THE EFFECT OF THE TRADITIONAL MEDICINE PHELA ON *P*-GLYCOPROTEIN AND MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 2 DRUG TRANSPORTERS IN THE GASTROINTESTINAL TRACT OF A RAT MODEL

BY

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ABSTRACT

Phela is the herbal preparation of four African traditional medicinal plants, and is under the development by the Medical Research Council (MRC) as an immune stimulant for immune compromised individuals. Patients might use Phela with other medicines; therefore, the herb-drug interactions profiling of Phela is important. Membrane drug-transporters such as *P*-glycoprotein (*P*-gp) and multidrug resistance-associated protein 2 (MRP2) are considered important factors in determining the pharmacokinetic parameters of drugs such as paclitaxel (PTX) and methotrexate (MTX), respectively. Inhibition or induction of transport might result in drug interactions with other drugs transported by these respective transporters. Moreover, significant herb-drug interactions involving *P*-gp and MRP2 have been described. Therefore, the effect of Phela on *P*-gp and MRP2 in the gastrointestinal tract of a rat model was investigated here.

First, a high performance liquid chromatography (HPLC) method for determination of PTX in plasma was developed. It involved liquid-liquid extraction of 100 µl plasma, spiked with PTX, extracted with diethyl ether: dichloromethane (2:1), followed by centrifugation. The supernatant was evaporated to dryness under a stream of nitrogen, reconstituted, and 100 µl was injected into the HPLC. The sample was eluted with a mobile phase of sodium phosphate buffer (pH 2): acetonitrile (60:40, v/v) over a $C_{8\ (1)}$ (4.6 X 250 mm) 5 µ analytic column at 1 ml/min. PTX was detected by UV at 230 nm. Docetaxel (DTX) was used as the internal standard. Under these conditions, DTX and PTX eluted at retention times of 6.595 and 6.038 minutes, respectively. The average calibration curve (0-15 µg/ml) was linear with a regression equation of $y = 0.1931x + 0.0705$, and correlation coefficient (r) of 0.9973. The method was used successfully in animal experiments to measure PTX in the plasma of treated rats.

Thereafter, a preliminary experiment was conducted *in vitro* to establish whether Phela has a direct/ physical effect on PTX, using a direct drug interaction testing experiment in buffer, as well as Slide-A-Lyzer® dialysis. During the direct drug interaction experiment, buffer was spiked with 10 µg/ml of PTX with or without 3.85 mg/ml Phela, and PTX concentrations were determined by HPLC. Then, using a Slide-A-Lyzer® dialysis

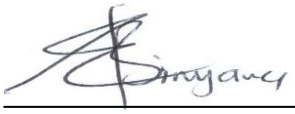
cassette, the time of equilibrium of PTX was determined by monitoring the changes in PTX concentrations over 12 hours, in plasma containing 230 µg/ml PTX and buffer. Thereafter, the potential of an interaction was tested by adding 88.55 mg/ml Phela to the same experiment after 8 hours of incubation, and monitoring PTX concentrations after 10 and 12 hours by HPLC. In the first experiment, Phela had no direct effect on PTX concentrations, while in the second experiment the time of equilibrium of PTX was estimated at 8 hours. After Phela was added, PTX concentrations and its free fraction (*fu*) remained unchanged. Therefore, it was concluded that there is no interaction between Phela and PTX *in vitro*.

This final part of the study was undertaken to investigate the effect of Phela on *P*-gp and MRP2 transporters. PTX and cyclosporin A (CyA) were used as the respective substrate and inhibitor of *P*-gp, while MTX and probenecid (PRO) were those of MRP2. Ethical approval was obtained and male Sprague-Dawley (SD) rats (200-250 g) were used. The animal experiment was divided into two parts. In Part I, three groups of 40 rats each received a one-off oral dose of PTX-only (10 mg/kg); PTX & CyA (10 mg/kg); or PTX & Phela (15.4 mg/kg), while in Part II, three groups of 40 rats each received a one-off oral dose of MTX-only (10 mg/kg); MTX & PRO (20 mg/kg); or MTX & Phela (15.4 mg/kg). For each group, 5 rats were sacrificed after 0.5, 1, 2, 4, 6, 8, 10, and 12 hours. Blood was analysed for full blood count, liver function, and PTX and MTX concentrations. CyA and PRO increased the area under the plasma concentration-time curve (AUC) of PTX and MTX, respectively, whereas Phela had no effect on the AUC of PTX or MTX.

Overall, no direct interaction between PTX and Phela was observed both *in vitro* and *in vivo*, and there were also no interactions between MTX and Phela *in vivo*. Phela did not inhibit *P*-gp or MRP2. This implies that Phela will most probably not be involved in herb-drug interactions of membrane transporter origin. Therefore, the doses of drugs that are transported by *P*-gp and MRP2 need not be adjusted when co-administered with Phela.

DECLARATION OF INDEPENDENT WORK

I, Moleboheng Emily Binyane, hereby declare that the master's research dissertation that I herewith submit at the University of the Free State, is my own independent work and that I have not previously submitted it for a qualification at another institution of higher education. I am aware that the copyright is vested in the University of the Free State.



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10 November 2015

Date

SUPERVISOR'S DECLARATION

I, Professor A. Walubo, the supervisor of this dissertation entitled: The effect of the traditional medicine Phela on *P*-glycoprotein and multidrug resistance-associated protein 2 drug-transporters in the gastrointestinal tract of a rat model, hereby certify that the work in this project was done by Moleboheng Emily Binyane at the department of Pharmacology, University of the Free State.

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

Signature

10 November 2015

Date

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I dedicate this work to my late father and mother, Pule and Nkutloang Binyane. They worked hard to see me succeed. Even though they are no more, their love and teachings have kept me firm.

Lastly and foremost, I thank Almighty God, Jehovah Jireh, for making this journey possible and successful!

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ABBREVIATIONS

ABC	ATP-binding cassette
ALP	alkaline phosphatase
ALT	alanine transaminase
AST	aspartate aminotransferase
AUC	area under the curve
BCRP	breast cancer resistance protein
Cal	calibration
CLp	clearance
Cmax	maximum concentration
CV	coefficient of variation
CyA	cyclosporin A
CYP2C8	cytochrome P450 enzyme 2C8
CYP2C19	cytochrome P450 enzyme 2C19
CYP3A4	cytochrome P450 enzyme 3A4
CYP450	cytochrome P450
EMI	enzyme multiplied immunoassay
FPIA	fluorescence polarization immunoassay
GIT	gastrointestinal tract
HPLC	high performance liquid chromatography
IS	internal standard

Ke	elimination rate constant
LC-MS-MS	liquid chromatography tandem mass spectrometry
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MDR	multidrug resistant
MRC	Medical Research Council
MRP2	multidrug resistance-associated protein 2
MRT	mean residence time
MTX	methotrexatre
OATP1B1	organic anion-transporting polypeptide 1B1
P-gp	*P*-glycoprotein
PRO	probenecid
PTX	paclitaxel
RIA	radioimmunoassay
T½	half-life
Tmax	time to reach maximum concentration
UV	ultraviolet
Vd	volume of distribution
WHO	World Health Organization

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CHAPTER 1
GENERAL INTRODUCTION

Research has taken interest in membrane transporters, particularly because of their role in determining pharmacokinetic, safety and efficacy profiles of drugs (Giacomini and Sugiyama, 2006). Membrane transporters are membrane-associated proteins that govern the transport of influx and efflux ions, nutrients, and drugs (Huang *et al.*, 2004). In particular, more than 400 membrane transporters in two major super families, *i.e*., ATP-binding cassette (ABC) and solute carrier (SLC), have been described in the human genome. Many different drug-transporters are expressed in various tissues, such as the epithelial cells of the intestine and kidney, hepatocytes, and brain capillary endothelial cells (Takano *et al.*, 2006).Transporters can play a vital role in determining drug concentrations in the systemic circulation, as well as in cells.

It is becoming increasingly evident that, among other transporters, the intestinal transporters play an important role in the oral absorption of compounds, with both influx and efflux transporters influencing drug absorption processes (Pang, 2003).Oral absorption of compounds can be limited by efflux transporters located in the intestine, such as *P*-glycoprotein (*P*-gp) or multidrug resistance-associated protein 2 (MRP2) (Chan *et al.*, 2004), while influx transporters such as the organic anion-transporting polypeptide (OATP) and peptide transporters (e.g., PEPT1), can aid intestinal drug absorption (Kim, 2003). Moreover, there appears to be an overlap in the substrate specificity between the efflux transporter *P*-gp and the influx transporter OATP, which could lead to opposing influences on the net absorption of a shared substrate (Kim, 2003).

Most known interactions between herbal extracts and drugs involve the inhibition of drug-metabolising enzymes, but little is yet known about the possible role of transporters in these interactions (Fuchikami *et al.*, 2006). Citrus juices, including grapefruit juice, have been reported to reduce the bioavailability of orally administered fexofenadine. This interaction is considered to be caused by the inhibition of intestinal OATPs (Banfield *et al.*, 2002). This decrease in exposure could result in reduced efficacy of

fexofenadine in patients (Kamath *et al.*, 2005). Therefore, it is necessary to screen traditional medicines for their effects on drug-transporters involved in drug absorption. In this study, more interest was placed particularly on Phela, a traditional medicine. Phela is the herbal mixture of four African traditional medicinal plants that has been used for decades in wasting conditions and, after successful observation studies in humans, is now being developed by the Medical Research Council (MRC) as an immune booster for patients with a compromised immune system (Lekhooa *et al.*, 2012). Due to the fact that Phela may be used by HIV patients who may be using other medications as well, it was therefore important to create a model to provide a method of predicting possible drug interactions of Phela. Here, Phela was screened for potential interaction with two efflux drug-transporters, *P*-gp and MRP2, involved in drug absorption, with a hope that this developed technique can also be applied for screening other products in development.

CHAPTER 2

LITERATURE REVIEW: PART I

AN OVERVIEW OF MEMBRANE TRANSPORTERS

2.1 Drug absorption

Absorption is the movement of a drug from the site of administration to the circulation, where the route of administration is the determinant of the rate and efficiency of absorption. When a drug is administered intravenously, the total dose is able to reach the bloodstream (*i.e*., absorption is complete), whereas an orally administered drug may undergo partial absorption which results in low bioavailability (Richard *et al*., 2009, p.7-8).

Although oral administration of drugs is more convenient and acceptable to patients, good oral bioavailability is important because it means the total drug can reach the circulation via the gastrointestinal tract. Therefore oral drug absorption is continuously being researched in order to improve bioavailability, taking into consideration both the kinetics and dynamics of the orally administered drug (Pang, 2003).

Due to their major role in first-pass metabolism, the liver and small intestine are important in the absorption of orally administered drugs. The small intestine has a large surface area and facilitates the majority of drug absorption due to the presence of villi and microvilli (Figure 2.1, letters B and C), which increases the absorptive area. The highest concentration of villi and microvilli is located in the duodenum and jejunum, while it is least in the ileum. Furthermore, the circulation of the intestine is unique, in that it is the vesicular portal system with which drugs are delivered to the liver.

Drug delivery rate and the extent of saturability of intestinal enzymes are affected by the amount of drug entering the intestine, as well as by the rate of blood flow. This eventually affects the rate of intestinal and hepatic first-pass metabolism. Moreover, absorption is also influenced by other variables such as drugs or food (Pang, 2003).

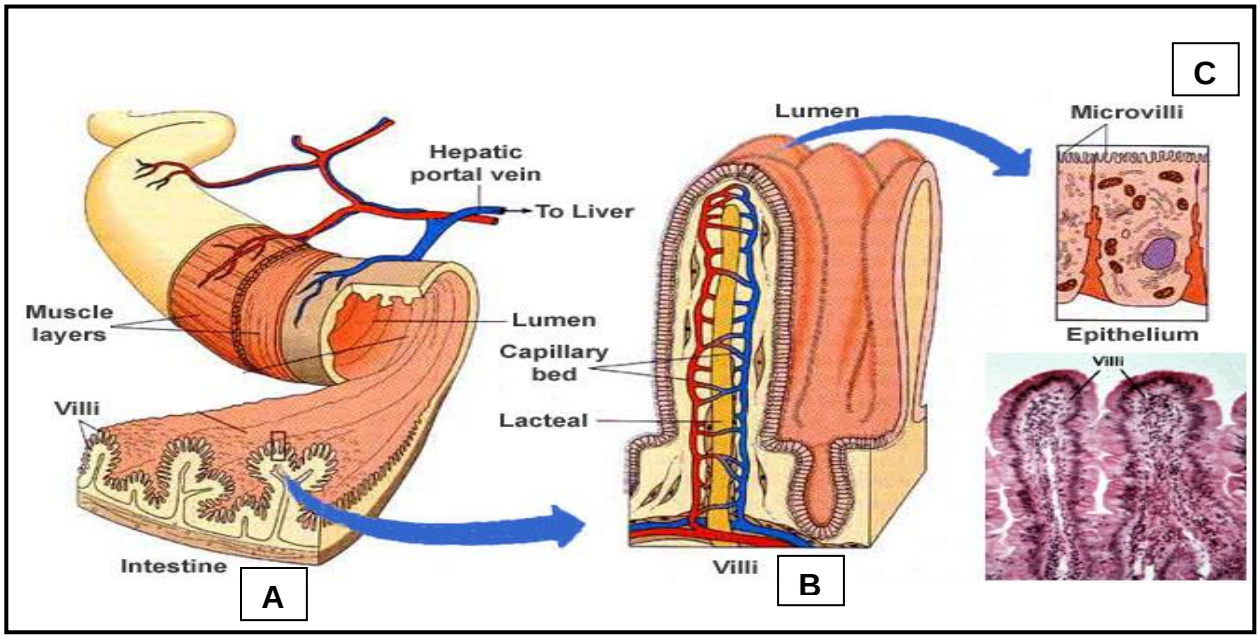


Figure 2.1: Cross section and structure of the small intestine (From: http://wwww.daviddarlinginfo/encyclopedia/copyright.htmlcited26/07/11)

Though it is believed that most drugs are absorbed by a simple diffusion mechanism via the gastrointestinal epithelium, direct and indirect evidence points to the participation of transporters in mediating absorption (Tsuji, 2006). Here, it was found that membrane-bound drug-transporters have absorbed or secreted several drugs that had poor bioavailability. Subsequently, this shifted the focus to the involvement of membrane transporters in the mechanism of drug absorption.

Transporters that are expressed in the intestines of humans are also found in rats (Mizuno *et al.*, 2003), meaning that transporters involved in drug absorption are found in the intestines of both humans and rats, therefore a rat model was appropriate for the purpose of this study.

2.2 Membrane transporters

Membrane transporters are defined as membrane-associated proteins responsible for the transport of solutes, including drugs and other xenobiotics, into and out of cells (Giacomini *et al*., 2010). Transporters are key determinants of drug concentrations in the bloodstream and in cells. There are two major super-families of membrane transporters,

namely: the ATP-binding cassette (ABC) and solute carrier (SLC) transporters (Figure 2.2).In order to understand the molecular characteristics of individual transporters belonging to these families, many transporters have been cloned and studied, thus extensive progress has been made. It is now known that some of these transporters govern drug transport in different tissues, and they may become main determinants of the pharmacokinetic characteristics of a drug as far as its intestinal absorption, tissue distribution, and elimination are concerned (Giacomini *et al.*, 2010).

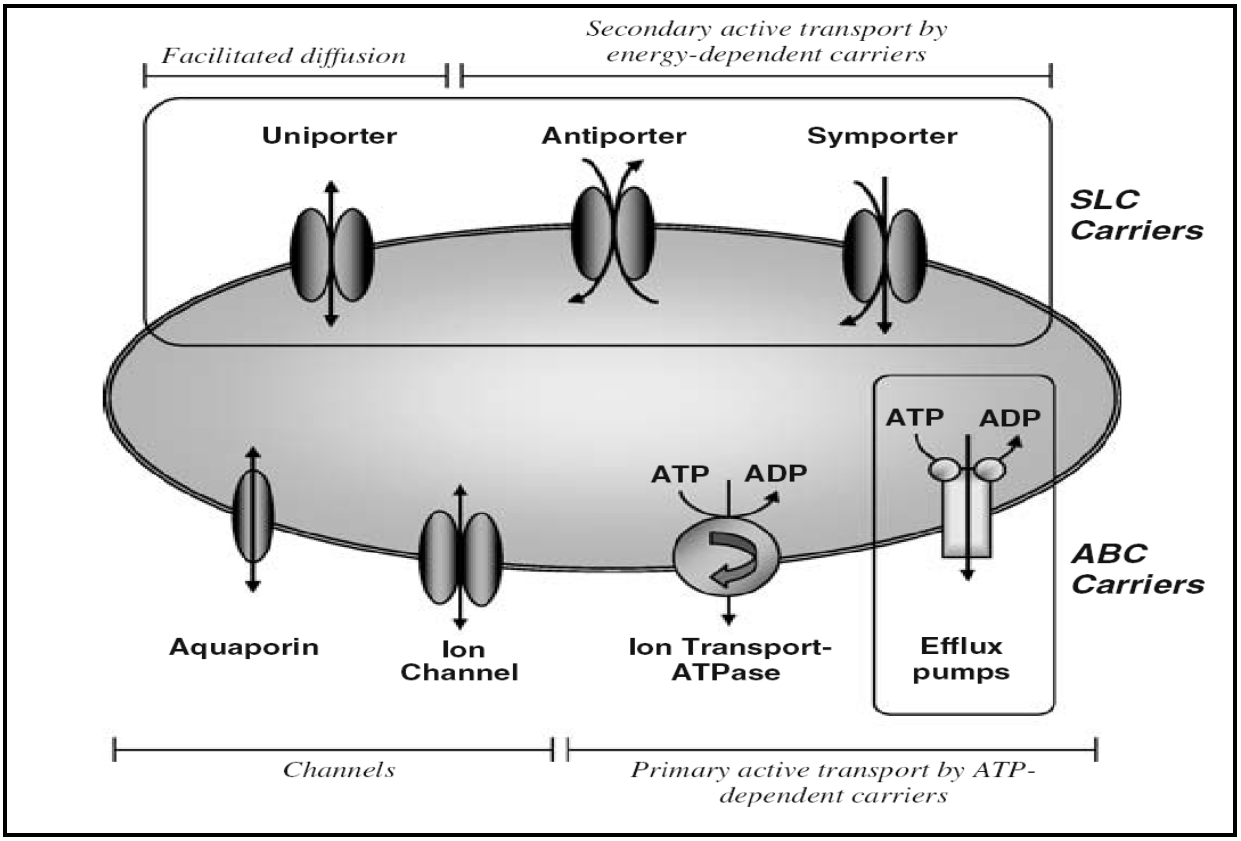


Figure 2.2: Illustration of solute carriers (SLC), ATP-dependent carriers (ABC-carriers), and channels in the plasma membrane (From: Mizuno *et al.,* 2003).

Different drug-transporters are located in various tissues (Figure 2.3), such as the epithelial cells of the intestine and kidney, hepatocytes, and brain capillary endothelial cells (Mizuno *et al.*, 2003). Transporters have been grouped as primary, secondary, or tertiary active transporters. Primary active transporters include: ATP-binding cassette

transporters, such as multidrug resistant (MDR), multidrug resistance-associated protein (MRP), and breast cancer resistance protein (BCRP), which are driven by the force of ATP hydrolysis. They are responsible for the efflux of potentially toxic endogenous and exogenous compounds from cells (Vlaming *et al*., 2011). Secondary active transporters include: organic anion transporter (OAT), organic anion-transporting polypeptide (OATP), sodium taurocholate co-transporting peptide (NCTP), organic cation transporter (OCT), and oligopeptide transporter (PEPT), which are driven by an exchange or co-transport of intracellular and/or extracellular ions (Mizuno *et al.*, 2003).

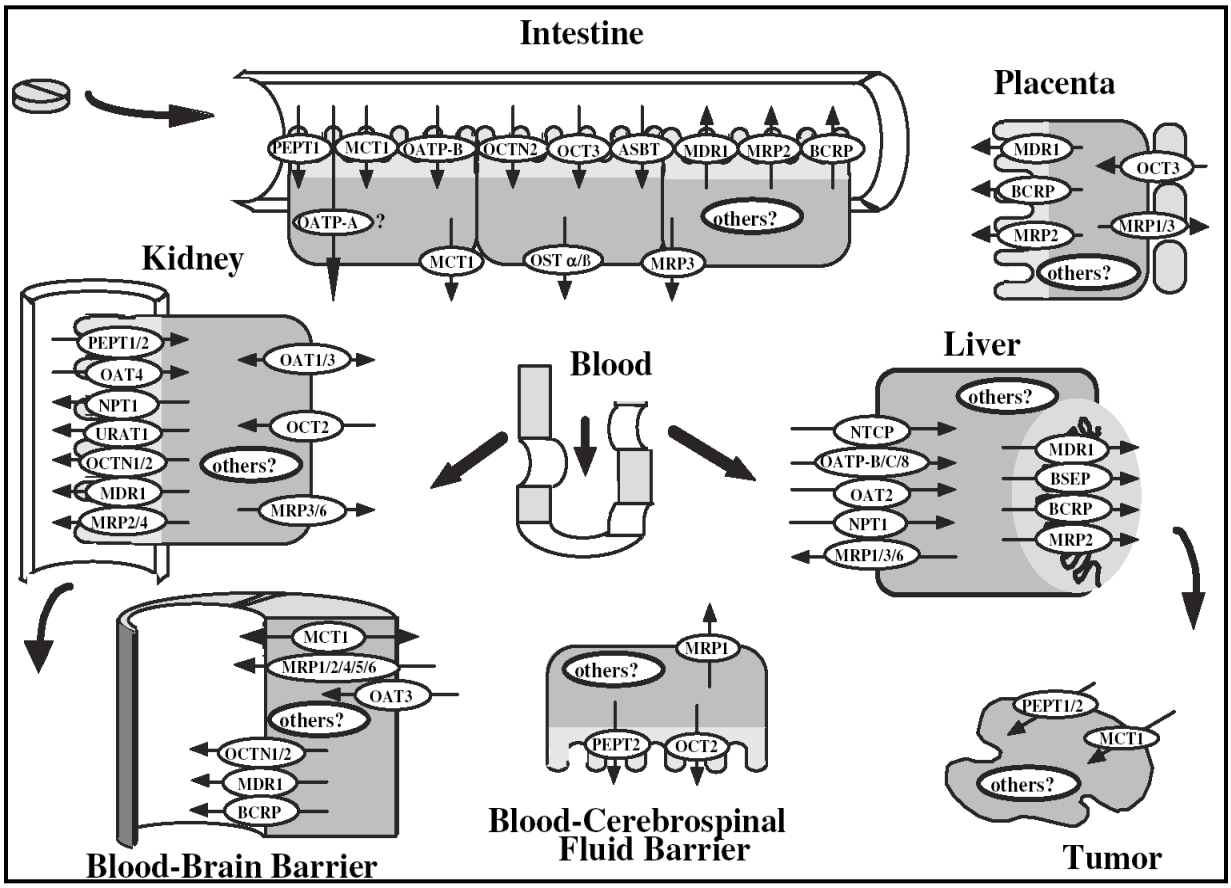


Figure 2.3: Illustration of organ distribution of transport proteins (From: Tsuji, 2006)

For purposes of this study, two primary active efflux membrane drug-transporters, multidrug resistant and multidrug resistance-associated protein, involved in absorption in the gastrointestinal tract, were reviewed.

2.2.1 P-glycoprotein

P-glycoprotein (*P*-gp), also known as multidrug resistant, is a type of ATPase, energy-dependent trans-membrane drug efflux pump which is one of the ABC transporters. Furthermore, *P*-gp is a glycoprotein that has a molecular weight of approximately 170 kDa. It appears as a single chain with two equal homologous portions, both containing six trans-membrane domains and two ATP-binding areas divided by an elastic linker polypeptide area between the Walker A and B motifs (Varma *et al*., 2003). *P*-gp was the first discovered transporter due to its ability to confer multidrug resistance to cancer cells (Juliano and Ling, 1976). Additionally, *P*-gp facilitates the ATP-dependent export of drugs from cells to the blood-stream. In the intestine, *P*-gp is situated in the apical membrane of mature enterocytes (*O*'Brien and Cordon-Cardo, 1996), where it mediates the transport of substrates out of the cell into the intestinal lumen, thereby forming a barrier to drug absorption. The level of expression and function of *P*-gp can be changed by inhibition and induction of the transporter itself, which can affect the pharmacokinetics, efficacy or tissue levels of *P*-gp substrates (Zhou, 2008). Paclitaxel is a known substrate of *P*-gp (Nakajima *et al.*, 2005), whereas cyclosporine (also known as cyclosporin A), verapamil, tamoxifen, quinidine, and phenothiazines are inhibitors of *P*-gp (Fisher and Sikic, 1995).

2.2.2 Multidrug resistance-associated protein 2

Multidrug resistance-associated protein 2 (MRP2) is a member of the ATP-binding cassette (ABC) family and is found mainly in the liver, kidneys and gut (Chen *et al*., 2002). MRP2 is expressed on the brush-border membrane of intestinal enterocytes, and excretes its substrates into the lumen, thereby limiting absorption (Taipalensuu *et al.*, 2001). Methotrexate and irinotecan are examples of substrates of MRP2, as are the glucuronide conjugates of paracetamol (Chen *et al*., 2002). Probenecid is a known inhibitor of several transporters, and among those are the multidrug resistance-associated proteins (MRPs; Tunblad *et al*., 2003).

2.2.3 Role of efflux transporters in oral drug absorption

Oral administration is the predominant route for drug administration since it is convenient (Chan *et al*., 2004). However, in the intestine, drug-transporter interactions involving the efflux transporters often result in poor absorption and low oral

bioavailability, as the drug is effluxed back into the intestinal lumen and subsequently excreted (Mitchell and Thompson, 2013).This means that absorption of orally administered compounds can be limited by efflux transporters such as *P*-gp and MRP2 (Kamath *et al*., 2005).

2.2.4 Efflux transporters inhibition associated interactions

Much attention has been paid to transporter-mediated processes, since these significantly modulate drug absorption, distribution, metabolism and excretion. Transporters are common sites for drug-drug interactions, as well as interactions of drugs with endogenous substrates, leading to drug toxicity and various adverse effects (Glavinas *et al.*, 2004). As such, one drug which interacts with a transporter might inhibit the transport of another drug, either in a competitive or in a non-competitive manner.

Should drug transport be inhibited, it usually results in increased bioavailability and decreased clearance, thereby markedly elevating the area under curve (AUC) of the affected drug (Stieger *et al.*, 2000).For example, it was observed that orally administered verapamil increases the peak plasma level, prolongs the elimination half-life and increases the volume of distribution of orally administered doxorubicin due to inhibition of *P*-gp (Kerr *et al*., 1986), while co-administration of tenofovir and didanosine increased the AUC of didanosine by 40 to 60 % due to MRP2 inhibition (Weiss *et al*., 2007). Therefore, these transporters play an integral role in drug absorption, and should always be borne in mind when developing newly discovered drugs. However, there is few data on the effects of herbal medicinal compounds on these drug transporters.

CHAPTER 2

LITERATURE REVIEW: PART II

SUBSTRATES AND INHIBITORS OF *P*-GLYCOPROTEIN AND MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 2 TRANSPORTERS

2.3 Substrates and inhibitors of *P*-glycoprotein

2.3.1 Paclitaxel

Paclitaxel is an antineoplastic agent which belongs to a group of cytotoxic agents, the taxanes (Martin *et al.*, 1998). Its main mechanism of action is mediated by the stabilization of cellular microtubules, and investigators have demonstrated paclitaxel activity against adult epithelial ovarian cancer, breast cancer and melanoma (Eric *et al*., 1995). During treatment, drug-related hypersensitivity reactions may occur (Britten *et al*., 2000). Nonetheless, paclitaxel has been identified as a substrate of *P*-glycoprotein (*P*-gp; Sparreboom *et al.*, 1997).

2.3.1.1 Physical properties

Paclitaxel (Figure 2.4) is a white to off-white crystalline powder with an empirical formula of $C_{47}H_{51}NO_{14}$ and molecular weight of 853.9 g/mol. It is slightly soluble in water, and melts between 216-217°C (Kumar *et al.*, 2009). Furthermore, the drug is available in an intravenous formulation, namely Taxol® (Kumar *et al*., 2009).

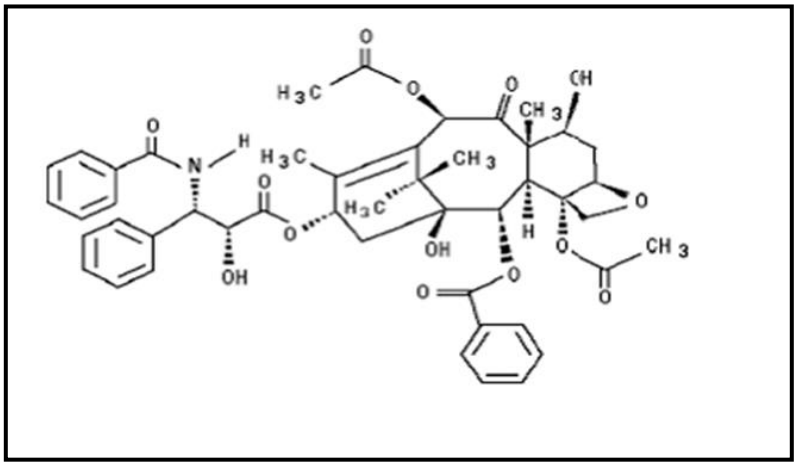


Figure 2.4: The chemical structure of paclitaxel (From: Kumar *et al.*, 2009)

2.3.1.2 Pharmacokinetics

Paclitaxel is administered intravenously since the oral formulation is considered problematic due to its poor absorption. Paclitaxel is widely distributed in the body and 95 % to 98 % is bound by plasma proteins, primarily albumin (Choi and Li, 2005).The metabolism of paclitaxel is catalyzed by cytochrome P450 (CYP) enzymes. Here, CYP2C8 is responsible for the formation of 6α-hydroxypaclitaxel, whereas the formation of 3'-*p*-hydroxypaclitaxel is catalyzed by CYP3A4 (Harris *et al.*, 1994), and 6α3'-*p*-dihydroxypaclitaxel is formed after stepwise hydroxylations by CYP2C8 and CYP3A4. The primary route of elimination of paclitaxel is by hepatic metabolism and biliary excretion (Monsarrat *et al.*, 1993).

2.3.1.3 Drug interactions

A number of clinically important drug interactions have been reported for paclitaxel. Firstly, co-administration of paclitaxel with lapatinib results in decreased clearance of lapatinib due to the inhibition of CYP3A4, CYP2C8 and *P*-gp. Also, cyclosporin A increases the absorption of paclitaxel by effectively blocking *P*-gp (Terwogt *et al*., 1999), while valspodar (an analogue of cyclosporin D) increases brain levels of paclitaxel by potently inhibiting *P*-gp as well. Furthermore, the total body clearance of paclitaxel and digoxin has been found to decrease substantially after co-treatment of verapamil in human subjects (Varma *et al*., 2003). Lastly, combination therapy of dexverapamil and paclitaxel in metastatic breast cancer patients showed increased mean peak paclitaxel concentrations and delayed clearance (Tolcher *et al*., 1996).

2.3.2 Cyclosporin A

Cyclosporin A is a lipophilic cyclic endecapeptide. The US Food and Drug Administration (FDA) approved cyclosporin A for treatment and/or prevention of transplant rejection, as seen with graft rejection in kidney, liver, heart, lung, and combined heart-lung transplantation. In addition, it is applied in bone marrow transplantation to prevent graft-versus-host disease, as well as in treatment of autoimmune conditions like psoriasis, atopic dermatitis, rheumatoid arthritis, and a variety of glomerular disorders. Cyclosporin A acts by binding to the cytosolic protein, cyclophilin A, the most abundant cyclophilin in T-lymphocytes (Kapturczak *et al*., 2004). Nephrotoxic effects, both acute and chronic, are among the most common side effects

of cyclosporin A therapy (Klintmalm *et al.*, 1981). Furthermore, cyclosporin A is a known inhibitor of *P*-gp (Britten *et al*., 2000).

2.3.2.1 Physical properties

Cyclosporin A has a chemical formula of $C_{62}H_{111}N_{11}O_{12}$ and molecular weight of 1203 g/mol (Figure 2.5). It is a cyclic, highly hydrophobic endecapeptide, and its purified form appears as white prismatic needles, which are neutral and only slightly soluble in water and saturated hydrocarbons. The drug is also slightly soluble in lipids and other organic solvents (Kapturczak *et al*., 2004). Furthermore, the drug is available in an oral formulation, namely, Sandimmune ®.

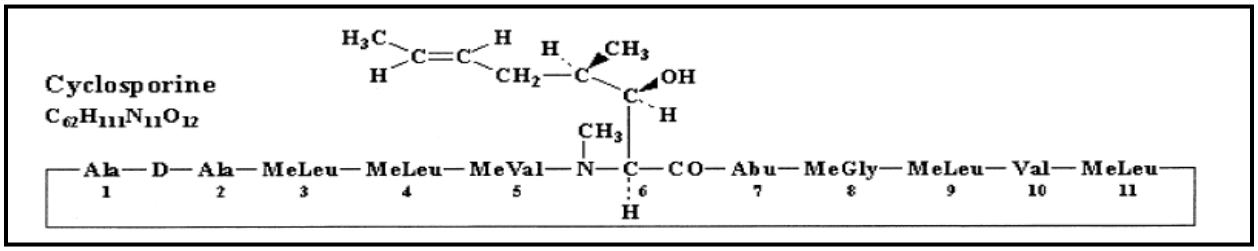


Figure 2.5: The chemical structure of cyclosporin A (From: Kapturczak *et al.,* 2004)

2.3.2.2 Pharmacokinetics

Due to its lipophilic properties, the majority of cyclosporin A leaves the blood-stream. The apparent volume of distribution of cyclosporin A varies between 4 and 8 L/Kg (Misteli *et al.*, 1990), while the noncellular fraction of blood cyclosporin A is carried mainly by lipoproteins (Urien *et al.*, 1990). Cyclosporin A is primarily metabolized by CYP3A4 (Shimada *et al.*, 1994) in the liver. CYP3A4 transforms cyclosporin A to more than 30 metabolites by hydroxylation, demethylation, sulfation, and cyclization (Christians and Sewing, 1993), and all metabolites display only minimal, if any, immunosuppressive activity (Radeke *et al.*, 1992). The average half-life of cyclosporin A is approximately 19 hours (Yee, 1991). It is primarily excreted in the bile with less than 1 % contribution of the parent drug, while urinary excretion accounts for 6 % of the oral cyclosporin A dose, of which 0.1 % is unaltered (Maurer and Lemaire, 1986). Furthermore, cyclosporin A crosses the plancenta and is excreted in human milk (Flechner *et al.*, 1985).

2.3.2.3 Drug interactions

Cyclosporin A influences the pharmacokinetics of sirolimus by increasing its bioavailability via competitive interaction with both CYP3A4 and *P*-gp (Christians *et al.*, 2003). As corticosteroids are part of most immunosuppressive regimens, they have shown to be substrates, inhibitors and inducers of CYP3A4, as well as potent inducers of *P*-gp (Salphati and Benet, 1998). Subsequently, they either lower or increase cyclosporin A requirements. Furthermore, cyclosporin A increases the plasma concentrations of atorvastatin and several other statins, probably by OATP1B1 inhibition (Neuvonen *et al.*, 2006).

2.4 Substrates and inhibitors of multidrug resistance-associated protein 2

2.4.1 Methotrexate

Methotrexate is a folic acid antagonist which is used in a wide array of clinical conditions. It was introduced for the treatment of acute lymphoblastic leukemia in 1948 (Farber and Diamond, 1948), and later on used for cancer monotherapy, and as an antineoplastic and immunosuppressive agent (Mohammad *et al.*, 1979).The drug is also effective for the treatment of psoriasis and rheumatoid arthritis (Oufi and Al-Shawi, 2014). Methotrexate acts by inhibiting the proliferation of malignant cells, primarily by preventing the *de novo* synthesis of purines and pyrimidines (Chan and Cronstein, 2002). The most common side effects observed after treatment with methotrexate are mucosal ulceration and nausea (Richard *et al*., 2009, p.461-464). Lastly, methotrexate is a known substrate of multidrug resistance-associated protein 2 (MRP2).

2.4.1.1 Physical properties

Methotrexate (Figure 2.6) is an orange yellow crystalline powder with empirical formula $C_{20}H_{22}N_8O_5$ and molecular weight of 454.4 g/mol (Basile *et al*., 2002). It is water soluble, and almost insoluble in alcohol, ether, and chloroform (Chan, 1988).The drug is available in intravenous and oral formulations.

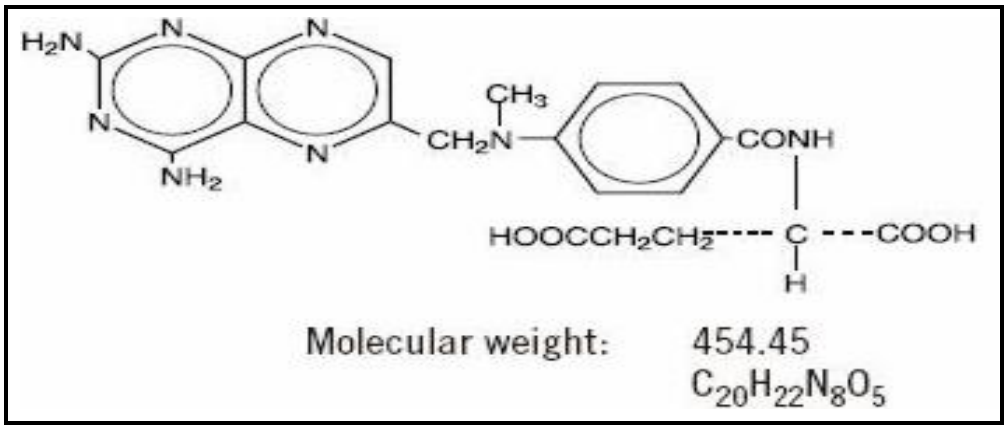


Figure 2.6: The chemical structure of methotrexate (From: Saxena *et al.*, 2009)

2.4.1.2 Pharmacokinetics

After oral administration, approximately 35 % of methotrexate is bound to plasma proteins and a larger amount is eliminated by the kidneys, whereas less than 10 % of the drug is metabolized to 7-hydroxymethotrexate in the liver (Chiang *et al*., 2005). Methotrexate is transported by efflux transporters: P-*gp*, MRPs and breast cancer resistance protein (BCRP) (Yokooji *et al*., 2007). At doses of 40 mg/m^2 or less, the bioavailability of methotrexate is about 42 %, and at doses greater than 40 mg/m^2 it is reduced to only 18 %.

2.4.1.3 Drug interactions

Some nonsteroidal anti-inflammatory drugs (NSAIDs) such as salicylate, piroxicam, ibuprofen, naproxen, sulindac, tolmetin, and etodolac, inhibit the renal tubular secretion of methotrexate by inhibiting OATP1 and 3, and MRP2 and 4, thus plasma methotrexate concentrations are increased to levels that can be potentially toxic (El-Sheikh *et al*., 2006). Furthermore, pantoprazole and omeprazole inhibit methotrexate transport by BCRP, thereby resulting in elevated methotrexate concentrations (Breedveld *et al*., 2004).

2.4.2 Probenecid

Probenecid is a uricosuric drug that increases uric acid excretion in the urine; therefore, it is primarily used for the treatment of gout and hyperuricemia. Unfortunately, probenecid competitively inhibits the renal excretion of some drugs, thereby increasing their plasma concentrations and prolonging their effects. Other than that, probenecid is

well tolerated, with only 2 % of patients developing mild gastrointestinal irritation, while 2 % to 4 % of patients may experience mild hypersensitivity reactions (Brunton *et al.*, 2008).

2.4.2.1 Physical properties

Probenecid (Figure 2.7) is a white or nearly white, fine, crystalline powder with empirical formula $C_{13}H_{19}NO_4S$ and molecular weight of 285.4 g/mol. It is a weak acid (pKa 3.7; Gutman *et al*., 2012) and is soluble in dilute alkali, alcohol, chloroform, and acetone, but practically insoluble in water and dilute acids (Xu and Madden, 2011). The drug is available in an oral formulation.

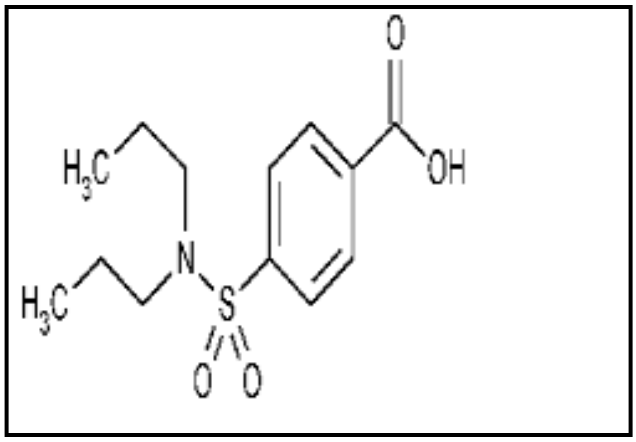


Figure 2.7: The chemical structure of probenecid (From: Himani *et al*., 2014)

2.4.2.2 Pharmacokinetics

Probenecid has an oral bioavailability of greater than 90 %, is 85 to 95 % bound to plasma albumin, and has a small apparent volume of distribution of 0.003-0.014 L/kg in humans. The maximum adult dose of probenecid is 3 g, and a single oral dose of 2 g (approximately 25 mg/kg) yields peak plasma concentrations of 150-200 µg/ml within 4 hours, while concentrations greater than 50 µg/ml are sustained for 8 hours. Following a 2 g dose, the half-life is 4-17 h, however the half-life is dose-dependent, and decreasing as the dose decreases to 500 mg. Probenecid is metabolized in the liver via oxidation and glucuronidation and is primarily excreted in the urine (75-85 %). Also, probenecid is transported by MRP and the uric acid transporter (Gutman *et al*., 2012).

2.4.2.3 Drug interactions

Namkoong *et al*. (2007) reported that co-administration of probenecid and irinotecan reduced irinotecan-induced late-onset toxicity in the gastrointestinal tissue as they inhibit the biliary excretion of irinotecan by MRP2 inhibition. Probenecid increases plasma concentrations of methotrexate by inhibiting drug efflux mediated by MRP, while at the same time inhibiting folate uptake. It also inhibits the tubular secretion of organic anion derivatives, such as penicillin, by inhibiting organic anion transporters (OATs). The drug is a weak inhibitor of CYP2C19 and blocks the renal transport of many compounds, including many classes of antibiotics, antivirals, and NSAIDs, leading to an increase in their mean plasma elimination half-life that can lead to increased plasma concentrations (Gutman *et al*., 2012).

Although the available literature provides much information regarding (western) drug-transporter interactions, less is known about potential interactions should natural products be consumed.

CHAPTER 2

LITERATURE REVIEW: PART III

SCREENING OF PHELA FOR POTENTIAL INTERACTION WITH MEMBRANE TRANSPORTERS

2.5 An overview on Traditional Medicine

The use of traditional medicines and natural health products is increasing among those living with HIV/ AIDS (Fairfield *et al.*, 1998), one of the reasons being that currently there is no cure for HIV/ AIDS, and the programs used to manage the pandemic are not always satisfactory. Secondly, most traditional medicines have been used for years, hence are often assumed to be safe and efficacious, and are recommended to be used with anti-retroviral treatments (Lekhooa *et al.*, 2010). According to the World Health Organization (WHO), TMs are defined as: “health practices, approaches, knowledge and beliefs incorporating plant, animal, and mineral based medicines, spiritual therapies, manual techniques and exercises, applied singularly or in combination, to treat, diagnose and prevent illness or maintain the well-being”. This is a broad definition that ensures that all types of traditional medicines are included. Other terminologies used in traditional medicine include complementary/alternate medicine (CAM), herbal medicines, and herbs (Crouch *et al.*, 2000).

2.5.1 Interactions with Traditional Medicines

The concomitant use of traditional medicines with prescription drugs may result in potential pharmacokinetic interactions mediated by drug-metabolizing enzymes or transporters, termed herb-drug interactions (Tomlinson *et al.*, 2008; Table 2.1). To date, citrus juices, especially grapefruit juice, have been reported to reduce the bioavailability of orally administered fexofenadine, an antihistamine. This interaction is considered to be caused by the inhibition of intestinal OATPs (Banfield *et al.*, 2002). On the other hand, the interaction of herbal dietary products with transporters has also received increasing attention. For example, repetitive administration of St. John’s wort induces the expression of not only CYP450 enzymes, but also *P*-gp, which decreases the

bioavailability of its substrates such as indinavir, cyclosporin A, and digoxin (Durr *et al.*, 2000). It was also demonstrated that extracts of green tea, garlic, and milk thistle inhibit the function of *P*-gp *in vitro* (Jodoin *et al.*, 2002). A study conducted by Honda *et al*. (2004) has reported that grape fruit and orange juice interact not only with *P*-gp, but also with MRP2, both of which are expressed at apical membranes and limit the apical to basal transport of vinblastine and saquinavir in Caco-2 cells. Therefore, MRP2, in addition to *P*-gp and CYP3A4, may contribute to the drug pharmacokinetic changes brought on by grape fruit and orange juices. Chiang *et al*. (2005) reported a life-threatening interaction between methotrexate (substrate of MRP2) and *Pueraria lobata* root decoction in rats. Here, PLRD significantly decreased elimination and resulted in markedly increased exposure of methotrexate.

Table 2.1: Herb-drug interactions mediated by drug-metabolizing enzymes or transporters (From: Mills *et al.*, 2005*; Yokooji *et al*., 2010)

Herb	Drug	Enzyme/ Transporter	Herb-drug interaction
Hypoxide	Verapamil	CYP3A4/ *P*-gp*	drug toxicity
Sutherlandia	Verapamil	CYP3A4/*P*-gp*	loss of therapeutic effect
Rhei Rhizhoma	2.4-dinitro-Phenyl-S-glutathione (DNP-SG)	MRP2	Increased peak concentration & Area under the curve of DNP-SG

2.6 Phela

Phela is the name given to the herbal preparation of four African traditional medicinal plants, *i.e., Clerodendrum glabrum*, *Polianthes tuberose*, *Rotheca myricoides* and *Senna occidentalis*. These plants have been used for decades in wasting conditions and for increasing energy in patients with various disease symptoms including: severe chest problems with pain and coughing; high fevers associated with shivers and headaches; severe loss of weight and appetite; vomiting and diarrhoea; bed-ridden patients with stiff posture, and lip wounds. Phela is currently under development by the

Medical Research Council (MRC) as an immune booster for patients with a compromised immune system (Lekhooa *et al*., 2012, p.27-39). Although the mechanism of action of Phela is unknown, an *in vivo* experiment conducted in rats showed that Phela stimulates or restores cyclosporine induced immune suppression, indicating possible IL-2 activation (Lekhooa *et al*., 2010). Results of clinical safety studies conducted on 40 healthy male participants revealed that participants reported no major side effects, and it was concluded that Phela is safe and well tolerated (Medical Research Council, Indigenous Knowledge Systems lead programme report, 2009).

2.6.1 Physical properties

Phela extract powder (Figure 2.8) is a brown to light brown coloured powder with uniform particle size (size 90 sieved). It is soluble in water and evaporates at around 105 °C (Medical Research Council, Indigenous Knowledge Systems lead programme report, 2009).

Figure 2.8: Phela extract powder

2.6.2 Oral formulation

The finely ground pre-mixed plant powders are encapsulated in a standardized 350 mg unit dose capsule (Medical Research Council, Indigenous Knowledge Systems lead programme report, 2009).

2.6.3 Pharmacokinetic parameters

The consumption of Phela has been calculated to equate to an adult dose of 1081 mg /70 kg/day which is equivalent to 15.4 mg/kg body weight (Medical Research Council, Indigenous Knowledge Systems lead programme report, 2009). The pharmacokinetic

parameters of Phela in rats were determined using this 15.4 mg/kg dose of Phela. From the results, the metabolite's half-life was 3.47±0.35 hours and reached maximum concentration at 4.67±1.15 hours. The concentration at steady state was estimated to be 47.52±5.94 PK-area/L, with no drug accumulation when a once daily dose of Phela is taken (Lekhooa *et al*., 2012, p.73-80).

2.6.4 Phela-drug interactions

From a previous departmental study it was observed that Phela has no significant effect on the activity of CYP450 isoforms (Medical Research Council, Indigenous Knowledge Systems lead programme report, 2009).

2.6.5 Conclusion

Although Phela has shown not to induce drug interactions via the CYP450 enzyme system, other factors must be taken into consideration concerning the transport of Phela, such as its effect on efflux membrane drug-transporters, and more specifically *P*-gp and MRP2, which are involved in drug absorption in the gastrointestinal tract. Since Phela is reported to be a potential immune modulator (Lekhooa *et al.*, 2012, p.73-80), it may benefit individuals with compromised immune systems such as HIV/AIDS patients. Therefore, there is a need to understand potential herb-drug interactions of Phela in order to predict its safety and toxicological effects that may occur.

CHAPTER 3

REVIEW OF ANALYTICAL METHODS

3.1 Review of analytical methods for the determination of paclitaxel and methotrexate in plasma

3.1.1 Paclitaxel

A number of analytical methods for the quantification of paclitaxel in plasma are described, and among those, high performance liquid chromatography (HPLC) and liquid chromatography tandem mass spectrometry (LC-MS-MS) are commonly used. Though LC-MS-MS have many advantages over HPLC, the latter is more convenient for paclitaxel analysis (Yonemoto *et al*., 2007).

LC-MS-MS methods described by Lian *et al*. (2013) and Rajender and Narayana (2010) are rapid, sensitive, and highly accurate for determination of paclitaxel in plasma. However, the method reported by Lian *et al*. (2013) was advantageous in that it required a small sample volume. Though these methods seemed more appealing than HPLC, they require highly specialized and expensive equipment, which are not available in our set-up, hence had to be dismissed.

Regarding HPLC methods, Martin *et al*. (1998) described a convenient assay for determination of paclitaxel in plasma. The method utilized docetaxel as internal standard and involved a liquid-liquid extraction with diethyl ether. Unfortunately, the mobile phase of ammonium acetate buffer-tetrahydrofuran resulted in inadequate elution of paclitaxel.

The method reported by Coudoré *et al*. (1999) was simple, and entailed a rapid single-step liquid-liquid extraction with dichloromethane. The mobile phase of distilled water-methanol resulted in poor paclitaxel elution, and plasma interfered with paclitaxel separation.

Another HPLC assay for paclitaxel was explained by Andersen *et al*. (2006), which involved a large sample volume of 4 000 µl and also used docetaxel as internal

standard. Furthermore, it made use of solid phase extraction, and a mobile phase of acetonitrile-sodium phosphate buffer, which showed promising separation of paclitaxel.

The reviewed methods could not be solely adopted; however, each method held appealing conditions which were selected as a starting point for method development. This includes: the internal standard from Martin *et al*. (1998) and Andersen *et al*. (2006); the analytic column and liquid-liquid extraction from Martin *et al*. (1998); as well as the mobile phase from Andersen *et al*. (2006).

3.1.2 Methotrexate

Several methods for methotrexate analysis have been reported. These methods are either expensive or time-consuming such as HPLC, radioimmunoassay (RIA), dihydrofolate reductase inhibition assay, enzyme immunoassay (EIA), fluorescence polarization immunoassay (FPIA), and enzyme multiplied immunoassay (EMI; Lobo and Balthasar, 1999).

An HPLC method for determination of methotrexate in plasma described by Lobo and Balthasar (1999) used a small sample volume of 100 µl, but showed low sensitivity. Uchiyama and co-workers (2012) reported methods which involved the use of post-column photochemical reaction, complex chemicals, and tedious extraction processes. Therefore, the use of HPLC was discarded.

Although RIA methods are sensitive, and proven to be technically simple, they are costly, and require time-consuming experimental procedures. Furthermore, they require antibodies and make use of radioactive material with short shelf-life and inconvenient disposal properties (Howell *et al*., 1980; Tracey *et al*., 1983). Enzyme assays such as EIA and FPIA make use of expensive enzymes and antibodies (Al-Bassam *et al*., 1979; Belur *et al*., 2001; Jolley *et al*., 1981), therefore cannot be considered for use.

In the Toxicology Laboratory of the Department of Pharmacology, University of the Free State, EMI has for years been the method of choice for patient therapeutic drug monitoring of methotrexate. It has proved to be accurate and reliable, therefore,

For the purpose of this study, it was felt appropriate to analyse methotrexate by this method.

3.2 Review of methods for determination of the protein-binding capacity of paclitaxel

Concerning the determination of free concentration and bound fractions of paclitaxel, various methods are described. These techniques are time-consuming, result in loss of analyte to membranes, produce errors due to protein leakage, and can create a shift in concentrations. Available methods include: ultrafiltration, ultracentrifugation, and equilibrium dialysis (Musteata and Pawliszyn, 2006).

Paál and co-workers (2001) reported a simple ultrafiltration method which was fast and utilized a small sample volume of 990 µl. Unfortunately, it was unreliable in that the binding was not temperature controlled, and the volume of ultrafiltrate was not sufficient for the drug assay.

Ultracentrifugation requires costly equipment, and sedimentation, back diffusion viscosity and binding to plasma lipoproteins in the supernatant fluid during the process, can cause errors in the estimation of the free drug concentration (Barré *et al*., 1985). The ultracentrifugation method reviewed which was described by Gapud *et a*l. (2004), was complicated and inconvenient, and thus discarded.

A reliable equilibrium dialysis method was described by Brouwer *et al*. (2000). The method involved the use of a small sample volume of 300 µl, which was dialysed against phosphate buffered saline at 37 °C for 24 hours, in a humidified atmosphere of 5 % carbon dioxide. Thereafter, paclitaxel was quantified by liquid scientillation.

A Slide-A-Lyzer® dialysis method was described by Zhao and co-workers (2010), which utilised a large sample volume of 2 000 µl, and dialysis against phosphate buffered saline at 37 °C and 100 rpm, after which paclitaxel was analysed by HPLC.

In reviewing the methods discussed above, it was concluded that none could be adopted, owing to equipment used. However, Slide-A-Lyzer® equilibrium dialysis, as described by Zhao *et al*. (2010), was considered, and used as such.

CHAPTER 4

OBSERVATIONS FROM THE REVIEW

4.1 Observations from the review

- There is a need to develop a model for screening traditional medicines for potential interaction with absorption transporters
- The interaction of herbal dietary products with transporters has recently received increasing attention.
- Phela, a traditional medicine, is under development by the Medical Research Council of South Africa as an immune booster.
- It is important to determine possible interactions between Phela and transporters.
- The discovery of membrane drug-transporters has led to renewed interest in, among others, the mechanism of drug absorption.
- Saturation or inhibition of influx (pump in) transporters leads to decreased drug absorption, while inhibition of the efflux (pump out) transporters leads to increased drug absorption and concentration.
- It is now established that drug-transporters are an important factor in the bioavailability of some drugs, hence are source of drug interactions.
- Knowledge of the possible interactions will help to determine the mechanism of action of Phela on the transporters, making it easier to predict the effects on the transport of Phela out of cells as well as its bioavailability.

4.2 Aim

- To investigate the effect of Phela, a traditional medicine, on drug-transporters *P*-gp and MRP2, in the gastrointestinal tract of a rat model.

4.3 Objectives

- To develop an HPLC method for the analysis of paclitaxel in plasma.
- Determination of a drug interaction between Phela and paclitaxel *in vitro*, by a direct drug interaction testing experiment and Slide-A-Lyzer® technique.
- Determining the effect of Phela on the pharmacokinetics of paclitaxel and methotrexate, after oral administration in rats using HPLC-UV and enzyme multiplied immunoassay, respectively.

CHAPTER 5

DETERMINATION OF PACLITAXEL IN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Summary

A high performance liquid chromatography (HPLC) method for the determination of paclitaxel in plasma was developed. It involved liquid-liquid extraction of 100 µl plasma, spiked with paclitaxel, extracted with diethyl ether: dichloromethane (2:1), followed by centrifugation. The supernatant was evaporated to dryness under a stream of nitrogen, reconstituted, and 100 µl was injected into the HPLC. The sample was eluted with a mobile phase of sodium phosphate buffer (pH 2): acetonitrile (60:40, v/v) over a $C_{8\ (1)}$ (4.6 X 250 mm) 5 µ analytic column at 1 ml/min and detected by UV at 230 nm. Docetaxel was used as the internal standard. Under these conditions docetaxel and paclitaxel eluted at retention times of 6.595 and 6.038 minutes, respectively. The average calibration curve (0 – 15 µg/ml) was linear with a regression equation of y = 0.1931x + 0.0705, and correlation coefficient (r) of 0.9973. The method was used successfully in animal experiments to measure paclitaxel in the plasma of treated rats.

5.1 Introduction

In this chapter, a high performance liquid chromatography assay is described.

5.2 Methods

A. Materials

5.2.1Apparatus

For weighing gram and milligram quantities of reagents and drug standards, a precision balance (SPB 52, Scaltec Instruments, Goettingen, Germany) and analytic balance (AS 220/C/2, Randwag, Random, Poland) were used. A vortex mixer (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY, U.S.A) and micro centrifuge (Minispin, Eppendorf, Hamburg, Germany) were used for mixing and spinning of the samples. Spectrophotometer (Biochrom Libra S12) was used to determine the wavelength of paclitaxel and docetaxel.

5.2.2 Reagents and chemicals

All standards and chemicals used were of analytic grade. Paclitaxel and docetaxel were obtained from Sigma-Aldrich Inc. (St. Louis, MO. U.S.A). Orthophosphoric acid (H_3PO_4), di-sodium hydrogen orthophosphate dehydrate ($Na_2HPO_4.2H_2O$), and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) were purchased from Merck laboratories (Darmstadt, Germany).HPLC grade acetonitrile (C_2H_3N), diethyl ether ($(C_2H_5)_2O$), dichloromethane (CH_2Cl_2), ethanol (C_2H_6O) and methanol (CH_4O) were from Honeywell Burdick and Jackson (Muskegon, MI, U.S.A).Fresh plasma was obtained from healthy volunteers after informed consent.

5.2.3 Chromatographic system

The HPLC system was an Agilent, Hewlett Packard 1100 Series, equipped with an 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 was collected using ChemStation software.

5.3 Preliminary experiments

5.3.1 Selection of a mobile phase

Initially, a mobile phase of distilled water (solvent A) and pure acetonitrile (solvent B) was tried in a ratio of 40:60 (A:B), but with little success, as paclitaxel eluted poorly.

Similarly, a mobile phase of distilled water (solvent A) and pure methanol (solvent B) in a ratio of 30:70 (A:B) did not yield good results as paclitaxel still eluted poorly.

Thereafter, a mobile phase of 20 mM sodium phosphate buffer at pH 2 (solvent A) and pure acetonitrile (solvent B) was tried with different gradients. Finally, paclitaxel and docetaxel showed satisfactory separation at a ratio of 40:60 (A:B), and the respective peaks were sharp and well resolved. As such, this mobile phase was selected for further evaluation in the subsequent experiments.

5.3.2 Preparation of standard solutions

Stock solutions of paclitaxel and docetaxel at a concentration of 0.2 mg/ml were

prepared in ethanol and methanol, respectively. These were diluted to working solutions of 50 µg/ml, using ethanol for paclitaxel and mobile phase for docetaxel.

5.3.3 Selection of an internal standard

The selection of a suitable internal standard was fortunately not a tedious task. A report by Martin and co-workers (1998) suggested the use of docetaxel, and it was tried as such. Luckily, docetaxel showed no interference with the paclitaxel peak. Therefore, it was selected as the appropriate internal standard.

5.3.4 Selection of a detection wavelength

In order to determine an appropriate wavelength at which to detect paclitaxel and docetaxel, UV-wavelength spectra of the respective drugs were performed. Here, both drugs showed maximum response at 230 nm; hence this wavelength was selected and used for the remainder of the experiments.

5.3.5 Selection of an analytical column

First a Phenomex® Luna $C_{18\ (2)}$ (4.6 X 150 mm) 5 µ analytic column was tried, but could not be utilized, as paclitaxel and docetaxel were poorly separated. Thereafter a Sphereclone ODS $_{(2)}$ (4.6 X 250 mm) 5 µ analytic column was tried, but not with success, as the peaks of paclitaxel and docetaxel were broad with longer retention times. Finally, a Phenomex® Luna $C_{8\ (1)}$ (4.6 X 250 mm) 5 µm analytic column was tried. The column produced satisfactory results as the peaks of paclitaxel and docetaxel were sharp and well resolved with preferred retention times of less than 10 minutes. As such, the mentioned column was selected and used to validate the method.

5.3.6 Sample preparation and extraction

5.3.6.1 Liquid-liquid extraction with ethyl acetate

A simple liquid-liquid extraction with ethyl acetate was tried. Unfortunately the chromatogram showed interfering peaks, and the method was discarded.

5.3.6.2 Liquid-liquid extraction with diethyl ether and dichloromethane

To plasma spiked with paclitaxel and internal standard, 3 ml of a diethyl ether and dichloromethane mixture (2:1) was added. The sample was vortexed for 30 seconds, shaken for 15 minutes and centrifuged at 2451 *g* (3 500 r.p.m) and 4 °C for 5 minutes.

Thereafter the supernatant was evaporated to dryness under a stream of nitrogen at 45 °C, reconstituted with 200 µl mobile phase, and 50 µl was injected into the HPLC. The chromatogram showed no interfering peaks, however the resolution of the paclitaxel and docetaxel peaks could still be improved.

First, the sample was acidified with 50 % phosphoric acid and then extracted as described above. This did not improve the chromatogram as the peaks still appeared the same; therefore acidification of the sample was discarded.

Secondly, the sample volume was tested at 100, 250, 500 and 1000 µl, and extracted under the same conditions. Here, the 100 µl sample produced best results and was selected for use.

Thereafter, the extraction procedure was optimized. The time of extraction (shaking) was tested at 10 and 15 minutes, respectively, where the 10 minutes of shaking showed satisfactory results. In addition, the volume of mobile phase with which the sample would be reconstituted was tested at 150 and 200 µl. Here, the volume of 150 µl produced best results, and was selected for use as such.

Lastly, the injection volume was tested at 50 and 100 µl, respectively, and the best chromatogram was achieved at 100 µl. Thereafter, these conditions were set as final for liquid-liquid extraction of the plasma sample, and proved to be reproducible.

5.4 Final conditions

5.4.1 Sample preparation and extraction

To 100 µl of plasma spiked with paclitaxel, 40 µl of internal standard was added and the sample was vortexed for 30 seconds. Thereafter the sample was purified by liquid-liquid extraction with 3 ml diethyl ether and dichloromethane (2:1). The sample was shaken for 10 minutes and centrifuged at 2451 *g* (3 500 r.p.m) and 4 °C for 5 minutes, after which it was evaporated to dryness under a stream of nitrogen at 47 °C, reconstituted with 150 µl mobile phase, and 100 µl was injected into the HPLC for analysis.

5.4.2 Chromatographic conditions

Chromatographic separation of paclitaxel and docetaxel was achieved by running the mobile phase at a flow rate of 1 ml/min over a Phenomex® Luna $C_{8\ (1)}$ (4.6 X 250 mm) 5 µ analytic column, coupled to a Phenomex® securityGuard™ C_{18} (4 X 3 mm) guard column (Torrance, CA, U.S.A). Compounds were detected by UV at a wavelength of 230 nm.

5.5 Method validation

The optimized method was validated by the determination of linearity, accuracy and stability.

5.5.1 Calibration/ Linearity

The linearity of the assay was tested to determine a proportional relationship of response versus analyte concentration, over the working range (Shabir, 2004). Here, calibration was performed by analysing samples spiked with paclitaxel at a concentration range of 1.25, 2.50, 5.00, 7.50, 10.00 and 15.00 µg/ml on different days, for 5 days. Calibration curves were created by plotting the peak area ratio of paclitaxel to docetaxel, against the spiked (known) concentrations of paclitaxel. The curves were analysed by linear regression using the GraphPad® Instat Statistical program.

5.5.2 Accuracy

Accuracy of the method was tested to determine whether the method can be repeated a number of times and still give similar results. Accuracy was tested at 1.25, 7.50 and 15.00 µg/ml. The test was repeated five times for each sample and accuracy values were derived from a calibration curve. The results obtained were used to calculate the coefficient of variation (CV) using the following formula: (Standard deviation/ mean) X 100.

5.5.3 Stability

Stability was tested to determine appropriate storage conditions for samples containing a specific drug. Stability of paclitaxel was determined at 1.25, 7.50 and 15.00 µg/ml. The samples were stored at room temperature, 4 °C and -20 °C, and analysed after short and long time periods.

5.5.3.1 Short-term stability

For short-term stability, samples were analysed after 24 and 48 hours.

5.5.3.2 Long-term stability

For long-term stability, samples were analysed after 1, 2 and 4 weeks.

5.5.4 Application of the validated method

The method was tested by analysing plasma samples of rats, after oral administration of paclitaxel. All details on the animal study and procedures are described in Chapter 7.

5.6 Results

5.6.1 UV-spectra analysis

Figures 5.1 a) – 5.1 c) are the representative UV-spectra for the mobile phase and standard solutions of paclitaxel and docetaxel. From the UV-spectra it was observed that paclitaxel and docetaxel exhibited maximum response at 230 nm.

Figure 5.1 a): UV-spectrum of mobile phase

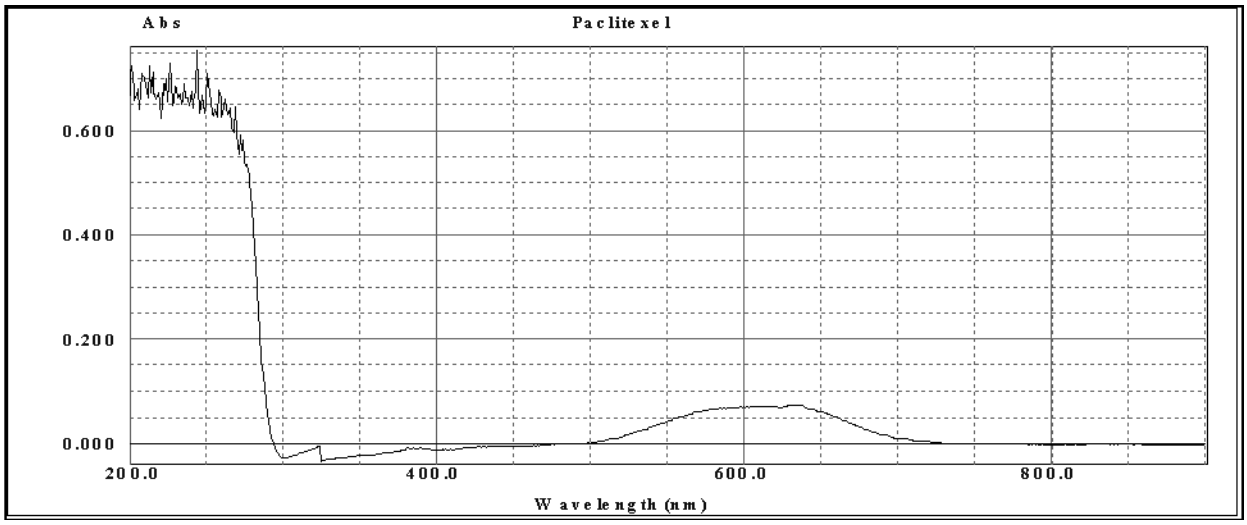


Figure 5.1 b): UV-spectrum of paclitaxel

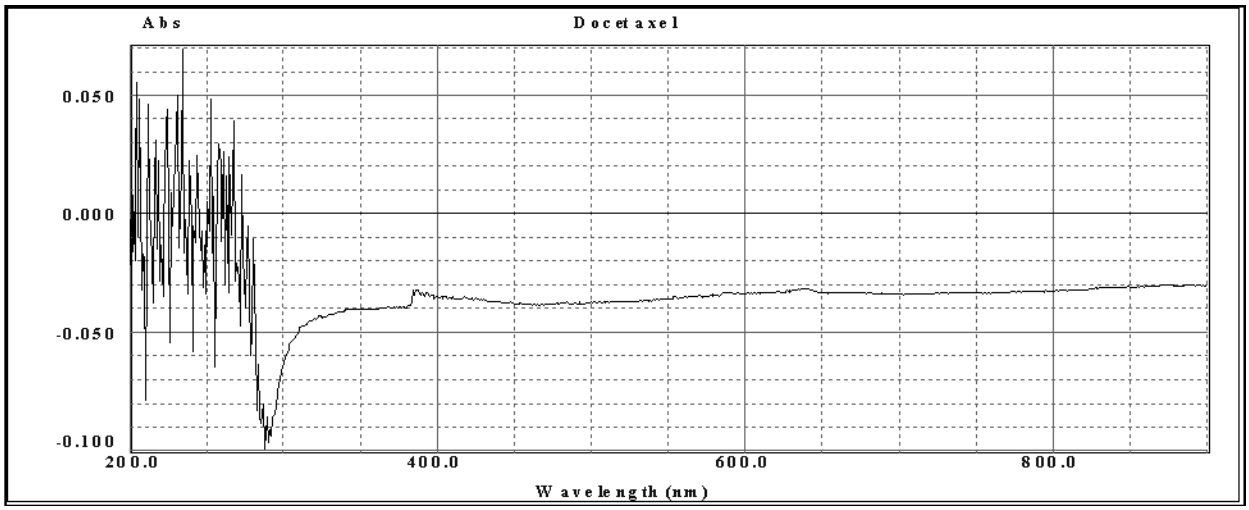


Figure 5.1 c): UV-spectrum of docetaxel

5.6.2 Chromatographic performance

Figures 5.2 a) – 5.2 e) are representative chromatograms for the mobile phase, standard solutions, blank plasma and spiked plasma. From the standard solutions it was observed that the peaks were well resolved, with the drugs eluting at the following retention times (in minutes): paclitaxel, 6.730 and docetaxel, 6.180. The blank plasma showed no interference from plasma. Retention times for plasma spiked with paclitaxel and docetaxel were 6.594 and 6.038 minutes, respectively. The total run time was 10 minutes.

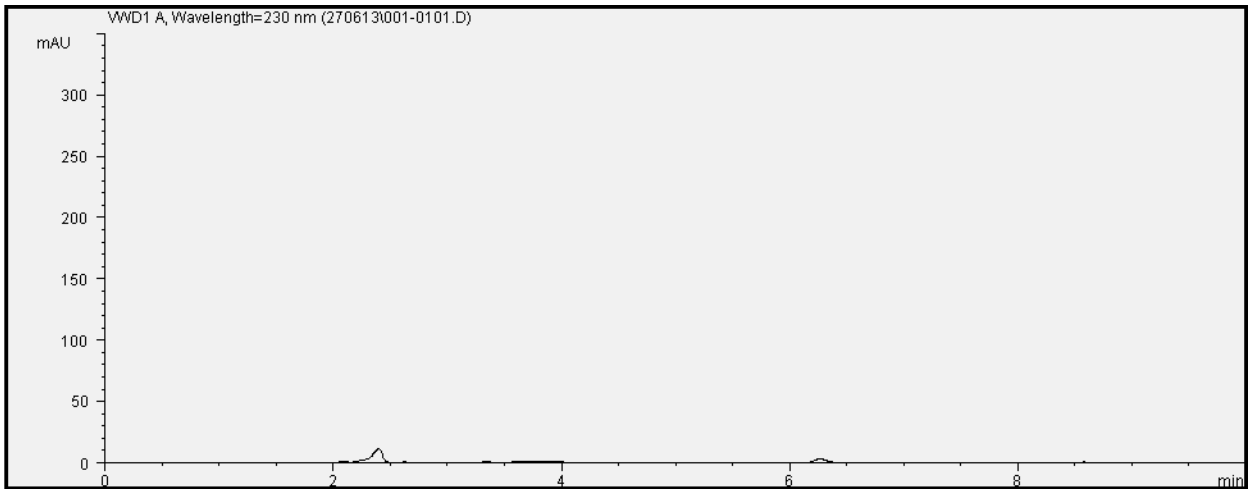


Figure 5.2 a): Chromatogram of mobile phase

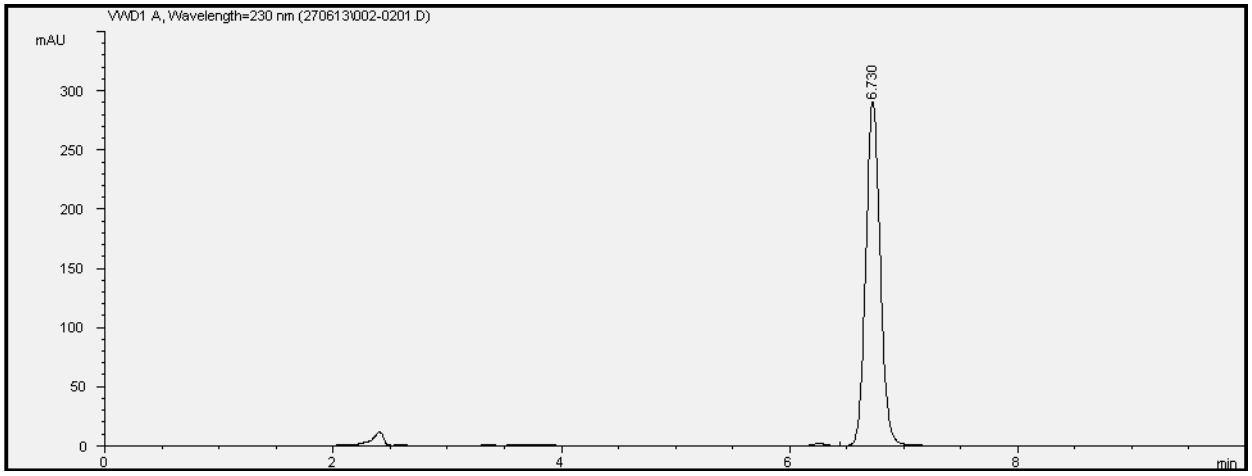


Figure 5.2 b): Chromatogram of mobile phase spiked with paclitaxel

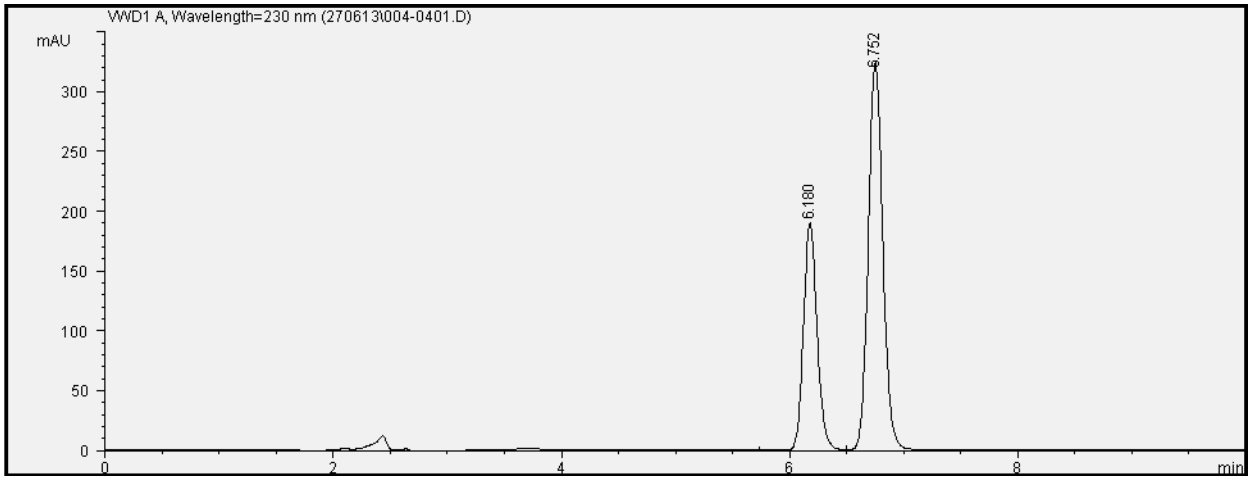


Figure 5.2 c): Chromatogram of mobile phase spiked with paclitaxel and docetaxel

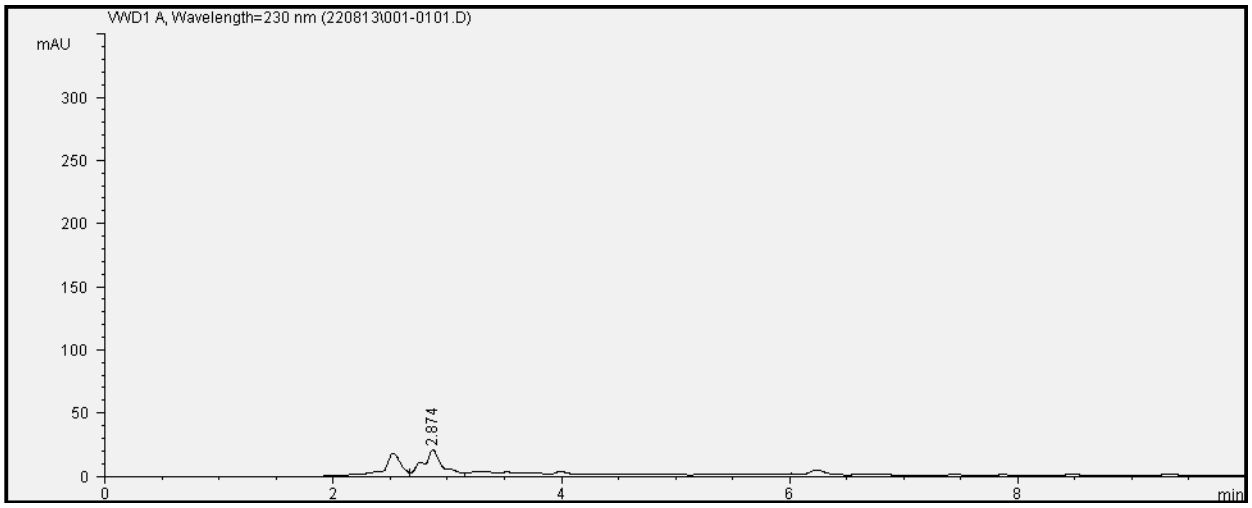


Figure 5.2 d): Chromatogram of a blank plasma sample

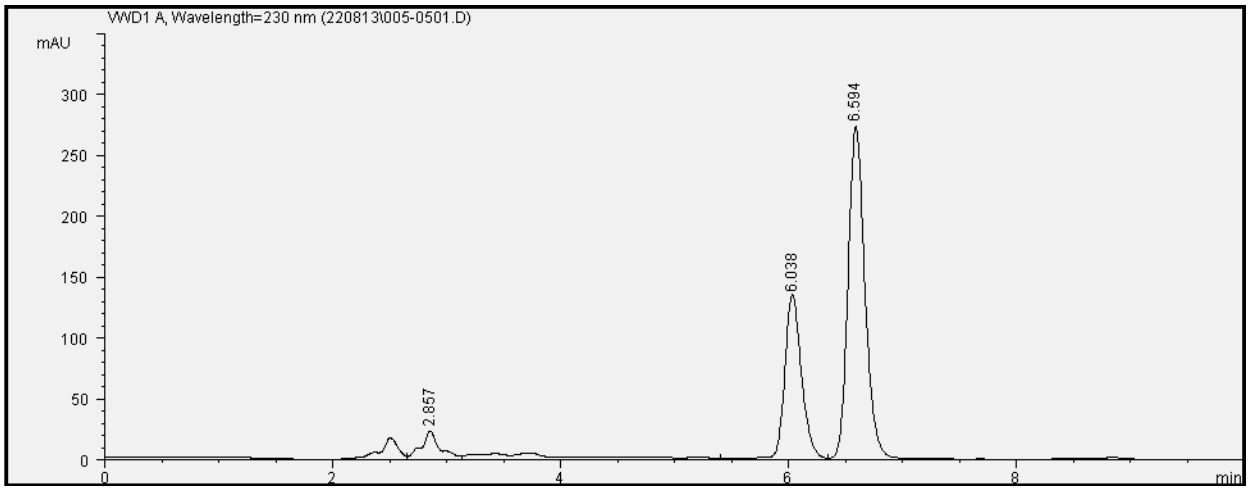


Figure 5.2 e): Chromatogram of a plasma sample spiked with 10 µg/ml paclitaxel and 10 µg/ml docetaxel

5.6.3 Standardization of the paclitaxel assay

5.6.3.1 Calibration

The summary data for the calibration over five days is shown in Table 5.1, while the average calibration curve is shown in Figure 5.3 (see Appedix A for individual calibrations). From the results, the calibration curve was linear with a regression equation of y=0.1931x + 0.0705 and correlation coefficient (r) of 0.9973, while the coefficient of variation percentage (CV %) was less than 15 %.

Table 5.1: HPLC calibrations for paclitaxel using ratio paclitaxel/ ratio docetaxel

Conc. (µg/m)	Cal. Day 1	Cal. Day 2	Cal. Day 3	Cal. Day 4	Cal. Day 5	Mean	SD	CV (%)
1.25	0.36	0.30	0.38	0.26	0.32	**0.32**	**0.04**	**13.63**
2.50	0.54	0.55	0.64	0.63	0.57	**0.58**	**0.04**	**7.58**
5.00	1.10	1.01	1.30	1.08	1.07	**1.11**	**0.11**	**10.01**
7.50	1.45	1.51	1.57	1.59	1.60	**1.55**	**0.06**	**4.13**
10.00	1.92	1.86	1.62	2.05	2.18	**1.93**	**0.21**	**10.91**
15.00	2.90	2.98	2.65	2.97	3.36	**2.97**	**0.26**	**8.63**

Conc. = concentration; Cal. = calibration; SD = standard deviation; CV = coefficient of variation

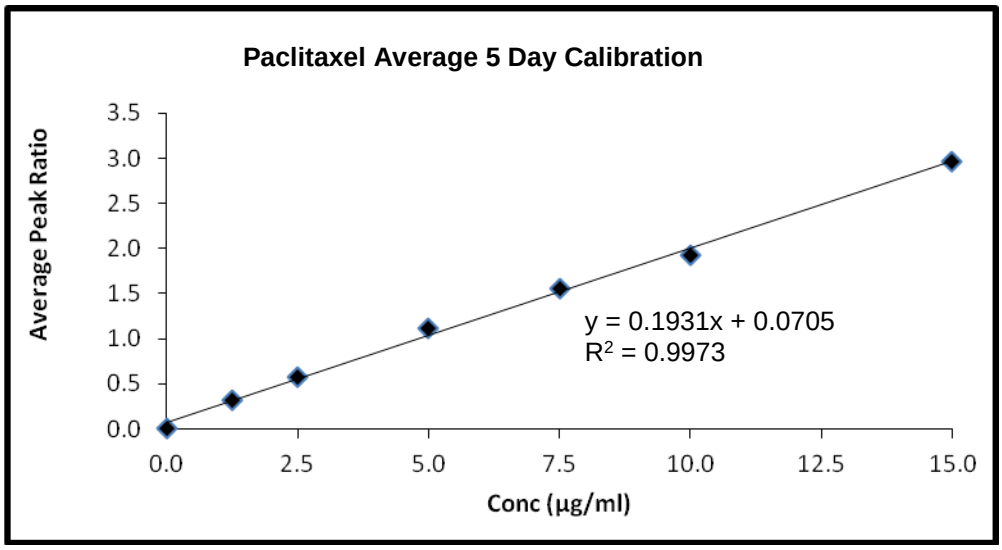


Figure 5.3: Average 5 day calibration curve of paclitaxel

5.6.3.2 Accuracy

According to the data in Table 5.2, accuracy was 97 %, 90 % and 97 % at 1.25, 7.50 and 15.00 µg/ml, respectively (see Appendix B for detailed accuracy results). The CV % was less than 15 % for all samples.

Table 5.2: Summary of accuracy data of paclitaxel in plasma at 1.25, 7.50 and 15.00 µg/ml

Conc. prepared (µg/ml)	Conc. measured (µg/ml)	Mean Accuracy (%)	SD	CV (%)
1.25	1.21	**97**	**0.13**	**10.78**
7.50	6.72	**90**	**0.85**	**12.71**
15.00	14.60	**97**	**0.61**	**4.17**

Conc. = concentration; SD = standard deviation; CV = coefficient of variation

5.6.3.4 Stability

5.6.3.4.1 Short-term stability

A variation in paclitaxel stability was observed over 48 hours at both medium (7.50 µg/ml) and high (15.00 µg/ml) concentrations (Table 5.3). It is advisable to immediately freeze samples just after blood collection, and to analyse these samples within 1-2 days (refer to Appendix C for detailed stability results).

5.6.3.4.2 Long-term stability

In view of the long-term stability of paclitaxel, it remained unstable over 4 weeks at all concentrations (Table 5.3). At all concentrations, paclitaxel showed a marked decay over 4 weeks, hereby further emphasising the importance of freezing samples immediately after blood collection. It is not recommended to store the samples for longer than 1-2 days, as samples have to be analysed as soon as possible (see Appendix C for detailed stability results).

Table 5.3: Summary of short- and long-term stability data of 1.25, 7.50 and 15.00 µg/ml paclitaxel in plasma at ambient temperature, 4 °C and -20 °C measured after 24 and 48 hours and 1, 2 and 4 weeks.

Temp.	24 hours		48 hours		1 week		2 weeks		4 weeks	
	Conc. Measured	Stab. (%)	Conc. Measured	Stab. (%)	Conc. Measured	Stab. (%)	Conc. Measured	Stab. (%)	Conc. Measured	Stab. (%)
1.25 µg/ml										
Ambient	1.2	96	2.1	168						
4 °C	1.2	96	2.3	184						
-20 °C	1.5	120	1.7	136	1.4	112	0.81	65	0.56	45
7.50 µg/ml										
Ambient	6.0	80	6.3	84						
4 °C	9.2	123	9.4	125						
-20 °C	8.6	115	7.5	100	9.5	127	4.42	59	3.26	43
15.00 µg/ml										
Ambient	12.1	81	12.2	81						
4 °C	17.6	117	18.5	123						
-20 °C	17.1	114	15.4	103	21.5	143	9.26	62	6.68	45

Temp. = temperature; Conc. = concentration; Stab. = stability

5.6.4 Application of the method

Figure 5.4 a) illustrates a chromatogram of blank rat plasma, while Figure 5.4 b) shows the chromatogram of paclitaxel in rat plasma. Paclitaxel was administered orally at a dosage of 10 mg/kg one-off and concentration was calculated as 0.74 µg/ml after 8 hours of treatment.

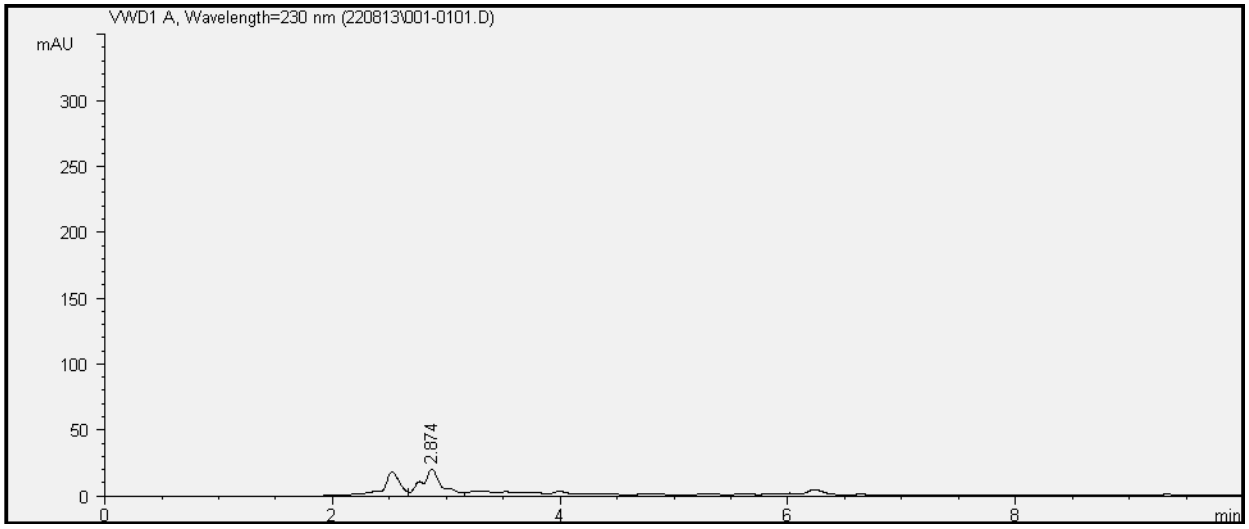


Figure 5.4 a): Chromatogram of blank rat plasma

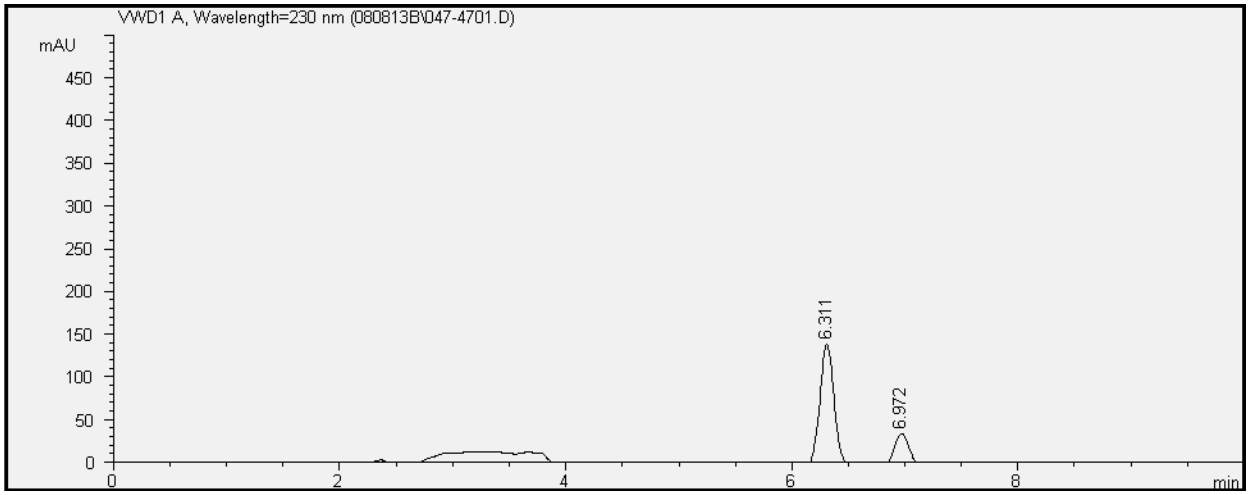


Figure 5.4 b): Chromatogram of paclitaxel (0.74 µg/ml) in rat plasma after 8 hours of 10 mg/kg one-off oral administration

5.7 Discussion

A robust and accurate HPLC method for determination of paclitaxel in plasma was successfully developed. Sharp symmetrical peaks of paclitaxel and docetaxel (internal standard) were observed in the chromatogram produced. The average calibration curve was linear (y = 0.1931x + 0.0705), with a CV % of less than 15 %. Accuracy at low, medium and high concentrations was 97 %, 90 % and 97 %, respectively. Paclitaxel was more stable at -20 °C. The method was used to monitor paclitaxel concentrations in the plasma of treated rats.

Unfortunately, paclitaxel proved to be unstable, delivering poor results. Thus, it is advisable to analyse paclitaxel plasma samples as soon as possible after blood collection. Also, repeated freeze-thaw cycles should be avoided. In spite of this shortcoming the method produced satisfactory results, as attention was paid to the time of storage of the samples.

The assay will be useful for plasma drug monitoring in patients treated with paclitaxel in the clinic and could be suitable for paclitaxel pharmacokinetic studies, and this is part of the objective of Chapter 7.

CHAPTER 6

DETERMINATION OF A POTENTIAL DRUG INTERACTION BETWEEN PACLITAXEL AND PHELA *IN VITRO*

Summary

The possibility of a direct interaction between paclitaxel (PTX) and Phela *in vitro* was investigated by monitoring the concentrations of PTX under different conditions. Firstly, during the direct interaction testing experiment, buffer was spiked with 10 µg/ml of PTX, in combination with 3.85 mg/ml Phela or without, and PTX concentrations were determined by high performance liquid chromatography (HPLC). Then, using a Slide-A-Lyzer® dialysis cassette, the time of equilibrium of PTX was determined by monitoring the changes in PTX concentrations over 12 hrs in plasma, containing 230 µg/ml PTX and buffer. Thereafter, the potential of an interaction was tested by adding 88.55 mg/ml Phela to the same experiment after 8 hours of incubation, and monitoring PTX concentrations after 10 and 12 hours by HPLC.

In the first experiment, Phela had no direct effect on PTX concentrations µg/ml: mean±SD, (PTX-only 4.93±0.22; PTX & Phela 4.94±0.19), while in the second experiment; the time of equilibrium of PTX was estimated at 8 hours. Phela had no effect on PTX concentrations (P=0.3887) and its free fraction percentage was 9.57±6.01 for the PTX-only group and that of the PTX & Phela group was 13.13±1.78. There was no significant change in PTX free fraction percentage after addition of Phela (P=0.3804). Therefore, it was concluded that there is no possible interaction between Phela and PTX *in vitro*.

6.1 Introduction

In this chapter, the potential for a drug interaction between Phela and paclitaxel was tested. *In vitro* experiments involved direct drug interaction testing of paclitaxel and Phela in buffer and a Slide-A-Lyzer® dialysis technique in both plasma and buffer. Analysis was performed by high performance liquid chromatography.

6.2 Materials and methods

6.2.1 Apparatus

For weighing gram and milligram quantities of reagents and drug standards, a precision balance (SPB 52, Scaltec Instruments, Goettingen, Germany) and analytic balance (AS 220/C/2, Randwag, Random, Poland) were used. A vortex mixer (Vortex Genie 2, Scientific Industries Inc., Bohemia, NY, U.S.A) and micro centrifuge (Minispin, Eppendorf, Hamburg, Germany) were used for mixing and spinning of the samples. Slide-A-Lyzer® Dialysis (Thermo Scientific, USA) with a membrane pore diameter of 10,000 MWCO was used to perform equilibrium dialysis. Plastic bags (110 mm X 100 mm) were used to add dialysis buffer. A horizontal shaker was used for mix shaking samples and a nitrogen evaporator coupled with a heater was used for evaporating samples.

6.2.2 Chemicals and reagents

All standards and chemicals used were of analytic grade. Phela was supplied by the Indigenous Knowledge Systems unit of the South African Medical Research Council. Paclitaxel and docetaxel were obtained from Sigma-Aldrich Co. (St. Louis, MO. USA). Orthophosphoric acid (H_3PO_4), di-sodium hydrogen orthophosphate dehydrate ($Na_2HPO_4.2H_2O$), and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4.H_2O$) were purchased from Merck laboratories (Darmstadt, Germany). HPLC grade acetonitrile (C_2H_3N), diethyl ether $(C_2H_5)_2O$), dichloromethane (CH_2Cl_2), ethanol (C_2H_6O) and methanol (CH_4O) were from Honeywell Burdick and Jackson (Muskegon, MI, U.S.A). Fresh plasma was obtained from healthy volunteers after informed consent.

6.2.3 Preparation of standard solutions

For paclitaxel, a standard solution of 2 mg/ml was prepared in methanol, and diluted to working solutions of 1 mg/ml and 50 µg/ml, respectively, with mobile phase. Docetaxel was prepared as described in section 5.3.2. Phela was prepared in water at a concentration of 300 mg/ml.

6.3 Procedures

6.3.1 Direct drug interaction testing experiment

The test samples (n=4) consisted of 100 µl buffer containing 10 µg/ml paclitaxel (PTX-only) or 10 µg/ml paclitaxel and Phela (3.85 mg/ml; PTX & Phela). For each experiment, the paclitaxel concentration was determined using the HPLC method developed earlier (Chapter 5). Here, paclitaxel concentrations were extrapolated from a calibration plot with known paclitaxel calibration standards.

6.3.2 Slide-A-Lyzer® equilibrium dialysis technique

a) Determination of the time of equilibrium of paclitaxel

The test sample consisted of 3 ml of plasma containing 230 µg/ml of paclitaxel which was injected via the syringe ports located at the top corner of the dialysis cassette (Figure 6.1 A). Air was removed from the cassette cavity (Figure 6.1 B). Thereafter, the cassette was placed in a plastic bag containing 20 ml of sodium phosphate buffer (pH 7.4), after which the plastic bag was tightly sealed, placed in a glass beaker containing water (Figure 6.1 C) and incubated at 37 °C in a shaking water bath for 12 hours in total. After 2, 4, 6, 8, 10, and 12 hours, the air was discharged into the cassette cavity (Figure 6.1 D), and a 100 µl plasma sample was drawn from the cassette chamber (Figure 6.1 E) as well as from the outer buffer in the plastic bag. After sample collection, paclitaxel concentrations were determined using the HPLC method as described in Chapter 5. Paclitaxel concentrations were extrapolated, as mentioned in section 6.3.1.

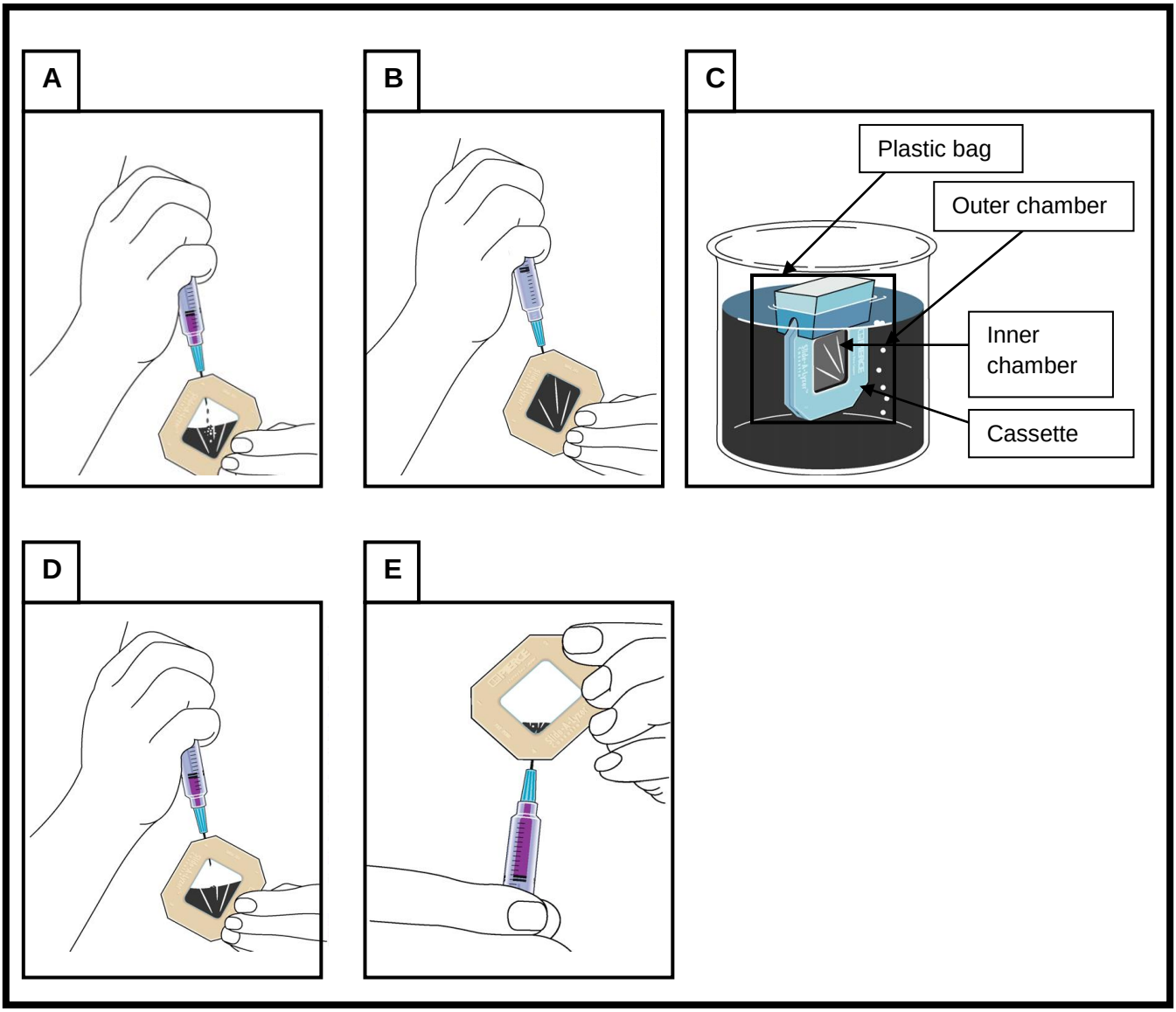


Figure 6.1: An illustration of Slide-A-Lyzer® dialysis technique

b) Determination of an interaction between paclitaxel and Phela

The test sample consisted of 3 ml plasma containing 230 µg/ml of paclitaxel. The sample was treated and incubated as described in section 6.3.2 a), for a total of 8 hours. Thereafter, 88.55 mg/ml of Phela was injected into the cassette and incubated for an additional 4 hours, during which a 100 µl sample was drawn after 2 and 4 hours from the cassette, and from the buffer. Paclitaxel concentrations were determined with the developed HPLC method, and were extrapolated as mentioned in Section 6.3.1, and the free fraction (*fu*) and bound fraction of paclitaxel, as well as the free fraction percentages (*fu* % and % bound), were calculated using the following equations:

$$Fu = \frac{[\text{Drug}]\,\text{buffer}}{[\text{Drug}]\,\text{plasma}} \qquad \text{Equation 1}$$

$$Fu\ \% = \frac{[\text{Drug}]\,\text{buffer}}{[\text{Drug}]\,\text{plasma}} \text{X } 100 \qquad \text{Equation 2}$$

$$bound = \frac{[\text{Drug}]\,\text{plasma} - [\text{drug}]\,\text{buffer}}{[\text{Drug}]\,\text{plasma}} \qquad \text{Equation 3}$$

$$\%\ bound = \frac{[\text{Drug}]\,\text{plasma} - [\text{drug}]\,\text{buffer}}{[\text{Drug}]\,\text{plasma}} \text{X } 100 \qquad \text{Equation 4}$$

6.4 Results

6.4.1 Direct drug interaction experiment

Table 6.1 shows results of paclitaxel concentrations after the direct drug interaction testing experiment, with and without Phela. Paclitaxel concentrations were similar in both experiments, and indicate that Phela did not have a direct effect on paclitaxel concentrations.

Table 6.1: Average (mean±SD) paclitaxel concentrations after the direct drug interaction testing experiment

Samples	**Conc. (µg/ml)**
PTX only	4.93±0.22
PTX & Phela	4.94±0.19

PTX = paclitaxel; Conc. = concentration

6.4.2 Slide-A-Lyzer® equilibrium dialysis

a) Time of equilibrium of paclitaxel

Table 6.2 shows the results of paclitaxel concentrations and the free fraction (*fu*) of time of equilibrium of paclitaxel, while Figure 6.2 is a graphical illustration of the same. Between 6 and 10 hours, there were no significant changes in the paclitaxel concentrations in the buffer, or in the free fraction percentage of paclitaxel, and from 12

hours these parameters remained constant. Therefore, the time of equilibrium of paclitaxel was estimated to be 8 hours (refer to Appendix D for detailed tables).

Table 6.2: Average (mean±SD) paclitaxel concentrations and its free fraction percentage

Time (hours)	PTX conc. in plasma (µg/ml)	PTX conc. in buffer (µg/ml)	Fu (%)
2	93.90	2.86	3.03
4	70.01	4.33	6.39
6	42.11	4.53	10.93
8	33.46	4.19	13.90
10	34.95	4.09	12.10
12	52.64	1.64	2.70

PTX = paclitaxel; Conc. = concentration; fu % = free fraction percentage

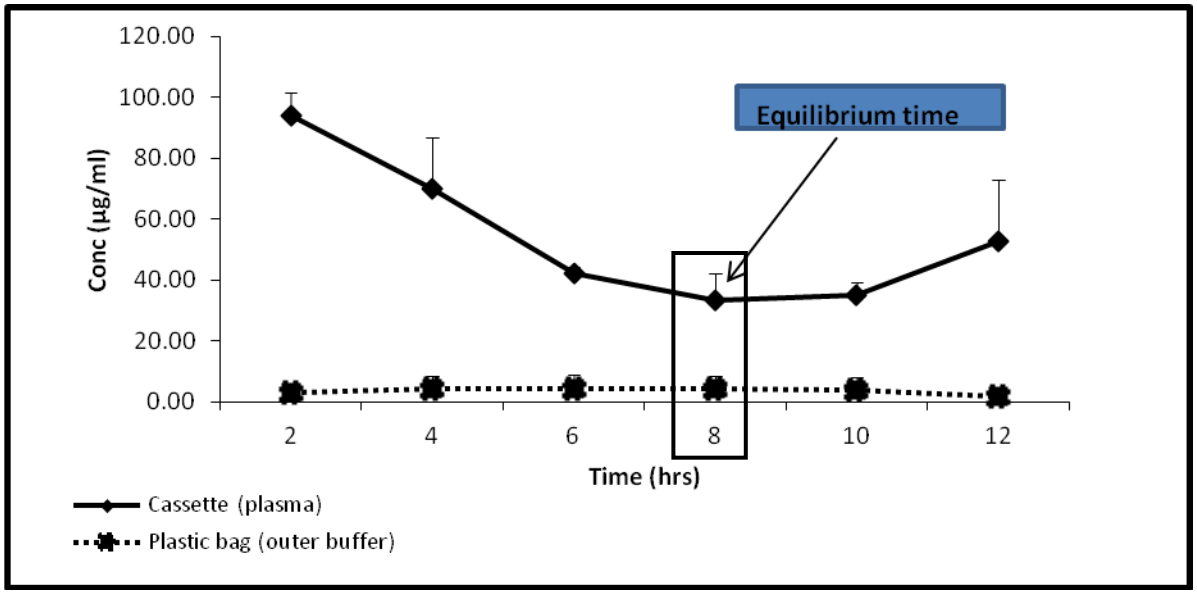


Figure 6.2: Plot of time of equilibrium of paclitaxel

b) Interaction between paclitaxel and Phela

Table 6.3 shows the results of paclitaxel concentrations with Phela, and its free fraction percentage, while Figure 6.3 is a graphical illustration of the same. After Phela was added, there were no significant changes in the paclitaxel concentrations (P=0.3887), or the free fraction percentage of paclitaxel (P=0.3804).

Table 6.3: Average (mean±SD) paclitaxel concentrations and its free fraction percentage after Phela

Time (hours)	**PTX conc. in plasma (µg/ml)**	**PTX conc. in buffer (µg/ml)**	**Fu (%)**
		PTX-only	
8	33.46	4.19	13.90
10	34.95	4.09	12.10
12	52.64	1.64	2.70
Mean	40.35	3.31	**9.57**
SD	10.67	1.44	**6.01**
		PTX & Phela	
8	33.46	4.19	13.90
10	37.21	3.85	11.10
12	31.96	4.56	14.40
Mean	34.54	4.20	**13.13**
SD	2.34	0.36	**1.78**

PTX = paclitaxel; Conc. = concentration; fu % = fraction unbound percentage

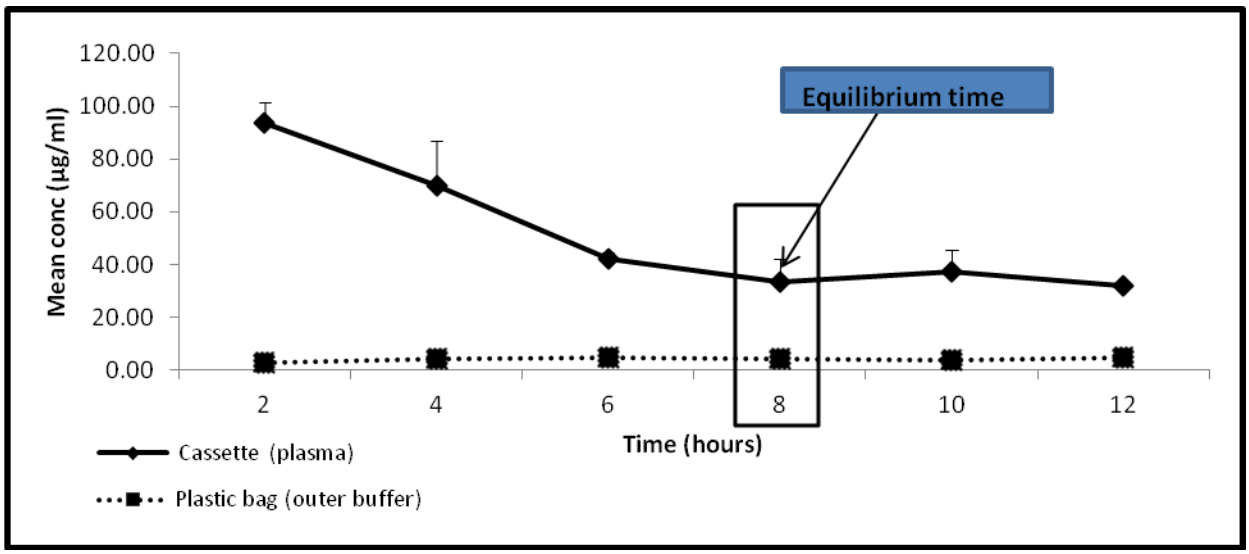


Figure 6.3 Plot of paclitaxel concentrations after the addition of Phela

6.5 Discussion

In vitro studies are one set of approaches to developing information about herb-drug interactions. As there is a correlation between *in vitro* and *in vivo* studies, it is always important to determine possible interactions using *in vitro* testing. Co-administration of traditional medicines with prescription drugs may result in potential pharmacokinetic interactions mediated by transporters such as herb-drug interactions (Tomlinson *et al.*, 2008)**.** In the current study paclitaxel, a known substrate of *P*-glycoprotein (*P*-gp; Sparreboom *et al.*, 1997), was used and *in vitro* drug interactions were studied between paclitaxel and Phela. Phela is a traditional medicine under investigation by MRC as an immune booster (Lekhooa *et al*., 2012).

The direct drug interaction testing experiment in buffer and Slide-A-Lyzer® equilibrium dialysis technique were used since they are convenient methods to determine drug interactions *in vitro* and are also inexpensive. Moreover, the results of dialysis experiment are obtained under equilibrium conditions and in that way the true nature of the interaction is studied. Only free fraction of the drug is available for pharmacological interactions (Banker *et al*., 2003). The results of this experiment have shown that Phela had no effect on the free fraction percentage of paclitaxel throughout the duration of the study. Observations have also shown that the chosen concentration of Phela (3.85 mg/ml) which was to be used for *in vivo* testing had no effect on paclitaxel concentration, where any change in paclitaxel

concentration and free fraction percentage would indicate an interaction. This implies that there is no direct interaction between Phela and paclitaxel, since the protein binding capacity of paclitaxel was not affected. However, the implications of these observations require further studies *in vivo* to confirm whether co-administration of Phela and paclitaxel are likely to cause a herb-drug interaction, and this is part of the objective of the next chapter.

The interaction between cyclosporin A and Phela was determined by Lekhooa *et al*. (2012, p.47-63). For Methotrexate and probenecid, enzyme multiplied immunoassay (EMI) was selected as a method of choice (mentioned in Chapter 3, section 3.1.2). EMI uses a small serum sample volume of 50 µl and has lower detection limits of 0.05 µM and 0.1 µM plasma, respectively (Lennard, 1999). As a result, *in vitro* experiments (drug interaction testing in buffer and Slide-A-Lyzer® dialysis) could not be conducted, since both require analysis in buffer, which might require further dilutions in plasma and affect the detection of drugs by kits.

CHAPTER 7

THE EFFECT OF PHELA ON *P*-GLYCOPROTEIN AND MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 2 TRANSPORTERS

Summary

Membrane transporters play an integral role in the determination of the pharmacokinetic, safety and efficacy profiles of drugs. Even so, little is known about the role of membrane transporters in herb-drug interactions. Therefore, the effect of Phela on intestinal *P*-glycoprotein (*P*-gp) and multidrug resistance-associated protein 2 (MRP2) was investigated in a rat model. Paclitaxel (PTX) and cyclosporin A (CyA) were used as the respective substrate and inhibitor of *P*-gp, while methotrexate (MTX) and probenecid (PRO) were those of MRP2.

Ethical approval was obtained and male Sprague-Dawley (SD) rats (200-250 g) were used. The animal experiment was divided into two parts. In Part I, three groups of 40 rats each received a one-off oral dose of PTX-only (10 mg/kg), PTX & CyA (10 mg/kg) or PTX & Phela (15.4 mg/kg), while in Part II, three groups of 40 rats each received a one-off oral dose of MTX-only (10 mg/kg), MTX & PRO (20 mg/kg), or MTX & Phela (15.4 mg/kg). For each group, 5 rats were sacrificed after 0.5, 1, 2, 4, 6, 8, 10, and 12 hours. Blood was analysed for full blood count, liver function, and PTX and MTX concentrations.

CyA and PRO increased the area under the plasma concentration-time curve (AUC) of PTX and MTX, respectively, whereas Phela had no effect on the AUC of PTX or MTX. Therefore, Phela did not inhibit *P*-gp or MRP2, and this implies that Phela will most probably not be involved in herb-drug interactions of membrane transporter origin.

7.1 Introduction

This study was aimed at determining the effect of Phela on two efflux drug-transporters, *P*-glycoprotein (*P*-gp) and multidrug resistance-associated protein 2 (MRP2) in the gastrointestinal tract of a rat model. Paclitaxel and cyclosporin A were used as the respective substrate and inhibitor of *P*-gp, while methotrexate and probenecid were those of MRP2. Paclitaxel concentrations were determined by high performance liquid chromatography, while those of methotrexate with enzyme multiplied immunoassay.

7.2 Methods

A. Materials

7.2.1 Apparatus

Rats were weighed with a precision balance (1213 MP, Sartorius, Göttigen, Germany), and feeding needles (16 G-3)", curved 3 mm ball; Popper and Sons Inc, NY, U.S.A, were used for oral gavage. Anaesthesia was administered with a gas anaesthetic machine (Ugo Basile, Comerio, VA, Italy), while blood was collected in the following tubes (Vacuette®, Greiner Bio-One): 4 ml K2EDTA tubes (Kremsmünster, Austria), 4 ml lithium heparin tubes separator tubes and 5 ml Z serum separator clot activator tubes (Chonburi, Thailand). Rat surgery was performed with a dissection kit (Lasec S.A., Bloemfontein, South Africa). For analysis of paclitaxel concentrations, the apparatus are the same as discussed in Chapter 5. Methotrexate concentrations were analysed by enzyme multiplied immunoassay (EMI) using an automated drug analyser (Siemens dimension Xpand plus, Siemens healthcare diagnostic, Syva Business, Newark, DE).

7.2.2 Chemicals

All standards and chemicals used were of analytical grade. Paclitaxel, docetaxel, cyclosporin A and probenecid were obtained from Sigma-Aldrich Co. (St. Louis, MO. USA). Phela was supplied by the Indigenous Knowledge Systems unit of the South African Medical Research Council. Paxitas (paclitaxel) injection (30 mg/ 5 ml; Accord Healthcare (Pty) Ltd, Rivonia, Gauteng, South Africa) and P&U METHOTREXATE CSV (methotrexate) injection (50 mg/ 2 ml; Pfizer Laboratories (Pty) Ltd, Sandton, Gauteng, South Africa) were purchased from a local pharmacy. Saline solution (0.9 %; Adcock Ingram Critical Care (Pty) Ltd, Johannesburg, South Africa) was kindly sponsored by the Department of Pharmacology, Toxicology Laboratory, University of the Free State, while olive oil was purchased from a local supermarket. ISOFOR (isoflurane) inhalation anaesthetic was obtained from Safe Line Pharmaceuticals (Pty) Ltd (Roodepoort, Gauteng, South Africa) and Emit® methotrexate assay kits were purchased from Siemens Medical Solutions Inc (Malvern, U.S.A).

7.2.3 Preparation of drugs for oral administration

All drugs were prepared in saline solution to attain a volume of 4 ml/kg: 20 mg/kg paclitaxel, 10 mg/kg cyclosporin A, 10 mg/kg methotrexate, 20 mg/kg probenecid and 15.4 mg/kg Phela.

B. Procedures

7.2.4 Experimental design

Total of 292 rats were used. Ten rats were not treated with any drug, and used for baseline data. Furthermore, the study was divided into two parts.

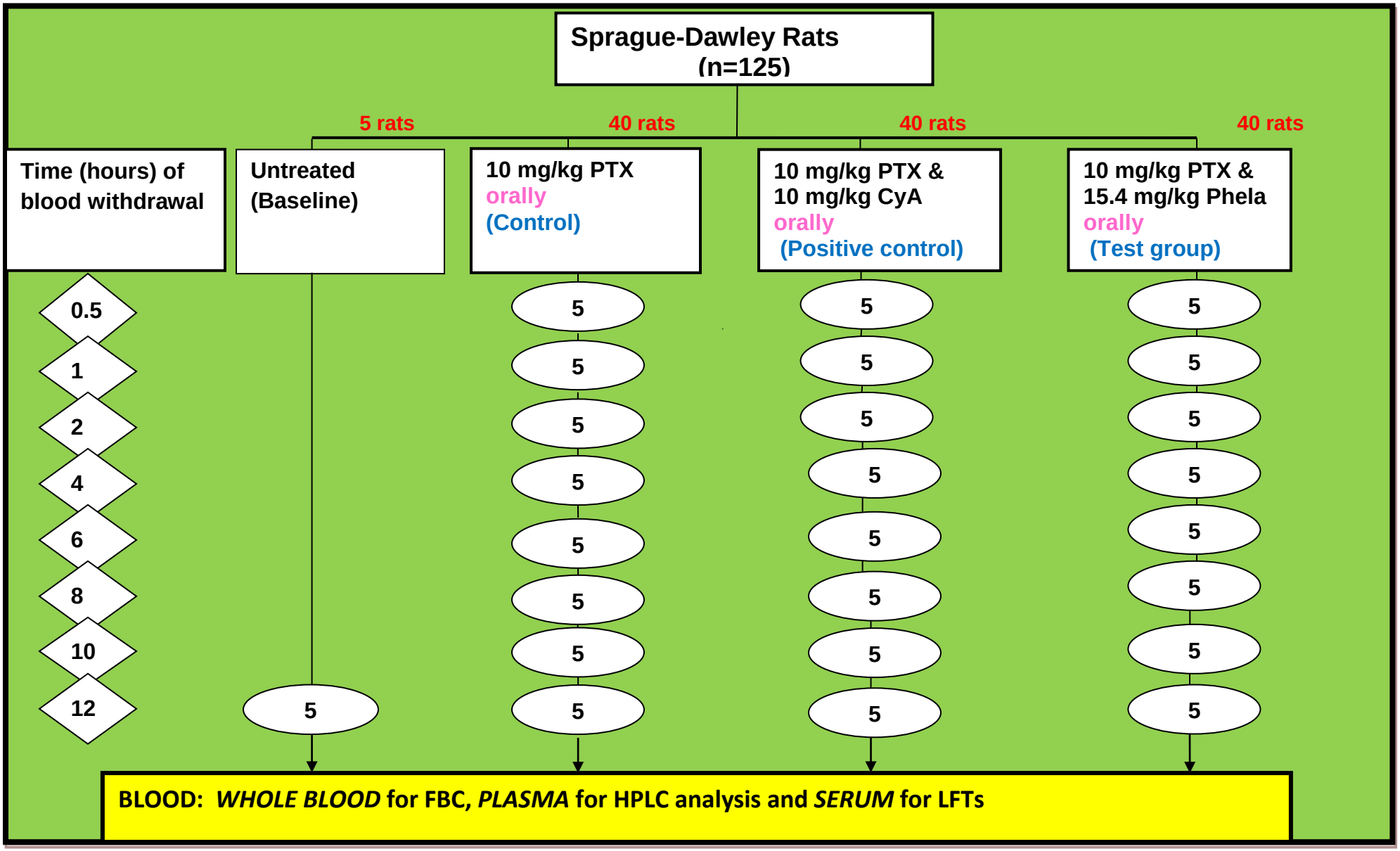
7.2.4.1 Part I – The effect of Phela on *P*-glycoprotein

Rats were divided into three groups of 40 animals each, namely: the PTX-only group (control), the PTX & CyA group (positive control), and the PTX & Phela group (test). Rats received paclitaxel only, paclitaxel and cyclosporin A, or paclitaxel and Phela in a one-off oral dose, and 5 rats were sacrificed after 0.5, 1, 2, 4, 6, 8, 10 and 12 hours. Paclitaxel, cyclosporin A and Phela were administered as follows (Figure 7.1):

- PTX-only group: 10 mg/kg paclitaxel (4 ml/kg, orally)
- PTX & CyA group: 10 mg/kg paclitaxel and 10 mg/kg cyclosporin A (4 ml/kg, orally)
- PTX & Phela group: 10 mg/kg paclitaxel and 15.4 mg/kg Phela

The doses of paclitaxel and cyclosporin A were as per van Asperen *et al*. (1998), while that of Phela was as per Lekhooa *et al*. (2012).

Figure 7.1: A schematic illustration of Part I of the animal experiment



7.2.4.2 Part II – The effect of Phela on multidrug resistance-associated protein 2

a) Preliminary experiment

Treatment was started for the group co-administered with methotrexate and probenecid (MTX & PRO). Here, a dose of 5 mg/ml probenecid was used. Unfortunately, this dosage was not sufficient as multidrug resistance-associated protein 2 (MRP2) was not inhibited. As such, the dose of probenecid, as well as the vehicle in which the drug would be prepared, had to be revised.

Rats were weighed and divided into six groups of six animals each, namely: the MTX-only (saline control); the MTX & PRO-10 group 1 (saline positive control); the MTX & PRO-20 group 1 (saline positive control); the MTX & OO group (olive oil control); the MTX & PRO-10 group 2 (olive oil positive control), and the MTX & PRO-20 group 2 (olive oil positive control). Rats received methotrexate only, methotrexate and probenecid in saline, or methotrexate and probenecid in olive oil in a one-off oral dose, and 3 rats per group were sacrificed after 0.5 and 4 hours. Methotrexate and probenecid were administered as follows (Figure 7.2):

- MTX-only group: 10 mg/kg methotrexate (4 ml/kg, orally)
- MTX & PRO-10 group 1: 10 mg/kg methotrexate and 10 mg/kg probenecid in saline (4 ml/kg, orally)
- MTX & PRO-20 group 1: 10 mg/kg methotrexate and 20 mg/kg probenecid in saline (4 ml/kg, orally)
- MTX & OO group: 10 mg/kg and 1 ml olive oil (4 ml/kg, orally)
- MTX & PRO-10 group 2: 10 mg/kg methotrexate and 10 mg/kg probenecid in olive oil (4 ml/kg, orally)
- MTX & PRO-20 group 2: 10 mg/kg methotrexate and 20 mg/kg probenecid in olive oil (4 ml/kg, orally)

After blood collection, methotrexate concentrations were quantified by enzyme multiplied immunoassay, and the results of the test groups were compared to their respective controls.

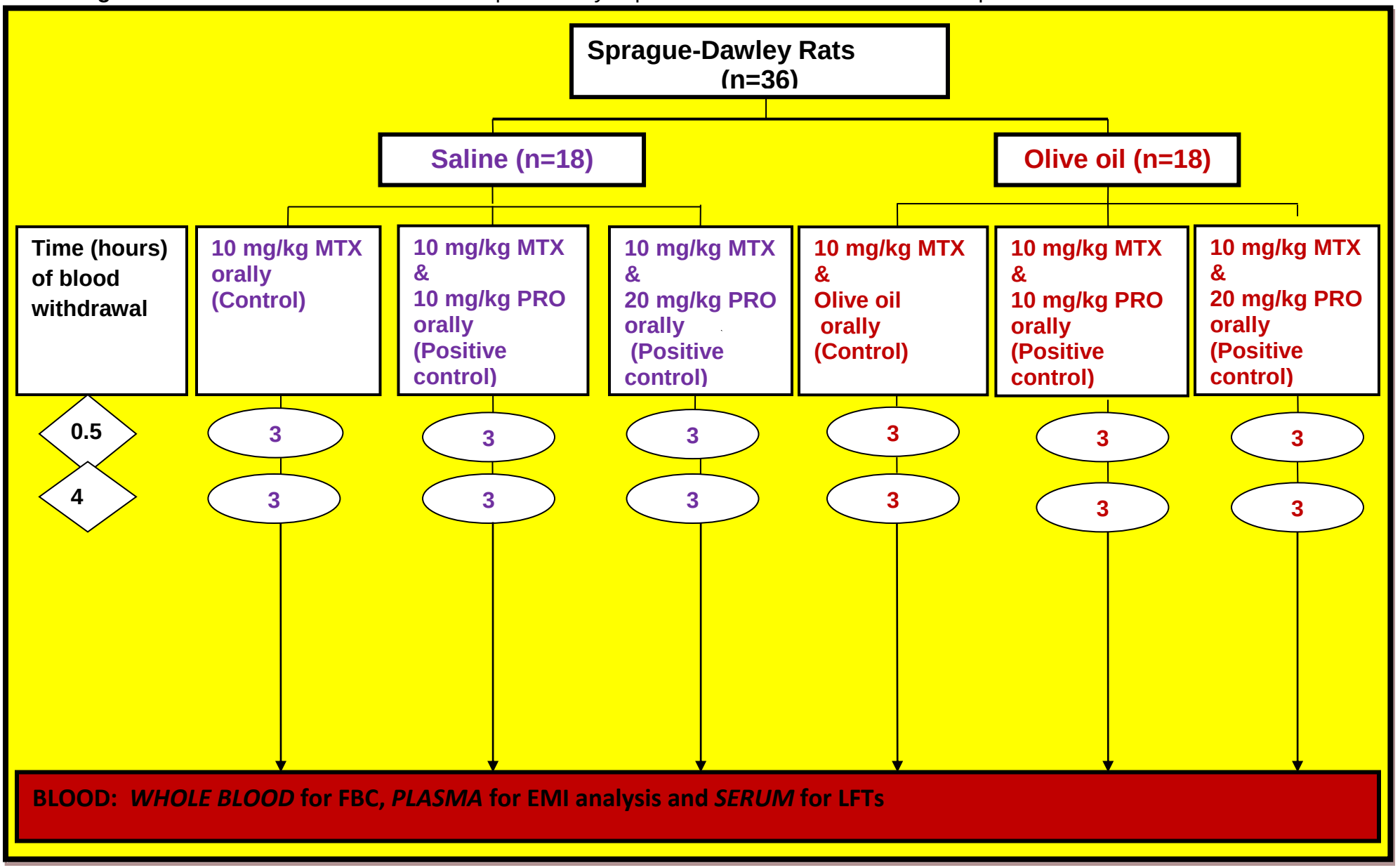
Table 7.1 shows a summary of methotrexate concentrations after 0.5 and 4 hours of co-treatment with probenecid dissolved in either saline or olive oil. Methotrexate concentrations after 0.5 hours were similar in both groups. Olive oil increased the methotrexate concentration in rats treated with methotrexate and olive oil, and in all groups that used olive oil as a vehicle. Even though the groups treated with 10 and 20 mg/kg of probenecid in olive oil exhibited higher concentrations of methotrexate, the MRP2 inhibition was a combined effect of probenecid and olive oil. As a result, olive oil as a vehicle was not considered further. The methotrexate concentration was increased in all test groups that used saline as vehicle, which indicates MRP2 inhibition by probenecid. The dose of 20 mg/kg of probenecid in saline provided sufficient inhibition of MRP2 and was used further.

Table 7.1: Average (Mean ±SD) methotrexate concentrations after 0.5 and 4 hours of co-treatment with probenecid

Time (hours)	Vehicle	Groups	Concentration (µg/ml)
0.5	**Saline**	MTX-only group	0.2±0.0
		MTX & PRO-10 group 1	0.2±0.1
		MTX & PRO-20 group 1	0.2±0.0
0.5	**Olive oil**	MTX & OO group	0.3±0.0
		MTX & PRO-10 group 2	0.4±0.1
		MTX & PRO-20 group 2	0.4±0.0
4	**Saline**	MTX-only group	0.2±0.0
		MTX & PRO-10 group 1	0.2±0.1
		MTX & PRO-20 group 1	0.3±0.0
4	**Olive oil**	MTX & OO group	0.3±0.0
		MTX & PRO-10 group 2	0.4±0.0
		MTX & PRO-20 group 2	0.4±0.2

MTX = methotrexate; PRO = probenecid; OO = olive oil

Figure 7.2: A schematic illustration of the preliminary experiment of Part II of the animal experiment



b) Ultimate experiment

Rats were weighed and divided into three groups of 40 animals each, namely: the MTX-only group (control), the MTX & PRO group (positive control) and the MTX & Phela group (test). Rats received methotrexate only, methotrexate and probenecid or methotrexate and Phela in a one-off oral dose and 5 rats per group were sacrificed after 0.5, 1, 2, 4, 6, 8, 10 and 12 hours. Methotrexate, probenecid and Phela were administered as follows (Figure 7.3):

- MTX-only group: 10 mg/kg methotrexate (4 ml/kg, orally)
- MTX & PRO group: 10 mg/kg methotrexate and 20 mg/kg probenecid (4 ml/kg, orally)
- MTX & Phela group: 10 mg/kg methotrexate and 15.4 mg/kg Phela

The doses of methotrexate and probenecid were as per Shin *et al*. (2013) and Tunblad *et al*. (2003), respectively, while that of Phela was as per Lekhooa and co-workers (2012).

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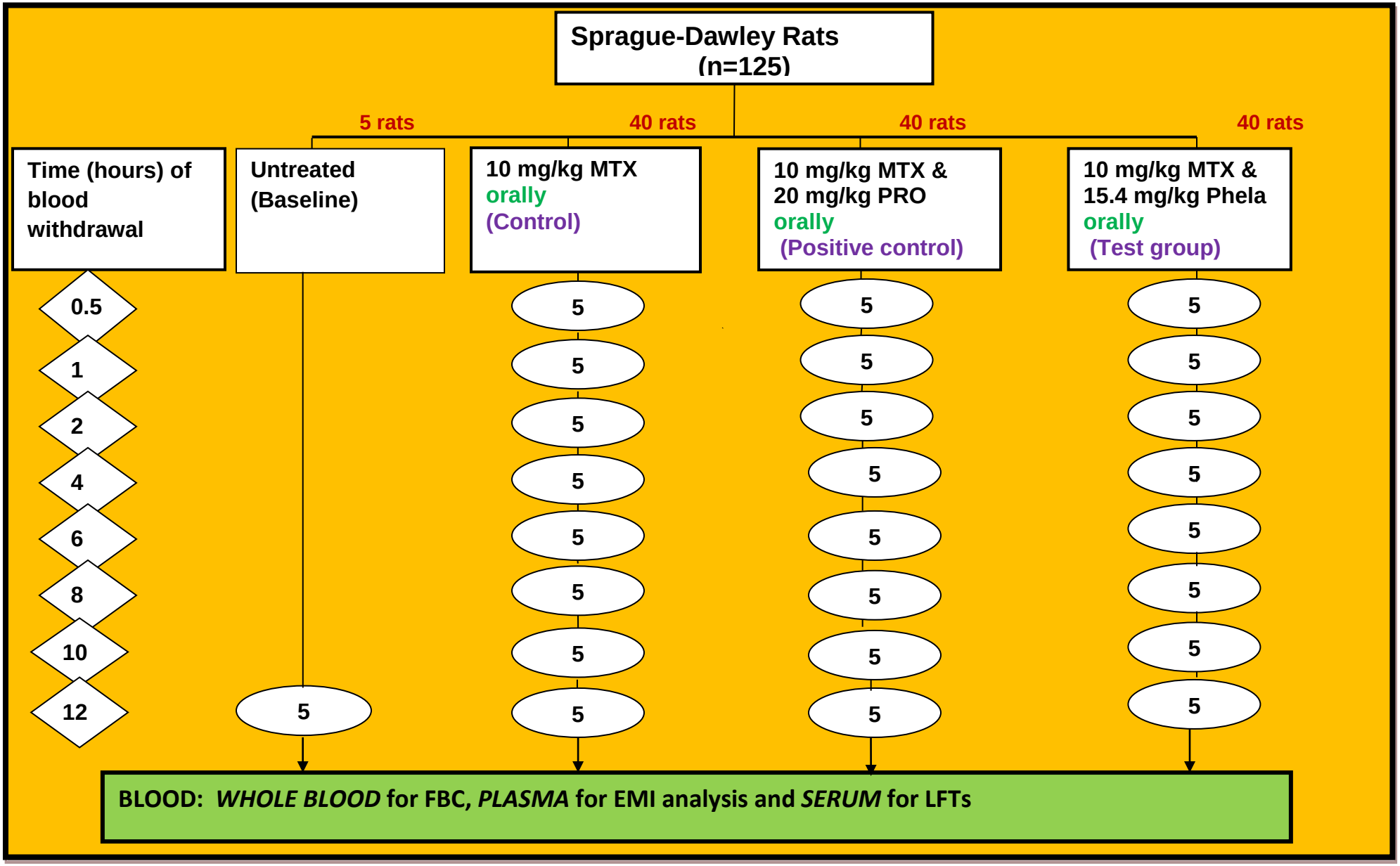
Figure 7.3: A schematic illustration of Part II of the animal experiment



7.2.5 Animal care

Ethical approval (Animal Experiment NR 12/2012) was obtained from the Animal Ethics Committee of the University of the Free State. Male Spraque-Dawley (SD) rats with a weight range of 200 – 250 g were used. Animals were housed at the Animal House of the University of the Free State, where they were fed and looked after by qualified staff, and their cages were cleaned once a week. Standard rat chow and water was available to the animals *ad libitum*. All drug administration took place at the Animal House, and animals were inspected for skin lesions and other visible adverse events throughout the experiment.

7.2.6 Animal weighing and blood collection

For all groups, animals were weighed before the day of the start of dosing, in order to prepare the required drug dosage per kg of body weight. During the treatment period the respective drugs were administered by oral gavage. Rats were sacrificed after 0.5, 1, 2, 4, 6, 8, 10 and 12 hours of a single dose. Anesthesia was performed with isoflurane. Firstly, anaesthesia was induced in a gas chamber at a concentration of 4 % isoflurane, after which the rat was transferred and secured to a surgical board. Secondly, anaesthesia was maintained with a cone diaphragm at a concentration of 2 % isoflurane. Under anaesthesia, central blood was drawn by direct cardiac puncture (Figure 7.4). Blood was collected in yellow top serum separator tubes for serum, green top lithium heparin tubes for plasma, and purple top EDTA tubes for full blood count. The plasma and serum tubes were centrifuged, the separated serum and plasma removed and stored at -20 °C and -85 °C for plasma and serum, respectively, until analysis. Blood for the full blood count was sent to an independent laboratory for analysis (Section 7.2.7).

After blood collection, animals were sacrificed by exsanguination whilst still under isoflurane anaesthesia.

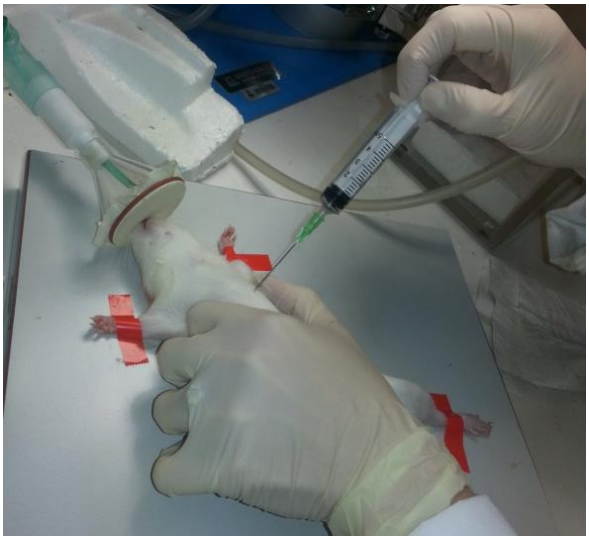


Figure 7.4: A photograph of blood collection via cardiac puncture

7.2.7 Analysis of function tests

Whole blood was sent to the National Health Laboratory Service (NHLS) for full blood count analysis.

Serum was sent to the Toxicology Laboratory at the Department of Pharmacology for liver function tests, *i.e.,* alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate aminotransferase (AST). All function tests were done after 12 hours of treatment.

7.2.8 Analysis of paclitaxel concentrations in rat plasma

Paclitaxel concentrations in plasma were determined by the high performance liquid chromatography assay, as described in Chapter 5. A standard/ calibration curve was generated from six known calibration standards, from which paclitaxel concentrations in rat plasma were extrapolated.

7.2.9 Analysis of methotrexate concentrations in rat plasma

The Toxicology Laboratory at the Department of Pharmacology determined methotrexate concentrations in rat plasma by enzyme multiple immunoassay.

7.2.10 Determination of pharmacokinetic parameters

Pharmacokinetic parameters were calculated by using plasma concentration versus time data. The non-compartmental pharmacokinetic analysis was done with a computerized template to calculate the area under the curve (AUC) of the plasma concentration (C_p) as a function of time (t). AUC was computed to reduce the errors associated with the trapezoidal

rule. The maximum plasma concentration (C_{max}) and the time to reach the maximum plasma concentration (T_{max}) were determined by a visual inspection of the experimental data. Other parameters were determined as follows:

- Elimination rate constant (k_e): calculated by regression analysis from the slope of the line
- Half-life:

$$Half - life(\frac{t1}{2}) = 0.693/ke \quad \text{Equation 1}$$

- Mean residence time (MRT_{oral}):

$$MRToral = 1/ke \quad \text{Equation 2}$$

- Volume of distribution (Vd):

$$Vd = Dose/\ AUC\ X\ ke \quad \text{Equation 3}$$

- Total plasma clearance after oral administration (CLp):

$$CLp = Dose/AUC \quad \text{Equation 4}$$

7.2.11 Statistical analysis

Data were analysed by non-parametric methods utilizing the Graphpad™Statistical program. Accordingly, parameters were reported as mean and standard deviation (SD), and the student's *t* test was used for data comparison between groups, with the level of significance set at the $P<0.05$.

7.3 Results

A. Part I: The effect of Phela on *P*-glycoprotein

7.3.1 Physiological observations

7.3.1.1 Full blood count

Table 7.2 shows results of the full blood count of the PTX-only, PTX & CyA, and PTX & Phela groups over 12 hours. Despite wide variations amongst groups, all the full blood count parameters were within normal range.

Table 7.2: Average (mean±SD) full blood count and platelets results of the PTX-only, PTX & CyA, and PTX & Phela groups over 12 hours

Parameters	Groups			
	Untreated	**PTX-only**	**PTX & CyA**	**PTX & Phela**
		Red blood cells		
RCC (X10^12/l)	6.3±0.2	6.8±0.2	4.8±0.5*	4.4±2.0
Haemoglobin (g/dl)	12.9±0.3	14.1±0.6	10.4±1.1*	9.4±4.3
Haematocrit (l/l)	0.4±0.0	0.4±0.0	0.3±0.0*	0.3±0.1
MCV (f/l)	63.5±2.5	63.1±1.1	64.1±2.2	65.1±0.9*
MCH (pg)	20.5±0.4	20.7±0.5	21.5±0.7	21.4±0.6
MCHC (g/dl)	32.3±0.8	32.8±0.3	33.5±2.1	32.9±0.9
Platelets (X10^9/l)	860.0±221.1	724.8±134.0	346.5±235.7*	409.0±212.4
		White blood cells		
WCC (X10^9/l)	7.0±2.7	7.5±2.0	6.4±2.1	3.0±1.3
Neutrophils (X10^9/l)	0.5±0.5	1.1±0.4	0.3±0.2	0.6±0.3
Lymphocytes (X10^9/l)	3.1±3.0	5.3±0.8	5.6±2.0	2.2±1.1
Monocytes (X10^9/l)	0.1±0.1	0.2±0.1	0.4±0.1	0.1±0.1
Eosinophils (X10^9/l)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Basophils (X10^9/l)	3.2±5.5	0.1±0.1	0.2±0.1	0.0±0.0

*P<0.05 control vs. positive control and test group, PTX = paclitaxel; CyA = cyclosporin A; RCC = red cell count; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; WCC = white cell count

7.3.1.2 Liver function tests

Table 7.3 shows the ALP, ALT and AST of the PTX-only, PTX & CyA, and PTX & Phela groups over 12 hours. Liver function was normal. There were no differences in the ALP, ALT and AST of all groups.

Table 7.3: Average (mean±SD) liver function test results of PTX-only, PTX & CyA, and PTX & Phela groups over 12 hours

Group		**LFT**	
	ALP	**ALT**	**AST**
(n=3)	**(U/L)**	**(U/L)**	**(U/L)**
Untreated			
0 hours	352.3±75.6	49.7±4.7	88.0±13.9
PTX-only			
12 hours	411.7±160.5	67.8±29.8	90.3±34.7
PTX & CyA			
12 hours	372.0±20.3	65.0±18.3	164.7±51.0
PTX & Phela			
12 hours	367.7±51.8	67.8±35.8	105.7±19.7

ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate aminotransferase; LFT = liver function test

7.3.1.3 Paclitaxel concentrations

Table 7.4 shows paclitaxel concentrations after 12 hours of co-treatment with Phela, while Figure 7.5 is a graphical illustration of the same. Cyclosporin A significantly increased paclitaxel concentrations throughout the 12 hours, and Phela had no effect on paclitaxel concentrations over 12 hours.

Table 7.4: Average (mean±SD) paclitaxel concentrations (µg/ml) after 12 hours of co-treatment with Phela

Time	Groups		
(hours)	**PTX-only**	**PTX & CyA**	**PTX & Phela**
0.5	4.61±0.13	5.93±0.49	4.60±0.12
1	4.04±0.07	5.90±0.40	4.03±0.27
2	1.06±0.15	2.24±0.14	1.23±0.12
4	1.30±0.08	2.69±0.15	1.66±0.18
6	1.02±0.09	2.20±0.03	1.31±0.09
8	0.74±0.04	1.91±0.11	1.01±0.02
10	0.51±0.10	1.71±0.10	0.71±0.17
12	0.30±0.07	1.70±0.16	0.60±0.10

PTX = paclitaxel; CyA = cyclosporin A

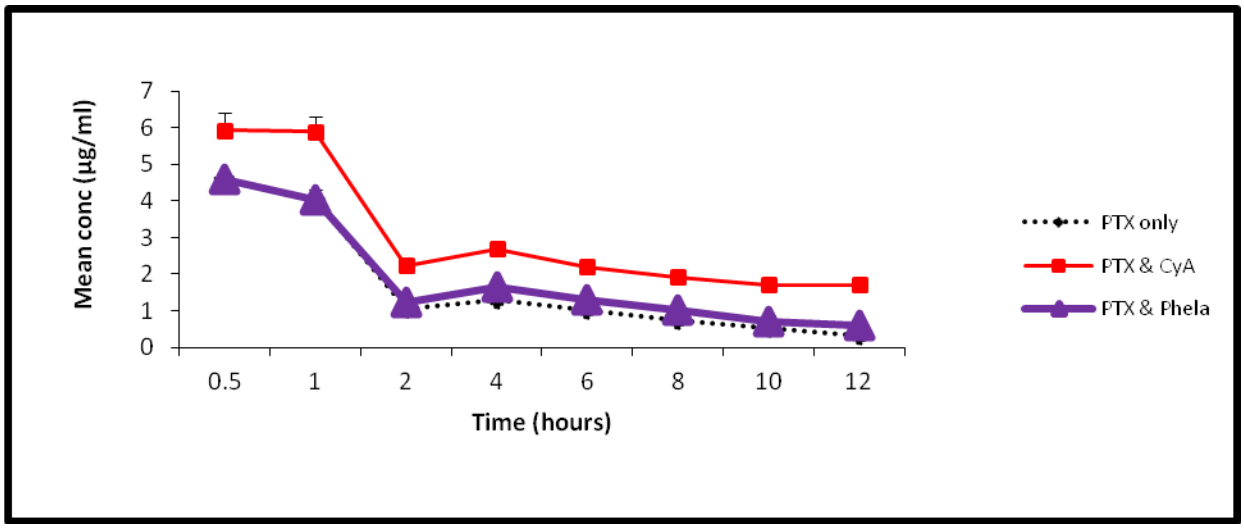


Figure 7.5: Paclitaxel concentrations after co-treatment with Phela over 12 hours

7.3.1.4 Paclitaxel pharmacokinetic parameters

Table 7.5 shows paclitaxel pharmacokinetic parameters after 12 hours of co-treatment with Phela. Cyclosporin A significantly increased paclitaxel AUC, whereas Phela did not affect paclitaxel AUC (P=0.084). Cyclosporin A also increased the half-life and mean residence time (MRT), whereas paclitaxel kinetics after co-administration with Phela were similar to that of the controls.

Table 7.5: Paclitaxel pharmacokinetic parameters in rats after 12 hours of co-treatment with Phela

Kinetic parameters	PTX-only (Control)	PTX & CyA (+ control)	PTX & Phela (Test group)
Wt (kg)	0.21±0.0	0.21±0.0	0.21±0.0
Dose (mg)	2.4±0.0	2.1±0.0	2.1±0.0
C_o (mg/L)	3.5±0.2	2.7±0.1	3.5±0.2
K_e (hr)	0.2±0.0	0.0±0.0*	0.2±0.0
Half-life (hr)	3.7±0.4	18.4±0.5*	4.4±0.4
AUC_{oral} (mg.hr/L)	18.5±1.9	70.4±1.4*	21.8±1.7
Vd (L)	0.7±0.0	0.8±0.0	0.6±0.4
CLp (L/hr)	0.1±0.0	0.0±0.0*	0.1±0.0
MRT (hr)	5.3±0.5	26.5±0.7*	6.3±0.6
Cmax (µg/ml)	4.6±0.1	6.2±0.3*	4.6±0.1
Tmax (hr)	0.5±0.0	0.8±0.3	0.5±0.0

PTX = paclitaxel; CyA = cyclosporin A; +control = positive control; Wt = weight; Co = concentration of the metabolite after instantaneous distribution at time zero; Ke = elimination rate constant of the metabolite; AUC_{oral} = area under the curve of the plasma concentration (C_{oral}) as a function of time; Vd = volume of distribution of the metabolite; CLp_{oral} = plasma clearance of the metabolite; MRT = mean residence time of the metabolite; Cmax = maximum plasma concentration; Tmax = the time to reach the maximum plasma concentration

B. Part II: The effect of Phela on multidrug resistance-associated protein 2

7.3.2 Physiological observations

7.3.2.1 Full blood count

Table 7.6 shows results of the full blood count of the MTX-only, MTX & PRO, and MTX & Phela groups, over 12 hours. Despite wide variations amongst groups, all the full blood count parameters were within normal range.

Table 7.6: Average (mean±SD) full blood count and platelets results of the methotrexate only, methotrexate and probenecid, and methotrexate and Phela groups over 12 hours

Tests	Groups			
	Untreated	**MTX-only**	**MTX & PRO**	**MTX & Phela**
		Red blood cells		
RCC (X10^12/l)	6.3±0.2	5.5±0.4	5.8±0.2	5.6±0.3
Haemoglobin (g/dl)	12.9±0.3	11.7±0.2	11.7±0.0	11.8±0.7
Haematocrit (l/l)	0.4±0.0	0.3±0.0	0.4±0.0	0.3±0.0
MCV (f/l)	63.5±2.5	61.8±2.4	64.2±0.8	59.4±1.1
MCH (pg)	20.5±0.4	21.3±0.8	20.5±0.4	20.9±0.2
MCHC (g/dl)	32.3±0.8	35.4±0.4	31.5±0.5	35.3±0.3
Platelets (X10^9/l)	860.0±221.1	554.3±37.4	522.3±53.4	713.7±50.6
		White blood cells		
WCC (X10^9/l)	7.0±2.7	5.2±0.8	5.2±1.2	4.9±0.7
Neutrophils (X10^9/l)	0.5±0.5	0.5±0.0	0.3±0.0	0.5±0.0
Lymphocytes (X10^9/l)	3.1±3.0	4.3±1.0	3.8±0.7	3.5±1.0
Monocytes (X10^9/l)	0.1±0.1	0.3±0.0	0.2±0.0	0.1±0.0
Eosinophils (X10^9/l)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Basophils (X10^9/l)	3.2±5.5	0.0±0.0	0.1±0.0	0.0±0.0

MTX = methotrexate; PRO = probenecid; RCC = red cell count; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; WCC = white cell count;

7.3.2.2 Liver function tests

Table 7.7 shows the ALP, ALT and ASP of the MTX-only, MTX & PRO and MTX & Phela groups, over 12 hours. Liver function was normal. There were no differences in the ALP, ALT, and AST of all groups.

Table 7.7: Average (mean±SD) liver function test results of MTX-only, MTX & PRO, and MTX & Phela groups, over 12 hours

Group	**LFT**		
	ALP	**ALT**	**AST**
(n=3)	**(U/l)**	**(U/l)**	**(U/L)**
Untreated			
0 hours	352.3±75.6	49.7±4.7	88.0±13.9
MTX-only			
12 hours	302.7±59.0	30.3±2.5	78.7±1.7
MTX & PRO			
12 hours	286.7±76.1	34.0±4.3	99.0±6.2
MTX & Phela			
12 hours	257.7±36.6	32.3±2.5	86.6±1.6

ALP = alkaline phosphatase; ALT = alanine transaminase; AST = aspartate aminotransferase; LFT = liver function test

7.3.2.3 Methotrexate concentrations

Table 7.8 shows methotrexate concentrations over 12 hours of co-treatment with Phela, while Figure 7.6 is a graphical illustration of the same. Probenecid significantly increased methotrexate concentrations throughout 12 hours (P=0.0026), while Phela had no effect on methotrexate concentrations over 12 hours (P=0.5790).

Table 7.8: Average (mean±SD) methotrexate concentrations after 12 hours of co-treatment with Phela

Time	Groups		
(hours)	**MTX-only**	**MTX & PRO**	**MTX & Phela**
0.5	0.44±0.01	0.60±0.02	0.53±0.08
1	0.34±0.13	0.43±0.01	0.40±0.02
2	0.31±0.02	0.56±0.12	0.28±0.10
4	0.18±0.04	0.26±0.01	0.20±0.04
6	0.12±0.04	0.24±0.03	0.16±0.05
8	0.15±0.07	0.18±0.07	0.08±0.06
10	0.12±0.01	0.14±0.03	0.11±0.03
12	0.04±0.01	0.09±0.01	0.04±0.02

MTX = methotrexate; PRO = probenecid

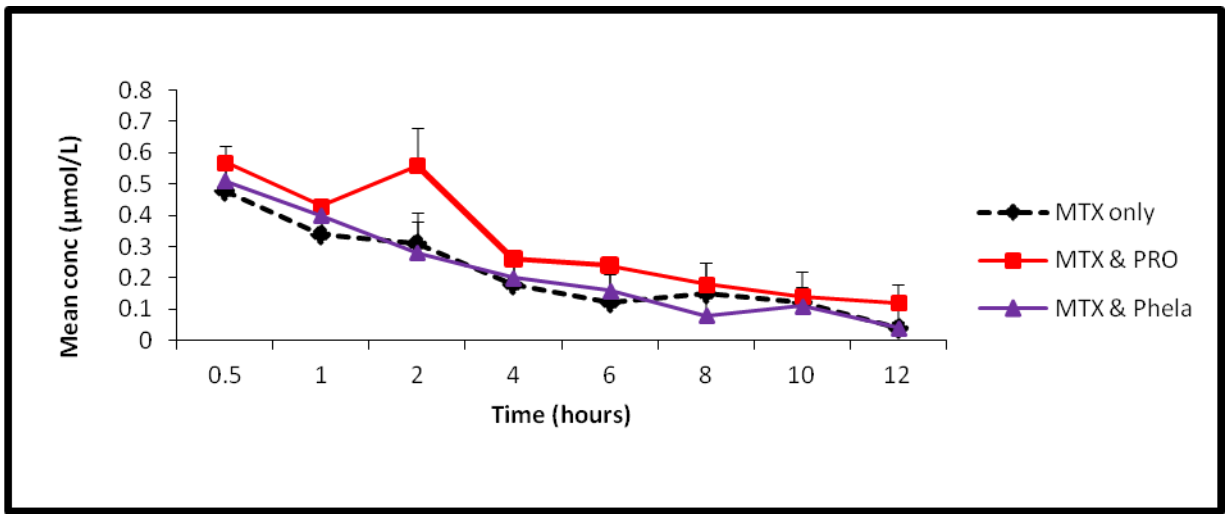


Figure 7.6: Methotrexate concentrations after co-treatment with Phela over 12 hours

7.3.2.4 Methotrexate pharmacokinetic parameters

Table 7.9 shows methotrexate pharmacokinetic parameters after 12 hours of co-treatment with Phela. Probenecid significantly increased methotrexate AUC (P=0.0086), whereas Phela did not have an effect on methotrexate AUC (P=0.9999). Methotrexate peak concentrations in the control and test group were not significantly different (P=0.2447), and that of the positive control group increased (P=0.0096).

Table 7.9: Methotrexate pharmacokinetic parameters in rats after 12 hours of co-treatment with Phela

Kinetic parameters	MTX-only (Control)	MTX & PRO (+ control)	MTX & Phela (Test group)
Wt (kg)	0.22±0.0	0.23±0.0	0.22±0.0
Dose (mg)	2.2±0.1	2.3±0.1	2.1±0.0
C_o (mg/L)	0.4±0.0	0.6±0.1	0.5±0.0
K_e (hr)	0.2±0.0	0.1±0.0	0.2±0.0
Half-life (hr)	4.0±0.5	5.0±1.1	3.7±0.4
AUC_{oral} (mg.hr/L)	2.5±0.3	4.1±0.5	2.5±0.3
Vd (L)	5.0±0.4	4.1±0.6	4.5±0.1
CLp (L/hr)	0.9±0.1	0.6±0.1	0.9±0.1
MRT (hr)	5.7±0.7	7.2±1.6	5.3±0.6
Cmax (µg/ml)	0.4±0.0	0.6±0.0	0.5±0.1
Tmax (hr)	0.5±0.0	0.8±0.3	0.5±0.0

MTX= methotrexate; PRO = probenecid; +control = positive control; Wt = weight; Co = concentration of the metabolite after instantaneous distribution at time zero; Ke = elimination rate constant of the metabolite; AUC_{oral} = area under the curve of the plasma concentration (C_{oral}) as a function of time; Vd = volume of distribution of the metabolite; CLp_{oral} = plasma clearance of the metabolite; MRT = mean residence time of the metabolite; Cmax = maximum plasma concentration; Tmax = the time to reach the maximum plasma concentration

7.4 Discussion

This part of the study has achieved its aim of determining the pharmacokinetics of paclitaxel and methotrexate when administered together with cyclosporin A and probenecid, respectively, or with Phela, thereby revealing no potential for transporter related herb-drug interaction. The results of this study are relevant because the rat model is commonly used for evaluating the pharmacokinetics of paclitaxel and methotrexate (Chen *et al*., 2002; Choi *et al*., 2005; He *et al*., 2002). Indeed, some of the kinetic parameters or trends and, as such, in the paclitaxel group were similar to those reported in humans. For instance, as shown in Table 7.5: the total clearance of 0.1±0.0 Litre/hour in rats versus 0.1±0.1 Litre/hour in humans, while the area under the curve (AUC) in the paclitaxel and cyclosporin A group in rats was 18.5±1.9 mg.hour/Litre

versus 13.2±6.7 mg.hour/Litre in humans (Britten *et al*., 2000). Another example is shown in Table 7.9: in the methotrexate group, the total clearance after oral methotrexate administration in rats was 0.9±0.1 Litre/hour compared to 0.8±0.1 in humans (Chládek *et al*., 1998). Though these observations are relevant, human studies will still have to be done for concrete answers.

Inhibition of transport: Previous studies have shown that cyclosporin A enhances absorption of paclitaxel by inhibiting intestinal *P*-gp (van Asperen *et al*., 1998), whereas probenecid increases intestinal methotrexate absorption by inhibiting MRP2 (Yokooji *et al*., 2007). The findings of the current study confirmed that co-administration of cyclosporin A with paclitaxel led to increased concentrations of paclitaxel, which was associated with increased AUC and the half-life of paclitaxel. The reduction in paclitaxel clearance demonstrates that this was due to inhibition of *P*-gp mediated efflux of paclitaxel in the lumen or elsewhere in the body. Similarly, co-administration of probenecid with methotrexate led to higher concentration, and AUC of methotrexate. The increase in the intestinal methotrexate absorption by probenecid was due to the suppression of methotrexate efflux. These results are proof of the validity of the results with Phela.

Herb-drug interactions: Co-administration of traditional medicines with prescription drugs may result in potential pharmacokinetic interactions mediated by transporters such as herb-drug interactions (Tomlinson *et al.*, 2008)**.** This study investigated the traditional medicine, Phela, and results revealed that co-administration of Phela with either paclitaxel or methotrexate had no effect on paclitaxel and methotrexate kinetics, which implies that Phela does not inhibit *P*-gp and MRP2 mediated effluxes in the lumen. Therefore, the doses of drugs that are transported by *P*-gp and MRP2 need not be adjusted when co-administered with Phela.

CHAPTER 8

CONCLUSIONS AND FUTURE STUDIES

8.1 Conclusions

The objectives of this study were achieved as follows:

- A method for determining paclitaxel in plasma by high performance liquid chromatography was successfully developed and applied in the study.
- No direct chemical interaction between paclitaxel and Phela was observed *in vitro*.
- When Phela and paclitaxel or methotrexate were co-administered in rats, Phela did not affect the pharmacokinetics of paclitaxel or methotrexate, revealing no effect on the transporters, *P*-glycoprotein (*P*-gp) and multidrug resistance-associated protein 2 (MRP2).

8.2 Future studies

- Regarding the animal studies, there is a need to evaluate the effect of Phela on transporters *P*-gp and MRP2 for longer periods to determine whether the same results will be observed.
- There is also a need to undertake studies in humans to determine whether Phela does not interact with the respective transporters.
- A similar approach should be used to investigate other transporters.

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APPENDICES

APPENDIX A

HPLC CALIBRATION OF PACLITAXEL OVER 5 DAYS

Appendix A-1: Calibration, day 1

Table A-1: Calibration data, day 1

Concentration (µg/ml)	Ratio
1.25	0.36
2.50	0.54
5.00	1.10
7.50	1.45
10.00	1.92
15.00	2.90

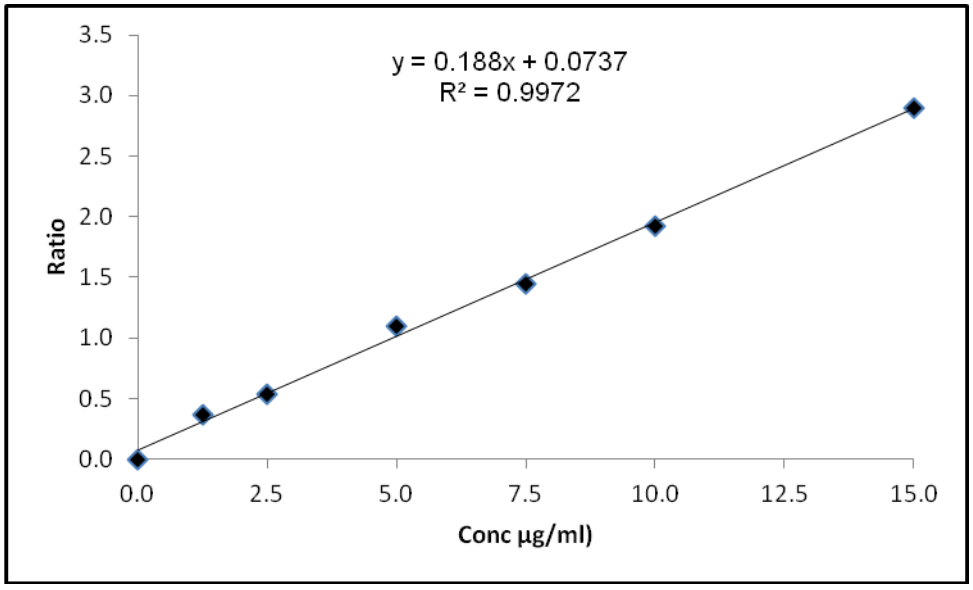

Figure A-1: Calibration curve, day 1

Appendix A-2: Calibration, day 2

Table A-2: Calibration data, day 2

Concentration (µg/ml)	Ratio
1.25	0.30
2.50	0.55
5.00	1.01
7.50	1.51
10.00	1.86
15.00	2.98

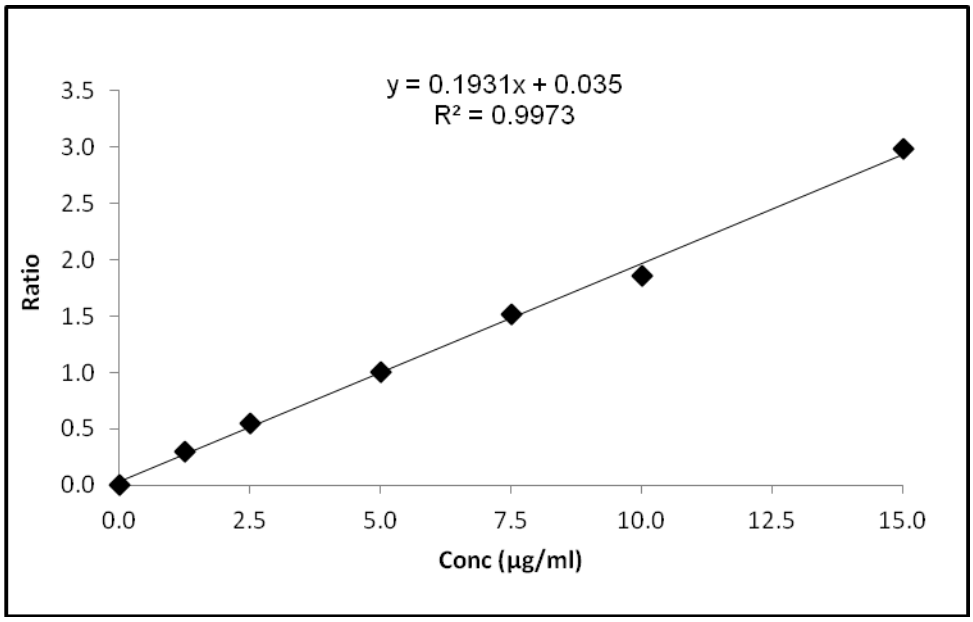

Figure A-2: Calibration curve, day 2

Appendix A-3: Calibration, day 3

Table A-3: Calibration data, day 3

Concentration (µg/ml)	Ratio
1.25	0.38
2.50	0.64
5.00	1.30
7.50	1.57
10.00	1.62
15.00	2.65

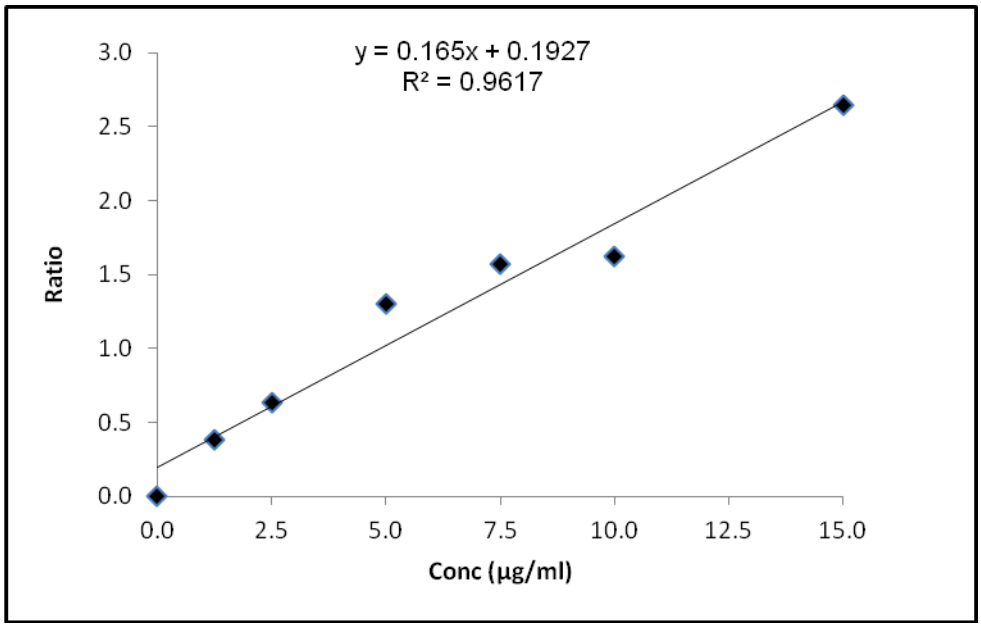

Figure A-3: Calibration curve, day 3

Appendix A-4: Calibration, day 4

Table A-4: Calibration data, day 4

Concentration (µg/ml)	Ratio
1.25	0.26
2.50	0.63
5.00	1.08
7.50	1.59
10.00	2.05
15.00	2.97

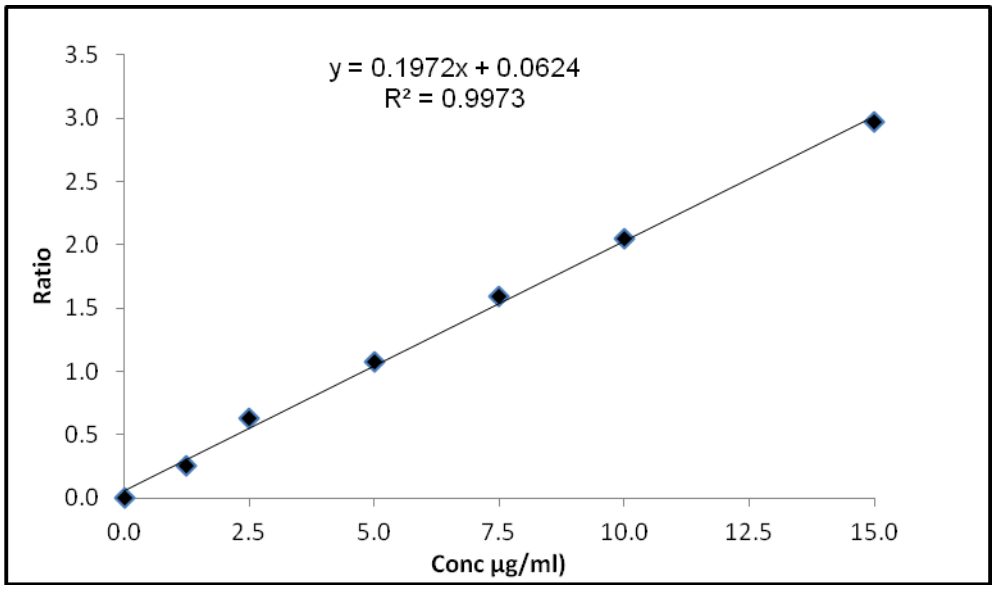

Figure A-4: Calibration curve, day 4

Appendix A-5: Calibration, day 5

Table A-5: Calibration data, day 5

Concentration (µg/ml)	Ratio
1.25	0.32
2.50	0.57
5.00	1.07
7.50	1.60
10.00	2.18
15.00	3.36

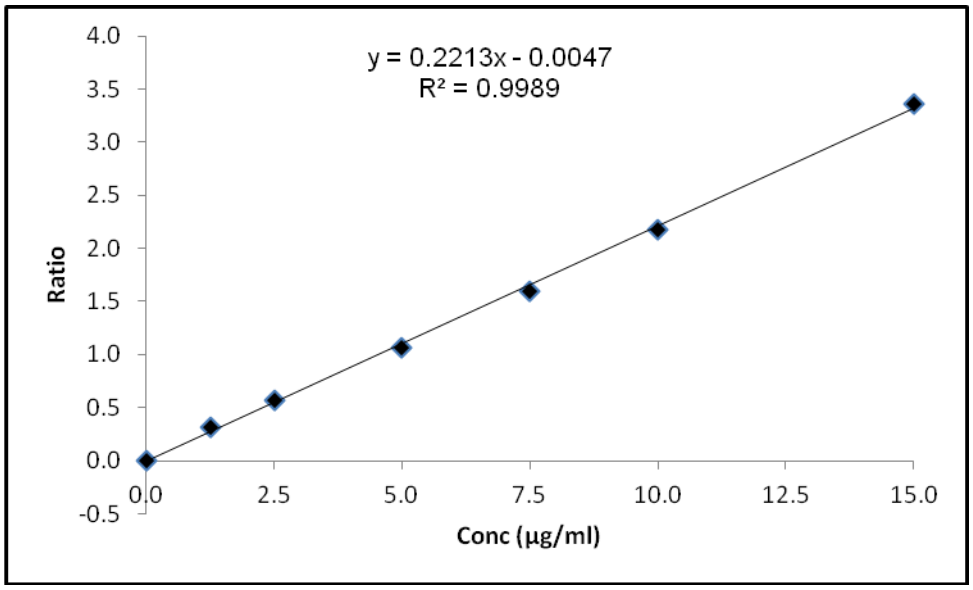

Figure A-5: Calibration curve, day 5

APPENDIX B

ACCURACY DETERMINATION OF PACLITAXEL

Appendix B-1: Accuracy of paclitaxel at 1.25, 7.50, 15.00 µg/ml repeated 5 times

Table B-1: Accuracy data at 1.25, 7.50 and 15.00 µg/ml

Prep Conc. µg/ml	Conc.	Mean Conc. 1 µg/ml	Mean Conc. 2 µg/ml	Mean Conc. 3 µg/ml	Mean Conc. 4 µg/ml	Mean Conc. 5 µg/ml	Mean	SD	% Acc	CV%
1.25		1.19	1.12	1.38	1.06	1.30	1.21	**0.13**	**97**	**10.78**
7.50		7.65	6.46	7.61	6.03	5.87	6.72	**0.85**	**90**	**12.71**
15.00		13.90	14.68	14.28	15.54	14.59	14.60	**0.61**	**97**	**4.17**

SD= standard deviation; Acc% = accuracy percentage; CV% = coefficient of variation

APPENDIX C

STABILITY DETERMINATION OF PACLITAXEL

Appendix C-1: Stability of paclitaxel (1.25, 7.50, 15.00 µg/ml) at room temperature, 4 °C and -20 °C after 24 hours of storage

Table C-1: Stability data after 24 hours of storage

Time	Prep	Temp	Measured	Measured	Mean	SD	% Recovery
24 hrs	**1.25**	Room Temp	1.2	1.1	1.2	**0.1**	**96**
		4 °C	1.4	1.0	1.2	**0.3**	**96**
		-20 °C	1.6	1.4	1.5	**0.1**	**120**
24 hrs	**7.50**	Room Temp	6.2	5.8	6.0	**0.3**	**80**
		4 °C	9.5	8.9	9.2	**0.4**	**123**
		-20 °C	8.5	8.6	8.6	**0.1**	**115**
24 hrs	**15.00**	Room Temp	12.4	11.7	12.1	**0.5**	**81**
		4 °C	17.5	17.6	17.6	**0.1**	**117**
		-20 °C	17.6	16.5	17.1	**0.8**	**114**

Prep = prepared; Temp = temperature; SD= standard deviation

Appendix C-2: Stability of paclitaxel (1.25, 7.50, 15.00 µg/ml) at room temperature, 4 °C and -20 °C after 48 hours of storage

Table C-2: Stability data after 48 hours of storage

Time	Prep	Temp	Measured	Measured	Mean	SD	% Recovery
48 hrs	**1.25**	Room Temp	2.3	1.9	2.1	**0.3**	**168**
		4 °C	2.4	2.1	2.3	**0.2**	**184**
		-20 °C	1.8	1.5	1.7	**0.2**	**136**
48 hrs	**7.50**	Room Temp	6.1	6.5	6.3	**0.3**	**84**
		4 °C	9.3	9.4	9.4	**0.1**	**125**
		-20 °C	7.3	7.7	7.5	**0.3**	**100**
48 hrs	**15.00**	Room Temp	13.2	11.1	12.2	**1.5**	**81**
		4 °C	19.1	17.8	18.5	**0.9**	**123**
		-20 °C	14.7	16.0	15.4	**0.9**	**103**

Prep = prepared; Temp = temperature; SD= standard deviation

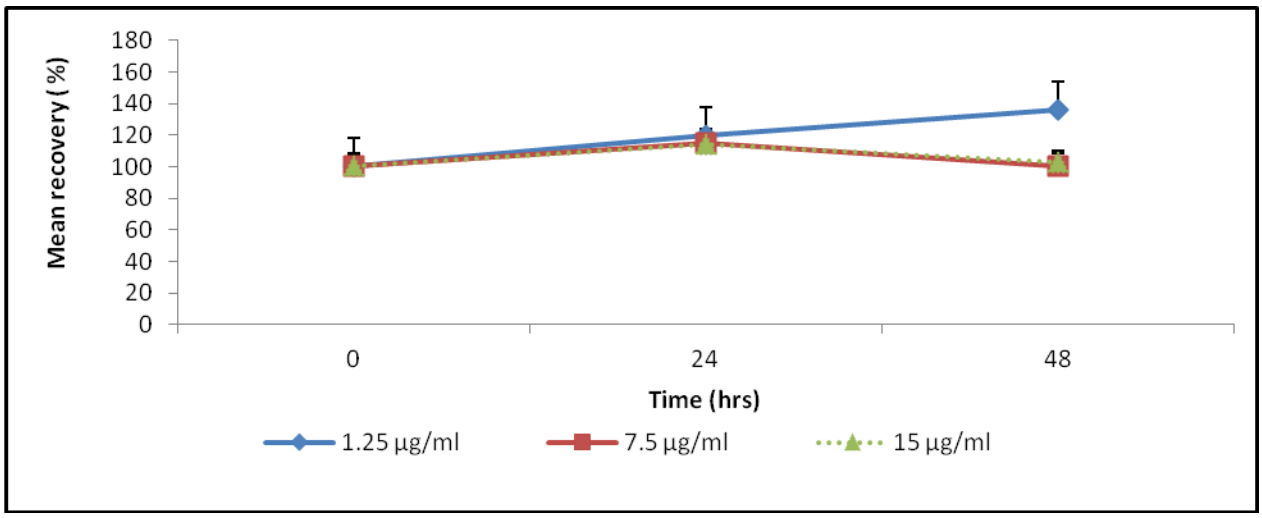

Figure C-2: Plot of stability of paclitaxel (1.25, 7.50 and 15.00 µg/ml) after 24 and 48 hours of storage in -20 °C

Appendix C-3: Stability of paclitaxel (1.25, 7.50, 15.00 µg/ml) at -20 °C after 1, 2 and 4 of storage

Table C-3: Stability data at room temperature, -4 °C and -20 °C after 24 hours of storage

Time	Prep	Temp	Meas.	Meas.	Meas.	Mean	SD	% Recovery
1 Week	**1.25**	**-20 °C**	**1.4**	**1.3**	**1.5**	**1.4**	**0.1**	**112**
	7.50	-20 °C	10.1	10.1	8.4	9.5	**1.0**	**127**
	15.00	-20 °C	20.1	21.2	23.2	21.5	**1.6**	**143**
2 Weeks	**1.25**	-20 °C	0.8	0.8	0.8	0.8	**0.0**	**65**
	7.50	-20 °C	4.3	4.5	4.5	4.4	**0.1**	**59**
	15.00	-20 °C	9.0	9.4	9.4	9.3	**0.3**	**62**
4 Weeks	**1.25**	-20 °C	0.7	0.6	0.5	0.6	**0.1**	**45**
	7.50	-20 °C	3.5	3.3	3.0	3.3	**0.3**	**43**
	15.00	-20 °C	6.7	6.8	6.6	6.7	**0.1**	**45**

Prep = prepared; Temp = temperature; Meas. = measured; SD= standard deviation

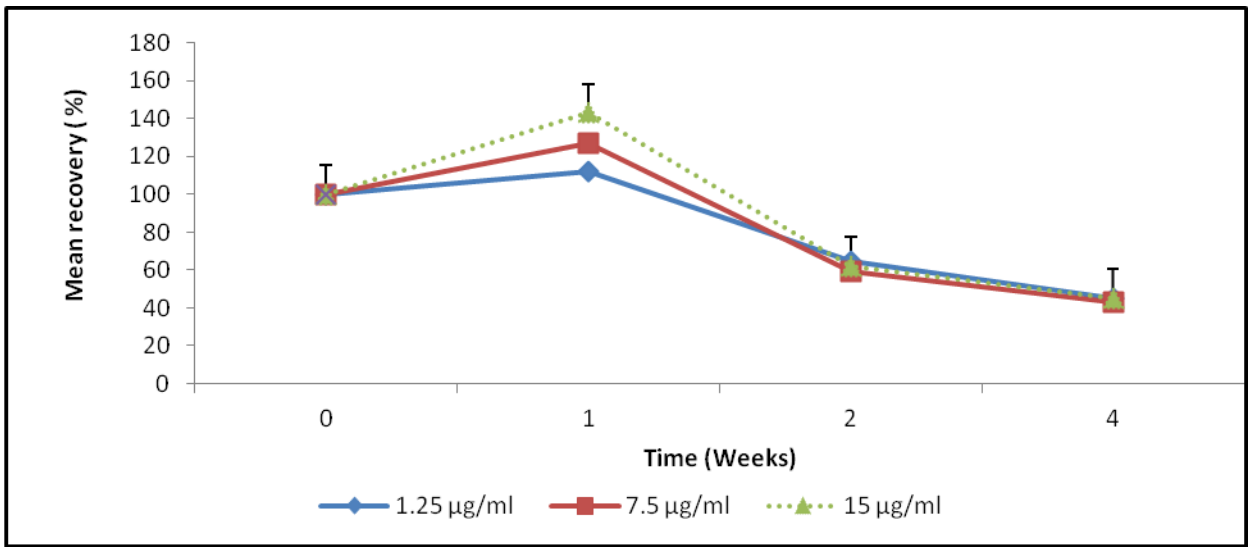

Figure C-3: Plot of stability of paclitaxel (1.25, 7.50 and 15.00 µg/ml) after 1, 2, and 4 Weeks of storage in -20 °C

APPENDIX D

CONCENTRATION AND FREE FRACTION DETERMINATION OF PACLITAXEL

Appendix D-1: Results of paclitaxel equilibrium dialysis experiment after 2, 4, 6, 8, 10 and 12 hours of incubation

Table D-1: Data of equilibrium dialysis experiment after 12 hours of incubation

Time Of Incubation (Hours)	Cassette Conc. (µg/ml)	Plastic bag Conc. (µg/ml)	Free Fraction Conc. (µg/ml)	Free Fraction %	Bound Conc. (µg/ml)	Bound %
2	93.90	2.86	0.03	3.03	0.97	**97.00**
4	70.01	4.33	0.06	6.39	0.94	**94.00**
6	42.11	4.53	0.11	10.93	0.89	**89.00**
8	33.46	4.19	0.13	13.90	0.87	**87.00**
10	34.95	4.09	0.12	12.10	0.88	**88.00**
12	52.64	1.64	0.03	2.70	0.97	**97.00**

Conc. = concentration

Appendix D-2: Results of paclitaxel equilibrium dialysis experiment after 2 and 4 hours of addition of Phela

Table D-2: Data of equilibrium dialysis experiment after 2 and 4 hours of addition of Phela

Time Of Incubation (Hours)	Cassette Conc. (µg/ml)	Plastic bag Conc. (µg/ml)	Free Fraction Conc. (µg/ml)	Free Fraction %	Bound Conc. (µg/ml)	Bound %
8	33.46	4.19	0.13	13.90	0.87	**87.00**
10	37.21	3.85	0.10	11.10	0.90	**90.00**
12	31.96	4.56	0.14	14.40	0.86	**86.00**

Conc. = concentration

APPENDIX E

RAT WEIGHTS

Appendix E-1: Rat weights during the determination of the effect of Phela on *P*-glycoprotein experiment

Table E-1: Rat weights of 10 mg/kg paclitaxel-only

Group	Time (Hours)	Rat 1	Rat 2	Rat 3	Mean	SD
PTX-only						
	0.5	231.0	229.5	229.4	230.0	0.9
	1	231.5	243.4	226.4	233.8	8.7
	2	227.4	233.4	228.3	229.7	3.2
	4	232.2	221.4	223.7	225.8	5.7
	6	248.0	248.8	250.0	248.9	1.0
	8	227.1	242.1	239.7	236.3	8.1
	10	221.9	234.9	228.8	228.5	6.5
	12	250.2	239.6	244.6	244.8	5.3

SD= standard deviation; PTX = paclitaxel

Appendix E-2: Rat weights during the determination of the effect of Phela on *P*-glycoprotein experiment

Table E-2: Rat weights of 10 mg/kg paclitaxel and 10 mg/kg cyclosporin A

Group	Time (Hours)	Rat 1	Rat 2	Rat 3	Mean	SD
PTX & CyA						
	0.5	227.5	206.6	212.4	215.5	10.8
	1	216.5	211.8	218.6	215.6	3.5
	2	212.5	213.8	210.1	212.1	5.2
	4	208.4	203.7	222.7	211.6	9.9
	6	204.1	203.2	200.0	202.4	2.2
	8	219.3	211.9	213.9	215.0	3.8
	10	218.5	217.4	202.6	212.8	8.9
	12	219.2	223.0	208.0	216.7	7.8

SD= standard deviation; PTX = paclitaxel; CyA = cyclosporin A

Appendix E-3: Rat weights during the determination of the effect of Phela on *P*-glycoprotein experiment

Table E-3: Rat weights of 10 mg/kg paclitaxel and 15.4 mg/kg Phela

Group	Time (Hours)	Rat 1	Rat 2	Rat 3	Mean	SD
PTX & Phela						
	0.5	202.8	203.1	211.1	205.7	4.7
	1	205.9	211.7	208.5	208.7	2.9
	2	202.3	209.3	203.5	205.0	3.7
	4	201.9	209.7	207.9	206.5	4.1
	6	207.7	207.8	201.8	205.8	3.4
	8	214.7	206.1	205.7	208.8	5.1
	10	224.7	229.8	214.7	223.1	7.7
	12	208.6	211.5	214.1	211.4	2.8

SD= standard deviation; PTX = paclitaxel

Appendix E-4: Rat weights during the determination of the effect of Phela on multidrug resistance-associated protein 2 experiment

Table E-4: Rat weights of 10 mg/kg methotrexate-only

Group	Time (Hours)	Rat 1	Rat 2	Rat 3	Mean	SD
MTX-only						
	0.5	236.0	238.2	232.8	235.7	2.7
	1	232.0	231.1	201.2	221.4	17.5
	2	228.0	238.5	221.5	229.3	8.6
	4	200.2	200.0	207.1	202.4	4.0
	6	208.7	212.6	209.9	210.4	2.0
	8	210.2	209.4	211.2	210.3	0.9
	10	201.4	207.0	209.2	205.9	4.0
	12	250.0	250.0	250.0	250.0	0.0

SD= standard deviation; MTX = methotrexate

Appendix E-5: Rat weights during the determination of the effect of Phela on multidrug resistance-associated protein 2 experiment

Table E-5: Rat weights of 10 mg/kg methotrexate and 20 mg/kg probenecid

Group	Time (Hours)	Rat 1	Rat 2	Rat 3	Mean	SD
MTX & PRO						
	0.5	248.8	207.7	247.0	234.5	23.2
	1	250.0	236.1	250.0	245.4	8.0
	2	216.2	212.3	250.0	226.2	20.7
	4	214.3	219.7	234.3	222.8	10.3
	6	243.7	246.5	234.5	241.6	6.3
	8	226.8	240.8	223.4	230.3	9.2
	10	244.1	244.9	246.4	245.1	1.2
	12	204.3	200.4	222.7	209.1	11.9

SD= standard deviation; MTX = methotrexate; PRO = probenecid

Appendix E-6: Rat weights during the determination of the effect of Phela on multidrug resistance-associated protein 2 experiment

Table E-6: Rat weights of 10 mg/kg methotrexate and 15.4 mg/kg Phela

Group	Time (Hours)	Rat 1	Rat 2	Rat 3	Mean	SD
MTX & Phela						
	0.5	217.4	223.9	229.0	223.4	5.8
	1	227.5	210.1	207.9	215.2	10.7
	2	238.6	227.7	232.5	232.9	5.5
	4	214.0	237.6	227.1	226.2	11.8
	6	226.9	234.6	212.8	224.8	11.1
	8	210.1	212.4	206.6	209.7	2.9
	10	219.7	200.0	200.0	206.6	11.4
	12	240.5	250.0	250.0	246.8	5.5

SD= standard deviation; MTX = methotrexate

APPENDIX F

FULL BLOOD COUNT (FBC)

Appendix F-1: FBC after 12 hours of one-off oral treatment

Table F-1: FBC of PTX-only, PTX & CyA, and PTX & Phela groups after 12 hours of one-off oral treatment

Parameters	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
PTX-only						
White cell count	5.6	7.0	7.0	10.3	7.5	2.0
Red cell count	6.6	6.7	6.9	7.0	6.8	0.2
Haemoglobin	13.3	13.8	14.5	14.7	14.1	0.6
Haematocrit	0.4	0.4	0.4	0.4	0.4	0.0
MCV	61.8	62.7	63.4	64.4	63.1	1.1
MCH	20.2	20.5	21.1	21.1	20.7	0.5
MCHC	33.3	32.6	32.7	32.7	32.8	0.3
Platelets count	598.0	652.0	745.0	904.0	724.8	134.0
PTX & CyA						
White cell count	4.3	4.8	7.8	8.5	6.4	2.1
Red cell count	4.2	4.7	5.1	5.4	4.8	0.5
Haemoglobin	9.0	10.1	11.0	11.4	10.4	1.1
Haematocrit	0.3	0.3	0.3	0.4	0.3	0.0
MCV	61.9	62.8	65.2	66.8	64.2	2.2
MCH	20.5	21.6	21.6	22.2	21.5	0.7
MCHC	30.7	33.1	34.9	35.3	33.5	2.1
Platelets count	127.0	285.0	293.0	681.0	346.5	235.7
PTX & Phela						
White cell count	2.1	2.2	2.7	5.0	3.0	1.3
Red cell count	2.5	2.9	5.4	6.9	4.4	2.1
Haemoglobin	5.3	6.4	11.8	14.2	9.4	4.3
Haematocrit	0.2	0.2	0.4	0.5	0.3	0.1
MCV	64.0	64.9	65.6	66.0	65.1	0.9
MCH	20.7	21.2	21.8	21.9	21.4	0.6
MCHC	31.6	33.0	33.1	33.7	32.9	0.9
Platelets count	223.0	233.0	544.0	636.0	409.0	212.4

FBC = full blood count; SD = standard deviation; PTX = paclitaxel; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; CyA =cyclosporin A

Appendix F-2: FBC after 12 hours of one-off oral treatment

Table F-2: FBC of MTX-only, MTX & PRO, and MTX & Phela groups after 12 hous of one-off oral treatment

Parameters	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
MTX-only						
White cell count	4.3	4.9	5.8	5.9	5.2	0.8
Red cell count	5.1	5.8	6.0	-	5.5	0.4
Haemoglobin	11.4	11.8	11.8	-	11.7	0.2
Haematocrit	0.3	0.3	0.3	-	0.3	0.0
MCV	59.0	60.6	63.7	63.9	61.8	2.4
MCH	20.7	20.8	21.3	22.5	21.3	0.8
MCHC	35.1	35.2	35.2	35.9	35.4	0.4
Platelets count	513.0	564.0	586.0	-	554.3	37.4
MTX & PRO						
White cell count	4.2	4.2	5.7	6.7	5.2	1.2
Red cell count	5.7	5.8	6.0	-	5.8	0.2
Haemoglobin	11.7	11.7	11.7	-	11.7	0.0
Haematocrit	0.4	0.4	0.4	-	0.4	0.0
MCV	63.6	63.8	64.0	65.4	64.2	0.8
MCH	20.0	20.3	20.7	20.8	20.5	0.4
MCHC	30.7	31.6	31.8	31.8	31.5	0.5
Platelets count	473.0	515.0	579.0	-	522.3	53.4
MTX & Phela						
White cell count	4.0	4.8	5.3	5.7	4.9	0.7
Red cell count	5.5	5.5	6.0	-	5.7	0.3
Haemoglobin	11.3	11.4	12.6	-	11.8	0.7
Haematocrit	0.3	0.3	0.4	-	0.3	0.0
MCV	58.0	59.0	60.1	60.3	59.4	1.1
MCH	20.7	20.8	21.1	21.1	20.9	0.2
MCHC	35.1	35.2	35.3	35.7	35.3	0.3
Platelets count	682.0	687.0	772.0	-	713.7	50.6

FBC = full blood count; SD = standard deviation; MTX = methotrexate; MCV = mean corpuscular volume; MCH = mean corpuscular haemoglobin; MCHC = mean corpuscular haemoglobin concentration; PRO = probenecid

APPENDIX G

LIVER FUNCTION TESTS (LFTS)

Appendix G-1: LFTs after 12 hours of one-off oral treatment

Table G-1: LFTs of PTX-only, PTX & CyA, and PTX & Phela groups after 12 hours of one-off oral treatment

Parameters	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
PTX-only						
ALP	379.0	586.0	270.0	-	411.7	160.5
ALT	39.0	49.0	78.0	105.0	67.8	29.8
AST	80.0	129.0	149.0	-	119.3	35.5
PTX & CyA						
ALP	350.0	376.0	390.0	-	372.0	20.3
ALT	44.0	58.0	71.0	87.0	65.0	18.3
AST	106.0	190.0	294.0	-	196.7	94.2
PTX & Phela						
ALP	313.0	374.0	416.0.	-	367.7	51.8
ALT	39.0	54.0	58.0	120.0	67.8	35.8
AST	115.0	119.0	132.0	-	122.0	8.9

LFT = liver function test; SD = standard deviation; PTX = paclitaxel; ALP = alkaline phosphatase; ALT = Alanine transaminase; AST = aspartate aminotransferase; CyA =cyclosporin A

Appendix G-2: LFTs after 12 hours of one-off oral treatment

Table G-2: LFTs of MTX-only, MTX & PRO, and MTX & Phela groups after 12 hours of one-off oral treatment

Parameters	Rat 1	Rat 2	Rat 3	Rat 4	Mean	SD
MTX-only						
ALP	260.0	278.0	370.0	-	302.7	59.0
ALT	28.0	30.0	33.0	-	30.3	2.5
AST	78.5	80.5	77.2	-	78.7	1.7
MTX & PRO						
ALP	223.0	266.0	371.0	-	286.7	76.1
ALT	31.0	32.0	39.0	-	34.0	4.4
AST	94.0	97.0	106.0	-	99.0	6.2
MTX & Phela						
ALP	220.0	260.0	293.0	-	257.7	36.6
ALT	30.0	32.0	35.0	-	32.3	2.5
AST	85.7	85.6	88.4	-	86.6	1.6

LFT = liver function test; SD = standard deviation; MTX = methotrexate; ALP = alkaline phosphatase; ALT = Alanine transaminase; AST = aspartate aminotransferase; PRO =probenecid

APPENDIX H

PLASMA MONITORING

Appendix H-1: PTX concentrations after 12 hours of one-off oral treatment

Table H-1: PTX concentrations after 12 hours of one-off oral treatment with PTX-only, PTX & CyA, PTX & Phela

Time (hours)	Groups	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
0.5	**PTX-only**	4.46	4.68	4.69	-	-	**4.61**	**0.13**
1		3.97	4.07	4.09	-	-	**4.04**	**0.06**
2		0.94	0.98	1.02	1.05	1.33	**1.06**	**0.15**
4		1.23	1.23	1.29	1.32	1.42	**1.30**	**0.08**
6		0.88	1.00	1.05	1.09	1.09	**1.02**	**0.09**
8		0.67	0.72	0.75	0.77	0.77	**0.74**	**0.04**
10		0.40	0.42	0.51	0.61	0.63	**0.51**	**0.11**
12								
0.5	**PTX & CyA**	5.64	5.66	6.50	-	-	**5.93**	**0.49**
1		5.49	5.92	6.30	-	-	**5.90**	**0.41**
2		2.04	2.19	2.26	2.31	2.39	**2.24**	**0.13**
4		2.43	2.71	2.72	2.78	2.81	**2.69**	**0.15**
6		2.17	2.18	2.20	2.20	2.25	**2.20**	**0.03**
8		1.77	1.82	1.92	1.97	2.05	**1.91**	**0.11**
10		1.61	1.61	1.76	1.78	1.81	**1.71**	**0.10**
12		1.53	1.56	1.73	1.74	1.93	**1.70**	**0.16**
0.5	**PTX & Phela**	4.47	4.67	4.67	-	-	**4.60**	**0.12**
1		3.79	3.98	4.33	-	-	**4.03**	**0.27**
2		1.08	1.17	1.21	-	-	**1.23**	**0.12**
4		1.38	1.63	1.70	1.77	184	**1.66**	**0.18**
6		1.19	1.26	1.33	1.36	1.43	**1.31**	**0.09**
8		0.99	1.00	1.01	1.01	1.05	**1.01**	**0.02**
10		0.44	0.66	0.78	0.82	0.85	**0.71**	**0.17**
12		0.43	0.62	0.64	0.64	0.68	**0.60**	**0.10**

SD = standard deviation; PTX = paclitaxel; CyA = cyclosporin A

Appendix H-2: MTX concentrations after 12 hours of one-off oral treatment

Table H-2: MTX concentrations after 12 hours of one-off oral treatment with MTX-only, MTX & PRO, MTX & Phela

Time (hours)	Groups	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Mean	SD
0.5	**MTX-only**	0.43	0.44	0.56	-	-	**0.48**	**0.07**
1		0.19	0.40	0.42	-	-	**0.34**	**0.13**
2		0.30	0.30	0.33	-	-	**0.31**	**0.02**
4		0.15	0.17	0.22	-	-	**0.18**	**0.04**
6		0.09	0.11	0.16	-	-	**0.12**	**0.04**
8		0.08	0.14	0.22	-	-	**0.15**	**0.07**
10		0.11	0.12	0.12	-	-	**012**	**0.01**
12		0.03	0.03	0.05	-	-	**0.04**	**0.01**
0.5	**MTX & PRO**	0.51	0.58	0.61	-	-	**0.57**	**0.05**
1		0.42	0.44	0.44	-	-	**0.43**	**0.01**
2		0.45	0.54	0.68	-	-	**0.56**	**0.12**
4		0.26	0.26	0.27	-	-	**0.26**	**0.01**
6		0.21	0.24	0.27	-	-	**0.24**	**0.03**
8		0.12	0.18	0.25	-	-	**0.18**	**0.07**
10		0.12	0.13	0.18	-	-	**0.14**	**0.03**
12		0.08	0.09	0.19	-	-	**0.12**	**0.06**
0.5	**MTX & Phela**	0.47	0.47	0.58	-	-	**0.51**	**0.06**
1		0.38	0.40	0.42	-	-	**0.40**	**0.02**
2		0.21	0.24	0.40	-	-	**0.28**	**0.10**
4		0.17	0.20	0.24	-	-	**0.20**	**0.04**
6		0.12	0.14	0.21	-	-	**0.16**	**0.05**
8		0.04	0.06	0.15	-	-	**0.08**	**0.06**
10		0.09	0.10	0.14	-	-	**0.11**	**0.03**
12		0.03	0.04	0.06	-	-	**0.04**	**0.02**

SD = standard deviation; MTX = methotrexate; PRO = probenecid

APPENDIX I

PUBLICATIONS

A. Conference abstracts

1. **M.E Binyane,** M.R Lekhooa, J. du Plessis and A. Walubo. **Determination of paclitaxel in plasma by high performance liquid chromatography.** 17th World Congress of Basic and Clinical Pharmacology (WCP2014). CTICC in Cape Town, South Africa, 13-18 July 2014. Also at Faculty forum 2013; Faculty of Health Sciences; University of the Free State.

2. **M.E Binyane,** M.R Lekhooa, J. du Plessis, Matsabisa M.G, and A. Walubo. **Effect of Phela, a traditional medicine, on P-glycoprotein transporter in the gastrointestinal tract of a rat model.** 17th World Congress of Basic and Clinical Pharmacology (WCP2014). CTICC in Cape Town, South Africa, 13-18 July 2014.

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Introduction and aim: Paclitaxel (PTX) is an anti-cancer drug that is also used as a selective marker for *P*-glycoprotein, a recognized transporter that is responsible for limiting absorption of many drugs across the gastro-intestinal tract. In a bid to investigate the effect of herbal extract, Phela, on the intestinal *P*-glycoprotein using PTX, a method for analysis of PTX in plasma was required. Therefore, the aim of this study was to develop a method for determination of PTX in plasma by high performance liquid chromatography (HPLC), in the hope that this method can also be used for monitoring of patients on PTX in the clinic.

Methodology: To 100 µl of plasma spiked with PTX, docetaxel (IS) was added and the mixture was extracted with 3 ml diethyl ether and dichloromethane (ratio 2:1). The organic phase was evaporated and the residue was reconstituted with 150 µl of mobile phase of which 100 µl was injected on HPLC. The HPLC system consisted of an isocratic pump and a UV detector set at 230 nm. Separation was performed on a C_{18} (250mm x 4.6 mm x 5 µm) column. The mobile phase was acetonitrile: 20 m*M* sodium phosphate buffer pH 2 (60/40: v/v), and the flow rate was set at 1.0 ml/min with a run time of 10 min. The assay was validated by determination of linearity, accuracy and stability.

Results: PTX and IS eluted at the retention times of 6.751 and 6.178 min, respectively, and there was no interference with plasma. The method was linear with the regression equation of y = 0.1931x + 0.0705 and correlation coefficient (r) = 0.9973. The percentage recoveries for 1.25, 7.50 and 15.00 µg/ml were 97%, 90% and 97% respectively. Samples were more stable at -20°C.

Conclusion: A simple and reliable HPLC-UV method for determination of PTX in human plasma was successfully developed.

Effect of Phela a traditional medicine on *P*-glycoprotein transporter in the gastrointestinal tract of a rat model

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Background: Phela is a traditional medicine made of a mixture of four African traditional medicinal plants and is under development as an immune booster. *P*-glycoprotein is the efflux transporter that limits absorption of drugs across the gastro-intestinal tract (GIT). The aim of the study was to investigate the effect of Phela on the intestinal *P*-glycoprotein in a rat model, using paclitaxel (PTX) as the selective marker for *P*-glycoprotein.

Methods: Male Sprague-Dawley rats (120) were divided into 3 groups (n=40). Each group was administered orally one-off with either PTX only 10 mg/kg (control), PTX 10 mg/kg and cyclosporin A (CyA; positive control), and PTX 10 mg/kg and Phela 15.4 mg/kg (test group). Thereafter 5 animals were sacrificed and blood was withdrawn at 30 min, 1, 2, 4, 6, 8, 10, and 12 hrs of treatment. PTX plasma concentrations were measured via a validated high performance liquid chromatography (HPLC-UV) method.

Results: CyA (positive control) significantly increased the area under the plasma concentration-time curve (AUC) with P=0.0003, while Phela (test group) had no effect with P=0.0824. PTX AUC for the control was mg.hr/L: mean±SD, 18.45±1.86, versus Phela 21.81±1.71 and CyA 70.44 ± 1.35. Whereas the PTX peak concentrations in the control and test group were similar (4.63 ± 0.12 µg/ml), that of the positive control was increased (6.23 ± 0.31 µg/ml; P=0.0011). Co-administration of Phela and PTX in rats did not increase AUC of PTX, indicating that Phela does not inhibit *P*-glycoprotein mediated efflux of PTX in the lumen. Therefore, the dose of drugs that are transported by *P*-glycoprotein need not be adjusted when co-administered with Phela.

Conclusion: Phela did not inhibit *P*-glycoprotein transporter in the GIT of the rat model, thereby indicating lack of potential interactions with drug-substrates of *P*-glycoprotein.

SUMMARY

Key terms: Membrane drug-transporters, *P*-glycoprotein, multidrug resistance-associated protein 2, pharmacokinetic parameters, paclitaxel, methotrexate, herb-drug interactions, Phela, high performance liquid chromatography, the area under the plasma concentration-time curve.

Membrane drug-transporters such as *P*-glycoprotein (*P*-gp) and multidrug resistance-associated protein 2 (MRP2) are considered important factors in determining the pharmacokinetic parameters of drugs such as paclitaxel (PTX) and methotrexate (MTX), respectively. Inhibition or induction of transport might result in drug interactions with other drugs transported by these respective transporters. Moreover, significant herb drug interactions involving *P*-gp and MRP2 have been described. Therefore, the effect of Phela on *P*-gp and MRP2 in the gastrointestinal tract of a rat model was investigated here.

First, a high performance liquid chromatography (HPLC) method for determination of PTX in plasma was developed. It involved liquid liquid extraction of 100 µl plasma, spiked with PTX, extracted with diethyl ether: dichloromethane (2:1), followed by centrifugation. The supernatant was evaporated to dryness under a stream of nitrogen, reconstituted, and 100 µl was injected into the HPLC. The sample was eluted with a mobile phase of sodium phosphate buffer (pH 2): acetonitrile (60:40, v/v) over a $C_{8\ (1)}$ (4.6 X 250 mm) 5 µ analytic column at 1 ml/min. PTX was detected by UV at 230 nm. Docetaxel (DTX) was used as the internal standard. Under these conditions DTX and PTX eluted at retention times of 6.595 and 6.038 minutes, respectively. The average calibration curve (0-15 µg/ml) was linear with a regression equation of $y = 0.1931x + 0.0705$, and correlation coefficient (r) of 0.9973. The method was used successfully in animal experiments to measure PTX in the plasma of treated rats.

Thereafter, a preliminary *in vitro* experiment was conducted to establish whether Phela has a direct/ physical effect on PTX. Here, a direct drug interaction testing experiment, as well as Slide-A-Lyzer® dialysis were performed, and PTX concentrations measured by HPLC. Phela had no direct effect on PTX concentrations. The time of equilibrium of PTX was estimated at 8 hours. After Phela was added, PTX concentrations and its free

fraction (*fu*) remained unchanged. Therefore, it was concluded that there is no interaction between Phela and PTX *in vitro*.

This final part of the study was undertaken to investigate the effect of Phela on *P*-gp and MRP2 transporters. PTX and cyclosporin A (CyA) were used as the respective substrate and inhibitor of P-*gp*, while MTX and probenecid (PRO) were those of MRP2. Ethical approval was obtained and male Sprague-Dawley (SD) rats (200-250 g) were used. The animal experiment was divided into two parts. In Part I, three groups of 40 rats each received a one-off oral dose of PTX-only (10 mg/kg); PTX & CyA (10 mg/kg); or PTX & Phela (15.4 mg/kg), while in Part II, three groups of 40 rats each received a one-off oral dose of MTX-only (10 mg/kg); MTX & PRO (20 mg/kg); or MTX & Phela (15.4 mg/kg). For each group, 5 rats were sacrificed after 0.5, 1, 2, 4, 6, 8, 10, and 12 hours. Blood was analysed for full blood count, liver function, and PTX and MTX concentrations. CyA and PRO increased the area under the plasma concentration time curve (AUC) of PTX and MTX, respectively, whereas Phela had no effect on the AUC of PTX or MTX.

Overall, no direct interaction between PTX and Phela was observed both *in vitro* and *in vivo*, and there were also no interactions between MTX and Phela *in vivo*. Phela did not inhibit *P*-gp or MRP2. This implies that Phela will most probably not be involved in herb-drug interactions of membrane transporter origin. Therefore, the doses of drugs that are transported by *P*-gp and MRP2 need not be adjusted when co-administered with Phela.

OPSOMMING

Sleutelterme: Membraan-gekoppelde middel-transporters, *P*-glikoproteïen, multimiddel weerstand verwante proteïen 2, farmakokinetiese parameters, paclitaxel, metotreksaat, plant geneesmiddelinteraksies, Phela, hoëdrukvloeistof chromatografie, die area onder die plasma konsentrasie-tyd-kurwe.

Membraan gekoppelde middel transporters soos *P*-glikoproteïen (*P*-gp) en multimiddel weerstand verwante proteïen 2 (MRP2) word beskou as belangrike faktore in die bepaling van die farmakokinetiese parameters van middels soos paclitaxel (PTX) en metotreksaat (MTX), onderskeidelik. Inhibisie of induksie van vervoer kan lei tot interaksies met ander middels wat ook deur hierdie onderskeie transporters vervoer word. Verder is beduidende plant- geneesmiddelinteraksies met *P*-gp en MRP2 reeds beskryf. Daarom word die effek van Phela op *P*-gp en MRP2 in die spysverteringskanaal van 'n rot-model hier ondersoek.

Eerstens, is 'n hoëdrukvloeistof-chromatografie (HPLC) metode vir die bepaling van PTX in plasma ontwikkel. Dit het behels vloeistof-ekstraksie van 100 µl plasma, waartoe die PTX gevoeg is met diëtieleter: dichlorometaan (2:1), gevolg deur sentrifugering. Die supernatant is met ‘n stikstofstroom verdamp tot droog, hersaamgestel, en 100 µl is in die HPLC ingespuit. Die monster is geëlueer met 'n mobiele fase van natrium-fosfaat-buffer (pH 2): asetonitriel (60:40, v/v) oor 'n $C_{8\ (1)}$ (4.6 X 250 mm) 5 µ analitiese kolom teen 1 ml/min. PTX is bepaal deur UV by 230 nm. Docetaxel (DTX) is gebruik as die interne standaard. Onder hierdie toestande het DTX en PTX onderskeidelik geëlueer teen retensietye van 6,595 en 6,038 minute. Die gemiddelde kalibrasie-kurwe (0-15 µg/ml) was liniêr met 'n regressievergelyking van y = 0.1931x + 0.0705 en korrelasie-koëffisiënt (r) van 0.9973. Die metode is suksesvol in diere-eksperimente gebruik om PTX te meet in die plasma van behandelde rotte.

Hierna is 'n voorlopige *in vitro* eksperiment uitgevoer om te bepaal of Phela 'n direkte / fisiese effek het op PTX. 'n direkte middelinteraksie-eksperiment sowel as Slide-A-Lyzer® dialise is uitgevoer en PTX konsentrasies is gemeet met HPLC. Phela het geen direkte effek op PTX konsentrasies. Die tyd om ekwilibrium van PTX te bereik is geskat op 8 ure. Nadat Phela bygevoeg is, het PTX konsentrasies en die vry-fraksie (*fu*)

onveranderd gebly. Daarom word die gevolgtrekking gemaak dat daar geen interaksie tussen Phela en PTX *in vitro* is nie.

Die finale deel van die studie is onderneem om die effek van Phela op P-gp en MRP2 transporters te ondersoek. PTX en siklosporien A (CYA) is onderskeidelik gebruik as die substraat en die inhibeerder van P-gp, terwyl MTX en probenesied (PRO) soortgelyk gebruik was vir MRP2. Etiese goedkeuring is verkry en manlike Sprague-Dawley (SD) rotte (200-250 g) is gebruik. Die proefdier-eksperiment is in twee dele verdeel. In Deel I het drie groepe van 40 rotte elk 'n eenmalige dosis gekry van PTX-alleen (10 mg/kg); PTX & CYA (10 mg/kg); of PTX & Phela (15.4 mg/kg), terwyl in Deel II, drie groepe van 40 rotte elk 'n eenmalige dosis gekry het van MTX-alleen (10 mg/kg); of MTX & PRO (20 mg/kg); of MTX & Phela (15.4 mg/kg). Uit elke groep is 5 rotte geslag na 0.5, 1, 2, 4, 6, 8, 10, en 12 uur. Bloed is ontleed vir volbloedtelling, lewerfunksie, en PTX en MTX konsentrasies. CYA en PRO het die area onder die plasma-konsentrasie tyd-kurwe (AUC) van PTX en MTX, onderskeidelik verhoog, terwyl Phela geen effek op die AUC van PTX of MTX getoon het nie.

Algeheel, is daar geen direkte interaksie tussen PTX en Phela waargeneem beide *in vitro* en *in vivo* nie, en daar was ook geen interaksie tussen MTX en Phela *in vivo* nie. Phela het nie *P*-gp of MRP2 inhibeer nie. Dit impliseer dat Phela waarskynlik nie betrokke sal wees in plant-geneesmiddel-interaksies van membraan-vervoerder-oorsprong nie. Daarom hoef die dosisse van middels wat deur *P*-gp en MRP2 vervoer word, nie aangepas te word wanneer dit saam met Phela toegedien word nie.