 mg/kg, respectively.

7.4.1 a) Experimental design

The experiment consisted of 27 rats which were divided into 3 groups of 9 rats each. The groups were treated with 1000, 1725 and 1822 mg/kg of paracetamol respectively, and sacrificed after 24, 48 and 72 hours (Figure 7.1 – page 69).

7.4.1 b) Results

During surgery, the livers and kidneys of rats treated with different paracetamol concentrations were observed. At 24 hours, the livers of the rats in all doses showed moderate hepatotoxicity. However, at 48 hours with the dose of 1725 mg/kg, there was one mortality and one rat was critical and had blood in the urine.

Table 7.1 – page 71, shows the changes in animals' weights. The growth pattern in terms of body weight was satisfactory.

After 24 hours and at all dosages, the rat livers were slightly spotted and red in colour. After 48 hours the 1000 mg/kg dosage caused only slightly spotted livers. However, the dosage of 1725 mg/kg elevated ALT 837 (647 - 1026), AST 1359 (1073 - 1645) and ALP 296 (191 - 401). Upon physical observation, the livers had black spots and a yellowish appearance, while the kidneys were enlarged and discoloured (Figures 7.2 and 7.3). The dosage of 1822 mg/kg also produced elevated ALT 449 (64 - 728), AST 1201 (156 - 1651) and ALP 231(153 - 364). Finally, after 72 hours, a slight decrease in ALT 44 (42 - 47), AST 110 (88 - 114) and ALP 256 (130 - 285) was observed, and this might have been due to the death of hepatocytes.

The renal function tests (Table 7.1 – page 71) revealed reduced kidney function as the dose increased. One rat in the group dosed with 1725 mg/kg had an enlarged kidney after 48 hours and also bloody urine, while another was found dead on the day of sacrificing.

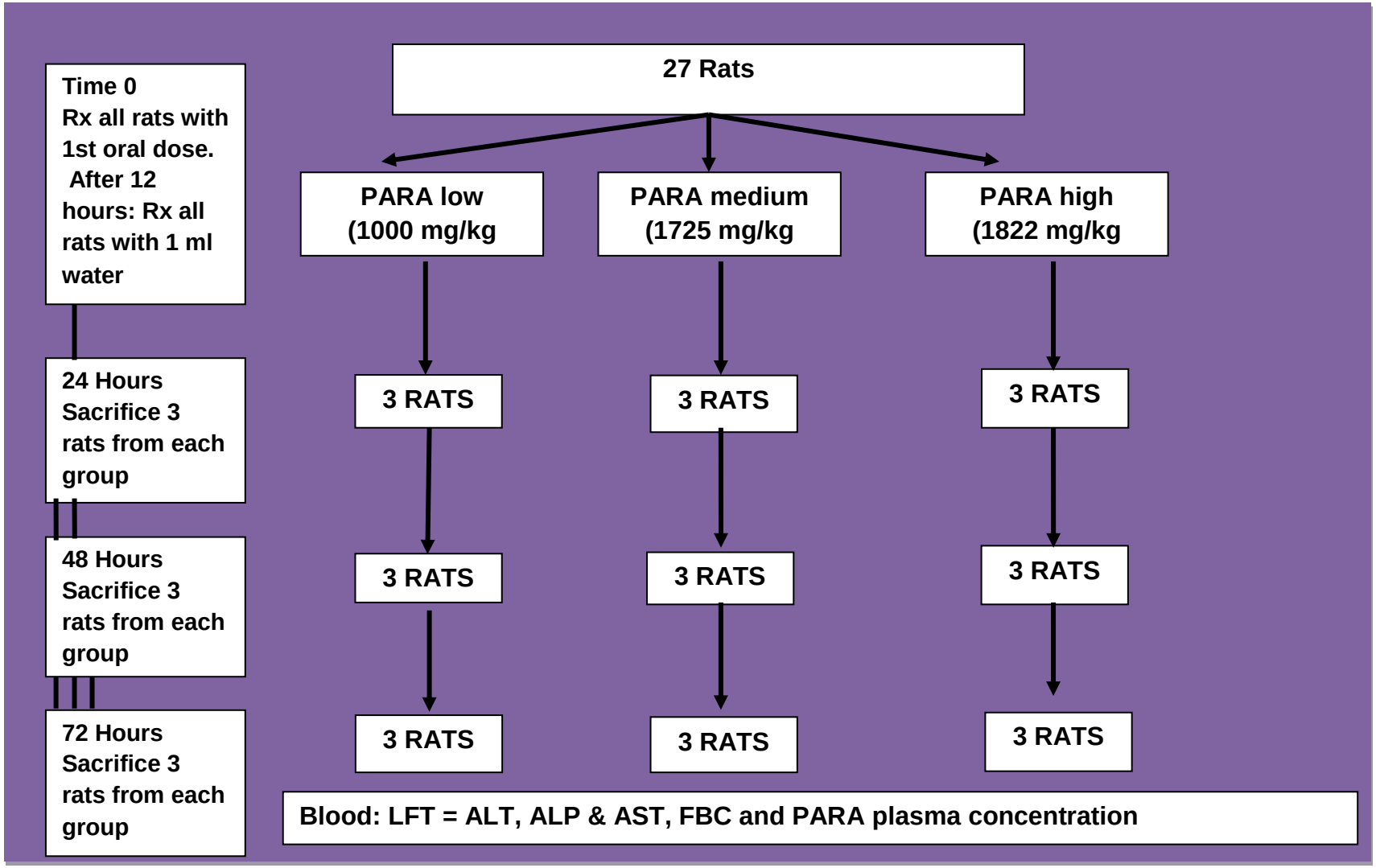

Figure 7.1: A schematic illustration of the experimental design for determining a hepatotoxic dose of paracetamol

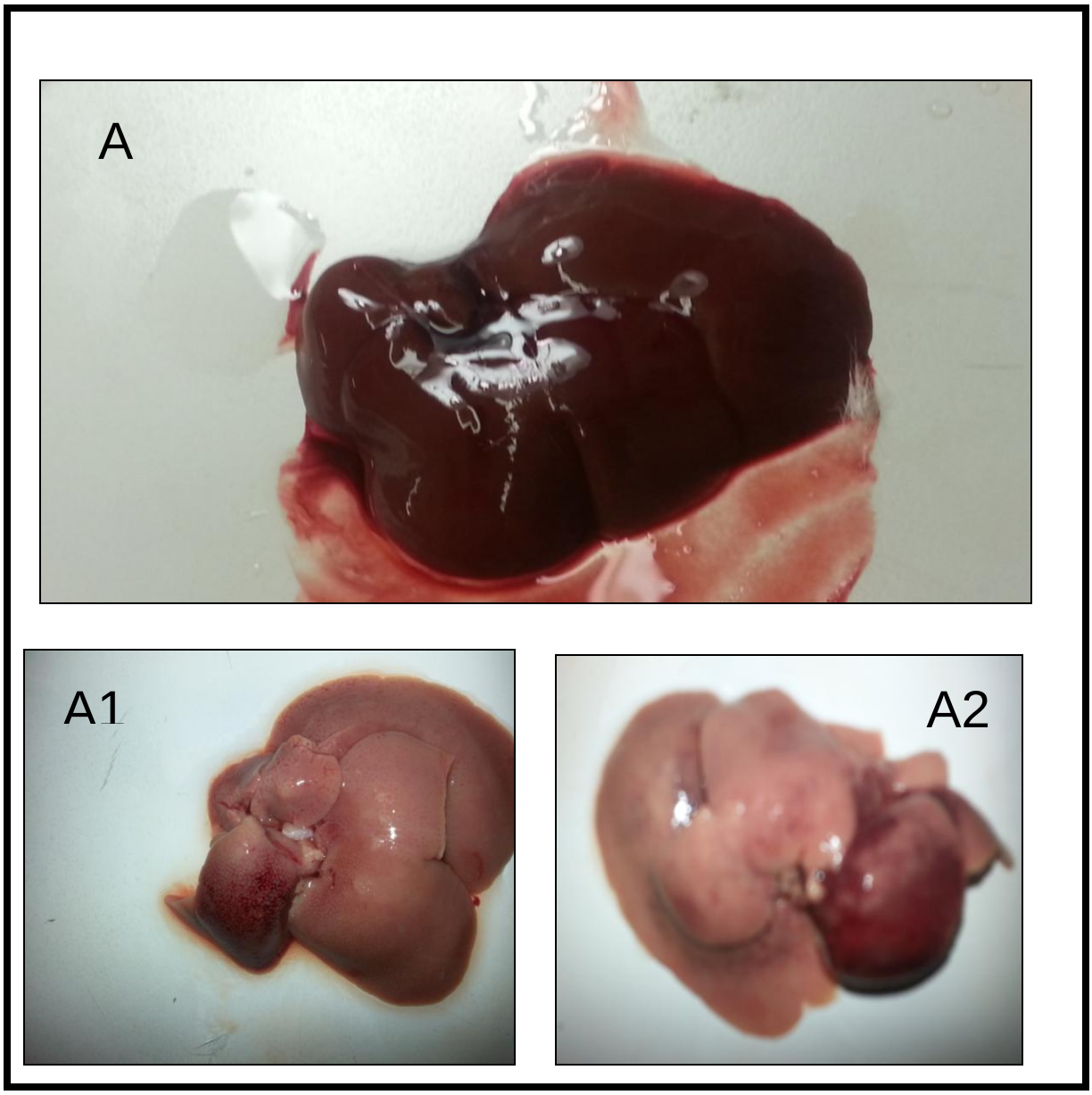

Figure 7.2: Photographs of rat livers: **A** is the liver an untreated rat while, **A1** and **A2** are the livers of rats treated with 1725 mg/kg (A1) and 1822 mg/kg (A2) which clearly indicates hepatotoxicity

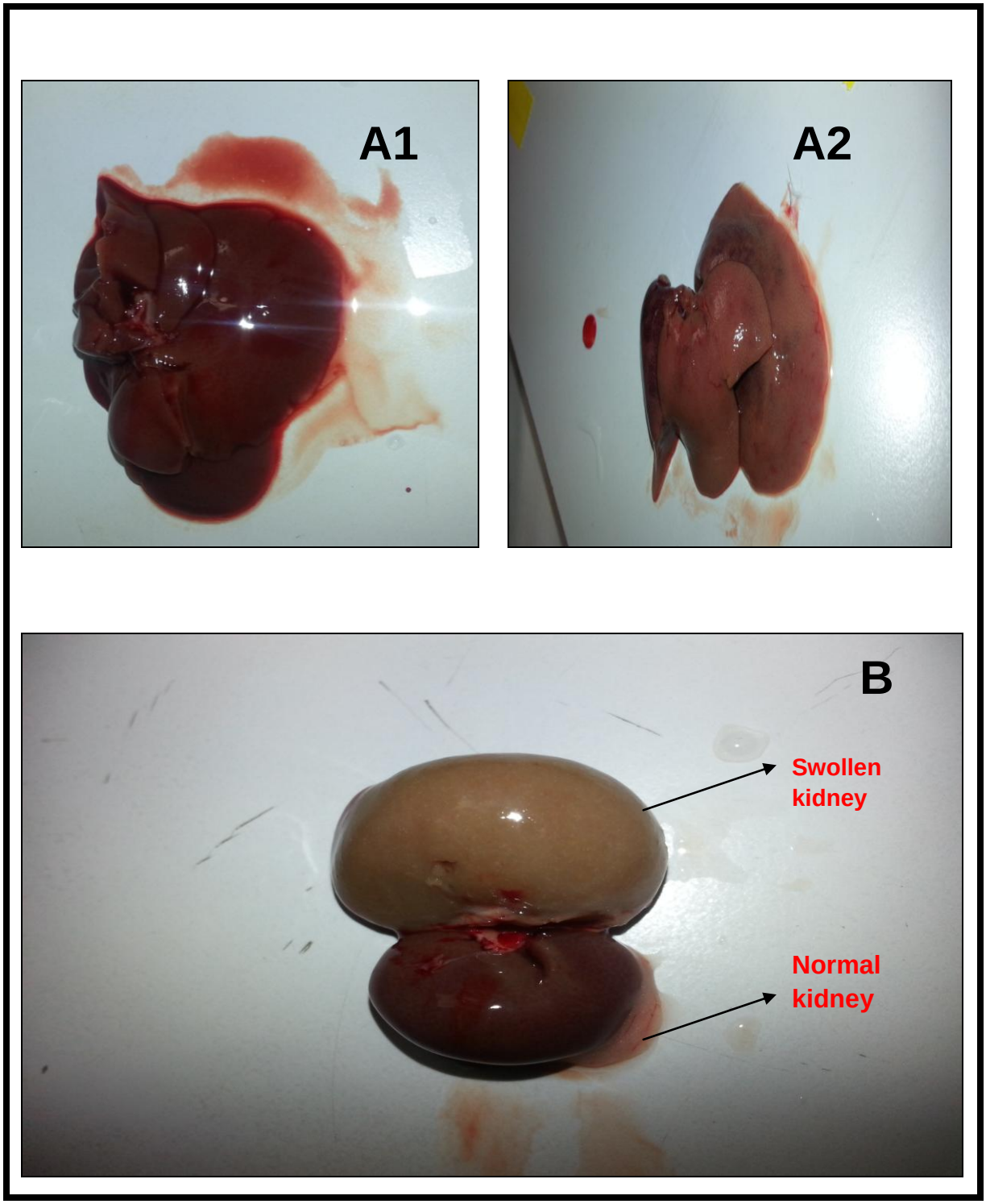

Figure 7.3: Photographs of a normal liver (**A1**) and an injured liver (**A2**); and kidneys (normal and injured) (**B**) of a rats after 48 hours at 1725 mg/kg paracetamol

Table 7.1: Average (Mean ± SD) values of rat weights, liver function tests and renal function tests of groups A (PARA low 1000 mg/kg), B (PARA medium 1725 mg/kg) C (PARA high1822 mg/kg).

Group	Weight (g)			Liver function tests (units/l)			Renal Function Tests	
(n = 3)	Before Rx	After Rx	Change	ALP	ALT	AST	Urea (µmol/L)	Creatinine (mmol/L)
A (PARA low)								
24 hours	230 ± 10.1	232 ± 10.0	**2**	295.3 ± 90.5	38.7 ± 5.7	103.7 ± 9.7	4.6 ± 0.5	28.0 ± 14.1
48 hours	218 ± 15.4	241 ± 1.8	**23**	174.0 ± 98.6	44.0 ± 4.6	91.3 ± 11.0	5.0 ± 0.7	28.0 ± 5.7
72 hours	240 ± 1.8	245 ± 4.3	**5**	243.3 ± 160.5	44.3 ± 4.0	122.0 ± 12.8	6.5 ± 0.9	32.7 ± 9.2
B (PARA medium								
24 hours	242 ± 1.6	243 ± 1.4	**1**	319.3 ± 2.1	63.3 ± 41.3	156.0 ± 76.2	5.0 ± 0.4	26.3 ± 12.2
48 hours	232 ± 9.7	246 ± 6.2	**14**	296.0 ± 148.5	836.5 ± 268.0	1359.0 ± 404.5	8.2 ± 1.8	40.5 ± 13.4
72 hours	234 ± 11.0	234 ± 6.6	**0**	260.0 ± 35.8	419.0 ± 585.7	512.0 ± 592.5	**57.8 ± 89.5**	**204.3 ± 296.8**
(PARA high)								
24 hours	245 ± 2.6	246 ± 2.6	**1**	150.3 ± 159.9	79.7 ± 63.5	254.0 ± 263.3	5.2 ± 0.3	17.5 ± 23.3
48 hours	223 ± 4.1	241 ± 3.1	**18**	249.3 ± 106.7	413.7 ± 333.4	1002.7 ± 767.0	**35.4 ± 48.0**	**157.3 ± 212.8**
72 hours	241 ± 2.6	242 ± 1.3	**1**	223.7 ± 82.4	44.3 ± 2.5	104.0 ± 14.0	6.3 ± 0.3	29.7 ± 9.1

Rx = treatment; **ALP** = alkaline phosphatase; **ALT** = alanine aminotransferase; **AST** = aspartate aminotransferase; Johnson-Delaney, 1996); **PARA** = paracetamol

Table 7.2: Average (Mean ± SD) values of full blood count results of groups A (PARA low, 1000 mg/kg), B (PARA medium, 1725 mg/kg), C (PARA high, 1822 mg/kg)

Group	Full blood count							
(n = 3)	**WCC**	**RCC**	**Hb**	**Hct**	**MCV**	**MCH**	**MCHC**	**Plt**
A (PARA low)								
24 hours	5.0 ± 1.3	6.6 ± 0.1	13.7 ± 0.2	0.401 ± 0.007	60.8 ± 1.5	20.7 ± 0.4	34.1 ± 0.2	1003 ± 47
48 hours	5.6 ± 1.2	7.0 ± 0.3	13.9 ± 0.4	0.434 ± 0.009	62.2 ± 2.0	20.0 ± 0.3	32.0 ± 1.0	**645 ± 76**
72 hours	5.4 ± 0.5	6.7 ± 0.3	13.2 ± 0.5	0.406 ± 0.015	60.8 ± 0.6	19.8 ± 0.3	32.5 ± 0.6	**579 ± 28**
B (PARA medium)								
24 hours	4.1 ± 1.1	6.0 ± 2.6	15.2 ± 0.5	0.439 ± 0.014	57.6 ± 0.6	19.9 ± 0.3	34.5 ± 0.2	1171 ± 81
48 hours	5.9 ± 0.5	7.5 ± 0.4	15.4 ± 0.8	0.464 ± 0.023	61.7 ± 0.2	20.5 ± 0.1	33.1 ± 0.8	**311 ± 305**
72 hours	4.7 ± 1.4	6.9 ± 0.9	13.6 ± 2.1	0.406 ± 0.077	58.9 ± 3.8	19.8 ± 0.6	33.7 ± 1.4	**326 ± 293**
C (PARA high)								
24 hours	4.7 ± 1.5	7.0 ± 0.4	13.9 ± 0.6	0.402 ± 0.021	57.5 ± 0.1	20.0 ± 0.3	34.7 ± 0.6	980 ± 93
48 hours	4.7 ± 2.8	6.1 ± 1.8	12.1 ± 3.6	0.366 ± 0.127	59.3 ± 3.1	19.7 ± 0.7	33.2 ± 1.7	**338 ± 474**
72 hours	5.2 ± 0.9	7.1 ± 0.5	13.8 ± 0.9	0.427 ± 0.030	60.4 ± 1.3	19.6 ± 0.5	32.4 ± 0.2	**636 ± 58**

PARA = paracetamol; **WCC** = white cell count; **RCC** = red cell count, **Hb** = Haemoglobin; **Hct** = Haematocrit; **MCV** = mean corpuscular volume; **MCH** = mean corpuscular haemoglobin; **MCHC** = mean corpuscular haemoglobin concentration; **Plt** = platelets (Johnson-Delaney, 1996; Giknis and Clifford, 2008)

7.4.1 c) Comment

The preliminary experiment was performed in order to determine a hepatotoxic dose of paracetamol to be used for the study. Paracetamol was tested at three dosages; 1000, 1725 and 1822 mg/kg respectively, whereby a one-off oral dose was given and animals sacrificed after 24, 48 and 72 hours.

By 48 hours, maximum liver enzyme elevation was observed at the dose of 1725 mg/kg, while at the dose of 1822 mg/kg, liver enzymes decreased. Therefore, it was decided to use the 1725 mg/kg dose for the remainder of the study.

7.4.2 Ultimate experimental design

The animal experiment was divided into two phases, namely, the control phase and the test phase (Figure 7.4 – page 76). For each phase, rats were weighed and divided into groups. The control phase had six groups of 15 animals each and the test phase had four groups of 15 animals each. For each group five rats were treated and sacrificed after 24, 48 and 72 hours. Paracetamol, grapefruit juice, bergamottin and saline solution were administered orally as follows for each phase:

7.4.2 a) Control phase

- Control A (Saline): 1 ml saline solution
- Control B (GFJ low): 2 ml grapefruit juice
- Control C (GFJ high): 3 ml grapefruit juice
- Control D (BGT low): 0.05 mg/kg bergamottin
- Control E (BGT high): 0.22 mg/kg bergamottin

7.4.2 b) Test phase

- Control A (PARA): 1725 mg/kg paracetamol
- Test B (PARA+GFJ low): 1725 mg/kg paracetamol (1 ml) and 2 ml grapefruit juice
- Test C (PARA+GFJ high): 1725 mg/kg paracetamol (1 ml) and 3 ml grapefruit juice
- Test D (PARA+BGT low): 1725 mg/kg paracetamol (1 ml) and 0.05 mg/kg bergamottin (1 ml)
- Test E (PARA+BGT high): 1725 mg/kg paracetamol (1 ml) and 0.22 mg/kg bergamottin (1 ml)

7.5 PROCEDURES

7.5.1 Surgical procedure and blood collection

All rats were sacrificed after 24, 48 and 72 hours following a one-off drug administration. The rats were anaesthetised with isoflurane, and blood was drawn by direct cardiac puncture (Figure 7.5 – page 77). Thereafter, the abdomen was opened and the liver exposed. A liver section was cut and stored in 10% formalin. The remainder of the liver was excised, removed and washed in a 1.5% potassium chloride solution, frozen with liquid nitrogen and stored at -85°C. The rats were sacrificed by exsanguination whilst still under isoflurane anaesthesia. Blood was collected in yellow top serum separator tubes for liver function tests, green top lithium heparin tubes for plasma, and purple top EDTA tubes for full blood count. The plasma and serum tubes were centrifuged and aliquots stored at -20°C until analysis. Blood for liver function tests and full blood count was sent off to an independent laboratory for analysis (Section 7.5.2)

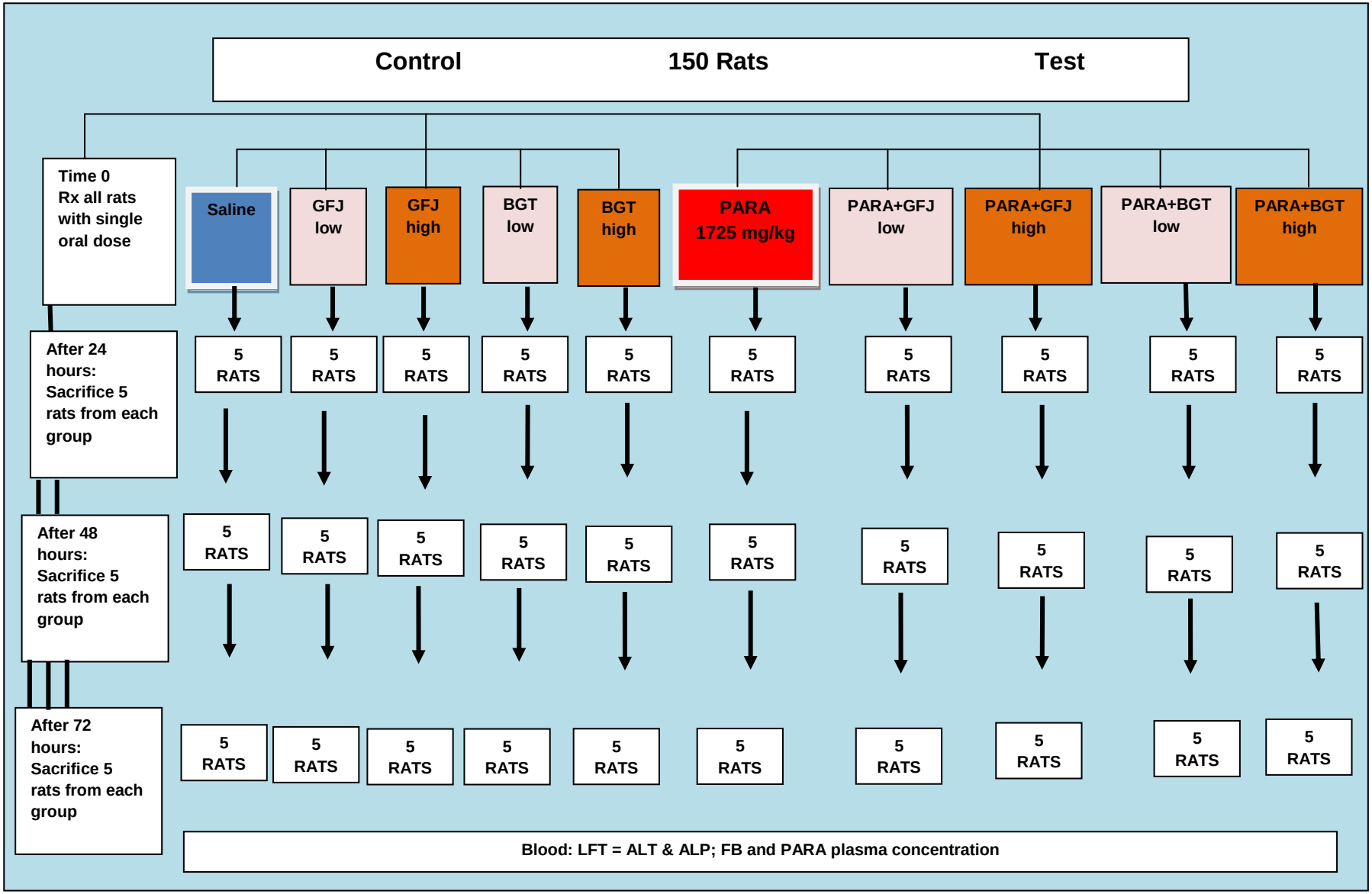

Figure 7.4: A schematic illustration of the ultimate experimental design

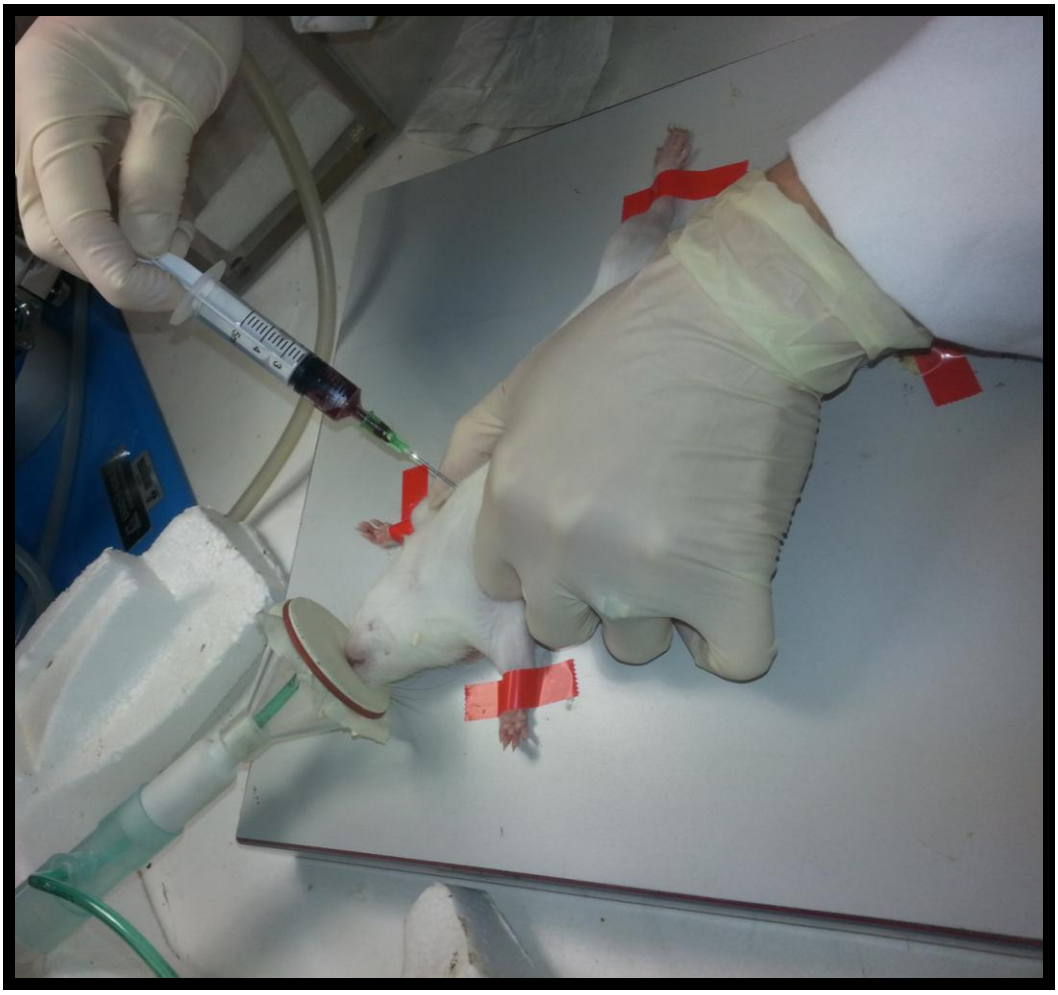

Figure 7.5: A photograph of direct cardiac puncture for blood collection under isoflurane anaesthesia

7.5.2 Analysis of liver function tests, full blood count and histopathology

All liver function tests were conducted and reported by the Toxicology laboratory of the department of Pharmacology, University of the Free State. The following serum transminase enzymes levels were measured on the day of surgery: alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP).

Full blood count was determined and reported by National Health Laboratory Services (NHLS). Histopathological diagnosis of the rat livers was performed and reported by an independent veterinary pathologist (Golden Vetpath, Idexx Laboratories, Johannesburg, South Africa). As mentioned in Section 7.5.1, liver sections were cut from the rat livers and placed in 10% neutral buffered formalin until analysis.

7.5.3 Analysis of paracetamol in rat plasma by the developed HPLC assay.

Paracetamol levels in rat plasma were monitored using the HPLC assay developed and described in Chapter 6. A standard curve was generated from 5 known paracetamol calibration standards, from which paracetamol concentrations in rat plasma were derived.

7.6 STATISTICAL ANALYSIS

Data were analysed by non-parametric methods using the GraphPad Instat statistical program. Accordingly, parameters were reported as mean and standard deviation (SD) and median and range, and the Kruskal-Wallis Test, as well as the Dunn's Multiple Comparisons Test, were used for data comparison with the level of significance set at $p < 0.05$.

7.7 RESULTS

7.7.1 Direct observations

The physical observation of livers and kidneys of rats treated with paracetamol + grapefruit juice and paracetamol + bergamottin was done. At all treatment times, the livers of the rats in all doses were normal with some showing slight spotting and discolouration.

However, the livers of the rats which were treated with paracetamol were highly discoloured while others exhibited goose bump like spots which were severe after 48 hours of treatment. The growth rate in terms of body weight was satisfying (Appendix E).

7.7.2 Haematology

The full blood count values are shown in table 7.3 a-b (pages 80-81) after 24, 48 and 72 hours one-off treatment with paracetamol only, paracetamol + grapefruit juice and paracetamol + bergamottin (see Appendix H). When paracetamol was administered alone, the rats exhibited thrombocytopenia (low platelet count) at 48 hours, while at 72 hours a gradual increase in platelet count was observed. Co-administration of paracetamol with either grapefruit juice or bergamottin may have protected the rats from hepatotoxicity as the platelet count was higher than when paracetamol was administered alone. The rest of the full blood count parameters were not affected.

Table 7.3 a: Mean ± SD values of FBC results of the control phase (saline, GFJ and BGT)

Group (n = 3)	FULL BLOOD COUNT WCC	RCC	Hb	Hct	MCV	MCH	MCHC	Plt
SAL (control)								
24 hours	4.8 ± 0.2	6.2 ± 0.1	13.3 ± 0.2	0.388 ± 0.011	62.2 ± 1.8	21.1 ± 0.2	33.9 ± 0.6	970 ± 129
48 hours	6.0 ± 0.4	6.3 ± 0.4	12.9 ± 0.8	0.387 ± 0.016	61.8 ± 1.4	20.6 ± 0.2	33.4 ± 0.6	1023 ± 162
72 hours	4.9 ± 1.5	6.5 ± 0.1	13.0 ± 0.1	0.392 ± 0.006	60.5 ± 0.4	20.1 ± 0.5	33.3 ± 0.6	957 ± 88
GFJ low								
24 hours	6.2 ± 1.3	6.1 ± 0.3	12.5 ± 0.8	0.369 ± 0.018	60.5 ± 0.3	20.6 ± 0.3	34.0 ± 0.5	933 ± 132
48 hours	5.9 ± 0.2	5.2 ± 0.6	11.2 ± 1.2	0.336 ± 0.022	65.2 ± 0.4	21.7 ± 0.3	33.2 ± 0.3	852 ± 99
72 hours	5.2 ± 0.7	6.3 ± 0.4	12.8 ± 0.2	0.376 ± 0.047	60.4 ± 3.7	20.4 ± 1.0	33.7 ± 0.6	1043 ± 91
GFJ high								
24 hours	4.8 ± 1.2	5.9 ± 0.6	12.4 ± 1.2	0.367 ± 0.038	62.4 ± 0.3	21.1 ± 0.2	33.7 ± 0.4	924 ± 158
48 hours	4.3 ± 1.1	5.7 ± 0.4	11.9 ± 1.0	0.358 ± 0.022	63.0 ± 2.4	20.9 ± 0.4	33.2 ± 1.0	875 ± 124
72 hours	4.6 ± 0.9	6.8 ± 0.3	13.7 ± 0.3	0.405 ± 0.010	59.5 ± 0.9	20.2 ± 0.3	33.9 ± 0.2	936 ± 140
BGT low								
24 hours	6.2 ± 1.0	6.3 ± 0.5	12.9 ± 1.1	0.396 ± 0.034	63.3 ± 0.7	20.6 ± 0.3	32.5 ± 0.4	840 ± 59
48 hours	5.2 ± 1.2	6.0 ± 0.3	12.0 ± 0.8	0.376 ± 0.026	63.3 ± 5.1	20.4 ± 1.7	32.3 ± 0.2	729± 79
72 hours	6.2 ± 0.8	6.4 ± 0.8	12.9 ± 1.6	0.393 ± 0.043	61.9 ± 1.7	20.3 ± 0.1	32.8 ± 0.8	1106 ± 76
BGT high								
24 hours	5.5 ± 0.8	5.9 ± 0.3	12.7 ± 0.8	0.387 ± 0.026	65.5 ± 2.5	21.6 ± 0.6	32.9 ± 0.4	690 ± 106
48 hours	4.7 ± 0.8	6.0 ± 0.2	13.0 ± 0.5	0.401 ± 0.018	66.9 ± 1.7	21.6 ± 0.5	32.3 ± 0.2	716 ± 98
72 hours	4.3 ± 1.5	6.2 ± 0.4	13.1 ± 0.6	0.404 ± 0.014	65.3 ± 4.5	21.1 ± 1.2	32.3 ± 0.6	837 ± 199

GFJ = grapefruit juice; **BGT** = bergamottin; WCC = white cell count; **RCC** = red cell count, **Hb** = Haemoglobin; **Hct** = Haematocrit; **MCV** = mean corpuscular volume; **MCH** = mean corpuscular haemoglobin; **MCHC** = mean corpuscular haemoglobin concentration; **Plt** = platelets (Johnson-Delaney, 1996; Giknis and Clifford, 2008)

Table 7.3 b: Mean ± SD values of FBC results of the test phase (PARA-only, PARA+GFJ and PARA+BGT)

Group (n = 3)	FULL BLOOD COUNT							
	WCC	**RCC**	**Hb**	**Hct**	**MCV**	**MCH**	**MCHC**	**Plt**
PARA								
24 hours	4.1 ± 1.3	7.6 ± 2.6	15.2 ± 0.5	0.439 ± 0.014	57.6 ± 0.6	19.9 ± 0.3	34.5 ± 0.2	1159 ± 81
48 hours	5.9 ± 0.2	7.5 ± 0.4	15.3 ± 0.8	0.464 ± 0.023	61.1 ± 0.2	20.4 ± 0.1	33.1 ± 0.4	**310 ± 305**
72 hours	4.7 ± 1.4	6.8 ± 0.9	13.6 ± 2.1	0.406 ± 0.077	58.9 ± 3.8	19.8 ± 0.6	33.7 ± 1.4	326 ± 293
PARA+GFJ low								
24 hours	6.4 ± 1.4	6.7 ± 0.4	13.4 ± 0.9	0.397 ± 0.025	59.6 ± 1	20.0 ± 0.5	33.6 ± 0.2	1101 ± 172
48 hours	5.1 ± 0.9	6.1 ± 1.5	12.8 ± 2.9	0.387 ± 0.090	63.4 ± 1	20.9 ± 0.3	33.0 ± 0.5	**333 ± 78**
72 hours	6.1 ± 1.1	6.2 ± 0.7	12.4 ± 1.3	0.389 ± 0.045	62.3 ± 1	19.8 ± 0.1	31.9 ± 0.5	627 ± 59
PARA+GFJ high								
24 hours	6.2 ± 1.9	6.6 ± 0.4	13.7 ± 0.8	0.405 ± 0.025	61.0 ± 1.5	20.6 ± 0.4	33.7 ± 0.4	1088 ± 85
48 hours	4.7 ± 1.4	6.1 ± 0.2	13.0 ± 0.5	0.395 ± 0.022	64.5 ± 2.2	21.2 ± 0.4	33.0 ± 0.5	**464 ± 74**
72 hours	6.7 ± 0.2	6.6 ± 0.2	13.1 ± 0.5	0.399 ± 0.022	64.9 ± 2.2	21.3 ± 2.4	32.8 ± 0.5	492 ± 163
PARA+BGT low								
24 hours	4.5 ± 0.4	5.8 ± 0.5	11.6 ± 1.2	0.353 ± 0.035	61.0 ± 2.7	20.1 ± 0.8	33.0 ± 0.3	944 ± 185
48 hours	6.5 ± 0.3	6.4 ± 1.1	13.5 ± 1.9	0.420 ± 0.059	65.6 ± 1.6	21.0 ± 0.3	32.0 ± 0.2	**587 ± 134**
72 hours	5.4 ± 1.3	6.2 ± 0.6	12.8 ± 1.4	0.380 ± 0.032	61.6 ± 0.5	20.7 ± 0.2	33.6 ± 0.1	1065 ± 122
PARA+BGT high								
24 hours	7.0 ± 1.1	5.9 ± 0.8	12.4 ± 1.6	0.382 ± 0.053	64.4 ± 0.8	20.8 ± 0.6	30.6 ± 0.8	923 ± 68
48 hours	6.6 ± 0.8	6.4 ± 0.8	13.5 ± 1.4	0.392 ± 0.032	61.7 ± 3.1	21.1 ± 0.7	34.2 ± 0.8	**773 ± 25**
72 hours	5.6 ± 0.9	6.7 ± 0.6	13.5 ± 1.3	0.415 ± 0.037	62.3 ± 3.0	20.3 ± 1.6	32.6 ± 0.2	780 ± 119

PARA = paracetamol; **GFJ** = grapefruit juice; **BGT** = bergamottin; WCC = white cell count; **RCC** = red cell count, **Hb** = Haemoglobin; **Hct** = Haematocrit; **MCV** = mean corpuscular volume; **MCH** = mean corpuscular haemoglobin; **MCHC** = mean corpuscular haemoglobin concentration; **Plt** = platelets (Johnson-Delaney, 1996; Giknis and Clifford, 2008)

7.7.3 Hepatotoxicity

The liver function test results are shown in table 7.4 a-b (pages 83-84), while, Figures 7.6 a) - 7.6 i - page 85) are the photographs of liver histopathology.

7.7.4 Liver Function Tests

There was a significant increase in the liver function test results (ALT and AST) in the rats which were treated with paracetamol only at 24 and 48 hours, which was followed by a decline at 72 hours (refer to Appendix F for all LFTs). When paracetamol was co-administered with grapefruit juice, a remarkable decrease in liver enzyme levels was observed at 48 and 72 hours. Similarly, co-administration of paracetamol with bergamottin decreased the levels of liver enzymes after 48 and 72 hours. The control group (saline) had lower liver enzyme levels than the paracetamol only group which indicated that the paracetamol only group might have experienced hepatotoxicity.

Table 7.4 a: Mean ± SD values of rat weights, liver function tests and renal function tests of the control phase

Group (n = 5)	Weight			Liver Function Tests (units/l)			`Renal Function Tests	
	Before Rx	After Rx	Change	ALP	ALT	AST	Urea (µmol/L)	Creatinine (mmol/L)
SAL (control)								
24 hours	223 ± 6.9	243 ± 7.2	20	226.3 ± 45.2	44.0 ± 10.6	60..0 ± 10.6	5.1 ± 1.1	50.7 ± 4.0
48 hours	217 ± 12.7	240 ± 13.3	23	365.7 ± 11.2	51.7 ± 7.2	147.0 ± 153.3	6.5 ± 0.2	65.2± 1.5
72 hours	214 ± 16.5	256 ± 18.4	32	136.3 ± 88.7	39.3 ± 0.7	54.3 ± 2.8	5.0 ± 0.1	49.7 ± 7.1
GFJ low								
24 hours	215 ± 4.7	227 ± 6.2	12	90.0 ± 89.6	36.0 ± 5.7	68.7 ± 36.1	4.2 ± 1.1	6.3 ± 3.5
48 hours	207 ± 7.1	235 ± 8.6	28	175.7 ± 107.0	43.3 ± 4.0	122.3 ± 53.0	5.9 ± 0.6	9.5 ± 0.7
72 hours	215 ± 2.6	248 ± 5.0	33	214.7 ± 10.7	42.7 ± 6.5	68.0 ± 9	6.5 ± 0.6	9.7 ± 9.1
GFJ high								
24 hours	229 ± 4.7	237 ± 4.3	8	209.0 ± 34.0	40.0 ± 14.1	77.7 ± 9.7	3.8 ± 1.0	9.7± 3.8
48 hours	206 ± 6.0	229 ± 9.4	23	60.3 ± 78.7	35.3 ± 7.8	59.3 ± 5.5	4.3 ± 1.2	7.0 ± 7.1
72 hours	231 ± 4.9	260 ± 6.9	29	133.7 ± 90.6	49.3 ± 10.1	88.7± 34.5	6.2 ± 1.2	7.7 ± 2.1
BGT low								
24 hours	215 ± 4.7	227 ± 6.2	12	341.3 ± 84.9	59.7 ± 7.8	171.0 ± 112.2	7.1 ± 0.7	72.7 ± 8.7
48 hours	210 ± 12.0	231 ± 13.8	21	290.0 ± 82.7	49.0 ± 11.3	234.0 ± 261.6	5.7 ± 1.6	60.0 ± 0.7
72 hours	209 ± 4.2	230 ± 3.8	21	Below assay range	53.7 ± 2.1	108.0 ± 4.2	6.4 ± 1.1	62.3 ± 3
BGT high								
24 hours	219 ± 1.0	228 ± 3.9	9	205.7 ± 8.5	42.3 ± 2.1	90.3 ± 2.8	6.0 ± 0.3	64.0 ± 4.9
48 hours	201 ± 1.5	221 ± 4.0	20	319.3± 21.9	43.3 ± 1.4	88.3 ± 26.9	5.1 ± 0.7	62.3 ± 1.4
72 hours	216 ± 10.9	241 ± 9.1	25	148.3 ± 7.7	50.3 ± 3.5	79.3 ± 2.8	5 .5± 0.1	57.7±5.7

Rx = treatment; **ALP** = alkaline phosphatase; **ALT** = alanine aminotransferase; **AST** = aspartate aminotransferase (Johnson Delaney, 1996); **SAL** = saline; **GFJ** = grapefruit juice; **BGT** = bergamottin

Table 7.4 b: Mean ± SD values of rat weights, liver function tests and renal function test of the test phase

Group (n = 5)	Weight			Liver Function Tests (units/l)		Renal Function Tests		
	Before Rx	After Rx	Change	ALP	ALT	AST	Urea (µmol/L)	Creatinine (mmol/L)
PARA-only								
24 hours	242 ± 1.6	243 ± 1.4	1	319.3 ± 2.1	63.3 ± 41.3	156.0 ± 76.2	5.0 ± 0.4	26.3 ± 12.2
48 hours	223 ± 4.1	241 ± 3.1	18	**296.0 ± 148.5**	**836.5 ± 268.0**	**1359.0 ± 404.5**	8.2 ± 1.8	40.5 ± 13.4
72 hours	234 ± 11.0	234 ± 6.6	0	266.7 ± 38.9	419.0 ± 690.1	512.3 ± 657.9	**57.8 ± 109.6**	**204.3±363.5**
PARA + GFJ low								
24 hours	242 ± 6.4	244 ± 6.3	2	62.3 ± 64.7	9.3 ± 2.5	69.0 ± 5.0	5.3 ± 0.9	31.3 ± 1.2
48 hours	206 ± 6.5	203 ± 9.0	-3	**312.0 ± 72.3**	**628.0 ± 764.5**	**640.7 ± 660.7**	7.5 ± 1.1	38.3±10.0
72 hours	204 ± 3.5	219 ± 7.3	16	284.7 ± 102.9	5.0 ± 2.8	84.7 ± 43.8	5.9 ± 0.2	28.0 ± 4.4
PARA+ GFJ high								
24 hours	227 ± 12.9	231 ± 10.4	4	212.3 ± 114.1	17.7 ± 7.6	141.0 ± 65.8	5.9 ± 0.6	18.5 ± 0.7
48 hours	207 ± 6.5	209 ± 13.2	2	**222.3 ± 34.0**	**34.0 ± 48.8**	**238.3 ± 220.5**	7.0 ± 0.5	33.7± 3.1
72 hours	215 ± 18.0	226 ± 4.8	11	327.0 ± 34.6	27.3 ± 26.1	147.0 ± 52.4	7.4 ± 0.6	37.7 ± 2.1
PARA + BGT low								
24 hours	208 ± 5.1	209 ± 13.1	1	194.3 ± 86.0	9.0 ± 3.0	94.3 ± 45.6	6.6 ± 1.1	31.7 ± 5.0
48 hours	221 ± 7.2	226 ± 12.9	5	**156.3 ± 104.6**	**17.0 ± 13.1**	**81.3 ± 7.0**	6.6 ± 0.1	30.3 ± 6.4
72 hours	205 ± 1.5	221 ± 6.6	16	283.3 ± 74.8	11.7 ± 7.1	82.7 ± 18.6	5.4 ± 0.1	32.7 ± 5.7
PARA + BGT high								
24 hours	207 ± 7.5	207 ± 17.3	0	268.0 ± 48.4	9.0 ± 4.4	104.0 ± 9.2	6.8 ± 1.5	35.0 ± 6.2
48 hours	212 ± 4.6	230 ± 36.2	18	**314.0 ± 36.8**	**22.0 ± 13.9**	**168 .3± 49.6**	6.9 ± 1.2	39.7 ± 2.1
72 hours	218 ± 8.1	233 ± 14.4	15	236.7 ± 18.6	11.7 ± 7.6	110.3 ± 38.2	6.2 ± 0.8	29.7 ± 4.0

Rx = treatment; **ALP** = alkaline phosphatase; **ALT** = alanine aminotransferase; **AST** = aspartate aminotransferase (Johnson Delaney, 1996); **PARA** = paracetamol; **GFJ** = grapefruit juice; **BGT** = bergamottin

7.7.5 Histopathology photographs and reports

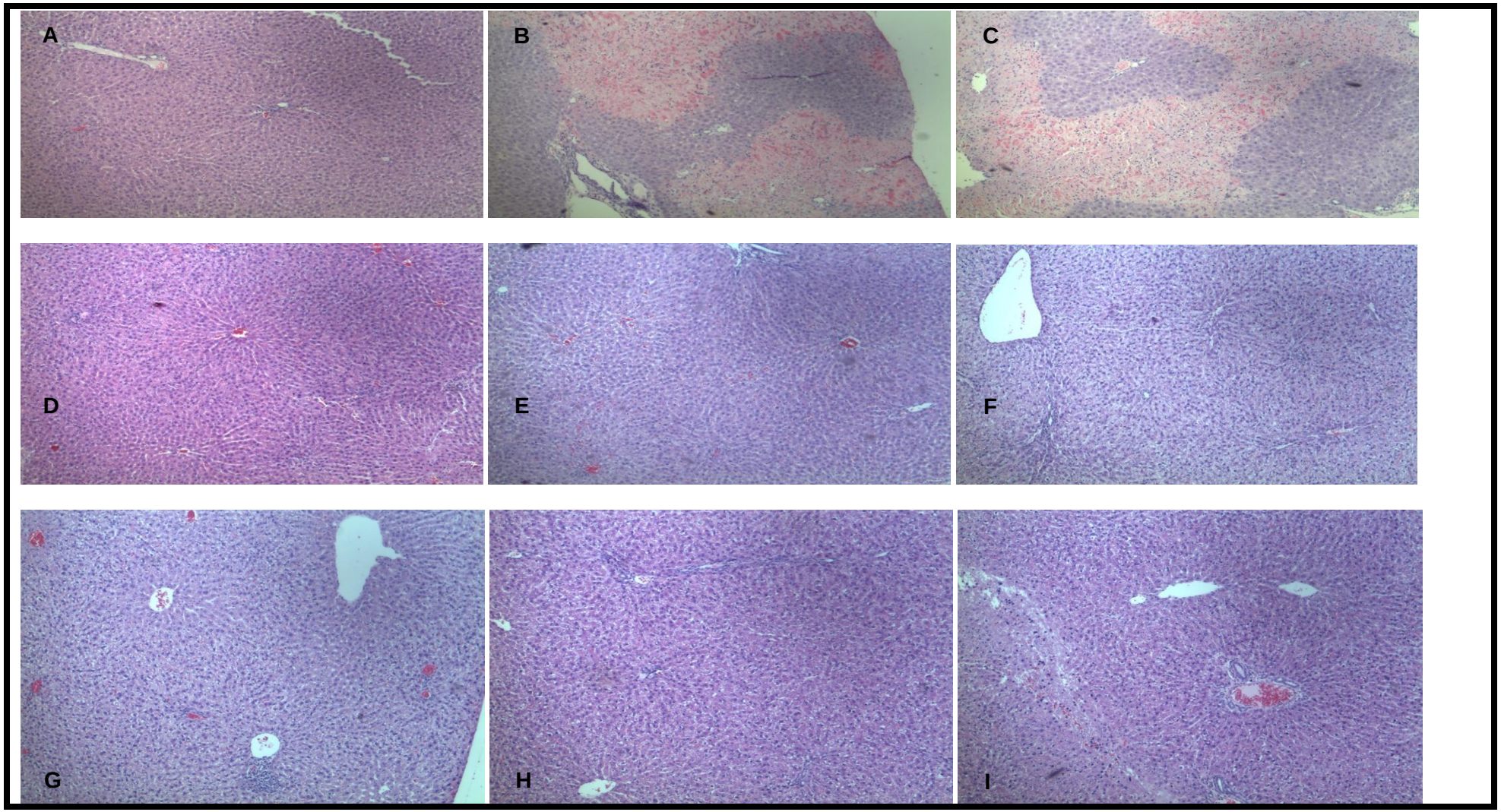

Figure 7.6: Representative histopathology slides (x 4) for paracetamol (PAR) treated rat livers and the corresponding pathology reports.

Sections of a rat liver from the group treated with single toxic dose of PAR (A, B, C), PAR+GFJ (D, E, F) and PAR+BGT (G, H, I):

A: 24 hrs after dosing with PAR.

- The section of liver reveals mild granular vacuolar degeneration and very mild swelling of the hepatocytes. The trabecular structure can still be visualized, as can the sinusoids, although slightly reduced in diameter. There is a mild mononuclear portal inflammatory infiltrate mainly composed of macrophages.

B: 48 hrs after dosing with PAR.

- Under low power (4 x magnification) severe centrilobular bridging necrosis can be seen. Large confluent areas of necrosis characterized by loss of hepatocyte nuclei, disarrangement of the cords and loss of structure is present in the centrilobular areas, with extension from the centrilobular regions through the zones to link up with other centrilobular areas. The hepatocytes are lightly eosinophilic and there is loss of the trabecular structure and the acute necrosis is associated with mild haemorrhage as well as acute inflammatory cells including polymorphonuclear cells and mononuclear cells. The rest of the hepatocytes between the bridging necrosis reveal mild to moderate, granular, vacuolar degeneration and mild fatty change.

C: 72 hrs after dosing with PAR.

- Severe centrilobular bridging necrosis is noticeable with severe centrilobular and periportal inflammation characterized by mononuclear inflammatory cells. The bridging necrosis is similar to that already described, characterized by eosinophilia of the hepatocytes with loss of nuclei and trabecular structure. Increased mitotic figures at approximately four mitoses per high power field (40 x magnifications) can be seen close to the necrotic zones, but within the preserved areas.

Some of the necrotic areas maintain an outline of the hepatocytes, suggesting acute coagulative necrosis. There is a mild leukostasis.

D: 24 hrs after dosing with PAR+GFJ:

- Mild vacuolar change and swelling, mild leukostasis and reduced numbers of mitoses can be seen as well as mild portal mononuclear inflammatory infiltration.

E: 48 hrs after dosing with PAR+GFJ:

- Noticeable mild to moderate vacuolar change and degeneration with mild, portal, mononuclear inflammatory infiltrates and very few mitotic figures.

F: 72 hrs after dosing with PAR+GFJ:

- There are few mitotic figures and mononuclear inflammatory infiltration.

G: 24 hrs after dosing with PAR+BGT:

- Moderate to severe vacuolar degeneration and severe swelling of the hepatocytes with a mild mononuclear portal cell infiltration has taken place.

H: 48 hrs after dosing with PAR+BGT:

- Moderate vacuolar degeneration and mild mononuclear portal infiltration with few mitoses has occured.

I: 72 hrs after dosing with PAR+BGT:

- Severe vacuolar degeneration and swelling of the hepatocytes with loss of trabecular structure and odd, small granules of bile pigment could be seen within the hepatocytes and sinusoids.

7.7.6 Paracetamol plasma concentrations

The plasma concentration of paracetamol was measured at 24, 48 and 72 hours after administration of paracetamol only, paracetamol + grapefruit and paracetamol + bergamottin (view Appendix I). At 48 hours, the concentration of paracetamol in the plasma of the rats treated with paracetamol only was higher than in the plasma of the rats treated with paracetamol + grapefruit juice. Similarly, the low plasma concentration levels were obtained in the group of rats treated with bergamottin. The plasma concentration levels were further decreased after 72 hours when paracetamol was co-administered with either grapefruit juice or bergamottin.

Table 7.5: Median (range) values of paracetamol plasma concentration of groups PARA-only, PARA + GFJ and PARA + BGT at 24, 48 and 72 hours.

Group (n = 5)	Paracetamol plasma conc (µg/ml)
PARA-only	
24 hours	5.72 (4.38 - 28.15)
48 hours	**4.45 (2.28 - 6.67)**
72 hours	1.61 (1.45 - 2.02)
PARA + GFJ	
24 hours	0.60 (0.16 - 3.01)
48 hours	**0.92 (0.92 - 1.42)**
72 hours	1.14 (0.99 - 1.67)
PARA + BGT	
24 hours	1.10 (0.88 - 1.24)
48 hours	**5.57 (5.26 - 16.93)**
72 hours	0.13 (0.00 - 0.13)

PARA = paracetamol; **GFJ** = grapefruit juice; **BGT** = bergamottin

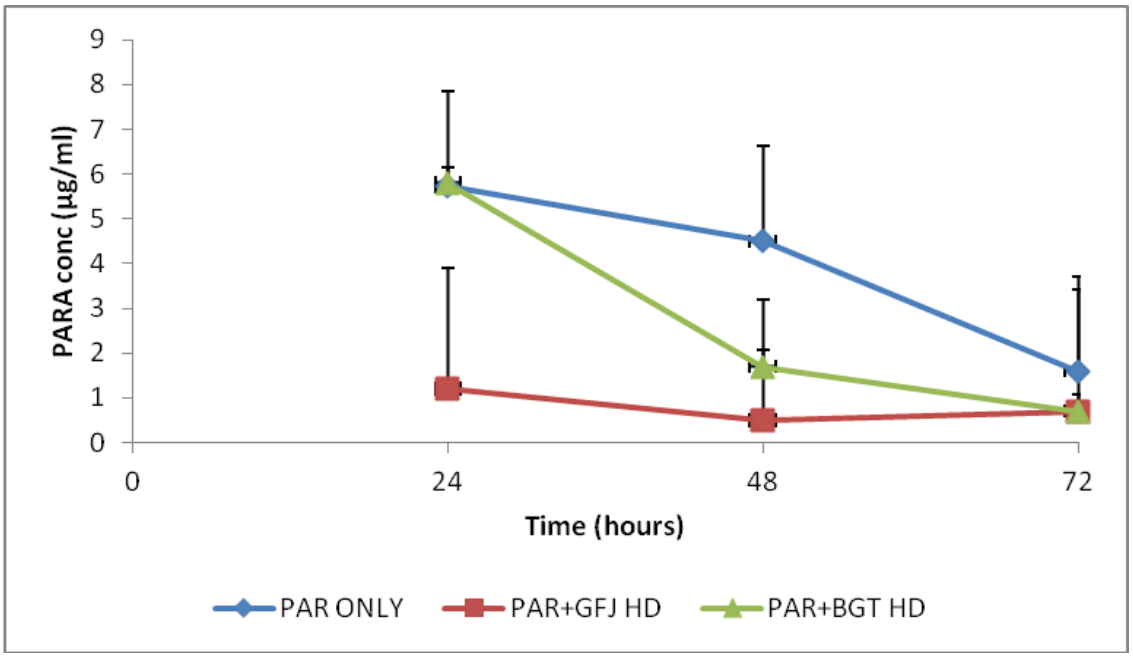

Figure 7.7: A graph of paracetamol plasma levels of groups PARA-only, PARA + GFJ and PARA + BGT at 24, 48 and 72 hours.

7.8 Discussion

Liver is an important organ which plays a vital role in detoxification, plasma protein synthesis and metabolism of drugs (Al-Awar *et al*., 2013). When gradually exposed to toxic agents, the liver may be prone to hepatic injury. As such, medicinal plants are being discovered for their therapeutic effects as they have shown to be able to protect the liver against hepatic injury (Gole and Dasgupta, 2002).

To assess liver damage, biomarkers such as liver enzymes AST and ALT are measured. These liver enzymes are released into the circulation due to liver damage emerging from necrosis. High levels of AST and ALT may indicate liver damage arising from viral hepatitis, cardiac infarction and muscle injury. However, ALT is more specific to the liver and has shown to be a better parameter for detection of liver injury (Al-Awar *et al*., 2013).

Paracetamol, a widely used antipyretic and analgesic drug, has been known to cause hepatotoxiciy after overdose. Currently, *N*-acetylcysteine (NAC) is the drug of choice for treatment of paracetamol overdose. It acts by replenishing the glutathione stores, thereby enhancing NAPQI detoxification. Of note, glutathione itself cannot be used because it does not cross cell membranes as a whole molecule, but can be transported across. NAC has been shown to prevent paracetamol-induced hepatotoxicity when administered soon after paracetamol overdose.

Unfortunately, NAC does not stop the production of the NAPQI and is associated with hypersensititvity reactions, which hampers its use in some patients (Walubo *et al*., 2004).

As such, a plethora of research has been carried out to find possible protection against paracetamol-induced toxicity.

As observed in the study, the administration of a toxic dose of paracetamol (1725 mg/kg) alone to the treatment control group showed an increase in the levels of ALT, AST, and ALP at 48 hours.

The levels of ALP usually increase remarkably in a disease which impairs bile formation and to a lesser extent, in hepatocellular diseases hence, this will not be reported in this study.

Following administration of paracetamol with grapefruit juice/bergamottin, a decrease in ALT and AST was observed. A decrease in the liver enzymes may be attributed to the hepatoprotective effect of furanocoumarins such as bergamottin, found in grapefruit juice.

The possible mechanism responsible for the protection of the paracetamol-induced liver damage by the grapefruit juice may be as a result of the grapefruit juice acting as an enzyme inhibitor by intercepting the enzymes involved in paracetamol metabolism. One other reason is the presence of phytochemicals in the grapefruit juice whereby a number of scientific reports have indicated the role of certain flavonoids and furanocoumarins, in this case bergamottin in a hepato-protection role against hepatotoxins. The presence of bergamottin in grapefruit juice may be responsible for the protective effect on paracetamol-induced liver damage in rats. Hence, the grapefruit juice was characterized initially before it was administered to rats to verify the presence of bergamottin in the dosing sample.

The hepatoprotective effect of grapefruit juice was further confirmed by histopathological examination of the liver. The histological observation proved the hepatoprotective effect of grapefruit juice by completely preventing development of cytonecrosis and centrolobular zonal necrosis, and almost prevented the development of hepatocyte mitosis.

The blood of rats was further analysed to determine the concentration of paracetamol before and after grapefruit juice was administered. The results of the HPLC indicated that when paracetamol was administered alone, the concentration was high at 24 hours, and there was a decline from 48 to 72 hours. However, co-administration of paracetamol with grapefruit juice produced a significant decline in the paracetamol plasma concentration at 48 hours and a further decline at 72 hours.

In the present study, hepatic damage was observed in rats treated with paracetamol as evidenced by an elevation of serum AST and ALT concentration in the liver. These changes may be due to increased production of a toxic metabolite, NAPQI, which consequently led to subsequent cell damage. Again, lower levels of serum AST and ALT were observed when paracetamol was co-administered with grapefruit juice, which suggests the possibility of grapefruit juice being able to give protection against paracetamol-induced hepatotoxicity.

The exact mechanism involved in the hepatoprotective activity of grapefruit juice against paracetamol-induced hepatotoxicity in rats is still not yet understood. However, direct inhibition of enzymes involved in the production of a toxic metabolite, NAPQI, could be the answer. The presence of flavonoids and furanocoumarins (bergamottin) in grapefruit juice play a very important role in this regard because a high consumption of flavonoids is known to protect the liver (Aniyathi *et al*., 2009).

7.9 Conclusion

In conclusion, the results of this study demonstrate that grapefruit juice has hepatoprotective action against paracetamol-induced hepatic damage in rats. The results indicate that the hepatoprotective effects of grapefruit juice may be due to its enzyme inhibition properties and the direct interaction which has an effect in the metabolism of paracetamol. Further investigation to determine the exact mechanism that is responsible for its hepatoprotective effect is recommended for future studies.

CHAPTER 8

CONCLUSIONS AND FUTURE STUDIES

The objectives of this study were achieved as follows:

1. Characterisation of grapefruit peel extract and juice using high performance liquid chromatography was successfully performed. The presence of bergamottin was confirmed only in the juice, while the preparation of peel extract may have caused the degradation of bergamottin due to rigorous heating by rotary evaporator.

2. A method for determining paracetamol in plasma by high performance liquid chromatography was successfully developed. This method was used to measure the concentration of paracetamol in rats treated with 1725 mg/kg of paracetamol at 24, 48 and 72 hours.

3. Evaluation of grapefruit juice for the prevention of paracetamol-induced hepatotoxicity was successfully performed in rats. Grapefruit juice has shown the ability to protect the liver against hepatic injury when a toxic dose of paracetamol was administered to rats. The severity of injury to the liver was minimal when grapefruit juice/bergamottin was co-administered with paracetamol as opposed to when paracetamol was administered alone.

Future studies

4. Further investigation to determine the exact mechanism that is responsible for the hepatoprotective effect of grapefruit juice is recommended.

CHAPTER 9

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APPENDICES

APPENDIX A: ENZYME INHIBITION EXPERIMENT

Appendix A-1: CYP1A2 assay template

Tube number	Sample name	pure CYP1A2 enzyme (µl)	HEPES pH 7.4 (µl)	EDTA 0.6 Mm (µl)	$MgSO_4$ 30 Mm (µl)	PARA 10 mg/ml vol (µl)	GFJ 25 Nm (µl)	E-RF 2.5 pmol/µl (µl)	RF (µl)	Distilled water (µl)	Pre-incubate 5 min	NADP (µl)	Incubate 20 min	Final vol (µl)
1	blank	10	62.5	25	40	0	0	12.5	0	50		50		250
2	CAL 1	10	62.5	25	40	0	0	12.5	5	45		50		250
3	CAL 2	10	62.5	25	40	0	0	12.5	10	40		50		250
4	CAL 3	10	62.5	25	40	0	0	12.5	15	35		50		250
5	CAL 4	10	62.5	25	40	0	0	12.5	20	30		50		250
6	CAL 5	10	62.5	25	40	0	0	12.5	25	25		50		250
7	**CNTRL**	**10**	**62.5**	**25**	**40**	**0**	**0**	**12.5**	**0**	**50**		**50**		**250**
8	**CNTRL**	**10**	**62.5**	**25**	**40**	**0**	**0**	**12.5**	**0**	**50**		**50**		**250**

Appendix A-2: CYP1A2 reaction rate results

Sample name	Metabolite per conc. (pmol/ml)	Metabolite final	Reaction rate pmol/min*mg	nmol/min*mg Mean	SD	% of control
Blank	0	0				
CAL 1	12.5	50				
CAL 2	25	100				
CAL 3	37.5	150				
CAL 4	50	200				
CAL 5	62.5	250				
CNTRL	**127.88**	**511.51**	**63.94**	**63.71**	**0.32**	**100**
CNTRL	**126.96**	**507.84**	**63.48**			

Reaction rate per group

Mean	SD	Median
63.71	**0.32**	**63.71**

Appendix A-3: CYP2E1 assay template

Tube number	Sample name	enzyme	0.1 M Phos-bf pH 7.4	10 mg/ml PARA	GFJ	0.848 mg/ml CZN	20 µg/ml 6-OHCZN	distilled water	Pre-incubate 5 min	NADP solution	Incubate 10 min	Final vol
1	blank	10	120	0	0	20	0	50		50		250
2	CAL 1	10	120	0	0	20	5	45		50		250
3	CAL 2	10	120	0	0	20	10	40		50		250
4	CAL 3	10	120	0	0	20	20	30		50		250
5	CAL 4	10	120	0	0	20	30	20		50		250
6	CAL 5	10	120	0	0	20	40	10		50		250
7	CAL 6	10	120	0	0	20	50	0		50		250
8	**CNTRL**	**10**	**120**	**0**	**0**	**20**	**0**	**50**		**50**		**250**
9	**CNTRL**	**10**	**120**	**0**	**0**	**20**	**0**	**50**		**50**		**250**

Appendix A-4: CYP2E1 reaction rate results

Sample name	Metabolite conc,	Metabolite per sample	Metabolite final conc	Reaction rate nmol/min*mg	Mean	nmol/min*mg SD	% of control
Blank	0.0	0.000	0.000				
CAL 1	0.1	0.141	0.760				
CAL 2	0.4	0.164	2.155				
CAL 3	0.8	0.259	4.310				
CAL 4	1.2	0.346	6.466				
CAL 5	1.6	0.528	8.621				
CNTRL			**0.860**	**0.11**	**0.12**	**0.02**	**100**
CNTRL			**1.08**	**0.13**			

Appendix A-4: CYP3A4 assay template

Sample name	enzyme	0.1 M phos-bf pH 7.4	100 mM MgCl	10 mg/ml PARA	GFJ	0.5 mg/ml MDZ	62.5 nM 1-OHMDZ	Distilled water	Pre-incubate 5 minutes	NADP	Incubate 10 min	final vol
blank	10	120	10	0	0	10	0	50		50		250
CAL 1	10	120	10	0	0	10	2.5	47.5		50		250
CAL 2	10	120	10	0	0	10	5	45		50		250
CAL 3	10	120	10	0	0	10	10	40		50		250
CAL 4	10	120	10	0	0	10	15	35		50		250
CAL 5	10	120	10	0	0	10	20	30		50		250
CNTRL	**10**	**120**	**10**	**0**	**0**	**10**	**0**	**50**		**50**		**250**
CNTRL	**10**	**120**	**10**	**0**	**0**	**10**	**0**	**50**		**50**		**250**

Appendix A-6: CYP3A4 reaction rate results

Sample name	Metabolite per sample (nmol/250 µl)	Metabolite final conc. (nmol/ml)	Metabolite final conc. (pmol/ml)	Reaction rate pmol/min*mg	Mean pmol/min*mg	SD	% of control
Blank	0.0000	0.000	0				
CAL 1	0.3125	1.25	1250				
CAL 2	0.6250	2.5	2500				
CAL 3	0.6250	5	5000				
CAL 4	1.8750	7.5	7500				
CAL 5	2.5000	10	10000				
CNTRL	**0.179**	**0.716**	**716**	**89.50**	**89.50**	**0.00**	**100**
CNTRL	**0.179**	**0.716**	**716**	**89.50**			

APPENDIX B: HPLC CALIBRATION OF PARACETAMOL OVER 5 DAYS

Appendix B-1: Calibration, day 1

Table B-1: Calibration data, day 1

Concentration (µg/ml)	Ratio
1	**0.11**
5	**0.44**
10	**0.78**
15	**1.06**
20	**1.38**

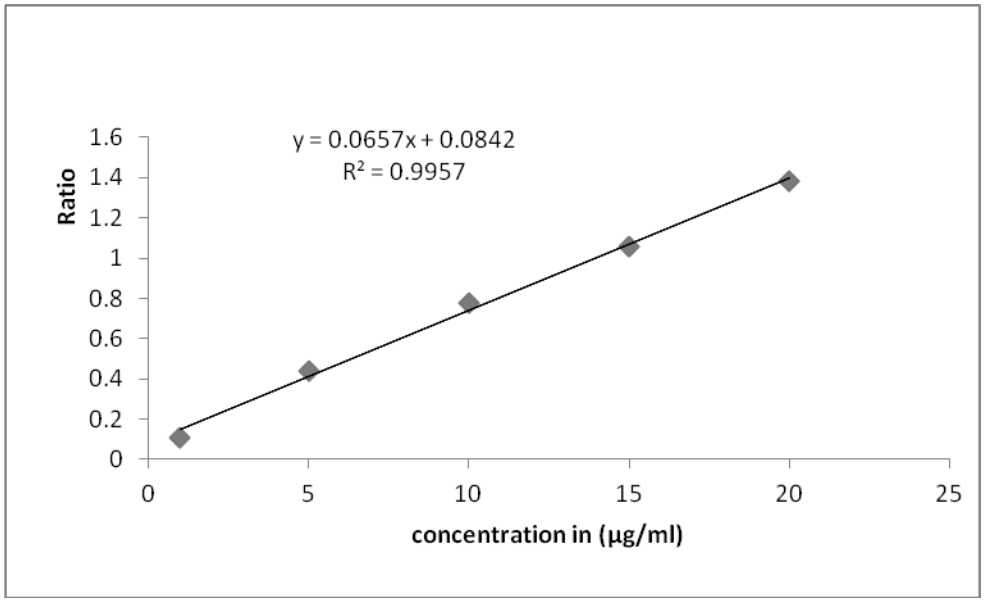

Figure A-1: Calibration curve, day 1

Appendix B-2: Calibration, day 2

Table B-2: Calibration data, day 2

Concentration (µg/ml)	Ratio
1	**0.13**
5	**0.42**
10	**0.76**
15	**0.91**
20	**1.07**

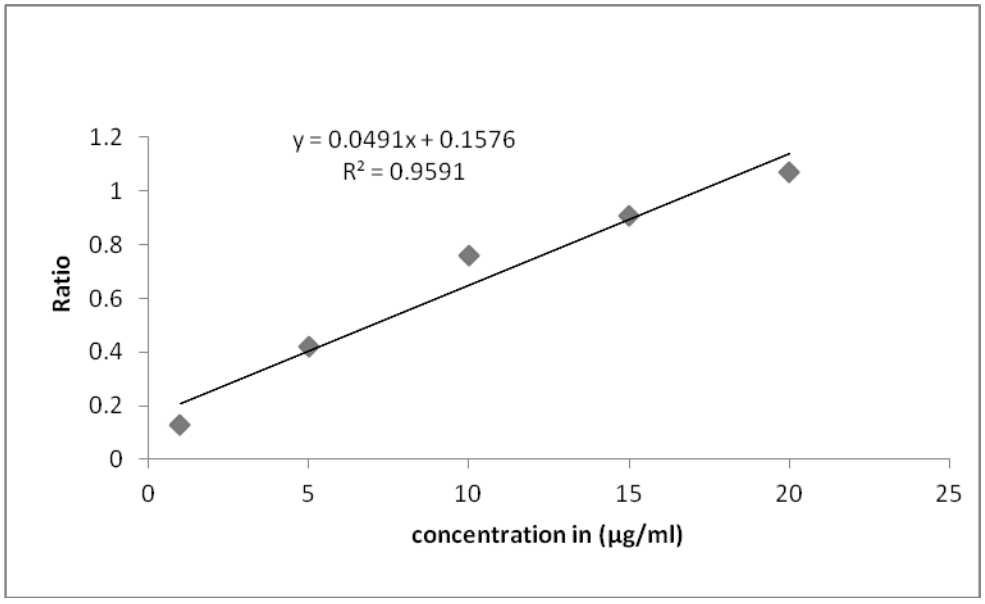

Figure B-2: Calibration curve, day 2

Appendix B-3: Calibration, day 3

Table B-3: Calibration data, day 3

Concentration (µg/ml)	Ratio
1	**0.14**
5	**0.39**
10	**0.74**
15	**1.06**
20	**1.34**

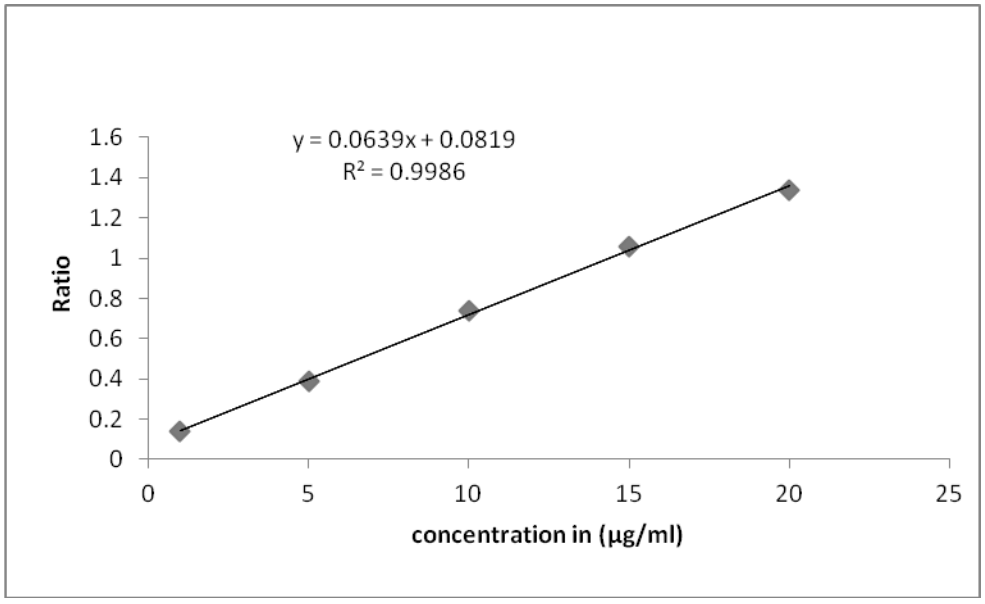

Figure B-3: Calibration curve, day

Appendix B-4: Calibration, day 4

Table B-4: Calibration data, day 4

Concentration (µg/ml)	Ratio
1	**0.13**
5	**0.35**
10	**0.74**
15	**0.99**
20	**1.32**

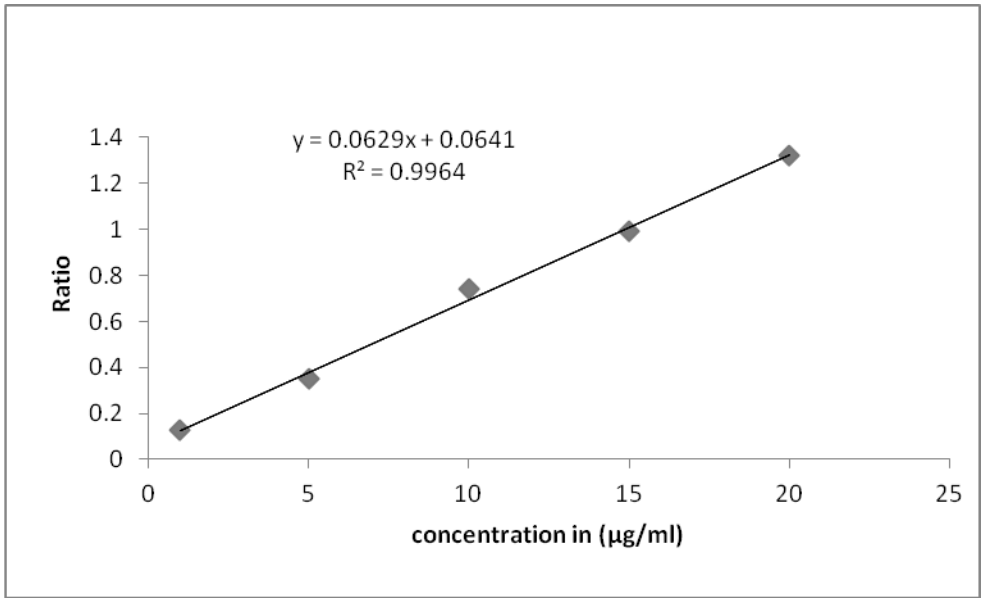

Figure B-4: Calibration curve, day 4

Appendix B-5: Calibration, day 5

Table B-5: Calibration data, day 5

Concentration (µg/ml)	Ratio
1	**0.11**
5	**0.41**
10	**0.71**
15	**0.90**
20	**1.30**

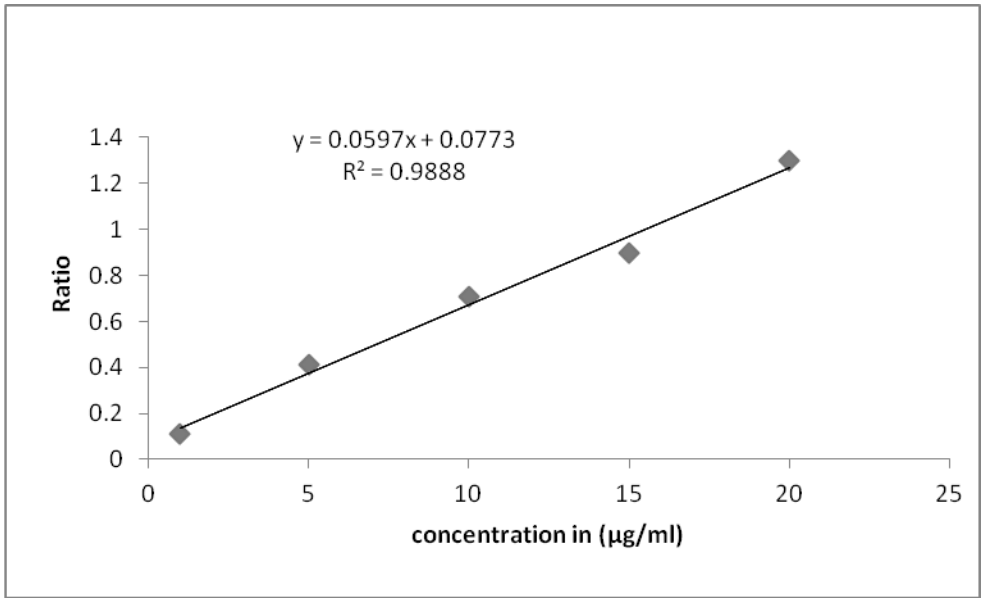

Figure B-5: Calibration curve, day 5

APPENDIX C: ACCURACY DETERMINATION OF PARACETAMOL

Appendix C-1: Accuracy of paracetamol HPLC assay

Table C-1: Accuracy data of paracetamol HPLC assay

Prep Conc. (µg/ml)	Meas Conc. 1 (µg/ml)	Meas Conc. 2 (µg/ml)	Meas Conc. 3 (µg/ml)	Meas Conc. 4 (µg/ml)	Meas Conc. 5 (µg/ml)	Mean	SD	% Acc	CV%
1	0.99	1.20	0.92	1.02	1.02	1.03	0.09	103	9.00
10	10.10	10.51	10.37	10.61	10.98	10.51	0.29	105	2.75
20	18.76	19.09	19.19	19.18	19.29	19.10	0.18	96	0.95

SD = standard deviation; **ACC =** accuracy; **CV% =** coefficient of variation

APPENDIX D: STABILITY DETERMINATION OF PARACETAMOL

Appendix D-1: Stability of paracetamol at room temperature

Table D-1: Table of stability data at room temperature at 8, 12 and 24 hours

Room Temperature							
Time	**Prep**	**Measd Conc. (µg/ml)**	**Measd**	**Mean**	**SD**	**%Stability**	**CV%**
8 hours	1	0.96	0.94	**0.95**	0.01	95	1.49
	10	9.45	9.75	**9.60**	0.21	96	2.21
	20	18.52	19.25	**18.89**	0.52	94	2.73
12 hours	1	0.95	0.99	**0.96**	0.05	96	5.18
	10	9.62	9.99	**9.81**	0.26	98	2.67
	20	18.26	18.98	**18.62**	0.51	93	2.73
24 hours	1	0.94	0.98	**0.96**	0.03	96	2.95
	10	9.96	10.03	**10.00**	0.05	100	0.47
	20	18.28	19.89	**19.09**	1.14	95	5.97

SD = standard deviation; **CV% =** coefficient of variation

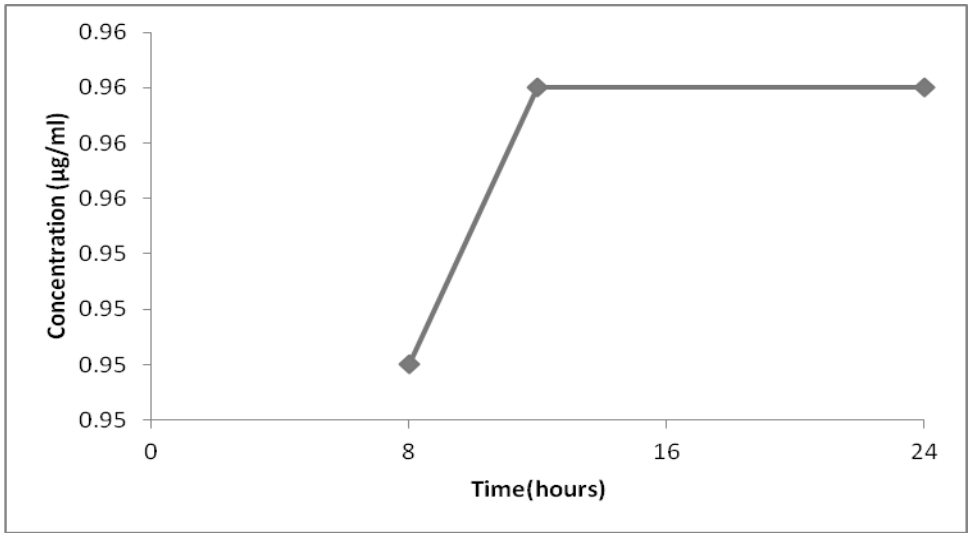

Figure D-1: Plot of stability of paracetamol (1 µg/ml) at room temperature over time

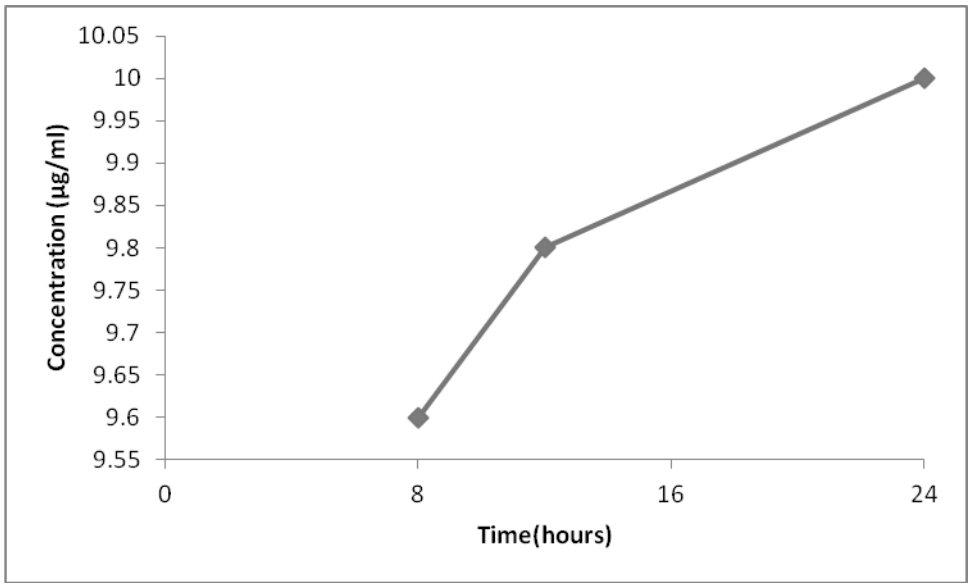

Figure D-2: Plot of stability of paracetamol (10 µg/ml) at room temperature over time

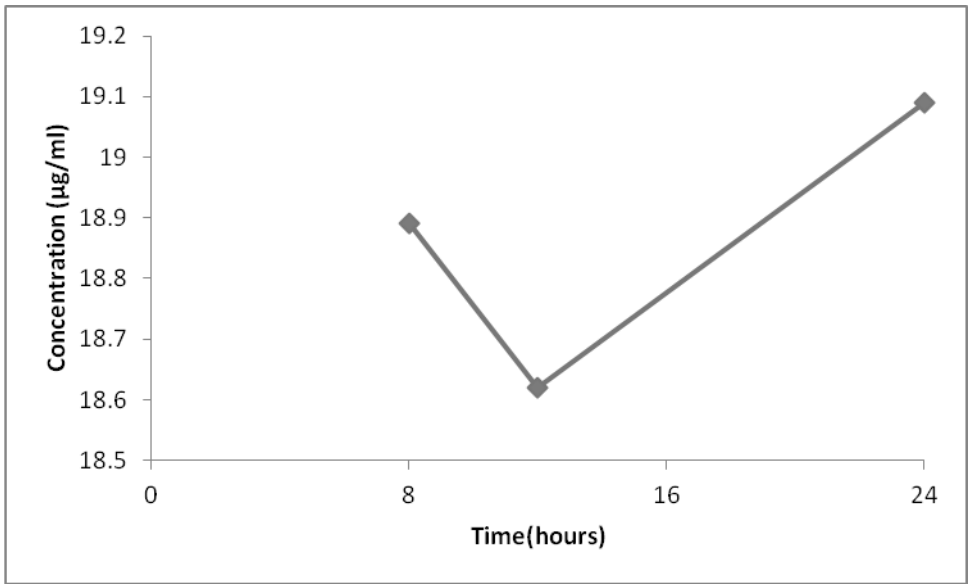

Figure D-3: Plot of stability of paracetamol (20 µg/ml) at room temperature over time

Appendix D-2: Stability of paracetamol at -20°C

Table D-2: Table of stability data at -20°C at 8, 12 and 24 hours, 14, 30 and 60 days

Time	Prep	Room Temperature Measd Conc. (µg/ml)	Measd	Mean	SD	%Stability	CV%
8 hours	1	0.93	0.95	**0.94**	0.01	94	1.50
	10	10.93	11.63	**11.21**	0.17	112	1.48
	20	20.74	19.29	**19.51**	0.62	98	3.17
12 hours	1	1.05	0.80	**0.93**	0.18	93	18.94
	10	11.26	11.69	**11.48**	0.30	115	2.64
	20	18.26	18.76	**19.78**	1.18	99	5.97
24 hours	1	0.90	0.91	**0.91**	0.01	91	0.78
	10	10.74	11.21	**10.98**	0.33	110	3.02
	20	19.55	18.44	**19.08**	0.78	95	4.13
14 days	1	0.92	0.93	**0.93**	0.01	93	0.76
	10	11.0	10.9	**10.92**	0.06	109	0.58
	20	18.4	17.9	**18.14**	0.31	92	1.73
30 days	1	0.58	0.51	**0.54**	0.05	54	9.90
	10	10.67	10.65	**10.66**	0.02	107	0.17
	20	19.04	19.71	**19.38**	0.48	97	2.47
60 days	1	0.55	0.58	**0.57**	0.02	57	3.75
	10	8.15	8.62	**8.42**	0.38	84	4.53
	20	19.62	18.05	**18.84**	1.11	94	5.89

SD = standard deviation; **CV% =** coefficient of variation

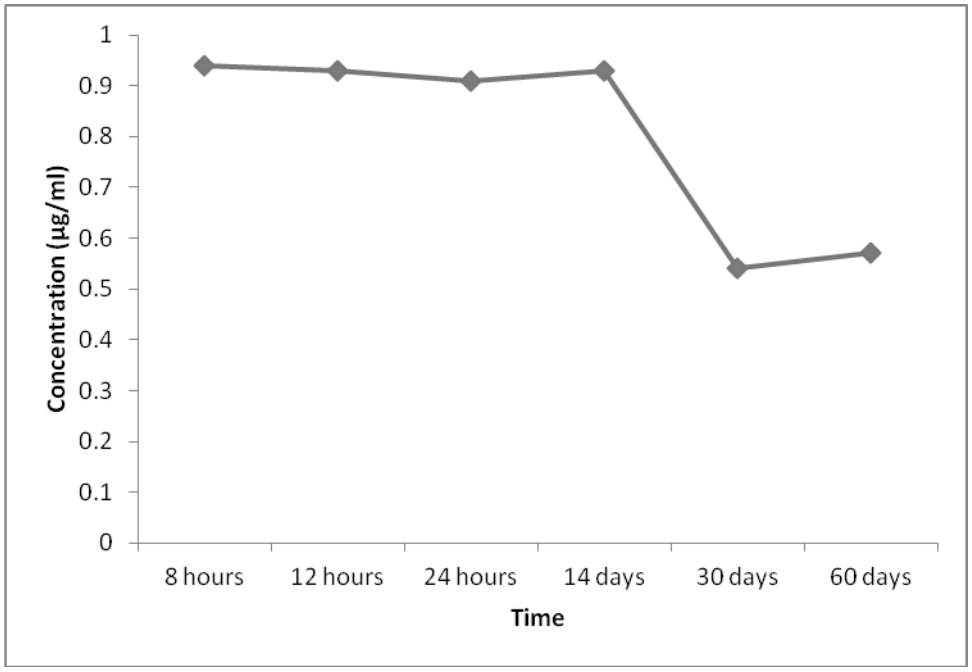

Figure D-3: Plot of stability of paracetamol (1 µg/ml) at -20°C over time

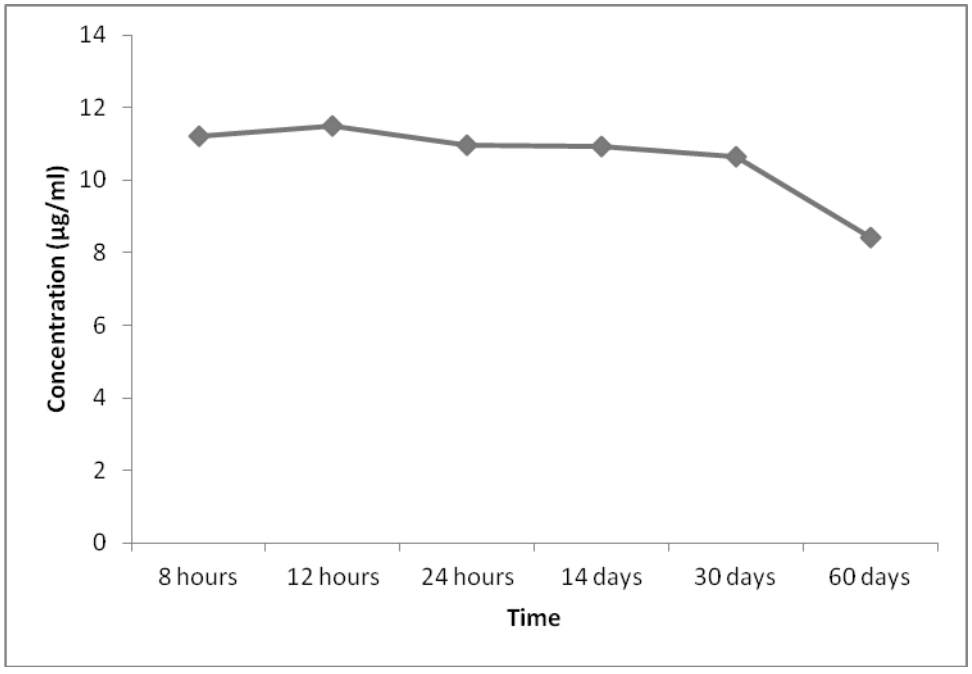

Figure D-3: Plot of stability of paracetamol (10 µg/ml) at -20°C over time

Figure D-3: Plot of stability of paracetamol (20 µg/ml) at -20°C over time

APPENDIX E: RAT WEIGHTS

Appendix E-1: Rat weights during the determination of a toxic dose experiment

Table E-1: Rat weights of groups **PARA 1000 mg/kg, PARA 1725 mg/kg** and **PARA 1822 mg/kg** at 24, 48 and 72 hours

Group	Time	Rat 1	Rat 2	Rat 3	Mean	SD
		PARA 1000 mg/kg				
0 hrs	**Before Rx**	240	220	231	**230**	10
24 hrs	**After Rx**	242	222	231	**232**	10
0 hrs	**Before Rx**	225	200	228	**218**	15
48 hrs	**After Rx**	241	239	242	**241**	2
0 hrs	**Before Rx**	241	238	242	**240**	8
72 hrs	**After Rx**	247	241	249	**245**	4
		PARA 1725 mg/kg				
0 hrs	**Before Rx**	241	244	242	**242**	2
24 hrs	**After Rx**	242	245	243	**243**	1
0 hrs	**Before Rx**	222	239	242	**234**	11
48 hrs	**After Rx**	229	239	___	**234**	7
0 hrs	**Before Rx**	219	222	227	**223**	4
72 hrs	**After Rx**	237	244	242	**241**	3
		PARA 1822 mg/kg				
0 hrs	**Before Rx**	239	241	244	**241**	3
24 hrs	**After Rx**	242	244	241	**242**	1
0 hrs	**Before Rx**	237	220	237	**232**	10
48 hrs	**After Rx**	249	239	250	**246**	6
0 hrs	**Before Rx**	239	241	244	**241**	3
72 hrs	**After Rx**	242	244	241	**242**	1

SD = standard deviation; **PARA** = paracetamol; **hrs** = hours; **Rx** = treatment

Appendix E-2: Rat weights during the ultimate experiment

Table E-2: Rat weights of groups **PARA, PARA+GFJ low** and **PARA+GFJ high** at 24, 48 and 72 hours

Group	Time	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
				PARA				
0 hrs `	**Before Rx**	241	244	242			**242**	2
24 hrs	**After Rx**	242	245	243			**243**	1
0 hrs	**Before Rx**	222	239	242			**234**	11
48 hrs	**After Rx**	229	239	___			**234**	7
0 hrs	**Before Rx**	219	222	227			**223**	4
72 hrs	**After Rx**	237	244	242			**241**	3
				PARA+GFJ low				
0 hrs	**Before Rx**	240	249	248	241	233	**242**	6
24 hrs	**After Rx**	242	251	240	244	235	**244**	6
0 hrs	**Before Rx**	201	208	210	206	204	**206**	3
48 hrs	**After Rx**	214	192	198	210	201	**203**	9
0 hrs	**Before Rx**	203	205	202	200	209	**204**	4
72 hrs	**After Rx**	213	225	212	218	229	**229**	7
				PARA+GFJ high				
0 hrs	**Before Rx**	229	206	227	234	240	**227**	13
24 hrs	**After Rx**	231	215	230	238	242	**231**	10
0 hrs	**Before Rx**	205	205	218	200	206	**207**	7
48 hrs	**After Rx**	224	191	200	216	212	**209**	13
0 hrs	**Before Rx**	225	209	220			**218**	8
72 hrs	**After Rx**	238	217	244			**233**	14

SD = standard deviation; **PARA** = paracetamol; **GFJ** = grapefruit juice; **hrs** = hours; **Rx** = treatment

Appendix E-3: Rat weights during the ultimate experiment

Table E-3: Rat weights of groups PARA+BGT low and PARA+BGT high at 24, 48 and 72 hours

Group	Time	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
				PARA+BGT low				
0 hrs	**Before Rx**	210	211	209	200		**208**	5
24 hrs	**After Rx**	200	198	214	225		**209**	13
0 hrs	**Before Rx**	223	227	213			**221**	7
48 hrs	**After Rx**	231	236	212			**226**	13
0 hrs	**Before Rx**	205	206	203	207		**205**	2
72 hrs	**After Rx**	217	218	230	218		**221**	7
				PARA+BGT high				
0 hrs	**Before Rx**	220	200	204	205	207	**207**	8
24 hrs	**After Rx**	202	189	207	202	236	**207**	17
0 hrs	**Before Rx**	208	217	212			**212**	5
48 hrs	**After Rx**	227	234	229			**230**	18
0 hrs	**Before Rx**	205	206	203	207		**205**	2
72 hrs	**After Rx**	217	218	231	218		**221**	16

SD = standard deviation; **PARA** = paracetamol; **BGT** = bergamottin; **hrs** = hours; **Rx** = treatment

APPENDIX F: LIVER FUNCTION TEST RESULTS

Appendix F-1: LFTs for the determination of a toxic dose

Table F-1: LFTs (u/l) of groups **PARA 1000 mg/kg (low), PARA 1725 mg/kg (medium)** and **PARA 1822 mg/kg (high)** at 24, 48 and 72 hours

Group	Parameter	rat 1	rat 2	rat 3	mean	SD
			PARA low			
24 hours	**ALP**	191	342	353	**295**	91
	AST	93	106	112	**104**	10
	ALT	34	45	37	**39**	6
48 hours	**ALP**	270	73	179	**174**	99
	AST	102	80	92	**91**	11
	ALT	39	48	45	**44**	5
72 hours	**ALP**	334	58	338	**243**	161
	AST	108	125	133	**122**	13
	ALT	49	42	42	**44**	4
			PARA medium			
24 hours	**ALP**	321	320	317	**319**	2
	AST	113	244	111	**156**	76
	ALT	41	111	38	**63**	41
48 hours	**ALP**	191	401	___	**296**	145
	AST	1073	1645	___	**1359**	404
	ALT	647	1026	___	**837**	268
72 hours	**ALP**	293	222	265	**260**	36
	AST	1189	259	88	**512**	593
	ALT	1094	118	45	**419**	586
			PARA high			
24 hours	**ALP**	334	42	75	150	160
	AST	96	108	558	**254**	263
	ALT	42	44	153	**80**	64
48 hours	**ALP**	231	153	364	**249**	107
	AST	1201	156	1651	**1003**	767
	ALT	449	64	728	**414**	333
72 hours	**ALP**	256	130	285	**224**	82
	AST	110	88	114	**104**	14
	ALT	47	42	44	**44**	3

LFT = liver function test; **ALP** = alkaline phosphatase; **AST** = aspartate aminotransferase; **ALT** = alanine aminotransferase; **PARA** = paracetamol; **SD** = standard deviation

APPENDIX G: LIVER FUNCTION TEST RESULTS

Appendix G-1: LFTs for the ultimate experiment

Table G-1: LFTs (u/l) of groups **saline** and **PARA** at 24, 48 and 72 hours

Group	Parameter	rat 1	rat 2	rat 3	mean	SD
			Saline			
24 hours	**ALP**	277	190	212	**226**	45
	AST	68	64	48	**60**	11
	ALT	56	40	36	**44**	11
48 hours	**ALP**	374	370	353	**367**	11
	AST	59	324	58	**147**	153
	ALT	47	60	48	**52**	7
72 hours	**ALP**	238	75	96	**136**	89
	AST	52	56	55	**54**	3
	ALT	38	37	43	**39**	1
			PARA			
24 hours	**ALP**	321	320	317	**319**	2
	AST	113	244	111	**156**	76
	ALT	41	111	38	**63**	41
48 hours	**ALP**	191	401	___	**296**	149
	AST	1073	1645	___	**1359**	405
	ALT	647	1026	___	**837**	268
72 hours	**ALP**	293	222	285	**267**	39
	AST	1189	259	89	**512**	658
	ALT	1094	118	45	**419**	690

LFT = liver function test; **ALP** = alkaline phosphatase; **AST** = aspartate aminotransferase; **ALT** = alanine aminotransferase; **PARA** = paracetamol; **SD** = standard deviation

Appendix G-2: LFTs of ultimate experiment

Table G-2: LFTs (u/l) of groups PARA+GFJ low and PARA+GFJ high at **24, 48 and 72 hours**

Group	Parameter	rat 1	rat 2	rat 3	mean	SD
			PARA+GFJ low			
24 hours	**ALP**	27	23	137	**62**	65
	AST	69	74	64	**69**	5
	ALT	7	12	9	**9**	3
48 hours	**ALP**	296	249	391	**312**	72
	AST	1386	126	410	**641**	661
	ALT	402	2	1480	**628**	765
72 hours	**ALP**	338	350	166	**285**	103
	AST	79	131	44	**85**	44
	ALT	3	7	___	**5**	3
			PARA+GFJ high			
24 hours	**ALP**	344	142	151	**212**	114
	AST	215	119	89	**141**	66
	ALT	23	9	21	**18**	8
48 hours	**ALP**	249	234	184	**222**	34
	AST	492	131	92	**238**	221
	ALT	90	11	1	**30**	49
72 hours	**ALP**	363	294	324	**327**	35
	AST	205	103	133	**147**	52
	ALT	56	21	5	**27**	26

LFT = liver function test; **ALP** = alkaline phosphatase; **AST** = aspartate aminotransferase; **ALT** = alanine aminotransferase; **PARA** = paracetamol; **GFJ** = grapefruit juice; **SD** = standard deviation

Appendix G-3: LFTs of ultimate experiment

Table G-3: LFTs (u/l) of groups PARA+BGT low and PARA+BGT high at **24, 48 and 72 hours**

Group	Parameter	rat 1	rat 2	rat 3	mean	SD
			PARA+BGT low			
24 hours	**ALP**	292	130	161	**194**	86
	AST	147	67	69	**94**	46
	ALT	9	12	6	**9**	3
48 hours	**ALP**	39	190	240	**156**	105
	AST	82	74	88	**81**	7
	ALT	23	26	2	**17**	13
72 hours	**ALP**	326	327	197	**283**	75
	AST	70	104	74	**83**	19
	ALT	11	21	3	**12**	7
			PARA+BGT high			
24 hours	**ALP**	215	310	279	**268**	48
	AST	102	96	114	**104**	9
	ALT	4	12	11	**9**	1
48 hours	**ALP**	280	353	309	**314**	37
	AST	164	121	220	**168**	50
	ALT	14	14	38	**22**	14
72 hours	**ALP**	235	256	219	**237**	19
	AST	90	176	65	**110**	58
	ALT	20	10	5	**12**	8

LFT = liver function test; **ALP** = alkaline phosphatase; **AST** = aspartate aminotransferase; **ALT** = alanine aminotransferase; **PARA** = paracetamol; **BGT** = bergamottin; **SD** = standard deviation

APPENDIX H: FULL BLOOD COUNT RESULTS

Appendix H-1: FBC of ultimate experiment

Table H-1: FBC of groups **PARA** and **PARA+GFJ high at 24 hours**

Group	Rat 1	Rat 2	Rat 3	Mean	SD
		PARA			
White cell count	4.3	5.1	3.0	**4.1**	**1.1**
Red cell count	7.5	7.6	7.8	**7.6**	**0.2**
Haemoglobin	14.6	15.3	15.6	**15.2**	**0.5**
Haematocrit	0.425	0.440	0.453	**0.439**	**0.0**
MCV	57.0	57.8	58.1	**57.6**	**0.6**
MCH	19.6	20.1	20.0	**19.9**	**0.3**
MCHC	34.4	34.8	34.4	**34.5**	**0.2**
Platelets count	**1245**	**1085**	**1148**	**1159**	**80.6**
		PARA+GFJ high			
White cell count	6.1	8.1	4.3	**6.2**	**1.9**
Red cell count	6.2	6.6	7.1	**6.6**	**0.4**
Haemoglobin	12.8	13.8	14.4	**13.7**	**0.8**
Haematocrit	0.377	0.414	0.425	**0.405**	**0.0**
MCV	60.4	62.7	60.0	**61.0**	**1.5**
MCH	20.5	20.9	20.3	**20.6**	**0.3**
MCHC	34.0	33.3	33.9	**33.7**	**33.7**
Platelets count	**996**	**1104**	**1164**	**1088**	**85.1**
		PARA+BGT high			
White cell count	7.9	7.2	5.8	**7.0**	**1.1**
Red cell count	6.9	5.4	5.5	**5.9**	**0.8**
Haemoglobin	14.3	11.3	11.7	**12.4**	**1.6**
Haematocrit	0.443	0.353	0.349	**0.382**	**0.1**
MCV	64.7	64.9	63.5	**64.4**	**0.8**
MCH	20.9	20.1	21.3	**20.8**	**0.6**
MCHC	32.2	32.0	33.5	**32.6**	**0.8**
Platelets count	**879**	**889**	**1001**	**923**	**67.7**

FBC = full blood count; **MCV** = mean corpuscular volume; **MCH** = mean corpuscular haemoglobin; **MCHC** = mean corpuscular haemoglobin concentration; **SD** = standard deviation

Appendix H-2: FBC of ultimate experiment

Table H-2: FBC of groups **PARA** and **PARA+GFJ high** at **48 hours**

Group	Rat 1	Rat 2	Rat 3	Mean	SD
		PARA			
White cell count	5.9	5.9	___	**5.9**	0.0
Red cell count	7.8	7.2	___	**7.5**	0.4
Haemoglobin	15.9	14.8	___	**15.3**	0.8
Haematocrit	0.480	0.447	___	**0.464**	0.0
MCV	61.5	62.8	___	**62.2**	0.9
MCH	20.4	20.5	___	**20.5**	0.1
MCHC	34.1	33.1	___	**33.1**	0.0
Platelets count	**256**	**95**	___	**311**	305
		PARA+GFJ high			
White cell count	4.7	3.3	6.2	**4.7**	1.4
Red cell count	6.4	5.6	6.3	**6.1**	0.4
Haemoglobin	13.2	11.9	13.9	**13.0**	1.0
Haematocrit	0.408	0.357	0.419	**0.395**	0.0
MCV	60.1	63.4	66.1	**64.5**	1.4
MCH	20.7	21.1	21.9	**21.2**	0.5
MCHC	32.4	33.3	33.2	**33.0**	33.7
Platelets count	**492**	**380**	**520**	**464**	**74.1**
		PARA+BGT high			
White cell count	7.5	6.3	6.0	**6.6**	0.8
Red cell count	6.9	5.6	6.8	**6.4**	0.7
Haemoglobin	14.0	12.0	14.6	**13.5**	1.4
Haematocrit	0.402	0.360	0.422	**0.395**	0.0
MCV	58.3	64.4	62.4	**61.7**	3.1
MCH	20.3	21.5	21.6	**21.1**	0.7
MCHC	34.8	33.3	34.6	**34.2**	0.8
Platelets count	**949**	**480**	**890**	**773**	255.5

FBC = full blood count; **MCV** = mean corpuscular volume; **MCH** = mean corpuscular haemoglobin; **MCHC** = mean corpuscular haemoglobin concentration; **SD** = standard deviation

Appendix H-3: FBC of ultimate experiment

Table H-3: FBC of groups **PARA** and **PARA+GFJ high** at **72 hours**

Group	Rat 1	Rat 2	Rat 3	Mean	SD
		PARA			
White cell count	3.1	5.0	6.0	**4.7**	1.5
Red cell count	5.8	7.7	7.1	**6.9**	1.0
Haemoglobin	11.2	15.0	14.6	**13.6**	2.1
Haematocrit	0.317	0.457	0.443	**0.406**	0.1
MCV	54.7	59.7	62.2	**58.9**	3.8
MCH	19.3	19.6	20.5	**19.8**	0.6
MCHC	35.3	32.8	33.0	**33.7**	1.4
Platelets count	**29**	**336**	**614**	**326**	292.6
		PARA+GFJ high			
White cell count	6.8	6.5	6.7	**6.7**	0.2
Red cell count	6.0	6.2	6.3	**6.2**	0.2
Haemoglobin	12.6	13.0	13.6	**13.0**	0.5
Haematocrit	0.380	0.395	0.423	**0.399**	0.0
MCV	63.5	63.7	67.4	**64.9**	2.2
MCH	21.1	21.0	21.7	**21.3**	0.5
MCHC	33.2	32.9	32.2	**32.8**	33.7
Platelets count	**321**	**509**	**646**	**492**	163.2
		PARA+BGT high			
White cell count	6.6	5.0	5.1	**5.6**	0.9
Red cell count	7.1	6.0	6.8	**6.7**	0.6
Haemoglobin	15.0	12.6	13.0	**13.5**	1.3
Haematocrit	0.457	0.389	0.400	0.415	0.0
MCV	63.6	64.4	58.9	**62.3**	3.0
MCH	20.9	20.9	19.1	**20.3**	1.0
MCHC	32.8	32.4	32.5	**32.6**	0.2
Platelets count	**643**	**850**	**847**	**780**	118.7

FBC = full blood count; **MCV** = mean corpuscular volume; **MCH** = mean corpuscular haemoglobin; **MCHC** = mean corpuscular haemoglobin concentration; **SD** = standard deviation

APPENDIX I: PARACETAMOL PLASMA CONCENTRATIONS

Appendix I-1: Paracetamol plasma levels of ultimate experiment

Table I-1: Paracetamol plasma levels (µg/ml) of groups **PARA, PARA+GFJ high and PARA+BGT high**

Group	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
			PARA				
24 hours	28.15	5.72	4.38	___	___	**12.75**	13.4
48 hours	2.22	6.67	___	___	___	**4.45**	3.1
72 hours	1.61	2.02	13.37	___	___	**6.33**	7.8
			PARA+GFJ high				
24 hours	18.81	3.99	12.87	___	___	**11.89**	7.5
48 hours	0.18	1.42	0.92	___	___	**0.84**	0.6
72 hours	1.14	1.67	0.99	___	___	**1.27**	0.36
			PARA+BGT high				
24 hours	6.84	7.78	8.60	___	___	**7.74**	0.88
48 hours	5.26	16.93	5.57	___	___	**9.25**	6.7
72 hours	0.16	0.13	0.00	___	___	**0.09**	0.08

PARA = paracetamol; **GFJ** = grapefruit juice; **BGT** = bergamottin; **SD** = standard deviation

APPENDIX J: P-VALUES

Appendix J-1: P-values of LFTs of groups saline, PARA and PARA+GFJ

Aspartate transminase (AST)

Kruskal-Wallis Test (Nonparametric ANOVA)

The P value is 0.0389, considered significant.

Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 16.251 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison			Mean Rank Difference		P value
saline 24	vs.	saline 48	-5.667	ns	P>0.05
saline 24	vs.	saline 72	2.333	ns	P>0.05
saline 24	vs.	para 24	-10.333	ns	P>0.05
saline 24	vs.	para 48	-19.667	ns	P>0.05
saline 24	vs.	para 72	-13.167	ns	P>0.05
saline 24	vs.	para+gfj 24	-9.167	ns	P>0.05
saline 24	vs.	para+gfj 48	-11.333	ns	P>0.05
saline 24	vs.	para+gfj 72	-10.333	ns	P>0.05
saline 48	vs.	saline 72	8.000	ns	P>0.05
saline 48	vs.	para 24	-4.667	ns	P>0.05
saline 48	vs.	para 48	-14.000	ns	P>0.05
saline 48	vs.	para 72	-7.500	ns	P>0.05

saline 48	vs.	para+gfj 24	-3.500	ns	P>0.05
saline 48	vs.	para+gfj 48	-5.667	ns	P>0.05
saline 48	vs.	para+gfj 72	-4.667	ns	P>0.05
saline 72	vs.	para 24	-12.667	ns	P>0.05
saline 72	vs.	para 48	-22.000	ns	P>0.05
saline 72	vs.	para 72	-15.500	ns	P>0.05
saline 72	vs.	para+gfj 24	-11.500	ns	P>0.05
saline 72	vs.	para+gfj 48	-13.667	ns	P>0.05
saline 72	vs.	para+gfj 72	-12.667	ns	P>0.05
para 24	vs.	para 48	-9.333	ns	P>0.05
para 24	vs.	para 72	-2.833	ns	P>0.05
para 24	vs.	para+gfj 24	1.167	ns	P>0.05
para 24	vs.	para+gfj 48	-1.000	ns	P>0.05
para 24	vs.	para+gfj 72	0.000	ns	P>0.05
para 48	vs.	para 72	6.500	ns	P>0.05
para 48	vs.	para+gfj 24	10.500	ns	P>0.05
para 48	vs.	para+gfj 48	8.333	ns	P>0.05
para 48	vs.	para+gfj 72	9.333	ns	P>0.05
para 72	vs.	para+gfj 24	4.000	ns	P>0.05
para 72	vs.	para+gfj 48	1.833	ns	P>0.05
para 72	vs.	para+gfj 72	2.833	ns	P>0.05
para+gfj 24	vs.	para+gfj 48	-2.167	ns	P>0.05
para+gfj 24	vs.	para+gfj 72	-1.167	ns	P>0.05
para+gfj 48	vs.	para+gfj 72	1.000	ns	P>0.05

Appendix J-2: P-values of LFTs of groups saline, PARA and PARA+BGT

Aspartate transminase (AST)

The P value is 0.0316, considered significant.

Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because exact calculations would have taken too long.

Kruskal-Wallis Statistic KW = 16.863

Dunn's Multiple Comparisons Test

Comparison			Mean Rank Difference	P value	
saline 24	vs.	saline 48	-5.667	ns	P>0.05
saline 24	vs.	saline 72	2.667	ns	P>0.05
saline 24	vs.	para 24	-11.000	ns	P>0.05
saline 24	vs.	para 48	-19.333	ns	P>0.05
saline 24	vs.	para 72	-13.333	ns	P>0.05
saline 24	vs.	para+bgt 24	-8.000	ns	P>0.05
saline 24	vs.	para+bgt 48	-12.667	ns	P>0.05
saline 24	vs.	para+bgt 72	-7.000	ns	P>0.05
saline 48	vs.	saline 72	8.333	ns	P>0.05
saline 48	vs.	para 24	-5.333	ns	P>0.05
saline 48	vs.	para 48	-13.667	ns	P>0.05
saline 48	vs.	para 72	-7.667	ns	P>0.05
saline 48	vs.	para+bgt 24	-2.333	ns	P>0.05
saline 48	vs.	para+bgt 48	-7.000	ns	P>0.05
saline 48	vs.	para+bgt 72	-1.333	ns	P>0.05

saline 72	vs.	para 24	-13.667	ns	P>0.05
saline 72	vs.	para 48	-22.000	ns	P>0.05
saline 72	vs.	para 72	-16.000	ns	P>0.05
saline 72	vs.	para+bgt 24	-10.667	ns	P>0.05
saline 72	vs.	para+bgt 48	-15.333	ns	P>0.05
saline 72	vs.	para+bgt 72	-9.667	ns	P>0.05
para 24	vs.	para 48	-8.333	ns	P>0.05
para 24	vs.	para 72	-2.333	ns	P>0.05
para 24	vs.	para+bgt 24	3.000	ns	P>0.05
para 24	vs.	para+bgt 48	-1.667	ns	P>0.05
para 24	vs.	para+bgt 72	4.000	ns	P>0.05
para 48	vs.	para 72	6.000	ns	P>0.05
para 48	vs.	para+bgt 24	11.333	ns	P>0.05
para 48	vs.	para+bgt 48	6.667	ns	P>0.05
para 48	vs.	para+bgt 72	12.333	ns	P>0.05
para 72	vs.	para+bgt 24	5.333	ns	P>0.05
para 72	vs.	para+bgt 48	0.6667	ns	P>0.05
para 72	vs.	para+bgt 72	6.333	ns	P>0.05
para+bgt 24	vs.	para+bgt 48	-4.667	ns	P>0.05
para+bgt 24	vs.	para+bgt 72	1.000	ns	P>0.05
para+bgt 48	vs.	para+bgt 72	5.667	ns	P>0.05

Appendix J-3: P-values of LFTs of groups saline, PARA and PARA+GFJ

Alanine transminase (ALT)

The P value is 0.0676, considered not quite significant.

Variation among column medians is not significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 14.590 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison			Mean Rank Difference		P value
saline 24	vs.	saline 48	-4.833	ns	P>0.05
saline 24	vs.	saline 72	1.667	ns	P>0.05
saline 24	vs.	para 24	-2.333	ns	P>0.05
saline 24	vs.	para 48	-11.667	ns	P>0.05
saline 24	vs.	para 72	-8.500	ns	P>0.05
saline 24	vs.	para+gfj 24	7.667	ns	P>0.05
saline 24	vs.	para+gfj 48	4.167	ns	P>0.05
saline 24	vs.	para+gfj 72	4.167	ns	P>0.05
saline 48	vs.	saline 72	6.500	ns	P>0.05
saline 48	vs.	para 24	2.500	ns	P>0.05
saline 48	vs.	para 48	-6.833	ns	P>0.05
saline 48	vs.	para 72	-3.667	ns	P>0.05
saline 48	vs.	para+gfj 24	12.500	ns	P>0.05
saline 48	vs.	para+gfj 48	9.000	ns	P>0.05

saline 48	vs.	para+gfj 72	9.000	ns	P>0.05
saline 72	vs.	para 24	-4.000	ns	P>0.05
saline 72	vs.	para 48	-13.33	ns	P>0.05
saline 72	vs.	para 72	-10.167	ns	P>0.05
saline 72	vs.	para+gfj 24	6.000	ns	P>0.05
saline 72	vs.	para+gfj 48	2.500	ns	P>0.05
saline 72	vs.	para+gfj 72	2.500	ns	P>0.05
para 24	vs.	para 48	-9.333	ns	P>0.05
para 24	vs.	para 72	-6.167	ns	P>0.05
para 24	vs.	para+gfj 24	10.000	ns	P>0.05
para 24	vs.	para+gfj 48	6.500	ns	P>0.05
para 24	vs.	para+gfj 72	6.500	ns	P>0.05
para 48	vs.	para 72	3.167	ns	P>0.05
para 48	vs.	para+gfj 24	19.333	ns	P>0.05
para 48	vs.	para+gfj 48	15.833	ns	P>0.05
para 48	vs.	para+gfj 72	15.833	ns	P>0.05
para 72	vs.	para+gfj 24	16.167	ns	P>0.05
para 72	vs.	para+gfj 48	12.667	ns	P>0.05
para 72	vs.	para+gfj 72	12.667	ns	P>0.05
para+gfj 24	vs.	para+gfj 48	-3.500	ns	P>0.05
para+gfj 24	vs.	para+gfj 72	-3.500	ns	P>0.05
para+gfj 48	vs.	para+gfj 72	0.000	ns	P>0.05

Appendix J-4: P-values of LFTs of groups saline, PARA and PARA+BGT

Alanine transminase (ALT)

The P value is 0.0069, considered very significant.

Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because at least one column has two or more identical values.

Kruskal-Wallis Statistic KW = 21.084 (corrected for ties)

Dunn's Multiple Comparisons Test

Comparison			Mean Rank Difference	P	value
saline 24	vs.	saline 48	-5.000	ns	P>0.05
saline 24	vs.	saline 72	1.667	ns	P>0.05
saline 24	vs.	para 24	-2.000	ns	P>0.05
saline 24	vs.	para 48	-10.167	ns	P>0.05
saline 24	vs.	para 72	-7.667	ns	P>0.05
saline 24	vs.	para+bgt 24	11.000	ns	P>0.05
saline 24	vs.	para+bgt 48	6.000	ns	P>0.05
saline 24	vs.	para+bgt 72	10.000	ns	P>0.05
saline 48	vs.	saline 72	6.667	ns	P>0.05
saline 48	vs.	para 24	3.000	ns	P>0.05
saline 48	vs.	para 48	-5.167	ns	P>0.05
saline 48	vs.	para 72	-2.667	ns	P>0.05
saline 48	vs.	para+bgt 24	16.000	ns	P>0.05
saline 48	vs.	para+bgt 48	11.000	ns	P>0.05
saline 48	vs.	para+bgt 72	15.000	ns	P>0.05

saline 72	vs.	para 24	-3.667	ns	P>0.05
saline 72	vs.	para 48	-11.833	ns	P>0.05
saline 72	vs.	para 72	-9.333	ns	P>0.05
saline 72	vs.	para+bgt 24	9.333	ns	P>0.05
saline 72	vs.	para+bgt 48	4.333	ns	P>0.05
saline 72	vs.	para+bgt 72	8.333	ns	P>0.05
para 24	vs.	para 48	-8.167	ns	P>0.05
para 24	vs.	para 72	-5.667	ns	P>0.05
para 24	vs.	para+bgt 24	13.000	ns	P>0.05
para 24	vs.	para+bgt 48	8.000	ns	P>0.05
para 24	vs.	para+bgt 72	12.000	ns	P>0.05
para 48	vs.	para 72	2.500	ns	P>0.05
para 48	vs.	para+bgt 24	21.167	ns	P>0.05
para 48	vs.	para+bgt 48	16.167	ns	P>0.05
para 48	vs.	para+bgt 72	20.167	ns	P>0.05
para 72	vs.	para+bgt 24	18.667	ns	P>0.05
para 72	vs.	para+bgt 48	13.667	ns	P>0.05
para 72	vs.	para+bgt 72	17.667	ns	P>0.05
para+bgt 24	vs.	para+bgt 48	-5.000	ns	P>0.05
para+bgt 24	vs.	para+bgt 72	-1.000	ns	P>0.05
para+bgt 48	vs.	para+bgt 72	4.000	ns	P>0.05

APPENDIX K: P-VALUES OF PARACETAMOL PLASMA CONCENTRATIONS

Appendix K-1: P-values of PARA plasma concentrations of groups PARA, PARA+GFJ and PARA+BGT

The P value is 0.0156, considered significant.

Variation among column medians is significantly greater than expected by chance.

The P value is approximate (from chi-square distribution) because exact calculations would have taken too long.

Kruskal-Wallis Statistic KW = 18.869

Dunn's Multiple Comparisons Test

Comparison			Mean Rank Difference	P	value
PARA 24	vs.	PARA 48	4.000	ns	P>0.05
PARA 24	vs.	PARA 72	4.667	ns	P>0.05
PARA 24	vs.	PARA+GFJ 24	-1.000	ns	P>0.05
PARA 24	vs.	PARA+GFJ 48	13.333	ns	P>0.05
PARA 24	vs.	PARA+GFJ 72	11.333	ns	P>0.05
PARA 24	vs.	PARA+BGT 24	-1.000	ns	P>0.05
PARA 24	vs.	PARA+BGT 48	0.6667	ns	P>0.05
PARA 24	vs.	PARA+BGT 72	17.000	ns	P>0.05
PARA 48	vs.	PARA 72	0.6667	ns	P>0.05
PARA 48	vs.	PARA+GFJ 24	-5.000	ns	P>0.05
PARA 48	vs.	PARA+GFJ 48	9.333	ns	P>0.05
PARA 48	vs.	PARA+GFJ 72	7.333	ns	P>0.05

PARA 48	vs.	PARA+BGT 24	-5.000	ns	P>0.05
PARA 48	vs.	PARA+BGT 48	-3.333	ns	P>0.05
PARA 48	vs.	PARA+BGT 72	13.000	ns	P>0.05
PARA 72	vs.	PARA+GFJ 24	-5.667	ns	P>0.05
PARA 72	vs.	PARA+GFJ 48	8.667	ns	P>0.05
PARA 72	vs.	PARA+GFJ 72	6.667	ns	P>0.05
PARA 72	vs.	PARA+BGT 24	-5.667	ns	P>0.05
PARA 72	vs.	PARA+BGT 48	-4.000	ns	P>0.05
PARA 72	vs.	PARA+BGT 72	12.333	ns	P>0.05
PARA+GFJ 24	vs.	PARA+GFJ 48	14.333	ns	P>0.05
PARA+GFJ 24	vs.	PARA+GFJ 72	12.333	ns	P>0.05
PARA+GFJ 24	vs.	PARA+BGT 24	0.000	ns	P>0.05
PARA+GFJ 24	vs.	PARA+BGT 48	1.667	ns	P>0.05
PARA+GFJ 24	vs.	PARA+BGT 72	18.000	ns	P>0.05
PARA+GFJ 48	vs.	PARA+GFJ 72	-2.000	ns	P>0.05
PARA+GFJ 48	vs.	PARA+BGT 24	-14.333	ns	P>0.05
PARA+GFJ 48	vs.	PARA+BGT 48	-12.667	ns	P>0.05
PARA+GFJ 48	vs.	PARA+BGT 72	3.667	ns	P>0.05
PARA+GFJ 72	vs.	PARA+BGT 24	-12.333	ns	P>0.05
PARA+GFJ 72	vs.	PARA+BGT 48	-10.667	ns	P>0.05
PARA+GFJ 72	vs.	PARA+BGT 72	5.667	ns	P>0.05
PARA+BGT 24	vs.	PARA+BGT 48	1.667	ns	P>0.05
PARA+BGT 24	vs.	PARA+BGT 72	18.000	ns	P>0.05
PARA+BGT 48	vs.	PARA+BGT 72	16.333	ns	P>0.05

APPENDIX L: PUBLICATIONS

Appendix L-1: Conference abstracts

Poster presentation

R. Baleni, Z. Bekker and A. Walubo. **Grapefruit Juice Prevented the Rise in Liver Enzymes After Paracetamol Overdose in Rats.** World Pharmacology Congress (WCP 2014), Cape Town, South Africa. 13 - 18 July 2014.

Faculty Forum 2014, Faculty of Health Sciences of the University of the Free State, Bloemfontein, South Africa, 28 - 29 August 2014.

Appendix L-2: Manuscripts in preparation

R.Baleni., Z. Bekker., A. Walubo and J.B Du Plessis: Co-administration of Fresh Grapefruit Juice (GFJ) and Bergamottin Prevented Paracetamol-Induced Hepatotoxicity After Paracetamol Overdose In Rats. Approved May 2015

Grapefruit juice prevented the rise in liver enzymes after paracetamol overdose in rats

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Objective:

Paracetamol (PARA) is a widely used analgesic and antipyretic agent. While it is generally safe for use at recommended doses, acute overdose of PARA can cause fatal liver damage. Despite the understanding that some cytochrome P450 isoforms are responsible for activation of PARA to the hepatotoxic metabolite, *N*-acetyl-*p*-benzoquinone-imine (NAPQI), the use of enzyme inhibitors for prevention and/or treatment of PARA hepatotoxicity is still not well researched. Therefore, grapefruit juice (GFJ), a well known enzyme inhibitor, was investigated for the prevention of hepatotoxicity after PARA overdose in rats.

Methods:

The study was approved by the animal ethics committee, which also imposed dosage limitations for this experiement. Twelve groups of 5 Sprague Dawley rats each were treated with single oral dose of either saline, PARA only 1000 mg/kg, PARA + GFJ low dose (2 ml) and PARA + GFJ high dose (3 ml). The remaining six groups were treated with bergamottin, a GPJ derivative and known enzyme inhibitor, instead. Thereafter, 5 rats from each group were sacrificed after 24, 48 and 72 hours and, on each occasion, blood samples were collected for determination of liver & renal function, electrolytes, FBC and PARA concentration. A piece of liver was sent for histopathalogy.

Results:

By 48 hrs the liver enzymes in the PARA only group were significantly ($P<0.05$) higher than in the GFJ + PARA and bergamottin + PARA groups. The concentrations, median (range), were: alkaline phosphate (ALP) 319 [71-328] u/L and alanine transaminase (ALT) 56 [50-59] u/L for PARA only, versus ALP 44 [39-58] u/L, ALT 40 [36-49] u/L for GFJ, ALP 34 [24-401] u/L, ALT 46 [41-49] for bergamottin, and ALP 96 [75 -238] u/L and ALT 38 [37-43] u/L for the control group.

Conclusion:

GFJ and bergamottin prevented PARA induced increase in liver enzymes after paracetamol overdose. Although the increase in liver enzymes did not meet the criteria for clinical hepatotoxicity, the results are promising, and call for use of a higher dose.

SUMMARY

Keywords: paracetamol, N-acetyl-*p*-benzoquinone-imine, liver damage, grapefruit juice, bergamottin, high performance liquid chromatography.

Paracetamol is a widely used analgesic and antipyretic agent. While it is generally safe for use at recommended doses, acute overdose of paracetamol can cause potentially fatal liver damage. Despite the understanding that some cytochrome P450 isoforms are responsible for activation of paracetamol to the hepatotoxic metabolite, N-acetyl-*p*-benzoquinine-imine (NAPQI), the use of enzyme inhibitors for prevention and/or treatment of paracetamol hepatotoxicity is still not well researched. Therefore grapefruit juice was evaluated for prevention of paracetamol-induced hepatotoxicity.

A high performance liquid chromatography (HPLC) method for the determination of paracetamol in plasma was developed. It involved protein precipitation of 50 µl of paracetamol spiked plasma with zinc sulphate followed by centrifugation. The supernatant was directly injected into the HPLC. The sample was eluted with a mobile phase of 0.01% trifluoroacetic acid in distilled water: acetonitrile (75: 25, v/v) over a Phenomenex C_{18} (4.60 x 250 mm) 5 µ analytical column at 1 ml/min. 4'Aminoacetophenone was used as the internal standard. Under these conditions paracetamol and 4'aminoacetophenone eluted at retention times of 4.2 minutes and 6.2 minutes, respectively. The average calibration curve (0 - 20 µg/ml) was linear with a regression equation of y = 0.0603x + 0.089, and regression coefficient of r^2 = 0.9957. The method was used to measure paracetamol concentrations in rat plasma.

Evaluation of grapefruit juice for the prevention of paracetamol-induced hepatotoxicity was performed with Sprague Dawley rats. The rats were treated with one-off oral dose of saline, paracetamol only, paracetamol + grapefruit juice low dose and paracetamol + grapefruit juice high dose.

A commercially available grapefruit derivative, bergamottin, was also evaluated. Thereafter, 5 rats from each group were sacrificed after 24, 48 and 72 hours.

After the treatment period the blood samples were collected for liver function tests, full blood count and paracetamol concentration. A piece of liver was sent for histopathology.

Administration of a toxic dose of paracetamol (1725 mg/kg) elevated the liver enzymes (ALT and AST) significantly when compared to the control group. Upon physical observation of the liver during surgery, the livers of the rats at 48 hours exhibited moderate to severe hepatic injury. The haematology results, especially platelet count, revealed a very low amount of platelets at 48 hours, which is indicative of thrombocytopenia. Paracetamol plasma concentrations of rats treated with paracetamol only were higher than at 24 hours and there was a slight decrease at 48 and 72 hours due to the drug metabolism.

However, the hepatic injuries of the rats treated with paracetamol only were antagonised by co-administration of paracetamol with grapefruit juice and also co-administration with bergamottin. The significant decrease in liver enzymes (ALT and AST) and the slight increase in platelet count at 72 hours revealed that grapefruit juice and bergamottin may have hepatoprotective effects against paracetamol-induced hepatotoxicity. Similarly, the concentration of paracetamol in the plasma of rats treated with paracetamol + grapefruit juice/bergamottin were lowered even further as opposed to when paracetamol was administered alone.

The results of this study imply that both grapefruit juice and bergamottin have enzyme inhibition ability and hence were able to play a role in inhibiting the enzymes which are involved in the production of a toxic metabolite known as NAPQI, which is produced in high quantity during overdose of paracetamol and is a major cause of liver injury.

OPSOMMING

Sleutelterme: parasetamol, N-acetyl-*p*-benzoquinine-imine, lewerskade, pomelosap, bergamottin, hoëdrukvloeistof-chromatografie.

Parasetamol word algemeen gebruik vir die behandeling van pyn en koors. Alhoewel dit veilig is om te gebruik teen die aanbevole dosis, kan `n akuute parasetamol-oordosering lei tot potensiële dodelike lewerskade. Ten spyte van die kennis dat die hepatotoksiese metaboliet van parasetamol, *N*-acetyl-*p*-benzoquinine-imine (NAPQI), gevorm word deur sitochroom P450 ensieme, is die kennis van ensieminhibeerders vir die voorkoming of behandeling van parasetamol hepatotoksisiteit baie beperk. Dus is pomelosap ondersoek vir die voorkoming van parasetamol-geïnduseerde hepatotoksiteit.

'n Hoëdrukvloeistof-chromatografie (HPLC) metode vir die bepaling van parasetamol in plasma is ontwikkel. Dit behels proteïenpresipitasie van 50 µl plasma, wat parasetamol bevat, met sinksulfaat, gevolg deur sentrifugering. Die boonste laag is direk in die HPLC ingespuit. Die monster is geëlueer met `n mobiele fase van 0.01% trifluoroasetaatsuur: gedistilleerde water (75: 25, v/v) deur 'n Phenomenex C_{18} (4.60 x 250 mm) 5 µ analitiese kolom teen 1 ml/min. 4'Aminoasetofenoon is as interne standaard gebruik. Onder die toestande het parasetamol en 4'aminoasetofenoon onderskeidelik teen 4.2 en 6.2 minute geëlueer. Die gemiddelde 5 dag kalibrasiekromme (0 – 20 µg/ml) was liniêr met 'n regressievergelyking van y = 0.0603x + 0.089, en korrelasiekoëffisiënt (r^2) van 0.9957. Die metode is suksesvol gebruik om parasetamolkonsentrasies in rotplasma te bepaal.

Die ondersoek van pomelosap vir die voorkoming van parasetamolgeïnduseerde hepatotoksiteit was in Sprague-Dawley rotte uitgevoer. Die rotte is behandel met `n eenmalige orale dosis soutoplossing, parasetamol-alleen, parasetamol + pomelosap lae dosis, en parasetamol + pomelosap hoë dosis.

`n Kommersiële pomelokomponent, bergamottin, is ook getoets. Daarna is 5 rotte uit elke groep geslag na 24, 48 and 72 uur.

Na die behandelingsperiode is bloedmonsters geneem vir lewerfunksietoetse, volbloedtelling en parasetamolkonsentrasies. `n Lewersnit was gestuur vir histopatologiese ondersoek.

Die toediening van `n toksiese dosis parasetamol (1725 mg/kg) het lewerensieme (ALT en AST) merkwaardig verhoog in vergelyking met die kontrolegroep. Na 48 uur en tydens fisiese ondersoek van die lewers gedurende chirurgie, is gesien dat daar matige tot ernstige lewerskade was. Die hematologie-uitslae en veral die plaatjietelling het teen 48 uur verlaag, wat aanduidend is van tromobositopenie. Parasetamol-plasmakonsentrasies van die rotte wat met parasetamol-alleen behandel is, was die hoogste teen 24 uur vanwaar dit effens verlaag het teen 48 en 72 uur as gevolg van metabolisme.

Aldus is die lewerskade van die rotte wat met parasetamol-alleen behandel is teengewerk deur die gelyktydige toediening van pomelosap, so wel as bergamottin. Die merkwaardige verlaging van lewerensieme (ALT en AST) en die effense verhoging van die plaatjietelling teen 72 uur beteken dat pomelosap en bergamottin `n beskermende effek teen parasetamolgeïnduseerde hepatotoksiteit mag hê. In ooreenstemming is die konsentrasie van parasetamol in die plasma van rotte wat met parasetamol + pomelosap/bergamottin behandel is, selfs verder verlaag in teenstelling met die toediening van parasetamol-alleen.

Die uitslae van die studie dui daarop dat beide pomelosap en bergamottin oor ensiem-inhiberende eienskappe beskik, en dus `n rol gespeel het in die inhibisie van die ensieme wat betrokke is by die vorming van die toksiese metaboliet, NAPQI, wat in groot hoeveelhede geproduseer word tydens parasetamol-oordosering en hoofsaaklik lei tot lewerskade.