AFRICAN TRADITIONAL MEDICINE-ANTIRETROVIRAL INTERACTIONS:

Effects of *Sutherlandia frutescens* on the Pharmacokinetics of Atazanavir

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ABSTRACT

In response to the urgent call for investigations into antiretroviral (ARV)-African traditional medicine (ATM) interactions, this research was undertaken to ascertain whether chronic administration of the ATM, *Sutherlandia frutescens* (SF) may alter the bioavailability of the protease inhibitor (PI), atazanavir (ATV), which may impact on the safety or efficacy of the ARV.

Prior to investigating a potential interaction between ATV and SF *in vitro* and *in vivo*, a high performance liquid chromatography method with ultraviolet detection (HPLC-UV) was developed and validated for the bioanalysis of ATV in human plasma and liver microsomes. An improved and efficient analytical method with minimal use of solvents and short run time was achieved in comparison to methods published in the literature. In addition, the method was selective, linear, accurate and precise for quantitative analysis of ATV in these studies.

Molecular docking studies were conducted to compare the binding modes and affinities of ATV and two major SF constituents, Sutherlandioside B and Sutherlandin C, with the efflux transporter, P-glycoprotein (P-gp) and the CYP450 isoenzyme, CYP3A4 to determine the potential for these phytochemicals to competitively inhibit the binding of ATV to these two proteins, which are mediators of absorption and metabolism. These studies revealed that modulation of P-gp transport of ATV by Sutherlandioside B and Sutherlandin C was not likely to occur via competitive inhibition. The results further indicated that weak competitive inhibition of CYP3A4 may possibly occur in the presence of either of these two SF constituents.

The Caco-2 cell line was used as an *in vitro* model of human intestinal absorption. Accumulation studies in these cells were conducted to ascertain whether extracts and constituents of SF have the ability to alter the absorption of ATV. The results showed that the aqueous extract of SF significantly reduced ATV accumulation, suggesting decreased ATV absorption, whilst a triterpenoid glycoside fraction isolated from SF exhibited an opposing effect. Analogous responses were elicited by the aqueous extract and a triterpenoid glycoside fraction in similar accumulation studies in P-gp overexpressing Madin–Darby Canine Kidney Strain II cells (MDCKII-MDR1), which signified that the effects of this extract and component on ATV transport in the Caco-2 cells were P-gp-mediated.

The quantitative analysis of ATV in human liver microsomes after co-incubation with extracts and components of SF was conducted to determine the effects of SF on the metabolism of ATV. The aqueous and methanolic extracts of SF inhibited ATV metabolism, whilst the triterpenoid glycoside fraction had a converse effect. Analogous effects by the extracts were demonstrated in experiments conducted in CYP3A4-transfected microsomes, suggesting that the inhibition of ATV metabolism in the liver microsomes by these SF extracts was CYP3A4-mediated. A combination of Sutherlandiosides C and D also inhibited CYP3A4-mediated ATV metabolism, which was in contrast to the response elicited by the triterpenoid fraction in the liver microsomes, where other unidentified compounds, shown to be present therein, may have contributed to the activation of ATV metabolism.

The *in vitro* studies revealed the potential for SF to alter the bioavailability of ATV, therefore a clinical study in which the effect of a multiple dose regimen of SF on the pharmacokinetics (PK) of a single dose of ATV was conducted in healthy male volunteers. The statistical

analysis showed that the 90 % confidence intervals around the geometric mean ratios (ATV + SF/ATV alone) for both C_{max} and $AUC_{0\text{-}24\ hours}$, fell well below the lower limit of the "no-effect" boundary of 0.8 – 1.25, implying that the bioavailability of ATV was significantly reduced in this cohort of subjects.

It may thus be concluded that if the reduction in bioavailability observed in this clinical study is found to be clinically relevant, co-administration of SF commercial dosage forms and ATV in HIV/AIDS patients may potentially result in subtherapeutic ATV levels, which may in turn contribute to ATV resistance and/or treatment failure. This research has therefore highlighted the potential risk for toxicity or lack of efficacy of ARV regimens which may result when ATMs and PIs are used concurrently and that patients and health care practitioners alike should be aware of these perils.

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% RE	Percentage relative error
% RSD	Percent relative standard deviation
ΔG	Free energy of binding
ABC	ATP-binding cassette
ADT	Autodock® tools
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
ART	Antiretroviral therapy
ARV	Antiretroviral
A_s	Peak asymmetry factor
ATM	African traditional medicine
ATV	Atazanavir
AUC	Area under the curve (plasma concentration-time profile)
BCL	Bicuculline
BCRP	Breast cancer resistance protein
BSA	Bovine serum albumin
CAM	Complementary/alternative medicine
CAR	Constitutive androstane receptor
CD4	Cluster of differentiation 4
CI	Confidence interval
CL	Clearance
C_{max}	Maximum plasma concentration
C_{min}	Minimum plasma concentration
CNS	Central nervous system
CNT	Concentrative nucleoside transporter
CO	Carbon monoxide
CSF	Cerebrospinal fluid
CV %	Co-efficient of variation
CYP	Cytochrome P450
DIAZ	Diazepam

DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-picrylhydrazyl
dTTP	Deoxythymidine triphosphate
EC_{50}	Concentration at 50% efficacy
EDTA	Ethylenediaminetetraacetic acid
EE	Extraction efficiency
ELISA	Enzyme-linked immunosorbent assay
EMEA	European Medicines Agency
ENT	Equilibrative nucleoside transporter
F	Bioavailability
FDA	Food and Drug Administration
FMLP	L-formyl-L-methionyl-L-leucyl-L-phenylalanine
FMO	Flavin monooxygenase
F_u	Fraction of the drug unbound in the plasma
GC	Gas chromatography
GCP	Good Clinical Practice
GR	Glucocorticoid receptor
HBSS	Hank's buffered salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HIV	Human immunodeficiency virus
HIV-RT	HIV reverse transcriptase
HPLC-UV	High performance liquid chromatography method with ultraviolet detection
IC_{50}	Concentration at 50% inhibition
ICH	International Conference on Harmonisation
ID	Internal diameter
IS	Internal standard
K-EDTA	Potassium edentate
K_{el}	Elimination rate constant
K_i	Inhibition rate constant
KTZ	Ketoconazole
LC-MS	Liquid chromatography-mass spectrometry

LD_{50}	50% mortality
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LTR	Long terminal repeat
MCC	Medicines Control Council
MDCKII-MDR1	Madin Darby Canine Kidney Strain II cells with MDR1 (P-gp) overexpressed
MDCKII-WT	Madin Darby Canine Kidney Strain II cells-wild-type
MDR	Multidrug resistance
MHC	Major histocompatibility complex
MRA	Medicines Regulatory Authority
MRC	Medical Research Council
mRNA	Messenger RNA
MRP	Multidrug resistance-associated protein
NADPH	Nicotinamide adenine dinucleotide 2′-phosphate reduced
NBD	Nucleotide binding domain
NNRTIs	Non-nucleoside Reverse Transcriptase Inhibitors
NRTIs	Nucleoside Reverse Transcriptase Inhibitors
NtRTIs	Nucleotide Reverse Transcriptase Inhibitors
OAT	Organic anion transporter
OATP	Organic anion transporter polypeptide
OCT	Organic cation transporter
P	Partition co-efficient
P_{app} (AP-to-BL: BL-to-AP)	Apparent permeability coefficient ratio (apical-to-basolateral: basolateral-to-apical)
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCT	Picrotoxin
PD	Pharmacodynamic
PDA	Photodiode array
PEPT	Peptide transporter
P-gp	P-glycoprotein

PI	Protease inhibitor
PK	Pharmacokinetic
polyA-DT	polyadenylic acid-oligodeoxythymidilic acid
PPARγ	Proliferator-activated receptor
PTZ	Pentylenetetrazole
PXR	Pregnane X receptor
QC	Quality control
QZ59-*RRR*	Cyclic-tris-(*R*)-valineselenazole
QZ59-*SSS*	Cyclic-tris-(*S*)-valineselenazole
RMSD	Root mean square deviation
RNA	Ribonucleic acid
RUESC	Rhodes University Ethical Standards Committee
RXR	Retinoid X receptor
SA	South Africa
SADC	Southern African Development Community
SD	Standard deviation
SF	*Sutherlandia frutescens*
SLC	Solute carrier
SPE	Solid-phase extraction
ssRNA	Single strands of RNA
STZ	Streptozotocin
$T_{½}$	Elimination half-life
Tat	Transactivator of transcription
TB	Tuberculosis
TCAM	Traditional/complementary/alternative medicine
TLC	Thin layer chromatography
TM	Traditional medicine
T_{max}	Time at C_{max}
TMD	Transmembrane domain
TPA	12-O-tetradecanoylphorbol-13-acetate
TPNH	Reduced triphosphopyridine nucleotide
TR	Therapeutic range
tRNA	Transfer RNA
UGT	Uridine diphosphate-glucuronosyltransferase

UNAIDS	Joint United Nations Programme on HIV/AIDS
V_d	Volume of distribution
WHO	World Health Organisation
WLSLR	Weighted least squares linear regression

This thesis is dedicated to my family, especially:

To my parents, Gloria and Cyril Müller, for their prayers, love, encouragement, support and patience during my long academic career

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CHAPTER 1
INTRODUCTION

1.1 Research Contextualisation

In his statement of support for the "Stop AIDS campaign" launch in the United Kingdom in 2008, the then General Secretary of the Trades Union Congress, Brendan Barber, mentioned that: "The HIV/AIDS pandemic is one of the most intractable problems facing mankind today. It poses a formidable challenge to all developmental efforts. It has certainly set the clock back by decades in some of the poorest regions in the world, nullifying painstakingly made progress in the economic and social well-being of millions of people [1]". It is poignant settings such as these which have propelled urgent research in various areas of HIV/AIDs with the ultimate goal being to ease the burden of the disease.

The latest epidaemiological report of the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimated that, at the end of 2008, 33.4 million people worldwide were living with HIV; 67% of these were in sub-Saharan Africa [2]. The southern African region has been declared the epicentre of the pandemic, where low socio-economic status together with the prevalence of other infectious and parasitic diseases has heightened the vulnerability of the majority of people [3]. The statistics showed a modest rise in HIV prevalence in South Africa (SA), from 15.6% in 2003 to 16.9% in 2008 [2]. At a glance, this appears to be a negative trend that suggests an increase in the incidence of HIV infections in the country; however, there being no cure for HIV infection, prevalence is largely dependent on mortality rates too. Since the public sector rollout in 2004, access to antiretrovirals (ARVs) in SA has improved, particularly for the impoverished, and this has had a significant role to play in curbing HIV mortality [2], which in turn contributed to the slight increase in prevalence recorded four years later. Equally encouraging is the more marked reduction in the prevalence of HIV among children aged 2 to 14 years old from 5.6% in 2002 to 2.5% in 2008 [4]. This may be attributed mainly to a lower incidence in this age group through the successful implementation of programmes to prevent mother-to-child transmission of the virus, [4], such as the provision of ARV prophylaxis [2]. The trends observed in these epidaemiological indicators provide direct evidence of the importance of ARVs in the successful prevention and management of HIV/AIDS. However, whilst access to ARVs is important, of grave concern too, is the rational use of these medicines. Ensuring that the

safety and efficacy of ARVs is not compromised is imperative in the accomplishment of this ideal.

1.2 Human Immunodeficiency Virus Infection

1.2.1 Viral Taxonomy

HIV belongs to the family of human retroviruses, *Retroviridae* [5]. The genetic material of these viruses is found in single strands of ribonucleic acid (RNA) and a salient feature is the reverse transcription of RNA to deoxyribonucleic acid (DNA) [5] which occurs during the life cycles. HIV is further classified under the subfamily, *Lentiviruses*, and is divided into two types, namely, HIV-1 and HIV-2 [5], the former being the leading cause of infections in most parts of the world [6].

1.2.2 Viral Morphology

HIV is structurally designed to efficiently and effectively achieve its goal of replication in host cells. The primary structural features, as described by Levy (2007) [7], are summarised below and a schematic representation of the virus is shown in Figure 1.1. The *envelope* of the virion is composed of host cell membrane and two glycoprotein molecules, namely gp41 and gp120. The *core* of the virion contains two single strands of RNA (ssRNA) and the HIV enzymes *viz* reverse transcriptase protein, integrase protein and protease protein. The ssRNA holds the genes which code for the production of HIV structural and regulatory proteins. The contents of the core are protected by the structural protein, a capsid, which is in turn surrounded by matrix protein.

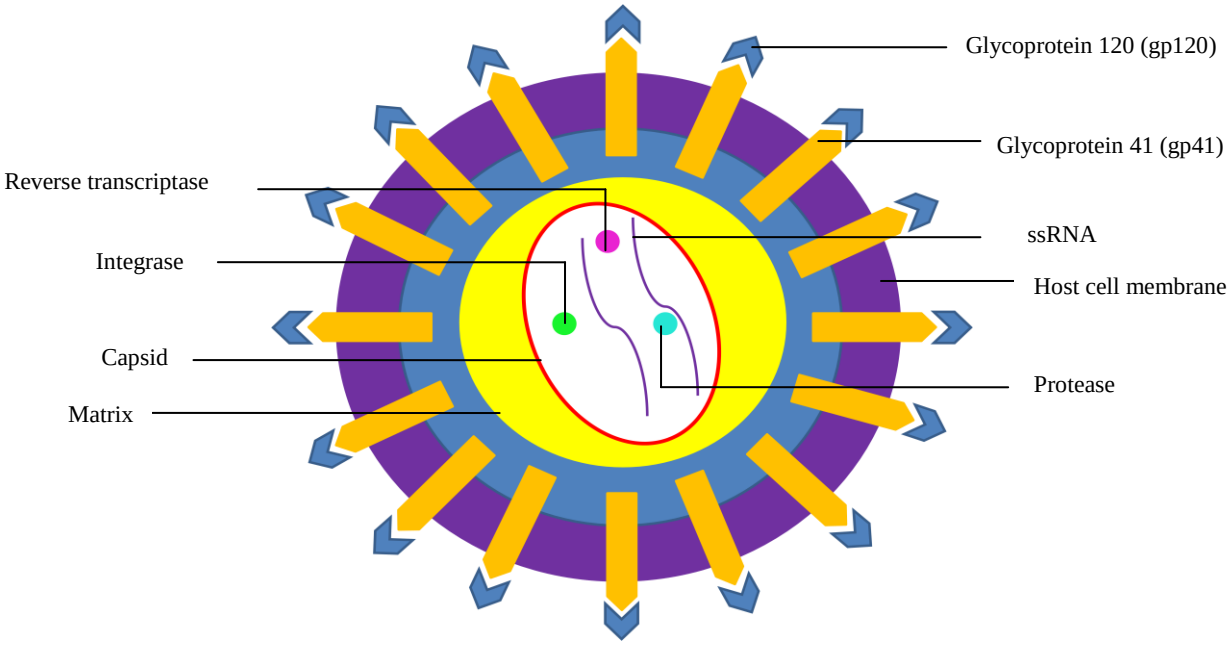


Figure 1.1: Schematic structure of HIV based on [8]

1.2.3 Replication Cycle of HIV

The replication cycle of HIV, which is shown in Figure 1.2 is key to understanding the immunopathology and management of the disease. A description of the cycle by Antoni *et al.* (1994) [9] is outlined in the following nine steps.

1.2.3.1 Step 1: Binding

The surface glycoprotein, gp120, recognises and binds to receptors for cluster of differentiation 4 (CD4), a glycoprotein found on the surface of host T-lymphocytes. This results in conformational changes in gp120, which in turn allows for interaction of gp120 with one of two coreceptors, C-C and C-X-C chemokine type 5 receptors, abbreviated to CCR5 and CXCR4 respectively.

1.2.3.2 Step 2: Fusion

Coreceptor binding facilitates conformational changes in gp41, which triggers fusion of the viral membrane with the host cell plasma membrane. The HIV genome and associated proteins are subsequently released into the cell.

1.2.3.3 Step 3: Reverse Transcription

Host nucleotides present in the cell are used by HIV reverse transcriptase to copy the single stranded viral RNA to produce double stranded viral DNA.

1.2.3.4 Step 4: Integration

Viral DNA enters the nucleus of the cell where it is incorporated into the host cell genome by the integrase enzyme. The cycle may be temporarily halted if integrated viral DNA remains latent in the nucleus. However, once the infected host cell is activated, the process resumes.

1.2.3.5 Step 5: Gene Expression and Transcription

During the transcription of host cell DNA by host RNA polymerase, the integrated viral DNA is also transcribed to form genomic viral RNA and messenger RNA (mRNA).

1.2.3.6 Step 6: Translation

The mRNA moves out of the nucleus into the cytoplasm of the host cell where it is translated via host transfer RNA (tRNA) to form long chains of viral proteins.

1.2.3.7 Step 7: Assembly

Two complete single strands of genomic viral RNA and the newly formed proteins assemble to form immature virions. The synthesised envelope proteins are inserted into the host cell membrane.

1.2.3.8 Step 8: Budding

The immature virions bud at the host cell membrane, thus acquiring an envelope containing HIV glycoproteins, gp120 and gp41.

1.2.3.9 Step 9: Maturation

After budding, HIV protease cleaves viral precursor proteins, converting the immature virion into a mature, infectious virus.

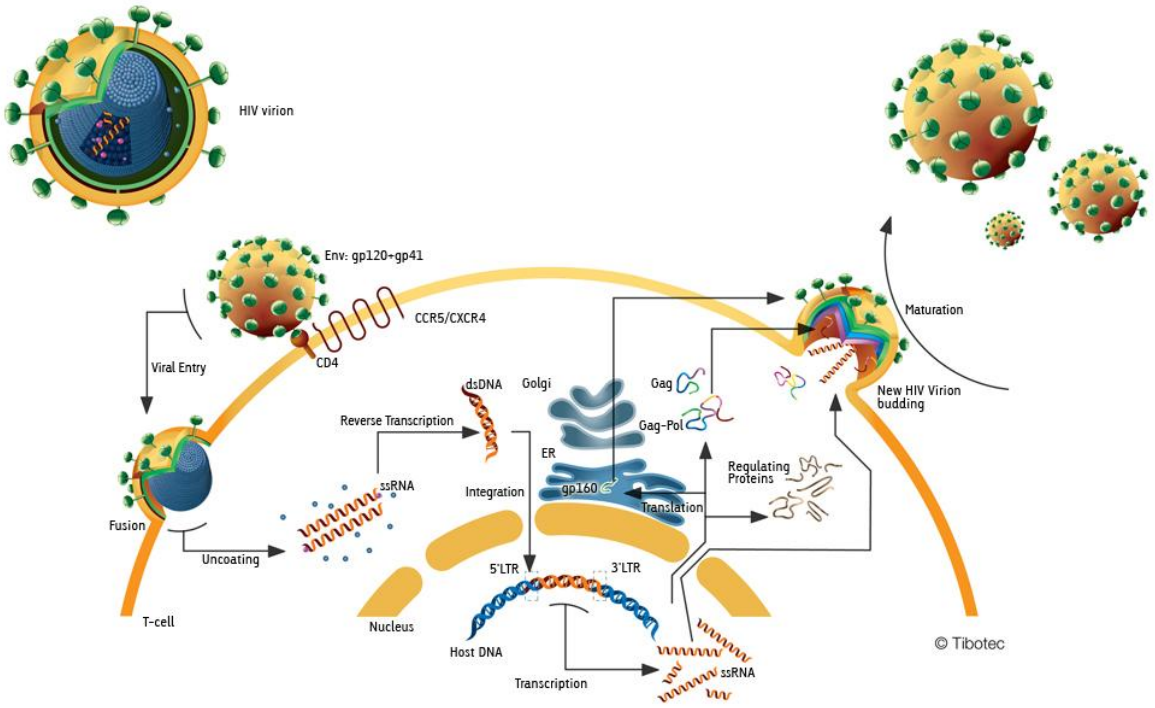


Figure 1.2: Illustration of HIV replication cycle [10]. Copyright Tibotec Pharmaceutics Ltd (2002). Permission for non-commercial use granted provided no modification or further reproduction of the content is performed.

1.2.4 Target Host Cells of HIV

As described in the discussion on the replication cycle of HIV (section 1.2.3), CD4 receptors and one of two coreceptor molecules are required for viral entry into a host cell, therefore any cell which expresses these may be infected. The main cells which fulfil these criteria are CD4 T-lymphocytes, as well as macrophages and dendritic cells [11]. These cells all have an important role to play in the normal functioning of the immune system in humans.

1.2.5 Immunopathology of HIV Infection

1.2.5.1 Definition

Since HIV infects immune cells, the pathophysiology of the disease is manifested in the immune system, and may therefore be termed the immunopathology of HIV infection [12].

However, this is complicated by the immune response to HIV infection, which may in itself be pathological [12].

1.2.5.2 Characteristics of the Immunopathology of HIV Infection

The hallmark of HIV immunopathology is the dysfunction and depletion of CD4 T-lymphocytes [12; 13]. Other features include the dysfunction and depletion of cytotoxic CD8 T-lymphocytes, the dysfunction of B cells, depletion of dendritic cells and cytokine dysregulation [12]. Furthermore, macrophages become reservoirs of HIV infection [14], which poses a great challenge in the suppression of HIV infection by ARVs.

1.2.5.3 Mechanisms of CD4 T-lymphocyte Depletion

The mechanisms which underlie the depletion of CD4 T-lymphocytes in HIV infection are several-fold, and only the primary pathways will be outlined here.

The replication of HIV in infected host cells has direct cytotoxic effects through accumulation of unintegrated viral DNA [13; 15; 16] and viral proteins [15]. Moreover, vital biochemical processes, particularly cellular protein synthesis, are disrupted [13] and important cell structures, such as the plasma membrane are damaged [15; 17]. Uninfected CD4 T-lymphocytes are not spared either, as many of these fuse with a single HIV-infected cell through the interaction between the CD4 receptor of the uninfected T-lymphocyte and gp120 present on the plasma membrane of the infected cell (see step 7 of replication cycle of HIV in section 1.2.3), thereby forming a multinucleated cell, termed a synctium, which is short-lived [13; 15].

The cell-mediated immune response to HIV infection may also contribute to the destruction of (i) infected and (ii) uninfected CD4 T-lymphocytes:

i. After the production of viral proteins in infected CD4 T-lymphocytes, these are presented as antigens on the cell surface in association with the class I major histocompatibility complex (MHC) [18]. Once activated by previous encounters with MHC class I containing HIV-specific antigen on infected CD4 T-lymphocytes, cytotoxic CD8 T-lymphocytes have the ability to recognise this HIV-specific antigen presented by the MHC class I on other infected CD4 T-lymphocytes [18]. The latter are subsequently lysed by the CD8 T-lymphocytes [12; 18].

ii. The envelope glycoprotein, gp120, is linked to the gp41 on the virus surface by non-covalent interactions and is frequently shed from infected cells or from virus particles. This free gp120 binds to uninfected cells via the CD4 receptor and may be processed by the cell and form part of the HIV-specific antigen, setting in motion its own fate of destruction by CD8 T-lymphocytes [12].

HIV-binding antibodies which are produced by B cells as part of the humoral immune response react with gp120 on the surface of HIV infected CD4 lymphocytes [18]. The antibodies serve as recognition sites to direct natural killer lymphocytes towards the infected CD4 T-lymphocytes [12; 13; 18]. The uninfected population of these host cells are once again potential targets if they happen to express free gp120 on their surface [12] (see ii above). The cross-linking of CD4, gp-120 and antibody to gp120 that occurs in the formation of this immune complex may also induce apoptosis (programmed cell death) of infected and uninfected CD4 T-lymphocytes [12; 13; 18].

1.2.5.4 Immunologic Consequences of the Depletion of CD4 T-lymphocytes

CD4 T-lymphocytes have a regulatory role to play in both the cellular and humoral immune systems [19]. Activation of CD4 T-lymphocytes by the antigen associated with the MHC class II molecules found on the surface of antigen presenting cells triggers the release of specific cytokines [19]. These boost the microbicidal activity of macrophages, activate the cytotoxic capacity of CD8 T-lymphocytes and stimulate the production of antibodies by B cells [19]. Depletion of CD4 T-lymphocytes leads to the development of an inefficient immune system [12; 13]. Once the CD4 T-lymphocyte population is too low, various other pathogens which are ordinarily cleared by the immune system go “unrecognised” and the appropriate antibody- and cell-mediated immune responses are not executed [12; 13]. The ultimate consequence is the development of opportunistic infections [12; 13].

1.2.6 Diagnosis

The molecular basis of HIV infection alluded to in sections 1.2.4 and 1.2.5 directs the diagnosis of the disease. There are two main approaches which involve the detection of: (i) HIV antibodies and (ii) HIV components (HIV-RNA, HIV-DNA or HIV capsid protein) [20; 21]. The latter is the method of choice if it is possible that seroconversion has not yet occurred in an infected individual (usually less than 6 months after potential exposure to

HIV) [21] and also in children less than 18 months old who may still carry maternal antibodies to HIV, despite not being infected [20].

1.2.7 Immunological and Clinical Classification

Once HIV infection has been diagnosed, the signs and symptoms which the patient presents are used to classify the clinical stage of the disease. Broadly, the four stages are:

1. Asymptomatic
2. Mild symptoms
3. Advanced symptoms
4. Severe symptoms

World Health Organisation (WHO) stage I may be characterised by persistent generalised lymphadenopathy, whilst stages II to IV are defined by progressive weight loss and increasingly serious opportunistic infections [20]. As described in section 1.2.5.2, a consequence of the immunopathology of HIV infection is a decrease in the number of CD4 T-lymphocytes and this ultimately leads to progression of the disease through the four clinical stages [20]. The immune status of HIV-infected people may also be classified in order to determine the severity of HIV-related immunodeficiency [20]. The absolute number or percentage of CD4 cells/mm^3 of blood underlies this classification [20]. The normal absolute CD4 cell count in adolescents and adults ranges from 500 to 1500 cells/mm^3 blood [20]. The proposed immunological classification [20] in HIV-infected people, five years and older is:

1. No significant immunodeficiency (>500 cells/mm^3).
2. Mild immunodeficiency (350–499 cells/mm^3).
3. Advanced immunodeficiency (200–349 cells/mm^3).
4. Severe immunodeficiency (<200 cells/mm^3).

In general, the CD4 cell count decreases as HIV disease (clinical stage) progresses [20].

1.3 Management of HIV Infection

1.3.1 Rational Approach

Since the advent of ARVs in 1996, the underlying principles for the management of the HIV infection have remained unchanged. However, in 2002, in an effort to expand access, the

WHO released guidelines for a public health approach to scaling up ARV therapy (ART) in resource-poor settings [22], guidelines that formed the backbone of management strategies in most low- to middle-income countries. Over the years, this experience in resource-poor settings, new clinical evidence and observational studies with respect to safety and efficacy, as well as cost and availability of drugs, have led to several revisions of this original document [23–25]. To avoid treatment failure over prolonged periods, a combination of at least three ARV drugs with different specific sites and mechanisms of action has remained the rational approach to the treatment of HIV/AIDS [22–25].

1.3.2 Eligibility Criteria for the Initiation of ART

The immunological and clinical classifications are used as a guide to determine the eligibility of HIV-infected people for initiation of ART [23]. The WHO recommends that ART be commenced for the management of HIV in WHO stage III or IV (regardless of immunological classification) and in WHO stage I or II only if immunodeficiency is advanced or severe [23]. The guidelines used in the public sector health system in SA are somewhat more conservative. Patients who are in clinical WHO stage IV or who have been diagnosed with multi-drug resistant tuberculosis (TB) or extremely drug resistant TB are eligible for ART, irrespective of the immune status of the patient [26]. Patients with advanced or severe immunodeficiency with concurrent pregnancy or TB are started on ART irrespective of WHO clinical stage [26].

Those patients not yet eligible for ART in the public sector in SA are transferred to a wellness programme for regular follow up every six months mainly to determine clinical and immunological staging to re-asses their eligibility for ART initiation [26]. TB prophylaxis with chronic isoniazid is prescribed in these patients, until ART is initiated [26].

1.3.3 Classes of ARVs

The molecular basis for the mechanisms of action of ARVs lies in the inhibition of the replication cycle of HIV. The drugs are categorised, as shown in Table 1.1, according to site (specific step in replication cycle) and mechanism of action.

1.3.3.1 Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and Nucleotide Reverse Transcriptase Inhibitors (NtRTIs)

The first groups of ARVs to be developed were the NRTIs and the NtRTIs. The structures of these drugs closely resemble natural nucleosides and nucleotides except for the hydroxyl group at the 3' position which is replaced with any functional group unable to form 5' to 3' phosphodiester linkages [27]. Intracellular phosphorylation by kinases is required for conversion to the active 5'-triphosphate form [27]. Reverse transcription, step 3 of the HIV replication cycle (see section 1.2.3) is blocked due to two actions of the drugs:

i. The binding of the natural substrate (nucleoside or nucleotide) to reverse transcriptase is inhibited, due to competition for the same binding site with the NRTI or NtRTI [27].
ii. The drug moiety becomes incorporated into viral DNA, but the inability to form phosphodiester linkages results in chain termination, preventing the synthesis of a complete double strand of viral DNA [27].

1.3.3.2 Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)

The NNRTIs also act at step 3 of the replication cycle (see section 1.2.3), inhibiting reverse transcriptase through non-competitive binding to the enzyme at an allosteric site. This results in a conformational change to the active site, thereby preventing the binding of natural nucleosides required for the formation of viral DNA [27].

1.3.3.3 Protease Inhibitors (PIs)

The PIs inhibit the protease enzyme during maturation of HIV in step 9 of the replication cycle (see section 1.2.3). Most contain a synthetic analogue of the phenylalanine-proline sequence at positions 167 and 168 of the gag-pol polyprotein, which is cleaved by the protease enzyme [28]; therefore, like the NRTIs and NtRTIs, the PIs act by competitive inhibition. Structurally disorganised and immature virions, which are non-infectious are formed in the presence of PIs [27].

1.3.3.4 Integrase Inhibitors

The current integrase inhibitors available prevent integration, step 4 of HIV replication cycle (see section 1.2.3). These drugs inhibit the strand transfer reaction of integrase in which the enzyme nicks the host cell DNA on each strand and covalently links the 5'-phosphate of this

DNA to the processed 3'-OH of viral DNA [29]. The incorporation of viral DNA into the host cell DNA is thus prevented.

1.3.3.5 HIV Entry Inhibitors

Attachment inhibitors are sulphated polysaccharides, which have been investigated as vaginal microbicides [30]. These polymers are anionic and may react with the positively charged sites on the V3 loop of the viral gp120 thus interfering with the binding thereof to the CD4 receptor of T-lymphocytes [30], which is the first step in the HIV replication cycle (see section 1.2.3). *Inhibitors of gp120/CD4 interaction* are investigational humanised monoclonal antibodies which also disrupt gp120/CD4 binding through the formation of immune complexes with the CD4 receptor or one of the two coreceptors on the host cell surface [31]. *CCR5 and CXCR4 inhibitors* are antagonists at these coreceptors, therefore interactions with CD4 receptors are blocked [31]. The latter are also still under various stages of development. *Fusion inhibitors* bind to gp41, preventing viral fusion [27], step 2 of the HIV replication cycle (see section 1.2.3).

Table 1.1: Classes of ARVs

Class of antiretroviral	Drugs
Nucleoside reverse transcriptase inhibitors	Zidovudine, Didanosine, Zalcitabine, Stavudine, Lamivudine, Abacavir, Emtricitabine
Nucleotide reverse transcriptase inhibitors	Tenofovir
Protease inhibitors	Saquinavir, Indinavir, Ritonavir, Nelfinavir, Amprenavir, Lopinavir, Atazanavir, Fosamprenavir, Duranavir, Tipranavir, Brecanavir
Non-nucleoside reverse transcriptase inhibitors	Nevirapine, Delavirdine, Efavirenz
Integrase inhibitors	Raltegravir, Elvitegravir
HIV entry inhibitors	
Attachment inhibitors	Dextrin-2-sulphate
Inhibitors of gp120/CD4 interactions	PRO 542, TNX 355
CCR5 inhibitors	Maraviroc, Vicriviroc
CXCR4 inhibitors	AMD 11070, KRH 2731
Fusion inhibitors	Enfuvirtide, Tifuvirtide

1.3.4 ART Regimens

The WHO suggests that first-line regimens for ART-naïve individuals should contain two NRTIs/NtRTIs (which are analogues of different nucleosides/nucleotides) and one NNRTI. The two currently recommended regimens are shown in Table 1.2.

Table 1.2: Current WHO first-line ART regimens

WHO Regimen 1	WHO Regimen 2
Zidovudine	Tenofovir
Lamivudine	Lamivudine/Emtricitabine
Efavirenz/Nevirapine	Efavirenz/Nevirapine

The South African guideline stipulates that the WHO Regimen 2 is preferred except in patients who have concurrent renal disease, in which case tenofovir is contra-indicated [26]. It is notable that stavudine-based regimens are no longer recommended due to the unpleasant and life-threatening adverse effects of this NRTI [23]. However, in SA, patients should remain on such regimens if well tolerated unless high risk factors are evident, such as older females with a high body mass index and those who have concomitant TB [26]. Nevirapine rather than efavirenz should be prescribed for ART-naïve women of child-bearing age, especially where reliable contraception is in doubt or in those in their first trimester of pregnancy, due to the teratogenicity of the latter [23; 26]. This also applies to patients who are using psychoactive drugs simultaneously [26]. In contrast, efavirenz is the NNRTI of choice for patients on TB treatment and those with hepatoxicity, to limit the potential for a pharmocokinetic interaction with rifampicin and to avoid further liver damage, both of which may predominate with the use of nevirapine [26].

The success or failure of ART regimens may be determined by assessing the viral, immunologic and clinical status of patients on treatment [32]. The three types of treatment failure may occur alone or together, although in general, virologic failure occurs first, followed by immunologic failure, and then clinical progression [32]. Establishing virologic failure is therefore most appropriate as it provides the earliest sign of treatment failure and allows for a more prompt change in the patient management strategy. Viral load, a measurement of HIV RNA copies/ml blood is used to determine virologic failure [23; 26]. This test should be routinely conducted on blood samples of HIV-infected patients on ART, at least every six months [23]. Virological failure is defined by the WHO as a viral load persistently >5000 copies/ml [26], whilst the SA guidelines characterise it as >1000 copies/ml on two occasions despite a good adherence record [26]. The first-line ART regimen in patients who experience treatment failure should be switched to one of the second-line regimens, which consist of a ritonavir-boosted PI and two NRTIs/NtRTIs [23], as shown in Table 1.3. When a second-line regimen is introduced, care should be taken to always switch the use of zidovudine- and tenofovir-based regimens. For example, if Regimen 1 (Table 1.2) was used as first-line, then Regimen 4 (Table 1.3) should be prescribed [23] as second-line ART.

Table 1.3: Current WHO second-line ART regimens

WHO Regimen 3	WHO Regimen 4
Zidovudine	Tenofovir
Lamivudine	Lamivudine/Emtricitabine
Ritonavir boosted lopinavir/atazanavir	Ritonavir boosted lopinavir/atazanavir

In SA, lopinavir is the PI of choice, unless gastrointestinal adverse effects are intolerable or there is an increased risk of others, such as hyperlipidaemia (serum total cholesterol >6 mmol/L) and hyperglycaemia (blood glucose >6 mmol/L) [26]. In these patients, atazanavir (ATV) which has a better adverse effect profile, is used instead [26].

1.3.5 Other Conventional Management Strategies

The body weight of HIV-infected patients should be regularly checked [26], as it is used as an indicator for the WHO clinical staging. Furthermore, if a patient is on ART, a loss in weight may be a sign of a potential opportunistic infection, particularly TB [26]. In addition to following standard treatment guidelines for any opportunistic infection diagnosed, a multi-vitamin is also recommended in those patients who have a low nutritional status [26].

Patients who exhibit any signs or symptoms should be screened and treated for common opportunistic infections [26]. A newly acquired stage four opportunistic infection or a new HIV-linked complication for patients on ART for more than three months may be significant signs of treatment failure [26]. The viral load should be determined, interpreted and appropriate action taken as described in section 1.3.4.

Cotrimoxazole prophylaxis for *Pneumocystis jivoreci* pneumonia (previously *Pneumocystis carinii* pneumonia), toxoplasmosis, many bacterial infections, malaria, and diarrhoea caused by *Isospora belli* or any *Cyclospora* species should be provided for patients with severe immunodeficiency or who are in WHO stage I, II or III [26]. This should be continued until the patient is on ART with an improved immune status of >200 cells/mm^3 [26].

1.3.6 Traditional Medicines

1.3.6.1 Definitions

The WHO defines the practice of traditional medicine (TM) as: "The sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment of physical and mental illness [33]".

When any form or adaptation of this practice is undertaken by non-indigenous users anywhere in the world, and is not part of the mainstream health care system, it may be termed alternative medicine [33]. The latter may be specifically referred to as complementary medicine when used in conjunction with the conventional practice of medicine. The two together are grouped under the umbrella term "complementary/alternative medicine (CAM)".

1.3.6.2 The Use of TCAM by HIV/AIDS Patients

The use of at least one form of traditional/complementary/alternative medicine (TCAM) by HIV/AIDS patients, as shown in Table 1.4, is a well established phenomenon in Africa [34–37], Asia [38-40], Australia [41], Europe [42–44] and North [45–47] and South America [48]. In a review of reports on the use of CAM amongst HIV-positive people, it was found that reasons for use include: alignment with cultural and/or religious beliefs, as a safe alternative to ART, for synergism with ART, to alleviate ART adverse effects, as a general way to promote health and quality of life and to treat HIV-related fatigue, nausea and pain [49]. Most of the surveys summarised in Table 1.4 included participants who were on ART and amongst those who practised TCAM, the use of herbal medicines was common. In addition, less than 40% of patients had informed any health care provider of their use of herbal medicines (in each of the three surveys where this was determined). In summary, the information gathered in Table 1.4 suggests that there are HIV-positive people who use ART and herbal remedies concomitantly, sometimes without the knowledge of their health care providers (doctors, nurses, pharmacists). In two of the South African studies, specific herbal remedies in the practice of African traditional medicine (ATM) which were reportedly used by participants included *Hypoxis hemerocallidea* (African potato) [34; 36], *Cocos nucifera* (coconut) [36] and *Aloe vera* (aloe) [34].

In addition to cross-sectional surveys, there have also been several anecdotal reports on the use of the indigenous southern African plants, African potato and *Sutherlandia frutescens* (SF) by HIV/AIDS patients [50], and the use thereof was even recommended at a consultative meeting of representatives of the Ministries of Health of the member states of the Southern African Development Community (SADC), in 2003 [51].

1.3.6.3 Preclinical Evidence of the Effectiveness of ATMs

There are many reviews in the literature [52–57] on laboratory-based studies investigating the anti-HIV activity of medicinal plants. In particular, countless plants indigenous to those parts

of the world with a rich history of TM, such as China [56], India [55] and Africa [53; 58–64], have been screened for anti-HIV properties. Such studies in South Africa appear to focus on those NOT traditionally used by HIV/AIDS patients. There is perhaps more intrigue and interest in the pursuit of novel anti-HIV activity in unexplored medicinal plants rather than to establish whether the current use of ATMs such as African potato and SF for HIV/AIDS is in fact valid.

Table 1.4: Cross-sectional surveys on the use of TCAMs by HIV/AIDS patients in different parts of the world

Year of study	Location	Number of patients	Prevalence: female	Prevalence: ART	Prevalence: TCAM practice	Prevalence: herbal medicine as % of total TCAM practice	Use of herbal medicine known by health care provider	Reference
2004	South Africa	44	None	68.2%	ND	32.8%	ND	[34]
2007	Pretoria, South Africa	180	68.9%	All	8.9% (plus OTC)	50% (TM only)	ND	[36]
2007	Western Uganda	137	70.1%	50.4%	ND	63.5% (total) 59.4% (on ARVs) 67.6% (ARV-naïve)	ND	[35]
2008	KwaZulu-Natal, South Africa	618	70.9%	none	51.3%	55.8%	10.2%	[37]
2005	North Eastern Thailand	132	72.0%	18.9%	ND	33.0%	ND	[38]
2007	India	1667	31.3%	22.9%	40.4%	80.1%	ND	[40]
2009	China	29	24.1%	All	>70%	ND	ND	[39]
2002	Australia	924	9.1%	72.6%	55.0%	34.6%	ND	[41]
2003	Europe	1066	30.3%	84.8%	59.3%	18.0%	ND	[44]
2006	Europe	632	35.0%	ND	15.8%	63%	ND	[42]
2008	London, UK	293	ND	All	68.9%	40.6%	ND	[43]
2003	Ontario, Canada	104	47.1%	86.7%	76.9%	40.0%	38.0%	[46]
2005	USA	226	None	87.6%	64.0%	26.2%	ND	[47]
2007	Multi-state, USA	914	32.4%	69.7%	14.9% (on ARVs) 17.3% (ARV-naïve)	ND	ND	[45]
2008	Brazil	128	ND	ND	ND	52.3%	23%	[48]

ND not determined

Table 1.5: Laboratory studies on anti-HIV activities of South African medicinal plants in the last six years

Year of study	Genus and species	Mechanism of action	Model used	Type and concentration of preparation	Comparison (IC_{50}/statistical analysis/positive control)	Reference
2004	*Terminilia sericea*	Inhibition of HIV RT-1	Cell-free radioactive-based assay measuring the incorporation of radiolabelled dTTP by recombinant HIV RT-1 using polyA-dT as a template-primer	Methanol extracts dissolved in DMSO (0-100 µg/ml)	IC_{50} of 7.2 µg/ml	[58]
2008		Inhibition of α-glucosidase (prevent glycosylation of HIV envelope proteins)	Cell free colorimetric enzyme assay measuring p-nitrophenol, a product of ρ-nitrophenyl-α-D-glucopyranose, in the presence of α-glucosidase	Organic extract (not specified) (0-200 µg/ml)	IC_{50} of 92 µg/ml < IC_{50} of 131 µg/ml (acarbose-positive control)	[63]
		Inhibition of HIV RT-1	Cell-free system using a HIV-RT colorimetric ELISA kit	Organic extract (not specified) (0-200 µg/ml)	IC_{50} of 43 µg/ml < IC_{50} of 100 µg/ml (adriamycin-positive control)	[63]
2005	*Lobostemon trigonus*	Inhibition of HIV RT-1	Cell-free system using a HIV-RT colorimetric ELISA kit	Aqueous, methanol and dichloromethane extracts of leaves dissolved in DMSO (0.2 mg/ml)	>50% inhibition for all; IC_{50} of 49 µg/ml for aqueous extracts with tannins and sulphated polysaccharides removed	[65]
		Inhibition of α-glucosidase and β-glucuronidase (prevent glycosylation of HIV envelope proteins)	Cell free colorimetric enzyme assay measuring p-nitrophenol, a product of ρ-nitrophenyl-α-D-glucopyranose and ρ-nitrophenyl-β-D-glucuronide, in the presence of α-glucosidase and β-glucuronidase respectively	Aqueous, methanol, ethanol and acetone extracts of leaves dissolved in DMSO (0.2 mg/ml)	>50% inhibition	[65]
2005	*Sutherlandia frutescens*	Inhibition of β-glucuronidase (prevent glycosylation of HIV envelope proteins)	Cell free colorimetric enzyme assay measuring p-nitrophenol, a product of ρ-nitrophenyl-β-D-glucuronide, in the presence of β-glucuronidase	Leaves (methanol, ethanol, acetone and dichloromethane extracts) and flowers (methanol, ethanol and acetone extracts) dissolved in DMSO (0.2 mg/ml)	>50% inhibition	[65]
2005	*Peltophorum africanum*	Inhibition of HIV RT-1	Cell-free system measuring the incorporation of radiolabelled dTTP by recombinant HIV RT-1 using polyA-dT as a template-primer	Methanol extract of roots and bark (0-100 µg/ml)	IC_{50} of 8 µg/ml (roots) IC_{50} of 3.5 µg/ml (bark)	[59]

Table 1.5: (continued)

2006	*Bridelia micrantha*	Inhibition of HIV RT-1	Cell-free system measuring the incorporation of radiolabelled dTTP by recombinant HIV RT-1 using polyA-dT as a template-primer	Ethyl actetate and n-butanol fractions of methanol extract	IC_{50} of 7.3 µg/ml (n-butanol) IC_{50} of 10.4 µg/ml (ethyl acetate)	[60]
2009	*Bulbine alloides*	Inhibition of HIV-1 infected CEM.NK^R-CCR5 cell proliferation	HIV-1 infected CEM.NK^R-CCR5 cell viability determined using a Cell Proliferation Kit II (XTT)	Aqueous extract of roots dissolved in DMSO (2 µg/ml)	~20% inhibition Student's t-test ($p<0.05$)	[62]
	Leonotis leonurus	Inhibition of HIV-1 protease	Cell free HIV-1 protease assay performed using a fluorogenic octapeptide substrate, HIV-fluorescence resonance energy transfer and recombinant HIV-1 protease	95% ethanol extract of leaves dissolved in DMSO (0.2 mg/ml)	>50% inhibition Student's t-test $p<0.05$)	[62]
		Inhibition of HIV RT-1	Cell free HIV RT-1 colorimetric ELISA	Aqueous extract of leaves dissolved in DMSO (0.2 mg/ml)	>50% inhibition Student's t-test $p<0.05$)	[62]
2009	*Hypoxis sobolifera*	Inhibition of HIV RT-1	Cell free HIV RT-1 colorimetric ELISA	95% ethanol extract of corms dissolved in DMSO (0.2 mg/ml)	>50% inhibition Student's t-test $p<0.05$)	[62]
		Inhibition of HIV-1 protease	Cell free HIV-1 protease assay performed using a fluorogenic octapeptide substrate, HIV-fluorescence resonance energy transfer and recombinant HIV-1 protease	Aqueous extract of corms dissolved in DMSO (0.2 mg/ml)	>50% inhibition Student's t-test $p<0.05$)	[62]
2008	*Elaeodendron transvaalense*	Inhibition of HIV cellular transcription factor NF-ƙB	NF-ƙB-dependent luciferase assay in a Jurkat-derived clone cell line stably transfected with a plasmid containing the firefly luciferase gene driven by the HIV-LTR promoter	Chloroform and ethyl actetate extracts of bark (15 µg/ml)	>70% inhibition (<84% of mesuol-positive control)	[63]
		Inhibition of HIV regulatory protein tat	Tat-dependent luciferase assay in HeLa cells transfected with plasmid $pcDNA_3$-TAT with a reporter plasmid LTR-Luc	Ethyl acetate extracts of bark (15 µg/ml)	>70% inhibition (>72.3% of mesuol-positive control)	[63]

HIV RT	HIV reverse transcriptase	dTTP	deoxythymidine triphosphate	polyA-dT	polyadenylic acid-oligodeoxythymidilic acid
DMSO	dimethylsulphoxide	IC_{50}	Concentration at 50% inhibition	ELISA	enzyme-linked immunosorbent assay
LTR	long terminal repeat				

Table 1.5 shows the various *in vitro* experiments and associated results of anti-HIV activity of extracts of South African plants published in the past six years. Despite being so popularly used and endorsed by health authorities, African potato did not feature in any of these studies, whilst only one showed some promise for SF. Several organic leaf extracts prevented the glycosylation of HIV proteins by a 50% or greater inhibition of β-glucuronidase in comparison to the control [65]. However, as with most of the studies in the literature highlighted in Table 1.5, a positive control was not used to determine the relative potency of the extracts and thus a sound scientific basis for the true anti-HIV potential of SF was not established. It is noteworthy that Tshikalange *et al.* (2008) [63] used positive controls for all the experiments involving *Terminilia sericea* and *Elaeodendron transvaalense.* Extracts of the former exhibited 50% inhibition (IC_{50}) at concentrations lower than the positive controls in assays of both α-glucosidase and HIV reverse transcriptase activity. Similarly, extracts of *Elaeodendron transvaalense* had a slightly greater % inhibition of the HIV regulatory protein, transactivator of transcription (tat), than the positive control, mesuol, when used at the same concentration. Inhibition of the HIV cellular transcription factor, NF-ƙB by these same extracts was however not as effective as mesuol.

One study published over a decade ago alluded to the immunomodulatory activity of β-sitosterol and β-sitosterol glucoside which are purported to be found in extracts of African potato [66]. This effect was shown by stimulation of the proliferation of human peripheral blood lymphocytes [66]. However, since then, no studies have attempted to verify these results in HIV-infected lymphocytes nor to discuss the possible implications of the potential proliferation of such infected lymphocytes on the immunopathology of the disease. Moreover, based on laboratory findings, Nair and Kanfer, 2008 [67] have subsequently argued that the content of β-sitosterol and β-sitosterol glucoside in African potato is likely lower than that reported by others and lower than that required to achieve the claimed therapeutic efficacy, including the immunomodulatory activity. Interestingly, Table 1.5 also illustrates the HIV reverse transcriptase and HIV protease inhibitory activities of *Hypoxis sobolifera* which belongs to the same genus as African potato [62]. The ethanolic extract had a greater than 50% inhibition of HIV reverse transcriptase in comparison to the control, whilst a similar effect on HIV protease by an aqueous extract was observed.

1.3.6.4 Clinical Evidence of the Safety and Effectiveness of ATMs in SA

A general notice on a draft policy on ATM for SA published in the government gazette on 25 July 2008 [68] states that SA has no legislative framework for the registration of and therefore the regulation of ATMs. However, ATMs most probably fall under the definition of medicine, which, according to the Medicines and Related Substances Act [69] is: "any substance or mixture used or suitable for use or manufactured or sold for use in the diagnosis, treatment or prevention of disease or its symptoms". This statute does not directly include ATMs, although it impacts on the use and practice thereof [68], which indicates that, with respect to current legislation, the quality, safety and efficacy of ATMs may be questioned. Contributors to the draft policy point out that current medicine registration requirements are more suitable for allopathic medicine and may require amendment for appropriate application to ATMs [68]. The promulgation of the Traditional Health Practitioners Act [70] which has ratified the regulation of the practice of ATM as a whole, may be the stepping stone to the establishment of a legislative framework which includes the registration of ATMs. However, this act may also need to be amended to allow for appropriate harmonisation and thus implementation of the legislation contained in the two aforementioned acts [68]. Currently, the Medicines Control Council (MCC) is responsible for registration of medicines in SA. However, a ministerial task team of the new Medicines Regulatory Authority (MRA) has made certain proposals for the regulation of ATMs and CAMs [68]. The recommendations include seven regulatory categories: i) pharmaceuticals ii) medical devices iii) vaccines iv) blood-derived products for medicinal purposes v) radiopharmaceuticals vi) food products with medicinal claims and medicinal content and vii) cosmetics with medicinal claims and medicinal content [68]. The first category would be further subdivided into prescription, non-prescription, complementary and ATMs, and each should have separate guidelines and/or requirements for registration [68].

There are several potential compromises to the safety and efficacy of ATMs [68], some of which have arisen due to the commercialisation of the traditional crude form of the plants by the manufacture of herbal medicinal products. These include but are not necessarily limited to:

a) Intentional addition of any substance which may be responsible for a therapeutic and/or adverse event.
b) Unintentional substitution of the species that has medicinal properties with a toxic species.

c) Environmental contamination of the plant with chemicals and/or pathogens.
d) Sub-optimal or varying amounts of active and/or toxic constituents.
e) Adverse effects due to a constituent or a combination of constituents.
f) Interference with the activity of allopathic medicines used concomitantly with the potential for treatment failure and/or toxicity.
g) Confounding factors leading to the misinterpretation of the efficacy of an ATM.

There is dearth of clinical data on the safety and efficacy of ATMs [68]. The knowledge derived therefrom may be used to develop policies/guidelines and hopefully even regulations which could prevent the issues highlighted above from occurring. Nonetheless, the contributors to the draft policy argue that this should not preclude the use of ATMs, which have been used over the centuries by South Africans [68]. Clinical trials should be conducted not to justify use but rather to develop a knowledge base on the safety and efficacy of such medicines [68], and this information may be particularly important in the use of manufactured herbal medicinal products.

The draft policy goes on to suggest that the design of clinical trials for ATMs should be based on traditional use [68]. The study population should be from the same communities that traditionally use the medicines and the treatment should be prepared in the customary manner [68]. Under these circumstances, the contributors believe that these trials could be performed with minimal risk to the participants and be based on limited preclinical data [68]. However new plants or manufactured herbal medicinal products of these ATMs should be subject to full scale preclinical and clinical research [68]. Clinical investigators should also be cognisant of the fact that unlike allopathic medicines, the active constituent of an ATM may not be known, or the efficacy may be due to a complex mixture of constituents, and that the alleviation of symptoms as a primary therapeutic endpoint should be considered [68]. With regard to safety, reported and documented adverse effects of the ATM or constituents during previous anecdotal or unregistered use of the ATM or manufactured herbal medicinal product should be taken into account [68].

Despite there being no previous or current legislated requirement for clinical data on the safety and efficacy of ATMs in SA, some scientists who support the use of SA medicinal plants either in crude (traditional) form and/or as manufactured herbal products, have conducted clinical trials [71–74] to assess the appropriateness of the use thereof by HIV-

infected patients. This is most likely an attempt to convince patients and cynics from the scientific community that there is sufficient evidence for the safe and effective use of these medicines in the treatment and management of the disease.

In the only randomised, double-blind, placebo-controlled trial which tests a South African ATM and is published in a peer-reviewed journal [73], the safety of capsules containing 400 mg of SF leaf powder (400 mg of plant material per capsule; 600 µg of canavanine per capsule) administered twice daily for three months (recommended dosage), was investigated. This followed a preclinical study, conducted by the SA Medical Research Council (MRC) in vervet monkeys, which found that up to nine times the recommended dose of 9mg/kg/day SF plant material for three months produced no significant changes in haematological, biochemical and physiological parameters [75]. The other objective of the clinical study was "to contribute to establishing procedures for ethical and scientifically rigorous clinical trials of African indigenous medicines" [73].

The clinical researchers chose to use healthy volunteers as is common in Phase I clinical trials of experimental drugs, where safety is being assessed. This would in any case be in agreement with the draft policy on the regulation of ATM, which, as previously mentioned, recommends full clinical trial procedures for manufactured herbal medicinal products. Another feature of this study which alludes to the potential for successful harmonisation of legislation for allopathic medicine and ATM registration is that the SF capsules were standardised in terms of the content of one of the plant's constituents, L-canavanine, and were assessed for pharmaceutical quality by measuring content uniformity, stability, and release characteristics, as well as microbial, chemical and heavy metal contamination.

Participants were provided with capsules containing either the placebo material or SF which was self-administered for the three month period. A limitation of the study was that adherence could not be precisely measured since it was a self-reported observation by participants, together with adverse effects and concomitant medication. Participants may not always be reliable, especially if full remuneration depends on such adherence; information regarding remuneration was not disclosed in the article. Moreover, the researchers mention that the small sample size of 12 participants per treatment group was also a limitation. This number of participants is sufficient to detect a power of 80% in a two-sample Student's *t* test. However, this estimation of power is only applicable to quantitative data, therefore

qualitative data such as the occurrence of rare adverse effects may have required larger sample sizes for statistically meaningful conclusions to be drawn. The authors in this study therefore acknowledged that it was particularly unlikely that rare adverse events would have been observed as statistically significant.

The authors stated that baseline data for vital, physical, haematological, biochemical and endocrine parameters were similar, which provides some evidence that the distribution of participants was random, although no statistical analysis was conducted to confirm this. Nevertheless, the results of this study showed that there were no significant differences between the 'placebo' and 'SF' groups with respect to categories of adverse events associated with organ systems, such as gastro-intestinal and central nervous system, aside from increased appetite (which many may not consider an adverse effect). The change in some haematological parameters from baseline to post-study treatment was significantly greater and respiratory rate significantly lower in the "SF" group, but the respective measurements all fell within the normal physiological ranges and were therefore not considered clinically relevant. L-canavanine, a potentially auto-immune disease-inducing constituent of SF was undetected in the plasma of all participants. Although this correlates to "no adverse events" and "normal physiological ranges" and is therefore most probably an indication of safety, the limit of detection and the plasma concentration of L-canavanine that may produce toxicity were not mentioned. Furthermore, the study did not fulfil all the criteria usually required for a full scale Phase I clinical trial. The editorial commentary of the paper points out that dose escalation and pharmacokinetic (PK) study data were not collected and may have been important in determining the ultimate safety of the medicinal product. Contributors to the draft policy on ATM regulation may argue against applying these additional restrictions to the regulation for assessing safety of ATM. Despite a few shortcomings, this pilot study was the first to provide a scientific basis for the potential safety of SF and in fact any ATM or manufactured herbal medicinal product in healthy human volunteers in SA.

In the only other randomised, double-blind, placebo-controlled trial of a South African ATM, which formed part of a doctoral dissertation [72], unpublished in the scientific literature, the efficacy of a herbal mixture containing extracts of SF and a Turkish traditional medicinal plant, *Nerium oleander* in HIV-infected patients was investigated. The author stated that the mixture is "effective in increasing the CD4 counts of HIV-positive individuals with initial CD4 counts of less than 400 cells/mm^3 in a meaningful way over a 60-day period" [72].

Although the participants in this study signed a consent form, no mention was made of ethical approval obtained for the study. This brings into question whether the potential risk to patients was adequately reviewed. One exclusion criterion was that each participant was not currently receiving ART, but no criteria were set to exclude patients who may have previous (especially recent) ART experience, which may influence baseline CD4 counts.

It was also not certain if the herbal mixture was prepared in a traditional manner and if the cohort of study participants came from a community which commonly uses SF and/or *Nerium oleander*. This, according to the draft policy recommendations may reduce risk to the participants. Moreover, no information was given regarding the preparation of the herbal mixture and the exact composition of the fortified soy milk, which was given as the placebo and which is the vehicle for the administration of the herbal mixture in the treatment group. Thus, the doses may not have been standardised and uniform, which may have compromised the quality, and ultimately the safety and efficacy thereof. Again, according to the suggestions by contributors to the draft policy on ATMs, this may not have been a major issue if the herbal mixture was a traditional preparation of the medicinal plants.

It was acknowledged by the author that a limitation of the study was that, since the dosing was not directly observed, doses may have been missed. However, the extent to which this could affect results was not discussed. The author instead made the following comment for which no evidence was found: "Fortunately, unlike the case with ARVs, this does not affect the effectiveness of the mixture and is another reason why it is preferable to ARVs." The author also acknowledged that 60 days was not long enough to determine the long-term effectiveness of the herbal mixture and that a follow up study would be advisable. It is important that an attempt was made to perform a randomised, placebo-controlled double-blinded study. The validity of the randomisation and placebo was however anonymously questioned in a forum on curezone.com [76], since the anonymous critic alleges that there appeared to be a statistically significant difference in CD4 cell count between the placebo and treatment groups at baseline, which contradicts the reported randomisation of participants to treatment groups. Moreover, the critic further alleges that the rate of decline of the CD4 cell count in the placebo group was greater than would be expected in HIV-infected patients not on any form of treatment, implying that the "placebo" of soy milk may in fact be detrimental. In the author's defence, the anonymous writer did not substantiate this with specific references, and to date literature searches have revealed many studies which investigate the

change in CD4 cell counts in patients who are ART-naïve at baseline but ARV-experienced at the end of the study, therefore an evidence-based comparison could not be drawn. The anonymous writer has boldly suggested that to confirm the validity of the results, a study should perhaps be performed which includes two additional comparative arms. Participants in these groups should i) be ART-naïve and not take any form of treatment during the study and ii) should be ART-naïve at baseline and start ART and maintain therapy during the study. The statistical analysis in the study also appeared flawed as, despite a null and alternate hypothesis being stated, no statistical test was performed, and a visual assessment of the differences between quantitative data appeared to be the basis for rejecting the null hypothesis.

Although attempts have been made to prove the safety and efficacy of some ATMs, critical evaluations of these studies have revealed that there are inadequacies in the study design and statistical analyses, which have led to much debate amongst the "proponents" and the "cynics" of the use of ATMs. Perhaps the take home point is that this in itself underscores the need for modified guidelines and/or regulations for clinical trials on ATMs, which may allow for a mutually beneficial interface between allopathic medicines and ATMs in the management of HIV/AIDs in SA.

1.3.7 Concomitant Use of ART and Herbal Medicines

1.3.7.1 The Risk of Interactions

As previously suggested (section 1.3.6.2), there may be a large cohort of HIV-infected people the world over who use ART and herbal medicines simultaneously. As is the case in any instance of polypharmacy, such patients are at risk of experiencing significant interactions between two or more of the medicines. However, the distinguishing features of this type of polypharmacy are that: i) the health care provider concerned is often not aware of the use of herbal medicines by the patient and can therefore not anticipate a potential or known interaction and ii) even if the health care provider is aware of the use of these medicines by the patient, there is usually not sufficient preclinical and clinical evidence to unequivocally advise the patient against the use of a particular herbal medicine with ART.

1.3.7.2 Definitions

The term "interaction" in pharmacology refers to the physical, chemical, physiologic or pathophysiologic interplay between two xenobiotics [77] in a living system. It is only

considered significant if an alteration in clinical response of one or both xenobiotics arises as a consequence thereof [77]. The clinical response may be positive or negative and occurs either due to changes in the pharmacodynamics (PD) or PK of the affected xenobiotic [77]. The former alludes to a direct physiologic effect, whilst the latter to a change in disposition (absorption, distribution and elimination) of the xenobiotic [77], which in turn impacts indirectly on the physiologic effect.

1.3.7.3 Clinical Significance of Pharmacodynamic Interactions with ARVs

As mentioned in section 1.3.4, viral load is used as a measure of efficacy of an ART regimen in HIV-infected patients. A positive synergistic effect is evident if any concomitant medication shifts the viral load to <1000 copies/ml (threshold for treatment failure in SA, see section 1.3.4) in comparison to ART alone, and has shown preclinical potential for ARV activity [78]. Conversely, if the viral load is increased above the threshold in comparison to the ART alone, then ARV activity is negated [78].

The adverse effects associated with the different classes of ARVs are generally well known. For example, the PIs are known to cause hyperlipidaemia [79]. With respect to PI-containing ART regimens, any concomitant agent which precipitates hyperlipidaemia independently would aggravate this effect, and a lipid-lowering agent would relieve it [78].

1.3.7.4 Clinical Significance of Pharmacokinetic Interactions with ARVs

A strong relationship exists between plasma concentrations of PIs and NNRTIs and ARV activity [80]. Plasma concentration versus time data are used to determine the AUC and C_{max}, which may be used as indicators of bioavailability. Subtherapeutic plasma concentrations of these classes of ARVs, which may occur due to reduced absorption and/or increased elimination, are associated with inadequate ARV activity and therefore treatment failure and the emergence of resistance [80]. In contrast, plasma concentrations above the therapeutic range through enhanced absorption and/or decreased elimination may lead to a higher prevalence of adverse or toxic effects [80]. The three scenarios of sub-therapeutic, therapeutic and potentially toxic plasma concentrations after a single dose of a drug are depicted in Figure 1.3.

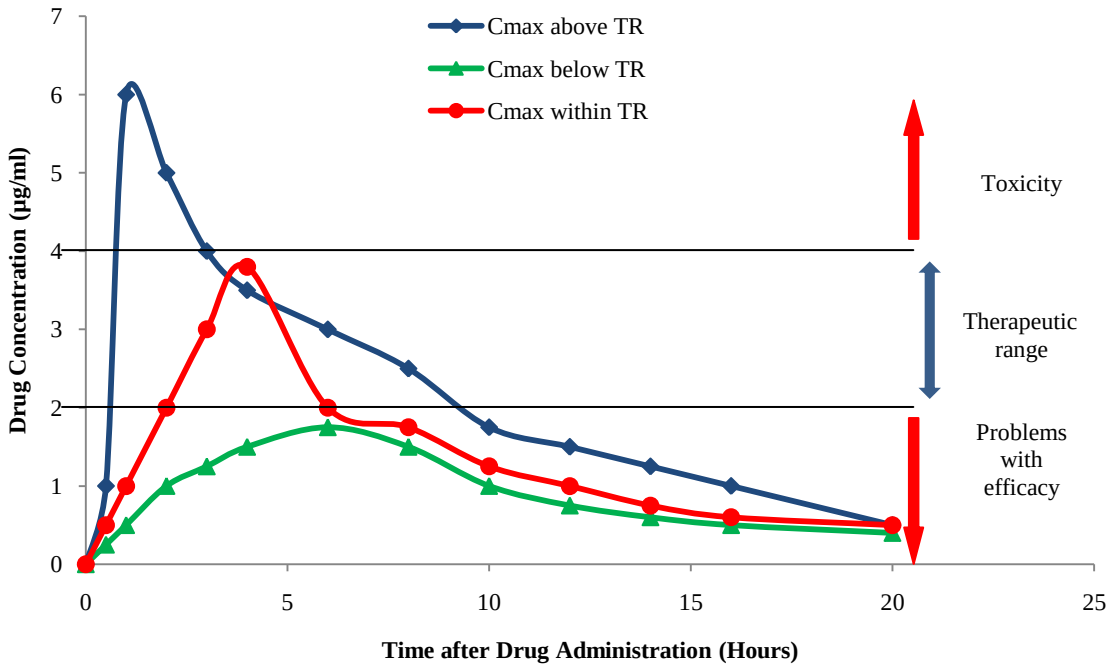


Figure 1.3: Concentration-time profiles where C_{max} remains within the therapeutic range (TR), exceeds the therapeutic range (TR) and and falls short of the therapeutic range as indicated.

1.3.7.5 Drug Transporters

Drug transporters are expressed in numerous human epithelial tissues [81]. In the enterocytes of the jejunum, these have a role to play in the absorption of drugs, whilst the opposing process of elimination is driven in part by the transporters located in the hepatocytes and renal tubular cells of the liver and kidney respectively

Influx transporters are responsible for the movement of drug substrates across plasma membranes and into cells [82]. These belong to the solute carrier (SLC) superfamily, members of which share 20–25% amino acid sequence identity [83]. This superfamily may be further subdivided into families, such as the organic anion transporter polypeptides (OATP), the organic anion transporters (OAT), the organic cation transporters (OCT) and the peptide transporters (PEPT), concentrative nucleoside transporters (CNT) and equilibrative nucleoside transporters (ENT) [84]. Conversely, efflux transporters, which are members of the ATP-binding cassette (ABC) protein superfamily, extrude drugs from the cell cytosol into the extracellular environment [85]. The two main families of interest with respect to drug

efflux are the multidrug resistance (MDR) and the multidrug resistance-associated protein (MRP) transporters [85].

The level of gene expression (mRNA) of drug transporters in the human PK organs, jejunum, liver and kidney has been used as an indirect predictor of the protein expression and functional activity thereof [81]. Figure 1.4 provides schematic impressions of the localisation of the transporters, of which ARVs are known to be substrates and which also have a high or moderate level of gene expression [81] and therefore potential activity in the jejunum, liver and kidney. For the jejunum (Figure 1.4 A), MDR1, commonly known as P-glycoprotein (P-gp), MRP2 and breast cancer resistance protein (BCRP) of another ABC family are found in the apical (luminal) membrane and are therefore involved in the efflux of substrates from the enterocytes back into the lumen of the jejunum [85] with the intention of faecal elimination. In the liver (Figure 1.4 B), primarily influx transporters such as OATP1B1, OCT1 and OAT2 are located on the basolateral (sinusoidal) membrane of hepatocytes to drive the transport of ARV substrates from the blood into the cells for detoxification by metabolism [84]. MRP3, MRP4 and MRP5 are also positioned there for movement of substrates in the opposite direction into the systemic circulation [84]. The apical (canalicular) membranes of two adjacent hepatocytes form the walls of a canaliculus. Many canaliculae combine to form the bile duct which joins the gastro-intestinal tract at the duodenum. Several efflux transporters on this apical membrane, namely P-gp, MRP2 and BCRP, excrete substrates from the hepatocytes into the canaliculae for biliary [84; 85] and ultimately faecal elimination. The basolateral membrane of renal tubule epithelial cells (Figure 1.4 C) is traversed by OAT (1,3) and OCT2 which carry substrates from the systemic circulation into the cells for secretion into the urine in the renal tubules by P-gp, MRP2 and MRP4 found on the apical (luminal) membrane for renal elimination [84].

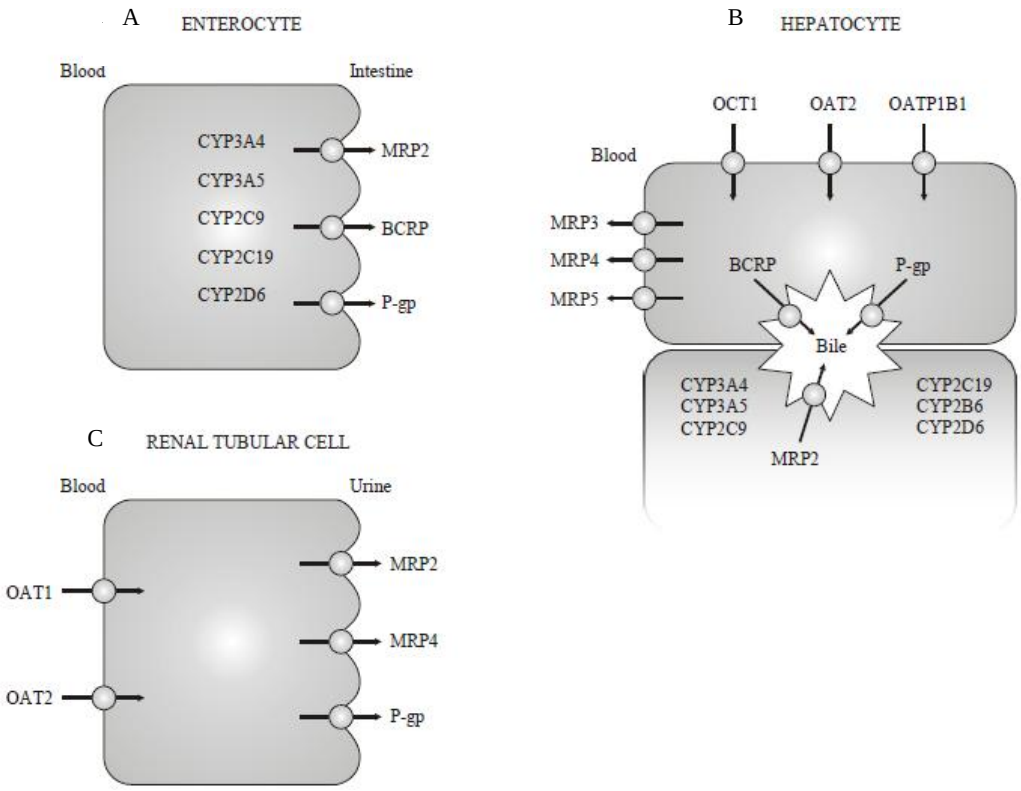


Figure 1.4: Schematic representation of the localisation of drug transporters and CYP enzymes with moderate or high gene expression in A) an enterocyte, B) a hepatocyte and C) a renal tubular cell. Based on [86].

Table 1.6 summarises the three main classes of ARVs which are known substrates of those drug transporters (and metabolising enzymes) that are highly and moderately expressed in the enteroctyes of the small intestine, the hepatocytes of the liver and renal tubular cells of the kidney. In general, it appears that the NNRTIs and PIs are substrates of more transporters, in comparison to the NRTIs, and may therefore have a greater susceptibility to transporter-dependent alterations in oral bioavailability. It is particularly noteworthy that the mRNA for P-gp was consistently expressed at moderate to high levels in all the cells shown in Figure 1.4. Moreover, between the ARV classes, NNRTIs and PIs, the latter are clearly the more prominent substrates of P-gp (see Table 1.6). It is thus plausible that transporter-dependent changes in ARV oral bioavailability are most likely to occur with PIs rather than any other ARV class, primarily via the modulation of P-gp activity as opposed to other transporters.

Table 1.6: ARVs as substrates of membrane-bound transporters and intracellular enzymes expressed in moderate to high levels in enterocytes, hepatocytes and renal epithelial tubule cells (transporters only)

ARV class and drug	ABC transporters	SLC transporters	Phase I metabolising enzymes (CYP450s)	References
PIs				
Amprenavir	P-gp		CYP3A4, CYP3A5	[84; 87]
Atazanavir	P-gp, MRP2	OATP (non-specific)	CYP3A4, CYP3A5	[84; 88]
Darunavir	P-gp	OATP1B1	CYP3A4 , CYP3A5	[84; 89]
Indinavir	P-gp, MRP2		CYP3A4, CYP3A5	[84; 90]
Lopinavir	P-gp, MRP2	OATP1B1	CYP3A4, CYP3A5	[84; 91]
Nelfinavir	P-gp		CYP3A4, CYP3A5, CYP2C19	[84; 92]
Ritonavir	P-gp, MRP2	ND	CYP3A4, CYP3A5, CYP2D6	[84; 90]
Saquinavir	P-gp, MRP2	OATP1B1	CYP3A4, CYP3A5	[84; 93]
Tipranavir	P-gp	ND	CYP3A4, CYP3A5	[84; 94]
NRTIs and NtRTIs				
Abacavir	P-gp, MRP4, BCRP	ND	ND	[84]
Didanosine	BCRP	ND	ND	[84]
Emtricitabine	ND	ND	ND	
Lamivudine	BCRP	OCT1, OCT2	ND	[84]
Stavudine	BCRP, MRP5	ND	ND	[84]
Tenofovir	P-gp, MRP4, BCRP	OAT1, OAT3	ND	[84]
Tenofovir Disoproxil Fumarate	P-gp	ND	ND	[84]
Zidovudine	MRP4, BCRP	OAT1, OAT2, OAT3	ND	[84]
NNRTIs				
Delavirdine	ND	ND	CYP3A4, CYP3A5, CYP2D6	[95]
Efavirenz	ND	ND	CYP3A4, CYP3A5, CYP2B6	[96]
Etravirine	ND	ND	CYP3A4, CYP2C9, CYP2C19	[97]
Nevirapine	ND	ND	CYP3A4, CYP2B6	[98]

ND Not determined

1.3.7.6 Metabolising Enzymes

In addition to mediation by drug transporters, the bioavailability of many drugs, such as ARVs, after oral administration is also influenced by intracellular metabolic enzymes present on the endoplasmic reticulum in the cytosol of enterocytes and hepatocytes [85]. Lipophilicity facilitates absorption, but conversely, this property also hinders elimination of these xenobiotics [99]. In the process of metabolism, substrates are converted to metabolites that have a greater polarity, which promotes elimination via the urine or faeces [99]. However, first pass metabolism in the intestine and liver consequently reduces the bioavailability of the parent drug [99]. Drug metabolic enzymes are classified according to the type of reactions which are catalysed and therefore the type of products formed. Most Phase I enzymes are members of the cytochrome P450 (CYP) superfamily that catalyse reactions in which hydrophilic functional groups are introduced into the drug molecules [99]. These reactions include oxidation, reduction and hydrolysis [99]. CYPs showing greater than

40% amino acid sequence homology are grouped into a family, indicated by an Arabic numeral [99]. Sub-families have greater than 60% homology and are designated with a letter [99]. This is then followed by another Arabic numeral representing the individual enzyme (isoenzyme), which is assigned in the order of discovery [99]. Approximately 18 CYP enzymes in families 1, 2 and 3 are responsible for the metabolism of drugs although less than half are sufficiently expressed to enable a significant contribution to drug metabolism [99].

Phase II reactions involve the conjugation of hydrophilic substances such as glucuronic acid, sulphate, glutathione or even amino acids on a suitable functional group of the parent drug or Phase I drug metabolite [99]. The Phase II enzymes which catalyse these reactions include glucuronyl transferase, sulphotransferase, glutathione-S-transferase and N-acetyl transferase [99].

A meta-analysis of studies on the abundance of CYP enzymes in human hepatic microsomes revealed that the subfamily CYP3A (CYP3A4 and CYP3A5) is the most abundant followed by CYP2C9, CYP2E1, CYP1A2, CYP2A6, CYP2C8 and CYP2C19 in order of rank [100]. CYP2B6 and CYP2D6 were the two least abundant [100]. A similar trend was observed in human intestinal microsomes with respect to CYP3A4, CYP2C9, CYP2C19 and CYP2D6 [101]. In this study, the rank of 2E1, 1A2, 2A6 and 2B6 could not be determined as values of P450/mg protein for these were found to be below the limit of detection [101]. CYP3A is present at approximately double [100] and six-fold [101] the concentration of CYP2C9 (the second ranked CYP) in human hepatic and intestinal microsomes respectively. This implies that drugs that are substrates of CYP3A (particularly CYP3A4, which is the most abundant in this subfamily) will be more susceptible to first pass metabolism in the intestine and liver in comparison to those which are substrates of any of the other CYPs. The CYP enzymes expressed in the small intestine and liver for which the three main classes of ARVs are substrates are tabulated (Table 1.6) and depicted in the schematic representations of the enterocyte, hepatocyte and renal tubular cell in Figure 1.4. Table 1.6 and Figure 1.4 B show that the six enzymes involved in the metabolism of ARVs are CYP3A4, CYP3A5, CYP2C9, CYP2C19, CYP2B6 and CYP2D6 in order of abundance in the liver. Those also present in the small intestine are depicted in Figure 1.4 A. Analogous to the transporters, it appears that the PIs and NNRTIs are more susceptible to CYP-dependent alterations in oral bioavailability since they are substrates of the highly expressed CYP3A4 and CYP3A5 in the small intestine and liver.

1.3.7.7 Combined Action of Drug Transporters and Metabolising Enzymes

Following the path of orally administered drugs in the small intestine and liver reveals that the drug transporters and metabolising enzymes in these sites probably act in concert to determine the oral bioavailability of drugs [85]. After drug release from the dosage form and dissolution into the gastrointestinal fluids, carrier-mediated (facilitated by influx transporters) or passive transport of the drug across apical membranes of enterocytes in the small intestine occurs. In the enterocytes, the parent drug may be subject to (i) efflux back across apical membrane for re-absorption or faecal elimination, (ii) metabolism by intracellular enzymes or (iii) efflux across the basolateral membrane into the hepatic portal blood system [85]. Drug metabolites formed in ii) may also undergo efflux as described in (i) or (iii) [85]. Similarly, parent drug and drug metabolites in the hepatic portal blood system may undergo active or passive influx into the hepatocytes, wherein the three processes of (i) efflux back into the systemic circulation, (ii) metabolism or (iii) efflux into the canaliculae for biliary excretion, into the small intestine, may occur [85]. The latter, once again, ultimately results in either re-absorption, termed entero-hepatic cycling, or faecal elimination of the drug or its metabolites.

1.3.7.8 Mechanisms of PK interactions

The mechanisms which underlie PK interactions with drugs involve alterations to the normal functioning of the transporters and/or metabolising enzymes for which these are substrates [85; 102]. Xenobiotics may have the ability to modify levels of transcription, translation or translocation of these proteins [85], thereby changing the number available for activity at the sites of action. Alternatively or additionally, xenobiotics may directly alter the activity of transporters and enzymes already present at the site of action through inhibition or activation of one or more of the steps required in the process of transport and metabolism of substrates by the proteins [85].

1.3.7.8.1 Modulation of the Transcription of Drug Transporters and Metabolising Enzymes

Nuclear receptors such as, pregnane X receptor (PXR), peroxisome proliferator-activated receptor (PPARγ), glucocorticoid receptor (GR) and constitutive androstane receptor (CAR) regulate the transcription of drug transporters and metabolising enzymes [103]. PXR and CAR are not only the most commonly involved, but more importantly, they modulate transcription of CYP3A4 and P-gp which were identified as the most likely enzyme and transporter respectively to influence interactions with ARVs (sections 1.3.7.5 and 1.3.7.6).

Gene expression determined by mRNA levels has shown that PXR and CAR are likely present in the tissue of the small intestine, liver and kidney, with both having highest relative abundance in the liver [104]. PXR appears to be more highly expressed in the small intestine than it is in the kidney, whereas for CAR, this ranking order is reversed [104].

Nuclear receptors are activated by ligands, including endogenous compounds and xenobiotics in the cytosol of cells [103]. Upon binding, the receptors translocate to the cell nucleus, where homodimerisation or alternatively, the formation of heterodimers with the common nuclear hormone partner, retinoid X receptor (RXR), occurs [103]. These dimeric complexes in turn bind to conserved DNA sequences known as response elements within the regulatory regions of the target genes to activate transcription [103]. In general, increased transcription (formation of mRNA) of the relevant gene in the nucleus results in an increase in translation of the corresponding transporter and/ or enzyme in the cytosol. This elevation in the availability of individual transporters and enzymes results in more transport and metabolism of drugs, thereby potentially reducing the rate of absorption and increasing the rate of elimination of these substrates. This mechanism of induction requires chronic exposure to the ligands [105] which activate PXR and/or CAR.

1.3.7.8.2 Modulation of the Translocation of Drug Transporters

It has been shown that xenobiotics may increase the activity of transporters and metabolising enzymes, without any change to the gene (mRNA) expression [85]. This implies that the mechanism of action involves an increase in individual proteins which is independent of transcription. Transporter expression in membrane vesicles may alter after short-term exposure to the xenobiotic if the latter has the ability to regulate the intracellular translocation of transporters [85]. The drug transporters are produced by translation in the cytosol of the enterocytes or hepatocytes and are then stored in intracellular vesicles until signalling molecules such as cyclic AMP and protein kinase C stimulate translocation to the plasma membrane (apical or basolateral) [106]. When the stimuli are withdrawn or replaced, transporters are removed from the membrane by endocytosis until restimulation occurs, when these are once again expressed in the membranes [106]. Eventually, these proteins are no longer recycled and are translocated to lysosomes in the cytosol for catabolic degradation [106]. Regulation of translocation may potentially occur at the level of the signalling molecules [85]. If the translocation of transporters in response to the xenobiotic is directed to

the membrane, then increased activity is expected, whereas the opposite would hold true if this process occurs in reverse.

1.3.7.8.3 Modulation of Catabolism of Drug Transporters and Metabolising Enzymes

As with transporters, metabolic enzymes are also subject to catabolism by peptidases in the lysosomes of the cytosol. Thus regulation of the activity of both types of proteins may occur through changes in the rate of catabolism [105]. In addition to altering the translocation of the proteins to the lysosomes, as described in section 1.3.7.8.2, xenobiotics may also influence the actual catabolic degradation process of the proteins [105]. A decrease in the rate of catabolism is termed post-translational stabilisation and increases the number of individual transporters and enzymes in the cell available for translocation and ultimately activity [105]. Similarly, an increase in the rate of catabolism would ameliorate this activity.

1.3.7.8.4 Inhibition of Drug Transporters and Metabolising Enzymes

Inhibition of transporters or metabolising enzymes is associated with direct interference with one of the steps in the catalytic cycle of the proteins.

1.3.7.8.4.1 Competitive Inhibition

Xenobiotics acting as competitive inhibitors bind at the same site on the transporter or enzyme as the drug substrate, preventing the interaction of the latter until the inhibitor dissociates [107; 108]. The binding affinity of the substrate for the transporter or enzyme is thus lower in the presence of the inhibitor [107; 108]. Since the inhibitor and substrate compete for the same site, raising the substrate concentration leads to displacement of the inhibitor from the binding site and the inhibitory effects may thus be overcome [107; 108].

1.3.7.8.4.2 Non-competitive Inhibition of Drug Transporters and Metabolising Enzymes

Non-competitive inhibition of transporters and enzymes is brought about by the binding of a xenobiotic to a site on the protein which is different to the one at which the substrate binds [107; 108]. This either inactivates the active site through conformational changes of the protein or it may prevent activation steps in the enzyme or transporter cycle, such as haeme or ATP binding respectively [107; 108]. Therefore, even if the drug substrate binds at the active site, transport or metabolism thereof is hindered.

1.3.7.8.4.3 Mechanism-based Inhibition of Drug Metabolising Enzymes

The mechanism-based inhibition of enzymes involves the inactivation of the protein via the formation of metabolites of the substrate which bind tightly and irreversibly to the enzyme [109]. For CYPs specifically, this may occur due to covalent bonding and thus chemical modification of the enzyme itself or the coenzyme, haeme [109]. The salient features of this type of inhibition are that (i) it is irreversible, thus it cannot be overcome even by increasing the concentration of the substrate and (ii) the metabolism of any concomitantly administered substrates would also be reduced and not only that of the metabolite responsible for the reaction.

1.3.7.8.5 Activation of Drug Transporters and Metabolising Enzymes

It has been shown that xenobiotics may enhance the activity of transporters and enzymes even after acute exposure to cells or microsomes [110–113]. This alludes to a mechanism independent of increased protein expression via induced transcription and translation. Analogous to non-competitive inhibition, perhaps binding of xenobiotics to allosteric sites may activate the transporter or enzyme via direct conformational changes or indirectly by facilitating activation steps such as binding of ATP or coenzymes, such as haeme.

1.3.7.9 Preclinical Evidence for ARV-ATM PK Interactions

A relatively recent, comprehensive review of the literature has revealed that there is preclinical evidence that many herbal medicines, commonly used all over the world, have the potential to be involved in PK interactions with ARVs, due to the ability of these to interfere with the activity of transporters and enzymes responsible for the translocation and metabolism of PIs and NNRTIs [102]. In the context of this thesis, the role of ATMs in this regard is most relevant and therefore, studies involving these [114–120] have been highlighted in Table 1.7. African potato and the nine Tanzanian medicinal plants were the most thoroughly investigated and were found to have significant effects on the functional activity of several CYPs as well as P-gp and CYP3A4-associated PXR.

The combination of CYP and P-gp inhibition and CYP3A4-associated PXR and P-gp induction, shown by African potato raises the possibility of bidirectional interactions with drugs which are substrates of each of these proteins. In such instances, the net effect may be substrate-dependent and probably difficult to predict *in vitro*. Experiments using an ARV as a specific substrate were undertaken to assess the ability of African potato and SF to alter the

apparent permeability coefficient (P_{app}) ratio (apical-basolateral: basolateral-apical) of the NNRTI, nevirapine, in Caco-2 cells [121]. The fact that nevirapine alone has a ratio less than 1 [121] indicated that it may be subject to efflux by transporters. In comparison, verapamil (100 µM), a known potent inhibitor of P-gp increased this ratio to 2.44 [121], thus implying that intestinal absorption of nevirapine is modulated by P-gp-mediated efflux. This is however contradictory to other reports which indicate that nevirapine is not a substrate of P-gp [122; 123]. Nevertheless, Brown *et al.* (2008) [121] found that an aqueous decoction (40 mg/ml) of African potato caused a P_{app} ratio of nevirapine even higher than that of verapamil, implying that it exhibits greater P-gp inhibitory effects than the latter [121]. A similarly prepared decoction (20 mg/ml) of SF and one of its components, L-canavanine (1.22 mg/ml), also revealed a P-gp inhibitory effect, albeit considerably weaker than the positive control (verapamil) [121].

An *in vivo* study was conducted using male Sprague Dawley rats to determine the effects of short- (12 mg/kg single dose) and long-term (12 mg/kg once a day for five days) administration of aqueous decoctions of SF on the bioavailability of nevirapine (6 mg/kg) [124]. Although no significant differences were found in the oral bioavailability of nevirapine before and after short-term administration of SF, a 50% decrease in bioavailability parameters, AUC and C_{max} after 5 day treatment was evident [124]. Furthermore, the mechanism which underlies this reduction in bioavailability was found to most likely be due to increased CYP3A4 metabolism of the NNRTI, since a 2-3 fold increase in the expression of CYP3A4 mRNA was observed in both the small intestine and liver of the animals [124]. It may be concluded that the increased *in vitro* absorption of nevirapine observed in the study by Brown *et al.* (2008) [121] after exposure to SF was of no consequence in this *in vivo* animal model. As stated in section 1.3.7.4, if this ARV-herb interaction were to occur in humans too, subtherapeutic levels of nevirapine may be achieved which could possibly lead to drug resistance and treatment failure.

Table 1.7: Laboratory studies showing the effect of ATMs on CYP, P-gp and PXR activity

Herbal medicinal plants of African origin	Extract/constituent tested	Effect on CYP enzymes	Effect on P-gp	Effect on PXR	References
Madagascan medicinal plant: *Catharanthus roseus*	Two triterpenes and three alkaloids present in the plant	Inhibitors of CYP2D6: ajmalicine (IC_{50} of 0.0023µM), vindolene (IC_{50} of 15.9µM), serpentine (IC_{50} of 3.51µM); inhibitors of CYP3A4: vindolene (IC_{50} of 20.1µM), serpentine (IC_{50} of 48.1µM) in human liver microsomes, *in vitro*	Not determined	Not determined	[114]
Southern African medicinal plant: *Hypoxis hemerocallidea*	Aqueous and methanolic plant extracts (25-100mg/ml); formulation extracts (10mg/ml), hypoxoside (3.6mg/ml), rooperol (50µg/ml) and stigmasterol (2.5mg/ml) present in the plant	Inhibitors (>50%) of CYP3A4 (extracts, rooperol, stimasterol), CYP3A5 (extracts, rooperol) and CYP2C19 (extracts) in CYP-transfected microsomes, *in vitro*	Inhibitors of P-gp ATPase (<50%), in P-gp-transfected membranes, *in vitro* (extracts) Inducers of P-gp (<50%), in Caco-2 cells, *in vitro* (extracts, rooperol, stigmasterol	Inducers of PXR activity, ($p<0.05$) using a a CYP3A4 luciferase reporter gene construct in HepG2 cells, *in vitro*	[116; 117]
Southern African medicinal plant: *Sutherlandia frutescens*	Aqueous and methanolic plant extracts (100mg/ml)	Inhibitors of CYP3A4 (>50%), in CYP3A4-transfected microsomes, *in vitro*	Inhibitors of P-gp (<50%), in P-gp-transfected membranes	Inducers of PXR activity ($p<0.01$), using a CYP3A4 luciferase reporter gene construct in HepG2 cells, *in vitro*	[116]
Southern African medicinal plant: *Hypoxis obtusa*	Aqueous and methanolic plant extracts (1 mg/ml for assay of CYP activity and 250 µg/ml for assay of P-gp activity)	Inhibitors of CYP1A2 (80−95%), CYP2C9 (60−80%), CYP2C19 (methanolic 85%), CYP2D6 (60–95%), CYP3A4 (85–95%) in CYP-transfected microosomes Inducer of CYP2C19 activity (aqueous 15%) in CYP-transfected microsomes	No effect on P-gp activity in Caco-2 cells *in vitro*	Not determined	[125]
Southern African medicinal plant: *Dicoma anomala*	Aqueous and methanolic plant extracts (1 mg/ml for assay of CYP activity and 250 µg/ml for assay of P-gp activity)	Inhibitors of CYP1A2 (20−30%), CYP2C9 (10−40%), CYP2C19 (5−40%), CYP2D6 (2–35%), CYP3A4 (30–70%) in CYP-transfected microsomes	No effect on P-gp activity in Caco-2 cells *in vitro*	Not determined	[125]
Southern African medicinal plant: *Moringa oleifera*	Aqueous and methanolic leaf extracts (0.01−10 mg/ml)	Inhibitors of CYP3A4 (IC_{50} of 0.5 and 2.5 µg/ml for methanolic and aqueous extracts respectively)	Not determined	Not determined	[120]

Table 1.7: (continued)

West African medicinal plants: *(i)Aframomum cuspidatum, (ii)Aframomum meligueta, (iii)Harrisonia abyssinica, (iv)Jutropha curcas, (v)Lipia multiflora,(vi)Persia americana, (vii)Phyllanthus amarus, (viii)Piper guineense (ix)Lonchocarpus sericeus, (x) Oxytenanthera abyssinica, (xi)Talinum triangulare*	Aqueous (100mg/ml) and methanolic (10mg/ml) plant extracts	Inhibitors (>50%) of CYP3A4, CYP3A5 (i,ii,iii,v,vii,viii, ix only) and CYP3A7, in CYP-transfected microsomes, *in vitro*	Not determined	Not determined	[118]
Tanzanian medicinal plants: *(i)Cyphostemma hildebrandtii, (ii) Acacia nilotica, (iii)Acacia robusta ssp. Usambarensis, (iv)Agauria salicifolia, (v)Elaeodendron buchananii (vi)Sclerocarya birrea* Sond, (vii) *Sterculia africana, (viii) Turraea holstii, (ix) Annickia kummeriae*	Methanolic plant extracts re-dissolved in DMSO	Inhibitors (IC_{50} <10µg/ml) CYP2C9 (i-v only), CYP2C19 (i,ii,iii only), CYP2D6 (i only), CYP3A4 (i-v only) in CYP-transfected microsomes, *in vitro*	Inhibitor of P-gp (ii, ixonly) in Caco-2 cells, *in vitro*	Inducers of PXR (EC_{50} <50µg/ml) (i,iv,v,vi,vii,viii only), using a CYP3A4 luciferase reporter gene construct in HepG2 cells, *in vitro*	[115; 119]

1.3.7.10 Clinical Evidence for ARV-herb PK Interactions

As with any potential PK interaction between allopathic drugs, extrapolation of preclinical data showing the potential for PK interactions between ARVs and herbal medicines to the clinical setting is speculative. Such data should rather serve only to signal the need to pursue further investigations by means of clinical studies. *In vitro* results may not necessarily correlate to clinical data because the PK parameters of absorption and elimination which influence bioavailability are complicated by additional factors not simulated in the *in vitro* systems. For example, the distribution of the interfering drug to the PK organs may be such that the resulting concentration at these sites is insufficient to achieve the predicted effect on transporters and enzymes [126]. This may depend on the dosage form, dosage, dosing regimen, route of administration and duration of treatment of the herbal product [102]. Moreover, effects observed in *in vivo* animal models may be due to species-specific substrate selectivity as well as expression and activity of transporters and enzymes in the PK organs and may therefore not be applicable to humans [127; 128]. Patient-related factors, such as gender, age, drug compliance, disease factors, concomitant medications and genetic polymorphisms of CYPs and transporters may also play a role [102].

Over the past decade, several ARV-herb drug interaction clinical studies have been conducted [129-138]. These have been summarised in Table 1.8 with respect to the ARV, the herbal product, the study design and the effects on the ARV AUC and C_{max} (if available). There are several observations from the tabulated information which should be underscored. It is remarkable that all but one study involved the use of a PI, most commonly indinavir. In fact, three independent studies were conducted with indinavir and milk thistle [132; 134; 136]. Only one of the studies involved an ATM as the herbal product [129]. There also appeared to be different approaches to the study design and in most, tests of significance were used to conclude whether there were differences in AUC and C_{max} of the ARV in the absence and presence of the herbal product; on this basis, only three of the studies [131; 135; 137] exhibited statistically significant differences in one or both of these systemic exposures. In these studies, St John's wort [137] and vitamin C [131] altered AUC and C_{max} of indinavir whilst garlic [135] modified AUC and C_{max} of saquinavir.

Table 1.8: Clinical studies of ARV-herb PK interactions

ARV drug	Herbal product	Study design	Effect on AUC and C_{max}	Reference
Indinavir (steady state)	St John's wort (Hypericum Buyer's Club)	Two-treatment, two-phase (I ARV alone, II ARV+ herb) crossover, one-sequence 8 healthy subjects (6 male)	Phase I to Phase II AUC_{0-8} decrease of 57% (p = 0.0008) C_{max} decrease of 28%	[137]
Indinavir (steady state)	Milk thistle (Thisilyn)	Two-treatment, three-phase (I ARV alone, II ARV + herb, III ARV alone) crossover, one-sequence 10 healthy subjects (6 male)	Phase I to Phase II AUC_{0-8} decrease of 9% (NS) C_{max} decrease 9% (NS) Phase I to Phase III AUC_{0-8} decrease of 22% C_{max} decrease of 24%	[136]
Saquinavir (steady state)	Garlic caplets (Galipure)	Two-treatment, three-phase (I ARV alone, II (ARV + herb, III ARV alone) crossover, one-sequence 10 healthy subjects (4 male)	Phase I to Phase II AUC_{0-8} decrease of 51% (p = 0.007) C_{max} decrease of 54% (p = 0.006) Phase I to Phase III AUC_{0-8} decrease of 35% C_{max} decrease of 39%	[135]
Indinavir (steady state)	Milk thistle (General Nutrition Center)	Two-treatment, two-phase (I ARV alone, II ARV+ herb) crossover, one-sequence 10 healthy subjects (7 male)	Phase I to Phase II AUC_{0-8} decrease of 6% (NS) C_{max} decrease of 11% (NS)	[134]
Ritonavir (single dose)	Garlic (Natural Source)	Randomised, two-treatment (I ARV alone, II ARV + herb) crossover, two-phase, two-sequence 10 healthy subjects (5/group) (5 male)	Treatment I to Treatment II $AUC_{0-\infty}$ decrease of 17% (NS) C_{max} decrease of 1% (NS)	[133]
Indinavir (single dose)	Goldenseal (Nature's Way)	Two-treatment, two-phase (I ARV alone, II ARV+ herb) crossover, one-sequence 10 healthy subjects (6 male)	Phase I to Phase II C_{max} increase of 3% (NS)	[130]
Indinavir (steady state)	Milk Thistle (Kare and Hope)	Randomised, two-treatment (I Control, II Active), three-phase, one-sequence. Crossover for Active group only 16 healthy males (8/group)	Control to Active Phase II AUC_{0-8} increase of 14% (NS) C_{max} increase of 9% (NS) Control to Active for Phase III AUC_{0-8} increase of 25% C_{max} increase of 7% (NS)	[132]
Indinavir (steady state)	Vitamin C	Two-treatment, two-phase (I ARV alone, II ARV+ herb) crossover, single-sequence 7 healthy subjects (6 male)	Phase I to Phase II $AUC_{0-\infty}$ decrease of 14% (p=0.04) C_{max} decrease of 20% (p=0.05)	[131]
Efavirenz (single dose)	Aqueous decoction (15mg/kg/day hypoxoside) of African potato	Two-treatment, two-phase (I ARV alone, II ARV+ herb) crossover, single-sequence 10 healthy males	Phase I to Phase II AUC_{0-48} GM ratio CI [89.04-118.80] (NS) C_{max} GM ratio CI [78.81-120.14] (NS)	[129]
Atazanavir, ritonavir, saquinavir (steady state)	*Uncaria tomentosa* (cat's claw) preparation	Case study (1 female HIV-positive patient)	C_{min} of each PI was 1.5-, 3- and more than 10-fold higher than the recommended target C_{min} for atazanavir, ritonavir and saquinavir respectively. These values normalised after withdrawal of cat's claw	[138]

Several studies of indinavir and milk thistle were conducted possibly in an attempt to determine whether the use of different study designs influences the outcome of the results. Two studies used a crossover design [134; 136], where two different treatments (ARV alone and ARV + herb) in two or three separate phases were administered to each subject. The studies both followed a one-sequence design, therefore each treatment was administered to all subjects in two phases. In the study by Piscetelli *et al.* (2002) [136], which had three phases, the third was ARV alone again after a washout period, to ascertain whether the systemic exposures of the ARV had returned to baseline and thus whether period effects may have played a role if significant changes were observed. In the third study [132], subjects were randomised to a "control" and an "active" group both of whom participated in three phases. The "control" group received ARV alone in all three phases, whilst the "active" group was subject to two crossovers. These subjects received ARV alone in Phase I, ARV + herb in Phase II and ARV alone again in Phase III. The third phase of the "active" group and all three phases of the "control" group once again provided a means to account for period effects whilst a comparison between the groups may also have considered bias due to individual effects. Interestingly, the results of all three studies showed no significant changes in AUC or C_{max} of indinavir.

According to the Guidance for Industry, "*In vivo* drug metabolism/drug interaction studies" compiled by the Food and Drug Administration (FDA) [139], it is more appropriate to use confidence intervals (CIs) of each systemic exposure which "convey a probability of the magnitude of the interaction", rather than tests of significance to conclude whether there is a significant interaction or not. Statistically significant differences derived from tests of significance may not necessarily be clinically relevant [139]. The use of CIs requires that "no effect" boundaries be defined and set [139]. No significant interaction would be concluded only if the CI falls entirely within the limits set. Even though all the studies in Table 1.8 did calculate CIs for % change in AUC and C_{max}, these were not tabulated, except for one study, since the authors of the other studies did not use the CIs as the criteria to reject "no interaction". Mogatle *et al.* (2008) [129] determined the CIs of the geometric mean ratios of AUC and C_{max}, setting the bioequivalence limits of 0.8–1.25 as "no effect" boundaries and since the CIs fell within these limits, the authors concluded that an aqueous decoction of African potato did not alter the single dose systemic exposure of efavirenz [129]. Application of this type of statistical analysis to the other studies may have allowed for more conclusive results with some insight into the clinical relevance thereof.

The last entry in Table 1.8 described a case study of one female HIV-positive patient with cirrhosis associated with hepatitis C infection [138]. The patient was on an ARV regimen of abacavir, lamivudine, ATV, ritonavir and saquinavir all administered once daily [138]. The C_{min} values of the PIs were found to be 1.22, 6.13 and 3.4 µg/ml for ATV, ritonavir and saquinavir, which were all above the recommended C_{min} levels of 0.8, 0.25 and 2.1 µg/ml respectively [138]. A patient history revealed that the patient was taking a herbal immune-boosting remedy commonly known as cat's claw [138]. The patient was advised to stop self-medication with this herbal medicine and fifteen days later, the C_{min} levels had dropped to 0.3, 0.92 and 0.64 µg/ml for ATV, ritonavir and saquinavir respectively, suggesting that the systemic exposure of the PIs was raised when cat's claw, a CYP3A4 inhibitor [140], was co-administered [138]. Clinical studies are required to delineate these results further in a cohort of patients or in healthy volunteers.

1.4 Summary

Despite an increase in access to ARVs in SA since the public sector rollout in 2004, the safe and effective use of these drugs remains a great challenge. The morphology, life cycle and immunopathology of HIV infection are important determinants of the molecular basis of the disease, which directs the pharmacological action required of drugs, like ARVs for successful management. Moreover, the immunologic and clinical states which arise therefrom guide the clinical use of ARVs in the form of ART.

Due to firm cultural or traditional beliefs or the perceived notion that TCAMs may provide safe adjunctive therapy for HIV-associated illnesses, the concomitant use of herbal medicines with ART by HIV/AIDs patients all over the world appears to be a common phenomenon. Even though preclinical evidence suggests that some southern African medicinal plants do exhibit anti-HIV properties, for the most part, suitably designed clinical trials have not been conducted to delineate the safety and efficacy profiles of these traditionally prepared or commercially manufactured medicines in the clinical setting. Current legislation in SA does not obligate providers of these medicines to furnish the regulatory authority with such information nor evidence on the impact which these medicines may have on the safety and efficacy of the co-administered ARVs. Such interactions may often go undetected because health care providers who prescribe or dispense the ARVs may not be aware that the patient is using an ATM. Alternatively or additionally, there may not be enough preclinical and

clinical data on the concomitant use of specific combinations of ARVs and ATMs to advise patients accordingly.

Interactions between xenobiotics, such as allopathic drugs and herbal medicines may be classified as either PD or PK, both of which ultimately impact on the physiological effect and therefore the safety and/or efficacy of one of the drugs. PK interactions appear to be prominent with the use of herbal medicines since these have a propensity to, through direct or indirect mechanisms, alter the activity of transporters and metabolising enzymes in PK organs which direct the absorption and elimination of drugs. Several medicinal plants indigenous to Africa have shown such effects in *in vitro* experiments. The bioavailability of drugs that are substrates of the affected transporters and/or enzymes may therefore be modified. If it increases above the TR, the patient may be more susceptible to adverse or toxic reactions, whilst a reduction to subtherapeutic levels may manifest as drug resistance and treatment failure. NNRTIs and PIs are particularly predisposed to such interactions with herbal medicines because these classes of ARVs are substrates of CYP3A4 and/or P-gp, which are the most abundantly expressed CYP enzyme and transporter respectively, and thus alterations in bioavailability are expected to be more significant than with others. A further step in this regard has been taken with *in vitro* experiments involving the southern African plants, African potato and SF. The impact of extracts of these plants on the *in vitro* absorption of the NNRTI, nevirapine, was undertaken and was found to increase the P_{app} ratio probably through P-gp inhibition. This effect of SF was however found to be negligible in an *in vivo* animal model where chronic exposure (5 days) to an aqueous decoction of the plant reduced the oral bioavailability through increased metabolism brought about by greater expression of CYP3A4 in the liver.

Since extrapolation of preclinical data to the clinical setting is highly speculative, clinical studies should be undertaken to confirm potential ARV-herb interactions. Several such studies have already been conducted with many of the older PIs, such as indinavir and saquinavir. The herbs used were mainly the western herbal CAMs, milk thistle, garlic and St John's Wort. The latter two altered oral bioavailability as observed by statistically significant, but not necessarily clinically relevant changes in AUC and C_{max}. Only one such study has been conducted on the effects of an ATM on an ARV. A two-week dosing regimen of an aqueous decoction of African potato did not change the AUC and C_{max} of the NNRTI,

efavirenz, in a clinically significant manner. This result indicates that predictions of significant ARV-herb interactions cannot be made on the premise of preclinical data.

1.5 Research Aims

This comprehensive review of the literature has revealed that the potential for a southern African ATM to compromise the safety and/or efficacy of a PI through PK effects is not known and thus a gap in the knowledge of ATM-ARV interactions exists. The primary aim of this work was thus to establish whether concurrent administration of SF changes the PK profile of ATV. ATV is particularly relevant as it is one of the newer PIs which have been recommended for use in the public sector of SA in HIV/AIDs patients on second-line therapy, for whom lopinavir is contra-indicated. A systematic approach was used by carrying out suitable *in silico*, *in vitro* and clinical investigations. A schematic diagram of this approach and how it is usually applied in drug interaction screening during the drug development process is shown in Figure 1.5. To minimise the complications associated with the use of a natural product due to the presence of so many phytochemical constituents, the procedure was modified for use in the context of this thesis. In particular, *in silico-in vitro* and *in vitro-in vivo* correlations were not attempted since only one or two concentrations of the SF phytochemical constituents could be tested *in vitro*, excluding the possibility of calculating the necessary K_i, IC_{50} and/or EC_{50} values required for such correlations to be drawn. Since both ATV and SF are known to be safe in humans, the pre-clinical *in vivo* studies were omitted. The rationale for this approach in this thesis is to start with an *in silico* method which is comparatively lower in cost despite the lack of clinical relevance in order to ascertain whether it is worth pursuing more expensive *in vitro* and *in vivo* experiments.

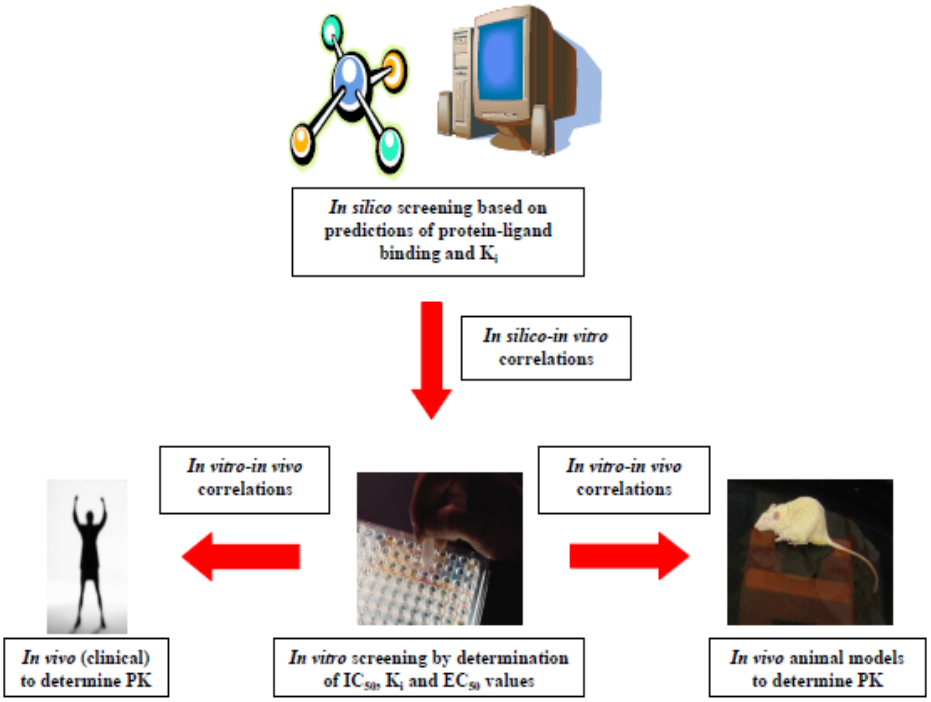


Figure 1.5: Drug interaction screening during the drug development process. Adapted from Rodriguez and Lin, 2001 [141]

Several specific aims were set and these are outlined below.

1. To develop and validate methods for the bioanalysis of ATV in the presence of SF.
2. To determine the potential for competition between some constituents of SF and ATV at the active site of CYP3A4 and P-gp, using *in silico* molecular docking.
3. To assess the potential effect of extracts and constituents of SF on human intestinal absorption of ATV, using an *in vitro* model.
4. To determine if any effect of SF on human intestinal absorption of ATV is P-gp-mediated.
5. To evaluate the potential effect of extracts and constituents of SF on human liver metabolism of ATV, using an *in vitro* model.
6. To establish whether the potential mechanism through which any effect of SF on human liver metabolism of ATV occurs via modulation of CYP3A4 activity.
7. To investigate whether SF changes the bioavailability of ATV, clinically, following administration of these medicines to human subjects.

It is intended that this study will provide more information on the potential for a PK interaction between SF and ATV, which may have important implications for the safety and efficacy of the ARV. The creation of a platform to encourage further work in this largely unexplored specific area of research into ARV-ATM drug interactions is also anticipated.

CHAPTER 2
ATAZANAVIR

2.1 Introduction

The search for potent PIs with a reasonable bioavailability following oral administration and which have activity against mutant strains of HIV prompted the design of a series of azadipeptide analogues including GCP-73547, in 1998 [142]. Novartis initiated preclinical and early clinical studies on this new chemical entity up until November 1999, when, under licence to Bristol Myers Squibb, it was relinquished and became known as BMS-232632 [143]. In 2003, BMS-232632 became the seventh PI to be approved in the USA under the name ATV [144].

2.2 Synthesis

Several publications describe different approaches to the synthesis of ATV [142; 145-147], for which low aqueous solubility (<1 µg/ml at 24 °C) and poor bioavailability in animals remained a challenge [147]. The synthetic procedure which involves the formation of an acid salt from the free base, ATV, sparks the greatest interest in the context of this research, in which changes in the degree of bioavailability of ATV is paramount. A sulphate salt was selected since it exhibited the highest solubility of 4-5 mg/ml and was obtained by stirring ATV with concentrated sulphuric acid in ethanol at room temperature [147]; *n*-heptane was used as a recrystallisation solvent to produce an easily filterable solid, ATV sulphate, with a yield of 85% [147].

2.3 Description

The chemical name for ATV sulphate is (3*S*,8*S*,9*S*,12*S*)-3,12-Bis(1,1-dimethylethyl)-8 hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)phenyl] methyl]-2,5,6,10,13 penta-azatetradecanedioic acid dimethyl ester, sulphate (1:1) [144]. The molecular formula is $C_{38}H_{52}N_6O_7 \bullet H_2SO_4$, which corresponds to a molecular weight of 802.9 (sulphuric acid salt). The free base, ATV, has a molecular weight of 704.9 [144]. To the naked eye, ATV sulphate appears as a white to pale yellow crystalline powder. The structure of ATV sulphate is shown in Figure 2.1 [144].

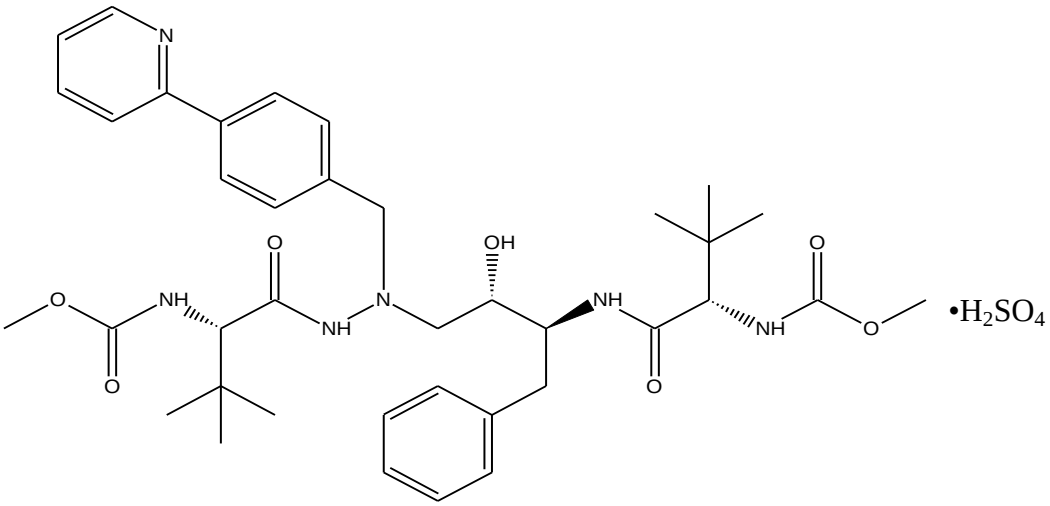


Figure 2.1: Structure of ATV sulphate

2.4 Structure-activity Relationships

HIV-1 protease is known to be a 22 kDa homodimer composed of two identical 99-amino acid subunits [148]. As shown in Figure 2.2, these monomers of HIV-1 protease dimerise such that C2 symmetry is achieved and the active site as well as the primary substrate binding pockets, S_1 and S_1', are created [148]. Each monomer contributes one aspartate residue to the active site [148]. Substrate recognition and binding is dependent on hydrogen bonding and van der Waal's forces between at least six hydrophobic subsites (S_3, S_2, S_1, S_1', S_2', and S_3') lining the walls of the binding cleft and the backbone of the substrate as well as corresponding side chains of the substrate which were analogously termed, P_3, P_2, P_1, P_1', P_2', and P_3' [148].

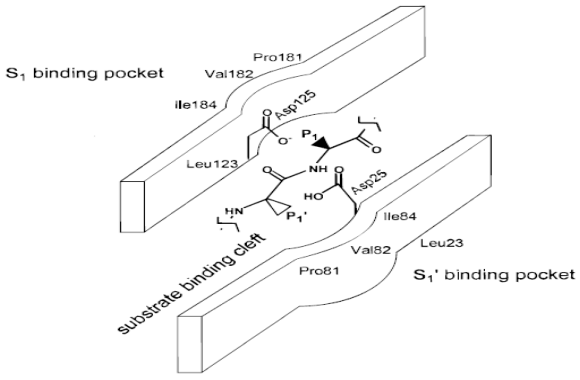


Figure 2.2: Schematic representation of the active site of HIV-1 protease, composed of the substrate binding cleft and S_1 and S_1' binding pockets. By convention, amino acid residues of one monomer are numbered 1–99, while the residues from the other monomer are numbered 101–199.Two amino acids of the substrate flanking either side of the scissile bond are shown in the centre. Reprinted with permission from [148]. Copyright 2000 American Chemical Society.

The first step in the design of an inhibitor of HIV-1 protease is the formation of a non-scissile dipeptide isostere [149]. A hydroxyethylene core [149], shown in Figure 2.3, is present for this purpose in all peptidic PIs.

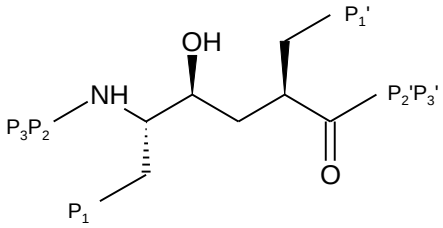


Figure 2.3: Hydroxyethylene dipeptide isostere

The C2-symmetry of HIV-1 protease also prompted the design of dihydroxyethyl amine dipeptide isostere-based experimental inhibitors [149] (see Figure 2.4).

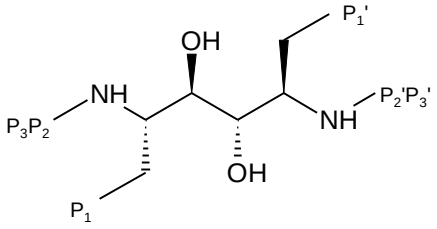


Figure 2.4: Dihydroxyethyl amine dipeptide isostere

Fässler *et al.* (1996) [149] further investigated a pseudosymmetric dipeptide isostere, shown in Figure 2.5, which is analogous to an azadipeptide. The carbon atom bearing the P_1' substituent of the dihydroxyethyl amine isostere was replaced by a nitrogen atom of a hydrazine group, which has high reactivity, thus a wider variety of substituents at the P_1' and P_2' position may be formed [149]. This core is termed a hydroxyethyl hydrazine azapeptide isostere [149].

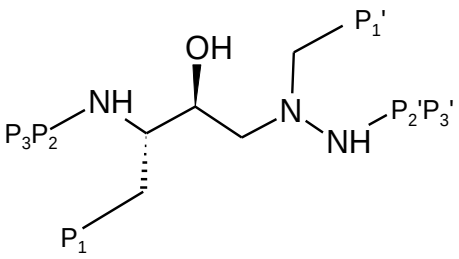


Figure 2.5: Hydroxyethyl hydrazine azapeptide isostere

GCP53820 (Figure 2.6) is an example of a pseudosymmetric azapeptide analogue, which was cocrystallised with the HIV-1 protease enzyme [150]. Results of computational modelling showed that the hydroxyl group of GCP53820 which has an *R* configuration was symmetrically arranged between the two active site aspartate residues and the phenyl (P_1) and cyclohexyl (P_1') substituents were accommodated in the S_1 and S_1' pockets of the enzyme [150]. The central dipeptide isostere was symmetrically acylated with valine substituents (boxed with a red outline in Figure 2.6), such that P_3P_2 and P_2'P_3' were MeOC-Val and Val-COMe respectively [150]. All amine and carboxyl functional groups of the backbone of GCP53820 were arranged within hydrogen bonding distances of H-bond donors and acceptors of the enzyme, affording high affinity [150]. However, Fassler *et al.* (1996) [149] found that in a cellular assay, the antiviral activity was only moderate as evidenced by an ED_{90} of 1 µM. In a preclinical PK study, GCP53820 levels in the plasma of mice were below the level limit of detection two hours after oral administration [149].

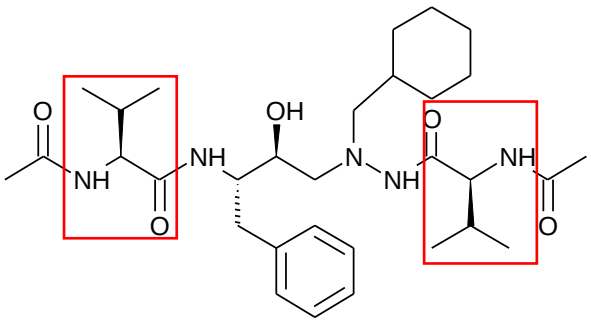


Figure 2.6: Structure of GCP53820 with valine substituents boxed with a red outline

Optimisation of both bioavailability and antiviral activity of this lead compound was undertaken [149]. *R* configuration of the transition state was maintained for further studies since the *S* epimer generated a ten-fold higher IC_{50} [149], implying lower potency. High bioavailability with moderate antiviral activity was obtained for compounds in which the valine substituents in P_3P_2 and P_2'P_3' were esterified to form carbamates, with a greater effect demonstrated for ethyl carbamates than methyl carbamates [149]. Conversely, compounds with an alkyl group rather than a cyclohexyl as a P_1' substituent exhibited good antiviral activity but poor bioavailability [149].

Computational data generated by Bold *et al.* (1998) [142] suggested that there should be available space in the substrate binding site S_1' for larger P_1' substituents to improve physicochemical properties required for enhanced bioavailability, while maintaining

appropriate affinity for the enzyme to ensure adequate antiviral activity. Enzyme activity and cellular antiviral assays showed that biphenyl substituents at P_1' did indeed improve protease inhibitory and antiviral activity, but not bioavailability [142]. Replacement of the valine substituents at P_3P_2 and $P_2'P_3'$ by *tert*-leucine increased the cellular antiviral activity even further with higher plasma levels after oral administration in mice [142]. Substitutions of the 4-biphenyl for 4-heterocyclyl-phenyl substituents, such as 4-pyridinyl-phenyl at position P_1' which imparted some hydrophilicity to the compound further improved the PK profile to form the optimal chemical entity now known as ATV [142].

2.5 Dosage Forms

According to the initial scientific discussion for the approval of Reyataz® by the European Medicines Agency (EMEA) [151], Reyataz® is available in two pharmaceutical forms, namely a hard capsule and a powder for reconstitution, both for oral use. Further discussion will only include the capsules which were used in this research. The capsules contain ATV sulphate equivalent to ATV dose strengths of 100 mg, 150 mg and 200 mg [151]. Hard gelatine capsule shells are used, and excipients incorporated with ATV sulphate in the capsules include lactose monohydrate as a diluent, crospovidone as a disintegrant and magnesium stearate as a lubricant [151].

2.6 Physicochemical Properties

2.6.1 pKa

As previously mentioned, ATV is a weak base and it therefore has the ability to ionise in aqueous-based media. The pKa values of ATV give an indication of the extent to which such ionisation may occur depending on the pH of the media. Two pKa values of 4.65 ± 0.25 and 11.11 ± 0.46 have been calculated for ATV [152] and these, particularly the first, have important implications with respect to the solubility of ATV sulphate or ATV, which have been outlined in more detail in section 2.6.2 below.

2.6.2 Solubility

Information relating to the solubility of ATV sulphate and ATV in various solvents has a number of important applications in the context of this research. Such data may direct the choice of solvents used: (i) to dissolve ATV sulphate for *in vitro* experiments and for the spiking of samples in bioanalytical method development and validation (ii) in the extraction

procedure of ATV from biological samples and (iii) as sample solvent and mobile phase in any chromatographic-based bioanalytical method which may be utilised.

As previously mentioned, the solubility of ATV sulphate in distilled water is 4-5 mg/ml resulting in a solution with pH of 1.9 at 24 ± 3 ºC. This suggests that this is the pH at which maximum ionisation and thus solubility of the free base ATV may occur. According to the Henderson–Hasselbalch equation, the basic pKa of 4.65 ± 0.25 indicates that the degree of ionisation and solubility of ATV as a free base will decrease by ~50% in a solution with a pH in the range 4.4–4.9 and further by ~99% in a solution with a pH in the range 6.4–6.9. In the interests of conserving the amount of ATV sulphate available, instead of determining the solubility of ATV sulphate in various solvents, concentrations of ATV sulphate required to prepare the stock solutions (maximum concentrations) for the experiments as described in (i) to (iii) above were first determined. Further investigations were then conducted to ascertain whether ATV sulphate was soluble in relevant solvents at these concentrations by observation with the naked eye. Only solvents and pHs suitable for use in reversed phase high performance liquid chromatography (HPLC) were tested. Table 2.1 shows that distilled water, buffers in a pH range 2–4 and combinations of all buffers (pH 2–8) with methanol or acetonitrile in a ratio of 50:50 had the ability to dissolve adequate amounts of ATV sulphate to achieve the concentration of the stock solution (5 mg/ml) required in conducting the experiments in this research project.

2.6.3 LogP

Since the partition co-efficient (P) of a drug is a good predictor of its ability to pass through biological membranes, it may, together with solubility and dissolution rate, provide clues as to what type of absorption, distribution and elimination profiles of the drug are likely to be prevalent [153]. The LogP value for ATV has been calculated as 5.198 ± 0.744 [152], which indicates that ATV partitions in favour of n-octanol by five orders of magnitude greater than it does in favour of water. ATV clearly exhibits high permeability, therefore absorption of this drug should be largely dependent on its *in vivo* dissolution rate.

Table 2.1: Solubility of ATV sulphate (5 mg/ml) in various solvents

Solvent	Soluble (yes/no)
Water	Yes
Methanol	No
Acetonitrile	No
Acetontrile: Water (50:50)	Yes
Methanol: Water (50:50)	Yes
Phosphate buffer, pH 2	Yes
Formate buffer, pH 3	Yes
Acetate buffer, pH 4	Yes
Acetate buffer, pH 5	No
Phosphate buffer, pH 6	No
Phosphate buffer, pH 7	No
Phosphate buffer, pH 8	No
Acetonitrile: Phosphate buffer, pH 2	Yes
Acetonitrile: Formate buffer, pH 3	Yes
Acetonitrile: Acetate buffer, pH 4	Yes
Acetonitrile: Acetate buffer, pH 5	Yes
Acetonitrile: Phosphate buffer, pH 6	Yes
Acetonitrile: Phosphate buffer, pH 7	Yes
Acetonitrile: Phosphate buffer, pH 8	Yes
Methanol: Phosphate buffer, pH 2	Yes
Methanol: Formate buffer, pH 3	Yes
Methanol: Acetate buffer, pH 4	Yes
Methanol: Acetate buffer, pH 5	Yes
Methanol: Phosphate buffer, pH 6	Yes
Methanol: Phosphate buffer, pH 7	Yes
Methanol: Phosphate buffer, pH 8	Yes

2.6.4 Ultra-violet (UV) Spectrum

The UV spectrum of ATV is important in deciding on an appropriate UV detection wavelength to use in the bioanalysis of the drug when HPLC is coupled to a UV detector. A wavelength at which an acceptable absorbance is achieved may assist in optimising the sensitivity of the analytical method.

ATV sulphate was dissolved in 50:50 acetonitrile: water, at a concentration of 10 µg/ml and was injected into an HPLC system which consisted of an Alliance 2695 Separations module, a Phenomenex® C_{18} (150 mm X 4.6 mm I.D) and a 2996 Waters photodiode array detector coupled to Empower data acquisition software (Waters, Milford, MA, USA). The UV spectrum of ATV which was generated is shown in Figure 2.7. ATV exhibited highest absorbance at 200 nm, which decreased drastically to ~225 nm. Two absorbance peaks or local maxima were present at 248.5 and 282.8 nm.

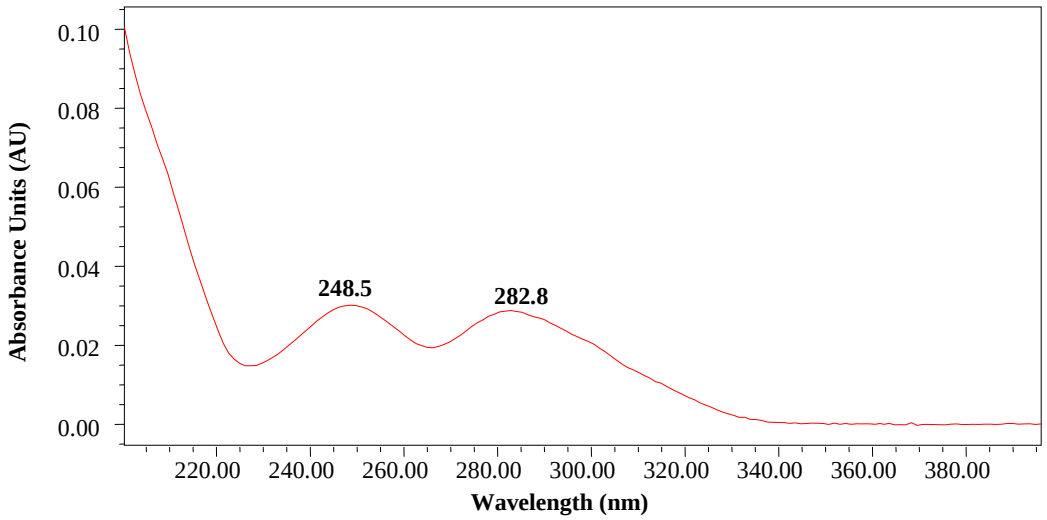


Figure 2.7: UV spectrum of ATV in 50:50 acetonitrile: water

2.6.5 Stability

2.6.5.1 Solid State Stability

A solid state stability study of ATV sulphate showed that after exposure to dry heat at 60 ºC for 60 days and to UV light with an overall illumination of >1.2 million lux hours and an energy of at least 200 Wh/m, no degradation products were observed in the samples which were assessed using a stability-indicating HPLC method [154]. Storage of ATV sulphate reference standard in the original container at room temperature should therefore be adequate to limit degradation of ATV.

2.6.5.2 Solution Stability

Forced degradation studies were carried out on working solutions generated from stock solutions (500 µg/ml) of ATV sulphate in 0.02 M ammonium acetate buffer and acetonitrile in a ratio of 45:55 respectively [154]. Acid hydrolysis in 1 N hydrochloric acid at 80 ºC for 8 hours and 2 hours revealed 86 and 12% degradation respectively [154]. In analogous alkaline conditions using 1 N sodium hydroxide, degradation was lower after the 8 hour exposure (24%) but higher after the 2 hour exposure (16%) compared to acid hydrolysis [154]. No degradation was observed under neutral conditions at 80 ºC for 12 hours [154]. Conditions to predispose stock solutions to oxidation were achieved by treating these samples with 30 and 10% hydrogen peroxide at room temperature for 24 hours [154]. Degradation of <0.5% was evident only on exposure to 30% hydrogen peroxide [154]. The results of these forced degradation studies indicate that ATV sulphate in solution may be most susceptible to degradation under acidic conditions. It further alludes to the need for stability studies to be

conducted on ATV sulphate stock solutions and spiked plasma samples, particularly if the solvents used to prepare stock solutions contain an acidic buffer.

2.6.5.3 Dosage Form Stability

Reyataz® capsules have a shelf-life of two years when stored at or below 25 ºC in the original container which is a high-density polyethylene bottle [144]. These conditions were thus adhered to by the storage of capsules purchased for use in this research project in an air-conditioned laboratory at 22 ± 2 ºC.

2.7 Clinical Pharmacology

2.7.1 Indication and Dosage

REYATAZ® (atazanavir sulphate) is indicated in combination with other ARVs for the treatment of HIV-1 infection [144]. In treatment-naïve patients, ATV 400 mg daily may be administered, whereas in those who have previously received ART, ATV 300 mg boosted with ritonavir 100 mg is indicated. These recommendations are based on results from several clinical studies:

i. Similar efficacy measured by plasma HIV-1 RNA levels as a primary endpoint was evident in ART-naïve patients over a 48-week period between ATV 400 mg daily and efavirenz 600 mg daily where both treatment arms also received a fixed-dose combination of zidovudine/lamivudine (300/150 mg) twice daily [155].

ii. In an analogous study also in ART-naïve patients carried out over a 96-week period, with the same primary endpoint, efficacy between ritonavir-boosted ATV (300/100 mg) daily and ritonavir boosted lopinavir (400/100 mg) twice daily was comparable, where both treatment arms also received a fixed-dose combination of tenofovir/emtricitabine (300/200) once daily [156].

iii. In patients who had experienced virological failure on only one PI-containing regimen, a 48 week study was conducted to compare ATV 400 mg once daily to lopinavir/ritonavir 400/100 mg twice daily, each in combination with two NRTIs [144]. The results showed that efficacy based on HIV-RNA levels was inferior in the former regimen in comparison to the latter one [144].

iv. The efficacy of an ATV-based regimen was comparable to a lopinavir-based regimen in treatment-experienced patients with a previous history of virological failure on ART which included PIs, NRTIs and NNRTIs. This was established in a 48-week study which was conducted as described in (ii) above, except that any NRTI was co-administered with emtricitabine [157]. These findings indicate that in treatment-experienced patients, boosting by ritonavir was required for the effectiveness of ATV.

2.7.2 Mechanism of Action

Like other PIs, ATV selectively inhibits the HIV protease enzyme, which is required for the processing of viral *gag* and *gag-pol* precursor polyproteins. The interruption of this latter step in the HIV life-cycle prevents the efficient processing of viral structural proteins, thus preventing the formation of an infectious and mature viral particle [158].

2.7.2.1.1 *In Vitro* Antiviral Activity

Pre-clinical studies showed that ATV has an IC_{50} of 2–5 nmol/l, an IC_{90} of 9–15 nmol/l and an inhibition rate constant (K_i) of less than 1 nmol/l [158]. The concentration at 50% efficacy (EC_{50}) increased five-fold in the presence of 40% serum due to a reduction in the availability of free ATV as a result of protein binding [158]. Despite this, ATV still exhibited a potency superior to that of older PIs [158].

2.7.2.1.2 Resistance

Genotypic and phenotypic analyses of clinical isolates in patients resistant to ATV have revealed that an isoleucine-to-leucine substitution at amino acid residue 50 (I50L) of the HIV-1 protease was the unique mutation observed in patients when treated with this PI without ritonavir boosting [159]. Notwithstanding, less than 2% of patients developed these ATV-resistant mutants over a 62 week period, in three trials with treatment-naïve patients receiving ATV [159]. Furthermore, although this mutation reduced susceptibility to ATV, it enhanced susceptibility of the HIV-1 protease to other PIs [160]. Mutations in HIV-1 protease in resistance isolates from treatment-experienced patients receiving boosted ATV were identified as I84V, L90M, A71V/T, N88S/D, and M46I [144; 161]. Additional data suggest that significant cross-resistance to unboosted ATV occurs in patients with strains of HIV-1 protease resistant to multiple PIs [162].

2.7.3 Biopharmaceutics and Pharmacokinetics

The PK of ATV in humans is influenced by the absence or presence of HIV-infection and by co-administration with ritonavir as a booster or not. This is depicted, for these respective groups, in Table 2.2, by values [144] for AUC, C_{max}, C_{min}, T_{max} and $T_{1/2}$ at steady state.

Table 2.2: PK parameters for ATV in HIV-positive patients and HIV-negative subjects

HIV status and sample number	Once daily dose	AUC ($ng/ml/hr^{-1}$)	C_{max} (ng/ml)	C_{min} (ng/ml)	T_{max} (hr)	$T_{1/2}$ (hr)
HIV-negative (n = 14)	400 mg ATV	29308 ± 8263	5358 ± 1731	218 ± 191	2.5	7.9 ± 2.9
HIV-negative (n = 28)	300 mg ATV/ 100 mg ritonavir	61435 ± 22911	6450 ± 2031	1441 ± 757	2.7	18.1 ± 6.2
HIV-positive (n = 13)	400 mg ATV	22262 ± 20159	3152 ± 2231	273 ± 298	2.0	6.5 ± 2.6
HIV-positive (n = 10)	300 mg ATV/ 100 mg ritonavir	53761 ± 35294	5233 ± 3033	862 ± 838	3.0	8.6 ± 2.3

All values represent mean ± SD, except t_{max} which is indicated by the median.

The underlying cause of lower C_{max}, C_{min}, AUC and $t_{1/2}$ values in HIV-infected patients in comparison to healthy subjects may be due to differences in gastro-intestinal pH. Kelly *et al.* (2010) [163] showed that HIV-infected patients exhibited hypochlorhydria. As described in section 2.6.1, an increased pH decreases the solubility of ATV, which limits absorption across intestinal membranes. Busti *et al.* (2004) [164] suggested that HIV-infection may alter hepatic and intestinal metabolism and/or transport of ATV. However, reduced bioavailability of ATV in HIV-infected patients implies enhanced transport and/or metabolism, yet the findings of Gotzkowsky *et al.* (1999) [165] that CYP3A4 activity is decreased in these patients contradict this. The reason for the discrepancy thus remains unclear. As expected, boosting by ritonavir which inhibits CYP3A4 and P-gp, increases the bioavailability of ATV irrespective of HIV-status. A C_{min} of 150 ng/ml of ATV has been found to be necessary for successful viral suppression [166], whilst an upper limit of 850 ng/ml has been associated with a risk of increased unconjugated bilirubin and incidences of hyperbilirubinaemia due to uridine diphosphate-glucuronosyltransferase (UGT) 1A1 inhibition [166], which is described as an adverse effect in more detail below, in section 2.7.3.

2.7.3.1 Absorption

ATV has a bioavailability of ~60–68% [167]. When ATV, as a 400mg single dose was administered with a high-fat and a light meal to 32 healthy volunteers, the AUC increased by 70% and 35% respectively. The corresponding co-efficients of variation (CV %) in AUC decreased from 69% in the fasted state to 43% with a high fat meal and 37% with a light meal

[168]. ATV should therefore be administered with a food to enhance bioavailability and minimise the variability associated therewith.

2.7.3.2 Distribution

ATV is 86% protein bound to albumin and α-1 acid glycoprotein. This is a concentration-independent effect which compares favourably to older PIs [169]. This indicates that interactions with highly protein bound drugs are not likely, and plasma concentrations of ATV would probably not be influenced by changes in plasma protein concentrations [164]. The central nervous system (CNS) and testes are potential sanctuary sites for HIV replication, therefore, it is important to determine whether ARVs such as ATV reach these sites at effective concentrations. In a phase II substudy [170], seminal and cerebrospinal fluid ATV concentrations were determined in 10 and 18 patients respectively. Median concentrations at 12 weeks for patients receiving 400 mg ATV daily was 132.3 ng/ml in semen and 5.8 ng/ml in CSF, with a CSF: plasma ratio of 0.006. Penetration to these sanctuary sites was deemed acceptable, since the levels were above the EC_{50} (1 ng/ml) for the wild-type virus isolated in the treatment-naïve patients in the study [170].

2.7.3.3 Metabolism

Five metabolites of ATV (an N-dealkylation product, two metabolites resulting from carbamate hydrolysis, a hydroxylated product and a keto-metabolite) have been detected and identified in the plasma of 12 patients [171]. The latter product was the most abundant in each case [171]. Glucuronidation has been considered a minor biotransformation pathway for ATV [144], although no such metabolites were observed clinically [171]. *In vitro* studies suggest that ATV is metabolised by CYP3A and that none of the metabolites exhibit anti-HIV activity [144].

ATV has been found to competitively inhibit CYP2C8, UGT1A1 and CYP3A, in descending order of rank with respect to the inhibition rate constant (K_i) [144]. The inhibition of CYP3A4 and UGT1A1 appears to be clinically relevant [164] since the K_i values of ~1300 ng/ml and ~700 ng/ml [144], can be achieved with approved dosing of ATV [164]. None of the major ATV metabolites inhibit the CYP enzymes [144]. In a multiple-dose study, ATV did not increase the urinary ratio of endogenous 6β-hydroxycortisol to cortisol, implying that CYP3A enzymes were not induced by ATV [144].

2.7.3.4 Elimination

The estimated clearance for the 400-mg dose is 25.3 L/hour [144]. Biliary elimination of ATV predominates, as 79% of a [^{14}C]-labelled dose was recovered in the faeces, while renal elimination accounted for a total of only 20% of the dose, 7% as unchanged ATV and 13% in the form of ATV metabolites [144].

2.7.4 Adverse Effects

During all clinical trials of ATV [155; 172–175], common adverse effects (≥20% incidence) were: infection (46–60%), nausea (25–30%), vomiting (15–20%), diarrhoea (23–31%), abdominal pain (22–31%), headache (21–26%), peripheral neurologic symptoms (20–25%), and rash (12–22%). Most notably, it was observed that ATV had a favourable adverse effect profile in comparison to other PIs and other ARVs. Diarrhoea was experienced by 56–61% of patients taking nelfinavir as opposed to 20–30% in the ATV groups [174; 175]. Furthermore ARVs, particularly PIs are known to alter serum lipids significantly [155; 172–175], yet the percentage change from baseline levels for total cholesterol, low density lipoprotein, high density lipoprotein and triglycerides was significantly lower with ATV (400 mg unboosted daily) than when nelfinavir [174; 175], ritonavir-boosted lopinavir [176] or efavirenz [155] was used in the ART regimen instead. Preliminary investigations also revealed that ATV had little effect on lipodystrophy [155; 174].

Hyperbilirubinaemia is a distinct feature of ATV treatment and probably occurs due to competitive inhibition of UGT1A1 by ATV [169]. It may be asymptomatic or manifest clinically as jaundice or *sclera icterus*. In two studies, 7% of patients in the ATV treatment arm presented with these conditions in comparison to none reported in other treatment arms or studies in which nelfinavir and efavirenz were being administered instead of ATV [155; 174; 175].

2.7.5 Drug Interactions

Based on the PK and PD profile of ATV, many potential interactions with comedications may be predicted; however the clinical relevance is often unknown. Only those for which clinical evidence exists will be mentioned here and these clinical studies include case studies and retrospective and prospective studies where participants were healthy human volunteers or patients and where plasma concentrations and/or clinical endpoints/outcomes were used to conclude clinical significance and clinical relevance of interactions, as appropriate.

2.7.5.1 Other ARVs

In an open-label pilot study, the influence of nevirapine on ATV levels was evaluated in 11 patients who had received 300/100 mg ATV/ritonavir once daily for 2 weeks or longer [177]. Nevirapine was added at a dose of 200 mg once daily from Days 0 to 14 and 200 mg twice daily from Days 14 to 28. ATV C_{min} levels were determined and compared at Days 0 and 28 [177]. A geometric mean ratio of 0.59 (95% CI, 0.38–0.80; P = 0.026) indicated that nevirapine reduced the levels of ATV in a significant manner [177]. Despite this observed effect, these ATV levels were higher than the minimum effective concentration in 80% of the participants, thus the authors concluded that the interaction may not have clinical relevance [177]. It was recommended that if this drug combination is used, therapeutic drug monitoring of ATV should be undertaken to identify individual instances where ATV dose changes may be necessary [177].

In a separate study by Dailly *et al.* (2006) [178], the influence of nevirapine, efavirenz and tenofovir co-administration on ritonavir-boosted ATV PK was investigated in HIV-infected patients. A significant increase in ATV clearance (CL/F) was found in each treatment group in comparison to the control arm, which was ritonavir-boosted ATV with NRTIs ($p<0.001$) [178]. However, the decrease in the C_{min} of ATV was significant only after co-administration of nevirapine ($p<0.001$), but as in the previously mentioned study, the plasma concentration of 0.210 ± 0.013 µg/ml was above the minimum effective concentration of ATV [178]. Therapeutic drug monitoring of ATV when these ARVs are co-administered was also suggested [178].

The outcomes of these studies imply that the co-administered ARVs enhance the metabolism and efflux of ATV and may, in part, have directed the decision not to include PIs (such as ATV) with either of the NNRTIs, nevirapine and efavirenz in the same ART regimen (see chapter 1, section 1.3.4).

2.7.5.2 Lipid-lowering Drugs

Although ATV has a favourable effect on serum lipids in comparison to other PIs, the prescription of lipid-lowering drugs such as the statins may still be necessary. Rhabdomyolysis and acute renal failure have been reported in an HIV-infected patient taking 80 mg simvastatin at bedtime (initiated 27 days earlier), amiodarone at a dose of 400 mg daily for 7 days, then 200 mg daily (initiated 19 days earlier). The ART regimen included

400 mg ATV daily (initiated at least 2 years previously) [179]. These adverse effects were reduced after discontinuation of all drug therapy [179]. Since simvastatin is metabolised by CYP3A4 and amiodarone and ATV are recognised CYP3A4 inhibitors, the risk of rhabdomyolysis, a common side effect of the statins is increased in the presence of these drugs [179]. An alternative lipid-lowering drug should be considered in such instances [179].

Healthy adult volunteers received single 10-mg doses of rosuvastatin at baseline and after 6 days of ritonavir-boosted ATV [180]. In each phase, blood samples were drawn over a 24 hour period and plasma concentrations of rosuvastatin were determined therefrom [180]. In comparison to baseline, the $AUC_{0\text{-}24h}$ and C_{max} of rosuvastatin increased by 213 and 600%, respectively [180]. The underlying mechanism was not clear although it was most likely related to the inhibition of UGT1A1 by ATV, since this enzyme is responsible for the glucuronidation of rosuvastatin [180]. The authors emphasise that dose limitations of rosuvastatin with ATV/ritonavir may be required [180].

2.7.5.3 Anti-tubercular Drugs

Tuberculosis is a common opportunistic infection among HIV-infected people, the first-line treatment for which includes rifampicin. A prospective study was performed in three HIV-infected patients with TB, treated with a rifampicin-containing regimen (rifampicin 600 mg per day) and ART including only NRTIs with ritonavir-boosted ATV [181]. Since rifampicin is a known inducer of CYP3A4 activity, the primary aim of this study was to ascertain whether inhibition of CYP3A4 by ritonavir could supersede the inducing effect by rifampicin [181]. In all three cases, the ATV level was very often (>50%) below the minimum effective plasma concentration of 0.15 µg/ml [181]. Subtherapeutic concentrations of ATV were achieved, thus, even when boosted with ritonavir, ATV should not be concomitantly administered with rifampicin [181]. There is dearth of data on interactions between ATV and other anti-TB drugs, such as isoniazid, pyrazinamide and ethambutol.

2.7.5.4 Neuro-active Drugs

Clinicians caring for patients infected with HIV and diagnosed with psychiatric co-morbidities must be aware of potential drug-drug interactions, particularly with PI-based ART.

Opioid-dependent, buprenorphine/naloxone-maintained, HIV-negative volunteers (n= 10 per PI) participated in a two-phase study to determine the PK of buprenorphine alone and buprenorphine in combination with ATV (400 mg daily) or ATV/ritonavir (300/100 mg daily) following administration for 5 days [182]. Objective opiate withdrawal scale scores and mini-mental state examinations were determined prior to and following ARV administration to determine if there is a correlation between the PD and the PK observed [182]. ATV, unboosted and boosted significantly increased concentrations of buprenorphine ($p<0.001$) [182]. Three buprenorphine/naloxone-maintained participants reported increased sedation with ATV/ritonavir [182]. The direct relationship between the PK and PD confirms the significance of this interaction and indicates that buprenorphine doses may need to be reduced in HIV-infected patients on an ATV-based ART regimen [182].

Two case studies have been reported in which adverse effects of quetiapine (weight gain and mental confusion in each case respectively) developed after simultaneous administration of this antipsychotic with ritonavir-boosted ATV [183]. The symptoms resolved when quetiapine therapy was withdrawn in each case [183]. Since quetiapine is primarily metabolised by CYP3A4, ATV and ritonavir may have inhibited its metabolism and it is reasonable to expect that quetiapine concentrations would have increased when these drugs were used concurrently, which would have been the likely cause of the toxicities in these two patients [183].

2.7.5.5 Azole Antifungals

The azole antifungal agents are an important part of the management strategy of HIV-infected patients in SA [26]. A randomised, crossover, drug interaction study was conducted to assess the PK effects of co-administration of an azole antifungal, posaconazole (400 mg twice daily), with ATV (300 mg/d alone) and with ritonavir (100 mg/d) in healthy subjects [138]. Posaconazole increased C_{max} and AUC of ATV by 2.6 and 3.7 times, respectively when given unboosted and 1.5-fold and 2.5-fold, respectively when boosted with ritonavir [138]. Most subjects who received ATV (with and without ritonavir) and posaconazole experienced clinically relevant increases in total bilirubin [138]. Increases in ATV exposure gave rise to the adverse effect of hyperbilirubinaemia (see section 2.7.3.4) and were probably related to CYP3A4 inhibition by posaconazole, a distinct feature of many azole antifungals [184]. Frequent monitoring of adverse events and toxicity related to increased ATV exposure

was advised in the event of co-administration of posaconazole and ATV with or without ritonavir [138].

2.7.5.6 Proton Pump Inhibitors

The PK of ATV may potentially be affected by co-administration with proton pump inhibitors, which reduce gastric pH, since the solubility and thus presumably the bioavailability of the former are pH-dependent (see section 2.6.1). An open-label randomised crossover study was conducted in which 10 healthy adult subjects received a single oral dose of ATV (400 mg) alone in Phase I and in Phase II. Two oral doses of lansoprazole (60 mg) were given 24 hours apart, the second of which was administered with another single oral dose of ATV (400 mg) [185]. Blood samples were collected from each subject in each phase over a 24-hour period. A 94 and 96% decline in ATV AUC_{0-24} and C_{max} (both $p<0.01$) was observed from Phase I to Phase II, indicating that absorption of the PI was significantly reduced when it was co-administered with lansoprazole [185]. Based on these results, it was suggested that ATV should not be co-administered with lansoprazole or other proton pump inhibitors [185].

In one PK study in an HIV-infected 65-year-old man on ritonavir-boosted ATV, a marked reduction in AUC_{0-24} and C_{min} of the PI was observed after simultaneous administration of the proton pump inhibitor, esomeprazole [183; 186]. Since data describing clinical outcomes associated with interactions between ATV and proton pump inhibitors were limited, viral load suppression, associated with the concurrent use of ritonavir-boosted or unboosted ATV and proton pump inhibitors, was determined in a retrospective study [187]. The primary outcome was achievement/maintenance of a viral load less than 400 copies/ml for two or more months during concomitant ATV and proton pump inhibitor therapy [187]. These criteria were obtained from nine of twelve subjects [187]. The interaction between ATV and once-daily proton pump inhibitors may thus not be clinically relevant, particularly in adherent patients [187].

2.7.5.7 Corticosteroids

A 14-year-old HIV-infected female on ritonavir-boosted ATV, rapidly developed cushingoid features often associated with corticosteroid therapy, within 2 weeks of receiving inhaled fluticasone/ salmeterol for asthma treatment [188]. After withdrawal of all drugs, symptoms of adrenal insufficiency were evident [188]. Cushing syndrome and adrenal suppression were

possibly caused by elevated steroid systemic concentrations resulting from inhibition of CYP3A4-mediated metabolism of the corticosteroid by ATV. Such an effect on fluticasone metabolism has been shown to occur *in vitro* with the CYP3A4 inhibitor, ketoconazole shown *in vitro* by the known inhibitor, ketoconazole [189] and calls for caution when these drugs are used concomitantly.

2.7.5.8 Herbal Medicines

An interaction between ATV and *Uncaria tomentosa* (cat's claw) was first mentioned in chapter 1 (section 1.3.7.10) of this thesis. An unusually high C_{min} of 1.22 µg/ml was observed for ATV, as well as other PIs (ritonavir and saquinavir) in the ART regimen in a 45 year-old HIV-infected woman who also presented with liver cirrhosis [138]. Before assuming the effect to be due to limited activity of CYP3A4 as a result of liver disease, interactions with other pharmacological treatments were first considered by questioning the patient on the use of CAM [138]. The patient reported the use of a cat's claw preparation during the preceding two months [138]. The patient was then asked to stop using this CAM and the ATV C_{min} measured 15 days later revealed that it had normalised to 0.3 µg/ml. CYP3A4 inhibition by this herbal remedy which has been established *in vitro* [140] was thought to be the underlying cause [138]. Despite the extensive use of herbal medicines by HIV/AIDS patients (see chapter 1, section 1.3.6.2), no other drug interaction studies between these and ATV were found.

2.7.6 Contra-indications

ATV is contra-indicated in patients with previously demonstrated clinically significant hypersensitivity (eg, Stevens–Johnson syndrome, erythema multiforme, or toxic skin eruptions) to this PI [144]. Contra-indications to components and excipients of the specific dosage forms of ATV should also be considered [144].

ATV is also contra-indicated in patients with certain comorbidities, such as severe hepatic impairment and end stage renal disease managed with haemodialysis [144].

An exhaustive list of co-administered drugs which exhibit CYP3A4 or UGT1A1 metabolism and for which elevated plasma concentrations are associated with serious and/or life-threatening events has been compiled [144]. As previously mentioned, ATV inhibits the activities of these enzymes, thus potential as well as clinically established interactions with

these drugs have been highlighted and forms the basis of this list of drugs contra-indicated for concurrent use with ATV [144].

2.8 Summary

A monograph detailing aspects of the synthesis, description, structure-activity relationships, dosage forms, physicochemical properties and clinical pharmacology of ATV which are relevant in the context of this research has been presented.

The synthetic procedure involved in the conversion of ATV into an acidic salt was of interest. ATV sulphate was prepared by stirring ATV with concentrated sulphuric acid in ethanol at room temperature, using *n*-heptane as a recrystallisation solvent.

The chemical name for ATV sulphate is (3*S*,8*S*,9*S*,12*S*)-3,12-Bis(1,1-dimethylethyl)-8 hydroxy-4,11-dioxo-9-(phenylmethyl)-6-[[4-(2-pyridinyl)phenyl] methyl]-2,5,6,10,13 penta-azatetradecanedioic acid dimethyl ester, sulphate (1:1). It appears as a white to pale yellow crystalline powder with a molecular formula of $C_{38}H_{52}N_6O_7 \bullet H_2SO_4$, which corresponds to a molecular weight of 802.9 g/mole (sulphuric acid salt). The free base, ATV, has a molecular weight of 704.9 g/mole.

Structure-activity relationships for ATV were developed and showed that the design of this PI is based on the C2 symmetry of the HIV protease enzyme as well as on the structure of common substrates. A novel pseudosymmetric dihydroxyethyl amine dipeptide isostere resulted in the formation of a lead compound GCP-53820, which only had moderate antiviral activity and which, in preclinical PK studies, did not reach detectable levels in the plasma of mice. Structure-activity optimisation showed that esterification of the valine substituents found on either end of the molecule, which interact with subsites S_3, S_2, S_2' and S_3' of HIV protease, increased bioavailability but decreased antiviral activity, whilst an alkyl group replacing cyclohexane at the position which interacts with S1 greatly improved antiviral activity at the expense of good bioavailability. In further studies, the use of *tert*-leucine as opposed to valine substituents increased antiviral activity and improved bioavailability. The substitution of the alkyl/cyclohexyl group with a 4-pyridinyl-phenyl group drastically increased plasma levels in preclinical PK studies and also further enhanced antiviral activity. These last two manipulations resulted in the structure of ATV.

ATV sulphate, under the brand name Reyataz® is available in two pharmaceutical forms, namely, a capsule, which is formulated in three dose strengths of 100 mg, 150 mg and 200 mg of ATV, and a powder containing 50 mg ATV, both for oral administration.

In distilled water ATV sulphate had a solubility of 4-5 mg/ml, producing a solution with a pH of 1.9 at 24 ± 3 ºC, indicating that this is the pH of maximum ionisation and solubility of the free base ATV. Since ATV is a weak base with a pKa of 4.65 ± 0.25, it is expected that ionisation and solubility would have decreased from 100% at pH 1.9 to about 1% at pH 6.4–6.9. ATV sulphate was not soluble in methanol or acetonitrile at a concentration of 5 mg/ml, but was soluble at this same concentration in buffers in the pH range 2–4, as well as mixtures of either of the two organic solvents with any of the buffers (pH range 2–8) in a ratio of 50:50. An UV spectrum showed that this compound exhibited highest absorbance at 200 nm, which decreased sharply to ~225nm. Two absorbance peaks or local maxima were present at 249.7 and 280.5 nm.

ATV sulphate exhibited photostability and thermal stability in the solid state. Forced degradation studies indicated that ATV sulphate in solution may be labile under acidic conditions. Stability of ATV sulphate in Reyataz® capsules is maintained for the shelf-life of the dosage form, which is two years, if it is stored at or below 25 ºC in the original container which is a high-density polyethylene bottle.

Reyataz® (ATV sulphate) is indicated in combination with other ARV agents for the treatment of HIV-1 infection. In treatment-naïve patients, ATV 400 mg daily may be administered, whereas in those who have received ART previously, ATV 300 mg boosted with ritonavir 100 mg is indicated.

Preclinical studies showed that ATV has an IC_{50} of 2–5 nmol/l, an IC_{90} of 9–15 nmol/l and an inhibition rate constant (K_i) of less than 1 nmol/l [158]. The EC_{50} increased five-fold in the presence of 40% serum due to a reduction in the availability of free ATV as a result of protein binding. Despite this, ATV still exhibited a potency superior to that of older PIs.

Analyses of clinical isolates in patients resistant to ATV have revealed that mutations occur in the amino acid sequence of HIV-1 protease. A unique mutation in ART-naïve patients on ATV 400 mg daily was an isoleucine-to-leucine substitution at amino acid residue 50 (I50L);

however this occurred in less than 2% of these patients. Significant cross-resistance to unboosted ATV occurred in patients with strains of HIV-1 protease resistant to multiple PIs. Boosted ATV gave rise to a wider range of mutations in HIV-1 protease in treatment-experienced patients.

The PK of ATV in humans is influenced by the absence or presence of HIV-infection and by co-administration with ritonavir as a booster or not. Lower C_{max}, C_{min} AUC and $t_{1/2}$ values were evident in HIV-infected patients and when ATV was administered unboosted. The reason for the former is inconclusive whilst the latter is known to be due to the inhibition of CYP3A4 and P-gp by ritonavir which increases bioavailability. The minimum plasma concentration required for effective viral suppression was predicted to be 150 ng/ml. ATV has a low bioavailability, which exhibits high inter-patient variability. Both effects may be negated by taking the dose with food. ATV is 86% protein bound, but is able to distribute to HIV sanctuary sites such as the seminal fluid and CSF in adequate concentrations. ATV readily undergoes CYP3A metabolism and, to a lesser extent, glucuronidation. ATV may influence the metabolism of other drugs since it is able to inhibit CYP2C8, UGT1A1 and CYP3A in ascending order of rank. The estimated clearance for a 400 mg dose of ATV is 25.3 L/hour. Most of an ATV dose (79%) is eliminated via the bile, whilst the rest is usually renally excreted.

The most common adverse effects experienced by patients and which could be attributed to ATV were: nausea, vomiting, diarrhoea, abdominal pain, headache, peripheral neurologic symptoms and rash. Most notably, it was observed that ATV had a favourable adverse effect profile in comparison to other PIs and other ARVs, particularly with respect to serum lipid profiles. Hyperbilirubinaemia is a distinct feature of ATV treatment and probably occurs due to competitive inhibition of UGT1A1 by ATV. It may be asymptomatic or manifest clinically as jaundice or *sclera icterus*, although this only occurs in 7% of patients.

Many potential PK interactions with ATV may be predicted, only some of which have been reported. Nevirapine and rifampicin appeared to induce the metabolism of ATV resulting in reduced bioavailability. This was of particular concern with rifampicin used in TB therapy where subtherapeutic plasma levels of ATV were achieved.

Two statins, namely simvastatin and rosuvastatin exhibited increased bioavailability when co-administered with ATV. Two different mechanisms underlying this effect in each case were proposed, *viz* inhibition of CYP3A4 and UGT1A1 by ATV respectively. Neuro-active drugs such as buprenorphine and quetiapine are also CYP3A4 substrates, thus increased exposure through CYP3A4 inhibition by ATV has resulted in an increase in adverse effects of these drugs. Remarkably, an analogous scenario was evident even after co-administration of inhaled fluticasone with oral ATV. Cushingoid signs and symptoms associated with increased systemic exposure of corticosteroids were prevalent as a result of CYP3A4 inhibition by ATV. Conversely, azole antifungals such as posaconazole increased ATV exposure via CYP3A4 inhibition, and in turn, the incidence of hyperbilirubinaemia was also raised. A similar effect may have been at play in a case study of a patient on ATV and a herbal medicine, cat's claw.

Proton pump inhibitors reduced the acidity of the gastro-intestinal tract, reducing the solubility of ATV. Consequently, the absorption of ATV which is dissolution rate-limited was also curtailed. The effects observed in healthy subjects indicated that this has the potential to compromise the efficacy of ATV, but results from a case study of one patient were not in agreement with this observation.

ATV is contra-indicated in patients with previously demonstrated clinically significant hypersensitivity to this PI, as well as in patients with certain comorbidities, such as severe hepatic impairment and end stage renal disease managed with haemodialysis. An exhaustive list of co-administered drugs, which exhibit CYP3A4 or UGT1A1 metabolism and for which elevated plasma concentrations are associated with serious and/or life-threatening events, has been compiled. Since ATV inhibits the activities of these enzymes, potential as well as clinically established interactions with these drugs have been highlighted and form the basis of the list of drugs contra-indicated for concurrent use with ATV.

The established clinical evidence for ATV to both induce and be susceptible to PK interactions with co-administered drugs further alludes to the potential for ATV to be involved in such interactions with a wide variety of orally administered xenobiotics.

CHAPTER 3

Sutherlandia frutescens

3.1 Introduction

SF is a Southern African plant which has a long history of use in the practice of TM, particularly by the Zulu, Sotho, Xhosa and Khoi-San people [190; 191]. Aqueous decoctions prepared from this plant have been used to treat a variety of symptoms and illnesses, such as pain and fever, wounds, stomach ailments, stress and anxiety, cancer and diabetes [190; 191].With the advent of the HIV/AIDS crisis in Southern Africa in recent times, SF has become popular as an "adaptogenic tonic", which anecdotal reports claim to be useful for alleviating the cachexia (muscle-wasting) [190] commonly observed in patients at the end stages of the disease. This, in turn, has provoked much interest in the plant, both commercially and scientifically [190]. Scientists are driven in the search for preclinical evidence of the safety and efficacy of SF which may or may not underlie the anecdotal reports of success in treating each of the above-mentioned ailments. Pharmaceutical dosage forms, such as tablets, capsules and creams, are currently readily available for purchase in pharmacies, health shops and even online. These dosage forms generally contain powdered herb, composed of the stems and leaves obtained from commercialised cultivation of SF, which was first initiated by Phyto Nova (Pty) Ltd, Cape Town, SA.

3.2 Taxonomy

The genus *Sutherlandia* belongs to the tribe Galegeae and the family Fabaceae [190]. This genus is closely related to the genera *Astragalus* L. and *Lessertia* DC. [190]. Currently, the following six species of *Sutherlandia* are formally recognised: (1) *S. frutescens* (L.) R. Br.; (2) *S. humilis* E. Phillips & R. A. Dyer; (3) *S. microphylla* Burch. ex DC.; (4) *S. montana* E. Phillips & R. A. Dyer; (5) *S. speciosa* E. Phillips & R. A. Dyer; (6) *S.tomentosa* Eckl. & Zeyh. [192]. Using enzyme electrophoresis, Moshe *et al.* (1998) [193] showed that these six species may be very similar and suggested that only two should be distinguished, namely *S. tomentosa* and *S. frutescens*, with all other species mentioned above included in the latter. The introduction of three subspecies, *viz* subsp. *frutescens*, subsp. *speciosa* and subsp. *microphylla* of *S. frutescens* was also recommended [193]. Goldblatt and Manning (2000) [194] proposed an alternative classification system by transferring *Sutherlandia* to the genus

Lessertia DC. The two genera may be similar, but no morphological or genetic analyses have been undertaken to verify this [190].

3.3 Nomenclature

3.3.1 Scientific Name

To take into account the two formal classification systems described in section 3.2, the full scientific name for the plant is: *Sutherlandia frutescens* (L.) R. Br. [syn. *Lessertia frutescens* (L.) Goldblatt & J. C. Manning] [190]. Informally, both names, *Sutherlandia frutescens* and *Lessertia frutescens* are considered correct.

3.3.2 Common Names

A wide variety of common names for SF in colonial and vernacular languages of SA are known. Afrikaans terms include *Kankerbos, Gansies, Grootgansies, Wildekeur(tjie), Keurtjie, Rooikeurtjie, Kalkoen(tjie)bos, Kalkoenblom, Belbos, Kalkoenbelletjie, Klapperbos, Jantjie-bêrend, Bitterbos, Wildekeurtjie, Eendjies* and *Hoenderbelletjie.* The English names were generally generated by direct translation of the Afrikaans names; for example, Cancer Bush, Sutherlandia, Balloon Pea and Turkey Flower. The most prominent vernacular names are *Musa-pelo, Musa-pelo-oa-nōka* and *Motlepelo* (Sesotho); *Phetola* (Setswana); *Insiswa* and *Unwele* (isiZulu, isiXhosa) [190; 191].

3.4 Physical Description

SF is a perennial shrub which may be positioned erect or slightly drooped, thus varying in height from 0.2 to 2.5 m. Van Wyk and Albrecht (2008) [190] describe the leaves as, "shortly petiolate, stipulate and pinnate, with ± 8 pairs of opposite leaflets plus a terminal leaflet." The shape of and the hair growth on the leaflets range from ovate to oblong, and hairless to densely-haired, respectively [190]. The leaves may thus have a silvery appearance depending on the extent of hair growth [190; 191]. The flowers are usually red [190; 191] with long, boat-shaped keel petals, small wing petals and a large, "apically recurved standard petal, typically marked with white lines" [190]. The fruits are large, balloon-like pods [191], which, like the leaflets may be ovate to oblong in shape, depending on the origin. Each pod carries many brown, kidney-shaped seeds [190]. The flowers and leaves of a typical SF plant are shown in Figure 3.1.

Figure 3.1: Flowers and leaves of SF [195]

3.5 Geographical Distribution

SF is indigenous to Southern Africa [190]. The geographical distribution covers at least four countries, namely, the western, central and eastern parts of South Africa, most of Lesotho, the southern parts of Namibia and south-eastern Botswana [190].

3.6 Preparations and Doses

3.6.1 Traditional

Anecdotal recordings of recipes for aqueous decoctions of SF for oral administration suggest that 2.5–5 g dry leaves infused in a cup of boiling water is the common traditional dose [190].

3.6.2 Commercial

For commercial products, 300 mg of dried plant material is administered twice a day, which is equivalent to ~9 mg/kg/day [73; 75]. Dosage forms (tablets, capsules) usually contain 300 mg per unit.

3.7 Phytochemical Constituents

A diverse range of compounds are found in plants in general and these may be categorised as either primary or secondary metabolites [196]. The former are essential for the growth, development and reproduction of the plant, whilst the latter are important for long-term survival [196]. Secondary metabolites are usually phylogenetically linked, therefore, certain combinations of these are unique to taxonomical families and become even more specific for

the genus, species and geographical population [196]. Secondary metabolites serve a protective function and are therefore bio-active [196].

3.7.1 Non-protein Amino Acids

Non-protein amino acids are very similar in structure to essential amino acids [197], therefore these compounds may interfere in two ways with protein biosynthesis in an organism which has consumed the plant. Competition for uptake may reduce the absorption of essential amino acids required [197]. Furthermore, competition for incorporation into proteins reduces the production of *bona fide* proteins and increases the production of faulty forms of these macromolecules [197].

3.7.1.1 L-canavanine

L-canavanine (Figure 3.2) is an anti-metabolite of the essential amino acid, L-arginine and is commonly found in the seeds of plants belonging to the Fabaceae family [190]. The only report of L-canavanine being present in SF plant material is in a thesis by Moshe (1998) [198] which has not been published in the scientific literature. Moshe (1998) [198] found L-canavanine present in the leaves of different species of *Sutherlandia* at concentrations between 0.42 and 14.5 mg/g dry weight, although it was not clear which concentration specifically related to SF. In tablets which contained 300 mg of SF plant material, Tai *et al.* (2004) [199] found 3 mg L-canavanine per tablet. However it is not known whether these dosage forms were perhaps fortified with L-canavanine, in which case, the L-canavanine content would not solely be due the presence of L-canavanine in the SF plant material incorporated into the tablets.

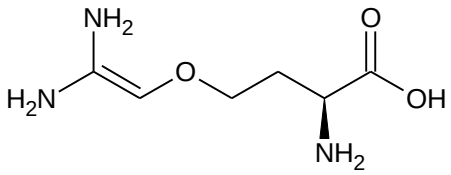


Figure 3.2: L-canavanine

3.7.1.2 L-γ-aminobutyric acid (L-GABA)

L-GABA is a compound which is not foreign to organisms, as it is an inhibitory neurotransmitter produced *in vivo* from the amino acid glutamate, which is also an excitatory neurotransmitter [200]. Analogous to L-canavanine, the presence of L-GABA (Figure 2.3) in

Sutherlandia plant material of the various species at concentrations of up to 17 mg/g dry weight, documented by Moshe (1998) [198], has not been reported in the scientific literature and again, the specific concentration in SF was not clear. It has also been detected in SF containing tablets [199], although this cannot be assumed to be due to the presence of this sugar in the SF plant material, since fortification of the tablets with L-GABA may have been undertaken by the manufacturers. A review by van Wyk *et al.* (2008) [190] reported different, yet also unpublished L-GABA concentrations of 0.23–0.85 mg/g dry weight of SF.

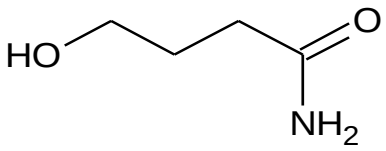


Figure 3.3: L-GABA

3.7.2 D-pinitol

The review by van Wyk *et al.* (2008) [190] reported data from a thesis by Snyders, 1965 [201], also not published in the literature, which showed the presence of the cyclitol, D-pinitol (Figure 3.4) in the leaves of *Sutherlandia microphylla*, at a concentration of 2.8 mg/g dry weight. In recent years it was found to be present in tablets containing SF in the parts per million range [199], but the question of fortification arises again. The chromatographic (HPLC) analyses in the thesis by Moshe (1998) [198] indicate that concentrations of up to 14 mg/g dry weight of D-pinitol may be present in the leaves of various species of *Sutherlandia*, with the concentration specific to SF again being unknown.

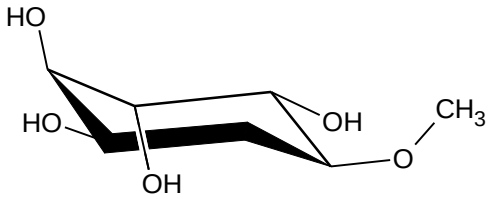


Figure 3.4: D-pinitol

3.7.3 Triterpenoid Glycosides

Van Wyk *et al.* (2008) [190] claim that triterpenoid glycosides were first detected in *Sutherlandia microphylla* leaves over thirty years ago in the thesis of Brummerhof (1969) [202], who proposed structures for two tetracyclic triterpenes, which upon acid hydrolysis produced aglycones with molecular masses of 472 and 490 g/mol respectively. This data was however not published in the literature and in yet another such thesis compiled by Gabrielse (1996) [203], compounds with similar fragmentation products to that found by Brummerhof (1969) [202] were reported. Using thin layer chromatographic (TLC) analysis, the thesis by Moshe (1998) [198] reported a complex pattern of triterpenoids in the various species of *Sutherlandia*. Mass spectrometric analyses of one of these compounds revealed a molecular mass of 562 g/mol [198] suggesting that this triterpenoid was different to those found previously [202; 203]. Structure elucidation of triterpenoid glycosides in SF was first published by Fu *et al.* (2008) [204]. The structures of the four compounds, which were termed Sutherlandioside A, B, C and D are shown in Figure 3.5. In an analysis of different SF plant samples, Avula *et al.* (2010) [205] found these to be present at concentrations of 0.606 ± 1.267, 2.75 ± 0.234, 0.221 ± 0.312, and 0.644 ± 0.524 % w/w, respectively. Sutherlandioside B was thus the most abundant of the four triterpenoid glycosides. Sutherlandia Su1™ tablets (Phyto Nova Pty Ltd, Cape Town) purchased from a pharmacy in Grahamstown, South Africa and which were used in the clinical component of this research were sent to Dr Bharathi Avula of the National Centre for Natural Products Research, Research Institute of Pharmaceutical Sciences at the University of Mississippi for analysis of triterpenoid glycoside content, and a similar trend was observed as reported in the commercial product's certificate of analysis [206]. Sutherlandioside A, B, C and D were present at 0.05, 3.02, 0.93, and 0.46 mg/tablet respectively.

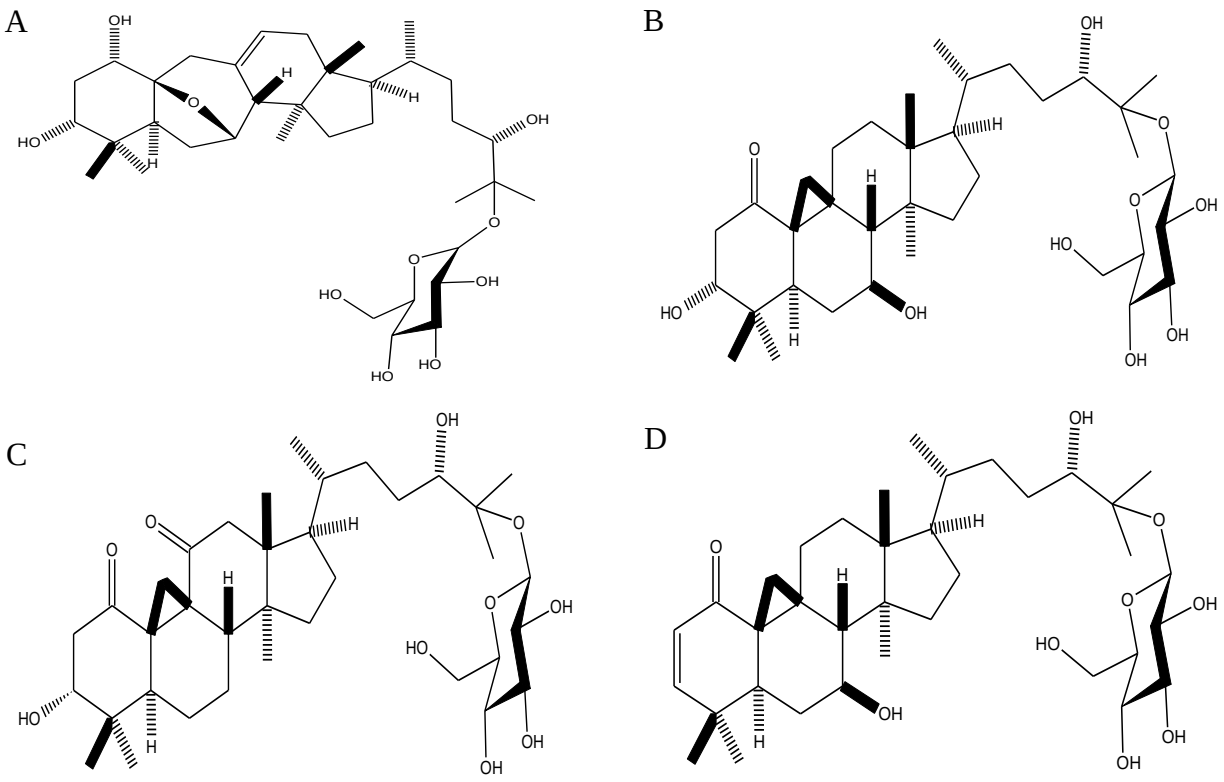
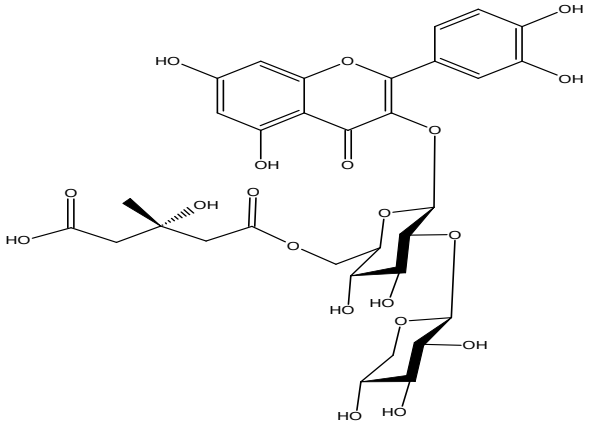


Figure 3.5: Sutherlandiosides (A–D)

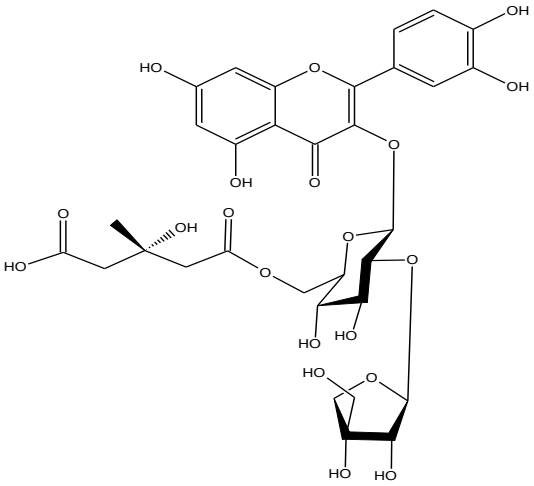
3.7.4 Flavonol Glycosides

By TLC and HPLC analysis, in addition to the triterpenoid glycosides, Moshe (1998) also found at least six unidentified flavonoids in the various species of *Sutherlandia* [198], four of which were recently isolated and named as Sutherlandin A, B, C and D [207]. Sutherlandin A and B are quercetin 3-*O*-glycosides, whilst Sutherlandin C and D are kaempferol 3-*O*-glycosides [207]. The concentrations of these (shown in Figure 3.6) in SF were found to be 0.531 ± 0.821, 0.485 ± 0.047, 0.943 ± 1.544 and 0.542 ± 0.524 % w/w, respectively [205] in samples of dried plant material. The same certificate of analysis [206] mentioned in section 3.7.3 showed that Sutherlandia Su1™ tablets contained 0.63, 0.67, 1.49 and 0.99 mg/tablet of Sutherlandin A, B, C and D respectively, indicating that Sutherlandin C was the most abundant.

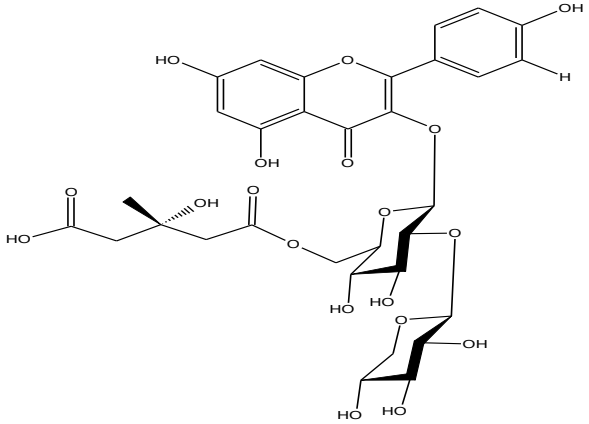
A



B



C



D

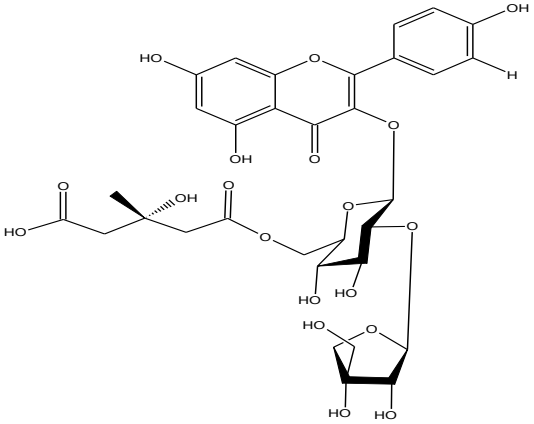


Figure 3.6: Sutherlandins (A–D)

3.8 Preclinical Studies

3.8.1 Pharmacological Activity

As previously mentioned in section 3.1, in the past decade, scientists have been in search of the pharmacological basis for the reported efficacy of SF in the practice of ATM.

3.8.1.1 Anti-oxidant Activity

The first attempt to elucidate the anti-oxidant potential of SF was undertaken by Tai *et al.* (2004) [199] who showed that a 70% ethanol SF extract has weak anti-oxidant activity due to its hydroxyl radical scavenging properties in a cell-free system. Concentrated extracts were toxic to leukaemic (RAW 264.7) cells, whilst a highly diluted extract did not have the ability

to inhibit lipopolysaccharide-induced nitric oxide production by these cells [199]. However, in contrast, the purported phytochemical constituents of SF, D-pinitol (10 mM) and L-canavanine (0.5 and 2 mM) produced this latter effect. These findings suggest that these two constituents, if confirmed to be present in SF plant material, may not attain concentrations in the extract high enough to exhibit anti-oxidant effects.

Two months later, a more comprehensive study by Fernandes *et al.* (2004) was published. The anti-oxidant activity of a hot water extract of SF in luminol- and lucigenin-enhanced chemiluminescence by L-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP)-stimulated neutrophils was determined [208]. Similarly, superoxide and hydrogen peroxide-generating cell-free systems were also used to examine for analagous effects [208]. The results indicated that SF hot water extract had the ability to scavenge superoxide and hydrogen peroxide in cell-free systems, as well as reactive oxygen species in general in stimulated neutrophil systems [208]. The authors concluded that this implies that SF may be an effective immunomodulator for the treatment of diseases associated with the overproduction of reactive oxidants by human phagocytes [208].

Katerere and Eloff (2005) [209] used the 2,2-diphenyl-picrylhydrazyl (DPPH) radical assay to assess the free radical scavenging ability of extracts and components of SF. The latter were obtained by separation of several organic extracts using thin layer chromatography [209]. The acetone, ethanol and aqueous extracts exhibited free radical scavenging activity, whilst those of hexane, dichloromethane and ethylacetate did not [209]. Polar constituents in the acetone, ethanol, aqueous and ethylacetate extracts were found to have the most significant activity, whilst non-polar components in dichloromethane, ethyl acetate and hexane only showed some activity [209]. The authors stated that anti-oxidants which scavenge free radicals in this assay are commonly phenolic compounds [209], which alludes to the possibility of the flavonol glycosides being the bio-active constituents in this respect.

3.8.1.2 Anti-cancer Activity

The anti-mutagenic potential of SF extracts were determined in a cell-free system using two histidine (His)-requiring strains of *Salmonella typhimurium* in the absence and presence of a liver metabolising system [210]. The assay acts by detecting back mutations in the His^- operon ($\rightarrow His^+$) when growing *S. typhimurium* bacteria in a histidine-poor medium [210]. The dichloromethane extract (5 mg/ml) demonstrated anti-mutagenic activity [210] which

implies that phytochemical constituents therein may have anti-carcinogenic (anti-cancer) properties.

The influence of 70% ethanol SF extract dilutions of between 1/400 and 1/1200 on cell growth of cultured breast (MCF and MDA-MB-468) and leukaemia (Jurkat and HL60) cell lines has been assessed [199]. Cell growth was evaluated by counting viable cells by trypan blue exclusion with a haemocytometer and anti-cancer activity was interpreted by the estimation of the IC_{50} values which were found to be ~1/250, 1/200, 1/150 and 1/200 dilutions for these cell lines, respectively [199]. The results thus showed varying anti-proliferative effects of the SF extract on breast and leukaemia cell lines, with slightly higher activity on the former [199]. Analysis using flow cytometry showed that the cell lines were arrested at different phases of the cell cycle, suggesting that different active constituents were involved [199]. The addition of L-arginine to MCF7 cells significantly reduced the anti-proliferative activity of 2 mM L-canavanine [199]. However, the same treatment did not affect activity of SF extract, confirming that L-canavanine, if present in SF, as claimed, is only one of many possible active constituents [199] with respect to anti-cancer activity.

The cytotoxicity of aqueous extracts of SF from the Western Cape in SA has been evaluated in Chinese hamster ovary (CHO) and Caski (cervical carcinoma) cells by histological analysis [211]. Morphological changes such as cell shrinkage, cell disintegration and reduction in cell number indicated that the SF extract induced cell death in both cell types [211]. The extract then tested positive for apoptosis-induced cytotoxic potential in CHO cells using crossmon trichome staining and an apoptosis assay kit [211]. A DNA fragmentation assay and flow cytometry showed that SF performed similarly to known inducers of apoptosis in CHO and Jurkat (leukaemic) cells, respectively [211]. In contrast, Steenkamp and Gouws (2006) [212] found that aqueous extracts of SF had no significant cytotoxic effect on three human cancer cell lines, namely, DU-145 prostate cancer cells, malignant MDA-MB-468 and MCF breast cancer cells. The discrepancy in results between these two studies may be due to different cancerous cell lines having unique susceptibilities to aqueous SF extracts, or variability in phytochemical content of the plants used. Importantly, however, it may simply be related to the manner in which the aqueous extracts were prepared.

Stander and co-workers [213] confirmed the anti-proliferative effect of a 70% ethanol SF extract on MCF7 cells, first claimed by Tai *et al.* (2004) [199], using time-dependent studies

at the pre-determined IC_{50} value. Histological analyses of SF treated cells revealed less dense cell populations as well as hallmarks of apoptosis, such as hyper-condensed chromatin and the formation of apoptotic bodies. Changes in gene expression profiles verified that molecules which have important roles to play in apoptosis, growth inhibition and NFκB signalling were potentially involved.

An abnormally elevated expression of cyclooxygenase-2 (COX-2) has been implicated in the pathogenesis and progression of carcinogenesis [214]. Na *et al.* (2004) [214] found that methanol extracts of SF significantly inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 expression in human breast epithelial (MCF10A) cells and in mouse skin *in vivo*, alluding to an anti-carcinogenic potential for SF. The molecular basis of the COX-2 inhibition was determined by evaluating effects of SF on activation of NF-κB which is one of the major transcription factors responsible for regulating COX-2 expression [214]. The DNA binding of NF-κB activated by TPA in MCF10A cells was attenuated in a dose-dependent manner [214]. The results indicated that SF may have chemopreventive or chemoprotective activity. In a similar set of experiments, several natural and semisynthetic triterpenoids of the cycloartane-type, like the triterpenoid glycosides in SF [204], exhibited anti-tumour promoting and initiating effects *in vitro* and *in vivo* [215]. This indicates that the anti-cancer activity of SF extracts may be attributed, at least in part to these phytochemical constituents.

3.8.1.3 Anti-bacterial Activity

The anti-bacterial properties of various organic extracts of SF were investigated by measuring the minimum inhibitory concentrations (MIC) using a serial dilution microtitre assay method as well as bioautography [209]. In the microtitre assay, *Staphylococcus aureus* was the most sensitive bacterium, being inhibited by all extracts except the acetone fraction [209]. Hexane showed the greatest activity against this bacterium with an MIC of 0.3 mg/ml [209]. The hexane fraction also showed some activity against *Escherichia faecalis* and *E. coli* [209]. In general, extracts of the more polar solvents, namely, acetone, ethanol and water were inactive, except acetone which had weak activity against *E. coli*, *Pseudomonas aeruginosa* and *E. faecalis* [209]. The results of the bioautography assay confirmed that the non-polar constituents of SF have activity against *S. aureus*, as observed in the microtitre assay. However, no activity against *E. coli* was detected in this assay which may suggest that such activity observed in the microtitre assay is not significant.

3.8.1.4 Anti-diabetic Properties

The anti-diabetic action of SF was first examined in a streptozotocin (STZ)-induced rat model of diabetes [216]. The hypoglycaemic effects of 800 mg/kg SF compared similarly to the recommended dose of the allopathic hypoglycaemic agent, chlorpropamide [216].

Oral administration of D-pinitol to STZ-induced diabetic rats showed a significant ($p<0.05$) decrease in the levels of blood glucose as well as other parameters associated with the metabolic syndrome, such as total cholesterol, triglycerides, low- and very low-density lipoproteins, free fatty acids, and phospholipids in serum, liver, kidney, heart, and brain [217]. Moreover, Sivakumar *et al.* (2009) [218] found that D-pinitol reduced oxidative stress and increased endogenous anti-oxidant enzymes in the pancreas of STZ-induced diabetes. In another study, oral administration of D-pinitol showed a marked decrease in the levels of blood glucose, glycosylated haemoglobin and an increase in plasma insulin, body weight and hepatic glycogen, coupled with enhanced activities of the glycogen-producing hepatic enzymes such as hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase and glycogen synthase in a diabetic rat model [219]. Conversely, glycogen catabolic enzymes, such as glucose-6-phosphatase, fructose-1,6-bisphosphatase, lactate dehydrogenase and glycogen phosphorylase activities were significantly decreased [219]. The results of the two aforementioned studies were statistically comparable with gliclazide, a standard hypoglycemic drug [218; 219]. The findings of all three of these studies suggest a biochemical basis for the anti-diabetic effect of D-pinitol and further imply that the anti-diabetic effect of SF may be attributed to this sugar if present in SF, as claimed.

Chadwick *et al.* (2007) [220] assessed the effect of an aqueous brew of SF in a prediabetic rat model, characterised by insulin resistance, in which hyperinsulinaemia without hypoglycaemia was observed. SF (0.01 ml aqueous tea/g rat weight/day) decreased blood insulin levels to those of the control, suggesting that SF may have been able to promote glucose uptake and/or to reduce glucose intestinal absorption [220]. In contrast, the known antidiabetic agent, metformin (0.012 mg tablet/g rat weight/day) increased blood insulin levels [220]. An increase in blood glucose clearance and a decrease in urine glucose levels were observed in prediabetic rats given each treatment, which further alluded to increased glucose uptake by systemic tissues or reduced intestinal absorption of glucose as a means for SF to decrease insulin levels [220]. These were confirmed as mechanisms of the hypo-insulinaemic effects of SF, by measuring glucose uptake in muscle, the epididymal fat,

kidney and intestinal tissue [220]. D-pinitol was not able to regulate blood glucose and insulin levels in mice with severe insulin resistance [221], which indicates that other phytochemical constituents and not this sugar, if present in SF, may be involved in alleviating this prediabetic state.

3.8.1.5 Analgesic Properties

The analgesic effect of an aqueous extract of SF shoots, which are the aerial parts of the plant used in ATM, was evaluated using the hot-plate and acetic acid test models of pain in mice [216]. The extract (50-800 mg/kg i.p.) produced significant ($p<0.05$-0.001) analgesic effects against both the thermally- and chemically-induced nociceptive pain stimuli in the mice [216]. These effects may explain the anecdotal reports of the analgesic properties of SF.

3.8.1.6 Anti-inflammatory Properties

The anti-inflammatory capacity of SF has been determined *in vitro* and in an *in vivo* animal model. Leukaemic cells (RAW 264.7 cells) incubated with 70% ethanol SF extract had no significant effect on the levels of expression of pro-inflammatory cytokines, TNF-α and IL-1β [199]. In contrast, Ojewole, 2004 [216] found that in a fresh egg albumin-induced pedal (paw) oedema rat model of inflammation, the SF plant extract (50-800 mg/kg p.o) significantly ($p<0.05$-0.001) inhibited acute inflammation, which was comparable to the effects of 100mg/kg diclofenac. Since D-pinitol (2.5-10 mg/kg, i.p.) attenuated inflammation in carrageenin-induced paw oedema in rats, the highest dose being comparable to phenylbutazone (100 mg/kg, i.p.), this constituent of SF, if present in the plant as claimed, may contribute to its anti-inflammatory effect [222].

Another study aimed to investigate the effect of a methanolic extract of SF on the expression of COX-2 in mouse skin stimulated with a prototype tumour promoter TPA [223]. SF inhibited TPA-induced COX-2 expression after *in vivo* application to mouse skin [223]. Potential mechanisms which underlie this were found to be inhibition of the activation of transcription factors, such as activator protein-1 and cyclic AMP response binding protein, as well as inhibition of the catalytic activity of extracellular signal-regulated protein kinase [223]. These results indicate an anti-inflammatory role for SF and also offer an additional mechanism of the anti-tumour promoting potential of this plant.

3.8.1.7 Anti-convulsant Activity

Aqueous extracts of the shoots of SF (aerial parts of the plant) (25–400 mg/kg i.p.) were tested for activity against pentylenetetrazole (PTZ)-, picrotoxin (PCT)- and bicuculline (BCL)-induced seizures in mice and were compared to standard anti-convulsant drugs, phenobarbitone and diazepam [224]. Extracts (50–400 mg/kg i.p.) reduced the number and onset of PTZ- and PCT-induced seizures, whilst a dose of at least 100 mg/kg i.p. of SF extract was required for significant effect with respect to those which were BCL-induced [224]. SF was however not as effective as phenobarbitone or diazepam [224]. Since PTZ and PCT induce seizures via GABA antagonist activity, the underlying mechanism of SF extracts in such seizures may be through GABA-ergic activity [224]. The likelihood of this proposed mechanism may only be substantiated if L-GABA is present in SF, as claimed.

3.8.1.8 Anti-HIV Activity

The anti-HIV activity of SF has already been alluded to in Chapter 1, section 1.3.6.3 and Table 1.4. Harnett *et al.* (2005) [65], using a cell-free HIV-reverse transcriptase (HIV-RT) colorimetric ELISA assay, found that aqueous extracts of SF leaves, but not other parts of the plant nor other organic solvent extracts, exhibited at least 50% inhibition of HIV-RT. This effect was consistent, even after removal of sulphated polysaccharides, which are known to have anti-HIV activity [65]. However, after addition of bovine serum albumin, the inhibition of HIV-RT was reduced to ~30% inhibition, indicating that the effect is, in part, due to the presence of tannins [65]. In a separate study, Bessong *et al.* (2006) [60], found that acetyl acetate and n-butanol fractions (100 µg/ml) stimulated HIV-RT activity by 68 and 15% respectively. Weak inhibition was observed with IC_{50} values of 410.4 and 296.3 µg/ml, respectively [60]. It may therefore be concluded that only the aqueous extracts and not those of organic solvents have the ability to significantly inhibit HIV-RT activity.

In another cell-free assay, no part or extract of SF had the ability to reduce HIV-protease activity by greater than ~20% [65]. Activities of three glycohydrolase enzymes with glycosylated HIV envelope proteins were also assessed in the presence of SF extracts [65]. All extracts (ethanol, acetone, methanol and dichloromethane), except the aqueous one from the leaves and flowers of SF, demonstrated at least 50% inhibition of β-glucuronidase, which was retained even after removal of tannins [65]. Tannins were therefore not involved in executing the observed effect. Furthermore, in contrast to the HIV-RT activity, the organic

but not aqueous extracts have β-glucuronidase inhibitory activity which may contribute to anti-HIV activity of SF.

3.8.1.9 Anti-stress Properties

Dysregulation of the physiological response to stress is associated with elevated glucocorticoid levels, therefore the effects of SF on these levels in a rat model of chronic intermittent immobilisation stress was investigated [225]. Animals treated with SF extract showed significantly decreased glucocorticoid levels ($p<0.005$) [225]. The authors also sought to determine the underlying mechanism of this effect [225]. Since the biosynthesis of glucocorticoids is catalysed by CYP enzymes in the adrenal glands, the influence of SF extracts thereon was evaluated in ovine adrenocortical microsomes and mitochondria, using spectral binding and enzyme conversion assays [225]. Aqueous, chloroform, and methanol extracts showed inhibition of substrate binding to cytochrome P450 21-hydroxylase (CYP21) and cytochrome P450 11-β-hydroxylase (CYP11B1) as well as inhibition of progesterone and pregnenolone metabolism [225]. This alluded to the inhibition of the activity of these adrenal CYP enzymes as the underlying mechanism of reduced glucocorticoid levels in rats after treatment with an SF extract.

In a follow up study, Prevoo *et al.* (2008) [226] went on to conduct a more detailed examination of SF in the regulation of CYP17 and CYP21 activity. Reduced progesterone and pregnenelone metabolism via CYP17 inhibition by the extract was confirmed using COS1 cells which overexpress CYP17 [226]. Aqueous and methanol extracts inhibited the type I progesterone-induced difference spectrum ($p<0.05$), but not that induced by pregnenolone binding ($p = 0.25$) in adrenal microsomes [226], suggesting that inhibition of pregnenelone by these extracts was not competitive in nature. The triterpenoid fraction extracted from SF inhibited both the type 1 pregnenolone- and progesterone-induced difference spectra and elicited a type II difference spectrum in the absence of the substrates, implying that the triterpenoids may have acted by both competitive and non-competitive inhibition [226]. These results allude to the potential for SF to inhibit or interfere with the activities of CYP enzymes including those involved in the metabolism of drugs.

3.8.2 Toxicity

A recently published study investigated the apoptotic effects of SF extracts on healthy human lymphocytes *in vitro* [227]. The IC_{50} of a 7.5 mg/ml 30% ethanol extract diluted with culture medium was calculated from cell viability assays carried out for 3, 6 and 12 hours [227]. Common features of apoptosis in the total lymphocyte population and in $CD4^+$ cells, such as increased phosphatidylserine translocation, caspase-3/7 activity, and decreased ATP content, were observed after 12 hour incubations with 7.5 mg/ml SF [227]. The underlying mechanism of this apoptotic cell death may be activation-induced lymphocyte cell death, since after 12 h, the SF extract doubled the number of cells expressing the early activation marker, CD69 [227]. The authors stated that "these results are in conflict with preliminary clinical evidence which has suggested SF extracts are possibly beneficial in the treatment of HIV infection and that more extensive evaluations of the effects of SF extracts on the immune system in such subjects are urgently needed" [227]. These effects may in part be attributed to L-canavanine, if present in SF, as claimed, which was found to be cytotoxic to peripheral blood mononuclear cells in culture and to impair B-cell function in auto-immune mice [228].

Renal elimination of polar bioactive constituents may increase susceptibility to toxicity in the kidney. Phulukdaree *et al.* (2010) [229] investigated the effects of aqueous SF extracts on endogenous anti-oxidant potential, lipid peroxidation, mitochondrial membrane potential and apoptotic activation in proximal (LLC-PK cell line) and distal (MDBK cell line) tubule epithelia, after a 48 hour incubation. Cell viability, determined using the MTT assay resulted in IC_{50} values of 15 mg/ml and 7 mg/ml for LLC-PK and MDBK, respectively [229]. At the IC_{50}, the SF extract significantly increased caspase 3 and 7 activity in both cell lines indicating that cell death may have occurred by apoptosis [229]. Mitochondrial dysfunction determined by measuring mitochondrial membrane potential using a flow cytometric JC-1 Mitoscreen assay demonstrated that cytotoxicity of SF extracts may be induced by membrane depolarisation [229]. In addition, SF extracts reduced intracellular glutathione content and increased lipid peroxidation, indicating that oxidative stress may also contribute to cell death [229]. This potential toxicity of SF warrants further investigation *in vivo*, using animal models.

In a study sponsored by the Medical Research Council and the National Research Foundation of SA, the potential toxicity of SF consumption was assessed in vervet monkeys *in vivo* [75].

As determined from haematological, biochemical, urine and physiological parameters, SF was not associated with toxic or other side effects at doses up to nine times the recommended daily dose of 9 mg/kg/day [75]. Statistically significant differences were observed between the treatment groups and the control group for some parameters [75]. However, the authors did not consider these as clinically significant primarily due to fluctuations in the control group [75], although the validity thereof may be questioned since such concessions were not catered for *a priori*.

It is noteworthy that in the investigation of the anti-convulsant properties of SF, Ojewole (2008) [224] demonstrated that the dose which resulted in 50% mortality (LD_{50}) in mice was 1.825 g/kg weight. This dose of SF is considerably higher (~200 times) than the recommended daily dose and may allude to the safety of oral consumption of the plant material at the recommended dose.

Over twenty years ago, the toxicity of L-canavanine was investigated in Sprague Dawley rats [230]. In an acute study, following a single subcutaneous (s.c.) injection, the LD_{50} was 5.9 ± 1.8 g/kg in adult rats and 5.0 ± 1.0 g/kg in 10-day-old rats [230]. Single oral doses of L-canavanine were less toxic to adult rats than s.c. injections [230]. Chronic s.c. administration (daily for seven days) exhibited more serious toxic effects, such as weight loss, alopecia and loss in appetite [230]. Histological studies of tissues from adult rats treated with 3.0 g/kg L-canavanine daily for six days revealed pancreatic acinar cell atrophy and fibrosis, which was initially associated with higher levels of serum amylase and lipase levels, followed by depletion of these enzymes after three days. Elevations in serum glucose and urea nitrogen, and depletion of cholesterol, were also observed [230]. The activities of liver enzymes, serum aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase were most significantly affected [230]. Less than 1% of a 2.0 g/kg dose of l-[*guanidinooxy*-^{14}C] canavanine was incorporated into the proteins of adult and neonatal rats 4 or 24 hr following administration [230]. L-canavanine has an LD_{50} ~5 times greater than that of SF in mice which indicates that if L-canavanine is indeed present in SF, as claimed, then it is not the only phytochemical constituent contributing to the toxic potential of SF.

3.8.3 Biopharmaceutics and Pharmacokinetics

3.8.3.1 Absorption

Sutherlandioside B was found to be unstable in simulated gastric fluid at pH 1.2 (46% degradation in 30 minutes) and stable in simulated intestinal fluid at pH 6.8 (5% degradation in 120 minutes) [231]. Efflux after *in vitro* intestinal transport in Caco-2 cells was also observed [231]. The latter was blocked by verapamil and MK-571 which are P-gp and MRP inhibitors respectively, indicating that these transporters may be involved in this process [231]. It also alludes to the potential for competitive inhibition with other substrates of P-gp and MRP. The bioavailability of a 2.0 g/kg s.c. dose of L-canavanine in adult rats was 72%, whilst that of the same dose administered orally was lower at 43% [230].

3.8.3.2 Distribution

Protein binding studies measured by equilibrium dialysis showed that Sutherlandioside B is 50% unbound in human plasma [231] with a volume of distribution at steady state of 0.154 litres [231].

3.8.3.3 Metabolism

Madgula *et al.* (2008) [231] further demonstrated that Sutherlandioside B has a half life of 495 minutes in human liver microsomes suggesting that this compound was not rapidly metabolised [231]. A weak inhibition of CYP3A4 activity, with a IC_{50} of 20 µM was observed, with no effect on CYP2D6 [231], which may suggest that this *in vitro* effect on CYP3A4 activity may not be clinically significant.

3.8.3.4 Elimination

Following a single intravenous (i.v.) dose of 2.0 g/kg, the systemic clearance value for L-canavanine in adult rats was 0.114 litres/hour, with a half-life of 1.56 hr [230]. Nearly half of this dose (48%) was excreted unaltered in the urine, whilst 16% of an s.c. dose was recovered in the urine [230]. In contrast, only 1% of an orally administered dose of L-canavanine was renally excreted and nearly a quarter (21%) remained in the gastro-intestinal tract 24 hours post-dose [230]. This long transit time in the gastro-intestinal tract suggests that if L-canavanine is present in SF as claimed, any effects it may have on efflux transporter and CYP enzyme activity in the enterocytes may be prolonged, increasing the risk for interactions with co-administered substrates.

3.8.4 Drug Interactions

As shown in Table 1.7, section 1.3.7.9 of Chapter 1, aqueous and methanolic extracts of SF inhibit CYP3A4 and P-gp, and also activate CYP3A4-associated PXR [116]. This indicates that if SF is used concomitantly with drugs which are substrates of any of these proteins, there is a potential for increased or decreased bioavailability of the latter. An additional study showed that *in vitro*, P-gp inhibition by an aqueous extract of SF, as well as by L-canavanine may result in increased absorption of the NNRTI, nevirapine, implying a possible elevation in bioavailability [121]. However, PK studies in rats revealed that such aqueous extracts had no effect after short-term administration *in vivo* [124], whilst a five day regimen reduced the bioavailability of the NNRTI as a result of CYP3A4 induction in the small intestine and liver of the animals [124].

3.9 Clinical studies

3.9.1 Safety

Following the preclinical study on vervet monkeys, as outlined in section 3.8.2, the safety of capsules containing 400 mg of *Sutherlandia* leaf powder (400 mg of plant material per capsule; 600 µg of L-canavanine per capsule) administered twice daily to healthy adult human volunteers, for three months (recommended dosage), was investigated [73]. A detailed appraisal of this study was included in Chapter 1, section 1.3.6.4. The authors concluded that this regimen of SF was safe for use by healthy adults, since there were no clinically relevant differences with respect to adverse effects and measured haematological, biochemical and physiological parameters between the placebo and treatment groups [73]. These results may not, however, be extrapolated to a general or a specific patient population.

3.9.2 Efficacy

As mentioned in chapter 1, section 1.3.6.4, only one clinical study, which formed part of a PhD dissertation and has not been published in the scientific literature [72], has been conducted in an attempt to provide an evidence-based rationale for the use of a medicinal product containing SF in HIV/AIDS patients. The author stated that a mixture of extracts from SF and a Turkish traditional medicinal plant, *Nerium oleander*, is "effective in increasing the CD4 counts of HIV-positive individuals with initial CD4 counts of less than 400 cells/mm^3 in a meaningful way over a 60-day period" [72]. However, the critical analysis of the study in section 1.3.6.4 has shown that the evidence on which this statement was based may be inadequate, particularly since the statistical analysis of the data was flawed.

Reduction of fatigue was noted by researchers in Italy who conducted a clinical trial using SF at the recommended dose described in section 2.3.6 in 16 cancer patients [232]. This suggests that the alleviation of fatigue may be an additional advantage if SF is administered to cancer patients based on its chemotherapeutic potential.

3.10 Summary

SF is a Southern African plant which belongs to the Galegeae tribe and the Fabaceae family. It has an indigenous geographical distribution in various parts of southern Africa, where it has a long history of medicinal use, ranging from pain, fever and gastro-intestinal disturbances to cancer and diabetes. SF plants have red flowers with balloon-like pods as fruit, which contain brown, kidney-shaped seeds. Each leaf is composed of approximately eight pairs of opposite leaflets and a terminal leaflet. Hair growth, if present, imparts a silvery appearance to the leaves. Many of the common names for SF, such as "balloon pea" and "cancer bush" are based on the salient features of its physical appearance or its traditional medicinal uses. Anecdotal recordings of recipes for aqueous decoctions of SF for oral administration suggest that 2.5-5 g dry plant material infused in a cup of boiling water is the common traditional dose.

In recent years in southern Africa, SF has become popular as an "adaptogenic tonic", which anecdotal reports claim to be useful for alleviating the cachexia (muscle-wasting) commonly observed in patients at the end stages of the disease. This has provoked much interest in the plant both commercially and scientifically. Commercial SF products are produced from the leaves and stems of cultivated plant which are milled to form dried plant powder. This powder, usually in aliquots of 300 mg, is then incorporated into each dosage unit, such as capsules and tablets. The recommended dose is one dosage unit twice a day which is equivalent to ~9 mg/kg/day.

The phytochemical constituents which have been reported to be present in SF include non-protein amino acids, such as L-canavanine and L-GABA, D-pinitol, as well as triterpenoid and flavonol glycosides, although the presence of the first three in SF plant material (not dosage forms) has only been recorded in unpublished theses. The amounts of all these phytochemical constituents present per gram of plant material seem to vary greatly and may be related to geographical location. Sutherlandioside B is at least three times more abundant

than the other triterpenoid glycosides in a number of samples of dried plant material, as well as in Phyto Nova ™ tablets, whilst Sutherlandin C is the most abundant flavonol glycoside.

Numerous preclinical studies have been conducted to ascertain whether there is a pharmacological basis for the anecdotal reports of the alleviation of the signs and symptoms of the various ailments for which SF was traditionally used, as well as underlying more recent claims of usefulness in HIV/AIDS patients. Extracts and constituents of SF at different concentrations have been shown to possess anti-oxidant, anti-cancer, anti-bacterial, antidiabetic, analgesic, anti-inflammatory, anti-convulsant, anti-HIV and anti-stress properties.

With respect to toxicity, the LD_{50} of dried SF plant material in mice was 1825 mg/kg weight and extracts of SF were found to cause cytotoxicity of healthy T-lymphoctyes from human blood and of epithelial cells from a kidney cell line. However, as determined from haematological, biochemical, urine and physiological parameters, SF was not associated with toxic or other side effects at doses up to nine times the recommended daily dose of 9 mg/kg/day after administration to vervet monkeys for three months.

The biopharmaceutics and PK of some constituents of SF has been delineated *in vitro*. Sutherlandioside B was stable in simulated intestinal fluid but not in simulated gastric fluid and was found to be subject to efflux by P-gp. Protein binding studies measured by equilibrium dialysis showed that this compound is 50% unbound in human plasma and that the volume of distribution at steady state was 0.154 litres. Furthermore, Sutherlandioside B has a half life of 495 minutes in human liver microsomes suggesting that this compound is not rapidly metabolised. A weak inhibition of CYP3A4 activity, with an IC_{50} of 20 µM was observed, with no effect on CYP2D6.

The bioavailability of a 2.0 g/kg oral dose of L-canavanine in adult rats was 43%, 1% of which was renally excreted. Nearly a quarter (21%) remained in the gastro-intestinal tract 24 hours post-dose, which may result in the prologation of any toxicity which may manifest *in vivo*.

Aqueous and methanolic extracts of SF inhibit CYP3A4 and P-gp, and also activate CYP3A4-associated PXR, which indicates that if SF is used concomitantly with drugs which

are substrates of any of these proteins, there is a potential for increased or decreased bioavailability of the latter. An additional study showed that *in vitro*, P-gp inhibition by an aqueous extract of SF, as well as L-canavanine may result in increased absorption of the NNRTI, nevirapine, implying a possible elevation in bioavailability. Pharmacokinetic studies in rats however revealed that such aqueous extracts had no effect after short-term administration *in vivo*, whilst a five day regimen reduced the bioavailability of the NNRTI, which was found to be as a result of CYP3A4 induction in the small intestine and liver of the animals.

Attempts have been made to elucidate the clinical significance and/or relevance of some of the results obtained in the preclinical studies by means of clinical trials. The safety of capsules containing 400 mg of *Sutherlandia* leaf powder (400 mg of plant material per capsule; 600 µg of canavanine per capsule) administered twice daily to healthy adult human volunteers, for three months (recommended dosage), was investigated. The authors concluded that this regimen of SF was safe for use by healthy adults, since there were no clinically relevant differences with respect to adverse effects and measured haematological, biochemical and physiological parameters between the placebo and treatment groups. These results may however not be extrapolated to a general or a specific patient population.

A mixture of extracts from SF and a Turkish traditional medicinal plant, *Nerium oleander*, was found to be "effective in increasing the CD4 counts of HIV-positive individuals with initial CD4 counts of less than 400 cells/mm^3 in a meaningful way over a 60-day period". However, a critical analysis of the study has shown that the evidence on which this statement was based may be inadequate. Reduction of fatigue was noted by researchers in Italy who conducted a clinical trial using SF at the recommended dose in 16 cancer patients, which may be an added advantage for its use in such patients.

Although a clinical study has shown that SF administration to healthy human volunteers may be safe, the preclinical evidence for the potential for SF to alter P-gp and CYP3A4 activity, as well as to enhance CYP3A4 protein expression, suggests that it may in turn affect the safety and/or efficacy profiles of drugs, which are P-gp and/or CYP3A4 substrates, such as PIs. This substantiates the urgent need for studies to delineate the true potential for a PI-SF PK interaction.

CHAPTER 4
BIOANALYSIS OF ATAZANAVIR

4.1 Background

Bioanalysis involves the quantitative or qualitative analysis of endogenous compounds, drugs or drug metabolites in biological matrices, derived either *in vitro*, for example, in biological media, biological buffer and liver microsomes or *in vivo*, in fluids such as plasma and urine [233; 234]. The bioanalyst persistently faces a challenge to develop bioanalytical methods with good selectivity, high sensitivity and faster throughput [235; 236]. Bioanalytical techniques and instruments, which have evolved over the last few decades to improve inherent selectivity and sensitivity, are discussed below.

4.1.1 Bioanalytical Techniques

4.1.1.1 Radio-assay

Radio-assay was a popular bioanalytical technique in the 1960s due to the potential for very high sensitivity [237-257]. Drugs are radiolabelled by substitution of one of the atoms in the structure for a radioactive isotope, such as ^{14}C and ^{3}H, and the radiation emitted therefrom is recorded by a scintillation counter [258; 259]. A small amount of radiolabelled compound with a large amount of the same compound unlabelled, allows for tracing of the compound in a biological system. Quantitation is possible due to the direct relationship between the concentration of the radiolabelled drug present and the amount of radiation emitted [258; 259].

Reviews published in the last 30 years [260-262] indicate that radio-assay has been and is still used in the drug development process to determine the PK parameters of absorption, distribution, metabolism and elimination of new chemical entities in cell lines, animal models and in early clinical studies in humans. For drug metabolism studies, coupling to a chromatographic technique is required, since the radioactive tag will be present on the parent compound as well as on its metabolites; therefore, without separation, there is no way of distinguishing between these [258].

4.1.1.2 Competitive Protein-binding Radio-assay and Radio-immunoassay

Adaptations of radio-assay emerged in the form of (i) competitive protein-binding radio-assay and (ii) radio-immunoassay [263]. In these methods, a known quantity of a radiolabelled compound is added to a known quantity of (i) a protein to which the compound specifically binds or (ii) an antibody to the compound [264–267]. The radiolabelled compound and the protein/antibody bind, and when a biological sample containing the same compound but unlabelled and in unknown quantities is added, competition for binding sites on the protein or antibody occurs between the labelled and unlabelled forms of the compound. Radiolabelled compound which is bound precipitates with proteins in the matrix, whilst that which is unbound is present in the supernatant and the radioactivity thereof is measured [264–267]. The higher the concentration of unlabelled compound present in the biological sample, the greater the displacement of the radiolabelled form from the protein/antibody binding sites and thus the greater the fraction unbound present in the supernatant and the higher the radioactivity count [264–267]. Although the specificity of these applications of radio-assay is improved, it is by no means guaranteed. Metabolites of the unlabelled compounds may however also interact with the protein or antibody, therefore the results obtained therefrom should still be interpreted with caution [268].

4.1.1.3 Non-radioactive Methods in Immunoassay

The use of radiolabelled substances requires additional policies and procedures to be put in place to avoid exposure to radioactivity during the analysis and to ensure safe disposal of radioactive waste [269]. This called for the development of immunoassay methods with non-radioactive labels [269; 270]. The most common of these which have been applied to bioanalysis include chemiluminophores [271–273], fluorophores [274–276] and enzymes [277–285]. Enzyme levels are quantitated through the ability of these to form coloured [277–279], fluorescent [282–285] or luminescent [280; 281] compounds from neutral substrates [269; 270].

4.1.1.4 Chromatography [286]

Chromatography is a separation method which was first described by a Russian botanist in the early years of the 20th century. Chromatography has revolutionised bioanalysis due to the high selectivity which may be achieved due to the separation of individual analytes prior to detection and quantitation. Analytes are injected onto a column containing a stationary phase, through which a mobile phase is passed. Differential affinity of analytes between the two

phases occurs due to differences in physicochemical properties and this, in turn, results in separate elution of the analytes from the column. As each analyte elutes, it is directed through a flow cell where detection of the analyte occurs. The signal response obtained therefrom is directly proportional to the concentration or mass of the analyte present in the detector.

4.1.1.4.1 Gas Chromatography

In gas chromatography (GC), the mobile phase is an inert gas and the analytes are also required to either be in gaseous form or a thermostable, volatile form. This chromatographic technique is thus limited in its usefulness in bioanalysis [286]. Chemical derivatisation of samples for conversion of the analytes into substances with the above mentioned properties may be undertaken prior to GC analysis, but this labour-intensive and time consuming complication has been the main contributing factor where GC has been largely superseded by high performance liquid chromatography (HPLC) in bioanalysis [268; 286].

4.1.1.4.2 High Performance Liquid Chromatography with Conventional Detection Methods

The versatility of high performance liquid chromatography (HPLC) in comparison to GC in bioanalysis lies in the wider range of analytes which may be separated on the column as this may be achieved when these remain dissolved in a sample solvent as it is carried through by a liquid mobile phase [286]. Furthermore, there is a greater variety of detectors which are appropriate for the quantitative analysis of compounds in liquid form [287; 288]. In the 1960s and 1970s attempts to convert GC detectors for HPLC were unsuccessful since efficient nebulisers were required for evaporation of mobile phase, and sufficient advancements in this type of technology had not yet been made [288]. Since then, several detection methods suitable for HPLC have been employed in bioanalysis, particularly UV, fluorescence and electrochemical detection.

4.1.1.4.2.1 UV Detection

The UV detector which is the most commonly used detector in HPLC was introduced by Horvárth and Lipsky in 1966, with further developments made by Kirkland in 1968 [288]. Through the 1970s, single wavelength (254 nm) UV detectors were available and in the subsequent decade, a variable wavelength detector was used [288]. The most recent adaptation is the photodiode array (PDA) detector [287–289]. In each case, the detector is set

to one or more wavelengths of UV light and responds to analytes which absorb light at these wavelengths [287–289].

As the name suggests, a variable wavelength detector may be set to any specific wavelength at any one time. Some may be programmed to change wavelengths for the analysis of different analytes within the same run [288; 290]. In a variable wavelength detector, white light is directed through a slit and onto a diffraction grating which splits the light into component UV wavelengths [287–289]. Another slit thereafter only allows the passage of the light of the set wavelength to the flow cell, through which the analytes pass. Light not absorbed strikes a photodetector, from which a signal response is generated [287–289].

The PDA detector has a similar set-up, except that white light passes through the flow cell before the diffraction grating, which spreads the UV spectrum of light across an array of photodiodes [287; 288]. The intensity of the signal from individual photodiodes depends on the extent of absorption of the corresponding wavelength of light by the analyte in the flow cell. These signals are processed to generate a UV spectrum of the analyte. A PDA detector may be programmed to collect data at one or more wavelengths simultaneously in the same run or at all UV wavelengths to generate a full spectrum [288–290]. Thus, three-dimensional data may be collected for each peak, namely retention time, wavelength spectrum and absorbance unit response [290]. The use of two different wavelengths to analyse each of two compounds eluted in the same run may provide for appropriate selectivity to compensate for inadequate chromatographic separation [288; 289]. Peak identification and peak purity may also be determined by comparing the spectra obtained at different times (beginning, middle and end) [287–290].

A limitation of the use of the UV detector is that the compounds of interest must have a UV chromophore to enable a signal response and thus detection of the compounds [287–290]. Furthermore, solvents and components of the mobile phase must transmit UV light to allow the light to reach the UV absorbing analytes dissolved therein [287; 288]. Solvents of HPLC grade available today, in particular, acetonitrile, have a UV transmission-cutoff wavelength as low as 200 nm. Despite these shortcomings, UV detection has remained a common method of detection in bioanalysis probably because, in general, it compares favourably to other methods in terms of reliability and robustness [287; 288].

4.1.1.4.2.2 Fluorescence Detection

Fluorescence detectors may be used for solutes which fluoresce when excited by UV light [287; 288]. This limits the range of analytes which may be detected, although chemical derivitisation of analytes may be undertaken to obtain compounds which meet these criteria. Ironically, it is this limitation which enhances the selectivity of fluorescence detection, since few endogenous co-eluting interferences are likely to exhibit this property [287; 288]. Sample extraction and pre-treatment steps may thus be simpler. Sensitivity is also about three orders of magnitude greater than UV detection [287].

The excitation wavelength of UV light is selected by setting a monochromator or filter [287; 288]. This particular wavelength of UV light illuminates the analyte in the sample as it passes through the flow cell. The emission wavelength is set by another monochromator or filter, thus light of that wavelength emitted by the analyte is directed to a photodetector to effect a signal response [287; 288]. Analogous to UV detection, the mobile phase should have the ability to transmit UV light.

4.1.1.4.2.3 Electrochemical Detection

Electrochemical detection is limited to analytes of interest which are subject to oxidation or reduction and to the use of mobile phases which are electrically conductive [287; 288; 291]. The flow cell in an electrochemical detector contains three electrodes, namely, the reference, working and counter electrodes. A potential is applied to the working electrode which is standardised by the reference electrode [287; 288; 291]. The potential allows for electrochemical reactions to occur at the surface of the working electrode, from which a current is generated. The electrons for compound reduction are provided by the auxiliary electrode whilst free electrons are released from the analyte to this same electrode upon oxidation. In either case, the electrical current generated is converted to a signal response [287; 288; 291]. Compounds that are electrochemically active are more common than those which fluoresce, thus electrochemical detection is more versatile, but less selective than fluorescence detection. Electrochemical detectors are more sensitive than UV or fluorescence detectors; however, there is a high risk of electrode contamination and background noise as a result of certain mobile phase components [288].

4.1.1.4.3 Liquid Chromatography-mass spectrometry

A hyphenated technique is formed when an independent analytical instrument is coupled to HPLC. Liquid chromatography coupled to mass spectrometry has in the last decade become popular in bioanalysis.

A single mass detector measures a single ionic species for each analyte, usually the molecular ion and this hyphenated technique is termed LC-MS [288]. In multiple-reaction monitoring, the molecular ion is fragmented further and one or more of these are monitored and the analogous acronym is LC-MS/MS [288; 292]. The latter is more commonly used in bioanalysis due to the greater selectivity, sensitivity and throughput [292].

Ionic species of analytes are required for detection using MS [287; 288; 293]. The high ionisation efficiency and easy fragmentation of many drugs contributes to the high sensitivity of LC-MS/MS [292]. A variety of ionisation techniques are used, most of which excite the neutral analyte molecule which then ejects an electron to form a radical cation [287; 288; 293]. Others involve ion molecule reactions that produce adduct ions. Electron ionisation and chemical ionisation are only suitable for gas phase ionisation, which requires a liquid-to-gas phase conversion of the column eluent prior to ionisation. Fast atom bombardment, secondary ion mass spectrometry, electrospray, and matrix assisted laser desorption have the ability to ionise liquid samples directly [287; 288; 293].

After ions are formed in the source region they are accelerated into a mass analyser by an electric field [287; 288; 293]. The mass analyzer separates these ions according to a mass-to-charge ratio (*m/z*). The most commonly used mass analyser is the quadrupole which filters the ions according to their *m/z* value so that only a selected single *m/z* ion is allowed passage to the detector at any one time [287; 288; 293]. In LC-MS/MS, also termed tandem MS, a triple quadrupole is used. The first quadrupole filters a selected single ion, the second one fragments the ion and the third isolates a specific ion fragment, which is detected. It is the detection of specific ion fragments which contributes significantly to the high selectivity of LC-MS/MS [287; 288; 292; 293]. The higher selectivity of LC-MS/MS compared to HPLC-UV may also allow for higher throughput since chromatographic separation between analytes and endogenous interferences may not be necessary, allowing shorter run times to be set [292].

4.1.2 Methods for the Bioanalysis of ATV

There are several methods described in the literature for the quantitative analysis of ATV in biological matrices, either alone [294–300] or in combination with other ARVs [301–329]. Most reported the use of LC-MS/MS [294; 296; 305; 311; 312; 314; 315; 317–322; 324–327; 329] or HPLC with UV detection [297–304; 306; 307; 309; 310; 313; 316; 323; 328; 330; 331]. An enzyme immunoassay has also been described. In this method, acetylcholinesterase was the enzyme label and acetylthiocholine iodide the substrate, the product of which reacts with dithiobisnitrobenzoate to form a yellow coloured compound, detected spectrophotometrically at 410 nm [295]. This latter method together with LC-MS/MS were the only techniques sensitive enough for the assay of intracellular levels of ATV in peripheral blood mononuclear cells (PBMCs) and T-lymphocytes after oral administration of the PI to healthy human subjects or patients [294; 315; 326]. The use of radiolabelled ATV with consequent radio-assay has been popularly used for cell uptake and accumulation studies of ATV in cultures of T-lymphocytes, the Caco-2 cell line and the human brain microvessel endothelial cell line [332–335].

The application of the ATV bioanalytical method in this research does not warrant the use of LC-MS/MS. Selective, sensitive and high throughput HPLC-UV methods for the quantitative analysis of ATV in plasma have been successfully developed even in the presence of co-administered ARVs, which, in the present study, is of no consequence. The robustness and reliability of HPLC-UV was thus favoured ahead of the general limitations often associated with its use. This chapter details the development and validation of an HPLC-UV method for the quantitative analysis of ATV in human plasma and liver microsomes.

4.2 Development of an HPLC-UV Method for the Analysis of ATV

4.2.1 Introduction

The individual strategies used in the development of an HPLC method may vary greatly from one chromatographer to another. However, a systematic approach would usually include the following steps: (i) acquiring information on the sample and defining separation goals, (ii) choosing an appropriate detector, (iii) choosing an initial HPLC method and (iv) optimising the HPLC method [336]. Information on the chemical composition of the samples and the physicochemical properties of these components as well as the goals of separation allows for a theoretical rather than an empirical approach to the other three steps in HPLC method development [336]. In this research, the ultimate goal was to develop an HPLC-UV method for the quantitative analysis of ATV in human plasma and liver microsomes. Since steps one and two have already been established, a theoretical approach could be followed in which published HPLC-UV methods with isocratic elution for the quantitative analysis of ATV in plasma (Table 4.1) were used to direct the choice of a suitable initial HPLC method and the optimisation thereof.

Parameters which constitute the chromatographic conditions for HPLC-UV include the: column, mobile phase, column temperature, flow rate, injection volume, sample solvent, UV detection wavelength and internal standard. These have an impact on the sensitivity, selectivity and throughput of the method [292]. Optimal chromatographic conditions are achieved when the combination of these produces a low limit of detection, short retention times and good resolution with symmetrical peaks. It is evident from Table 4.1, that a wide variety of configurations for these parameters have been used by others for the analysis of ATV in plasma.

Table 4.1: Published isocratic HPLC-UV methods for the quantitative analysis of ATV in human plasma

Column	Column Temperature (ºC)	Mobile phase	Flow rate (ml/min)	Detection wavelength (nm)	Extraction method	Injection Volume (µl)	Internal standard (IS)	Retention time of ATV (min)	Retention time of IS (min)	[Reference] ARVs quantitated
Allsphere C_6 5 µm (150 X 4.6 mm i.d.)	NR	Acetonitrile-methanol-sodium dihydrogen phosphate buffer (15 mM, pH 4.5) (35: 20: 45, v/v/v)	1	215	LLE	30	A86093	11.7	20.3	[301] ATV/other PIs
Ultrasphere C_8 4 µm (150 X 4.6 mm i.d.)	30	Water-methanol-acetonitrile (45: 20: 35, v/v/v)	1	260	SPE	30	Diazepam	13	6.5	[300] ATV alone
XTerra C_{18} 3.5 µm (150 X 4.6 mm i.d.)	30	Acetonitrile-phosphate buffer (52.5mM, pH 6) (43: 57, v/v)	1.5	203	SPE	20	NR	16.2	N/A	[299] ATV alone
S3 YMC C_8 (150 X 3.0 mm i.d.)	35	(52/48) Acetonitrile-monobasic Potassium phosphate buffer (25mM, pH 4.9) (48: 52, v/v)	0.4	212	LLE	25	Delavirdine mesylate	10.1	5.7	[302] ATV/otherPIs/NNRTIs
Kromasil C_{18} (150 X 3.0 mm i.d.)	NR	Acetonitrile-water (38/62, v/v)	1	210	SPE	50	Prazepam	18.5	22	[298] ATV alone
Nova-Pak C_{18} 4 µM (100 X 8.0 mm i.d.)	30	Phosphate buffer-methanol-acetonitrile (50 mM, pH 5.9) (39/22/39, v/v/v)	1.8	205	LLE	25	5-Thiazolylmethyl ester	8.2	15.2	[303] ATV/other PIs

NR: Not recorded

Columns with different organic surface layers usually bonded to silica-based packings are available in a variety of configurations. Totally porous silica microspheres each with a diameter of 3−10 µm and pore diameters of 7−12 nm (70−120 Å) have the greatest utility for small molecules (<10 000 daltons) [337]. The smaller the pore diameter, the shorter the retention times with equivalent resolution [337]. However, low pore diameter columns tend to plug easily, reducing column lifetime [337]. Silica exhibits high solubility at pH ≥9 which limits the use of high pH mobile phases [337; 338]. Another undesirable characteristic of silica is the acidic surface free silanol groups that bind basic compounds such that peak broadening and tailing results [337; 338]. To reduce this silanol effect, mobile phases with acidic pH should be used when separating basic compounds [337; 338]. However, modern silica-based columns have associated or hydrogen-bonded silanols that minimise this negative effect [337; 338]. The silanol groups of reversed phase HPLC columns are typically alkylated and it is the length of the alkyl chain (C_1–C_{18}) that predicts the degree of retention offered by the column [337; 338]. Generally, in columns where silanol effects are of no consequence, hydrophobic interactions predominate and therefore, the longer the alkyl chain, the greater the retention and thus the longer the retention times of the analytes [337; 338].

The internal diameter (ID) of a column is of particular significance with respect to the sensitivity of the method. Chromatographic theory shows that the limit of detection (LOD) is directly proportional to the square of the column radius [339], but is associated with a higher risk of extra-column peak broadening, especially if the ID is not complemented with the appropriate connectors, tubing and flow cell of the HPLC system [337]. Column efficiency is largely dictated by HPLC column lengths which range from 30–250 mm [337]. A longer column provides a greater area over which separation may occur and is thus more suited for complex mixtures and longer retention times are expected [337; 340].

Mobile phases used in reversed-phase chromatography are mixtures of organic and aqueous-based solvents [341; 342]. The former are usually acetonitrile and methanol, whilst the latter may be water or buffers in the pH range 2–8 [341; 342]. The analytes partition between the mobile phase and the C_8 or C_{18} stationary phase of the column. Acetonitrile exhibits greater elution strength than methanol and as the organic solvent strength increases, the earlier the analyte elutes [341; 342]. Resolution, peak shape, and retention time may be influenced by the type of organic solvent used, the organic solvent strength and even by using ternary mixtures, composed of both organic solvents [341; 342]. The use of buffers as the aqueous

component in mobile phases also impacts on these parameters provided that the analytes are ionisable [341; 343]. When the pH of the buffer in a mobile phase results in the partial or complete ionisation of the analyte, the retention time drops in comparison to that obtained in the absence of a buffer but with the same organic solvent strength [341; 343]. The peak shape also often improves [342].

An increase in column temperature enhances the kinetic energy of analyte molecules during interactions with both the stationary and mobile phases. For neutral compounds retention times of analytes relative to each other are not usually significantly affected [342]. In contrast, since pKa and pH are temperature-dependent, the ionisation state of ionisable analytes may be increased as temperature is elevated and since the effect on each may be unique, retention times relative to each other may be altered, which may in turn change peak resolution and shape [343]. Flow rate is indirectly proportional to retention time although higher flow rates may compromise resolution due to shorter times available for interaction of the analytes in the mobile phase with the column which facilitates separation. A higher injection volume increases the sensitivity of the method, although care should be taken not to surpass the linear range of the detector [344]. Furthermore, column overload should be avoided as this may contribute to peak tailing and peak broadening [344]. These undesirable peak distortions may also be prevalent with the use of a sample solvent which is stronger than the mobile phase [345]. Changing the UV detection wavelength may alter the sensitivity and/or selectivity of the method. A wavelength at which an analyte exhibits higher absorptivity enhances sensitivity, whilst an analogous wavelength for a co-eluting interferent may reduce resolution.

An internal standard is necessary to compensate for changes in sample size or concentration which may occur during sample pre-treatment/extraction or due to variation in instrument performance [346]. A challenge which faces chromatographers is to find an internal standard (IS) which behaves similarly to the analytes during sample pre-treatment procedures and under the same chromatographic conditions [346].

The main aims of sample preparation are to obtain an analyte-containing aliquot that is compatible with the mobile phase of the HPLC method and free of interferences that may affect the selectivity of the method and the longevity of the column [347–349]. Another advantage is that a preconcentration step may be included if necessary, which enhances the

sensitivity of the method [348; 349]. Procedures with higher throughput are also preferred [348; 349].

Typically, there are three most commonly used pretreatment methods for liquid samples [350]. Dilution is the most economical of these in terms of time and cost and involves the dilution of the sample with a solvent compatible with the mobile phase to avoid column overload and to ensure that the linear range of the detector is not surpassed [350]. This "dilute and shoot" approach may be modified for bioanalysis by ensuring that the solvent used is also a protein precipitant [347; 349]. A centrifugation step is then added and an aliquot of the supernatant is injected onto the column [347; 349]. Protein precipitation is simple, fast and may be applied to both hydrophilic and hydrophobic analytes [347; 349]. However, it is often not sufficient to remove biological components which interfere with resolution and/or reduce the longevity of the column and may lead to low extraction efficiency (EE) due to analyte coprecipitation [349].

In liquid-liquid extraction (LLE), a sample is partitioned between two immiscible phases [350]. An organic solvent, or mixture of solvents, is added to aqueous samples and *vice versa* [350]. These are selected based on a high differential solubility of the analytes of interest between the phases [347–349]. Such differential solubility may be manipulated and thus maximised through the use of additives such as buffers for pH adjustment, salts for ionic strength, complexing agents and ion-pairing agents [350]. Analytes in the organic phase are easily recovered by evaporation of the solvent. Reconstitution of residues in an appropriate mobile phase compatible solvent provides an opportunity to concentrate the analyte to elevate the sensitivity of the method [347–350]. Aliquots of the aqueous phase may often be injected directly onto the column for analysis of hydrophilic analytes [350]. Biological samples are aqueous in nature; therefore, immiscible organic solvents are used to facilitate extraction [347; 349; 350]. The aim is usually to extract the analytes of interest into the organic phase to eliminate the aqueous biological components that may cause selectivity issues [347; 349; 350]. This is another advantage of LLE over protein precipitation, although it also means that LLE is not suitable for the extraction of hydrophilic analytes [347; 349]. Some practical problems may occur with the use of LLE in bioanalysis. The formation of an emulsion close to the interface between the two phases may trap analyte molecules and thus reduce analyte EE [348; 350]. Similarly, binding of the analyte to proteins may also reduce recovery [350].

Emulsion formation is commonly prevented by adding a salt to the aqueous phase, or by heating or cooling the extraction vessel [350].

Solid-phase extraction (SPE) is a chromatographic procedure, therefore the efficiency of the separation of the analytes from biological interferences is superior to both LLE and protein precipitation [349; 350]. This also potentially translates to a higher EE in comparison to LLE [350]. In reversed phased-SPE, small cartridges or columns are usually packed with C_{18}-silica [350]. A liquid sample is added to the cartridge/column and a wash solvent is passed through which results in either strong retention or weak retention of the analyte on the sorbent [350]. Interferences with differing affinities for the SPE column/cartridge are conversely manipulated and thus separated from the analytes of interest. In bioanalysis, strong retention of the analytes is preferred and these are subsequently eluted using very small volumes of a strong elution solvent [350]. This preconcentrated eluent may thus be redissolved in a solvent compatible with the HPLC mobile phase [347–350] and saves on the evaporation time which is inevitable for concentration of samples in LLE. SPE cartridges may only be used once before discarding [347; 349; 350], since potential interferences may remain thereon [350]. Furthermore, cartridges with a variety of different sorbents may initially be required during method development [350]. In essence therefore, this form of sample pretreatment, although efficient, may not be as cost-effective. However, it may be argued that more thorough removal of biological components may extend the life of the HPLC column, avoiding frequent replacement thereof. Reproducibility of SPE separations and thus selectivity and EE may be jeopardised due to batch-to-batch variability of the sorbent in the SPE cartridges or columns [349; 350].

In previously published isocratic HPLC-UV methods for the quantitative analysis of ATV (see Table 4.1), SPE and LLE have been used for sample preparation with equal frequency. There is a good track record of the extraction of PIs and NNRTIs from plasma using organic solvents such as diethylether, ethyl acetate, *tert*-butyl methyl ether and hexane [347]. High recoveries and clean extracts with negligible matrix effects are obtained in most of the cases [347]. Thus, due to a reasonable balance between efficiency and cost-effectiveness, the three LLE methods of extraction of ATV from plasma were examined and these are delineated in Table 4.2. All methods [301–303] involved initial alkalinisation of the plasma to convert all the basic analytes including ATV into an unionised form to ensure the differential solubility and affinity of these for the organic phase which was subsequently added. Two of the

methods used a mixture of hexane and ethyl acetate as the organic phase, although the volumes utilised varied [301; 303]. Verbesselt *et al.* (2007) [301] included a back extraction for added selectivity.

Using the introductory information provided as a basis, the following experiments were designed to establish optimal chromatographic conditions and an extraction method in standards of ATV dissolved in mobile phase. Samples of the analyte in the intended biological matrices (plasma and liver microsome) were deliberately avoided at this stage to optimise the method without the complication of matrix effects.

Table 4.2: Liquid-liquid extraction procedures used in previously published isocratic HPLC-UV methods for the bioanalysis of ATV in plasma

Takahashi *et al*., 2005 [303]	**Verbesselt *et al.*, 2007** [301]	**Weller *et al*., 2007** [302]
1. 500 µl plasma + 1 ml sodium carbonate (0.5 M) + 2 ml hexane-ethylacetate (50:50, v/v), containing IS. 2. Vortex. 3. Centrifuge for 5 minutes at 3500 x g. 4. Separate organic layer. 5. Evaporate organic phase to dryness. 6. Dissolve dry residues in 100 µl of mobile phase. 7. Centrifuge at 13000 x g for 5 minutes 8. Inject 25 µl supernatant onto column.	1. 500 µl plasma + 50 µl IS + 500 µl sodium tetraborate buffer (0.05 M, pH 9) + 5ml hexane-ethylacetate (50:50, v/v). 2. Shake for 10 minutes. 3. Centrifuge for 5 minutes at 1286 x g. 4. Freeze aqueous layer in a cooling mixture of acetone and dry ice. 5. Evaporate organic phase to dryness with an airstream in a waterbath at 40 ºC. 6. Dissolve dry residues in 300 µl acetonitrile-potassium phosphate buffer (15 mM, pH 2.4) with 0.05% triethylamine (45:55, v/v). 7. Wash reconstituted residues for 15 s with 2 ml hexane. 8. Centrifuge and freeze aqueous layer as above. 9. Discard hexane layer. 10. Thaw aqueous layers and remove hexane with a cold airstream for 2 minutes 11. Inject 30 µl onto column.	1. 200 µl plasma + 25 µl IS + 200 µl 0.01 M sodium hydroxide. 2. Vortex. 3. Add 2 ml methyl-*tert*-butyl ether. 4. Vortex for 10 s. 5. Centrifuge at 1000 x g for 5 minutes. 6. Freeze aqueous layer in a dry-ice isopropyl alcohol bath for 10 minutes. 7. Dry organic layer to dryness under nitrogen at 40 ºC. 8. Dissolve dry residues in 200 µl mobile phase. 9. Inject 25 µl supernatant onto column. .

4.2.2 Materials and Methods

4.2.2.1 Chemicals and Reagents

ATV sulphate (100.9%) was donated by Aspen Pharmacare (Port Elizabeth, South Africa) and diazepam (DIAZ) was obtained from the Biopharmaceutics Research Institute (Rhodes University, Grahamstown, South Africa). HPLC grade acetonitrile was purchased from Romil Ltd (Cambridge, United Kingdom). Water was purified by reverse osmosis and filtration through a Milli-Q purification system (Millipore, Milford, Massachusetts, USA). Sodium carbonate (99.5%) and ethyl acetate (99–101%) were provided by BDH laboratory reagents (Poole, England), whilst n-Hexane (≥98%) was purchased from Merck (Darmstadt, Germany).

4.2.2.2 Instrumentation and Equipment

The HPLC system consisted of an Alliance 2695 Separations module and a 2996 Waters photodiode array UV detector coupled to Empower data acquisition software (Waters, Milford, MA, USA). There were two columns available for chromatographic separation, which differed only in ID: Luna C_{18} (2) (5 µm, 150 X 4.6 mm ID) and Luna C_{18} (2) (5 µm, 150 X 2.0 mm ID) columns (Phenomenex®, USA). The columns were protected by a Luna C_{18} guard column (Phenomenex®, USA) with the same ID. Mobile phases were filtered under reduced pressure through a 0.45 µm (PVDF) membrane (Durapore®, Millipore, Bedford, MA, USA) and degassed using an Eyela Aspirator A-25 (Tokyo Rikakikai Co Ltd, Tokyo, Japan) prior to use. A Mettler Toledo AG135 analytical balance, supplied by Microsep (Pty) Ltd (Sandton, Gauteng, South Africa) was used to accurately weigh drug samples and "A" grade measuring cylinders (Hirschmann Laborgeräte GmbH & Co KG, Eberstad, Germany) and volumetric flasks (Glassco, Beckenham, United Kingdom) were used to prepare (i) mobile phases and (ii) stock solutions and working standard solutions respectively. Samples for HPLC analysis were transferred to 0.1 ml micro-inserts in 1.5 ml vials with caps, all purchased from Separation Scientific SA Pty Ltd (Johannesburg, South Africa). A Mixmate® vortex mixer from Eppendorf AG (Hamburg, Germany), a Ministar Plus® centrifuge from Hangzhou Allsheng Instruments Co Ltd (Hangzhou City, China) and an N-EVAP® Analytical Evaporator from Organomation Associates Inc (Massachusetts, USA), as well as 2 ml microcentrifuge tubes (Optima Scientific, Cape Town, South Africa) were used during extraction procedures.

4.2.2.3 Preparation of Stock Solutions

A stock solution of ATV was prepared by dissolving an accurately weighed amount of ATV sulphate in acetonitrile-water (40: 60, v/v) to provide the equivalent of 1.0 mg/ml ATV. A stock solution of diazepam (100 µg/ml) was prepared likewise. All stock solutions were stored in a refrigerator at 4 ± 2 °C.

4.2.2.4 Preparation of Mobile Phases

Appropriate volumes of each component of the binary mobile phases were accurately measured to result in the desired ratios to prepare a total volume of 500 ml, which was filtered and degassed.

4.2.2.5 Preparation of Samples

The stock solutions were used to prepare several working standard solutions of ATV in a concentration range 0.1–50 µg/ml, by serial dilution with mobile phase. Each standard was spiked with DIAZ stock solution to achieve a final concentration of 2.5 µg/ml. Each sample solution was transferred to an HPLC vial and loaded onto the autosampler of the HPLC system.

4.2.2.6 Initial Chromatographic Conditions

The initial chromatographic conditions, shown in Table 4.3, were chosen mainly based on previously published HPLC-UV methods for the quantitation of ATV in human plasma, depicted in Table 4.1, as well as the physicochemical properties of ATV described in Chapter 2, section 2.6.

Table 4.3: Initial chromatographic conditions

Parameter	Configuration
Type of column	Luna C_{18} (2) (5 µm, 150 X 4.6 mm i.d.)
Mobile Phase	Acetonitrile-water (40: 60, v/v)
Flow rate	1.5 ml/min
Injection volume	20 µl
UV detection wavelength	280 nm
Internal standard	Diazepam
Column temperature	30 ºC

According to Snyder *et al.* (1997) [337], analytical methods are usually best developed with columns with an internal diameter (ID) of 4.6 or 3.0 mm, packed with particles in the range of 2 to 10 µm. Thus, of the two types of columns available, the column with a 4.6 mm ID was

preferred for the initial chromatographic conditions. This column offered the potential for a greater column efficiency and thus resolution compared to the 2.0 mm ID one, due to its relative insensitivity to extra-column effects.

The simplest mobile phase in the previously published methods found in Table 4.1 was a binary mixture of acetonitrile and water in the ratio 38: 62. As an initial mobile phase in the present study, this ratio was rounded off to 40:60. Using the ratio 38:62, a flow rate of 1 ml/min and a column with a slightly smaller ID of 3 mm, Cateau *et al.* (2005) [298] obtained a retention time of 18.5 minutes for ATV. In an attempt to reduce this retention time to allow for a shorter run time, a higher flow rate of 1.5 ml/min was selected. To minimise column load, a 20 µl injection volume was chosen. Two of the three methods which involve the quantitation of ATV alone used benzodiazepines, namely DIAZ and prazepam as internal standards (IS) and a column temperature of 30 ºC. DIAZ as an IS and this column temperature were therefore opted for. As a compromise between maximal detection sensitivity of ATV with minimal absorption of interferents, one of the local λ_{maxima} of ATV, namely, 283 nm, depicted in the UV spectrum in Chapter 2, Figure 2.7, was the basis for the selected detection wavelength of 280 nm.

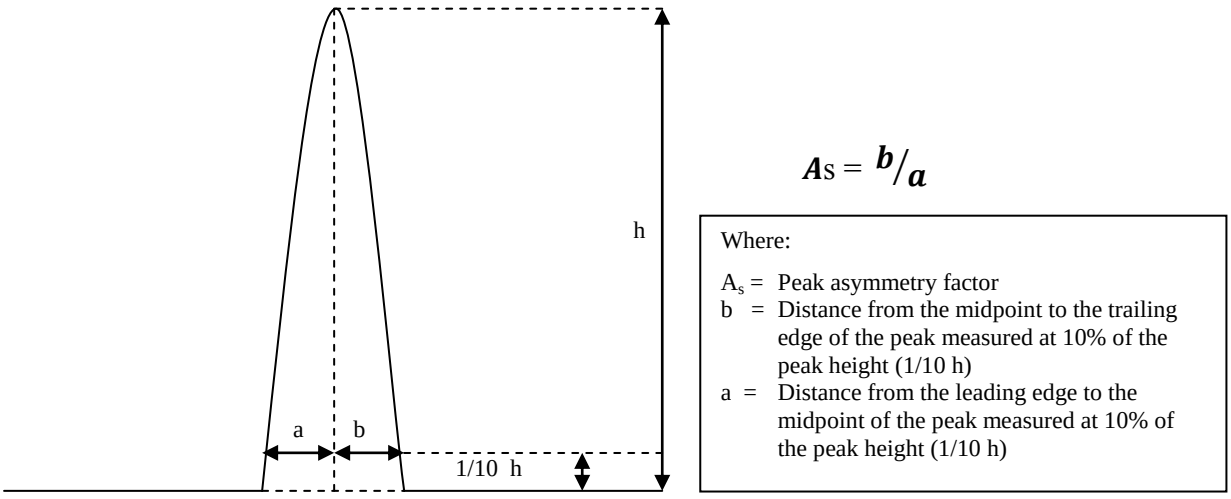


Figure 4.1: Schematic of a chromatogram and the corresponding equation to show the calculation of the peak asymmetry factor.

The initial chromatographic conditions were applied and the chromatogram generated was analysed to determine whether resolution, shape and retention times of ATV and IS peaks were adequate. Retention times were obtained by integrating the peaks of interest using the

data acquisition software, whilst resolution was subjectively determined. The shape of each peak was assessed for symmetry by calculating a peak asymmetry factor, as shown in the illustration and equation in Figure 4.1.

4.2.2.7 Optimisation of Chromatographic Conditions

If insufficiencies were evident in the analysis of the chromatogram in section 4.2.2.6 above, optimisation of the chromatographic conditions was required.

The first step in this optimisation process was to change the selection of mobile phase. The proportions of acetonitrile and water were first altered and changes in the chromatography with respect to the inadequacy identified in section 4.2.2.6 were graphically represented. Furthermore, if the chromatography was still not satisfactory, buffers with pHs in the range 2–8 were considered in place of water in the mobile phase.

To maximise the detection sensitivity of the method, larger injection volumes, lower wavelengths at which ATV exhibits greater UV absorbing capacity (see Chapter 2, Figure 2.7) and the use of a narrow-bore column with an ID of 2.0 mm were investigated. It has been shown that for the same sample mass, detection sensitivity may be substantially increased by using a column with a smaller ID [337; 338]. To obtain the same retention times for analytes using a column with a different ID, it is important to ensure that the same linear velocity of the mobile phase through the column is maintained [351]. This is achieved by adjusting the flow rate in proportion to the column cross-sectional area, which is, in turn, directly proportional to the square of the ratio of column diameter. Thus, according to Equation 4.1, a change from a 4.6 mm ID column to a 2.0 mm ID column requires a five times reduction in the flow rate. In the present study, a flow rate of 0.3 ml/min would thus be appropriate when the column of 2.0 mm ID is used.

$$No.of\ times\ to\ reduce\ flow\ rate\ by\ = \left(\frac{ID\ column\ 1}{ID\ column\ 2}\right)^2 \qquad \textbf{Equation 4.1}$$

$(4.6/2.0)^2 = 5.29 \approx 5$

Sensitivity was quantified by determining the LOD, which was defined as a signal-to-noise ratio of 3:1, for each combination or set of chromatographic conditions. Furthermore, chromatograms were carefully analysed to determine whether extra-column effects,

previously alluded to with the use of the narrow bore column, lead to unacceptable changes in peak shape and resolution. The final optimised chromatographic conditions were consequently selected.

4.2.2.8 Initial Extraction Method

Two previously published methods for the analysis of ATV in plasma have successful used ethyl acetate- n-hexane (50:50, v/v) in LLE (see Table 4.2), after alkalinisation of the plasma samples. The initial extraction method, shown in the flow chart (Figure 4.2) below is modified from the method by Takahashi *et al.* (2005) [303].

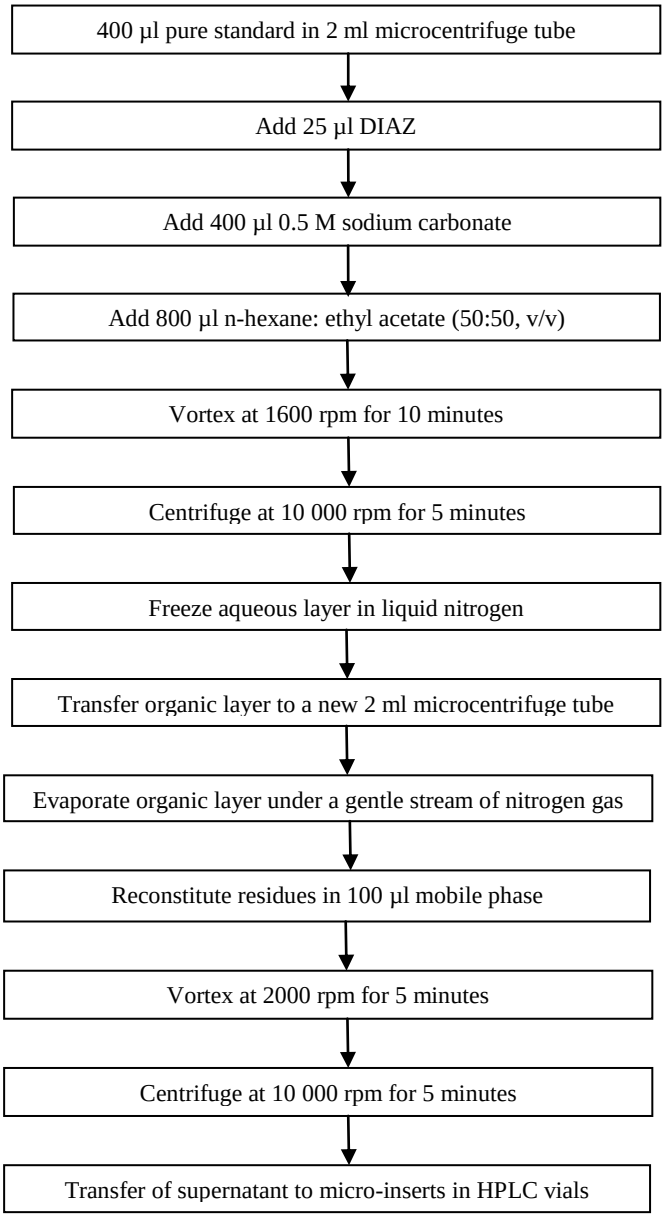


Figure 4.2: Flow chart showing the steps in the initial extraction procedure

In an effort to achieve concomitant multi-sample preparation, the method was modified from the procedures described by Takahashi *et al.* (2005) [303], such that the volume of a 2ml microcentrifuge tube was adequate for the extraction of each sample. A sample volume of 400 µl instead of 500 µl was used and the ratio of sample: sodium carbonate: ethyl acetate-n-hexane (50:50, v/v) was reduced from 1: 2: 4 to 1: 1: 2, giving a total extraction volume of 1.6 ml, instead of 3.5 ml.

4.2.2.9 Extraction Method Optimisation

The concentration of sodium carbonate was first varied to determine that which produced the greatest EE for both ATV and the IS, DIAZ. Similarly, the proportions of n-hexane and ethyl acetate in the organic extraction solvent were varied to determine the most appropriate ratio to use to maximise EE.

4.2.3 Results and Discussion

Figure 4.3 is a typical chromatogram obtained under the initial chromatographic conditions, which showed that DIAZ and ATV eluted at retention times of 12.5 and 25.8 minutes respectively. The peaks were well resolved from each other and had peak asymmetry factors of 1.16 and 1.10 respectively. The elution of DIAZ before ATV was expected since based on logP values, the hydrophobicity of ATV (see Chapter 2, section 2.6.3) is greater than that of DIAZ [352], and it therefore has a greater affinity for the hydrophobic C_{18} bonded phase of the column. The retention time of ATV was longer than that observed by Cateau *et al.* (2005) [298] where a similar mobile phase was used. It was retrospectively identified that using Equation 4.1, theoretically, a higher flow rate of 2.35 ml/min should have been set using the 4.6 mm ID column to obtain similar retention times to that of these authors who used a column with an ID of 3 mm. An even higher flow rate would be required to achieve retention times of 5–10 minutes which was the target, but the HPLC system used in this work would not have been able to withstand such a high flow rate. Peak asymmetry factors of 1–1.2 indicate acceptable symmetry [337] and therefore despite the absence of an acidic buffer for the analysis of these basic analytes, the peak shapes of both DIAZ and ATV were deemed adequate and silanol effects, if any, were of no consequence.

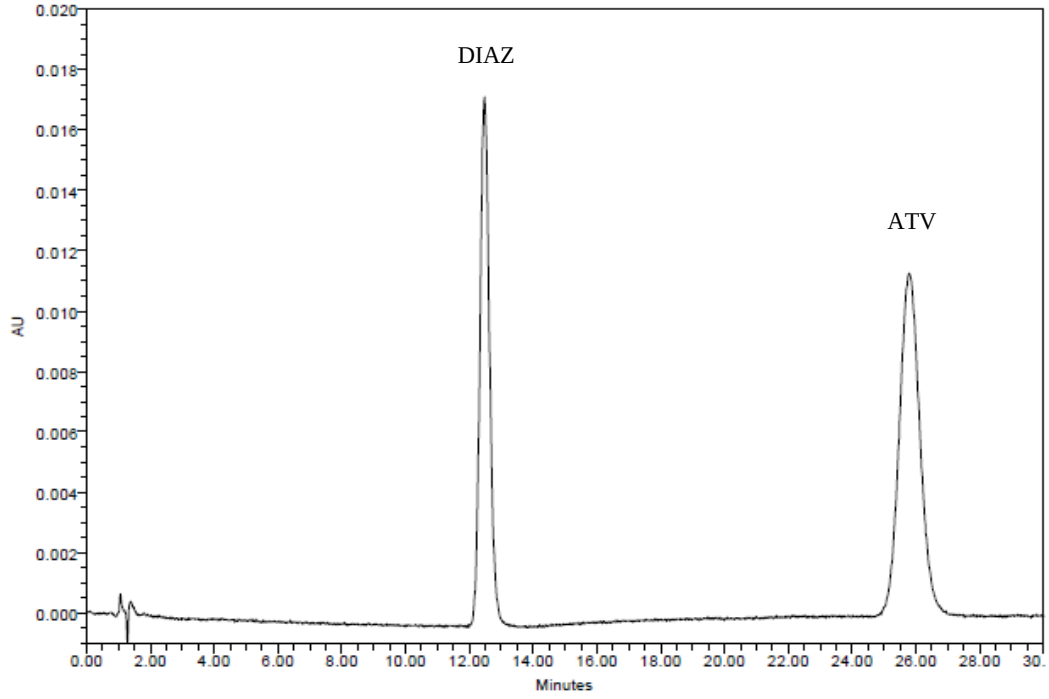


Figure 4.3: A typical chromatogram of ATV (10 µg/ml) and DIAZ (2.5 µg/ml) obtained using the initial chromatographic conditions

Increasing the percentage of acetonitrile from 40% to 50% in binary mobile phases consisting of mixtures of acetonitrile and water resulted in a reduction in the retention times of both DIAZ and ATV, as depicted in Figure 4.4. The retention time of ATV shifted from 25.8 to 7.08 minutes when the composition of acetonitrile was changed from 40 to 50%, whilst under the same conditions, the retention time of DIAZ moved from 12.5 to 5.3 minutes. The retention of DIAZ relative to ATV reduced with increasing strength of acetonitrile. ATV, being more hydrophobic than DIAZ, exhibited higher differential affinity between the C_{18} stationary phase and the initial mobile phase, and thus changes in the solvent strength produced more significant changes in retention for ATV than for DIAZ. Lower retention times when 50% acetonitrile was used were coupled with adequate peak resolution and slightly improved peak asymmetry factor values of 1.14 and 1.10 for DIAZ and ATV respectively. The mobile phase, acetonitrile-water (50:50, v/v) was thus selected for further development of other chromatographic parameters.

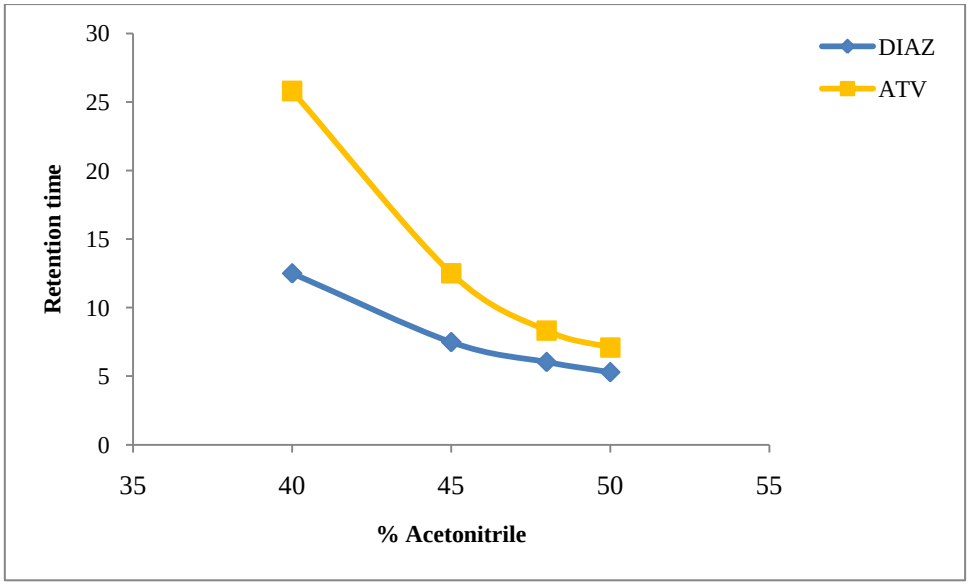


Figure 4.4: Effect of % acetonitrile in binary mobile phases of acetonitrile-water on the retention times of DIAZ and ATV

The optimised chromatographic conditions (Table 4.4) involved the use of a narrow-bore (2 mm ID) column with acetonitrile-water (50:50, v/v) as the mobile phase as well as the configurations for other parameters recorded in Table 4.4 below. These conditions gave rise to the lowest LOD (signal-to-noise ratio of 3:1) of 0.100 µg/ml. In comparison, using a 4.6 mm ID column at a flow rate of 1.5 ml/min instead, a higher LOD of 0.15 µg/ml was obtained which indicated that the narrower column did indeed exhibit greater sensitivity. It is evident from Figure 4.5 that under the chromatographic conditions stipulated in Table 4.4, the retention times for DIAZ and ATV at 5.5 and 6.9 minutes respectively were similar when samples were injected onto the 4.6 mm ID column at a flow rate of 1.5 ml/min (Figure 4.4, 50% acetonitrile). Peak asymmetry factor values were also similar at 1.15 and 1.12, for the two eluents respectively. An optimal UV detection wavelength of 210 nm was selected, since at this wavelength ATV clearly exhibited greater UV absorbing capacity (see Chapter 2, Figure 2.7) than at the initial wavelength of 280 nm and thus offered greater sensitivity, evident by the lower LOD of 0.100 µg/ml. The conditions listed in Table 4.4 were considered optimal for standards of ATV and were utilised in the search for an appropriate extraction method.

Table 4.4: Optimised chromatographic conditions

Parameter	Configuration
Type of column	Luna C_{18} (2) (5 µm, 150 X 2.0 mm i.d.)
Mobile Phase	Acetonitrile-water (50:50, v/v)
Flow rate	0.3ml/min
Injection volume	20 µl
UV detection wavelength	210 nm
Internal standard	Diazepam
Column temperature	30 ºC

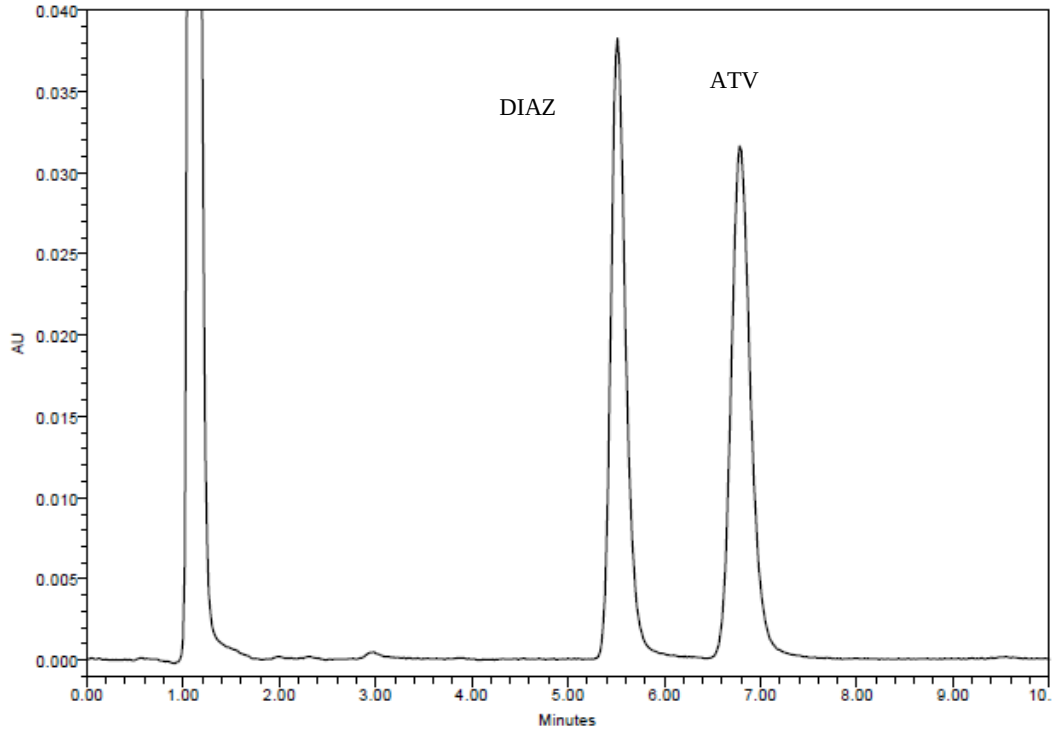


Figure 4.5: Typical chromatogram for ATV (10 µg/ml) and DIAZ (2.5 µg/ml)) obtained with the optimised chromatographic conditions.

The initial extraction method (Figure 4.2) modified from Takahashi *et al.* (2005) [303], which involved the use of 0.5 M sodium carbonate with ethyl acetate-n-hexane (50:50, v/v), resulted in an EE of 87.2 and 85.4%, for DIAZ and ATV from pure standards of ATV (10 µg/ml) and DIAZ (2.5 µg/ml) respectively (see Table 4.5). An increase in sodium carbonate concentration improved the EE, with 98.8 and 97.4% for DIAZ and ATV obtained with a concentration of 2 M, respectively. As previously mentioned, high salt concentrations are known to prevent emulsion formation, thus the higher EE was possibly due to reduced formation of emulsions at the interface of the two phases which was observed at lower concentrations of sodium carbonate. This, in turn may have avoided entrapment of ATV and DIAZ molecules in the droplets of organic phase present in the aqueous phase which form such emulsions. In contrast, the high EE deteriorated upon changing the proportion of ethyl acetate to more or less than 50%, indicating that both DIAZ and ATV have a greater

differential affinity between ethyl acetate-n-hexane (50:50, v/v) and the aqueous phase in comparison to such affinity when other solvent ratios were tested. The optimal LLE procedure differed from the initial method (shown in Figure 4.2) only in the concentration of sodium carbonate which changed from 0.5 M to 2 M.

Table 4.5: Change in EE with different concentrations of sodium carbonate and percentage ethyl acetate

Concentration of sodium carbonate	**EE (%)**		**Percentage ethyl acetate**	**EE (%)**	
(M)	DIAZ	ATV	**(%)**	DIAZ	ATV
0.5	87.2	85.4	25	80.3	76.2
1	92.7	90.2	50	98.8	97.4
2	98.8	97.4	75	89.5	85.9

Optimal chromatographic conditions and an acceptable LLE method suitable for the analytes of interest, namely ATV and the IS, DIAZ in mobile phase were thus developed. The application of this method to plasma and liver microsome samples was then investigated.

4.3 Development of an HPLC-UV Method for Analysis of ATV in Human Plasma and Liver Microsomes

4.3.1 Introduction

Liver microsomes and in particular, plasma are commonly encountered biological matrices in bioanalysis. The plasma is originally derived from blood, which, of all biological fluids, is of greatest analytical interest since it is the transport medium of drugs to the sites of action in humans. The concentration of a drug in the blood often correlates to the pharmacological action and may thus be used to predict the safety and efficacy thereof (Chapter 1, section 1.3.7.4). However, aside from the drugs of interest, blood also contains erythrocytes, monocytes, macromolecules such as proteins and lipids, and other endogenous or xenobiotic electrolytes and compounds [353]. Blood is usually collected in sample vials containing an anticoagulant such as ethylenediaminetetraacetic acid (EDTA) or heparin [353]. Cells are easily removed by centrifugation, and plasma, rich in lipids and lipoproteins, is harvested as the supernatant [353].

Selectivity and sensitivity issues have long been associated with the development of quantitative HPLC methods in plasma irrespective of the type of detection employed [292; 354]. Matrix effects, which may formally be defined as "the effect on an analytical method caused by all other components of the sample except the specific compound(s) to be quantified" often arise [354] and it is clear that the sources of these problems may largely be related to the presence of lipids, lipoproteins, the anticoagulant used during sample processing as well as dissolved endogenous and xenobiotic compounds. An even greater challenge with respect to developing selective and highly sensitive HPLC methods in plasma is that there may be inter- and intra-individual variability of these plasma constituents.

Fortunately, typical sample pretreatment strategies discussed in section 4.2.1 usually include a deproteination step. This may be achieved through: (i) a low pH by the addition of a strong acid, (ii) increase in ionic strength by the addition of salts, (iii) an increase in temperature, (iv) a change in dielectric constant by addition of organic solvents and (v) filtration and ultra-filtration [353].

Hydrophilic constituents of plasma are likely to be weakly retained on reversed-phase HPLC columns [337; 342] and thus elute within the first few minutes after injection. These are

usually of no consequence in terms of the selectivity of a method if retention times of the analytes are at least five minutes long. Co-eluting compounds possibly have one of two properties. These either exhibit a differential affinity between the column and the mobile phase, which is similar to that of the analyte, resulting in co-elution with the latter; or, if very hydrophobic, the compound may be strongly retained on the column inlet or on the column itself and co-elute with an analyte during the run time of a subsequent sample injection [337; 342]. Co-eluting compounds interfere with the selectivity of an HPLC-UV method if the UV absorbance is high at the selected wavelength [288]. This is particularly evident at wavelengths lower than 220 nm where a wide variety of compounds exhibit high absorbance [288]. The column packing may also be slightly modified by strongly retained interferents, which may affect column efficiency and thus diminish resolution and precipitate poor peak shape [337; 338].

Many hydrophilic and hydrophobic compounds are potential interferents when protein precipitation of plasma sample pretreatment is performed, whilst in LLE and SPE only, those compounds which are co-extracted with the analytes are available as potential interferents. Matrix effects on selectivity may be altered either by changing the chromatographic conditions, the sample pretreatment method or both. In order to avoid a concomitant drop in the sensitivity of the method, it may be prudent to change the mobile phase first before other parameters, such as the column, UV detection wavelength and injection volume on which sensitivity often depends. It is expected that the sensitivity of the method would be lower in the presence of the matrix [287; 292], but if severe impairment occurs, then the extraction method may need to be changed to include a more pronounced preconcentration step.

The liver microsomes purchased for studies detailed in Chapter 7 had an original protein concentration of 20 mg/ml in phosphate buffer. Subsequent steps during the experiment resulted in a 40 X dilution with phosphate buffered saline (0.1 M, pH 7.4) (PBS) to 0.5 mg/ml, therefore selectivity and sensitivity issues are less likely. Peak broadening may however result due to a possible incompatibility of PBS with the mobile phase [344].

4.3.2 Materials and Methods

4.3.2.1 Chemicals and Reagents

In addition to the chemicals and reagents used in section 4.2.2.1, ammonium formate (98%) from Riedel-de Haën (Seelze, Germany), formic acid (99.9%) from Associated Chemical Enterprises Pty Ltd (Johannesburg, South Africa), as well as phosphoric acid (85%) and potassium dihydrogen orthophosphate, both sourced from Saarchem (Pty) Ltd (Krugersdorp, South Africa), were also required. Fresh human plasma with potassium edentate (K-EDTA) as an anticoagulant was obtained from the South African National Blood Services, Eastern Cape Headquarters (Port Elizabeth, South Africa) and was stored at 4 ± 2 °C. Pooled human liver microsomes were purchased from BD Biosciences (Woburn, Massachusetts, USA) and were stored at -80 ºC. Phosphate buffered saline (PBS) (0.01 M, pH 7.4) was obtained from Sigma-Aldrich (Johannesburg, South Africa).

4.3.2.2 Instrumentation and Equipment

Other equipment required, aside from those described in section 4.2.2.2 included a Crison GLP21 pH meter (Barcelona, Spain).

4.3.2.3 Preparation of Mobile Phases

Mobile phases were prepared as described in section 4.4.2.5. However, if a deviation from the optimised chromatographic conditions found in section 4.2 was deemed necessary and a change in the mobile phase to one in which a buffer replaces the use of water was required, then an additional buffer preparation step was included. Buffers were prepared by dissolving an accurately weighed amount of the conjugate base in water to obtain the required buffer concentration. The buffer pH was adjusted by adding the corresponding acid dropwise until the desired pH, measured using a calibrated pH meter, was achieved.

4.3.2.4 Preparation of Plasma Standards

A stock solution of ATV was prepared by dissolving an accurately weighed amount of ATV sulphate in acetonitrile-water (50:50, v/v) (mobile phase) to provide the equivalent of 1.0 mg/ml ATV. A stock solution of the IS, DIAZ, was likewise prepared at a concentration of 100 µg/ml. All stock solutions were stored in a refrigerator at 4 ± 2 °C. A spiked plasma stock solution (20 µg/ml) was prepared by weight with blank plasma. The weight of blank plasma was converted to volume, using a plasma specific gravity of 1.025 g/ml [355]. Plasma

calibration standards were prepared from the plasma stock solution by weight, using serial dilution with blank plasma to provide plasma concentrations in the range 0.10–10 µg/ml.

4.3.2.5 Preparation of Liver Microsome Standards

A stock solution of ATV was prepared by dissolving an accurately weighed amount of ATV sulphate in acetonitrile-water (50:50, v/v) (mobile phase) to provide the equivalent of 5 mM ATV. A stock solution of the IS, DIAZ, was prepared in the same manner to obtain a concentration of 200 µg/ml. All stock solutions were stored in a refrigerator at 4 ± 2 °C. A liver microsome stock solution and a substock solution, both containing 50 µM ATV were prepared by diluting spiked liver microsomes to a protein concentration of 2 mg/ml and the ATV stock solution, respectively, using PBS, pH 7.4 as the diluent in each case. Two sets of calibration standards were prepared, one from the liver microsome stock solution and the other from the ATV substock solution (no microsomes present), both by serial dilution with mobile phase to produce concentrations of ATV in the range 2.5–15 µM.

4.3.2.6 Initial Plasma Sample Extraction Method and Chromatographic Conditions

The use of a simple protein precipitation extraction procedure was investigated by adding 800 µl mobile phase to a 400 µl plasma sample. This mixture was vortexed at 1600 rpm and centrifuged at 10 000 rpm, after which an aliquot of the supernatant was transferred to micro-inserts in HPLC vials. HPLC analysis was conducted under the optimised conditions described in Table 4.4.

4.3.2.7 Optimisation of Plasma Sample Extraction and Analysis

If selectivity and/or peak shape were compromised with the use of the "dilute and shoot" approach described above, then plasma samples were extracted using the optimised LLE procedure (discussed in section 4.2.3). The reconstituted residues were then analysed by HPLC-UV under the optimised chromatographic conditions.

If the method was no longer selective for the IS and/or ATV, or if unacceptable peak tailing was observed, a change in the mobile phase was considered, where buffers would be used in place of water. Since DIAZ and ATV are both weak bases with pKas of 3.4 ± 0.1 [352] and 4.65 ± 0.25 (see Chapter 2, section 2.6.1) respectively, buffers with pHs between 2 and 4, where one or both these drugs are at least 50% ionised, were selected.

Table 4.6: Buffers of desired pHs to be used in mobile phases for chromatographic separation of DIAZ and ATV

Desired pH	Buffer selected (Conjugate base/acid)	pKa of buffer	UV cutoff (maximum concentration)
2	Dibasic potassium phosphate/phosphoric acid	2.1	210 nm (5 mM)
2.5	Dibasic potassium phosphate/phosphoric acid	2.1	210 nm (5 mM)
3	Ammonium formate/formic acid	3.8	210 nm (10 mM)
3.5	Ammonium formate/formic acid	3.8	210 nm (10 mM)
4	Ammonium formate/formic acid	3.8	210 nm (10 mM)

The buffers shown in Table 4.6 were chosen for two main reasons:

i. The desired pH fell within ± 1 unit of the pKa of the buffer, which is necessary for effective buffering capacity [343].

ii. These buffers are optically transparent and therefore exhibit low absorbance at UV detection wavelengths as low as 210 nm, provided a maximum concentration of 5 mM for the phosphate buffer and 10 mM for the formate buffer is used [343]. Since a UV detection of 210 nm has been selected to enhance the sensitivity of the method, it would be prudent to use these buffers at these maximum concentrations.

Since the analytes will be partially or fully ionised at these pHs, it was expected that in a ratio of 50:50, the use of buffer instead of water with acetonitrile would result in shorter retention times with a possible loss of resolution between the DIAZ and ATV peaks, thus proportions of acetonitrile-buffer (45:55, v/v) were used in an attempt to maintain the resolution and retention times previously observed.

4.3.2.8 Liver Microsome Sample Extraction Method

The preparation of the liver microsome samples followed the procedure used to conduct the experiments in Chapter 7. Prior to analysis of samples, the original liver microsome solution (as purchased) would have been diluted 40 times. In light of this, direct injection of the samples was not expected to pose a problem with respect to selectivity and peak shape and was therefore first attempted. Another potentially positive spin off of this heavily diluted liver microsome solution was that the ATV concentrations contained therein could perhaps be calibrated using working standard solutions of an ATV substock solution which does not contain liver microsome solution, provided that the EE at each concentration is 100%. This approach, which has been utilised by Alkharfy *et al.* (2001) [356], offers more cost-effective use of the human liver microsomes. The feasibility of this approach was investigated by checking that the 99% CI for the EE (n = 3) at each concentration fell within the range 99–101%.

4.3.3 Results and Discussion

Figure 4.6 shows a chromatogram obtained from injection of a plasma sample containing ATV (10 µg/ml) and DIAZ (2.5 µg/ml) using the extraction procedure and chromatographic conditions described in section 4.2. Baseline resolution was clearly not exhibited between the DIAZ and ATV peaks and a shoulder on the ATV peak was observed. It therefore appeared that an interferent which had similar differential affinity between the stationary and mobile phases co-eluted with ATV and had high absorptivity at 210 nm. In addition, matrix components co-extracted with the analytes of interest may have reduced resolution between DIAZ and ATV by blocking or masking the column surface, altering its retention properties [357].

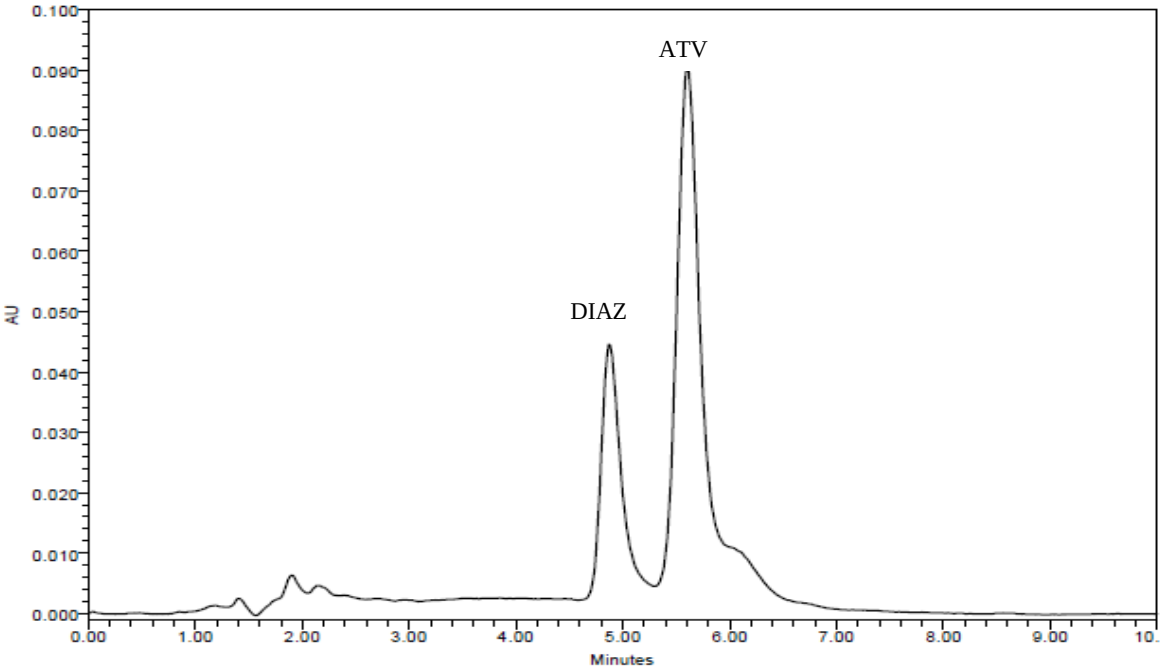


Figure 4.6: Typical chromatogram obtained from injection of a plasma sample containing ATV (10 µg/ml) and DIAZ (2.5 µg/ml) using acetonitrile-water (50:50, v/v) as the mobile phase.

In an attempt to regain selectivity, using the same extraction procedure and without affecting sensitivity, mobile phases of acetonitrile-buffer with composition, ionic strengths and pHs as described in section 4.3.2.7 were used. Those mobile phases with buffers of pH 2–3, but not those >3 eliminated the co-eluting interferent. At low pH (≤3), both DIAZ and ATV were partially or completely ionised (see section 4.3.2.7), which changed the differential affinity patterns for each between the stationary and mobile phases. On the other hand, at these pHs, the previous co-eluting interferent probably exhibits different physicochemical properties and

thus a differential affinity that was not the same as either analyte. Thus, this co-extracted compound no longer affected the selectivity of the method. Of these mobile phases, acetonitrile-ammonium formate (pH 3; 10 mM) (45:55, v/v) produced the best improvement in resolution between DIAZ and ATV and the shape of the peaks, as depicted in Figure 4.7. The retention times for DIAZ and ATV were 6.8 and 8.3 minutes respectively.

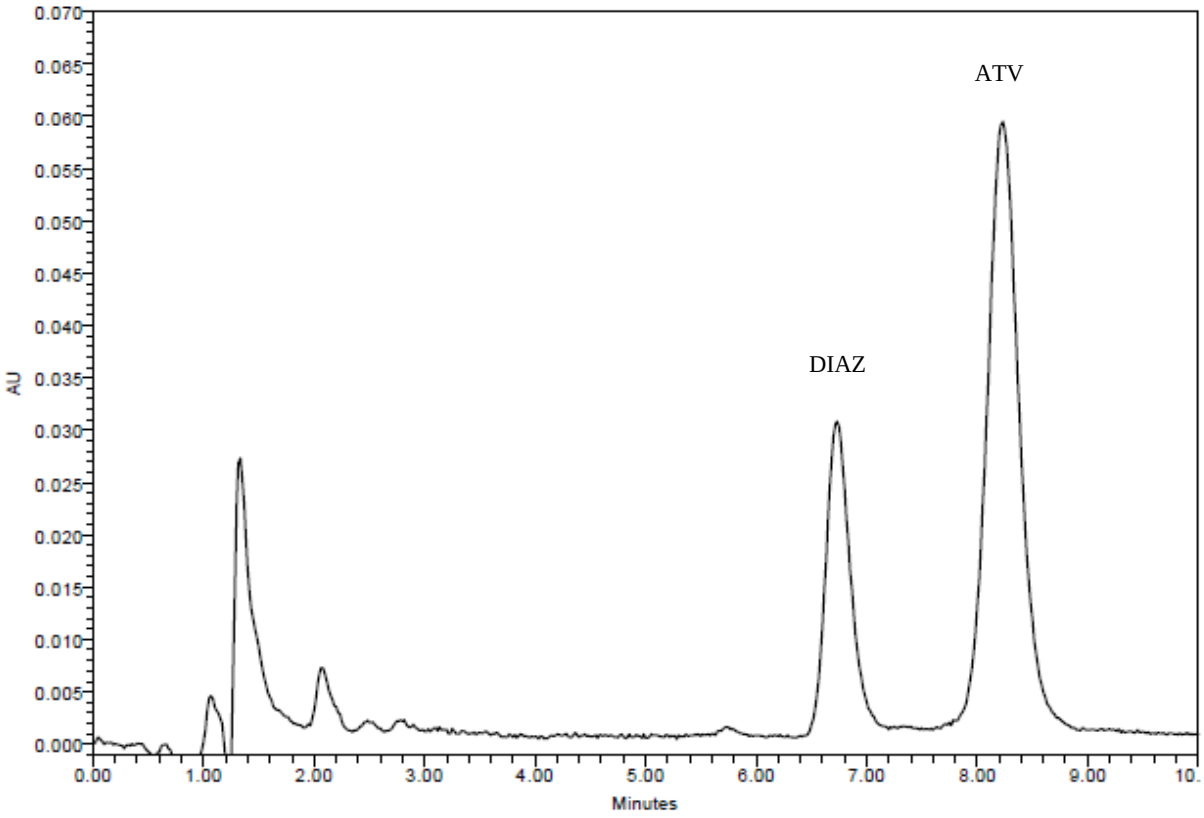


Figure 4.7: Typical chromatogram obtained from injection of a plasma sample containing ATV (10 µg/ml) and DIAZ (2.5 µg/ml) using acetonitrile-ammonium formate buffer (pH 3; 10 mM) (45:55, v/v) as the mobile phase.

Similarly when these chromatographic conditions with the "new" mobile phase were applied to liver microsomes samples, the peaks of the analytes were resolved and symmetrical. This is illustrated in Figure 4.8. Slightly longer retention times of 7.5 and 9.0 minutes were evident for DIAZ and ATV respectively, which may be attributed to the use of a different column with the same dimensions. The column packing between columns produced by the same manufacturers and with the same dimensions is known to be susceptible to variation, even under the same chromatographic conditions [337].

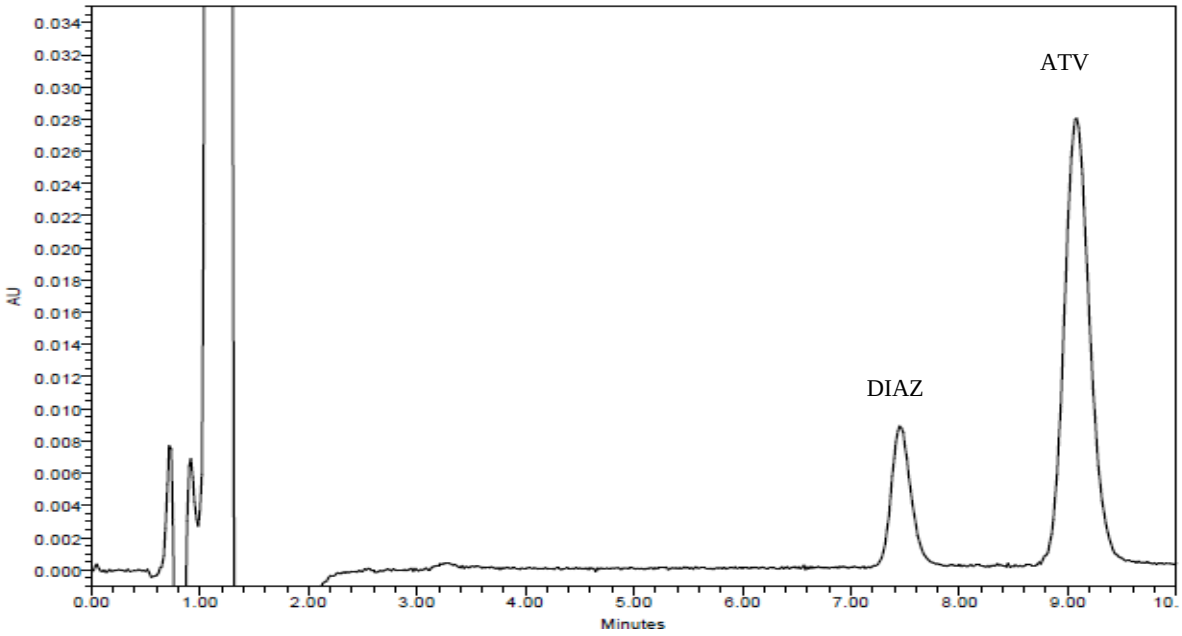


Figure 4.8: Typical chromatogram obtained from liver microsome sample containing ATV (30 µM) and DIAZ (2.5 µg/ml) using acetonitrile-ammonium formate buffer (10 mM, pH 3) (45:55, v/v) as the mobile phase.

For each concentration of ATV in liver microsomes, the 99% CI for the EE fell within the range 99–101%, with a general increase in the interval as the concentration of ATV decreased (see Table 4.7). This indicates that the "dilute and shoot" sample pre-treatment for liver microsome samples was suitable since the EE was reproducible. Furthermore, the 99% CIs all being within the range 99–101% showed that ATV recovery was not affected by the presence of microsomes and it would be acceptable to use standards of ATV in mobile phase as calibrator and QC samples as performed by Alkharfy *et al.* (2001) [356], and thus the expense of spiking liver microsomes for this purpose may be avoided.

Table 4.7: 99% CIs of the EE of ATV from human liver microsomes

Concentration of ATV (µM)	99% CI of EE
30	99.64–100.2
25	99.57–100.5
20	99.59–100.4
15	99.43–100.5
10	99.28–100.5
5	99.35–100.7
2.5	99.19–100.9

An HPLC-UV method for the quantitative analysis of ATV in the matrices, plasma and human liver microsomes was thus successfully developed. The method was optimised to attain selectivity and resolution in the presence of plasma, without a compromise on the throughput (short run time) and the sensitivity. A change in the chromatographic conditions, where a buffer (pH 3) was used as the aqueous component of the mobile phase and the proportion of acetonitrile was reduced from 50% to 45% was sufficient to achieve this. The following section details the validation procedures and the outcomes thereof.

4.4 Validation of an HPLC-UV Method for the Quantitative Analysis of ATV in Human Plasma and Liver Microsomes

4.4.1 Introduction

Federal regulations in the US require that "the analytical method used in an *in vivo* bioavailability or bioequivalence study to measure the concentration of the active drug ingredient or therapeutic moiety or its active metabolite(s) in the body fluids or excretory products, or the method used to measure an acute pharmacological effect shall be demonstrated to be accurate and of sufficient sensitivity to measure, with appropriate precision, the actual concentration of the active drug ingredient or therapeutic moiety or its active metabolite(s) achieved in the body" [358]. Such legislation alludes to validation as a prerequisite for the routine use of a bioanalytical method. Validation should involve a detailed investigation which proves that when an analytical method is applied, it produces results which are credible [359]. The pharmaceutical science community and the FDA developed a validation policy through much discussion during two Bioanalytical Workshops in 1990 and 2000 [360]. These policies resulted in the establishment of a final FDA guidance on bioanalytical method validation [361]. It states that the validation of a bioanalytical method before it is used for routine analysis should include a determination of: (i) selectivity, (ii) accuracy, precision, recovery, (iii) calibration curve, including an establishment of the lower limit of quantitation (LLOQ) and (iv) stability of the analyte in spiked samples.

Selectivity is the ability of the bioanalytical method to measure and differentiate the analytes in the presence of other components, such as metabolites, impurities, degradants, the matrix and co-administered drugs/foods which may be present [361]. Ideally, no interference with the analytes of interest is desirable, but this is not always possible [362; 363]. Whilst simple, subjective selectivity tests were conducted during development in sections 4.2 and 4.3, during validation, the impact of any interference at the LLOQ should be statistically determined [362]. The guidance suggests that the response of the LLOQ should be ≥5 times the response from the blank biological matrix and accuracy of interpolated concentrations at the LLOQ should be 80–120% with a corresponding precision thereat of ≤20% [361].

Interference by the matrix should be determined in a minimum of at least six independent sources [361], although some experts believe that since, in practice, interference in predose samples is a common problem with otherwise validated methods, evaluation of 10–20

sources of blank matrix is in fact necessary [362]. Impurities are often co-extracted during the sample pretreatment method. These may be identified by extracting standards in the absence of the biological matrix [362]. The potential for interference from metabolites should be determined by analysis of samples from subjects dosed with the analyte, or samples spiked with analyte previously co-incubated with human liver microsomes [362; 363]. Degradation products often arise as a result of acid- or base-catalysed hydrolysis in the gastro-intestinal tract. If subject samples are not available, samples spiked with analytes exposed to strong acid or base may be used [362; 363]. Investigation into the effects of co-administered drugs may be similarly assessed [362; 363].

Determining the accuracy and precision at the LLOQ involves the construction of full calibration curves in each independent matrix, which is uneconomical in terms of cost and time [362]. Thus the use of the signal-to-noise ratio of 5:1 may be a more practical way to initially define the LLOQ and once this has been established, it may then be confirmed by assessing whether accuracy and precision of interpolated concentrations fall within the set acceptance criteria [362]. It has been proposed that a more statistically relevant means of assessing the signal-to-noise ratio is by the construction of a 95% CI around the responses for the blank samples from the different matrix sources as well as samples of these spiked with analyte to obtain the potential LLOQ. The lower confidence limit for the LLOQ response should be ≥5 times the upper limit of the blank response [362].

Accuracy is the degree of agreement or closeness between the experimental value, obtained by replicate measurements, and the nominal value [359; 361]. It is usually determined by measuring the analyte in the biological matrix by interpolation and comparing the result with the nominal value. A minimum of three concentration levels prepared in pentaplicate in the range of expected concentrations (3 levels × 5 replicates per level = 15 determinations) is undertaken for bioanalytical methods [359; 361].

The determination of precision is the numerical expression of the random error or the degree of dispersion of a set of individual measurements by means of the standard deviation, the variance or the CV [359]. Precision is commonly further divided into repeatability, intermediate precision and reproducibility. In the determination of repeatability, global factors (human, preparation, instrumental and geographical) are kept constant over a short period of time [359; 364] and this is often known as intra-assay or intra-day precision [364].

Intermediate precision occurs over an extended period of time and is sometimes termed inter-assay or inter-day precision [359]. Reproducibility may involve a change in one or more global factors (usually inter-laboratory [364]) over a short or an extended period of time [359]. The same number of determinations should be undertaken as is required for accuracy [361], thus the same samples may be used for both accuracy and precision.

Recovery is the EE reported as a percentage of the known amount of an analyte carried through the sample extraction and processing steps of the method [361]. Absolute recovery is determined by the detector response obtained from an amount of the analyte spiked and extracted from the matrix compared to detector response obtained for the true concentration of unextracted standard [361]. It is influenced by the matrix and the extraction procedure. In contrast, relative recovery is determined using extracted standards instead and is thus only influenced by the matrix [363]. It is not necessary for recovery to be 100% as long as an adequate LLOQ may be obtained [361; 363].

A calibration curve used to generate bioanalytical data has an impact on the quality thereof. An unweighted linear regression is the most commonly adopted model, but if the range of concentrations is greater than one order of magnitude, it is expected that the variance at each data point is very different and that usually larger deviations present at the larger concentrations tend to influence (weight) the regression line more than the smaller deviations at lower concentrations [365; 366]. Such data exhibits heteroscedasticity rather than homoscedasticity (equal variances) and therefore accuracy at lower concentrations is poor [365; 366]. The use of a weighted least squares linear regression (WLSLR) usually adequately corrects this [365; 366]. Different weighting factors such as $1/x^{½}$, $1/x$, $1/x^2$, $1/y^{½}$, $1/y$, $1/y^2$ may be tested [365]. In order to determine which weighting factor would be required to fit the data using linear regression analysis, the percentage relative error (% RE), which expresses the deviation of the regressed (measured) concentration obtained for each weighting factor from the target (nominal) concentration, is determined. The weighting factor which produces the lowest sum of absolute % RE across the whole concentration range exhibits the best goodness of fit [365].

Drug stability experiments should be conducted such that these accurately reflect situations likely to be encountered during processing and analysis of study samples [361–363]. Drug stability at each point in processing and analysis is dependent on the storage conditions, the

container in which the sample is stored, the chemical properties of the drug and the matrix or solvent in which the analyte is dissolved [361–363].

Stability of analyte stock solutions under the intended storage conditions and conditions of use for appropriate lengths of time should first be determined [361–363]. Similarly, such stability for analytes in the matrix should also be evaluated, including samples which have undergone cycles of freezing and thawing [361–363]. In each case, it is advisable to assess stability at different time points and not only one so that the earliest time at which no stability issues are observed may be more easily determined [362; 363].

For processed sample stability, samples should be pooled, mixed and aliquoted for injection at time 0 and various time points thereafter [363]. Any differences observed are thus not a function of extraction reproducibility [363]. One evaluation should involve re-injection analysis [361], since the pierced vial directly exposes the sample to environmental conditions which may alter stability of the analyte.

Ideally, stability of the analyte in the matrix should be determined by analysis of pilot study samples when freshly collected and prepared, and then again after various times of storage [362]. After each time, fresh and stored interpolated values based on a calibration curve for each day should be compared [362]. However, pilot study samples are not always available. Furthermore, processing error inherent in any analytical method and not instability may account for some variability [362]. Analogously, bias error which contributes to deviation of actual concentrations from the nominal concentrations during preparation of the calibration curves and QC samples may also be misinterpreted as a stability issue [362].

To overcome the above mentioned limitations, spiked QC samples may be used and these may be stored and then analysed with freshly prepared samples on the same day and with the same calibration curve for interpolation [362]. Processing and bias errors are minimised since stored and fresh samples are processed on the same day using the same calibration curve [362]. The use of CIs about the mean for the ratio between the stored and fresh sample concentration to delineate stability is more likely to highlight relevant differences in stability [362; 367; 368] and not those which may simply be due to processing and bias errors [362]. The application of CIs was first introduced by Timm *et al.* (1985) [367] and was later refined

by Rudzki and Leś (2002) [368] to also allow for application of the method when variances of the stored and fresh concentrations are not equal.

Validation of the HPLC-UV method for the quantitative analysis of ATV in human plasma and liver microsomes was performed, in accordance with the stipulations in the FDA guidance on bioanalytical method validation.

4.4.2 Materials and Methods

4.4.2.1 Chemicals and Reagents

As described in section 4.3.2.1. In addition, Sutherlandia SU1™ tablets (Phyto Nova Natural Medicines, Cape Town, South Africa) each containing 300 mg *Sutherlandia frutescens* and Reyataz™ capsules (Bristol Myers Squibb, Bedfordview, South Africa) each containing ATV sulphate equivalent to 200 mg ATV were used to test for the selectivity of the method.

4.4.2.2 Instrumentation and Equipment

As described in section 4.3.2.2.

4.4.2.3 Preparation of Mobile Phase

As described in section 4.3.2.3.

4.4.2.4 Chromatographic Conditions

Chromatographic separation was achieved using a Luna C_{18} (2) (5 µm, 150 X 2.0 mm i.d.) column (Phenomenex®, USA) protected by a Luna C_{18} guard (4 X 2.0 mm i.d.) column (Phenomenex®, USA). Both columns were maintained at a temperature of 30 °C. The mobile phase consisted of acetonitrile-ammonium formate buffer (pH 3; 10 mM) (45:55, v/v), which was filtered under reduced pressure through a 0.45 µm (PVDF) membrane degassed prior to use. The mobile phase was pumped at a flow rate of 0.3 ml/min and the eluent was monitored at 210 nm.

4.4.2.5 Preparation of Plasma Calibration Standards and Quality Control (QC) Samples

Plasma calibration standards were prepared as described in section 4.3.2.4. QC plasma samples at low (~0.30 µg/ml), medium (~1.5 µg/ml) and high (~7.5 µg/ml) concentrations were prepared in the same manner, but from a separate plasma stock solution of ATV.

4.4.2.6 Preparation of ATV Substock for Calibration Standards and Quality Control (QC) Samples Used for the Validation of the Method in Liver Microsomes

ATV substock as calibration standards for liver microsome samples were prepared as described in section 4.3.2.5. QC samples at low (~7.5 µM), medium (~17.5 µM) and high (~27.5 µM) concentrations were prepared in the same manner, but from a separate ATV substock solution.

4.4.2.7 Plasma Sample Extraction Method

Plasma samples (400 µl) spiked with ATV were transferred to 2 ml microcentrifuge tubes. IS stock solution (25 µl), 2 M sodium carbonate (400 µl) and ethyl acetate-n-hexane (50:50, v/v) (800 µl) were added. The resultant mixture was then vortexed at 1600 rpm for 10 minutes and centrifuged at 10 000 rpm for 5 minutes. The aqueous layer was frozen in liquid nitrogen before the organic layer was transferred to a new set of 2 ml microcentrifuge tubes for evaporation under a gentle stream of nitrogen gas. The residues were reconstituted in mobile phase (100 µl), vortexed at 2000 rpm for 5 minutes and centrifuged at 10 000 rpm for 5 minutes. The supernatants were transferred to micro-inserts and 20 µl aliquots were injected onto the column.

4.4.2.8 Lower Limit of Quantitation (LLOQ)

The LLOQ for quantitation of ATV in plasma was determined based on a signal to noise ratio of 5:1 [361], from the determination of spiked plasma samples from six independent sources of plasma. 95% CIs were constructed about the mean peak area for the matrix blanks and the mean peak area for the LLOQ, both at the retention time of ATV (8.3 minutes) according to the Equations 4.2 and 4.3 below.

$$UL = X + t_{0.975}\left(\frac{s}{\sqrt{n}}\right) \qquad \textbf{Equation 4.2}$$

$$LL = X - t_{0.975}\left(\frac{s}{\sqrt{n}}\right) \qquad \textbf{Equation 4.3}$$

Where:

$t_{0.975}$ = the value of the Student's *t* distribution with n – 1 degrees of freedom

s = the standard deviation

n = the number of measured values.

The lower confidence limit for the LLOQ response should be ≥5 times the upper limit of the blank response.

Incubation of ATV (25 µM) in liver microsomes in a water bath at 37 ºC for 3 hours (three times longer than required for the actual experiments) was performed to determine whether it was suitable to set a concentration of 2.5 µM as the lowest point on the calibration curve.

4.4.2.9 Selectivity

Blank plasma samples and spiked LLOQ samples prepared from six different batches of plasma were extracted and checked for interference of ATV and the IS by endogenous compounds. A plasma sample from a healthy volunteer, following the oral administration for 5 days of a twice daily regimen of Sutherlandia SU1™ tablets each containing 300 mg SF and the oral administration of a single dose of 400 mg ATV, was collected 2.5 hours post ATV dose. This was to determine whether the metabolites of ATV and the components and/ or metabolites of SF would interfere with the assay of ATV.

4.4.2.10 Calibration Curve

Nine point calibration curves for the method in plasma were constructed in the range 0.1–10 µg/ml ATV. Each plasma calibration standard was extracted in triplicate and the mean peak area ratio, ATV/IS was plotted versus concentration of ATV with weighting factors of 1 (unweighted), $1/x^{½}$, $1/x$, $1/x^2$, $1/y^{½}$, $1/y$, $1/y^2$ [365]. In order to determine which weighting factor would be required to fit the data using linear regression analysis, the percentage relative error (% RE), which expresses the deviation of the regressed (measured) concentration obtained for each weighting factor from the target (nominal) concentration, was determined. The weighting factor that produced the lowest sum of absolute % RE across the whole concentration range of three sets of calibration standards, prepared and assayed on three different days, was selected [365].

Using an unweighted linear regression, calibration curves consisting of seven points were constructed in the range 2.5–30 µM ATV from calibration solutions prepared as described in section 4.4.2.6, to show linearity of the method for application in liver microsomes.

4.4.2.11 Accuracy and Precision

For the plasma and liver microsome applications of the method, accuracy was defined by the regressed concentration represented as a percentage of the target concentration. Intra-assay accuracy was determined from the analysis of five replicate samples at each of the low, medium and high QC concentrations. After intra-assay accuracy was assessed thrice, inter-assay accuracy was calculated from the fifteen samples at each of the high, medium and low QC concentrations. The percent relative standard deviation (% RSD) of the regressed concentrations was used to report precision. Intra-assay and inter-assay precision were assessed on the same samples used to determine intra- and inter-assay accuracy. For the plasma method only, intra- and inter-assay accuracy and precision were also determined at the LLOQ.

The limits for the accuracy values of plasma samples were set as the range, 85–115%, except at the LLOQ where values between 80 and 120% were accepted [361]. Similarly, precision for plasma samples were accepted if the % RSD fell within 15%, except at the LLOQ, where the limit was extended to 20%.

The QC and calibrator samples for application of the method in liver microsomes are pure samples (no liver microsomes present), therefore a higher degree of accuracy and precision is expected. To realistically account for this, values of 95–105% and 5% for accuracy and precision were set for the limits of accuracy and precision respectively.

4.4.2.12 Recovery

EE was determined from the ATV and DIAZ peak areas of the plasma QC samples in the low, medium and high QC concentrations and expressed as a percentage of ATV peak area of equivalent unextracted standards prepared in mobile phase. The EEs in liver microsome samples at each calibration concentration were already ascertained in the development of the method (see Table 4.7).

4.4.2.13 Stability

Five replicate plasma samples at each of the low and high QC concentrations were analysed for stability. Bench-top stability was assessed by storing unextracted plasma samples for 6 hours at ambient temperature (22 ± 3 °C), before extraction and analysis. Long-term stability was determined for 1 week, 1 month and 2 months after storage in a freezer at -10 ± 2 °C.

After three 24 hour freeze-thaw cycles, plasma samples were also extracted and analysed. Extracted plasma samples were tested for post-preparative stability, which included autosampler stability and re-injection analysis. For the former, samples were analysed after being stored in the autosampler at 22 ± 3 °C for 24 hours. The latter involved the re-assay of previously injected samples 24 hours after initial injection. All stored plasma samples were considered stable if the 90% CI of the regressed stored plasma sample concentrations calculated as a percentage of the regressed fresh plasma sample concentrations fell within the range 85–115% [368]. Stock solutions of ATV and DIAZ in mobile phase were assessed for stability at 22 ± 3 °C for 6 hours and following storage in a refrigerator at 5 ± 3 °C for 1 week. All stored stock solutions were considered stable if the 90% CI of the peak area of ATV from the stored sample calculated as a percentage of the peak area of ATV from a freshly prepared sample fell within 93–107% [369]. In each case, an *F*-test at the 0.01 level of significance was first performed to determine whether the stored and fresh data have equal variances or not. Equal variances between stored and fresh samples allowed the use of a pooled variance, as shown in Equations 4.4 and 4.5 whilst unequal variances require that individual variances be used (Equations 4.6 and 4.7).

$$LL = e^{lnx - lnz - t_{\alpha/2.df}\sqrt{s_p^2\left(\frac{1}{n_x}+\frac{1}{n_z}\right)}}.100\ \% \qquad \textbf{Equation 4.4}$$

$$UL = e^{lnx - lnz + t_{\alpha/2.df}\sqrt{s_p^2\left(\frac{1}{n_x}+\frac{1}{n_z}\right)}}.100\ \% \qquad \textbf{Equation 4.5}$$

$$LL = e^{lnx - lnz - t_{\alpha/2.df}\sqrt{\left(\frac{s_x^2}{n_x}+\frac{s_z^2}{n_z}\right)}}.100\ \% \qquad \textbf{Equation 4.6}$$

$$UL = e^{lnx - lnz + t_{\alpha/2.df}\sqrt{\left(\frac{s_x^2}{n_x}+\frac{s_z^2}{n_z}\right)}}.100\ \% \qquad \textbf{Equation 4.7}$$

4.4.3 Results and Discussion

4.4.3.1 LLOQ

The LLOQ in plasma was found to be 0.1 µg/ml, since, as shown in Table 4.8, it is the lowest concentration at which the lower limit of the 95% CI for the response is ≥5 times the upper limit of this same interval for the blank. This was similar to that of Takahashi *et al.* (2005) [303], who obtained an LLOQ of 0.09 µg/ml but was an improvement on the LLOQ of 0.156

µg/ml reported by Cateau *et al.* (2005) [298]. After a three hour incubation of ATV in liver microsomes at 37ºC, a concentration of 15 µM ATV was obtained, thus the lower limit of 2.5 µM was deemed acceptable.

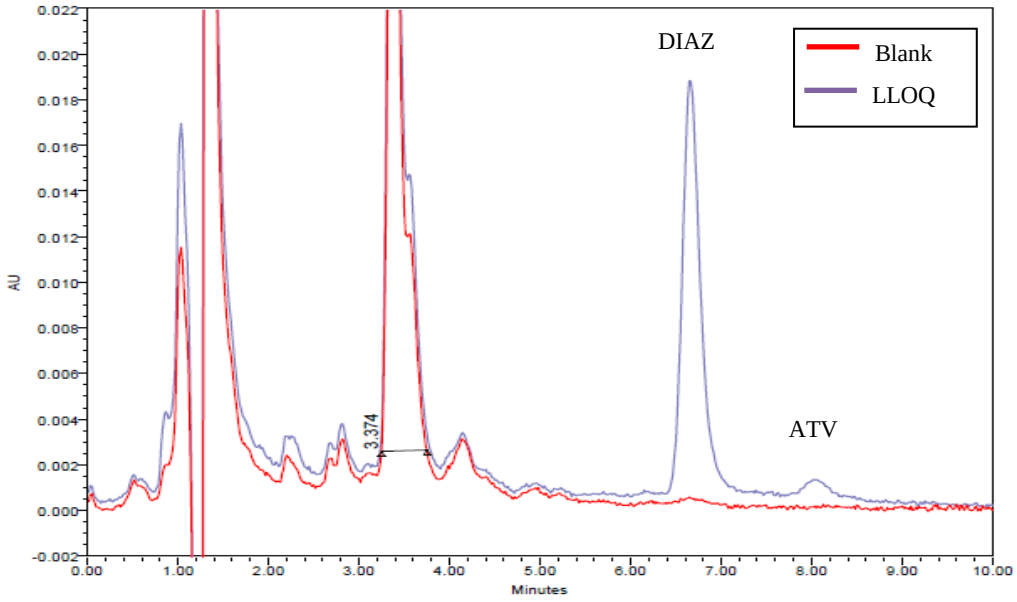
Table 4.8: Determination of LLOQ from 95% CI of responses at blanks and potential LLOQ

Concentration of potential LLOQ (µg/ml)	Blank		Potential LLOQ		Is UL of blank ≤5 times LL of potential LLOQ?
	LL of 95% CI of mean peak area	UL of 95% CI of mean peak area	LL of 95% CI of mean peak area	UL of 95% CI of mean peak area	
0.075	587	1697	7532	9045	No
0.100	587	1697	10 639	12 824	Yes
0.150	587	1697	15 903	17 498	Yes

4.4.3.2 Selectivity

Figure 4.9 depicts chromatograms showing (a) blank plasma extract overlayed on spiked plasma extract containing ATZ at the LLOQ and IS; (b) plasma extract of a healthy human volunteer 2.5 hours after a single oral dose of 400 mg ATV, where a twice daily regimen of 300 mg Sutherlandia SU1™ tablets had been administered for the preceding 5 days. There were no co-eluting interferents in any of the 6 different batches of human plasma, even at the LLOQ, nor in the plasma sample drawn from the healthy human volunteer.

A



B

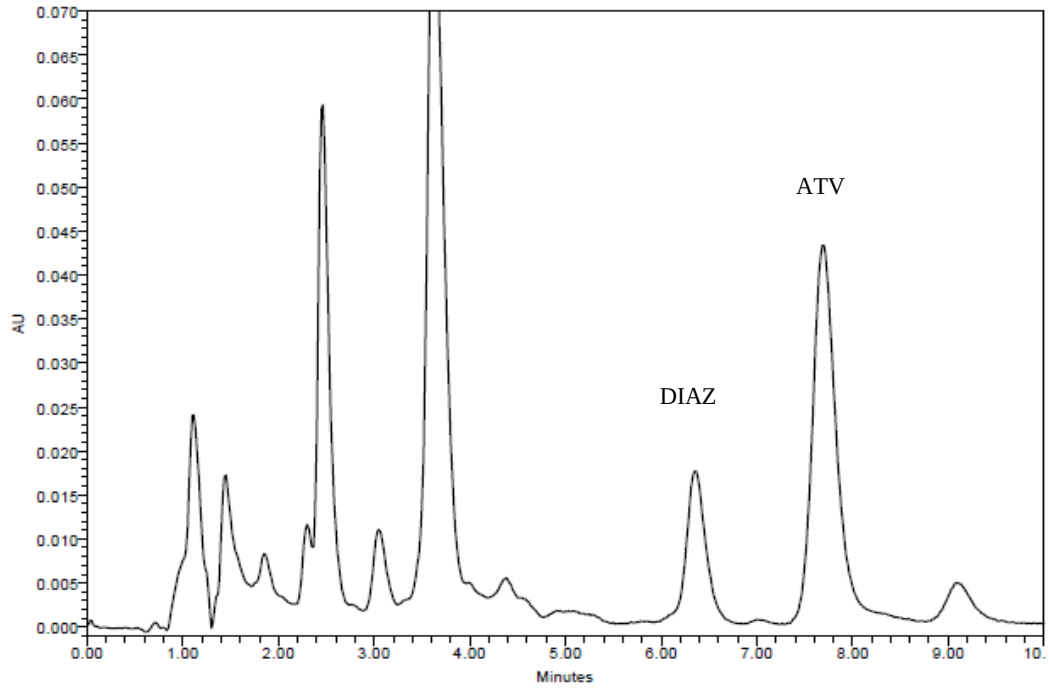


Figure 4.9: Chromatograms showing selectivity. (A) blank plasma extract (red) and spiked plasma extract containing ATZ at the LLOQ and DIAZ (purple); (B) plasma extract of a healthy human volunteer 2.5 hours after single oral dose of 400 mg ATV (2 X 200 mg Reyataz™ capsules) and a twice daily regimen of 300 mg Sutherlandia SU1™ tablets.

4.4.3.3 Calibration curve

On each of three days, $1/y^2$, $1/x^2$ and 1/y produced the lowest sum of absolute % RE across the whole concentration range of plasma calibrators, in ascending order. All calibration curves using a linear regression analysis with a weighting factor of $1/y^2$ were linear, with a correlation co-efficient (r) consistently ≥0.999, which relates to a co-efficient of determination (r^2) ≥0.998. The average slope of the calibration curves was 0.657 ± 0.0437.

For the liver microsome application of the method, an r^2 of at least 0.997 was obtained on each day with an average slope of 0.1298 ± 0.00709.

4.4.3.4 Accuracy and Precision

The intra- and inter-assay accuracies and precision of the LLOQ and QC plasma sample concentrations, shown in Table 4.9, were all within the acceptance criteria. The intra- and inter-assay accuracies at the LLOQ were found to be 97.1 ± 5.04% and 98.0 ± 11.3% respectively. The accuracies for the low, medium and high QC sample concentrations fell within the range 101 ± 4.48 – 104 ± 2.09%. The intra- and inter-day precision values were ≤11.6% RSD at the LLOQ and ≤6.78% RSD for all the QC sample concentrations.

Table 4.9: Accuracy and precision of LLOQ and QC plasma samples

QC concentrations (µg/ml)	Intra-assay (n = 5)		Inter-assay (n = 15)	
	Accuracy (Mean % ± SD)	Precision (% RSD)	Accuracy (Mean % ± SD)	Precision (% RSD)
LLOQ (0.1)	97.1 ± 5.04	5.18	98.0 ± 11.3	11.6
Low (0.3)	104 ± 2.09	1.88	101 ± 4.10	6.78
Medium (1.5)	101 ± 2.81	3.05	102 ± 4.11	4.10
High (7.5)	102 ± 1.47	1.44	101 ± 4.48	3.86

Table 4.10 shows that the intra- and inter-day accuracy and precision values for the liver microsomes application of the method fell within the limits set. Accuracy ranged from 97.4 ± 2.77 – 104 ± 1.19, whilst precision was ≤3.92.

Table 4.10: Accuracy and precision of QC samples for liver microsome application

QC concentrations (µM)	Intra-assay (n = 5)		Inter-assay (n = 15)	
	Accuracy (Mean % ± SD)	Precision (% RSD)	Accuracy (Mean % ± SD)	Precision (% RSD)
Low (7.5)	97.4 ± 2.77	2.85	98.9 ± 3.85	3.92
Medium (17.5)	99.4 ± 3.24	3.26	98.1 ± 2.82	2.88
High (27.5)	104 ± 1.19	1.15	101 ± 3.54	3.52

4.4.3.5 Recovery

EEs of ATV from plasma were 100 ± 2.22%, 95.0 ± 0.777% and 94.4 ± 1.07% at the low, medium and high QC sample concentrations respectively. These data show reasonably high recovery with high reproducibility as shown by the standard deviations of <5%. EEs of ATV from liver microsome samples were determined and described during method development (see Table 4.7).

4.4.3.6 Stability

The stability of stored stock solutions as well as stored plasma samples at low and high QC concentrations, as shown in Table 4.11, were all adequate according to the acceptance criteria. The 90% CI for ATV and DIAZ, after storing for 1 week in the refrigerator at 5 ± 3 °C was 96.8–99.1% and 96.4–98.2%, whilst the 90% CIs of unextracted and extracted plasma samples under all the storage conditions tested were within the range 85.0–110%.

Table 4.11: Stability of plasma samples and stock solutions

Storage Conditions	QC concentration (µg/ml)	90% CI (%)
Unextracted plasma		
6 hours at 22 ± 3 °C	0.3	89.4–110
	7.5	96.2–102
Three freeze-thaw cycles	0.3	90.6–102
	7.5	94.9–102
1 week at -10 ± 2 °C	0.3	90.0–97.2
	7.5	101– 106
1 month at -10 ± 2 °C	0.3	91.6–107
	7.5	94.2–108
2 months at -10 ± 2 °C	0.3	86.9–103
	7.5	85.6–91.8
Final extract		
24 hours at 22 ± 3 °C	0.3	85.6–103
	7.5	95.6–107
Re-injection analysis	0.3	84.5–101
	7.5	97.0–108
Stock solution (ATV)		
6 hours at 22 ± 3 °C		99.5–101
1 week at 5 ± 3 °C		96.8–99.1
Stock solution (DIAZ)		
6 hours at 22 ± 3 °C		100.9–102.2
1 week at 5 ± 3 °C		96.4–98.2

4.5 Conclusions

A relatively simple and cost-effective HPLC-UV method suitable for the determination of ATV in human plasma and liver microsomes was developed. A mobile phase of acetonitrile-buffer (10 mM, pH 3) (45:55, v/v), as opposed to a simpler mobile phase of acetonitrile-water (50:50, v/v) was required to maintain the selectivity of the method upon the analysis of plasma samples. However, this slight complication was compensated for since the chromatographic conditions and extraction method used introduced several advantages over previously reported methods for the quantitative analysis of ATV in human plasma. The use of a narrow-bore HPLC column allowed for the reduction in flow rate and consequently decreased the volume of expensive organic solvent used in the mobile phase. In addition, an existing LLE procedure for ATV in plasma was adapted such that the use of organic solvents in the extraction procedure was also minimised without jeopardising EE. This was demonstrated by a low total extraction volume of 1.6 ml, containing only 800 µl of ethyl acetate-n-hexane (50/50, v/v) per sample. In addition, despite the use of a low flow rate of 0.3 ml/min, relatively short retention times for the IS (6.8 minutes) and ATV (8.3 minutes) resulted. Hence a short run time of 10 minutes compared to previously reported run times of 15 minutes or longer was effected which will facilitate and enhance the efficiency of processing large numbers of plasma samples. The method for application in human plasma samples was validated according to the FDA guidelines for bioanalytical methods by fulfilment of the set criteria with respect to selectivity, a suitable linear regression, accuracy, precision, recovery and stability. Similarly, accuracy, precision and recovery were deemed acceptable for the analysis of ATV in human liver microsomes. Since liver microsomes had no effect on the EE of ATV, calibrator and QC samples free of liver microsomes could be used, thus enhancing the cost-effectiveness of the method for this particular application. The HPLC-UV method here described is thus suitable for the routine analysis of ATV in human plasma and liver microsome samples. A full account of this method was published in the Journal of Pharmaceutical and Biomedical Analysis in 2010 [370].

CHAPTER 5
PROTEIN-LIGAND INTERACTIONS

5.1 Background

In pharmacology, a drug often exerts its PD effect by acting as a ligand, which interacts with a binding site on a target protein [371; 372]. Similarly, such interactions may also affect the PK handling of the ligand by an organism if the protein involved in such interactions is a drug transporter or a drug metabolising enzyme [371; 372]. Binding between the protein and ligand is usually reversible and occurs by various intermolecular forces such as electrostatic interactions, hydrogen bonding interactions, $\pi - \pi$ and $\pi - \sigma$ interactions and/or hydrophobic interactions [373; 374]. The association of the ligand with the protein at a binding site often results in a change in the conformation of the protein, which in turn alters the functional state of the protein through which the PD or PK effect is executed [373; 374].

In the context of drug transporters and drug metabolising enzymes, ligands may be substrates, inhibitors, activators or products (only for enzymes) at binding sites within the protein [372; 375]. Binding of competitive inhibitors (see Chapter 1, section 1.3.7.8.4.1) occurs at the transport or metabolism competent active site, whilst that of non-competitive inhibitors (Chapter 1, section 1.3.7.8.4.2) and activators (Chapter 1, section 1.3.7.8.5) occurs at an allosteric site.

The degree of affinity which a ligand has for a protein is usually directly related to the strength of the intermolecular forces between the two [373]. A protein-ligand interaction with a high binding affinity is part of a more thermodynamically favourable system and therefore dissociation is far less likely to occur than in a protein-ligand interaction with a lower binding affinity. For drug transport or metabolism of a substrate, binding affinity is important to execute the intended effect, although for metabolism, the orientation of the substrate in the active site of the enzyme may be the important factor which might dictate which metabolite, if any, is formed [376; 377]. Similarly, the binding mode and affinity of other ligands determines the potential of these to act as modulators of the transport and metabolism of substrates [376; 377].

Computational simulations of protein-ligand interactions have had an important role to play in rational drug design over the last decade [378]. This *in silico* methodology, termed "Molecular Docking", is used to predict the potential binding mode (orientation) and binding affinity of a ligand in a protein [378]. For docking to be performed, the three dimensional (3D) structures of the proteins and ligands of interest are required. These protein and ligand structures are the inputs for a docking program, in which a search algorithm is set up to explore the conformational space where the binding of the ligand in the protein in different poses may occur [378]. During docking, the ligand and protein are some physical distance apart, each with an associated unbound internal energy [378]. For the ligand to be positioned in the relevant binding site of the protein, rotation of bonds in the ligands is required, during which torsional energy is expended [378]. The protein-ligand complex has associated internal and intermolecular energies, where the latter is usually inversely related to the strength of the intermolecular forces [379]. A docking scoring function estimates the magnitude of these energies [380]. AutoDock® 4 [380] computes the change in free energy of binding (ΔG) as intermolecular energy + torsional energy, which is directly related to an estimated dissociation constant of binding, denoted K_i, derived from exp (ΔG)/(R*T), where R and T are the gas constant and absolute temperature respectively [380]. These two parameters are inversely related to the binding affinity of the ligand for the protein [379]. Since torsional energy is largely dependent on the size of the ligand, a comparison of the ΔG and K_i of structurally unrelated ligands may not always be appropriate. Docking energy, which may be calculated as the intermolecular energy + internal energy of the bound protein and ligand [380] may thus be used to confirm or support the findings.

In addition to rational drug design, docking may also be used to screen for drug interaction potential of ligands. A comparison of the binding modes, ΔG, docking energy and K_i of the substrates and potential modulators in the binding site/s of the enzymes or transporters may be used to determine the likelihood of the occurrence of inhibition and/or activation of the metabolism or transport of the substrate [381–384].

It is important to note that molecular docking for drug transport and metabolism has limitations especially since the protein structure is static in one conformation of the respective catalytic cycles. Over recent years, docking programmes have become more sophisticated, which has permitted incorporation of flexible amino acid residues of the

protein into the search algorithm and may allow for a more accurate simulation [385–387]. However, this increases the number of degrees of freedom that have to be considered and thus complicates the scoring function, which may not result in appropriate predictions [385–387]. Similarly, neglecting protein flexibility may lead to poor binding poses, docking energy, ΔG and K_i estimations [385–387]. These limitations, among several others, indicate that molecular docking as an *in silico* method for determining drug-drug interaction potential is used only as a screening tool and that predictions may therefore not correlate with *in vitro* or *in vivo* outcomes. As described in Chapter 1, section 1.5, the rationale for the use of molecular docking in the context of this project is to hypothesise whether it is worth pursuing more expensive *in vitro* and *in vivo* experiments and not necessarily to predict what the specific outcomes in the latter experiments may be.

The sections of this chapter which follow deal with the comparison of potential binding modes as well as estimated docking energy, ΔG and K_i values of ATV after docking into P-gp and CYP3A4 with that of Sutherlandioside B and Sutherlandin C, which are the two major phytochemical constituents of SF (see Chapter 3, sections 3.7.3 and 3.7.4), belonging to the class, sutherlandioside and sutherlandin respectively. These two phytochemical constituents of SF, as previously explained were considered to be the most relevant examples of the specific phytochemical classes in view of their relative abundance

5.2 Comparison of Molecular Docking of ATV and Components of SF into P-gp

5.2.1 Introduction

Reduced cell membrane permeability as a mechanism for multidrug resistance was first demonstrated by Ling and Thompson (1974) [388]. Colchicine-resistant cell lines were isolated from cultured Chinese hamster ovary cells during successive single-step selections [388]. These cells showed a decreased permeability to colchicine, which correlated to the degree of resistance [388]. The cells were also found to exhibit multidrug resistance since uptake of other drugs such as actinomycin D and vinblastine into these cells was also reduced [388]. Preliminary observations by Juliano *et al.* (1976) [389] showed that a glycoprotein was associated with the plasma membrane of the resistant cell line, and further investigations by surface labelling studies revealed that the structure has an apparent molecular weight of 170 kDa and was not present in wild-type cells [390]. Since this glycoprotein was associated with altered drug permeability, Juliano and Ling (1976) [390] named it the P-glycoprotein (P-gp). Importantly, the relative amount of surface labelled P-gp correlated with the degree of drug resistance in a number of independent clones of drug-resistant Chinese hamster ovary cells [390], indicating that P-gp has a role to play in imparting such resistance to the cells.

P-gp is a 1280 amino acid protein containing two homologous portions of 610 amino acids residues [391], each with a transmembrane domain (TMD), comprised of six α-helices [391–393] and one nucleotide binding domain (NBD) located in the cytosol [391; 393] (see Figure 5.1). The two halves are separated by a flexible polypeptide linker region of 60 amino acid residues [391]. A substrate binding site is contained in each of two TMD α-helix pairs, namely 5 and 6, and 11 and 12 [394], whilst three glycosylation sites are located in the first extracellular loop which links TMD α-helix 1 and TMD α-helix 2 [395]. Each NBD contains an ATP-binding site [391; 393], where conserved amino acid motifs, named Walker A and Walker B are found [396]. A sequence motif, which is unique to ABC transporters [397] is positioned just outside the ATP-binding site downstream from the Walker B motif. This sequence motif has a significant role to play in the functioning of the catalytic cycle of P-gp.

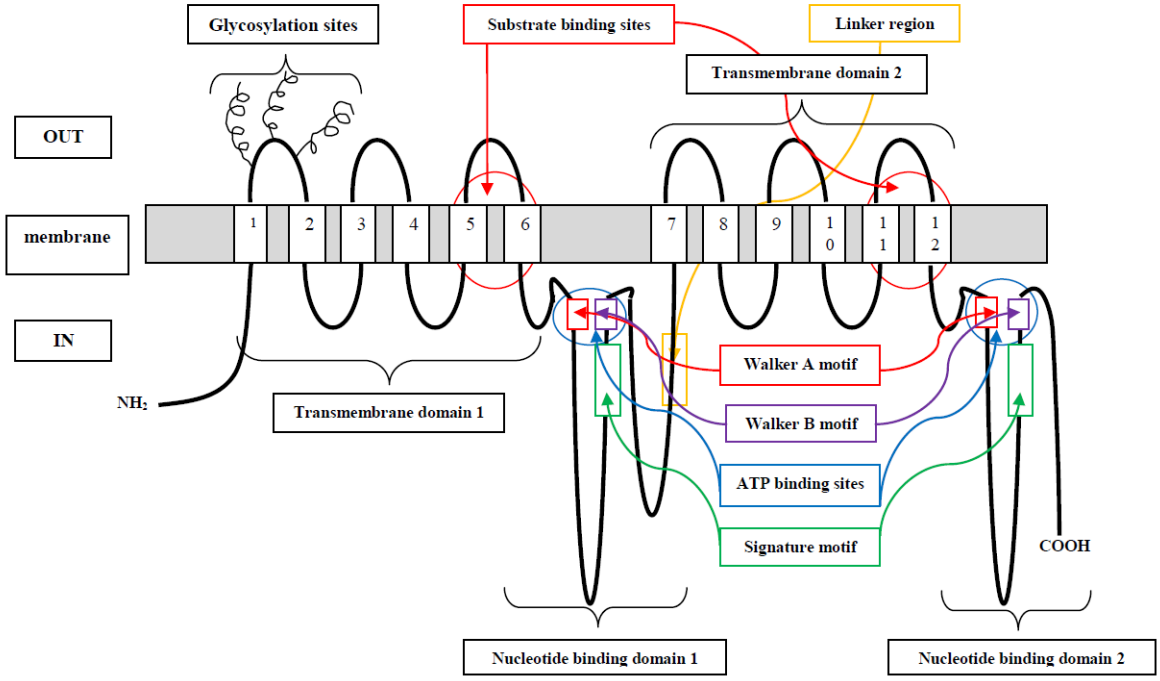


Figure 5.1: Two dimensional schematic representation of human P-gp showing the transmembrane and nucleotide binding domains. Adapted from Hrycyna (2001) [398] and Bozzuto *et al.* (2010) [399]

There are two main models, reviewed by Ambudkar *et al.* (2006) [400], which describe the transport cycle of P-gp. In both models, a protein-ligand interaction between P-gp and the substrate is required to initiate the catalytic cycle of this ABC transporter [401]. It has been suggested that the two substrate binding sites shown in Figure 5.1 are oriented such that these overlap to form a "large funnel-shaped binding pocket", with the open side facing the intracellular side of the membrane [400; 402–404]. Similarly, the binding of ATP at the two relevant binding sites (see Figure 5.1) is also required [401]. Drug and ATP binding for both models is shown in step 1 of Figure 5.2 (A) and Figure 5.2 (B).

Biochemical studies on P-gp and the crystal structures of the NBDs of other ABC transport proteins allude to the dimerisation of the two NBDs upon ATP-binding such that the ATP molecule of NBD 1 interacts with the Walker A and B motifs of NBD 1 and the signature motif of NBD 2 and *vice versa* [405; 406]. The Walker A and B motifs bind primarily at the γ-phosphate of the triphosphate moiety, the former directly by electrostatic interactions and the latter via hydrogen bonding with a co-ordinating divalent cation (usually Mg^{2+}) [407].

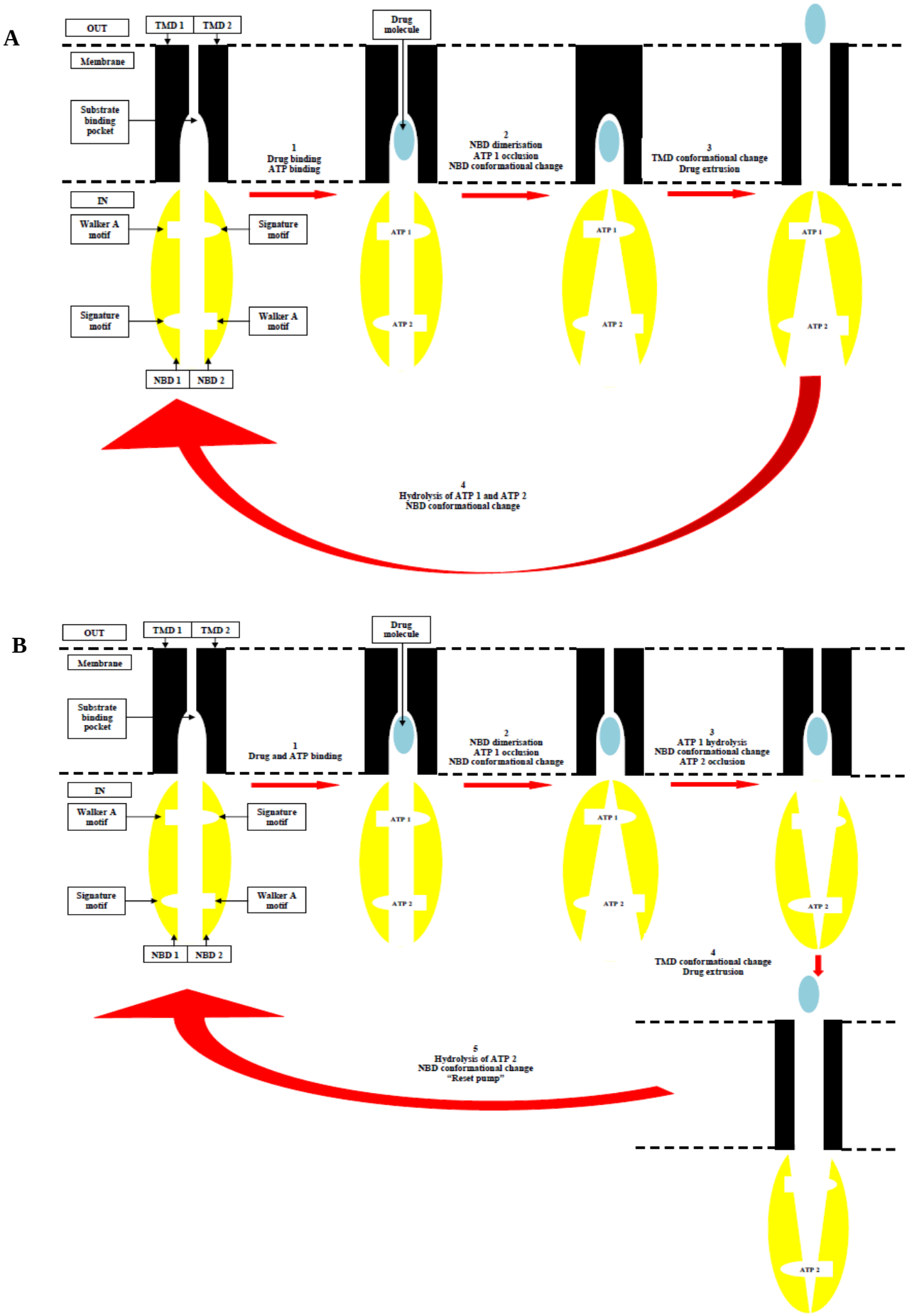


Figure 5.2: Model 1 (A) and Model 2 (B) proposed for the transport cycle of P-gp. Adapted from Ambudkar and Sauna, 2005 [400] and Sauna and Ambudkar, 2007 [401].

Closure of the ATP site of NBD 1 occurs as a result of interactions between the γ-phosphate and the adenine ring, and the signature motif of NBD 2, such that the Walker A motif of NBD 1 is adjacent to the signature motif of NBD 2, which occludes the ATP molecule at NBD 1 but leaves the ATP molecule at NBD 2 with minimal interaction with its surrounding motifs [406; 408; 409]. The closing of the ATP site at NBD 1 may be enhanced by drug binding at the substrate binding site [410]. For example, the binding of verapamil causes a conformational change in the TMDs which translates to the NBDs such that the distance between the Walker A motif of NBD 1 and the signature motif of NBD 2 is reduced [410]. NBD dimerisation, ATP 1 occlusion and the resultant NBD conformational change are depicted in step 2 of both models in Figure 5.2 (A) and Figure 5.2 (B).

In Model 1, Higgins and Linton, 2004 [411] suggested that NBD conformational change occurring as a result of the formation of the dimer is the main driving force behind the conformational change in the TMD. This in turn, opens the substrate binding pocket towards the extracellular environment, expelling the substrate out of the cell [401; 406]. These events are illustrated in step 3 of Figure 5.2 (A). The alternate model (Model 2), on the other hand, attributes this dominant driving force for TMD conformational change and subsequent extrusion of the drug, to the hydrolysis of ATP at one of the ATP-binding sites [412–414], as shown in step 3 of Figure 5.2 (B). Loss of the γ-phosphate ion during hydrolysis of occluded ATP at NBD 1 exposes the signature motif of NBD 2 to the divalent cation (Mg^{2+}) [406; 415; 416]. Electrostatic repulsion between this motif and the cation results in the disassembly of the occluded ATP site [406; 415; 416], which favours occlusion of the ATP site of NBD 2 and executes a conformational change in the NBDs [401]. This conformational change in the NBDs is transmitted to the TMDs resulting in drug extrusion, as portrayed in step 4 of Figure 5.2 (B).

In order for the same P-gp molecule to start a new transport cycle, energy is required to transform the molecule back into its original unbound state which may then bind another set of ATP and substrate molecules [401]. In Model 2, as depicted in step 5 of Figure 5.2 (B), the hydrolysis of ATP at NBD 2 is proposed to contribute to resetting the pump [412–414], although biochemical evidence indicates that this may not be sufficient [413; 414]. The requirement for two consecutive ATP hydrolytic processes, one at each binding site is thus suggested in Model 1 [411], as illustrated in step 4 of Figure 5.2 (A). Both models have

provision for an “alternating catalytic sites cycle” first proposed by Senior *et al.* (1995) [417], whereby hydrolysis of ATP may only occur at one site at a time which alternates between the two NBDs.

Modulation of the P-gp transport cycle may be accomplished in a number of ways. Inhibition of substrate extrusion by an inhibitor may occur as a consequence of (i) competitive inhibition at the same binding site in the binding pocket [418–421], (ii) non-competitive inhibition due to negative co-operativity at the two binding sites in the binding pocket [419–421], (iii) inhibition of basal ATP hydrolysis [422–425] and (iv) modified cell membrane integrity [426–428]. Conversely, basal ATP hydrolysis may be promoted by ligands [420; 421; 424; 429; 430] and activation of the efflux of one substrate by another via positive co-operativity [431–433] at the two binding sites of the binding pocket may also occur. In each case, as previously alluded to (section 5.1), the binding mode and binding affinity of the substrate and potential modulator in the substrate binding pocket of P-gp may dictate the effect of the latter, if any, on the P-gp transport of the former.

In competitive inhibition of P-gp transport at one of the binding sites in the binding pocket, both ligands, *viz* substrate and inhibitor, may be effluxed into the extracellular environment. A potent competitive inhibitor should thus have not only the same binding mode and a higher affinity than the substrate at the substrate binding pocket, but also a lower rate of efflux than the substrate [434]. Eytan *et al.* (1996) [435] suggested that immediately after being effluxed, the inhibitor is redirected to the hydrophobic interior of the membrane due to its hydrophobicity. The same inhibitor molecule is thus recycled and once again available for binding at the substrate binding site of a P-gp molecule. Eytan *et al.* (1996) [435] further proposed that this process occurs rapidly; therefore, the rate of efflux of the inhibitor is far lower than that of the substrate.

The exact mechanism by which ligands alter basal ATP hydrolysis has not been unequivocally established. Various findings in the literature indicate that the affinity of ATP for P-gp is not likely to be directly affected [413; 414; 436–439], neither is the activation energy of P-gp ATP hydrolysis [440–442]. Loo *et al.* (2003) [410] have provided the most plausible explanation of how drugs affect P-gp ATP hydrolysis. Cross-linking studies in a cysteine-less mutant of P-gp showed that ligands stimulate basal ATP hydrolysis by

decreasing the distance between the Walker A motif of one NBD and the signature motif of the other NBD, through conformational changes transmitted from the TMDs by drug binding at a binding site [410]. Basal ATP hydrolysis is attenuated by ligands which conversely increase the aforementioned distance. These data were further supported by experiments involving ADP-orthovanadate trapping by wild-type P-gp and ATP binding by catalytic carboxylate mutant P-gp conducted by Tombline *et al.* (2006) [443]. Theoretically, enhanced basal ATP hydrolysis would be expected to result in increased transport of a P-gp substrate. Experimental kinetic analysis, however, demonstrated that compounds which increase basal ATP hydrolysis may competitively or non-competitively inhibit the basal ATP hydrolysis induced by another [420; 421], indicating that inhibition of drug-binding may override the enhanced basal ATP hydrolysis. The converse may not necessarily be true since bromocriptine which inhibits basal ATP hydrolysis also inhibits verapamil-stimulated and progesterone-stimulated P-gp ATPase activity competitively and non-competitively, respectively [425].

Positive co-operativity for drug transport by P-gp was first proposed by Shapiro and Ling (1997) [431], who observed mutual transport stimulation by Hoeschst 33342 and rhodamine 123. Some P-gp substrates stimulated the transport of the former and inhibited the transport of the latter and *vice versa*, whilst others inhibited the transport of both [431]; therefore these authors concluded that the two sites where these substrates bind have distinct yet overlapping specificities. The same group of researchers later found that prazosin and progesterone stimulate the transport of both Hoechst 33342 and rhodamine 123 and that synergism of Hoechst 33342 transport occurs when rhodamine 123 and prazosin (or progesterone) are used in combination [433]. Moreover, Hoechst 33342 and either prazosin or progesterone interfere with each other, such that the stimulatory effect of the combination on rhodamine 123 transport is less than that produced by each agent alone [433]. Taken together, these findings allude to the presence of a third binding site in the drug binding pocket of P-gp.

Surfactants which inhibit P-gp disturb the integrity of membrane phospholipids by altering the membrane fluidity. This in turn disrupts the secondary and tertiary structure of the TMDs of P-gp resulting in the loss of P-gp function. This cascade of events which culminates in reduced P-gp function has been proposed by Hugger and co-workers [427; 428], who observed that the change in fluidity of cell membrane facilitates increased intracellular

accumulation of P-gp substrates by surfactants like polyethylene glycol, cremophor EL and Tween 80 in Caco-2 cells, without a significant effect on passive paracellular or transcellular permeation. The latter result is important as it excludes an elevation in these two influx modes as mechanisms of the enhanced P-gp substrate intracellular accumulation.

Several models have been suggested to predict P-gp substrate and/or inhibitor properties. Most of these point to neutral or basic compounds with a molecular weight >400 g/mol, a LogP ≥4 with a high number of hydrogen bond acceptors and rotatable bonds as likely P-gp substrates [444-447], suggesting that an interplay of hydrophobic and hydrogen bonding interactions may have a significant role to play in substrate binding. Similarly, the merging of five P-gp inhibitor pharmacophores revealed common areas for the presence of hydrophobic domains, aromatic ring features and hydrogen acceptors [444]. Since the internal cavity of the P-gp is composed primarily of non-polar amino acid residues [448], the hydrophobic interactions are likely to predominate to achieve high binding affinity of substrates and inhibitors. As previously mentioned, hydrophobicity for inhibitors may be particularly important for the recycling of the same inhibitor molecules via the hydrophobic interior of the membrane.

ATV is an established substrate of P-gp (see Chapter 1, Table 1.6) and therefore, according to the cycle of P-gp transport (Figure 5.5), this implies that interaction of this PI with a binding site in the substrate binding pocket of P-gp is required. Similarly, as described in Chapter 3, section 3.8.3.1, *in vitro* experiments indicated that Sutherlandioside B, the most abundant triterpenoid glycoside in SF is likely also a substrate of this ABC transporter. Since both these ligands are substrates of P-gp, there exists a potential for either ligand to competitively inhibit the P-gp transport of the other. Prerequisites for such competitive inhibition are that the ligands share a similar binding mode and that the ligand acting as the inhibitor has a higher binding affinity for P-gp than the other ligand. To investigate the potential for Sutherlandioside B, as well as the major flavonol glycoside in SF, Sutherlandin C, to competitively inhibit ATV binding at the P-gp substrate binding pocket, the binding modes (poses) and computationally derived docking energies, ΔG and K_i values of these three ligands in the P-gp substrate binding pocket were compared, using *in silico* molecular docking. The possibility that either of the three ligands enhance P-gp ATP hydrolysis or inhibited verapamil-stimulated ATPase activity was also explored by comparison of the

amino acid residues involved in these interactions with those in verapamil-P-gp and cyclic-tris-(*R*)-valineselenazole (QZ59-RRR)- and cyclic-tris-(*S*)-valineselenazole (QZ59-SSS)-P-gp interactions. These two stereoisomers are known inhibitors of verapamil-stimulated ATPase activity and calcein-AM export in mouse P-gp.

5.2.2 Methods

5.2.2.1 Molecular Structure of P-gp

An X-ray crystal structure of mouse P-gp, the only mammalian P-gp crystal structure available, was retrieved from the Protein Data Bank (www.pdb.org) [PDB ID: 3G5U [448]]. Mouse P-gp has 87% sequence homology with human P-gp [448]. In this crystal form which has a resolution of 3.8 Å, two P-gp molecules are present. Each molecule is ligand-free and in a drug-binding competent state or conformation. In line with the known substrate promiscuity of P-gp, a large binding pocket of ~6000 $Å^3$, which may easily accommodate at least two ligands simultaneously, is observed for each molecule. The three dimensional aspect of the structure was viewed using the "rotate tool" in Accelrys® Discovery Studio® Visualizer 2.5.5, where the protein sequence for one of the two P-gp molecules was removed to reveal the structure of a single P-gp molecule as shown in Figure 5.3.

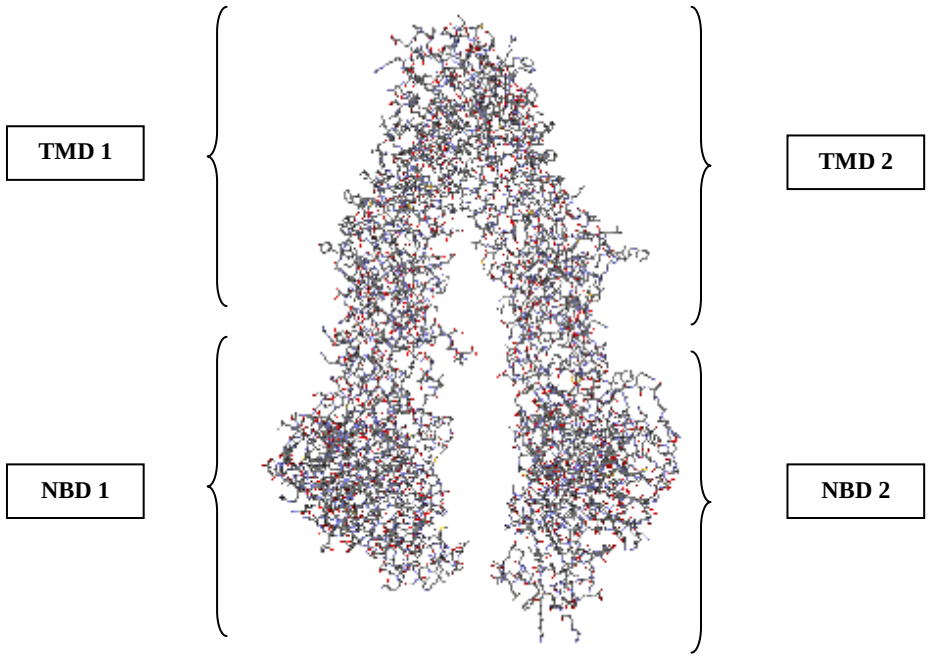


Figure 5.3: X-ray crystal structure of a P-gp molecule obtained from the Protein Databank [PDB ID No: 3G5U]. Note: Two P-gp molecules were present in the original crystal structure, one of which was removed and viewed in Accelrys® Discovery Studio® Visualizer 2.5.5.

5.2.2.2 Molecular Structures of Ligands

The 2D chemical structures of each ligand were constructed and the geometry optimised to 3D using ACD® Chemsketch® 12.0. These three dimensional structures were viewed using the "rotate tool" in Accelrys® Discovery Studio® Visualizer 2.5.5 (Figure 5.4).

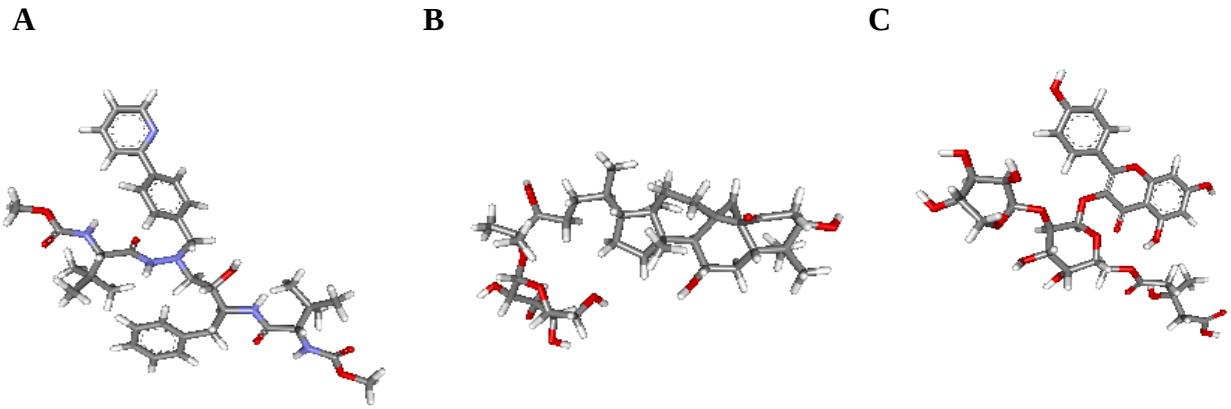


Figure 5.4: Structures of (A) ATV (B) Sutherlandioside B and (C) Sutherlandin C viewed in Accelrys® Discovery Studio® Visualizer 2.5.5

5.2.2.3 Molecular Docking

AutoDock® 4 with the AutoDock® tools graphical interface programme was used to prepare and run docking simulations and to read the scoring functions in the docking log. Accelrys® Discovery Studio® Visualizer 2.5.5 was used to view and analyse the binding modes (poses) of the ligands docked in the substrate binding pocket of P-gp.

5.2.2.3.1 Preparation of Ligand Files for use in Autodock®

Partial atomic charges, termed Gasteiger charges, were assigned by Autodock® tools (ADT) for the atoms in each ligand. ADT then merged non-polar hydrogens and assigned an "AutoDock type" to each atom. For example, nitrogens which can accept hydrogen bonds were assigned the type "NA", whilst those which cannot were assigned the type "N". ADT determined which atom was the best root, which formed part of the rigid portion of the ligand together with all atoms connected to it by non-rotatable bonds. These include amide, cyclic, aromatic and double bonds. Torsions were selected such that all rotatable bonds in each ligand were active (made rotatable).

5.2.2.3.2 Preliminary Docking Experiments

Due to the large size of the binding site pocket of P-gp, preliminary docking experiments, often termed "blind docking" were performed to determine the preferred region of binding of each ligand [380].

5.2.2.3.2.1 Preparation of the P-gp File for use in AutoDock®

P-gp was initially prepared as a rigid macromolecule. A similar assignment of charge, AutoDock® atom types and merging of non-polar hydrogens as performed for the preparation of the ligand files above was conducted in the preparation of the P-gp file.

5.2.2.3.2.2 Preparation of the Grid Parameter File and Running AutoGrid

AutoDock® requires pre-calculated 3D grid maps for each "AutoDock atom type" present in each ligand. These maps are generated by Autogrid within a defined space called a grid box which should surround the substrate binding pocket of the protein. The amino acid residues of P-gp which face the substrate binding pocket were defined by Aller *et al.* (2009) [448]. The grid box was positioned and sized such that all these residues fell within the boundaries of the box. The centre of this grid box had atomic co-ordinates of 20, 57 and -2.26 for x, y and z respectively, with a size of 50 X 60 x 50 Å. The spacing between gridpoints for the preliminary docking experiments was set at 0.75 Å. The gridbox positioned over the relevant part of P-gp is shown in Figure 5.5.

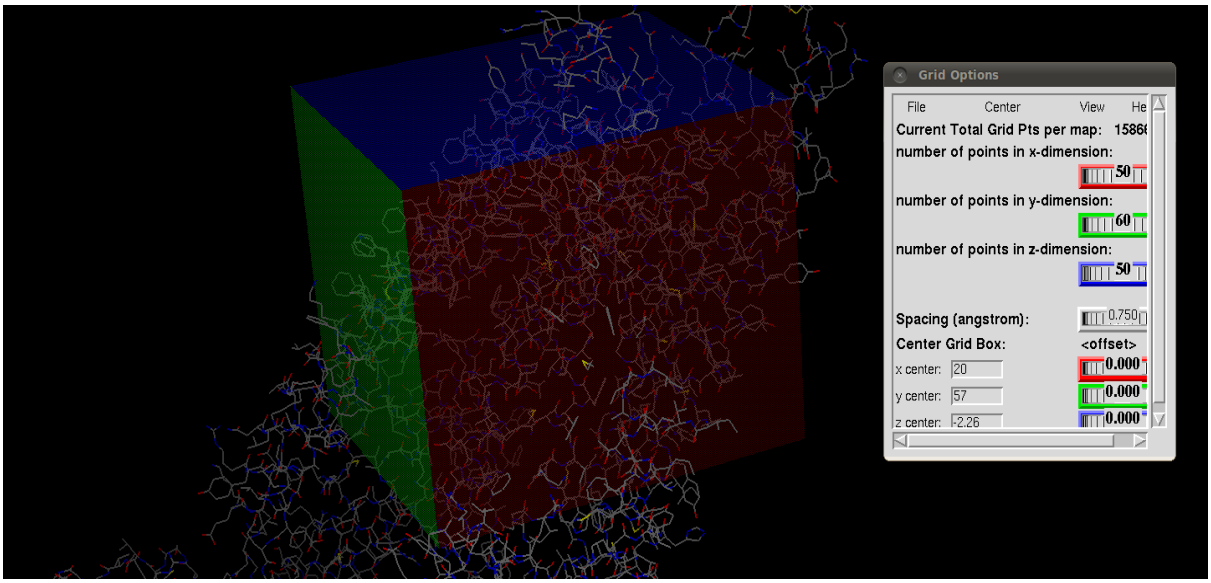


Figure 5.5: Part of screenshot of the gridbox positioned over drug binding pocket of a P-gp molecule in AutoDock® 4.

5.2.2.3.2.3 Preparation of the Docking Parameter File and Running AutoDock®

The docking parameter file contains all information on the protein, ligand and 3D grid maps generated in the preceding steps as well as instructions on which search algorithm to use to run Autodock®. The Lamarckian Genetic Algorithm [380] was used to search for different binding modes of each ligand from 100 runs. Each run started with a population of 500 random ligand conformations in random poses, with an energy evaluation being performed for each. Autodock® was programmed to select only one top individual to survive from one generation to the next. A maximum number of energy evaluations and generations were set at 25 000 000 and 50 000 respectively.

5.2.2.3.3 Second Round of Docking Experiments

The amino acid residues which each ligand most commonly interacted with, particularly in the lowest energy conformations, were identified. The specific amino acid residues involved in the conformational changes exhibited by P-gp upon substrate binding are not known; therefore P-gp in this round of docking experiments was also prepared as a rigid macromolecule. The grid box was positioned and sized such that all these residues fell within the boundaries of the box. The spacing between gridpoints for this second round of docking experiments was set at 0.375 Å instead of 0.75 Å. The same parameters for the Lamarckian Genetic Algorithm were set as described for the preliminary docking experiments.

5.2.2.3.4 Quality Assurance Analysis of AutoDock® Results

The parameters for a docking experiment are generally considered sufficient to find a best set of poses of the ligand in the protein if the results cluster into groups of similar conformations [380]. This assessment by AutoDock® involves the measurement of the root mean square deviation (RMSD) of the distance between selected atoms after superimposition of two conformations of the same ligand [380]. Poses with RMSDs which fall within a set tolerance level are grouped into the same cluster [380]. The best conformation (most probable binding mode) is usually selected as the lowest energy conformation of the most populated cluster [380]. An RMSD tolerance value of 2 Å was set for these docking experiments. The interactions between the protein and the best conformation of each ligand were also examined to evaluate the chemical reasonableness thereof.

5.2.2.3.5 Analysis of Conformations

The amino acid residues immediately adjacent to each ligand were identified using Accelrys® Discovery Studio® Visualizer 2.5.5, and those involved in the ATV interaction were independently compared to those in the Sutherlandioside B and Sutherlandin C interactions. The ΔG and K_i values associated with each conformation of a ligand were retrieved from the analysis of the docking log in AutoDock® and used to perform an analogous comparison of the binding affinities of these ligands in P-gp. Docking energy was calculated as intermolecular energy + internal energy. Key intermolecular forces, such as hydrogen and π bonding were also determined in Accelrys® Discovery Studio® Visualizer 2.5.5. Comparisons were also drawn between the residues involved in the P-gp-SF constituent interactions and those in the interaction between P-gp and the ATP hydrolysis stimulator, verapamil and between P-gp and QZ59-RRR and QZ59-SSS, inhibitors of verapamil-stimulated ATPase activity and calcein-AM export by P-gp.

5.2.3 Results and Discussion

Table 5.1 and Figure 5.6 (A) show that the best conformation of ATV interacted mainly with non-polar amino acid residues, the exceptions being Glutamine-343 and Glutamine-721. The 4-pyridinyl phenyl exhibited $\pi - \pi$ interactions with the aromatic side chain of Phenylalanine-339. Weak hydrophobic interactions may also have been at play between Phenylalanines-974, -332 and the other phenyl in ATV as the aromatic rings on both of these amino acid residues were in reasonable proximity to this group on ATV (see Figure 5.6 A). Valine-978 and Isoleucine-336 are both non-polar, neutral amino acids residues which were positioned adjacent to non-polar portions of ATV. Analogously, the polar side chain of Glutamine-343 was in proximity to the amide of one of the carbamates in ATV.

Hydroxyl groups on the sugar moiety of the best conformation of Sutherlandioside B and Serine-989 interacted via a hydrogen bond (Table 5.1 and Figure 5.6 B). A $\sigma - \pi$ interaction was observed between Sutherlandioside B and Phenylalanine-299, whilst another two phenylalanine residues (990 and 339) may have exhibited weak hydrophobic interactions as these were adjacent to non-polar portions of the triterpenoid glycoside. Sutherlandin C (see Table 5.1 and Figure 5.6 C), which has a highly hydroxylated structure exhibited five hydrogen bond interactions with P-gp, two with polar amino acid residues (Serine-989,

Asparagine-717) and three with non-polar residues which possess hydrogen-accepting groups (Phenylalanine-990, Methionine-982, Alanine-981).

The hydrophobicity of the internal cavity of P-gp may at least, in part, explain why ATV, which has greater potential for hydrophobic interactions, exhibited the highest binding affinity (lowest K_i in Table 5.1) followed by Sutherlandioside B and Sutherlandin C, where, in contrast, hydrogen bonding was more prevalent. In general, hydrophobic interactions may have a greater role to play in enhancing binding affinity of ligands in proteins, sometimes even at the expense of hydrogen bonds [449]. The K_i estimation was calculated by AutoDock® using ΔG, which was in turn calculated by addition of intermolecular energy and an estimate of the conformational entropy lost upon binding (torsional energy) [380]. Active torsions were highest in Sutherlandin C and lowest in Sutherlandioside B and in the case of the former, it may provide a further explanation for the higher ΔG and K_i observed in comparison to ATV and Sutherlandioside B, despite participating in a higher number of intermolecular interactions. The ranking for docking energy between the three ligands followed the same general trend as the ranking of ΔG and K_i, although there was more similarity in the former scoring function parameter between the ligands than the latter two. The ATV pose was therefore likely to be thermodynamically more favourable than Sutherlandioside B and Sutherlandin C. On the other hand, the difference between the binding affinity of Sutherlandioside B and Sutherlandin C may perhaps not be significant. These observations suggest that ATV was a more likely substrate of P-gp than the two phytochemical constituents of SF. From a comparison of the structures of ATV, Sutherlandioside B and Sutherlandin C and the probable physicochemical properties which may be inferred therefrom, it may be concluded that ATV was more likely to meet more of the criteria for the properties of a P-gp substrate and/or inhibitor. These were previously discussed (section 5.2.1) and the properties which are applicable in this respect relate particularly to the hydrophobicity/lipophilicity of the three ligands.

Previous studies have identified the amino acid residues of P-gp with which the substrate, verapamil interacts [450–452]. Many of these residues face the internal cavity of P-gp and are highly conserved in human and mouse P-gp, suggesting a common mechanism or mode of substrate recognition and binding [448]. Interaction with some or all of these residues may therefore be important for binding and/or subsequent initiation of ATP occlusion for

stimulation of ATP hydrolysis by verapamil and other substrates (see section 5.2.1). Only Valine-978 was involved in this verapamil-P-gp interaction [448] as well as the P-gp-ATV interaction. Moreover, there were no amino acids common to the verapamil-P-gp interaction and the P-gp interactions with either SF phytochemical constituent. These observations imply that none of the three ligands were likely to exhibit an ability to enhance ATP hydrolysis and initiate the P-gp transport cycle via this particular binding site (see Figure 5.2) to elicit ligand extrusion. The ability of any of these ligands to elicit such a response after binding in the binding modes determined in this study is not known and therefore such an effect, though not likely at the verapamil binding site, should not be unequivocally precluded.

Both QZ59-RRR and QZ59-SSS inhibit verapamil-stimulated ATPase activity as well as P-gp-mediated calcein-AM transport, despite having different binding modes to verapamil [448]. This alludes to negative co-operativity as an underlying mechanism of inhibition of these inhibitors of substrates which bind at the same site as verapamil. Interestingly, many of the amino acid residues involved in the binding of ATV (Table 5.1) were also involved in the binding of one or both of these inhibitors and may explain the known P-gp inhibitory ability of this PI, which was discussed in Chapter 2, section 2.7.3.3. A similar trend was noted for Sutherlandin C, although the comparatively higher docking energy, ΔG and K_i of -12.5 kcal/mol, -3.25 kcal/mol and 4.4 mM (see Table 5.1) respectively, indicated a relatively poor binding affinity for P-gp, which would reduce the efficiency of any P-gp inhibitory potential that the flavonol glycoside may possess. Since ATV had a different binding mode to verapamil, non-competitive inhibition of ATV by Sutherlandin C in the same manner as that of verapamil by QZ59-RRR and QZ59-SSS is also nevertheless unlikely. Sutherlandioside B on the other hand had little interaction with the binding modes of either QZ59-RRR or QZ59-SSS, possibly indicating that this triterpenoid glycoside is also unlikely to induce such negative co-operativity or non-competitive inhibition via the same binding site as these two inhibitors. Since other potential allosteric binding sites on P-gp are not known, non-competitive inhibition and even activation of the P-gp transport of ATV by Sutherlandioside B and Sutherlandin C may not be conclusively excluded.

Table 5.1 shows that the binding mode of ATV in P-gp was different to that of Sutherlandioside B and Sutherlandin C. Phenylalanine-339 was the only amino acid residue present in all three ligand-P-gp interactions, whilst Glutamine-721 was present in both ATV

and Sutherlandin C interactions. Since ATV binds to a different region of the binding site pocket of P-gp to that of either SF phytochemical constituent, simultaneous binding of ATV with each may occur, an observation which has previously been demonstrated with other P-gp substrates [453]. These *in silico* molecular docking results thus allude to the unlikelihood of competitive inhibition of P-gp-mediated ATV efflux by either Sutherlandioside B or Sutherlandin C.

In a review by Klepsch and Ecker (2010) [454], the drawbacks of the use of this recently published structure of mouse P-gp for docking studies was discussed. The resolution of the structure was relatively low at 3.8 Å, and the fact that only a specific snapshot of the transport cycle was reflected by the structure and the protein was kept rigid, despite P-gp being a highly flexible macromolecule, renders the docking and the associated scoring of binding modes quite risky [454]. These authors therefore suggest that docking studies with this mouse P-gp structure may be suitable for creating hypotheses but not for making definitive predictions. As mentioned in section 5.1 and in Chapter 1, section 1.5, the former but not the latter was the intention of this study.

Table 5.1: P-gp amino acid residues (adjacent to the ligand) and intermolecular forces involved in interactions with ATV, Sutherlandioside B and Sutherlandin C and the associated docking energy, ΔG and K_i

	ATV	Sutherlandioside B	Sutherlandin C
P-gp amino acid residues	Phenylalanine-332, Isoleucine-336, Phenylalanine-339, Glutamine-343, Glutamine-721, Phenylalanine-974, Valine-978,	Asparagine-292, Phenylalanine-299, Leucine-300, Phenylalanine-339, Serine-988, Serine-989, Phenylalanine-990,	Phenylalanine-299, Leucine-300, Phenylalanine-339, Asparagine-717, Glutamine-721, Phenylalanine-833, Alanine-981, Methionine-982, Valine-984, Glycine-985, Glutamine-986, Serine-989, Phenylalanine-990
Intermolecular Bonds	π – π with Phenylalanine-339	H-bond with Serine-989 σ – π bond with Phenylalanine-299	H-bonds with Serine-989, Phenylalanine-990, Asparagine-717, Methionine-982, Alanine-981
Docking energy	-15.5 kcal/mol	-13.7 kcal/mol	-12.5 kcal/mol
Gibb's free energy of binding (ΔG)	-6.62 kcal/mol	-6.13 kcal/mol	-3.25 kcal/mol
Binding affinity (K_i)	14.0 μM	32.0 μM	4.40 mM

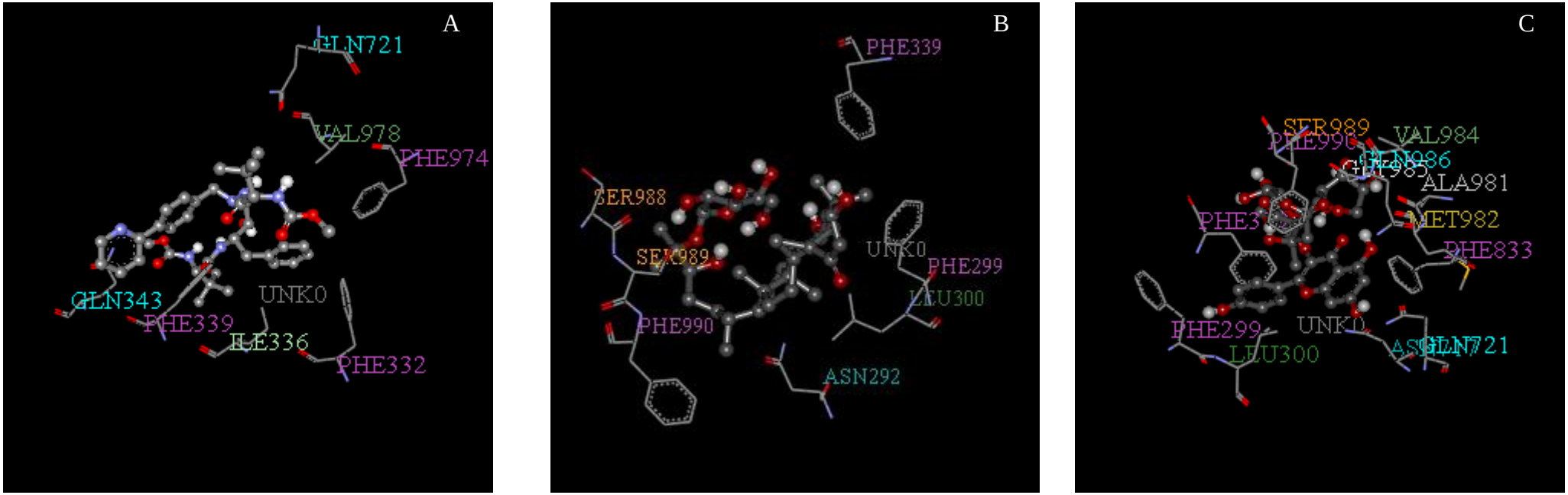


Figure 5.6: (A) ATV, (B) Sutherlandioside B and (C) Sutherlandin C docked into P-gp, where only the amino acid residues adjacent to the ligand are shown in each case. The ligands are shown as ball and stick figures and the amino acid residues as line figures. The amino acid residues are denoted by the standard three letter code and sequence number in the P-gp, whilst the ligand is symbolised by UNKO in each case.

5.3 Comparison of Molecular Docking of ATV and Components of SF into CYP3A4

5.3.1 Introduction

In 1956, Palade and Siekkevitz [455] discovered that rat liver microsomes contain phospholipids and a pigment and also exhibit reduced diphosphopyridine nucleotide-cytochrome c reductase activity. The pigment was found to participate in reduction-oxidation reactions, the reduced form of which bound carbon monoxide (CO) and exhibited a difference spectrum with an absorption maximum at 450 nm [456]. Omura and Sato [457; 458] provided evidence that the pigment is a cytochrome (haemoprotein) and named it Cytochrome P450 (CYP) [458; 459]. Meanwhile, in 1957, Ryan and Engel [460] found that the hydroxylation of steroids at carbon-21 by steroid-21-hydroxylase in bovine adrenocorticol microsomes is inhibited by CO and that this effect was reversed by monochromatic light. Aside from reduced triphosphopyridine nucleotide (TPNH), the reaction required molecular oxygen [460], one atom of which is incorporated into the product, whilst the other is reduced. Due to this phenomenon, enzymes like steroid-21-hydroxylase are classified as mixed-function oxidases [461]. A similar trend was observed by Cooper *et al.* (1965) [462] for the oxidative demethylation of codeine and monomethyl-4-aminopyrine and the hydroxylation of acetanilide by rat liver microsomes with maximum reversal occurring at 450 nm, which is the light absorption maximum of the CO-bound pigment of microsomes, previously demonstrated by Klingenberg (1958) [456]. These authors [462] were thus able to conclude that the pigment CYP is the terminal oxidase of the mixed-function oxidase systems in mammals which are responsible for the observed metabolism of these steroids and drugs.

Since the discovery of CYP enzymes, individual forms were isolated and characterised from animal and human livers. The growing number of these emphasised the need for a common nomenclature which was suggested by Nebert *et al.* (1987) [463], who assigned families, subfamilies and a number relating to the order of discovery, as described in Chapter 1, section 1.3.7.6. CYP3A4 was isolated and characterised as an inducible CYP [464] which is a 52kDa polypeptide [465] present in the human liver [464; 465]. Further research resulted in the determination of the gene code and thus the amino acid sequence of the enzyme [466–468]. In the early 1990s, using cDNA clones of CYP3A4, this isoenzyme was first transfected in cells and yeast [469–471]. This allowed for the novelty of the evaluation of specific metabolic transformations of steroids [468–470] and xenobiotics (including drugs)

[467–473] that CYP3A4 catalyses, which is now, 20 years later, a common research undertaking.

The first crystal structure of a mammalian microsomal CYP isoenzyme was elucidated by Williams *et al.* (2000) [474]. The isoenzyme binds to the endoplasmic reticulum at a hydrophobic surface formed by two portions of the polypeptide chain, such that the putative substrate access channel is in or near the membrane, adjacent to the haeme cofactor and perpendicular to the plane of the membrane to allow for interaction with NADPH P450 reductase [474].

CYP3A4 has the ability to interact with and metabolise a variety of ligands (drugs and xenobiotics) (see Chapter 1, section 1.3.7.6). Ueng *et al.* (1997) [475] suggested that this may be due to a large active site and multiple substrate binding sites on the enzyme [475]. Several human CYP3A4 crystal structures, either ligand-free or ligand-bound, have been described and published in the literature since the year 2004 [376; 476–478], in which such postulations were in part, confirmed. Yano *et al.* (2004) [476] found that CYP3A4 has an active site cavity of 1386 $Å^3$, which is bigger around the iron of the haeme moiety in comparison to CYP2C8, the significance of which is reflected in the ability of the former to metabolise bulky substrates. Several phenylalanine residues, namely, Phenylalanine-108, -213, -215, -220, -241 and -304 contribute to the roof of the active site above the haeme iron [476; 477]. The aromatic side-chains of this cluster of phenylalanine residues stack to form a prominent hydrophobic core [477]. This, according to Williams *et al.* (2004) [477], resulted in a smaller active site volume of 520 $Å^3$ whereas the findings of Yano *et al.* (2004) [476] led to postulations that conformational changes upon substrate binding may increase the size of this cavity [476]. However, Williams *et al.* [477] found no significant conformational change associated with the binding of metyrapone or progesterone, which have molecular weights of 226 and 315 g/mol respectively. Ekroos and Sjögren,(2006) [376] demonstrated that binding of larger substrates, namely, ketoconazole and erythromycin, with molecular weights of 531 g/mol and 734 g/mol, resulted in an increase in the volume of the active site cavity from 950 to 1650 $Å^3$ and ~2000 $Å^3$ respectively. X-ray crystallography, site-directed mutagenesis, molecular modelling and docking studies have revealed that amino acid residues which make up the catalytic site of CYP3A4 are: Valine-101, Asparagine-104, Arginine-105, Methionine-114, Serine-119, Leucine-211, Arginine-212, Asparagine-214, Asparagine-217, Proline-218,

Isoleucine-301, Alanine-305, Threonine-309, Isoleucine-369, Alanine-370, Leucine-373, Glutamate-374, Serine-478, Leucine-479 and the phenylalanine cluster already alluded to [476; 477; 479–481]. Furthermore, a peripheral binding site above the phenylalanine region was identified and was suggested to possibly also be involved in the binding of substrates or allosteric modulators [477].

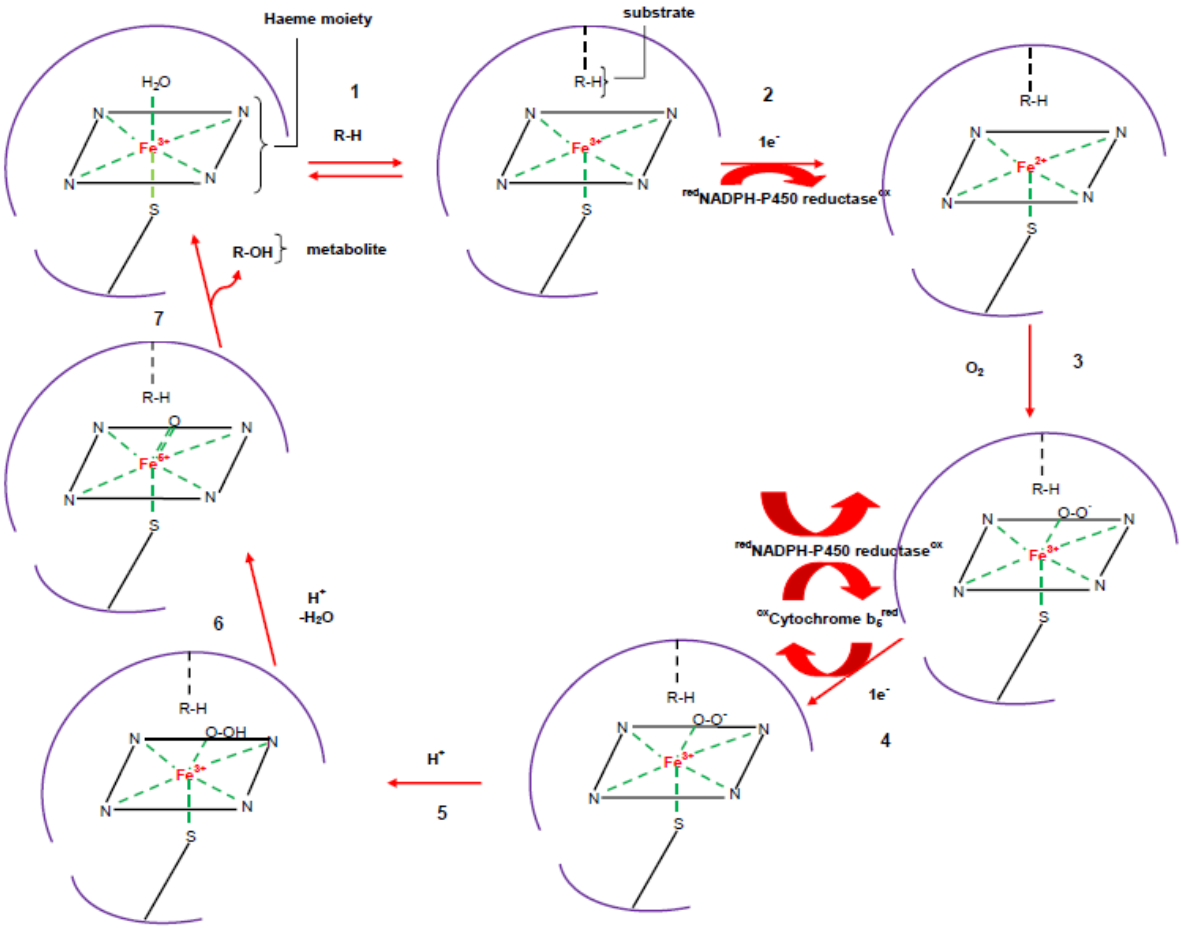


Figure 5.7: The catalytic cycle of CYP, adapted from Meunier *et al.* (2004) [482] and Guengerich, 2007 [483]

The catalytic cycle of any CYP, shown in Figure 5.7 is initiated (step 1) when the substrate binds at the active site [482–484], sterically displacing the axial water molecule involved in the hexa-co-ordinated complex with the Fe^{3+} of the haeme moiety [482]. The resulting conformational change facilitates the reduction of Fe^{3+} in step 2 of the cycle (Veitch and Williams, 1992, cited in [484]). The reduction of Fe^{3+} to Fe^{2+} occurs by the transfer of an electron to the haeme moiety from the flavoprotein, NADPH CYP reductase [482–484], imparting to the haeme a delocalised negative charge (not depicted in the figure) and efficient

reducing agent properties [482]. Amino acid residues which were found to form part of the electron transport pathway include Trp-574 of the flavoprotein and Proline-382 to Cysteine-400 of bacterial CYPBM-3 [485]. In step 3, the ferrous haeme is oxidised by reducing molecular oxygen, creating an Fe^{3+}-oxygen bond to form an Fe^{3+}-dioxy species, which is relatively stable [482–484]. The transfer of a second electron to haeme [482–484] in step 4 is facilitated by a coupled transfer from NADPH CYP reductase to cytochrome *b* reductase [483] and generates a short-lived nucleophilic negatively charged Fe-$^{3+}$-peroxo intermediate [482–484]. The latter is protonated in step 5 [482; 483] by Threonine-252 of bacterial CYP_{BM-3} [486] to form a Fe-$^{3+}$hydroperoxo complex [482; 483], a process which is also aided by Asparagine-251 of CYP [486]. Step 6 involves another protonation by Threonine-252, where a Fe^{5+}-oxygen double co-ordinate bond is formed, facilitated by the electron density accumulated in the first reduction step (due to delocalised negative charge), as well as by Asparagine-251, Lysine-178 and Arginine-186 [486]. An electrophilic Fe^{5+}-oxo species results with the loss of a molecule of water [482; 486]. In the final step of this cycle (7), nucleophilic groups on the substrate react with this electrophilic intermediate to form the metabolite [482]. Tyrosine-96 has a role to play in creating a polar environment to which bulk water molecules are attracted in the active site and through displacement, the metabolite is released from CYP [487]. The enzyme is reverted to the unbound conformation again [482–484], which is able to participate in a new cycle.

Molecular modelling of CYP3A4 based on homology with CYP102 [488] and CYP2C5 [489] has indicated that the majority of CYP3A4 substrates possess a hydrogen bond donor/ acceptor atom in proximity to the preferred site of metabolism. Further studies by these researchers showed that, like P-gp, a balance between hydrogen bonding [490] and hydrophobic interactions [490; 491] is important for substrates and inhibitors. Hydrophobic interactions in CYP3A4 are mediated primarily by the phenylalanine cluster in the active site which has the potential for extensive $\pi - \pi$ stacking interactions via the aromatic side chains [490]. In light of these findings, it is not surprising therefore that there is considerable overlap between substrates and inhibitors of CYP3A4 and P-gp.

Generally, most CYP-mediated metabolism follows simple Michaelis–Menten kinetics, in which it is assumed that the enzyme contains only one binding site where the enzyme reaction, which is in steady state and rapid equilibrium, occurs [108]. The kinetic constants

(K_m and V_{max}) for the substrate are easily derived, as are values for K_i and K_a, when a competitive or non-competitive inhibitor or an activator respectively, is added [108]. However, modulations of substrate metabolism by some CYP enzymes, in particular CYP3A4, exhibit unusual properties such as: (i) a substrate molecule activating the metabolism of another substrate molecule of the same type (autoactivation by positive co-operativity) [475; 492–495], (ii) a substrate inhibiting its own metabolism at high concentrations [496; 497], (iii) partial inhibition [494; 498; 499], which occurs when the enzyme is saturated with an inhibitor, but metabolism of a substrate is still achieved due to the formation of a substrate-enzyme-inhibitor complex which is productive, (iv) activation of one substrate in the presence of a second substrate [500–502] and (v) differential kinetics, where one substrate activates the metabolism of another substrate when positioned in one orientation in the binding site, but inhibits it when in a different binding mode [475; 503]. Multisite models, which make provision for 2 or 3 binding sites, are required to delineate the kinetic parameters and constants in these situations [108]. Failure to adequately account for multisite kinetic phenomena may compromise the appropriate interpretation of *in vitro* data in studies investigating drug-drug interaction potential [108].

Type I substrates of CYP promote electron transfer from CYP reductase by elevating the protein reduction-oxidation potential through the displacement of the water molecule co-ordinated to the iron of the haeme moiety [504; 505]. On the other hand, type II substrates of CYP3A4, such as ritonavir and ketoconazole [478] bind to the haeme iron more strongly than the water molecule, lowering the protein reduction-oxidation, thereby disfavouring reduction and preventing the turnover of the CYP catalytic cycle [504; 505], and inhibiting the metabolism of other substrates, even if these have access to the active site in appropriate binding modes.

Mutations to amino acid residues lining the active site of CYP3A4 such as Leucine-210 [506] and members of the phenylalanine cluster, namely, Phenylalanine-213, -215 and -304 [507], have been shown to alter the co-operativity exhibited by the enzyme. The peripheral binding pocket alluded to by Williams *et al.* (2000) [477] contains Asparagine-214 and Phenylalanine-219, -220, and is in proximity to some of the above mentioned amino acid residues, indicating that the pocket may be an allosteric binding site of CYP3A4.

An unusual feature of CYP3A4 is the presence of Arginine-212 in the active site cavity, the side chain of which is positioned such that hydrogen bonding with Phenylalanine-304 and the water molecules above the haeme moiety may occur [476]. Substrate binding may influence the orientation of the Arginine-212 side chain, if for example, there are intermolecular interactions between the two [476]. This may in turn alter the co-operativity if hydrogen bonding between Phenylalanine-304 and Arginine-212 is consequently affected.

The major metabolic pathways for substrates of CYP3A4 are aliphatic or aromatic hydroxylation and *O*- and *N*-dealkylation [508]. ATV is proposed to undergo N-dealkylation at three sites, as shown by M1, M2 and M3 in Figure 5.8, and aromatic hydroxylation as shown by M4 [171]. The exact structure of the major metabolite, M5 is not known, although it is thought to be a keto-metabolite with the oxygen present on either the tert-butyl or the methyl-carbamate moiety [171], as indicated by M5 of Figure 5.8.

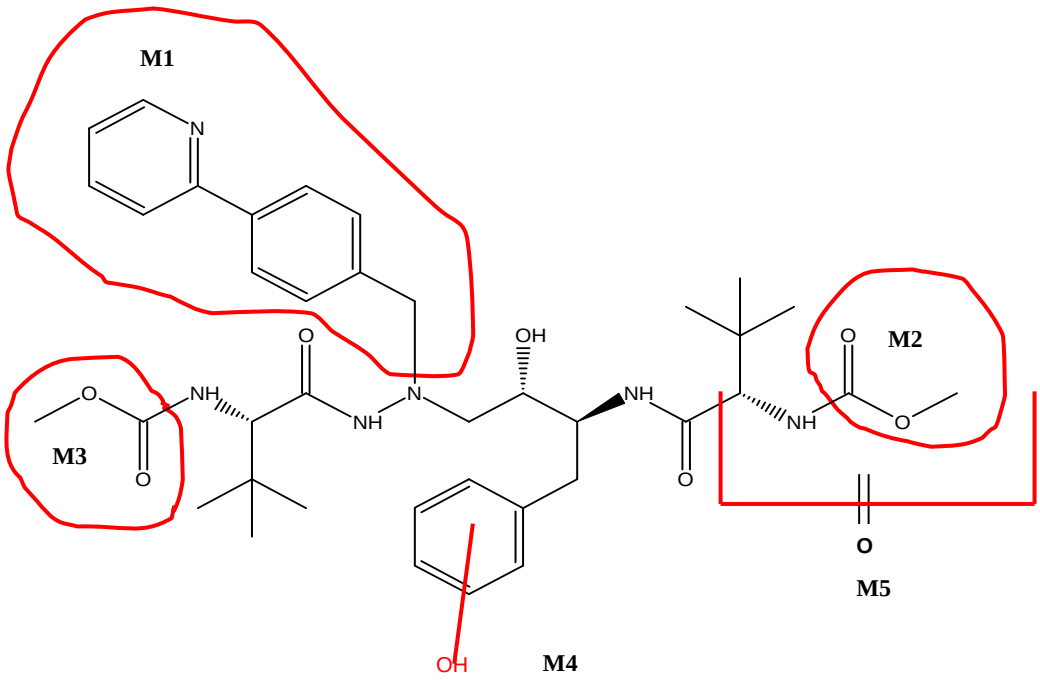


Figure 5.8: Structure of ATV showing the sites of *N*-dealkylation, aromatic hydroxylation and keto-metabolite formation [171]

To form each metabolite, ATV should be orientated in the binding site of CYP3A4 in such a way that the ligand sites of catalysis referred to in Figure 5.8 are in proximity to the iron (~6 Å apart) of the haeme moiety [382] from which the reaction required for the formation of the metabolites occurs, as indicated in the catalytic cycle of CYP shown in Figure 5.7. In addition to being a substrate of CYP3A4, ATV is also an inhibitor of this enzyme (see Chapter 2, section 2.7.3.3).

Methanolic and aqueous extracts of SF contain triterpenoid and flavonol glycosides, such as Sutherlandioside B and Sutherlandin C (see Chapter 3, section 3.7.3 and 3.7.4). These extracts [116], as well as Sutherlandioside B [231], have been shown to inhibit CYP3A4 activity. Since ATV is a CYP3A4 substrate, Sutherlandin C and/or Sutherlandioside B may have the ability to inhibit the metabolism of this PI. To investigate the potential for Sutherlandioside B, as well as the major flavonol glycoside in SF, Sutherlandin C, to competitively inhibit ATV binding at the CYP3A4 active site, the binding modes (poses) and computationally derived docking energy, ΔG and K_i values of these three ligands in the CYP3A4 active site were compared, using *in silico* molecular docking. The possibility that any of the three ligands are involved in the co-operativity of CYP3A4 was also investigated by ascertaining whether these interact with any of the amino acid residues implicated in this phenomenon.

5.3.2 Methods

The method of Jayakanthan *et al.* (2009) [381] was followed with a few modifications.

5.3.2.1 Molecular Structure of CYP3A4

A ligand-free x-ray crystal structure of human CYP3A4 with a resolution of 2.05 Å was retrieved from the Protein Data Bank (www.pdb.org) [PDB ID: 1TQN [476]]. The CYP3A4 contained a catalytic active site cavity with a volume of 1386 $Å^3$, which like P-gp is known to be able to accommodate at least two ligands simultaneously, with the co-factor haeme located therein. The structure, from which water molecules were removed to obtain a drug binding competent CYP3A4, was viewed in three dimensions using the "rotate tool" in Accelrys® Discovery Studio® Visualizer 2.5.5 (Figure 5.9).

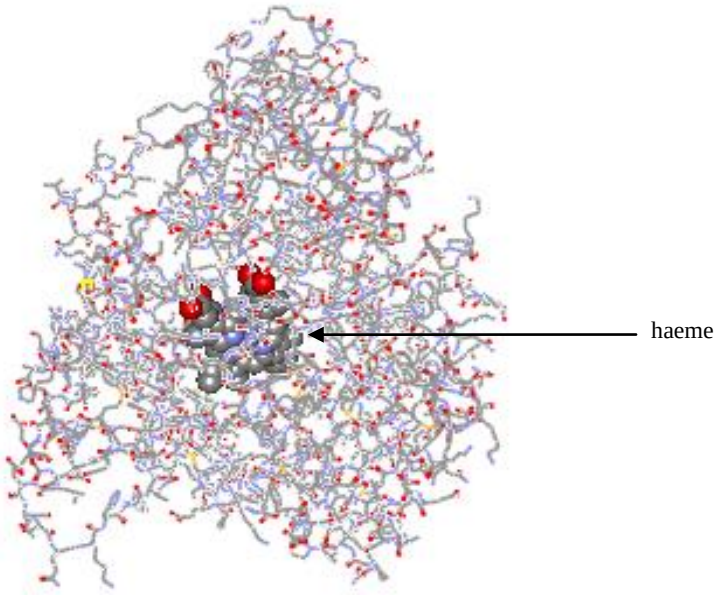


Figure 5.9: X-ray crystal structure of human CYP3A4 with co-factor haeme (shown in CPK display) obtained from the Protein Databank [PDB ID No: 1TQN] from which water molecules were removed. Viewed in Accelrys® Discovery Studio® Visualizer 2.5.5.

5.3.2.2 Molecular Structures of Ligands

The structures of ligands were constructed and viewed as described in section 5.2.2.2.

5.3.2.3 Molecular Docking

Molecular docking simulations were carried out as previously described (section 5.2.2.3) with a few minor differences. The grid box was centred on the iron atom of the haeme at x, y and z co-ordinates of -15.240, -22.717 and -11.539 respectively, with a size of 60 x 60 x 60 Å (see Figure 5.10). The spacing between grid points was set at 0.375 Å. For the clustering of the conformations, an RMSD tolerance value of 2 Å was set.

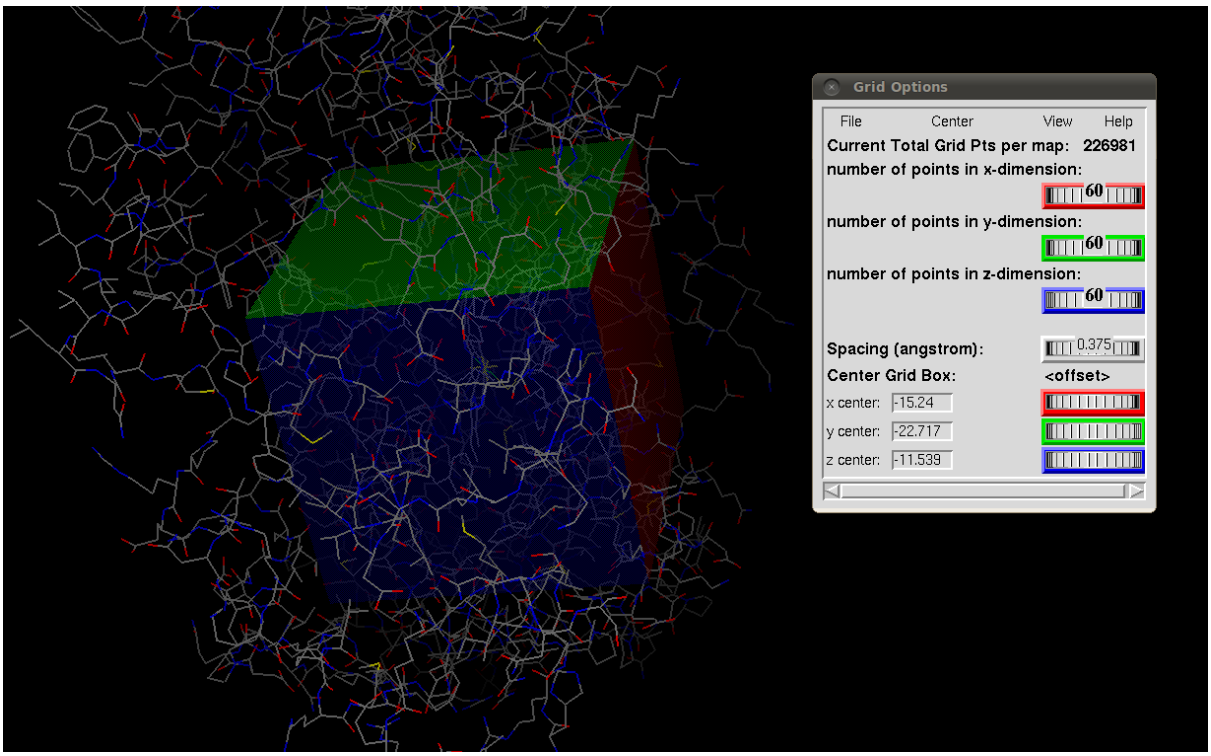


Figure 5.10: Part of a screenshot of gridbox positioned over active site of a CYP3A4 molecule in AutoDock® 4

5.3.2.4 Analysis of Conformations

The lowest energy conformation of ATV in each of the top five most populated clusters was first analysed to determine which of these clusters had an appropriate orientation to undergo CYP3A4-mediated metabolism. Teixeira *et al.* (2010) [382] recommended that the ligand/substrate site of catalysis should be a maximum of 6 Å apart from the iron of the haeme moiety. For the formation of ATV metabolites, these sites are: the respective nitrogen atoms for the formation of N-dealkylation ATV metabolites M1, M2 and M3, the aromatic carbons for the aromatic hydroxylation product, M4, and the amido carbonyl or nitrogen in the case of product M5. Accelrys® Discovery Studio® Visualizer 2.5.5 was used to determine if these atoms were ≤6 Å apart from the iron in the haeme cofactor. Only those conformations that fulfilled one or more of these criteria were used to compare binding modes and affinities with the lowest energy conformation in the most populated cluster for Sutherlandioside B and Sutherlandin C.

The amino acid residues which were immediately adjacent to each ligand were identified using Accelrys® Discovery Studio® Visualizer 2.5.5, whilst the ΔG and K_i values associated with each conformation of a ligand were retrieved from the analysis of the docking log in

AutoDock®. Docking energy was calculated as intermolecular energy + internal energy. Key intermolecular forces, such as hydrogen and π bonding were also determined in Accelrys® Discovery Studio® Visualizer 2.5.5.

5.3.3 Results and Discussion

Of the five ATV conformations which were viewed, three exhibited an appropriate orientation or binding mode within CYP3A4 such that one of the sites of catalysis, shown in Figure 5.8, was a maximum of 6 Å away from the haeme iron. The first of these three conformations is shown in Figure 5.11 (A) where the formation of M1 was favoured. Likewise, Figure 5.11 (B) and Figure 5.11 (C) depict conformations of ATV for which the production of M4 and M5, respectively, was possible. Table 5.2 shows that even though each of these three conformations was from a different cluster of the docking log, nine amino acid residues interacted with all three conformations of ATV. There was however a large difference in the ΔG which is reflected by the wide range in K_i values of 0.179–483 nM. The docking energies followed the same order of rank and also exhibited a wide range, indicating that the relative magnitudes of the ΔG and K_i values were plausible. However this range of K_i values falls below 1 µM which has arbitrarily been set as the maximum K_i for potent inhibitory activity *in vitro* [141], therefore the variation in K_i between the three may be of no consequence.

In molecular docking studies of ATV into the same 3D structure of CYP3A4 described by Jayakanthan *et al.* (2009) [381], a similar set of "interaction" amino acid residues was observed. Hydrogen-bonding between ATV and Serine-119 and Arginine-212 was also a salient feature of the docking results in both studies. Jayakanthan *et al.* (2009) [381] obtained a ΔG and docking energy of -7.40 kcal/mol and -13.08 respectively, which is similar to that obtained for the M5 favoured conformation (Table 5.2), but higher than that observed for M1 and M4.The discrepancy may reflect a difference in the search parameters set for the Lamarckian Genetic Algorithm. Jayakanthan *et al.* (2009) [381] used population size, maximum number of energy evaluations and maximum number of generations of 150, 250 000 and 1000 respectively in 10 runs, in comparison to the higher values used for each of these parameters in this study (see section 5.2.2.3.2.3). The "search" in the present study was thus run for longer, more binding modes were obtained and similar conformations could be grouped into several clusters. As a result, the reliability of the docking results in these

docking experiments is probably greater than that those undertaken by those authors. Furthermore, Jayakanthan and co-workers [381] did not investigate whether the ATV conformation selected is in fact appropriately positioned for the formation of any of the known ATV metabolites; therefore, despite having the lowest energy and thus the highest binding affinity, the ATV conformation that was presented may not be appropriate for metabolism of this PI.

Like P-gp, most of the amino acid residues which form the active site cavity of CYP3A4 are non-polar in nature, therefore it is plausible that hydrophobic interactions are favoured over hydrogen bonding as a determinants of the binding modes and affinities of a ligand in this enzyme. Intermolecular hydrogen bonding with polar amino acid residues was more prevalent in Sutherlandin C- and Sutherlandioside B-CYP3A4 interactions (See Figure 5.11 A and B and Table 5.3) than in ATV-CYP3A4 interactions (Table 5.2), where the hydrophobic interactions predominated. This may explain why the ΔG and K_i values were much higher for Sutherlandioside B and Sutherlandin C (Table 5.3) in comparison to ATV (Table 5.2), which was confirmed by a similar trend observed in the docking energy. According to the arbitrary scale for delineating CYP inhibitory potential [141], a weak CYP450 inhibitor has a K_i value greater than 20 µM, therefore the computationally derived K_i values of Sutherlandioside B and Sutherlandin C of 4.01 and 11.6 µM (Table 5.3) respectively may suggest that the SF phytochemical constituents may have intermediate CYP450 inhibitory potential and ATV may have a potent inhibitory potential.

Sutherlandioside B interacted with 60, 67 and 56% of the amino acid residues involved in the binding mode of ATV for the formation of M1, M4 and M5 respectively, which suggests that this major SF triterpenoid glycoside shared binding modes with the three metabolically favourable conformations of ATV. However, the ~10–20 000 times higher K_i value obtained for Sutherlandioside B, in comparison to the ATV conformations, implies that each ATV conformation was more likely to bind to CYP3A4 than Sutherlandioside B; therefore, despite its arbitrary classification as an intermediate CYP inhibitor, it is not likely that the latter would exhibit competitive inhibition of the metabolism of the former. This is particularly true in the case of the formation of M1 and M4, where K_i differences were ~1000 and ~20 000 respectively. Sutherlandin C interacted with 80, 83 and 78% of the amino acid residues involved in the binding mode of ATV for the formation of M1, M4 and M5 respectively, but

had an even lower binding affinity than Sutherlandioside B; therefore its potential to competitively inhibit the conversion of ATV to M1, M4 or M5 is even less likely.

All three conformations of ATV as well as Sutherlandioside B and Sutherlandin C interacted with Phenylalanine-215, whilst two ATV conformations and Sutherlandin C interacted with Phenylalanine-213. These same three ligands together with Sutherlandioside B also interacted with Phenylalanine-304. Since the phenylalanine residues referred to are thought to be implicated in co-operativity of CYP3A4 (see section 5.3.1), all the ligands may be substrates and/or effectors of this phenomenon by CYP3A4. Moreover, two of the ATV conformations and both SF phytochemical constituents formed hydrogen and/or π – cation interactions with Arginine-212, which may alter the Phenylalanine-304-mediated co-operativity of CYP3A4, as previously alluded to (section 5.3.1).

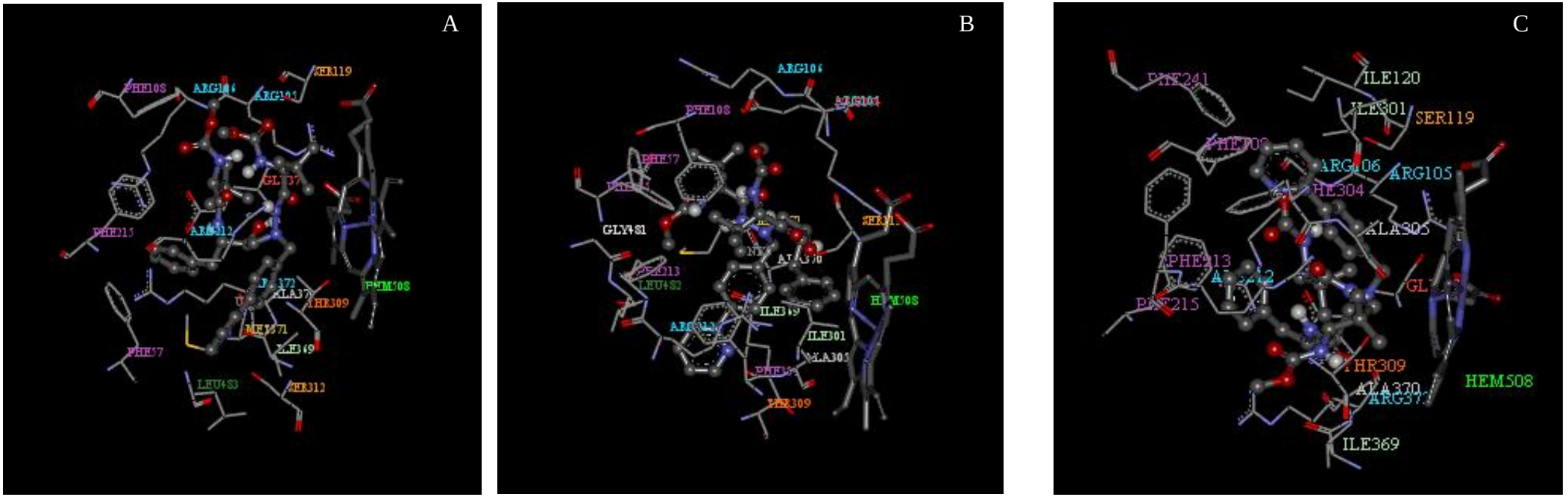


Figure 5.11: (A) ATV conformation for formation of M1, (B) ATV conformation for formation of M4 and (C) ATV conformation for formation of M5, docked into CYP3A4, where only the amino acid residues adjacent to the ligand (ATV) are shown in each case. Haeme, ATV and the amino acids residues are displayed as a stick, ball and stick, and line figures respectively. The amino acid residues are denoted by the standard three letter code and sequence number in the P-gp, whilst the ligand is symbolised by UNKO in each case.

Table 5.2: CYP3A4 amino acid residues (adjacent to the ligand) and intermolecular forces involved in interactions with ATV to form M1, M4 and M5 and the associated docking energy, ΔG and K_i

	ATV (M1)	**ATV (M4)**	**ATV (M5)**
CYP3A4 amino acid residues	Phenylalanine-57, Arginine-105, Arginine-106, Phenylalanine-108 Serine-119, Arginine-212, Phenylalanine-215, Threonine-309, Serine-312, Isoleucine-369, Alanine-370, Methionine-371, Arginine-372, Glutamate-374, Leucine-483	Phenylalanine-57, Arginine-105, Arginine-106, Phenylalanine-108, Serine-119, Arginine-212, Phenylalanine-213, Phenylalanine-215, Isoleucine-301, Phenylalanine-304, Alanine-305, Threonine-309, Isoleucine-369, Alanine-370, Methionine-371 Glutamate-374, Glycine-481, Leucine-482	Arginine-105, Arginine-106, Phenylalanine-108, Isoleucine-118, Serine-119, Isoleucine-120, Arginine-212, Phenylalanine-213, Phenylalanine-215, Phenylalanine-241, Isoleucine-301, Phenylalanine-304, Alanine-305, Threonine-309, Isoleucine-369, Alanine-370, Arginine-372, Glutamate-374
Intermolecular Bonds	H-bond with Serine-119 σ – π bond with haeme	H-bond with Serine-119 Cation – π bond with Arginine-212	H-bonds with Arginine-212 and Alanine-370 Cation – π bond with Arginine-212
Docking energy	-17.3 kcal/mol	-19.5kcal/mol	-11.3 kcal/mol
Gibb's free energy of binding (ΔG)	-11.5 kcal/mol	-13.3 kcal/mol	-8.62 kcal/mol
Dissociation constant of binding (K_i)	3.57 nM	0.179 nM	483 nM

Table 5.3: CYP3A4 amino acid residues (adjacent to the ligand) and intermolecular forces involved in interactions with Sutherlandioside B and Sutherlandin C and the associated docking energy, ΔG and K_i

	Sutherlandioside B	**Sutherlandin C**
CYP3A4 amino acid residues	Arginine-105, Phenylalanine-108, Serine-119, Arginine-212, Phenylalanine-215, Isoleucine-301, Phenylalanine-304, Alanine-305, Isoleucine-369, Alanine-370, Methionine-371, Leucine-482, Leucine-483	Phenylalanine-57, Arginine-105, Phenylalanine-108, Serine-119, Isoleucine-120, Arginine-212, Phenylalanine-213, Phenylalanine-215, Phenylalanine-241, Isoleucine-301, Phenylalanine-304, Alanine-305, Isoleucine-369, Alanine-370, Methionine-371, Arginine-372, Leucine-373, Glutamate-374, Leucine-482, Leucine-483
Intermolecular Bonds	H-bonds with Arginine-105, Arginine-212, Leucine-483	H-bonds with Arginine-105, Serine-119, Arginin-212, Alanine-370, Arginine-372, Glutamate-374, Cation – π bonds with Arginine-105, Arginine-212
Docking energy	-14.1 kcal/mol	-10.9 kcal/mol
Gibb's free energy of binding (ΔG)	-7.36 kcal/mol	-6.73 kcal/mol
Binding affinity (K_i)	4.01 μM	11.6 μM

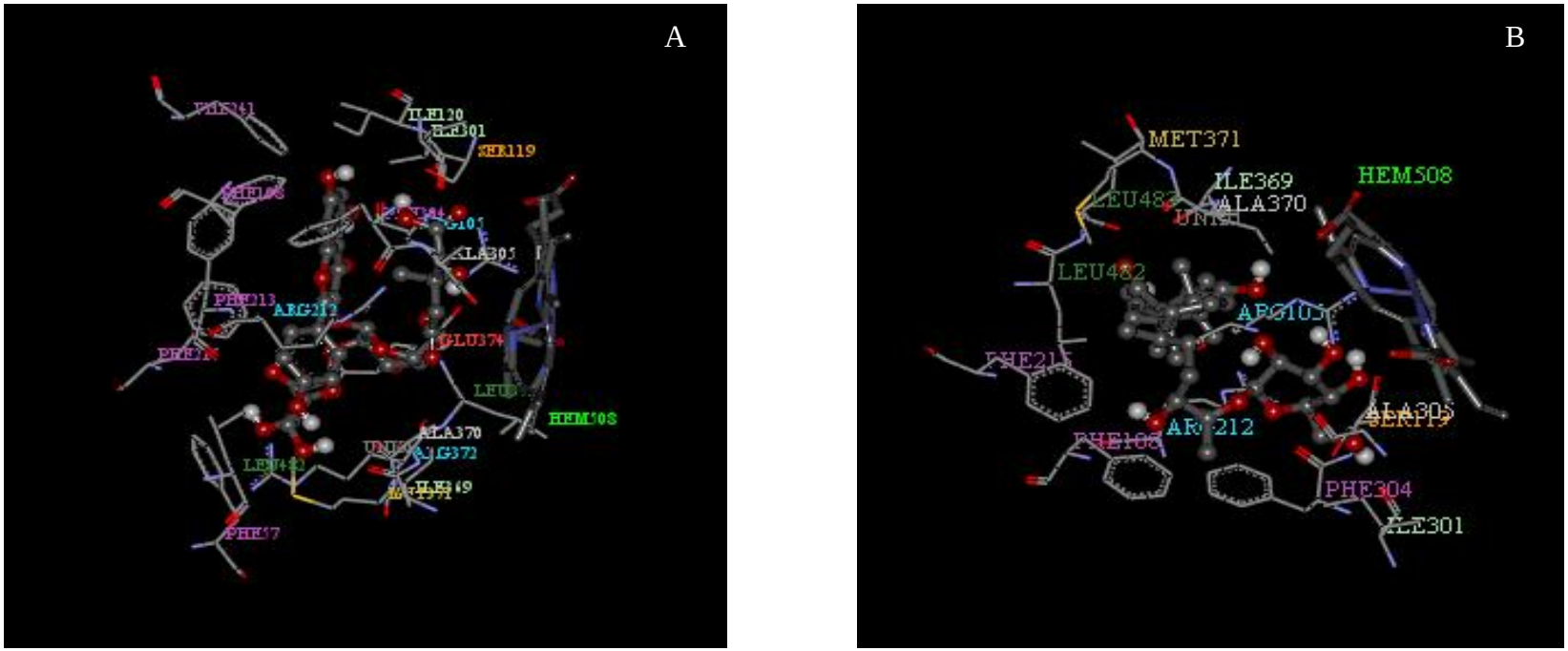


Figure 5.12: (A) Sutherlandioside B conformation and (B) Sutherlandin C conformation docked into CYP3A4, where only the amino acid residues adjacent to the ligand are shown in each case. Haeme, the ligand and the amino acids residues are displayed as stick, ball and stick, and line figures respectively. The amino acid residues are denoted by the standard three letter code and sequence number in CYP3A4, whilst the ligand is symbolised by UNKO in each case.

5.4 Conclusions

Protein-ligand interactions have an important role to play in drug transport and metabolism. Substrate binding at the active site initiates the catalytic cycles of both P-gp and CYP3A4, which, following a sequence of events, culminates in extrusion or metabolism of the same substrate. Competition between two different substrates for the same position in the active site may result in inhibition of the transport or metabolism of the substrate that has the lower binding affinity. Moreover, binding of ligands at allosteric sites may result in positive or negative co-operativity whereby conformational changes are elicited that facilitate or hinder binding of the substrate at the active site resulting in activation or non-competitive inhibition of the substrate, respectively.

The binding mode of the substrate in the active site of CYP3A4 dictates whether the ligand site of catalysis will be appropriately positioned, close to the iron of the haeme moiety, to allow for the reaction, in which the substrate is converted to the metabolite, to occur. Binding affinity determines whether the substrate remains associated with CYP3A4 or P-gp throughout a complete cycle of metabolism or transport, respectively. This parameter is usually dependent on the strength of the intermolecular interactions between the ligand and the protein. Hydrophobic interactions and hydrogen bonding both have a role to play in CYP3A4- and P-gp-ligand interactions, although the former usually predominate.

ATV is an established substrate and inhibitor of both CYP3A4 and P-gp, whilst Sutherlandioside B is a known P-gp substrate and CYP3A4 inhibitor. Aqueous and methanolic extracts of SF, which likely contain an abundance of the flavonol and triterpenoid glycosides, such as Sutherlandioside B and Sutherlandin C were found to inhibit P-gp and CYP3A4 activity, which suggested that SF constituents may have the potential to alter the P-gp transport and/or the CYP3A4 metabolism of ATV.

Molecular docking was used to screen for the drug interaction potential of SF phytochemical constituents with respect to the P-gp transport and CYP3A4 metabolism of ATV. The binding modes were compared by evaluating the intermolecular bonds and the amino acid residues of the protein involved in the protein-ligand interactions. Binding affinity was estimated by comparisons of the magnitude of the computationally derived ΔG, docking energy and K_i values. Interactions with amino acid residues potentially involved in the stimulation of ATP

hydrolysis (P-gp only) and/or the co-operative functioning of the proteins were also investigated.

ATV, Sutherlandioside B and Sutherlandin C each exhibited different binding modes in P-gp suggesting that simultaneous binding of the lowest energy conformations of these ligands may occur and that competitive inhibition of P-gp transport of ATV by either SF phytochemical constituents is unlikely. Sutherlandin C interacted with the same amino acid residues as QZ59-RRR and QZ59-SSS, two known non-competitive inhibitors of verapamil. However, since ATV had a different binding mode to verapamil, non-competitive inhibition of ATV by Sutherlandin C via this same binding site was unlikely. Sutherlandioside B had little interaction with the same amino acid residues as QZ59-RRR and QZ59-SSS, therefore this triterpenoid glycoside was unlikely to induce non-competitive inhibition via the same binding sites as these known inhibitors.

In contrast, the binding modes of three conformations of ATV suitably positioned for the formation of ATV metabolites M1, M4 and M5 all shared binding modes with Sutherlandioside B and Sutherlandin C which alluded to the potential for competitive inhibition of ATV in all three conformations by either SF constituent. Since the arbitrary scale for delineating CYP450 inhibitory potential suggests that a weak CYP450 inhibitor has a K_i value derived *in vitro* >20 µM, whilst a potent inhibitor has a K_i value <1 µM, the computationally derived K_i values for Sutherlandioside B and Sutherlandin C of 4.01 and 11.6 µM respectively may suggest that the SF phytochemical constituents have intermediate CYP450 inhibitory potential, whilst ATV with K_i values in the range 0.179–483 nM ATV has potent inhibitory potential. However, due to the large differences in K_i values between ATV and the SF constituents, with respect to competitive inhibition of ATV, the SF constituents were likely to exhibit a weak effect, if any, with the ATV conformation possible for the formation of M5, whilst no effect for the other two was expected. All three conformations of ATV as well as Sutherlandioside B and Sutherlandin C interacted with one or more of phenylalanine-213, -215 and -304, which are proposed to be involved in the co-operativity of CYP3A4, which indicates that one or more of the ligands may be substrates and/or effectors of this phenomenon by CYP3A4. Moreover, two of the ATV conformations and both SF phytochemical constituents formed hydrogen and/or π – cation interactions with Arginine-212, which may alter the Phenylalanine-304-mediated co-operativity of CYP3A4.

Sutherlandioside B and Sutherlandin C may thus modulate CYP3A4-mediated metabolism of ATV if, in such co-operative interactions, the former are the effectors and the latter is the substrate.

In summary, *in silico* data indicated that competitive inhibition of P-gp transport of ATV by Sutherlandioside B and Sutherlandin C is unlikely. The study did not provide evidence to exclude non-competitive inhibition or activation of P-gp transport of the PI by these SF constituents, since all possible allosteric sites of P-gp are unknown. It may therefore be concluded that any increase in ATV absorption which may be observed in the presence of these SF constituents, in *in vitro* models of this process, would either not be P-gp-mediated or, if P-gp-mediated, it would likely occur via non-competitive inhibition. Weak competitive inhibition of CYP3A4 may possibly account for a decrease in ATV metabolism *in vitro*, in the presence of Sutherlandioside B and to a lesser extent, Sutherlandin C. This was indicated by a lower binding affinity exhibited by the latter in comparison to the former. Non-competitive inhibition of CYP3A4-mediated metabolism of ATV by these SF components may however not be ruled out since analogous to P-gp, all allosteric sites on CYP3A4 where such non-competitive inhibition may be initiated, are not known. Enhanced ATV metabolism by these two SF constituents may possibly occur through activation of CYP3A4 metabolism since interactions with amino acid residues of CYP3A4 involved in positive co-operativity were evident.

CHAPTER 6
IN VITRO INVESTIGATIONS OF DRUG INTERACTIONS: INTESTINAL ABSORPTION

6.1 Background

The general definition of bioavailability (F) following the oral administration of a drug is: the rate and extent to which the drug reaches the systemic circulation [509; 510]. In Equation 6.1 below, F is dependent on the processes of absorption (f_a), metabolism in the gut (E_G) and metabolism and biliary excretion in the liver (E_H) [510].

$$F = f_a \cdot (1 - E_G) \cdot (1 - E_H) \qquad \textbf{Equation 6.1}$$

The process of absorption (f_a) includes drug release from the dosage form, drug dissolution and the transport of the drug across the apical/luminal and basolateral membranes of the enterocytes and thus into the blood stream [510]. These three components of absorption are in turn influenced by characteristics of the dosage form and the drug as well as the gastro-intestinal environment [510; 511]. Influences of the gastro-intestinal environment may be chemical or physiological in nature and include gastro-intestinal pH, the presence of certain foods, luminal digestive enzymes and luminal micro-organisms, gastric emptying time, intestinal transit time and multiple transport mechanisms between the intestinal lumen and the intestinal tissue [510; 511].

There are two primary routes by which dissolved drug molecules may move from the intestinal lumen into the enterocytes [85; 512; 513]. The paracellular pathway involves the movement of small hydrophilic, ionised drug molecules between two adjacent enterocytes, which is however usually limited by the formation of tight intercellular junctions [85; 512; 513]. In transcellular absorption, drug molecules move from the lumen across the luminal/apical membrane into the enterocytes [85; 512; 513]. Lipophilic drug molecules undergo passive transcellular transport, whilst hydrophilic drug molecules may require carrier-mediated transport by specific SLC influx transporters [85; 512; 513]. Drug molecules which have crossed the membrane by passive or active transport may be substrates of the ABC efflux transporters which extrude compounds back into the lumen to limit the absorption of potentially toxic xenobiotics as a defence mechanism [85; 512; 513]. In Chapter

1, section 1.3.7.5, a detailed account of the families and subfamilies of influx and efflux transporters was presented.

Since the absorption of a drug impacts its bioavailability (see Equation 6.1), PK drug interactions may occur as a result of one drug, or a xenobiotic, altering one or more of the processes in the absorption of another. Xenobiotics which alter gastro-intestinal pH may change the ionisation of weak basic and acidic drugs, thus modifying drug release, dissolution and drug permeability profiles [514–516]. Degradation of dissolved drugs susceptible to acid- or base-catalysed hydrolysis may also be aggravated or mitigated, thus changing the amount of parent drug available for uptake into the intestine [517–519]. A change in the expression of luminal digestive enzymes and the microflora in the gastro-intestinal tract may have a similar effect.

As described in Chapter 1, section 1.3.7.8, xenobiotics may have the ability to modify levels of transcription, translation or translocation of transporters [85], thereby changing the number available for functional activity on the membranes of the enterocytes. Alternatively or additionally, xenobiotics may directly alter the functional activity of the transporters already present on enterocytic membranes through inhibition or activation of one or more of the steps required in the transport of substrates by the transporters [85].

To identify potential PK interactions in which oral absorption of one drug is affected by another, various *in vitro* and *ex vivo* systems have been explored [520; 521]. The complexity of whole animal experiments is thus avoided since such models, although sometimes highly predictive, only allow for general effects on bioavailability to be determined and not the specific contribution of intestinal absorption, gut metabolism or hepatic metabolism [520; 521]. Furthermore, with respect to absorption, the experimental conditions may be simplified, controlled and standardised, such that only transport of the drug across the luminal/apical membrane of enterocytes is at play [520; 521]. In particular, cell culture models have bridged the gap between whole animal models and the most simple of models, *viz* isolated membrane fractions [520; 521].

6.2 Effect of Extracts and Components of SF on Accumulation of ATV in Caco-2 cells, *in Vitro*

6.2.1 Introduction

In order to determine the effect of extracts and components of SF on ATV absorption *in vitro,* a surrogate for the human small intestine is required. A cell culture system able to mimic the physical and physiological properties of the organ would be most suitable [522].

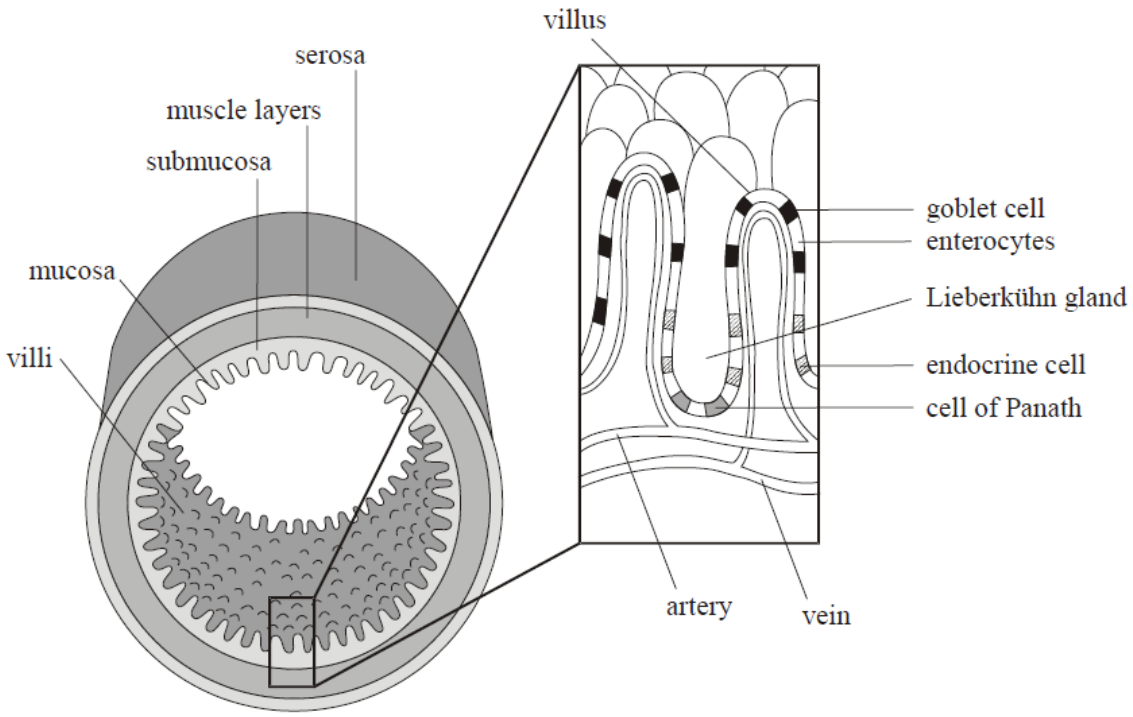


Figure 6.1: The anatomy of the human small intestine with an enlargement of the *villi*. Adapted from [523; 524].

As shown in Figure 6.1, a cross-section through the small intestine reveals several layers. The serosa is the outermost membrane which encapsulates longitudinal and circular smooth muscle layers, which in turn surround the submucosa and mucosa. To maximise its absorptive and secretory functions, the luminal surface area of the small intestine is enlarged by folding of the mucosal membrane into *plicae circulares* (also known as valves of Kerckring) on which protrusions called *villi* are located. Two *villi* are separated by a deep invagination termed the Lieberkühn gland. The intestinal mucosa is lined by columnar epithelia primarily composed of enterocytes with intermittent goblet cells. Enterocytes are the

absorptive cells whilst the goblet cells secrete mucous to promote movement and effective diffusion of gut contents. Micro*villi* (forming a brush border) are present on the apical/luminal surface membrane of enterocytes present on the *villus*. The Lieberkühn glands are lined by stem cells which differentiate into enterocytes and goblet cells in order to replenish the intestinal epithelium. The stem cells are protected by the surrounding Paneth cells, which secrete lysosomal enzymes into the lumen of the gland. Entero-endocrine cells which secrete hormones that regulate gastro-intestinal secretion or motility are also scattered, mainly in the crypt lining. Associated with each *villus* are blood and lymphatic (lacteal) capillaries from which drug molecules may diffuse to enter the systemic circulation [524–526].

The diagram above (Figure 6.1) clearly indicates that the small intestine is a very complex organ. However, the enterocytes present on the *villi* linings have the greatest influence on the bioavailability of drugs, as these cells are responsible for absorption (passive or carrier-mediated) and gut metabolism (see Equation 6.1). According to Kis *et al.* (2010) [84], transporters on the apical membrane of the enterocyte which facilitate drug uptake into enterocytes are OATP1A2, OATP2B1, OCT3 and CNT (1-3). However, jejunum samples pooled from five human subjects did not express genes for OATP1A2 and CNT3 or the expression was below the level of detection, whilst OATP2B1 and OCT 3 were expressed at very low levels [81]. Transporters which negate drug uptake by efflux back into the intestinal lumen are P-gp, MRP2, MRP4 and BCRP [84], the genes for which were all detected at high levels [81]. The Phase I CYP enzymes and Phase II enzymes involved in drug metabolism are present in the cytoplasm as well as digestive enzymes such as glycosidases, peptidases and alkaline phosphatase. Influx transporters, OCT1, OCT2, OAT2, ENT (1, 2) [84] on the basolateral membrane may carry parent drug from the systemic circulation back into the enterocyte for potential exposure to Phase I and/or II enzymes. Efflux transporters, MRP (1,3,5) and ENT (1,2) [84] on this membrane may efficiently move parent drug into the systemic circulation before possible metabolism by the CYP enzymes in the cytoplasm. The enterocytes which line the *villi* are thus said to be polarised because apical surfaces exposed to the lumen and basolateral surfaces in contact with the laterally adjacent epithelial cells have, as discussed above, different structures and functions, which allow for regulated and directed import and export of molecules [525; 526].

An *in vitro* cell culture system consisting of a monolayer of viable, polarised and fully differentiated *villi* enterocytes, similar to that found in the small intestine, would be a valuable tool in the study of drug absorption and/or gut drug metabolism. In 1983, Pinto *et al.* (1983) [527] first showed that the human colon carcinoma cell line, Caco-2, grown *in vitro* under standard culture conditions spontaneously exhibited such properties. Transmission and scanning electron microscopy showed columnar cells grown as a monolayer, attached to the growth surface at the basolateral membranes and covered with micro*villi* which extended perpendicularly to the surface [527]. The asymmetry of the cells and the presence of tight junctions suggested that the monolayer was polarised [527]. Riley *et al.* (1990) [528] found that conditions under which Caco-2 cells are cultured may modulate this morphology.

Gene expression for transporters and enzymes in any tissue or cells is the first clue of the presence of these, although this may only be confirmed by assessing protein expression. Similarly then, protein expression is a prerequisite for functional activity but does not guarantee it. This activity may be determined by conducting transporter and enzyme assays. The rank order correlation analysis of the expression of transporter genes in human jejunum and Caco-2 cells grown for 16 days indicated a good correlation between the two systems [81]. The five transporter genes which were expressed at the highest levels in the jejunum matched those expressed at high levels in Caco-2 cells although maximum levels were lower in the cell line [81]. Such correlations of gene expression of ABC efflux transporters between human jejunum and Caco-2 cells were also found by Taipalensuu *et al.* (2001) [529]. The 13 genes which were absent from the jejunum exhibited low or no expression in the Caco-2 cells [81]. Similar observations were reported by Englund *et al.* (2006) [530] and Seithel *et al.* (2006) [531].

After 5 days of culture, gene expression of a number of Phase I drug metabolising enzymes in cultured Caco-2 cells including CYP1A1, CYP1A2, CYP2C(8-19), CYP2D6, CYP2E1, CYP3A5 and epoxide hydrolase were found by Borlak and Zwadlo, 2003 [532]. Such expression of CYP3A4 and CYP2B6/7 was not detected [532]. Lampen *et al.* (1998) [533] compared the gene and protein expression of various CYP enzymes, as well as the CYP3A activity in fresh human enterocytes, human small intestinal microsomes and 18 day cultures of Caco-2 cells. The CYP1A1 gene was expressed in all three systems, whilst CYP1A2 and CYP2C9/10 genes were not detected in any. The CYP3A4 gene was expressed in fresh

enterocytes but was not detected in Caco-2 cells [533]. Protein expression of CYP1A and CYP3A was evident in the Caco-2 cells, the latter much lower than the former, whilst that of CYP1A1 and CYP3A was observed in human small intestinal microsomes [533]. CYP1A2 and CYP2C9/10 could not be detected in fresh enterocytes or in Caco-2 cells, whilst CYP2E1 protein was found in all three systems [533]. Caco-2 cells were able to produce at least three of the known CYP3A-mediated metabolites of tacrolimus, used as a CYP3A marker [533]. The fact that the CYP3A4 gene was not detected but that CYP3A protein was at very low levels may indicate that tacrolimus metabolism in Caco-2 cells was mediated by one or more of the CYP3A enzymes, which did not include CYP3A4.

Nakamura *et al.* (2002a) [534] found that Caco-2 cells at 5–7 days post-seeding had lower relative concentrations of MDR1, MRP1 and MRP2 mRNA in comparison to human duodenal enterocytes. Three of five different Caco-2 cell lines had no detectable CYP3A mRNA [534]. In those Caco-2 cell lines where CYP3A mRNA levels were present, these were lower than in human duodenal enterocytes [534]. Since CYP3A mRNA levels varied remarkably in the different Caco-2 cell lines, it was suggested that such expression, like the morphology of Caco-2 cells as described above, may be a function of culture conditions. These same authors therefore went on to explore the effects of culture period on the levels of MDR1 and CYP3A in Caco-2 cells [535]. The relative concentration of MDR1 mRNA decreased by 85% from day 6 to day 14, and thereafter remained almost constant up to day 28 [535]. In contrast, CYP3A mRNA levels in Caco-2 cells increased during culture periods and were 20 times higher from day 6 to day 28, although the level was far lower than the standard level observed in duodenal enterocytes [535]. These findings indicate that Caco-2 cells grown under standard conditions are unlikely to possess significant CYP3A activity. Although this may compromise the predictive power of Caco-2 cells to simulate human duodenal enterocytes, it may be a useful *in vitro* tool to determine the sole contribution of transporters to human intestinal absorption of drugs, without regard for CYP3A activity.

The different Phase II UGT enzymes had varying gene expressions in Caco-2 cells which were in agreement with that found in human small intestine in about 50% of cases. UGT1A1, UGT2B7 and UGT2B15 genes were highly expressed in both systems, whilst low levels were detected in both for UGT1A5, UGT1A7 and UGT1A9 [536]. UGT2B10 and UGT2B28 were undetectable in both human small intestine and Caco-2 cells [536]. UGT1A3, UGT1A4,

UGT1A8, UGT1A10 genes were detectable in both systems but a lower expression was exhibited in the Caco-2 cells compared to the human small intestine [536]. The opposite was true for UGT1A6, UGT2B4, UGT2B11 and UGT2B17 [536].

ATV is a known substrate of P-gp, MRP2, CYP3A4 and CYP3A5 (see Chapter 1, Table 1.6), whilst glucuronidation is a minor biotransformation pathway, the metabolites for which were not observed clinically (see Chapter 2, section 2.7.3.3). The oral absorption of ATV may thus be modified through any agent able to alter the activity of P-gp and/or MRP2, whilst gut metabolism may be similarly adjusted by CYP3A4 or CYP3A5 modulators. Since the gene and protein expression and functional activity of CYP3A (including CYP3A4) in Caco-2 cells appears to be insignificant, this cell line is particularly useful for the determination of ATV oral absorption, without the additional complexity of ATV metabolism. However, the interplay between the various transporters and the effects of xenobiotics thereon may not necessarily be an entirely true reflection of the dynamics of these transporters in the human small intestine, since although the rank order of gene and/or protein expression is similar, the magnitude of expression is often somewhat different in Caco-2 cells. The implication is that transporter activities may be different too, and this emphasises the circumspection required during the interpretation of all findings from Caco-2 transport studies.

The transporters with the greatest influence on drug absorption without regard for gut metabolism are, as previously mentioned, those which are present on the apical/luminal membrane of enterocytes, namely influx transporters, OATP1A2, OATP2B1, OCT3 and CNT (1-3) and efflux transporters, P-gp, MRP2, MRP4 and BCRP. Gene expression for all these, except CNT (1-3), has been detected in 16 day cultures of Caco-2 cells [81]. Caco-2 monolayers were thus grown and transport assays conducted thereon, in wells with an impermeable surface so that only drug transport across the exposed apical/luminal membrane is monitored, and therefore only the above mentioned transporters are likely to be involved. An assessment of drug transport was conducted by measuring the accumulation of drug in the cells.

Caco-2 cellular accumulation studies of ATV were first undertaken by Zastre and Bendayan, 2006 [537] in which 1 µM ATV was incubated for 120 minutes at 37 ºC in the absence and presence of various concentrations of P-gp inhibitors, PSC833, verapamil and quinidine,

MRP inhibitor, MK571 and BCRP inhibitor, Fumitremorgin C. ATV accumulation was enhanced approximately 3-fold in the presence of P-gp inhibitors, PSC833 and quinidine, and 2-fold by verapamil. The BCRP inhibitor, Fumitremorgin C had no effect on ATV accumulation, whereas the MRP inhibitor significantly decreased ATV accumulation [537]. These findings suggest that ATV absorption may be mediated by transporters.

A 20 mg/ml aqueous extract of SF was found to have no significant effect on the apparent permeability ratio (apical-to-basolateral: basolateral-to-apical) of nevirapine after a 120 minute incubation with Caco-2 cells grown as a monolayer on a permeable membrane with apical and basolateral chambers which allowed for the measurement of transepithelial flux [121]. In contrast, L-canavanine (0.0122 mg/ml) significantly increased the apparent permeability ratio, but to a considerably lower extent in comparison to verapamil (100 µM), which was the positive control [121]. The authors indicated that the effect may be due to modulation of transporters [121].

A review of the literature has revealed that ATV accumulation and thus absorption in Caco-2 cells may be transporter-mediated. Since L-canavanine had the ability to alter transepithelial flux of nevirapine, possibly through modulation of transporter activity, L-canavanine and possibly other extracts and components of SF may have the potential to alter accumulation of ATV in Caco-2 cells. Further investigations were thus deemed necessary.

6.2.2 Materials and Methods

6.2.2.1 Chemicals and Reagents

Caco-2 cells were obtained from the American Type Culture Collection (Manassas, Virginia, USA). [^{3}H] ATV (3 Ci/mmol) was purchased from Moravek Biochemicals (Brea, California, USA). Unlabelled ATV was obtained through the National Institutes of Health AIDS Research and Reference Reagent Programme, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (Bethesda, Maryland, USA). Hank's buffered salt solution (HBSS) containing 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM MgSO4, 5.3 mM KCl, 0.44 mM KH_2PO4, 138 mM NaCl, 0.34 mM Na_2HPO4, and 5.6 mM D-glucose, supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) and 0.01% bovine serum albumin (all from Sigma-Aldrich, St Louis, Missouri, USA) and adjusted to pH 7.4 was used to prepare conditioning and incubation buffers. The P-

gp inhibitor, PSC833 (i.e., valspodar) was generously donated by Novartis Pharmaceuticals (Dorval, Quebec, Canada). Triton X-100, phosphate buffered saline (PBS), L-canavanine, L-GABA and D-pinitol were all purchased from Sigma-Aldrich (St Louis, Missouri, USA). A detergent-compatible colorimetric protein assay kit (DC™ Protein Assay) was purchased from Bio-Rad (Mississauga, Ontario, Canada). The culture medium used for cell growth was Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 2 mM L-glutamine, 100 units/ml penicillin and 0.1 mg/ml streptomycin. Trypsin-EDTA (0.05%) in DMEM was used for the harvesting of cells. All these cell culture reagents were obtained from Invitrogen (Carlsbad, California, USA). PicoFluor 40 scintillation fluid was purchased from PerkinElmer Life and Analytical Sciences (Waltham, Massachusetts, USA). Leaves of *Sutherlandia frutescens* collected in Barrydale, Western Cape, South Africa were verified by the Selmar Schonland Herbarium affiliated to the Rhodes University Department of Botany and the Albany Museum in Grahamstown, Eastern Cape, South Africa. Powder obtained by the milling of *Sutherlandia frutescens* plant material was purchased from Zizamele Herbs, Onrus River, Western Cape, South Africa.

6.2.2.2 Preparation of SF Extracts and SF Flavonol Glycoside and Triterpenoid Glycoside Fractions

6.2.2.2.1 SF Extracts

SF is traditionally prepared for oral administration by the infusion of 2.5–5 g dry plant material in a cup of boiling water (see Chapter 3, section 3.6.1). To be able to examine the effect of the phytochemical constituents which may be present in such a decoction, an aqueous extract of SF for use in this *in vitro* experiment was prepared by boiling SF powder (5 g) in 250 ml water for 20 minutes on a hot plate. The brew was left to cool for 3 hours and was then centrifuged at 10 000 rpm for 15 minutes. The supernatant was transferred to a round-bottomed flask, shell-frozen in liquid nitrogen and freeze-dried. A methanolic extract was similarly prepared, except that SF powder in 250 ml methanol was heated at 50 ºC. The rationale for the use of the methanolic extract was to be able to determine the effects of phytochemical constituents which are less polar and unlikely to be present in the aqueous extract in any significant quantities. These constituents along with those present in the

aqueous extract are however likely to be amongst the various phytochemical components in commercial dosage forms which contain SF plant material.

6.2.2.2.2 Flavonol Glycoside and Triterpenoid Glycoside Fractions

This experiment was conducted prior to the availability of sutherlandiosides and sutherlandins present in SF plant material and isolated by Fu *et al.* (2008) [204] and Fu *et al.* (2010) [207], respectively (see Chapter 3, sections 3.7.3 and 3.7.4). Crude flavonol and triterpenoid glycoside fractions were therefore prepared for use as indicators of the presence of flavonol and triterpenoid glycoside in SF plant material and extracts thereof.

To prepare the flavonol glycoside and triterpenoid glycoside fractions, SF powder (100 g) was extracted with ethanol by soxhlet extraction and concentrated under vacuum. The concentrated mass was subsequently extracted with diethyl ether. The ethereal fraction was diluted with alcohol and chromatographed on a silica gel column, where dichloromethane was used to elute an organic fraction which contained the triterpenoid glycosides. The fraction which precipitated on the column and which contained the flavonol glycosides was eluted with ethanol. These crude fractions were further fractionated using a stationary phase of silica gel in dichloromethane and eluted with varying concentrations of ethanol in dichloromethane. To confirm that these fractions contained triterpenoid and flavonol glycosides, qualitative thin layer chromatography (TLC) and HPLC analyses were conducted. After development of TLC plates, these were sprayed with 1:1:10 v/v/v concentrated sulphuric acid: acetic anhydride: methanol and heated at 110 ºC for 15 minutes. A purple colour indicated the presence of triterpenoids. HPLC peaks were identified as flavonol glycosides based on qualitative similarities between the PDA-UV spectra of these and the known flavonol glycoside, quercetin-3-rutinoside. Moreover, subsequent to the recent isolation of sutherlandiosides and sutherlandins, qualitative HPLC analyses showed that these were in fact present in the triterpenoid and flavonol glycoside fractions (see Chapter 7, Figures 7.2 and 7.3).

6.2.2.2.3 Confirmation of the Presence of Triterpenoid and Flavonol Glycosides in SF Aqueous and Methanolic Extracts

To be able to draw valid conclusions regarding specific SF constituents which may be responsible for the responses elicited by the aqueous and methanolic extracts in this

experiment, it was deemed important to determine whether the aqueous and methanolic extracts (both at a concentration of 10 mg/ml) contained the relevant triterpenoid and flavonol glycosides and the relative quantities of these constituents. An HPLC-UV analysis was conducted using a method modified from that used by Prevoo *et al.* (2008) [226]. Chromatographic separation was achieved using a Luna C_{18} (2) (5 μm, 150 x 4.6 mm ID) (Phenomenex®, USA) protected by a Luna C_{18} guard column (Phenomenex®, USA) with the same ID. Samples were eluted at a flow rate of 1 ml/min with a gradient set from 98% solvent A (0.1% formic acid in water) to 100% solvent B (acetonitrile) for the first 30 minutes, after which solvent B (100%) was maintained for 5 minutes. The eluate was monitored at a UV detection wavelength of 254 nm.

6.2.2.3 Instrumentation and Equipment

Cells were grown in 75 cm^3 flasks (Sarstedt, St. Leonard, Quebec, Canada) and 48 well cell culture plates (BD Biosciences, Franklin Lakes, New Jersey, USA) in a Sanyo (MCO-17AC) incubator (Esbe Scientific Industries Inc, Markham, Ontario, Canada) as described in section 6.2.2.4 below. A Nikon TMS Inverted Phase Contrast Microscope fixed with a binocular eyepiece tube, quadruple revolving nosepiece and a plain stage (Nikon Instruments Inc, Melville, New York, USA) was used to view cells for haemocytometer cell counts during subculture and for the assessment of the degree of confluence and morphological change of growing cells. Aseptic manipulations were conducted under a NuAIR (Labgard 425 ES Class II Type A2) Biological Safety Cabinet (New Plymouth, Minnesota) and transport assays were performed on a Model DB-16525 hot plate (Thermo Fisher Scientific, Dubuque, Iowa, USA). Radioactivity was measured in 2 ml scintillation vials (Thermo Fisher Scientific, Dubuque, Iowa, USA) using a Beckman (Coulter LS6500) Scintillation counter (Fullerton, California, USA). Protein concentration was estimated by measuring UV absorbance at 750 nm using a SpectraMax 384 microplate reader (Molecular Devices, Sunnyvale, California, USA).

6.2.2.4 Cell Culture

Caco-2 cells used from passages 65 to 80 were grown as a monolayer at a density of 2000 cells/cm^2 on 75 cm^2 tissue culture flasks in culture media which was replaced every 2–3 days, in an incubator set at 37 ºC, 95% humidity and 5% CO_2. Cells from confluent cultures were harvested and seeded into 48 well plates at a seeding density of 60 000 cells/cm^2. Cells were

cultured in the plates under the same conditions described above for 21–24 days to allow cell differentiation into a tight intestinal epithelial monolayer.

6.2.2.5 Preparation of Conditioning and Incubation Buffers

Conditioning and incubation buffers were prepared using Hank's buffered salt solution (HBSS) containing 1.3 mM $CaCl_2$, 0.49 mM $MgCl_2$, 0.41 mM MgSO4, 5.3 mM KCl, 0.44 mM KH_2PO_4, 138 mM NaCl, 0.34 mM Na_2HPO4, and 5.6 mM D-glucose supplemented with 25 mM HEPES and 0.01% bovine serum albumin (BSA), which was adjusted to pH 7.4. SF extracts and components and corresponding controls were initially dissolved in solvents as shown in Table 6.1, after which the final concentrations in the conditioning and incubation buffers, also shown in Table 6.1, were prepared. PSC833 was used as the positive control for increased accumulation of ATV in Caco-2 cells [537]. Solvent content was limited to 1% since preliminary experiments showed that, at this concentration, water, methanol and dimethylsulphoxide (DMSO) had no effect on ATV accumulation in Caco-2 cells. In addition, the incubation buffer (but not the conditioning buffer) contained [^{3}H] ATV (0.1 µCi/ml) supplemented with unlabelled ATV to give a final concentration of 1 µM ATV.

Table 6.1: Solvents and concentrations of SF extracts and components and PSC833, and the corresponding controls

SF extract/component	Corresponding control
10 mg/ml aqueous extract in water	Water
100 µM L-canavanine in methanol-water (50:50, v/v)	Methanol-water (50:50, v/v)
500 µg/ml flavonol glycoside fraction in water	Water
100 µM L-GABA in water	Water
100 µM D-pinitol in water	Water
10 mg/ml methanolic extract in methanol	Methanol
500 µg/ml triterpenoid glycoside fraction in methanol	Methanol
1 µM PSC833 in DMSO	DMSO

6.2.2.6 Cellular Accumulation Experiments

To maintain maximum viability of the cells, culture media were replaced 24 hours before the experiment. Accumulation studies, conducted on a hot plate set at 37 ºC, were performed in triplicate for each group on the same cell culture plate. Culture media were aspirated and cells were initially washed and conditioned for 15 minutes with 250 µl warm (37 ºC) conditioning buffers prepared as described in section 6.2.2.5. Preliminary experiments showed that 1 hour incubations of Caco-2 cells with SF extracts and components, at these concentrations, under light microscopy at a magnification of 400 x, had no effect on the cell morphology; therefore

the viability of the Caco-2 cells was not likely affected by extracts and compounds for the duration of the incubation time used. The pre-incubation buffers were aspirated and the cells were then incubated with the incubation buffers prepared, as described in section 6.2.2.5, at 37 ºC for 1 hour. At the end of the incubation period, the reaction was stopped and the cells washed twice by the addition of ice-cold (4 ºC) 0.1 M PBS (500 µl) after aspiration of the incubation buffer. The cells in each well were solubilised with 1% Triton-X (500 µl) at 37 ºC for 30 minutes before transfer to scintillation vials. Scintillation fluid (2 ml) was added to each vial and the radioactivity of each sample was measured using a scintillation counter. In order to calibrate the cellular [^{3}H] ATV accumulation, the radioactivity of an aliquot (50 µl) of each incubation buffer was also measured. The latter samples were corrected for background radioactivity of blank buffer and ATV accumulation was initially represented as pmol ATV/well.

6.2.2.7 Protein Assay

Three wells of each plate which were only exposed to blank HBSS buffer throughout the experiment were set aside for the determination of protein concentration. Standard solutions of BSA (0.1, 0.25, 0.5, 0.75 and 1 mg/ml) were also prepared. The Bradford method [538] adapted for compatibility with detergents (Triton-X in this experiment) was applied to the samples and standard solutions according to the DC™ Protein Assay kit instructions (Bio-Rad, Mississauga, Ontario, Canada). Absorbances were measured on a UV spectrophotometer at 750 nm. A calibration plot was constructed and the unknown sample protein concentrations determined therefrom. The average protein concentration/well for each plate was calculated and ATV cellular accumulation could be standardised to this protein concentration and thus expressed in pmol/mg protein.

6.2.2.8 Data Analysis

The results were reported as mean ± SD of values from three independent experiments performed on cells from different passages. To determine the statistical significance of differences observed between control and treatment groups, an unpaired two tailed Student's *t*-test was computed using Graphpad Prism, version 3 (San Diego, California, USA). A *p*-value <0.05 was considered significant.

6.2.3 Results and Discussion

Chromatograms from the HPLC analysis (Figure 6.2) show that both the aqueous and methanolic extracts contain flavonol (peaks at 9–11 minutes) and triterpenoid (peaks at 13–17 minutes) glycosides. Both extracts contain small quantities of the triterpenoid glycosides relative to the triterpenoid glycoside fraction, used to indicate their presence. The aqueous extract contained a similar quantity of flavonol glycosides when compared to the flavonol glycoside fraction although the latter also contained a lower quantity of triterpenoid glycosides compared to the triterpenoid glycoside fraction. The methanolic extract, however, contained a much lower quantity of flavonol glycosides compared to either the aqueous extract or the flavonol glycoside fraction. These findings suggest that if the same effect is observed after treatment with the flavonol glycoside fraction and the aqueous extract, then the response of the latter may be attributed to the flavonol glycosides. On the other hand, the content of flavonol glycosides and triterpenoid glycosides in the methanolic extract is considerably lower than in the flavonol glycoside fraction and triterpenoid glycoside fraction respectively, therefore the same analogy does not necessarily apply with respect to those two fractions and the methanolic extract. The chromatograms of the flavonol and triterpenoid glycoside fractions further indicate that low quantities of several other compounds, besides the flavonol and triterpenoid glycosides were also present in these fractions and may thus contribute to any effects observed from treatment with these fractions.

Figure 6.3 shows that the aqueous extract ($p<0.05$) and D-pinitol ($p<0.01$) significantly attenuated accumulation of ATV, which was in contrast to the elevated accumulation observed in the presence of the triterpenoid glycoside fraction and PSC833 (both $p<0.0001$). The experiments were conducted on cells grown on an impermeable surface; therefore ATV accumulation is only dependent on the movement of ATV across the apical membranes and not across the basolateral membranes of the Caco-2 cells. Significant increases in ATV accumulation may therefore have reflected inhibition of one or more of the efflux transporters (P-gp, MRP2, MRP4) or enhanced activity of one or more influx transporters (OATP1A2, OATP2B1, OCT3) present on the apical membranes. Conversely, attenuated ATV accumulation was likely due to increased activity of these efflux transporters or inhibition of these influx transporters. With the use of inhibitors of P-gp, MRPs and OATPs in peripheral blood mononuclear cells (PBMCs), Janneh *et al.* (2009) [88] showed that ATV was a substrate of P-gp, MRPs and OATPs. Recently, Kis *et al.* (2010) [332] found no significant

difference in the uptake of ATV by the OATP2B1-overexpressing cells and the corresponding wild-type cells. In addition, established OATP2B1 inhibitors did not alter accumulation of ATV by these OATP2B1-overexpressing cells [332]. These findings suggest that ATV is not a substrate for OATP2B1; therefore the results of the present study were probably not due to modulation of OATP2B1 activity. In contrast, ATV, like other PIs was found to be a substrate of OATP1A2 in an over-expressing cell-line [539]. Similarly, studies conducted in a P-gp overexpressing cell line demonstrated that ATV was a substrate of P-gp, whilst those performed in an MRP-2 overexpressing cell line were inconclusive [540]. Changes in ATV accumulation were thus likely due to modulation of OATP1A2, P-gp and/or MRP2.

PSC833 has been shown to reduce the efflux ratio (P_{app} BL-AP/P_{app} AP-BL) of the P-gp probe, vinblastine with an IC_{50} of 0.15 µM in Caco-2 cells [541], suggesting that it has selective P-gp inhibitory activity in Caco-2 cells. It was thus a suitable candidate to use for the validation of the Caco-2 cell system used to conduct studies for the elucidation of transporter-mediated drug flux of ATV, a known P-gp substrate. The 2-3 fold increase in accumulation of ATV in the presence of 1µM PSC833 observed in the present study is in agreement with Zastre and Bendayan [537]. Comparative increases in the efflux ratio of ATV [537], as well as other PIs, indinavir, nelfinavir and saquinavir [542] in the presence of this same concentration of PSC833 have also been reported.

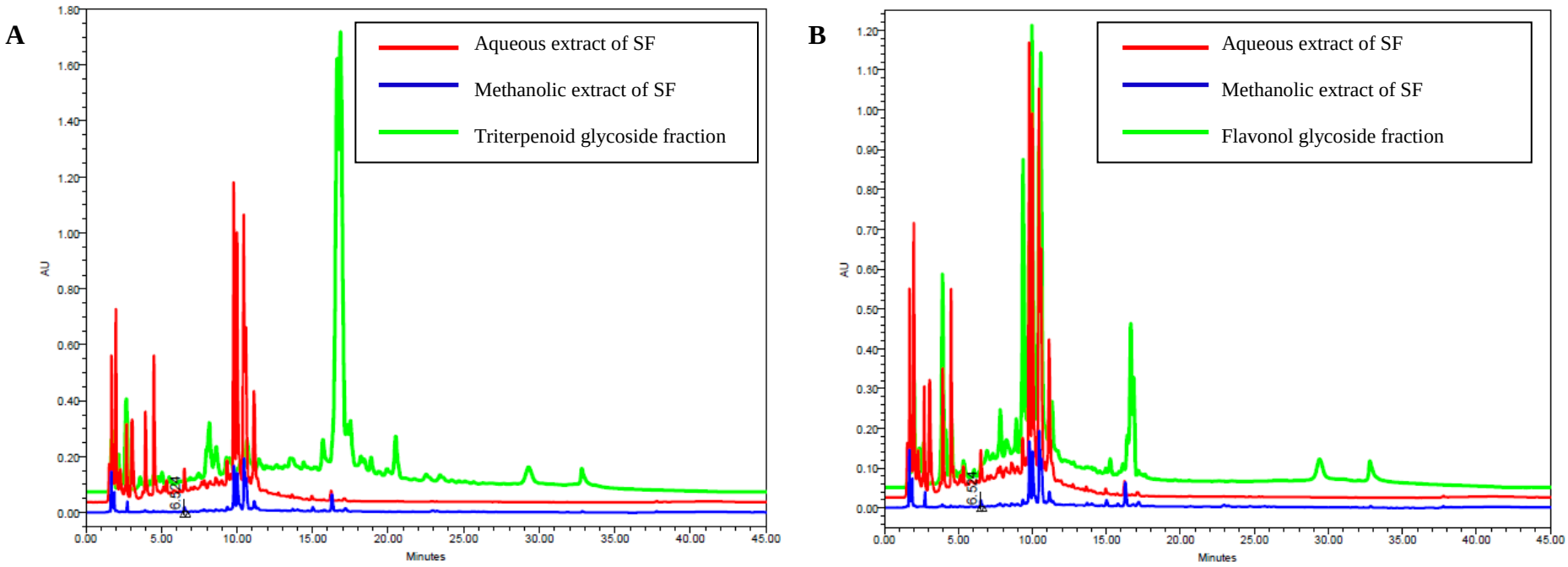


Figure 6.2: Overlay of chromatograms to show (A) the relative quantities of the triterpenoid glycosides (peaks at 13–17 minutes) and (B) the relative quantities of flavonol glycosides (peaks at 9–11 minutes) present in aqueous and methanolic extracts of SF.

The reduction in ATV accumulation by the 10 mg/ml aqueous extract of SF and 100 μM D-pinitol may be related, since the latter is a water soluble sugar which suggests that it was most likely present in the aqueous extract. D-pinitol, if present in SF as claimed, may therefore be the phytochemical constituent, or one of several in the aqueous extract, which was responsible for the observed effect. Whether D-pinitol exerts its effect by increasing the activity of P-gp and/or MRP2 or inhibiting OATP1A2, for which ATV is a likely substrate, is not clear. In skeletal muscle, D-pinitol stimulated the translocation of a glucose transporter from the cytosol to the plasma membrane [543] which has been considered as one of the mechanisms through which this sugar exhibits insulin-like properties (see Chapter 3, section 3.8.1.4). Such translocation of transporters from the cytosol to the plasma membrane has been described as one of the mechanisms of enhanced activity of influx and efflux transporters in enterocytes (see Chapter 1. section 1.3.7.8.2). D-pinitol may thus have exhibited analogous properties with respect to the efflux transporters located on the apical membrane of Caco-2 cells, whereby the number of P-gp and/or MRP2 transporters thereon were increased, thus resulting in a greater efflux and reduced accumulation of ATV.

Brown *et al.* (2008) [121] found that a higher concentration of SF aqueous extract (20 mg/ml) than that used in this study (10 mg/ml) produced no change in transepithelial flux of the NNRTI, nevirapine in Caco-2 cell monolayers. Conversely, a very low concentration of L-canavanine (12.2 μg/ml) significantly increased the efflux ratio of this NNRTI [121], whilst in the present study, a slightly higher concentration (100 μM = 17.6 μg/ml) had no effect. It recently emerged that nevirapine may be a substrate of OATP influx transporters, but not P-gp and MRP transporters [122], whilst ATV is a substrate of all three (see Chapter 1, Table 1.6). The aqueous extract of SF may thus have altered P-gp and/or MRP activity for which ATV but not nevirapine is a substrate. Moreover, the control results of Brown *et al.* (2008) [121] are in conflict with those of Störmer *et al.* (2002) [123], who found that there was no difference between BL-to-AP and AP-to-BL transport of nevirapine in Caco-2 cells and that verapamil had no effect on the transepithelial flux of this NNRTI. The change in transepithelial flux of nevirapine by L-canavanine [121] may thus possibly have been independent of the transporters and in particular, the efflux transporters, P-gp and MRPs.

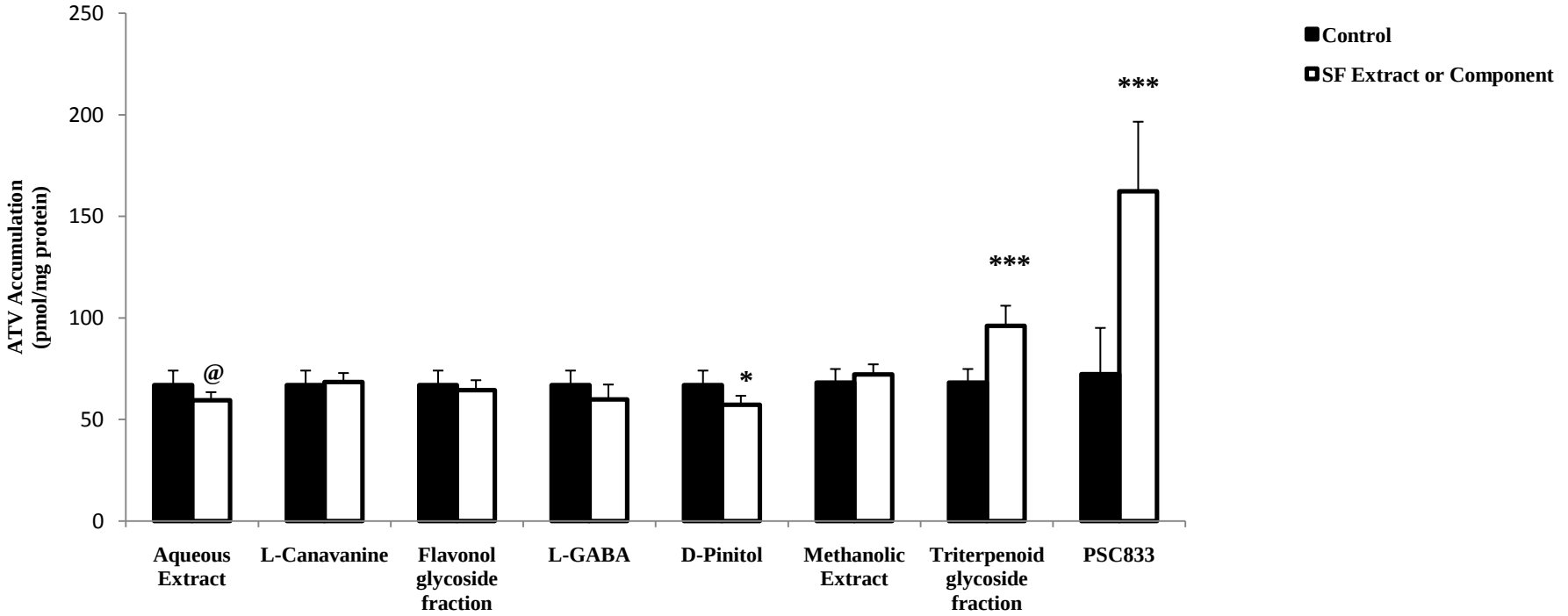


Figure 6.3: Effect of extracts and components of SF on ATV (1 µM) accumulation in Caco-2 cells, *in vitro*. PSC833 (1 µM) was used as a positive control. Each bar represents mean ± SD; n = 3. @ ($p<0.05$), * ($p<0.01$) and *** ($p<0.0001$) in comparison to control (Student's *t*-test).

The flavonol glycoside fraction of SF had no significant effect on ATV accumulation. This indicates that despite high quantities of flavonol glycosides present in the aqueous extract (see Figure 6.2 B), these did not likely contribute to the effect observed by this extract. Flavonol glycosides have exhibited low transepithelial flux in the apical-to-basolateral direction in Caco-2 cells in comparison to flavonol aglycones [544–547]. In one of these studies, this flux was increased in the presence of an ATP depletor [546] which suggests that the low transepithelial flux in Caco-2 cells was facilitated by efflux of the flavonol glycosides by the ABC transporters (P-gp, MRPs), which require ATP to function, rather than by poor passive uptake or low carrier-mediated influx transport (by OATPs, OCTs, CNTs). It was then demonstrated by several authors that flavonol glycosides may be MRP substrates, due to increased transepithelial flux observed in the presence of MRP inhibitors such as MK571 [544–546]. These findings point to the potential for such glycosides to competitively inhibit the efflux of other MRP substrates, which may include ATV. However, in one study, 3 out of 4 flavonol glycosides (100 μM) did not alter the transepithelial flux of cimetidine in Caco-2 cells, whilst all 4 corresponding flavonol aglycones did. It may thus be concluded that glycosylation of flavonols may limit the inhibitory capability of these compounds on the efflux of cimetidine in Caco-2 cells [548], which may also be true of those in SF with regard to ATV flux.

Fuchikami *et al.* (2007) [549] found that flavonol glycosides contained in *Gingko biloba* leaves and flavonol aglycones in green tea inhibited OATP2B1-mediated influx of OATP2B1 substrate, estrone-3-sulphate in an OATP2B1-overexpressing cell line. However, even if the flavonol glycosides in SF exhibited this property too, ATV, as previously mentioned, is not a substrate of OATP2B1, therefore such an effect would not be expected. Interestingly, the flavonoid aglycone, daidzen, increased ATV accumulation in human PBMCs [88], indicating that efflux transporters on the plasma membrane of PBMCs may have been inhibited. Conversely, another flavonoid aglycone, genistein, reduced ATV accumulation [88] implying that opposing mechanisms may be at play. Experiments using the corresponding glycosides were however not undertaken, thus it is not known whether, analogous to the reported results of Taur and Rodriguez-Proteau (2008) [548] discussed above and findings of the present study, no effect on ATV flux would be observed.

It has been demonstrated that flavonol glycosides may be converted to the corresponding aglycones in Caco-2 cells [547], which may activate potential efflux inhibitory activity of the phytochemicals. Nonetheless, the conversion occurs only after incubating flavonol glycosides with Caco-2 cells for at least six hours. This time period extends well beyond the incubation time of one hour used in the present study. The proposed conversion is thus of no consequence in this study, as are the effects associated therewith. Since deglycosylation of flavonol glycosides may occur in the gastro-intestinal tract [550], before they reach the site of intestinal absorption, it may be prudent to conduct further investigations into the effects of the aglycones of SF flavonol glycosides on ATV accumulation in Caco-2 cells.

The triterpenoid glycoside fraction significantly increased ATV accumulation, which demonstrates, as described earlier, that these compounds may have inhibited efflux transporters or increased the activity of influx transporters. Madgula *et al.* (2008) [231] found that Sutherlandioside B, the most abundant of the known triterpenoid glycosides in SF, was subject to low transepethelial flux or accumulation in Caco-2 cells which was blocked by the P-gp inhibitor, verapamil and the MRP inhibitor, MK-571. This suggests that the low transepithelial flux of this triterpenoid glycoside was P-gp- and/or MRP-mediated. Sutherlandioside B is thus probably a substrate of these transporters and may have exhibited competitive inhibition of P-gp and/or MRP2-mediated transport of ATV, thereby enhancing ATV accumulation. However, it is important to note that, contrary to the findings that Sutherlandioside B is the major component found in various samples of SF plant material by Avula *et al.* (2010) [205] and in Sutherlandia Su1™ tablets [206] (see Chapter 3, section 3.7.3), this sutherlandioside appeared to be present in lower quantities in the triterpenoid glycoside fraction used in this study in comparison to the other identified sutherlandiosides (see Chapter 7, Figure 7.2). In the present study, therefore, the other sutherlandiosides and other unknown constituents of the triterpenoid glycoside fraction may have had a greater role in imparting the observed effects of the fraction. Interestingly, low transepithelial flux in Caco-2 cells was also observed for astragaloside IV, also a cycloartane-type triterpenoid glycoside (like those in SF) which is an important constituent of the Chinese herbal remedy, Radix Astragali [551]. P-gp inhibitors, cyclosporin A and verapamil, did not alter the accumulation of astragaloside IV, which may indicate that the low transepeithelial flux of this compound (at a concentration of 50 µg/ml which is 10 times lower than the triterpenoid glycoside fraction used in this study) was not P-gp-mediated and that this triterpenoid

glycoside, unlike Sutherlandioside B [231], is thus not likely a substrate of P-gp. This suggests that despite belonging to the same class of compounds, these cycloartane-type triterpenoid glycosides exhibit different absorption capabilities in Caco-2 cells, which may be concentration-dependent, and also underscores the extent to which the effects of phytochemical constituents present in TCAMs and CAMs on drug bioavailability may vary.

Triterpenoid glycosides are present in the methanolic extract of SF in low quantities (see Figure 6.2 A); therefore ATV accumulation by this extract was not significantly different from the control probably because it did not contain sufficient quantities of the triterpenoid glycosides to elicit a significant response. Analogous to flavonol glycosides, it may be interesting to ascertain how the effect of the aglycones of the triterpenoid glycosides in SF on ATV accumulation compares to that of the corresponding glycosides.

The results obtained in this study show that SF has the potential to alter the absorption of ATV. However some circumspection is required in the interpretation of the findings. Even though the aqueous extract, D-pinitol and the triterpenoid glycoside fraction produced statistically significant changes in ATV accumulation, the effects may be considered relatively weak since they were far lower than the 2-3 fold increase observed in the presence of the positive control, PSC833, which is a potent inhibitor of P-gp at the concentration of 1 µM used in this study. Moreover, in some studies the presence of the OATP1A2 gene in human small intestinal tissue was not detected [81; 552] which indicates that even if OATP1A2 was expressed, it was likely at an undetectable level. The gene for this transporter was detected at a low level in Caco-2 cells [81], thus if any of the effects observed was due to modulation of OATP1A2 activity, the clinical impact may be of no consequence.

From the results of this study, it has emerged that traditional preparations of SF in the form of aqueous decoctions at a concentration of 10 mg/ml (see Chapter 3, section 3.6.1) may have the potential to decrease ATV absorption and thereby reduce the bioavailability of ATV. Commercial preparations of SF such as Sutherlandia Su1™ tablets (Phyto Nova) have been confirmed to contain sutherlandiosides (triterpenoid glycosides) and sutherlandins (flavonol glycosides) (see Chapter 3, sections 3.7.3 and 3.7.4). This may imply that since the flavonol glycosides did not elicit a significant response, the effect of inhibition of ATV transport by the triterpenoid glycosides, and thus increased absorption and bioavailability may

predominate. However, the SF constituents responsible for the response elicited by the aqueous extract may be D-pinitol, purported to be present in SF, and possibly other unknown constituents. One or more of these may be present in the tablets too, in which case co-administration of these SF tablets and ATV may result in a reduction in ATV absorption and bioavailability. The relative contribution of each constituent of SF to the overall effect of oral administration of SF traditional preparations or dosage forms on ATV absorption is thus not known and therefore only an *in vivo* drug-drug interaction study may unveil the true potential for SF to alter the bioavailability of ATV.

Drawing on the findings of this study and others in the literature discussed herein, it was proposed that the effects of extracts and constituents of SF, in particular, the triterpnoid glycosides, on ATV transport in Caco-2 cells were P-gp- mediated. On the other hand, the results of the molecular docking of ATV and sutherlandioside B in mouse P-gp alluded to the unlikelihood of such an effect by either competitive or non-competitive inhibition (see Chapter 5, section 5.2.3). A transport assay was therefore conducted in a P-gp-overexpressing cell line to investigate the effects of the same extracts and components of SF on P-gp-mediated ATV transport.

6.3 Effect of Extracts and Components of SF on Accumulation of ATV in MDR1-MDCKII cells, *in Vitro*

6.3.1 Introduction

Caco-2 cells are known to express multiple transporters, therefore the information obtained from the experiments conducted in section 6.2 was not specific enough to identify the transporter or transporters that may be involved in modifying ATV accumulation [553]. *In vitro* cellular drug transport models of specific human transporters may be formed by stable transfection of the human gene into a particular cell-line [554]. Models have been established using polarised epithelial cell lines [554], which are appropriate to use in the context of this research because the membrane localisation of the transfected transporter mimics that of the same endogenous transporter in the outer layer of the *villi* of the human jejunal epithelium. It may also be important for the model cell line to form tight junctions, preferably similar to those of Caco-2 cells, so that the paracellular and passive transcellular transport of compounds involved in the transport assay is comparable or at least relative.

Madin–Darby canine kidney (MDCK) strain II is an epithelial cell line which grows into a columnar epithelium with tight junctions within 3–5 days [555; 556]. Irvine *et al.* (2000) [556] found that the apparent permeability (P_{app}) values of 55 passively absorbed compounds in MDCKII cells correlated relatively well with those in Caco-2 cells ($r^2 = 0.79$). In the late 1980s, an MDCKII-MDR1 cell line was produced by transfection of the human MDR gene into MDCKII cells, which reportedly showed apical expression of P-gp [557]. Protein expression [558–562] and functional activity [558–561; 563; 564] of P-gp in these transfected cells has been confirmed by various authors over the last decade. Accordingly then, it would appear that MDCKII-MDR1 cells are suitable for high throughput screening of compounds for P-gp modulatory activity which may occur in human intestinal epithelium. ATV accumulation studies were thus conducted in MDCK-WT and MDCKII-MDR1 in the absence and presence of SF extracts and components to screen these for potential P-gp modulatory activity. A limitation of using this *in vitro* cell culture model of P-gp activity is that MDCKII cells are known to express endogenous transporters which may also contribute to accumulation or flux of P-gp substrates in the wild-type and transfected cell lines [553; 554; 559; 563; 565]. This complicates the interpretation of the results since it becomes difficult to ascertain the exclusive contribution of P-gp efflux activity to ATV accumulation.

6.3.2 Materials and Methods

6.3.2.1 Chemicals and Reagents

Madin–Darby canine kidney II cells wild type (MDCKII-WT) and Madin–Darby canine kidney II cells transfected with MDR1 (MDCKII-MDR1) were generously donated by Prof Dr Piet Borst of the Netherlands Cancer Institute, Amsterdam, The Netherlands. All other chemicals and reagents used were the same as those described in section 6.2.2.1. However, a different culture media, namely, DMEM with glutaMAX supplemented with 10% foetal bovine serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin, was used instead. All these reagents were purchased from Invitrogen (Carlsbad, California, USA).

6.3.2.2 Instruments and Equipment

As described in section 6.2.2.2.

6.3.2.3 Cell Culture

MDCKII-WT and MDCKII-MDR1 cells used from passages 5 to 15 were grown as a monolayer in culture media, at a density of 2000 cells/cm^2 on 75 cm^2 tissue culture flasks at 37 ºC, 95% humidity and 5% CO_2. Confluent cultures were subcultured after harvesting of cells with trypsin which were seeded into 48 well plates at a seeding density of 60 000 cells/cm^2. The cells were allowed to grow to confluence which took 3-4 days post-seeding with replacement of culture media performed every 2 days.

6.3.2.4 Cellular Accumulation Experiments

These transport assays were conducted in the same manner as those in the Caco-2 cells described in section 6.2.2.4. Experiments in the MDCKII-MDR1 were always conducted immediately before or after corresponding assays in MDCKII-WT.

6.3.2.5 Protein Assay

As described in section 6.2.2.5.

6.3.2.6 Data Analysis

The results for control groups from MDCKII-WT and MDCKII-MDR1 were reported as ATV accumulation in pmol/mg protein (mean ± SD), whilst SF extracts and component groups in both MDCKII-WT and MDCKII-MDR1 were represented as a percentage of the

corresponding control (mean ± SD). Values were obtained from three independent experiments performed on cells from different passages. To determine the statistical significance of differences observed between groups, an unpaired two tailed Student's *t*-test was computed using Graphpad Prism, version 3 (San Diego, California, USA). A *p*-value <0.05 was considered significant.

6.3.3 Results and Discussion

Figure 6.4 shows that in MDCKII-MDR1 cells, a lower accumulation of ATV was evident in comparison to that observed in MDCKII-WT cells ($p<0.0001$). This comparison serves to determine if the putative P-gp overexpression in MDCKII-MDR1 is functional. Cellular accumulation of ATV is dependent on active influx and efflux transporters (see section 6.1.1), thus it is expected that a cell line which overexpresses an efflux transporter for which ATV is a substrate, such as P-gp, would result in lower cellular accumulation of the PI compared to that in the corresponding WT cell line. An analogous effect has been observed for ATV in a P-gp overexpressed cell line [335] as well as for other substrates of cell lines which overexpress efflux transporters [565–567].

Several SF extracts (10 mg/ml aqueous and methanolic extracts) and components (flavonol glycoside and triterpenoid glycoside fractions), as well as PSC833, elevated ATV accumulation in MDCKII-WT (Figure 6.5). These results point to the functional expression of one or more endogenous canine transporters for which ATV is a substrate in the MDCKII cell lines used in this study. Since PSC833 is a P-gp selective inhibitor, the increase by PSC833 alludes to endogenous canine P-gp expression. On the other hand, the degree of specificity of the effects of SF extracts and components on the functional expression of transporters is unknown; therefore, the enhanced ATV accumulation evoked by each may occur as a result of predominant inhibition of one or more efflux transporters or alternatively, overriding enhanced activity of one or more influx transporters. Functional expression of canine MRP2 [553], P-gp [554] as well as non-specific influx [554] and efflux [559] transporters in MDCKII-WT cells have previously been reported, thus functional expression of any of these transporters for which ATV is a substrate may be implied.

In addition to the transfected human P-gp transporter, the apical membranes of the MDCKII-MDR1 cell line will also express the endogenous canine transporters localised thereon in the

MDCKII-WT cell line. Similar alterations in ATV accumulation due to effects of each SF extract or component as well as PSC833 on the endogenous canine transporters observed in MDCKII-WT is thus also expected in MDCKII-MDR1. In light of this, to ascertain the exclusive contribution of the transfected human P-gp efflux activity to ATV accumulation, the percentage of control observed in MDCKII-MDR1 cells for each SF extract or component was compared to that of the same treatment in MDCKII-WT cells. This evaluation (see Figure 6.5) revealed that the aqueous extract ($p<0.01$ for 10 mg/ml and $p<0.05$ for 2 mg/ml), the flavonol glycoside fraction ($p<0.01$) and the 10 mg/ml methanolic extract ($p<0.05$) ameliorated ATV accumulation, which alludes to enhanced P-gp efflux activity. Conversely, P-gp inhibition by the triterpenoid glycoside fraction ($p<0.01$) and PSC833 ($p<0.05$) was implied by the significantly higher ATV accumulation as a percentage of control observed in MDCK-MDR1 cells in comparison to that obtained in MDCKII-WT cells.

A similar P-gp inhibitory effect by PSC833 in MDCKII-MDR1 cells has previously been observed, although it was more pronounced probably because a different substrate, oseltamivir, was investigated and a higher concentration (5 µM) of PSC833 was used [568]. The P-gp inhibitory activity of the triterpenoid glycoside fraction and its influence on ATV accumulation was even greater than that of PSC833, which is known to be a selective and potent P-gp inhibitor when used at a concentration of 1 µM [569]. The effect of the triterpenoid glycoside fraction in the MDCKII-MDR1 cell line correlated to its effect in Caco-2 cells which may serve to confirm that the increased accumulation observed in the latter occurred as a result of inhibition of P-gp-mediated ATV transport. One or more of the three mechanisms which have been alluded to before (see Chapter 5, section 5.2.1) may underlie this effect, *viz* competitive inhibition, non-competitive inhibition or changes in the local lipid environment of the protein. Although the effects of other triterpenoid glycosides on P-gp activity in P-gp overexpressing cell lines have not previously been determined, P-gp inhibitory activity of triterpenoid aglycones has been observed in such cell lines [570; 571]. It is not known whether deglycosylation of the triterpenoid glycosides in the fraction used in the present study occurs in MDCKII cells, which suggests that the activity may be due to the triterpenoid glycosides, the corresponding aglycones or both.

In contrast to the triterpenoid glycoside fraction, the flavonol glycoside fraction enhanced P-gp activity in the MDCKII-MDR1 cells. The flavonols, kaempferol, quercetin, and galangin

used in the low micromolar range (for example, 5 µM), were found to be potent stimulators of the P-gp-mediated efflux of the P-gp substrate, 7,12-dimethylbenz(a)-anthracene in P-gp overexpressing cells derived from the human breast cancer cell line, MCF-7 [572]. Conversely, Kitagawa *et al.* (2005) [573] demonstrated that at higher concentrations, kaempferol and quercetin (100 µM) increased the accumulation of a different P-gp substrate, Rhodamine, in P-gp overexpressing KB-C2 cells, whilst two quercetin glycosides, namely rutin and quercetin-3-*O*-glucoside had no effect. A similar effect was observed for quercetin (100 µM) with respect to paclitaxel accumulation in another P-gp overexpressing cell line [574]. The effect of flavonols, such as the querectin- and kaempferol glycosides (sutherlandins) present in SF (see Chapter 3, section 3.7.4), on P-gp activity is thus difficult to predict since the same compounds produce varying effects on P-gp transport of different substrates and when different concentrations are used. Such anomalies have occurred in assays of P-gp transport, where modulators were not flavonol glycosides [575] and may arise due to the potential for co-operativity occurring at the multiple transport-competent and allosteric binding sites found in P-gp [576]. Furthermore, the glycosylation status of the flavonols is potentially a key factor too, since quercetin, but not two quercetin glycosides altered accumulation of the same P-gp substrate [573]. The only rational conclusion which may be drawn from the results with respect to the flavonol glycosides is that, at this relatively high concentration of 500 µg/ml, these compounds activate human P-gp transport of ATV probably by positive co-operativity, which, as described in Chapter 5, section 5.2.1, is commonly observed in the modulation of this transporter. There was however no correlation between these results and those obtained in the Caco-2 cell line, which demonstrates that the modulation of P-gp-mediated transport of ATV by SF flavonol glycosides may be of no consequence in human intestinal absorption. The effect of the flavonol glycosides on ATV transport mediated by other specific transporters also present on the apical membrane of the Caco-2 cells is not known. Modulation of other ATV transporters may have occurred in the Caco-2 cells, such that the combined effect of these and the enhanced P-gp transport of ATV resulted in no net change in ATV intracellular accumulation. It is particularly plausible that flavonol compounds may have the ability to inhibit MRP2-mediated transport of MRP2 substrates [577], like ATV

Flavonol glycosides are present in the aqueous extract of SF and the flavonol glycoside fraction in similar quantities (see Figure 6.2 B), therefore the increase in human P-gp-

mediated transport of ATV observed by the extract in MDCKII-MDR1 cells was likely attributable to these SF constituents. The 2 mg/ml concentration ($p<0.05$) of the aqueous extract probably contained a lower concentration of SF flavonol glycosides than the 10 mg/ml extract ($p<0.01$), which may explain the less potent effect observed after treatment with the former. Similarly, the methanolic extract also contained flavonol glycosides (Figure 6.2 B), which may have elicited the same effect. However, the quantities of these in this extract were much lower than those of the aqueous extract and the flavonol glycoside fraction (Figure 6.2 B) and may explain why, at the lower concentration of 2 mg/ml, the methanolic extract exhibited no effect. Analogous to the triterpenoid glycoside fraction, the effect of the aqueous extract on ATV transport in the MDCKII-MDR1 cell line correlated to the effect observed in Caco-2 cells and implies that the reduced ATV accumulation observed in the latter occurs as a result of increased activity of P-gp-mediated ATV transport. Despite the implication of the contribution of the flavonol glycosides present in the aqueous and methanolic extracts to the observed effects, alluded to by these results, the fact that the effects of the flavonol glycosides and the methanolic extract in the MDCKII-MDR1 cells and the Caco-2 cells did not correlate may serve to confirm that the aqueous extract may contain other unknown compounds which may act on other transporters in the Caco-2 cells to synergistically reduce ATV accumulation with the flavonol glycosides. The flavonol glycosides may not be potent enough to enhance ATV P-gp transport in a significant manner in Caco-2 cells at the concentration present in the flavonol glycoside fraction.

The enhanced accumulation of ATV by SF flavonol glycosides, the aqueous extracts and the methanolic extract (10 mg/ml only) in MDCKII-WT cells is indicative of inhibition or stimulation of an apically localised canine efflux or influx transporter respectively. The increased accumulation of ATV in the presence of PSC833 in MDCKII-WT cells confirmed the presence of endogenous canine P-gp in MDCKII-WT, although the availability or presence of other transporters at the membrane should not be excluded. If inhibition of canine P-gp is at play, then the discrepancy between these results and those observed for human P-gp (in the MDCKII-MDR1 cell line) may be related to inter-species differences in structure and/or function of human and canine P-gp [578–580]. The effects observed for the aqueous extract at both concentrations and the methanolic extract (10 mg/ml) in MDCKII-WT cells were less pronounced than that produced by the flavonol glycoside fraction. Since as previously mentioned, the flavonol glycoside fraction, aqueous extract and methanolic extract

all contain flavonol glycosides in decreasing order of magnitude (see Figure 6.2 B), which may account for the relative activity of the three test extracts/fractions.

The *in silico* molecular docking studies in Chapter 5 showed that even though the modulation of ATV P-gp transport by Sutherlandioside B and Sutherlandin C was not likely, it may not be conclusively ruled out (see Chapter 5, sections 5.2.3 and 5.4). The triterpenoid glycoside and flavonol glycoside fractions used to conduct these *in vitro* experiments contained several other constituents which belong to these two classes of compounds, besides Sutherlandioside B and Sutherlandin C. These other constituents may have had a greater contribution to the overall effects observed for the flavonol and triterpenoid glycoside fractions than Sutherlandin C and Sutherlandioside B, respectively. It has been shown that a change in the stereochemistry of the same compound may elicit different binding modes in mouse P-gp [448] which suggests a possible difference in the manner in which P-gp activity may be modulated. Moreover, as previously described in section 6.2.3, two cycloartane triterpenoid glycosides from two different plants may exhibit different P-gp transport patterns, therefore the potential for these two to exhibit modulation of the P-gp-mediated transport of any drug is also likely to vary. The notion that structurally similar compounds may also exhibit such varying abilities of these to modulate ATV P-gp transport is therefore plausible. Lastly, the *in silico* prediction may have been an artifact and therefore the true ability for Sutherlandioside B and/or Sutherlandin C to modulate ATV P-gp transport may not have been reflected.

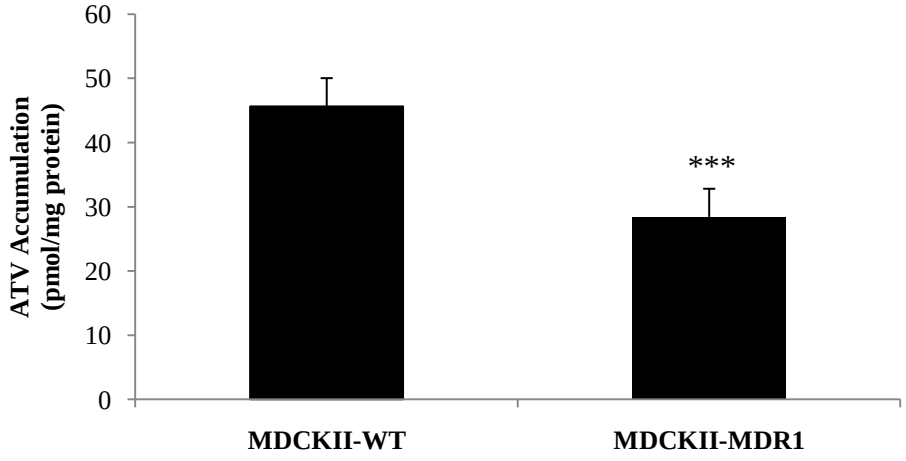


Figure 6.4: Atazanavir (1 µM) accumulation in MDCKII-WT and MDCKII-MDR1 cells, *in vitro*. Each bar represents mean ± SD; n = 3. *** ($p<0.0001$) in comparison to MDCKII-WT (Student's *t*-test).

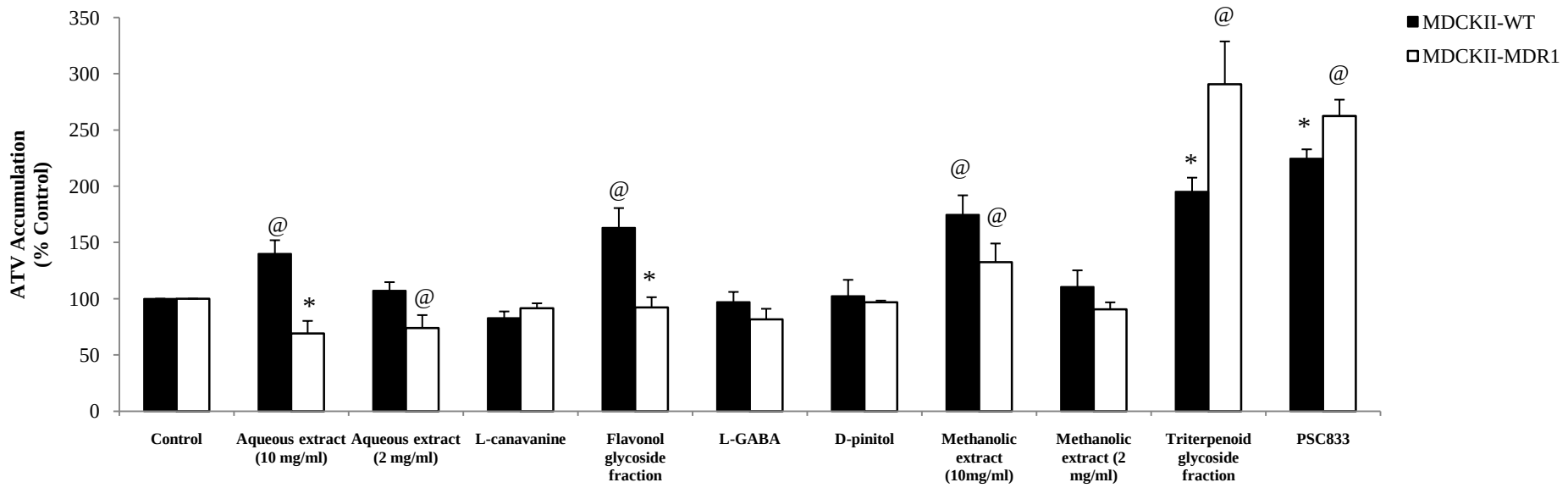


Figure 6.5: Effect of extracts (10 and 2 mg/ml) and components of SF (500 µg/ml) on atazanavir (1 µM) accumulation in MDCKII-WT and MDCKII-MDR1 cells, *in vitro*, depicted as a percentage of control. PSC833 (1 µM) is used as a positive control. Each bar represents mean ± SD; n = 3. @ ($p<0.05$); * ($p<0.01$) in comparison to MDCKII-WT control (those above black bars) and in comparison to corresponding SF treatment in MDCKII-WT (those above white bars). (Student's *t* test).

6.4 Conclusions

The bioavailability of a drug is dependent on its absorption across the luminal/apical and basolateral membranes of enterocytes in the small intestine. PK drug interactions may occur as a result of one drug or other xenobiotic altering one or more of the processes in the absorption of another. Apart from passive transcellular transport, drugs are translocated across the apical/luminal membrane and into the enterocytes of the intestine by influx transporters. Extrusion from the enterocytes back into the intestinal lumen may also occur to prevent the absorption of potentially toxic xenobiotics. Modulation of the activity of these influx and efflux transporters may therefore alter the bioavailability of a drug.

Caco-2 cells were used as an *in vitro* model of the human intestinal epithelium. To isolate transport across the apical/luminal membrane only, the cells were grown as a monolayer on an impermeable surface. Drug transport assays were conducted by incubating the cells with ATV in the absence and presence of SF extracts and components, as well as the positive control, PSC833. The results showed that the aqueous extract and D-pinitol significantly reduced ATV accumulation, implying a decrease in ATV bioavailability, whilst the opposite was true for the triterpenoid glycosides, which increased ATV accumulation. The relative contribution of each these active constituents to the overall effect that administration of traditional preparations or commercial dosage forms of SF may have on the absorption of ATV is not known. It is therefore evident that only an *in vivo* drug-drug interaction study may unveil the true potential for SF to alter the bioavailability of ATV.

Of the transporters present on the apical/luminal membrane of enterocytes, ATV is a known or potential substrate of the influx transporter OATP1A2 and the efflux transporters, P-gp and MRP2. This implies that the mechanism which underlies increased ATV accumulation may be stimulation of OATP1A2 activity or inhibition of P-gp and/or MRP2 activity, and the converse would be true for reduced ATV accumulation. However, since the presence of OATP1A2 may not always be expressed in human small intestinal tissue, any effects thereon in Caco-2 cells may be of no consequence in the clinical setting. P-gp was selected as a clinically relevant transporter in the context of ATV absorption to explore further in order to possibly elucidate the mechanisms which underlie the observed effects of SF extracts and components on ATV accumulation in Caco-2 cells.

MDCKII cells which overexpress the human efflux transporter P-gp were used to determine the effects of the SF extracts and components on P-gp-mediated ATV accumulation. Cell culture and drug transport assays were performed in a similar manner to that of the Caco-2 cell experiments. However, in order to evaluate human P-gp modulation exclusively, comparisons were drawn between the effects of the same extracts and components in MDCKII-MDR1 and MDCKII-WT cells. The SF methanolic and aqueous extracts, as well as the flavonol glycoside fraction significantly reduced ATV accumulation, which signifies that P-gp-mediated ATV transport was enhanced. Positive co-operativity was proposed as the specific mechanism which underlies this effect. On the other hand, the triterpenoid glycoside fraction increased ATV accumulation, which alludes to inhibition of P-gp-mediated ATV transport.

The results in this chapter demonstrate that although the methanolic extract, aqueous extract and flavonol glycoside fraction of SF may enhance P-gp-mediated transport, only the aqueous extract exhibited the same effect in the Caco-2 cells, which is the established model of human intestinal absorption. Similarly, a correlation between the Caco-2 and MDCKII-MDR1 cells for the results of the triterpenoid glycoside fraction was also evident. The observed effects of the methanolic extract and flavonol glycoside fraction in the MDCKII-MDR1 cells may have been offset in the Caco-2 cells where no net change in ATV accumulation was demonstrated. Such a counterbalance may possibly occur as a result of inhibition of other ATV efflux transporters, such as MRP2. This may be confirmed by conducting the experiments in an MRP2-overexpressing cell line.

The fact that the *in vitro* results in the P-gp-overexpressing cell line were not in agreement with the *in silico* molecular docking results in Chapter 5 indicate that other constituents of SF may have had a more profound effect than Sutherlandioside B and/or Sutherlandin C Alternatively the *in silico* findings were possibly not highly predictive of the true ability of SF extracts and components, such as Sutherlandioside B and Sutherlandin C to alter P-gp-mediated transport of ATV.

In summary, the aqueous extract and D-pinitol significantly reduced ATV accumulation, whilst the triterpenoid glycoside fraction increased ATV accumulation, which implied that ATV bioavailability may be reduced by the aqueous extract (non flavonol related effect) and

D-pinitol, and enhanced by the triterpenoid glycosides present in the triterpenoid glycoside fraction. The effects of the aqueous extract and the triterpenoid glycosides were P-gp-mediated. The relative contribution of each of the active constituents to the overall effect that administration of traditional preparations or commercial dosage forms of SF may have on the absorption of ATV is not known. It would therefore be prudent to undertake an *in vivo* drug-drug interaction study to ascertain the true potential for SF to alter the bioavailability of ATV.

CHAPTER 7
IN VITRO INVESTIGATIONS OF DRUG INTERACTIONS: LIVER METABOLISM

7.1 Background

Metabolism in the liver is one of several processes on which the bioavailability of a drug depends, following oral administration (see Chapter 6, section 6.1). As described in Chapter 1, section 1.3.7.7, subsequent to absorption in the intestine, a drug or xenobiotic is directed either to the liver via the hepatic portal vein or to the lymphatic system. Analogous to uptake into enterocytes, the entry of a drug into the hepatocytes of the liver is either by passive diffusion or by carrier-mediated transport. In the hepatocytes, the various Phase I and Phase II drug metabolising enzymes previously discussed (Chapter 1, section 1.3.7.6) and associated cofactors such as CYP reductase are present in the cytosol on the endoplasmic reticulum, where CYP substrates in particular undergo first pass biotransformation to metabolites via the catalytic cycle of these enzymes, as described in Chapter 5, section 5.3.1. These metabolites usually have a higher polarity than the parent drug, which promotes elimination via the urine or faeces (Chapter 1, section 1.3.7.6). This process is in dynamic interplay with drug efflux and serves primarily to reduce the toxic potential of xenobiotics.

It is thus evident that, like drug absorption, the hepatic metabolism of a drug may impact on its bioavailability. Similarly then, PK drug interactions may occur as a result of one drug or other xenobiotic altering the hepatic metabolism of another. As described in Chapter 1, section 1.3.7.8, xenobiotics may have the ability to modulate the expression of enzymes on the endoplasmic reticulum in the hepatocytes. Instead, or in addition, xenobiotics may directly alter the functional activity of these enzymes via various mechanisms of inhibition or activation. Specific examples of such mechanisms with respect to CYP3A4 have been described in Chapter 5, section 5.3.1.

In vivo animal studies for the investigation of potential PK interactions in which the hepatic metabolism of one drug may be affected by another are time-consuming and require relatively large amounts of the test compounds [581]. Moreover, the metabolism and PK of a drug may not be the same in animals and humans [581]. Human models of hepatic metabolism *in vitro* are thus preferred for delineating the potential for drug-drug interactions

before undertaking *in vivo* clinical studies [581]. Several of these *in vitro* models [582; 583], all derived from human liver tissue, have been developed over the past few decades and include:

(i) Perfused liver
(ii) Liver slices
(iii) Primary hepatocytes
(iv) Cell lines
(v) Transgenic cell lines
(vi) Liver microsomes,
(vii) Cytosolic fractions of liver tissue
(viii) S9 fractions of liver tissue and
(ix) CYP-transfected microsomes

Primary hepatocytes are the closest *in vitro* model to the human liver, since the metabolic profile of a drug which is produced therefrom is very similar to that found *in vivo* [584]. However, the major drawback of the use of these hepatocytes is that enzymatic activities are not stable for much longer than 24 hours [585]. This is also true for the other models which contain an intact, self-sustaining liver system, *viz* perfused liver, liver slices and cell lines [585]. On the other hand, liver microsomes, a subcellular fraction of liver tissue obtained by differential high-speed centrifugation, provide the most convenient way to study drug metabolism, particularly since the enzymes therein retain their activity for many years if stored at -70 ºC [585]. Due to a loss of cell integrity during the preparation of microsomes, some enzymes may be more labile and require exogenous cofactors, such as NADPH to maintain optimal activity [581; 582].

Advances in molecular biology led to the first stable expression of individual CYP enzymes and the enzymatic cofactor, CYP reductase, which were catalytically active in bacteria [586], yeast [587], insect [588] and mammalian [589] cells. Microsomes prepared from these genetically engineered cells (CYP-transfected cells), such as Supersomes™, may be useful to gain insight into the specific enzymes involved in the mechanisms which underlie any effect observed in human liver microsomes [581; 584; 585; 590].

Human liver microsomes and Supersomes™ are thus both valuable tools for the study of PK interactions modulated by hepatic metabolism.

7.2 Effect of Extracts and Components of SF on the Metabolism of ATV in Human Liver Microsomes, *in Vitro*

7.2.1 Introduction

Microsomes are primary constituents of the endoplasmic reticulum of hepatocytes and account for 10–13% of the total liver [591]. Enzymes present in microsomes include a variety of CYP450s, flavin monooxygenases (FMO), carboxyl esterases and epoxide hydrolase, and UGTs [582]. A meta-analysis of studies on the abundance of CYP enzymes in human hepatic microsomes revealed that the subfamily CYP3A (CYP3A4 and CYP3A5) was the most abundant, followed by CYP2C9, CYP2E1, CYP1A2, CYP2A6, CYP2C8 and CYP2C19 in order of rank [100]. CYP2B6 and CYP2D6 were the two least abundant. Several UGTs exhibited high levels of expression in adult human liver, *viz* five UGT1 isoforms (UGT1A1, 1A3, 1A4, 1A6 and 1A9) and five UGT2 family members (UGT2B4, 2B7, 2B10, 2B15 and 2B17) [592]. By use of known probe substrates, the activities of CYP450 enzymes—CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, CYP4A11 [582; 593]; of UGTs—UGT1A1, UGT1A4 and UGT1A9 and cofactors; and of CYP450 reductase and cytochrome b_5 [593] in human liver microsomes have been demonstrated. Inter-individual variability in the activity of drug metabolising enzymes is well known [582; 584; 592; 594], therefore the use of pooled human liver microsomes which have been prepared from the liver tissue of several individuals is advised.

Liver microsomes, unlike the S9 and cytosolic subcellular fractions of liver tissue, do not contain the soluble cytosolic enzymes, such as, N-acetyltransferase, sulphotransferase and glutathione *S*-transferase, which are involved in Phase II metabolism [582]; therefore interactions which occur through modulation of these enzymes may be overlooked and a qualitative correlation of PK interactions to *in vivo* hepatic metabolism may be compromised. As previously mentioned, microsomes are the primary constituents of the endoplasmic reticulum of hepatocytes that contain the metabolic enzymes and account for 10–13% of the total liver; therefore a higher concentration of CYP450 and UGT enzymes are obtained after isolation of the microsomal subcellular fraction than would be expected in the equivalent intact liver tissue from which the microsomes were derived [582]. A quantitative correlation to *in vivo* human hepatic metabolism may thus similarly not be appropriate.

ATV is a known substrate of CYP3A4 and CYP3A5 (see Chapter 1, Table 1.6), whilst glucuronidation is a minor biotransformation pathway (see Chapter 2, section 2.7.3.3). Huang, 2008 [595] evaluated the metabolism of ATV in human liver microsomes which revealed two major hydroxylated metabolites. Concentrations of 1, 10 and 30 µM ketoconazole (KTZ), a known CYP3A inhibitor of protease inhibitor metabolism [596–598] significantly reduced the formation of both metabolites formed from 50 µM ATV [595].

In Chapter 3, section 3.8.1.9, the effect of aqueous and methanol extracts and a triterpenoid glycoside fraction of SF on progesterone- and pregnenolone-induced type I as well as type II difference spectra in sheep adrenal microsomes, which contain CYP enzymes involved in steroidogenesis, was discussed. Aqueous and methanolic extracts inhibited type I progesterone-induced difference spectrum, but not that induced by pregnenolone binding in adrenal microsomes, suggesting that inhibition of pregnenelone metabolism by these extracts was not competitive in nature. The triterpenoid fraction extracted from SF inhibited both the type 1 pregnenolone- and progesterone-induced difference spectra and elicited a type II difference spectrum in the absence of the substrates, implying that the triterpenoids may have inhibited the metabolism of both steroids, by both competitive and non-competitive inhibition.

CYP enzymes have a common catalytic cycle (see Chapter 5, section 5.3.1), therefore aqueous and methanolic extracts, the triterpenoid glycoside fraction and possibly other components of SF may exhibit a similar ability to inhibit the activity of CYP enzymes in human liver microsomes, as observed by Prevoo *et al.* (2008) [226] in sheep adrenal microsomes. This may in turn interfere with the metabolism of known substrates of CYP enzymes, such as ATV.

7.2.2 Materials and Methods

7.2.2.1 Chemicals and Reagents

ATV sulphate (100.9%) was donated by Aspen Pharmacare (Port Elizabeth, South Africa) and diazepam (DIAZ) was obtained from the Biopharmaceutics Research Institute (Rhodes University, Grahamstown, South Africa). Pooled human liver microsomes were purchased from BD Biosciences (Woburn, Massachusetts, USA). PBS (0.01 M, pH 7.4), β-Nicotinamide adenine dinucleotide 2′-phosphate reduced (NADPH) tetrasodium salt, L-

canavanine, D-pinitol, BSA and Bradford reagent for protein assay were obtained from Sigma-Aldrich (Johannesburg, South Africa). Ketoconazole was purchased from Oman Chemicals and Pharmaceuticals (Al Buraimi, Sultanate of Oman). Leaves of *Sutherlandia frutescens* collected in Barrydale, Western Cape, South Africa were verified by the Selmar Schonland Herbarium affiliated to the Rhodes University Department of Botany and the Albany Museum in Grahamstown, Eastern Cape, South Africa. Powder obtained by the milling of *Sutherlandia frutescens* plant material was purchased from Zizamele Herbs, Onrus River, Western Cape, South Africa. Chemicals and reagents used to conduct the HPLC analysis were as described in Chapter 4, section 4.4.2.1.

7.2.2.2 Preparation of SF Extracts and SF Flavonol Glycoside and Triterpenoid Glycoside Fractions

These were prepared as previously described (section 6.2.2.2).

7.2.2.3 Preparation of Human Liver Microsomes

Pooled human liver microsomes (20 mg/ml protein as purchased) were thawed in a water bath at 37 ºC and diluted to 2 mg/ml protein with PBS.

7.2.2.4 Preparation of Incubation Buffers

A stock solution of ATV was prepared by dissolving an accurately weighed amount of ATV sulphate in mobile phase, *viz* acetonitrile-ammonium formate buffer (10 mM, pH 3) (45:55, v/v), to provide the equivalent of 5 mM ATV. Similarly, a stock solution of NADPH (10 mM) was prepared in PBS. Using these solutions, an incubation buffer containing 0.05 mM ATV and 2 mM NADPH was prepared in PBS. SF extracts and components and corresponding controls were initially dissolved in solvents as shown in Table 7.1, after which concentrations of these, also shown in Table 7.1, were prepared in the incubation buffer. KTZ was used as the positive control for inhibition of ATV metabolism in human liver microsomes [595].

Table 7.1: Solvents and concentrations of SF extracts and components and KTZ and the corresponding controls

SF extract/component	Corresponding control
20 mg/ml aqueous extract in water	Water
200 µM L-canavanine in methanol-water (50:50, v/v)	Methanol-water (50:50, v/v)
1000 µg/ml flavonol glycoside fraction in water	Water
200 µM L-GABA in water	Water
200 µM D-pinitol in water	Water
20 mg/ml methanolic extract in methanol	Methanol
1000 µg/ml triterpenoid glycoside fraction in methanol	Methanol
20 µM KTZ in methanol	Methanol

7.2.2.5 Metabolism Experiments

The human liver microsomes and incubation buffers prepared in sections 7.2.2.3 and 7.2.2.4 were transferred to separate 2 ml microcentrifuge tubes (Optima Scientific, Cape Town, South Africa) and pre-incubated at 37 ºC for 15 minutes in a Labotec (Pty) Ltd model 132 water bath (Johannesburg, South Africa) fitted with a Julabo PC/1 thermostat (Seelbach, Germany). Incubation buffers were then transferred to the microcentrifuge tubes containing the liver microsomes to obtain final incubation concentrations of 1 mg/ml protein, 25 µM ATV, 1 mM NADPH, 10 mg/ml SF aqueous and methanolic extracts, 100 µM L-canavanine, L-GABA and D-pinitol, 500 µg/ml flavonol glycoside and triterpenoid glycoside fractions and 10 µM KTZ, each in a total volume of 250 µl. These final concentrations of SF extracts and components were thus the same as those used in the transport assays described in Chapter 6. Each different sample was prepared in triplicate. The tubes were vortexed and incubated for 1 hour at 37 ºC. The reaction was stopped by addition of ice cold (4 ºC) mobile phase (250 µL), *viz* acetonitrile-ammonium formate buffer (10 mM, pH 3) (45:55, v/v) containing the IS, DIAZ (2 µg/ml).

7.2.2.6 HPLC Analysis

Calibration standards of ATV were prepared in the range 2.5–15 µM ATV by serial dilution of the stock solution as prepared in section 7.2.2.4 with 50:50 PBS: mobile phase, containing 1 µg/ml DIAZ, as the IS. These standards and the samples from the experiment were vortexed and transferred to micro-inserts in HPLC vials. The HPLC analysis was run using the validated method described in Chapter 4. A calibration plot was constructed using an unweighted linear regression analysis, and the concentration of ATV in the samples was determined accordingly. The amount of ATV present in each sample was represented as nmol/sample.

7.2.2.7 Protein Assay

Three samples which contained blank incubation buffer were set aside for the determination of protein concentration (mg/ml) and standard solutions of BSA (0.25, 0.5, 0.75, 1 and 2 mg/ml) were prepared. The Bradford method [538] for protein assay was applied to the samples and standard solutions, according to the Bradford reagent instructions (Sigma-Aldrich, Johannesburg, South Africa). UV absorbances were read at 595 nm on a Shimadzu UV mini-1240 UV-Vis spectrophotometer (Kyoto, Japan). The average protein concentration/sample was calculated and the amount of ATV per sample from section 7.2.2.6 was standardised to this protein concentration and thus expressed in nmol/mg protein.

7.2.2.8 Data Analysis

The results were reported as the mean ± SD of values from three independent experiments using different batches of pooled human liver microsomes. To determine the statistical significance of differences observed between control and treatment groups, an unpaired two tailed Student's *t*-test was computed using Graphpad Prism, version 3 (San Diego, California, USA). A *p*-value <0.05 was considered significant.

7.2.3 Results and Discussion

The results depicted in Figure 7.1 show that KTZ ($p<0.05$), as well as the aqueous ($p<0.01$) and methanolic ($p<0.05$) extracts of SF, significantly increased the ATV levels in human liver microsomes, whilst the triterpenoid glycoside fraction was the only test compound which exhibited the opposite effect ($p<0.01$). These results imply that the metabolism of ATV was reduced in the presence of KTZ and aqueous and methanolic extracts of SF but enhanced in the presence of the triterpenoid glycoside fraction. Since ATV is a known CYP3A4 and CYP3A5 substrate, interference of ATV metabolism by human liver microsomes likely occurred by modulation of the catalytic cycle of either of these two isoenzymes.

Inhibition of ATV metabolism by KTZ is in agreement with Huang, 2008, who found that ATV metabolites were reduced in the presence of KTZ [595]. The suggested inhibition of ATV metabolism by both the aqueous and methanolic extracts of SF may indicate that it is the effect of a component common to both extracts which predominated. The specific constituent which elicited the response is however unknown, since none of the SF

phytochemical constituents tested produced a corresponding effect. A similar effect was observed by Prevoo *et al.* (2008) [226] in sheep adrenal microsomes where lower concentrations of aqueous (2.4 mg/ml) and methanolic extracts (4.1 mg/ml) of SF inhibited a type I progesterone-induced difference spectrum, implying competitive inhibition of the metabolism of the steroid. The effect, as in the present study, was greater for the aqueous extract than the methanolic extract, suggesting that the unknown active constituents were present in larger quantities in the former extract.

The effects of triterpenoid glycosides as a class of phytochemical constituents on drug metabolism in human liver microsomes have not previously been reported. The triterpene backbone of these compounds resembles that of the steroids. It has been demonstrated that nevirapine 2- and 12-hydroxylations and carbamazepine 10,11-epoxidation in human liver microsomes were strongly activated by many endogenous steroids, especially androgens such as androstenedione, testosterone, and dehydroepiandrosterone [599]. If the triterpene backbone was instrumental in conferring the properties required for enzyme activation, then an analogous effect may have been elicited by the triterpenoid glycoside fraction in the present study, which resulted in reduced ATV levels. In Chapter 5, section 5.3.1, the co-operativity of CYP3A4 was described, and since ATV is a CYP3A4 substrate, it is likely through this CYP450 isoenzyme that such positive co-operativity and therefore activation of ATV metabolism was elicited. Other CYP enzymes do not commonly exhibit co-operativity (see Chapter 5, section 5.3.1), which may account for differences in the effects of the triterpenoid glycoside fraction in this study and in the study by Prevoo *et al.* (2008) who used bovine adrenal microsomes which contain CYP450 isoenzymes involved in steroidogensis, such as CYP450 17 α-hydroxylase and CYP450 21 hydroxylase [226]. Effector responses are also known to often be concentration-dependent [499], to which differences in the studies may also be attributed, since a three-fold higher concentration of the triterpenoid glycoside fraction was used in this study in comparison to that used by Prevoo *et al.* (2008). Moreover, Nakamura *et al.* (2002) [599] further found that the same endogenous androgens mentioned above inhibited erythromycin *N*-demethylation, implying that co-operativity is substrate-dependent, which may also explain the differences in the findings of the two studies with respect to the triterpenoid glycoside fraction.

The sutherlandins (flavonol glycosides) found in SF are glycosylated forms of quercetin and kaempferol (see Chapter 3, section 3.7.4). It has been shown that quercetin (100 µM) inhibited the CYP3A4-mediated hydroxylation of quinine in human liver microsomes by 55% [600]. He and Edeki (2004) [601] found that quercetin inhibited CYP3A4-mediated testosterone-6β-hydroxylation with an IC_{50} value of 38 µM, whilst much lower values of 6.51 ± 1.01 µM and 5.74 ± 1.16 µM were observed by Tsujimoto *et al.* (2009) [602], for kaempferol and quercetin respectively. The ability of cell-free extracts from human small intestine and liver to deglycosylate various flavonoid glycosides has also been investigated. Quercetin 4′-glucoside, naringenin 7-glucoside, apigenin 7-glucoside, genistein 7-glucoside and daidzein 7-glucoside were rapidly deglycosylated by both tissue extracts, whereas quercetin 3,4′-diglucoside, quercetin 3-glucoside, kaempferol 3-glucoside, quercetin 3-rhamnoglucoside and naringenin 7-rhamnoglucoside remained unchanged [603]. Deglycosylation at the 3-position may therefore be impeded in human liver microsomes and it is thus possible that the sugar moiety of the sutherlandins (which is present at that position) has masked the ability of these flavonol glycosides to inhibit CYP3A4-mediated drug metabolism as observed by others for the aglycones, quercetin and kaempferol [600–602]. The flavonol glycoside fraction similarly demonstrated no effect on progesterone and pregnenelone metabolism in sheep adrenal microsomes [226]. Likewise, L-canavanine, L-GABA and D-pinitol had no effect in both studies.

The findings of this study suggest that traditional preparations of SF in the form of an aqueous decoction at a concentration of 10 mg/ml (see Chapter 3, section 3.6.1) may have the potential to inhibit ATV metabolism and thereby increase the bioavailability of ATV. This is in contrast to the results observed in Caco-2 cells, where the potential for this same concentration of an aqueous extract to reduce the bioavailability of ATV was implied (see Chapter 6, section 6.2.3). Commercial preparations of SF such as Sutherlandia Su1™ tablets (Phyto Nova) are known to contain sutherlandiosides (triterpenoid glycosides) and sutherlandins (flavonol glycosides) (see chapter 3, sections 3.7.3 and 3.7.4). The flavonol glycoside fraction had no effect and, since the triterpenoid glycoside fraction enhanced ATV metabolism, a reduction in the bioavailability of ATV would be expected. In this instance, as suggested for the aqueous extract, an opposing effect of the triterpenoid glycoside fraction on ATV bioavailability to that inferred in Caco-2 cells is prevalent (see Chapter 6, section 6.2.3). However, the SF plant material present in these tablets likely also contained the unknown

phytochemical constituents responsible for inhibition of ATV metabolism by the aqueous extract. If these are present in the tablets in sufficient quantities, then inhibition of ATV metabolism and a potential increase in ATV bioavailability may instead predominate. Therefore, analogous to ATV absorption (Chapter 6, section 6.2.3), the relative contribution of each constituent of SF to the overall effect of the oral administration of SF traditional preparations or dosage forms on ATV hepatic metabolism is unknown. This further substantiates the call for an *in vivo* drug-drug interaction study (see Chapter 6, section 6.2.3) to determine the true potential for SF to alter the bioavailability of ATV.

ATV is a CYP3A4 substrate (see Chapter 1, Table 1.6) and methanolic and aqueous extracts of SF (100 mg/ml) have been shown to inhibit CYP3A4 [116]; therefore the effect of increased metabolism of ATV by these extracts at a lower concentration of 10 mg/ml in human liver microsomes may be CYP3A4-mediated. Moreover, the predictions of the molecular docking of ATV and Sutherlandioside B in human CYP3A4 suggested that competitive inhibition of CYP3A4-mediated metabolism of ATV by Sutherlandioside B is not likely, but that co-operative influences (whether inhibition or activation) of the latter on the CYP3A4-mediated metabolism of the former may not be ruled out. Drug metabolism experiments in CYP3A4-transfected Supersomes™ were therefore conducted to investigate the effects of the extracts and components of SF on CYP3A4-mediated ATV metabolism.

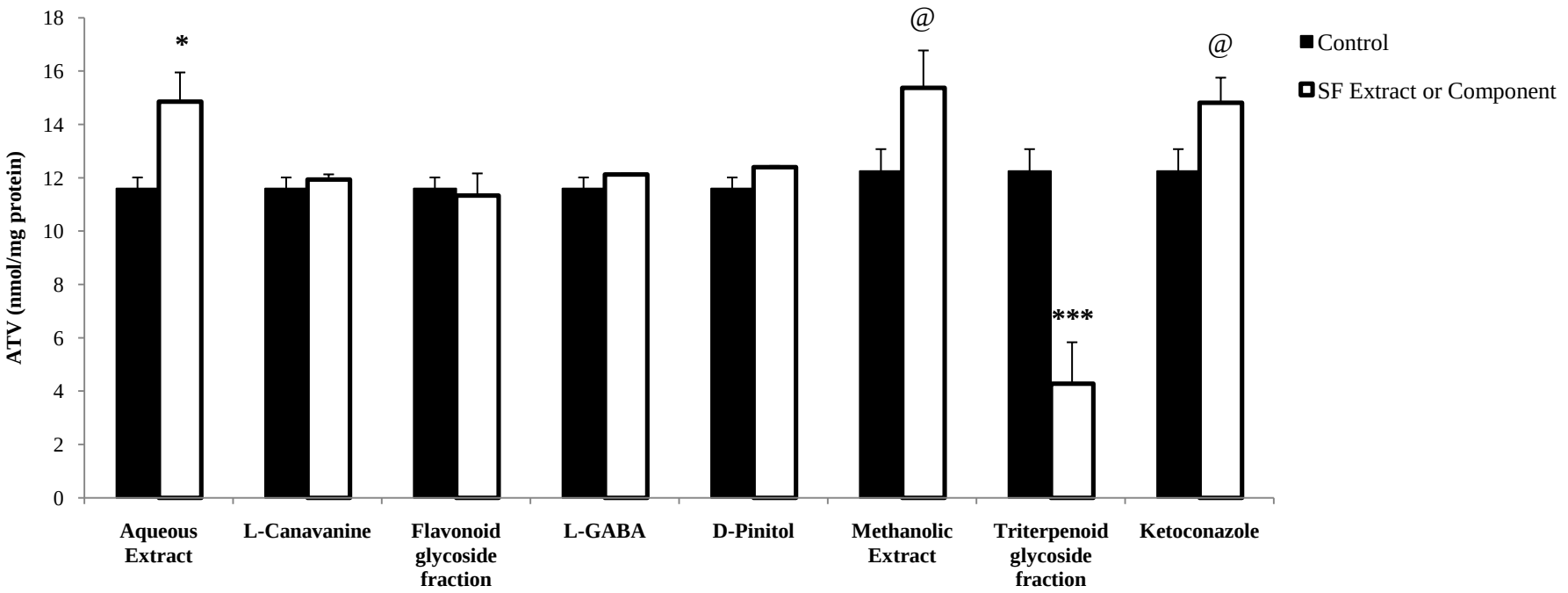


Figure 7.1: Effect of extracts (10mg/ml) and components of SF on ATV (25 µM) metabolism in human liver microsomes, *in vitro.* KTZ (10 µM) is used as a positive control. Each bar represents mean ± SD; n = 3. @ ($p<0.05$), * ($p<0.01$) and *** ($p<0.0001$) in comparison to control (Student's *t*-test).

7.3 Effect of Extracts and Components of SF on the Metabolism of ATV in CYP3A4-transfected Microsomes, *in Vitro*

7.3.1 Introduction

Since human liver microsomes contain a variety of Phase I and Phase II drug metabolising enzymes, it is not possible to identify or confirm which specific ATV metabolising enzyme is being modulated by the SF extracts and the triterpenoid glycoside fraction, as observed in section 7.1. Such information may be important if the enzyme involved is subject to genetic polymorphisms. In such instances, individuals with a particular polymorph of an enzyme, which manifests as a certain phenotype, may be more or less susceptible to drug-drug interactions, should the effect be observed *in vivo* [604]. Anderson *et al.* (2009) [605] showed that the clearance of ATV after seven days was 1.39-fold faster and the C_{min} was halved in healthy human volunteers who were CYP3A5 expressors versus those who were non-expressors. Modulation of CYP3A4, which is the only other CYP450 enzyme known to metabolise ATV may therefore have greater significance in CYP3A5 non-expressors, where metabolism of ATV is solely reliant on CYP3A4. Determining whether the ability of SF extracts and the triterpenoid fraction to alter ATV metabolism in human liver microsomes is CYP3A4-mediated is therefore an imperative step in the process of elucidating the potential for SF to interact with the metabolism of ATV.

The catalytic activity of CYP3A4 purified from human tissues is often lost, therefore heterologous gene expression systems have been utilised as an alternative source of functional protein [582; 606]. A recombinant system is most useful when catalytic properties resemble the native enzyme with respect to kinetics, regioselectivity, and modulation by inhibitors and/or activators [604; 606]. As previously mentioned, recombinant CYP3A4 has been expressed in bacteria, yeast, insect and mammalian cells (see section 7.1). Insect cells, like mammalian cells, are eukaryotic and therefore contain the cell organelles which target recombinant proteins to their appropriate subcellular compartments [606; 607]. An additional advantage of using insect cells is that the wild-type cells do not contain endogenous CYP, CYP reductase or cytochrome $b_{5,}$ which may complicate the interpretation of results. Lee *et al.* (1995) [606] first demonstrated the co-expression of human CYP3A4 and CYP reductase in *Spodoptera fugiperda* and *Trichlplusia ni* insect cells via infection with baculovirus

containing both cDNAs and further found that properties and catalytic activities with respect to testosterone metabolism were comparable to native CYP3A4 in human liver microsomes.

Insect microsomes expressing human CYP3A4, CYP reductase and cytochrome b_5 were thus used to determine possible effects of SF aqueous and methanolic extracts as well as isolated sutherlandiosides and sutherlandins, alone or in combination, on CYP3A4-mediated ATV metabolism.

7.3.2 Materials and Methods

7.3.2.1 Chemicals and Reagents

Chemicals and reagents used were as described in section 7.2.2.1. However, instead of pooled human liver microsomes, Supersomes™ prepared from insect cells transfected with baculoviruses containing cDNA-expressed human CYP3A4, human P450 reductase and cytochrome b_5 (CYP3A4-Supersomes™) were purchased from BD Biosciences (Woburn, Massachusetts, USA). Control-Supersomes™, also from BD Biosciences (Woburn, Massachusetts, USA), did not contain any cDNA. L-canavanine, flavonol glycoside fraction, L-GABA and the triterpenoid glycoside fraction were not used. Instead, Sutherlandioside A (98.73%), Sutherlandioside B (99.10%) and combinations of Sutherlandioside C (98.34%) and D (98.53%), Sutherlandin A (93.33%) and B (94.81%) and Sutherlandin C (96.65%) and D (93.46%), which were kindly donated by Drs T. Smillie and B. Avula of the National Center for Natural Products Research, Research Institute of Pharmaceutical Sciences at the University of Mississippi, USA, were utilised.

7.3.2.2 Preparation of SF Extracts

Aqueous and methanolic extracts of SF were prepared as previously described (section 6.2.2.2).

7.3.2.3 Confirmation of the Presence of Sutherlandiosides and Sutherlandins in the Triterpenoid and Flavonol Glycoside Fractions

To be able to draw valid comparisons between the study in human liver microsomes (section 7.2), where the crude triterpenoid and flavonol glycoside fractions were used, and this study involving specific sutherlandiosides and sutherlandins, it was necessary to determine whether the latter, at the final incubation concentrations used (see section 7.3.2.6), were present in the

crude fractions at a concentration of 500 µg/ml as per in the human liver microsome study. A qualitative HPLC-UV analysis was conducted, as described in section 6.2.2.2.3, where the presence of triterpenoid glycosides and flavonol glycosides in the aqueous and methanolic extracts was determined. Although a method for the quantitative determination of the sutherlandiosides and sutherlandins has recently been developed [205], the previous method (section 6.2.2.2.3) was used so that the findings of the relevant analyses could be compared and thus conclusions drawn regarding the presence of the specific triterpenoid glycosides (sutherlandiosides) and flavonol glycosides (sutherlandins), not only in the triterpenoid and flavonol glycoside fractions but also in the aqueous and methanolic extracts of SF.

7.3.2.4 Preparation of Insect Cell Microsomes

CYP3A4-Supersomes™ (1 nmol/ml CYP450 content as purchased) were thawed on ice and diluted to 100 pmol/ml CYP450 content with PBS. Equivalent dilutions were carried out for Control-Supersomes™.

7.3.2.5 Preparation of Incubation Buffers

Incubation buffers containing aqueous and methanolic extracts and KTZ with corresponding controls were prepared as described in section 7.2.2.4. sutherlandiosides and sutherlandins were initially dissolved in methanol and then diluted with PBS to a concentration of 100 µg/ml. These compounds were used either individually or as combinations, as provided by the donors, *viz*:

(i) Sutherlandioside A
(ii) Sutherlandioside B
(iii) Combination of Sutherlandiosides C and D
(iv) Combination of Sutherlandins A and B
(v) Combination of Sutherlandins C and D

7.3.2.6 Metabolism Experiments

The metabolism experiments were conducted as previously described (section 7.2.2.5) in both CYP3A4-Supersomes™ and Control-Supersomes™. The incubation concentrations of the aqueous and methanolic extracts and KTZ were the same as that used in the human liver microsomes study (see section 7.2.2.5). The incubation concentration of the individual or combined sutherlandiosides and sutherlandins (i–v above) was 50 µg/ml. This concentration

is equivalent to 69 and 77 µM for Sutherlandioside A and B respectively, the only two individual compounds available as all the others were mixtures of either two triterpenoid glycosides or two flavonol glycosides.

7.3.2.7 HPLC Analysis

The quantitative bioanalysis of ATV in the Supersomes™ was conducted as described in section 7.2.2.6. The result of each treatment was expressed as amount of ATV present after metabolism as a percentage of control.

7.3.2.8 Data Analysis

The results were reported as mean ± SD of values from three independent experiments using different batches of CYP3A4-transfected and Control Supersomes™. To determine the statistical significance of differences observed between control and treatment groups for the Control Supersomes™ and between Control Supersomes™ and CYP3A4-transfected Supersomes™ for each treatment group, an unpaired two tailed Student's *t*-test was computed using Graphpad Prism, version 3 (San Diego, California, USA). A p-value <0.05 was considered significant.

7.3.3 Results and Discussion

Figures 7.2 and 7.3 show that the isolated sutherlandiosides and sutherlandins, either alone or in combination are all likely present in the triterpenoid glycoside and flavonol glycoside fractions respectively. In Figure 7.2 A, Sutherlandioside A eluted at a retention time of 16.6 minutes which corresponded to the presence of that triterpenoid glycoside in the triterpenoid glycoside fraction. A similar observation was made for Sutherlandioside B, which eluted at 13.9 minutes (Figure 7.2 B). In Figure 7.2 C, only one peak at 16.5 minutes was evident for the combination of Sutherlandiosides C and D, which indicates that, unlike the recently published HPLC method for the quantitative determination of sutherlandiosides and sutherlandins [205], the qualitative HPLC method used as modified from Prevoo *et al.* (2008) [226] was unable to resolve Sutherlandioside C and D. Nevertheless, the retention time of the peak which represents both of these compounds fell within the triterpenoid glycoside cluster of peaks (13–17 minutes). A similar trend was observed in Figure 7.3 A, for the combination of Sutherlandins A and B, where one peak between 10 and 11 minutes was present which is within the retention time range of the flavonol glycosides (9–11 minutes) in the flavonol

glycoside fraction. Two unresolved peaks were evident in Figure 7.3 B for the combination of Sutherlandins C and D between 9 and 10 minutes which also eluted within the retention time range of the flavonol glycosides in the flavonol glycoside fraction. Since these sutherlandiosides and sutherlandins are amongst the triterpenoid and flavonol glycosides present in the triterpenoid and flavonol glycoside fractions respectively, which were in turn found to be present in the aqueous and methanolic extracts of SF (see Chapter 6, Figure 6.2), it may thus further be concluded these sutherlandiosides and sutherlandins are likely present in the aqueous and methanolic extracts of SF. These findings indicate that any effects of Sutherlandioside A, Sutherlandioside B or a combination of Sutherlandiosides C and D in this study, which correlate to the effects on ATV metabolism elicited by the triterpenoid fraction in human liver microsomes (section 7.2), may possibly be related. Likewise, the same analogy may be drawn with respect to the response generated by the flavonol glycoside fraction in section 7.2 and those of the combinations of Sutherlandins A and B and Sutherlandins C and D in this study. The chromatograms also indicate that the concentration of sutherlandiosides and sutherlandins in the triterpenoid and flavonol glycoside fractions respectively may be considerably higher than the concentrations present in the individual or combined samples of these compounds, except for Sutherlandioside B where the opposite is true. These findings may account for differences observed between the effects of the crude fractions in the human liver microsome study (section 7.2) and the individual or combined samples of sutherlandiosides and sutherlandins. As previously mentioned (section 6.2.3), the chromatograms of the flavonol and triterpenoid glycoside fractions further indicate that low quantities of several other compounds, besides the flavonol and triterpenoid glycosides were also present in these fractions which were used for the experiments in Chapter 6 and in section 7.2, thus it is plausible that these additional compounds may also contribute to any differences in responses elicited.

Figure 7.4 shows that there was no statistically significant difference between control and treatment groups within the Control Supersomes™. This finding was anticipated since Insect Control Supersomes™ are reported to not contain endogenous CYP450, CYP450 reductase or cytochrome b_5, therefore ATV metabolism, whether in the absence or presence of extracts or constituents of SF was not expected to occur and ATV levels remained unchanged. A comparison of Control Supersomes™ and CYP3A4-transfected Supersomes™ for each treatment group (see Figure 7.4) revealed higher amounts of ATV (as a percentage of control)

for both the aqueous ($p<0.05$) and methanolic ($p<0.01$) extracts, for the combination of Sutherlandiosides C and D ($p<0.05$) and for KTZ ($p<0.0001$) in the CYP3A4-transfected Supersomes™. This implies that these test compounds had the ability to inhibit the CYP3A4-mediated metabolism of ATV.

The crystal structure of a CYP3A4-KTZ complex showed that KTZ can bind to the haeme iron of the enzyme via the imidazole nitrogen [376] which classifies it as a type II substrate/inhibitor (see Chapter 5, section 5.3.1). Non-competitive inhibition of the metabolism of ATV, a CYP3A4 substrate, by KTZ, may have thus occurred since the iron in the haeme moiety was not free to interact with the ATV or to participate in the redox reactions required to initiate the catalytic cycle of CYP3A4 (see Chapter 5, section 5.3.1).

The increase in ATV observed as a percentage of control in the presence of the aqueous and methanolic extracts of SF and the implied inhibition of ATV metabolism associated therewith qualitatively correlates to the increase in ATV demonstrated by these same extracts in human liver microsomes (section 7.2.3). This suggests that the effect in the latter may, at least in part, be attributed to inhibition of CYP3A4-mediated metabolism of ATV by these extracts. The major implication of this finding with respect to the aqueous extract is that CYP3A5 non-expressors may be more susceptible to an interaction between ATV and SF than CYP3A5 expressors, if the effect is observed *in vivo* following co-administration of ATV and traditional aqueous decoctions of SF. Inhibition of the metabolism of CYP3A4 substrates by SF methanolic and aqueous extracts has previously been demonstrated by Mills *et al.* (2005) [116] at higher concentrations of 100 mg/ml. The present study shows that the unknown active constituents in these extracts were present in sufficient quantities to elicit the response at concentrations as low as 10 mg/ml. Moreover, the increase in ATV also observed for the combination of Sutherlandiosides C and D may indicate that one or both of these sutherlandiosides may have contributed to the inhibition of ATV metabolism observed in the aqueous and methanolic extracts, which likely contain the sutherlandiosides, as discussed above. The low concentrations likely present does however indicate that other unknown constituents of SF in these two extracts may also contribute to the observed effect by synergism.

Interestingly, Sutherlandioside B which has been shown to inhibit CYP3A4 metabolism of a fluorescent substrate with an IC_{50} of 20 µM [231] had no effect on CYP3A4-mediated metabolism of ATV at a higher concentration of 77 µM. This is, however in agreement with the molecular docking study which found that even though ATV and Sutherlandioside B shared similar binding modes, the binding affinity of ATV to CYP3A4 was considerably greater than that of Sutherlandioside B (see Chapter 5, section 5.3.3) which minimised the likelihood of Sutherlandioside B being able to competitively inhibit ATV metabolism. The flourescent substrate used in the study by Madgula *et al.* (2008) [231] may have had a lower affinity for CYP3A4 than ATV and this may explain why weak inhibition of CYP3A4-mediated metabolism by Sutherlandioside B of that substrate (IC_{50} of 20 µM) was observed.

The effect of enhanced ATV metabolism in human liver microsomes observed in the presence of the triterpenoid glycoside fraction (section 7.2), which contains sutherlandiosides (see Figure 7.2) differs from that of the individual or combined sutherlandiosides, which showed either no effect (Sutherlandiosides A and B) or inhibition of CYP3A4-mediated metabolism of ATV (combination of Sutherlandioside C and D). The discrepancy in the results may indicate that other unknown components of the triterpenoid glycoside fraction used in section 7.2 may be responsible for the activation of ATV metabolism in human liver microsomes, possibly via a CYP3A4-mediated mechanism. Alternatively, components of the triterpenoid glycoside fraction, including the four sutherlandiosides may have enhanced the activity of CYP3A5, the other major isoenzyme in the liver for which ATV is a substrate (see Chapter 1, Table 1.6). Co-operativity, which would be required for such activation, has however not been reported for CYP3A5. It is therefore more plausible that the activation of ATV metabolism by the triterpenoid glycoside fraction in the human liver microsomes was in fact CYP3A4-mediated. The enhanced ATV metabolism by unknown constituents of the triterpenoid glycoside fraction may have predominated over any inhibitory effect which may have been elicited by Sutherlandioside C and/or D present in the triterpenoid glycoside fraction. The difference in the results may also relate to the fact that the quantity of the sutherlandiosides A, C and D in the triterpenoid glycoside fraction at the concentration used in the human microsome study (section 7.2) is considerably higher than that present in the concentration of individual or combined sutherlandiosides used in the present study (see Figure 7.3). Responses of CYP3A4 effectors are known to be concentration-dependent [499], and this may also explain why activation of ATV metabolism (which may be CYP3A4-mediated) ocurred in the presence of the triterpenoid glycoside fraction which contained

higher concentrations of most of sutherlandiosides (triterpenoid glycosides) (see Figure 7.2) in comparison to no effect (Sutherlandiosides A and B) or inhibition of CYP3A4-mediated metabolism of ATV (combination of Sutherlandioside C and D) observed in the presence of these sutherlandiosides at lower concentrations.

The findings that the combinations of Sutherlandins A and B and Sutherlandins C and D exhibited no effect on CYP3A4-mediated metabolism of ATV are in agreement with the results obtained for the determination of the effect of the flavonol glycoside fraction on ATV metabolism in human liver microsomes (section 7.2.3), despite the likely difference in concentration of the sutherlandins in the flavonol glycoside fraction and in the combined sutherlandin samples (see Figure 7.3). It may thus be concluded that the flavonol glycosides likely have no effect on ATV hepatic metabolism whether CYP3A4-mediated or not.

The molecular docking study alluded to the unlikelihood of Sutherlandioside B and Sutherlandin C having the ability to competitively inhibit the CYP3A4-mediated metabolism of ATV (see Chapter 5, section 5.3.3). These *in silico* findings therefore corroborate the *in vitro* results obtained in the present study, where these compounds produced no significant effect on CYP3A4-mediated metabolism in comparison to the control.

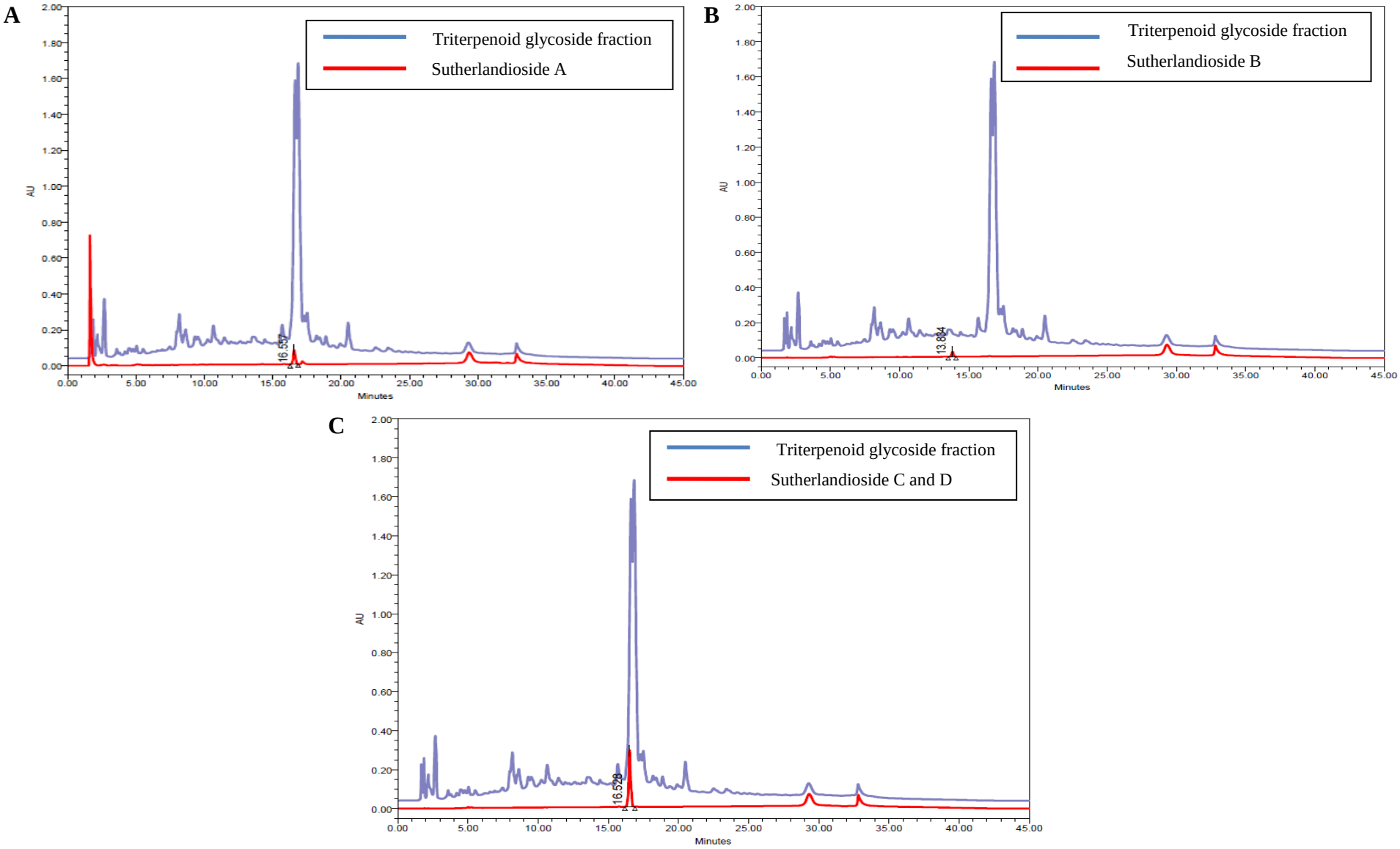


Figure 7.2: Overlay of chromatograms to show that (A) Sutherlandioside A (B) Sutherlandioside B and (C) a combination of Sutherlandioside C and D are present in the triterpenoid glycoside fraction. Peaks occurring at retention times greater than 28 minutes are common unidentified components.

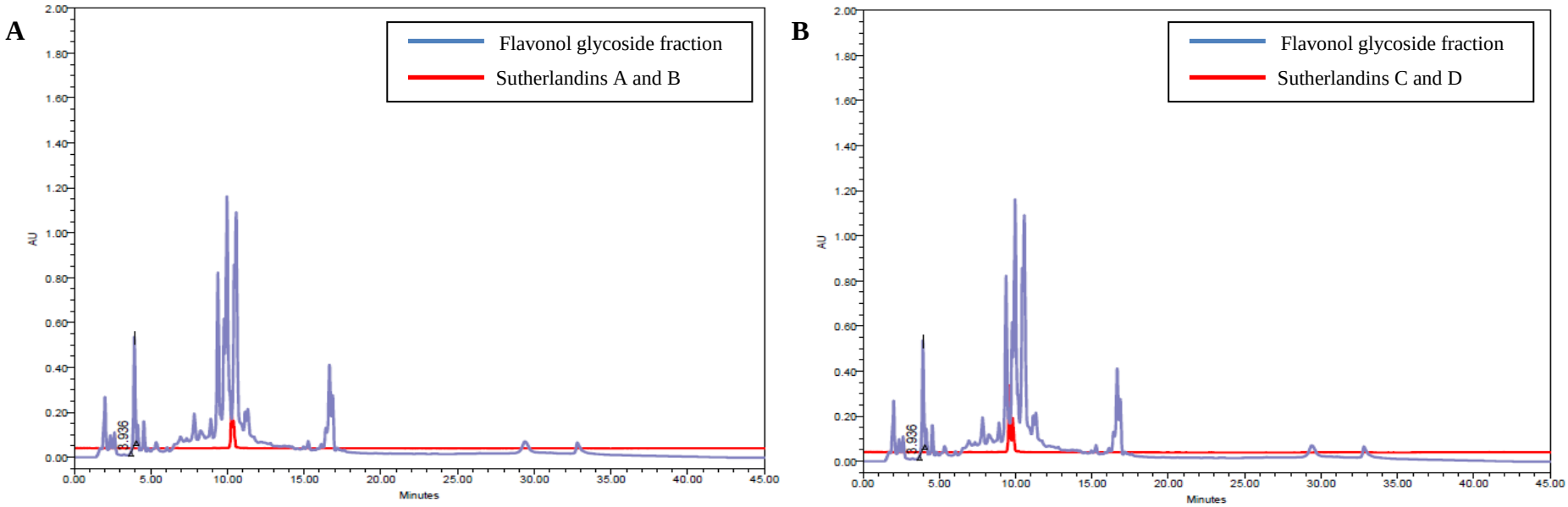


Figure 7.3: Overlay of chromatograms to show that (A) a combination of Sutherlandin A and B and (B) a combination of Sutherlandin C and D are present in the flavonol glycoside fraction

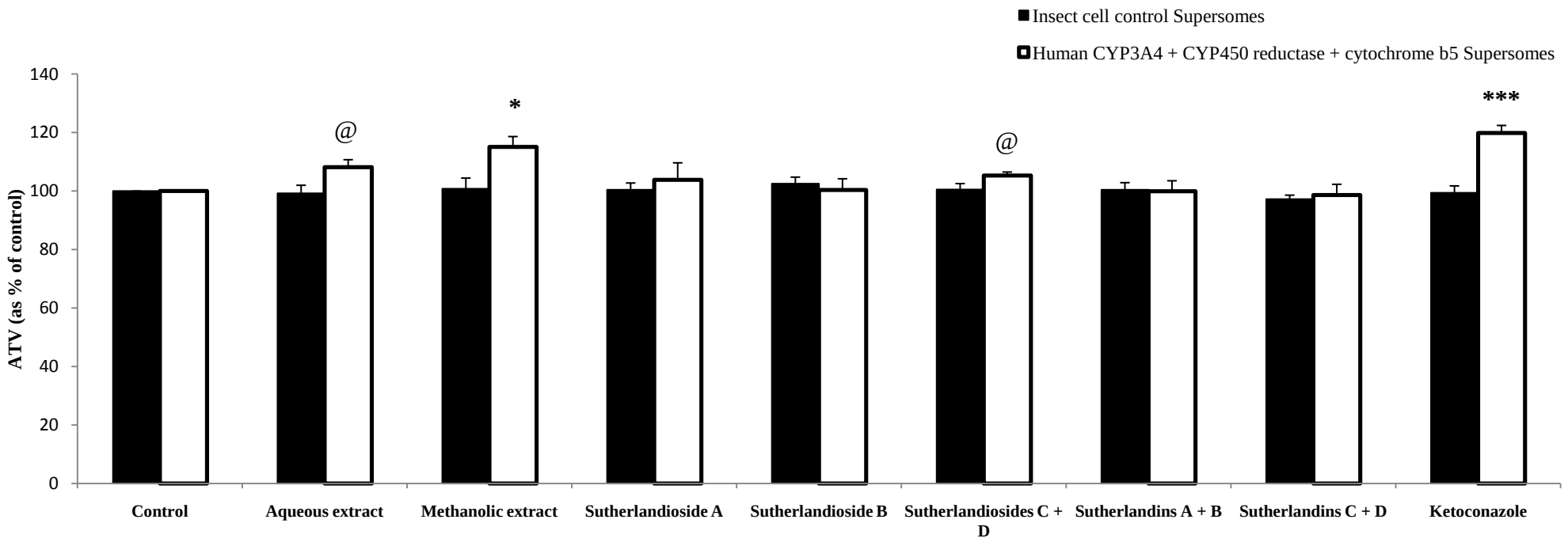


Figure 7.4: Comparative effects of extracts (10mg/ml) and components of SF on ATV (25 µM) metabolism in Control and CYP3A4-transfected Insect Supersomes™ , *in vitro.* KTZ (10 µM) is used as a positive control. Each bar represents mean ± SD; n = 3. @ ($p<0.05$), * ($p<0.01$) and *** ($p<0.0001$) in comparison to control of Insect cell control Supersomes™ (Student's *t*-test).

7.4 Conclusions

Since drug bioavailability is influenced by hepatic first pass metabolism, PK drug-drug interactions may occur due to the modulation of the metabolism of one drug or xenobiotic by another.

Human liver microsomes were used as an *in vitro* model of human hepatic metabolism, in which incubations of the microsomes were conducted with ATV in the absence and presence of SF extracts and components, as well as the positive control, KTZ. The results showed that the aqueous and methanolic extracts and KTZ inhibited ATV metabolism, whilst the converse was true for the triterpenoid glycoside fraction. The findings in this study suggest that traditional preparations of SF in the form of an aqueous decoction at a concentration of 10 mg/ml (see Chapter 3, section 3.6.1) may have the potential to inhibit ATV metabolism and thereby increase the bioavailability of ATV, whilst commercial dosage forms of SF such as Sutherlandia Su1™ tablets (Phyto Nova) may exhibit inhibition of ATV metabolism too if effects of phytochemical constituents responsible for this inhibition in the aqueous and methanolic extracts predominate. On the other hand, elevation of ATV metabolism may occur if effects observed by the triterpenoid glycoside fraction predominate. With respect to both the traditional SF preparations and the commercial dosage forms, the potential effects on ATV metabolism may not alter the bioavailability of ATV in the same manner as that which may occur through modulation of ATV absorption. The need for an *in vivo* drug-drug interaction study to determine the true potential for SF to alter the bioavailability of ATV is therefore necessary.

Evaluating whether the effects of SF extracts and components on ATV metabolism observed in human liver microsomes were CYP3A4-mediated was considered important since CYP3A5 non-expressors rely solely on CYP3A4 for ATV metabolism and are thus potentially more susceptible to drug-drug interactions involving ATV. Experiments were similarly conducted in control and CYP3A4-transfected insect cell Supersomes™, where individual or combinations of sutherlandiosides and sutherlandins were used instead of the triterpenoid and flavonol glycoside fractions used in the human liver microsome study. Qualitative HPLC-UV analysis was used to confirm the presence of these in the triterpenoid and flavonol glycoside fractions respectively. This analysis also revealed that the fractions

contained unidentified compounds in addition to the four sutherlandiosides and four sutherlandins.

The findings for the SF extracts, combinations of Sutherlandins A and B and Sutherlandins C and D and the positive control, KTZ, were in agreement with results from the human liver microsomes studies, implying that where statistically significant differences were observed, these were CYP3A4-mediated. The implication of this finding, particularly with respect to the aqueous extract is that CYP3A5 non-expressors may be more susceptible to an interaction between ATV and SF if the effect is observed *in vivo*. Sutherlandiosides A and B, showed no effect, whilst CYP3A4-mediated inhibition of ATV metabolism was exhibited by a combination of Sutherlandioside C and D. This was contrary to the finding of activation of ATV metabolism by the triterpenoid glycoside fraction, which contains the sutherlandiosides, in human liver microsomes. The discrepancy in these findings was considered most likely to be due to a possibly concentration-dependency of the response or due to the presence of additional unidentified compounds in the triterpenoid fraction which may have enhanced CYP3A4 activity and therefore reduced ATV levels in human liver microsomes. If this effect predominated, then any inhibitory activity of the Sutherlandioside C and/or D, present in the triterpenoid glycoside fraction, on ATV metabolism in human liver microsomes would have been masked.

The results of these studies were also corroborated by the molecular docking study which showed that Sutherlandioside B and Sutherlandin C were not likely to exhibit competitive inhibition of ATV metabolism by CYP3A4.

In summary, the aqueous and methanolic extracts inhibited ATV metabolism, whilst the converse was true for the triterpenoid glycoside fraction. The effects of both the aqueous and methanolic extracts were CYP3A4-mediated. The implications of these findings are that traditional preparations of SF in the form of an aqueous decoction at a concentration of 10 mg/ml (see Chapter 3, section 3.6.1) may have the potential to inhibit ATV metabolism and thereby increase the bioavailability of ATV, whilst commercial dosage forms of SF such as Sutherlandia Su1™ tablets (Phyto Nova) may also exhibit this same response if effects of phytochemical constituents responsible for this inhibition in the aqueous and methanolic extracts predominate. On the other hand, elevation of ATV metabolism may occur if effects

observed by the triterpenoid glycosides predominate. Thus, analogous to the proposed effects of SF on absorption of ATV, the relative contribution of each of the active constituents to the overall effect that administration of traditional preparations or commercial dosage forms of SF may have on the metabolism of ATV is not known, and an *in vivo* drug-drug interaction study is needed to reveal the potential for SF to alter the bioavailability of ATV.

CHAPTER 8
IN VIVO PHARMACOKINETIC INTERACTIONS

8.1 Background

Bioavailability, the rate and extent to which a drug reaches the systemic circulation, may be used as an indicator of the potential PD response which may lead to therapeutic or toxic effects of the drug. Changes in the bioavailability of a drug in an individual as a result of a disease condition or an interaction with drug/food/herbal medicine may thus alter these PD responses, thereby impacting on the efficacy or toxicity of the drug [608].

In vivo animal studies are often used in the drug discovery process to determine the toxicological profile of a drug and its metabolites that may provide useful information from which the clinical implications of potential drug-drug interactions may be predicted [609]. However, as previously mentioned in Chapter 7, section 7.2.1, inter-species differences in the PK handling of a drug often preclude meaningful extrapolation of drug-drug interaction data from animal surrogates.

Clinical drug interaction studies are designed to compare substrate drug levels with (S + I) and without (S) the interacting drug in humans [139; 608; 609]. Various study designs are acceptable depending on the specific objectives of the *in vivo* drug interaction study. In a parallel design, (S) is administered to one group of subjects and (S + I) to another group. In contrast, a crossover study is divided into two periods, in which all subjects participate, receiving one of the treatments, either (S) or (S + I) during each period [139; 609]. As the name suggests, in a one-sequence crossover study, all subjects receive the same treatment, either (S) or (S + I) during the same period, whereas in a randomised crossover study, half the subjects receive (S) followed by (S + I) in two periods and the other receive (S + I) followed by (S). The major advantage of crossover studies is that each individual subject acts as his/her own control and comparisons are based on intrasubject variability, which is smaller than the inter-subject variability associated with the use of parallel designs [139; 609; 610]. Crossover studies therefore have greater statistical power to detect a drug-drug interaction for a given number of subjects than do studies of parallel design [609; 610]. Parallel studies require the participation of more subjects but for a shorter period of time than crossover studies, therefore the former, despite their shortcomings, may be preferred for drugs with long

elimination half-lives where the time taken for steady state to be reached or for "washout" to be achieved is high [608]. A randomised crossover study is usually favoured over a one-sequence crossover study since it exhibits a higher sensitivity to sequence, period and carryover effects than a one-sequence crossover study [609]. The dosing regimen combinations of the substrate drug and interacting drug (S/S + I) may also be varied: single dose/single dose, single dose/multiple dose, multiple dose/single dose and multiple dose/multiple dose studies [139]. The criteria for the selection of a particular combination for a specific study include: (i) whether the agents are used acutely or chronically in the target patient population, (ii) whether multiple dosing of either agent may be toxic and (iii) the PD and PK characteristics of the agents, such as half-life, lag time to therapeutic effect and enzyme-inducing properties [609].

Several subject or patient groups may be considered for use as a study population in a drug-drug interaction study [139; 609]. The use of healthy subjects who meet certain inclusion and exclusion criteria increases the homogeneity of the cohort and thereby assists in reducing the variability in absorption and metabolic processes that are often associated with covariates such as age, gender, disease conditions, diet and recreational drug intake habits (e.g., smoking tobacco and drinking alcohol). On the other hand, the use of subjects drawn from a more general population or patients for whom the drugs are intended forms a more heterogenous cohort and may result in data which correlates better to the clinical situation [608]. If the substrate drug is metabolised by an enzyme which exhibits polymorphism, then genotyping of subjects to identify which polymorph is manifested in each individual may be useful in describing how metabolic status may influence the extent of a drug-drug interaction [139; 608; 609; 611]. It may also explain large inter-individual variability and the existence of outliers [609]. Conversely, selection bias may occur and therefore it may not be appropriate to extrapolate the findings to a general subject or patient population [609].

The most commonly used PK parameters of the substrate drug in the analysis of PK drug-drug interaction data include: the area under the plasma concentration vs time curve (AUC), the peak plasma concentration (C_{max}) [139; 608; 609], the time to reach C_{max} (t_{max}), the trough concentration (C_{min}) [139; 609], the clearance (CL) [139; 608; 609], the volume of distribution (V_d) [139; 609], elimination rate constant (k_{el}) [609] and elimination half-life ($t_{½}$)

[139; 608; 609]. The fraction of the drug unbound in the plasma (f_u) may also be useful when the substrate drug is highly protein bound with a low V_d [609].

The ultimate goal of a drug-drug interaction study is to determine the clinical significance of any change in one or more of the above-mentioned PK parameters of the substrate drug when administered in the presence of a possible interacting drug, *viz* S vs S + I. How PD effects (therapeutic and toxic) relate to each of these PK parameters for (S) might dictate which of these parameters are most appropriate to investigate [139; 608; 609]. An analogy may be drawn between drug-drug interaction and bioequivalence studies in that both seek to ascertain whether a clinically significant change in bioavailability of a drug has occurred when administered under different conditions [139; 608; 609; 612], where such conditions are either the co-administration of another drug (drug-drug interactions) [139; 608; 609] or the delivery of the same drug in a different formulation (bioequivalence) [612]. The US [139], Canadian [609] and European [608] drug-drug interaction guidances therefore all recommend that the statistical approach for bioequivalence be applied to drug-drug interaction studies. There are several of these approaches for bioequivalence which have been suggested, such as: the power approach, Hauck and Anderson's procedure [613], Westlake's shortest CI [614], and Schuirmann's two one-sided test procedure [615]. All are based on the principle that for given lower and upper tolerance limits, bioequivalence (or no drug-drug interaction) is claimed if the difference between the mean of the test and reference (or S and S + I) is within the tolerance limits with some degree of certainty [616]. Schuirmann (1987) [615] demonstrated that the two one-sided test procedure is superior to the power approach and may be considered analogous to Westlake's CI approach. In these two tests, equivalence (or no drug-drug interaction) claims are based on central *t*-distributions [616].

The two null and alternate hypotheses which relate to these two preferred approaches are:

$$H_{01}: \mu_T - \mu_R \leq \Theta_1$$
$$H_{11}: \mu_T - \mu_R > \Theta_1$$
and
$$H_{02}: \mu_T - \mu_R \geq \Theta_2$$
$$H_{12}: \mu_T - \mu_R < \Theta_2$$

Where:

μ_T = Mean of PK parameter in test product or mean of PK parameter in (S +I).

μ_R = Mean of PK parameter in reference product or mean of PK parameter in (S).

Θ_1 = Lower tolerance limit.

Θ_2 = Upper tolerance limit.

The overall null hypothesis, H_0 is rejected and thus equivalence of μ_T and μ_R (or no drug-drug interaction between S and I) is concluded, only if both H_{0l} and H_{02} are rejected at a chosen level of significance, α [615]. The two one-sided tests procedure and the CI approach are thus integrated due to the reasoning that if the results of the two one-sided tests have concluded that $\Theta_1 < \mu_T - \mu_R$ and $\mu_T - \mu_R < \Theta_2$, it is effectively implied that $\mu_T - \mu_R$ is in the interval, Θ_1,Θ_2 [615].

In crossover bioequivalence studies, the CI approach is usually used where a 90% CI is constructed around the geometric mean ratio (difference of means on natural log scale) between test and reference products for the AUC and C_{max} of each subject [612]. A "no effect" boundary, which is the interval within which the two products are deemed bioequivalent, is set to allow a 20% difference between the products. This translates to an interval of 0.8–1.25 using log-transformed data [612]. The CI and limits may thus be similarly constructed and set for drug-drug interaction studies, where test and reference products are replaced with (S + I) and (S) respectively. The CI is calculated according to Equation 8.1 [610].

$$x \pm t_{0.95;\, N-2}\sqrt{MSE\ (\frac{2}{N/2})} \qquad \textbf{Equation 8.1}$$

Where:

x	=	mean of the ratio of log-transformed data.
$t_{0.95;\, N-2}$	=	*t* value for a one-sided 95% CI with degrees of freedom of N – 2.
MSE	=	Mean sum of squares of the error.
N	=	Number of subjects per period/phase

The MSE is determined from an analysis of variance (ANOVA) and excludes error or variability due to inter-subject, -treatment and -period differences, such that only the intra-subject error is represented, and thereby included in the interval [610]. In a one-sequence crossover study, error due to period effects are not distinguishable from error due to treatment differences, which explains why this design, as previously mentioned, is less sensitive to period effects than the randomised crossover design.

The desire of the sponsors of bioequivalence or drug-drug interaction studies is usually to show bioequivalence or lack of an interaction [611]. In Equation 8.1, the smaller the MSE and the closer *x* is to unity (1), the greater the chance that the CI will fall within the limits set. The probability of committing a Type I statistical error and concluding bioequivalence (or no drug-drug interaction) when products are in fact bioinequivalent (or there is in fact a drug-drug interaction) [617; 618] is fixed at an α of 0.05, and the risk to the patient is thus minimised [617]. Conversely, the probability of concluding bioinequivalence (or a drug-drug interaction) when products are in fact bioequivalent (or there is in fact no drug-drug interaction), a Type II error, β [617; 618], may be set at the discretion of the sponsor [617]. Power, $1 - \beta$, is thus the probability of concluding bioequivalence (or no drug-drug interaction) when the products are in fact bioequivalent (or there is in fact no drug-drug interaction) [617; 618]. The larger the power, the more sensitive is the test [618]. A power of 0.8 (80%) is generally considered adequate for the sensitivity of statistical approaches used in bioequivalence studies [610; 618].

The number of subjects used in a clinical drug-drug interaction study should be sufficient to detect clinically significant differences between treatment groups with adequate statistical power [139; 608–610], usually 80% as discussed above. It is generally recommended that a minimum of 12 subjects in total for a crossover design, or 12 subjects per group for a parallel design, be used [611]. However, when conducting bioequivalence or drug-drug interaction studies on highly variable drugs, a large sample size may need to be considered [619]. When MSE and *x* of the particular formulation (in the case of bioequivalence studies) or the drug (in the case of drug-drug interaction studies) is known from previous studies, then the subject sample size appropriate for pivotal studies may be calculated. If not, pilot studies, with a small number of subjects may provide the data needed to establish the pool of subjects for further formal (pivotal) studies [619]. Marzo *et al.* (2004) [619] analysed the degree of

predictivity of pilot trials on six subjects and found that the average predictivity index was 71.1% for C_{max} and 82.9% for AUC, which suggested that pilot trials with as few as six subjects may be useful for predicting the pool size of subjects in bioequivalence studies.

By convention, therefore, an estimate of the number of subjects required to achieve a power of 80% is determined *a priori* [611; 618]. Liu and Chow (1992) [620] proposed approximate formulae for the total number of subjects, for the two one-sided tests procedure in bioequivalence, which were then modified by Hauschke *et al.* (1992) [621] for application to a log-normal distribution.

$$N = 2n \qquad \textbf{Equation 8.2}$$

If $x = 1$, then:

$$n \geq [t(\alpha, 2n - 2) + t(\beta/2, 2n - 2)]^2 \left[CV/(ln1.25)\right]^2 \qquad \textbf{Equation 8.3}$$

If $1 < x < 1.25$, then:

$$n \geq [t(\alpha, 2n - 2) + t(\beta, 2n - 2)]^2 \left[CV/(ln1.25 - ln\Delta)\right]^2 \qquad \textbf{Equation 8.4}$$

If $0.8 < x < 1$, then:

$$n \geq [t(\alpha, 2n - 2) + t(\beta, 2n - 2)]^2 \left[CV/(ln0.8 - ln\Delta)\right]^2 \qquad \textbf{Equation 8.5}$$

Where:

N	=	Total number of subjects (subjects per period)
n	=	Number of subjects per sequence
CV	=	$\sqrt{\exp(MSE) - 1}$
x	=	mean of the ratio of log-transformed data.

The x and CV is usually obtained from pilot studies and n is enumerated because the degrees of freedom (2n – 2) are unknown [620]. From Equations 8.4 and 8.5, it is evident that the smaller the CV and the closer x is to unity, the smaller the sample size required to obtain a given power (usually 80%) at a given level of significance (usually 0.05).

In a drug-drug interaction study, if either one or both CI limits fall outside the “no effect” boundary of 0.8–1.25, then a clinically significant interaction is implied. However, unlike bioequivalence studies, where bioinequivalence is directly inferred, the clinical relevance or impact of a clinically significant drug-drug interaction is not known and may only be proposed, particularly when a healthy study population has been used. A clinically significant interaction is considered clinically relevant when the PD (therapeutic activity and/or toxicity) is altered to such an extent that a dosage adjustment [139; 609], and/or medical intervention, is necessary [609].

8.2 Effect of Chronic Administration of Sutherlandia SU1™ tablets on the PK of ATV in Healthy Male Human Subjects

8.2.1 Introduction

The *in vitro* studies conducted in this thesis have alluded to the potential for traditional aqueous decoctions, as well as commercial preparations of SF, to modulate the functional activity of P-gp and CYP3A4 in the human small intestine and liver respectively. López Galera *et al.* (2008) [138] reported that the herbal medicine, cat's claw, was the likely cause of an elevated ATV C_{min} in one patient, which was in agreement with the known CYP3A4 inhibitory activity of the herbal medicine, *in vitro* [140]. This study highlights the susceptibility of ATV to drug-herb interactions due to altered functional activity of transporters and/or drug metabolising enzymes previously proven, *in vitro*.

Other researchers have demonstrated the potential for SF extracts to activate PXR *in vitro* [116] and to increase mRNA expression of CYP3A4 in both the liver and small intestine after 5 day administration of SF (12 mg/kg) to rats [124]. The increase in mRNA expression correlated with a 50% decrease in the AUC and C_{max} of co-administered nevirapine in comparison to nevirapine administered alone. Together, the findings of these two studies suggest that long-term administration of SF may cause induction of intestinal and hepatic CYP3A4, possibly through activation of PXR, which has, in turn, been shown to result in a decrease in the bioavailability of the CYP3A4 substrate, nevirapine. It is therefore plausible that such a reduction in bioavailability may occur for other CYP3A4 substrates too, such as ATV, when these are co-administered with SF.

The preclinical studies in this thesis, as well as those in the literature mentioned above, point towards the potential for SF to induce bidirectional interactions with ATV, since some results imply an increase in the bioavailability of ATV whilst for others, the opposite is true. Only a clinical drug interaction study may shed light on whether there is a significant interaction between ATV and SF and, if so, which of the two opposing effects prevails. This is further complicated by the fact that each effect may have an opportunity to predominate at different occasions during the co-administration of ATV and SF, which may be dependent on the dosing regimen combination of the two, as the timing of drug co-administration and sequence of administration may affect the type and magnitude of the interaction [609].

The PK of ATV has been found to be influenced by CYP3A5 and P-gp genetic polymorphisms [605]. CYP3A5 expressors had a faster CL/F which resulted in a lower C_{min} of ATV than non-expressors. Individuals who possess the wild-type ABCB1 (P-gp) CGC haplotype had a slower CL/F of ATV. As previously mentioned (Chapter 7, section 7.3.1), the primary implication of CYP3A5 polymorphism for ATV-drug PK interactions is that CYP3A5 non-expressors may be more susceptible to CYP3A4-mediated interactions, whether through altered activity or expression, since these individuals rely entirely on CYP3A4 for metabolism of ATV. The association of wild-type ABCB1 (P-gp) CGC haplotype with slower CL/F may suggest that, in individuals who possess this P-gp genetic polymorph, P-gp-mediated transport of ATV is less significant compared to those who exhibit other P-gp polymorphs. These individuals may therefore be at a lower risk of ATV-drug interactions mediated through P-gp.

Several lines of evidence discussed here emphasise the need for a suitably designed drug-drug interaction study to reveal the true potential for SF to alter the PK/bioavailability of ATV. An ATV single dose/ SF multiple dose, one sequence crossover drug-drug interaction study in healthy male subjects was therefore conducted.

8.2.2 Methods and Procedures

8.2.2.1 Ethical and Institutional Review

A study protocol was compiled (See Annexure I) and submitted to the Rhodes University Ethical Standards Committee (RUESC) for approval. Once the approval was obtained, all procedures were conducted in accordance with the principles of the Declaration of Helsinki (1964) and its subsequent amendments [622] and the International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) guidelines [623].

8.2.2.2 Study Population

An advert approved by the RUESC for the recruitment of non-smoking, healthy male subjects between the ages 18 and 55 was posted on various noticeboards on the Rhodes University campus. Interested respondents were provided with verbal and written information which outlined the rationale for the study as well as the study procedures. All these respondents were given 24 hours to consider the information provided to them, after which informed consent forms were completed and signed in duplicate by the first 20 respondents who

subsequently agreed to participate in the study. One copy of each informed consent form was filed as part of the study records and the other was issued to the subject.

Within one month prior to commencement of the study, a medical and physical screening was performed on each subject who had signed an informed consent form.

The first twelve (plus three reserves) who passed this screening according to the inclusion and exclusion criteria described in Table 8.1 were enrolled into the study. The screening included laboratory tests for liver function, hepatitis B and C, and HIV, as well as blood biochemistry and urinalysis. Key demographic data of the enrolled subjects are shown in Table 8.2.

Table 8.1: Exclusion and inclusion criteria for enrolment into the study

	Inclusion Criteria
1.	Subjects should be mentally competent and able to give informed consent by signing the informed consent form. They should be available for the entire study period and willing to adhere to the protocol requirements.
2.	Subjects should be males between the ages of 18 and 55 years.
3.	Subjects should have a body mass index (BMI) of between 19 and 30.
4.	Subjects should be non-smokers who have not smoked for at least two months.
5.	The medical history of the subject should be satisfactory. Any abnormality is only acceptable if the Study Physician considers it to be clinically insignificant.
6.	The physical examination of the subject at the pre-study medical screening should be satisfactory. Any abnormality is only acceptable if the Study Physician considers it to be clinically insignificant.
7.	The laboratory test values of the subject should be within the laboratory's stated normal range. Any abnormality is only acceptable if the Study Physician considers it to be clinically insignificant.
	Exclusion Criteria
1.	Subjects should not have any history of hypersensitivity or idiosyncratic reaction to ATV or SF
2.	Subjects should not have any history of cardiovascular dysfunction or disease.
3.	Subjects should not have any history of liver dysfunction or disease.
4.	Subjects should not have any history of anaemia or cytopaenia.
5.	Subjects should not have any history of renal dysfunction or disease.
6.	Subjects should not have any history of adrenal or pituitary insufficiency.
7.	Subjects should not have any history of chronic asthma, bronchitis or other bronchospastic conditions.
8.	Subjects should not have any history of epilepsy or other convulsive disorders.
9.	Subjects should not have any history of psychiatric disorders.
10.	Subjects should not have any history or other condition which the Study Physician regards as clinically significant (including fainting upon blood sampling).
11.	Subjects should not have any history of drug or alcohol abuse. They should not have smoked tobacco within two months of the start of the study.
12.	Subjects should not have received treatment with any drug known to have a well-defined potential for toxicity to one of the major organs, especially the kidneys or the liver, within three months of the start of the study.
13.	Subjects should not have received treatment with any drug known to modify renal excretion of other drugs (e.g. probenecid) within one month of the start of the study.
14.	Subjects should not be on a restricted or abnormal diet for longer than one week within one month of the start of the study.
15.	Subjects should not be on maintenance therapy or on chronic medication.
16.	Subjects should not have had a major illness considered to be clinically significant by the Study Physician within three months of the start of the study.
17.	Subjects should not have participated in another clinical study or donated one pint or more of blood within one month of the start of the study.
18.	Subjects should not test positive for Hepatitis B, Hepatitis C or HIV.
19.	Subjects should not receive treatment with any prescription drug within one week of the start of the study, unless the drug is considered to be clinically insignificant by the Study Physician.

Table 8.1: (continued)

20.	Subjects should not receive treatment with any over-the-counter drug within one week of the start of the study, unless the drug is considered to be clinically insignificant by the Study Physician.
21.	Subjects should not consume alcohol or any other agents which have the potential to inhibit or induce CYP450 enzymes and drug efflux transporters (e.g. grapefruit juice) within 96 hours of the start of the study.
22.	Subjects' urine should not test positive for any drug of abuse at the pre-study medical screening.

Table 8.2: Demographic data of subjects

Subject Number	Age (Years)	Race	Weight (Kgs)	Height (m)	Body Mass Index
1	19	White	72.3	1.78	23.0
2	19	White	78.1	1.80	24.2
3	24	Black	69.8	1.86	20.2
4	22	Black	67.4	1.69	23.6
5	22	Black	68.5	1.76	22.1
6	21	Black	68.5	1.82	20.8
7	23	White	91.2	1.87	26.2
8	23	Black	83.8	1.82	25.4
9	30	Black	87.9	1.79	27.6
10	30	Black	72.9	1.81	22.3
11	23	Indian	70.0	1.70	24.4
12	21	Black	71.1	1.77	22.7
Min	19		67.4	1.69	20.2
Average	23		75.1	1.79	23.5
Max	30		91.2	1.87	27.6

At check-in for each phase of the study, the urine of each subject was screened for drugs of abuse, including amphetamines, barbiturates, benzodiazepines, cocaine, methamphetamine, morphine, phencyclidine, tetrahydrocannabinol, tricyclic antidepressants and alcohol, using test kits. Subjects were also probed on compliance to the study restrictions, as shown in Table 8.3.

Table 8.3: Study Restrictions

Restricted Item	*Duration of Restriction*	*Examples of Restriction*	*Comments*
Prescription medicines	From 1 week before Phase I until the end of Phase II.	Antibiotics, vaccinations, anti-inflammatories, anti-asthmatics, anti-acne.	This included all long term medication.
Over-the-counter (OTC) medicines	From 1 week before Phase I until the end of Phase II.	Anti-flu medicines, sports supplements, paracetemol, vitamins and minerals.	This included all medication which may be bought, without a prescription, including herbal and traditional remedies.
Caffeine	From 2 days (48 hours) before the start of each phase until the last blood sample for that phase was withdrawn.	**Drinks:** coffee, tea, green tea, any cola drink (including coke), hot chocolate, tonics such as Bioplus®, Red Bull®. **Food:** Chocolates.	This includes all chocolate-containing foods, for example, chocolate cake, chocolate ice cream, choc-chip biscuits.
Grapefruit	From 2 days (48 hours) before Phase I until the last blood sample for Phase II had been withdrawn.	Grapefruit, grapefruit juice or foods containing either.	No grapefruit or grapefruit juice.
Alcohol	From 4 days (96 hours) before Phase I until the last blood sample for Phase II had been withdrawn.	All alcoholic drinks and alcohol-containing foods.	*It was very important that this requirement was taken seriously and observed, as alcohol may significantly affect the liver. Failure to comply with this requirement may have seriously affected the results of the study.*
Strenuous Physical Exercise	From 24 hours before the start of the each phase until the last blood sample for that phase had been withdrawn.	Rugby, squash, rowing, gym, tennis etc.	Light exercise such as walking was permitted.
Food and Fluid Intake	For the duration of the clinic stay for each phase as well as all the restrictions detailed above.	All food and fluids.	Food and fluid intake during the clinic stay was provided for all subjects and was standardised to minimise inter-individual variation.
Smoking	From at least two months prior to Phase I until the last blood sample for Phase II had been withdrawn.	The smoking of any tobacco.	

8.2.2.3 Study Design

The study had a one-sequence crossover, two phase design with a single dose/multiple dose regimen combination for ATV and SF respectively. Acute administration of a potential interacting drug may be adequate to reveal drug interactions which occur as a result of altered transporter/CYP450 activity, but modulation of protein expression requires chronic exposure to such an agent. Multiple dosing of SF was therefore used so that potential effects on ATV bioavailability due to either or both altered functional activity and protein expression could be assessed.

The night before the start of the study (Day 0), subjects checked into the clinic and were screened for drugs of abuse and probed to determine compliance with the study restrictions, as described in section Table 8.3. On Day 1 of the study (start of Phase I), subjects received a light meal before a single 400 mg (2 X 200 mg capsules) dose of ATV (Reyataz®, Bristol-Myers Squibb, Bedfordview, Gauteng, South Africa) was administered to each with a 240 ml glass of water. A mouth and hand check was conducted to confirm that the dose had been ingested. Subjects were not permitted to lie down or sleep until 4 hours post-dose, unless this was necessary due to an adverse event. Standard meals were provided until 24 hours post-dose. The time at which each subject commenced and ended each meal was recorded as well as the approximate amount consumed. Ten millilitre blood samples were collected into BD Vacutainer® blood collection tubes (Becton Dickinson, Woodmead, Gauteng, South Africa) containing potassium EDTA as the anticoagulant, at the following time intervals; before dosing (0) and at 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 9, 12, 18 and 24 hours post-dose. The exact time at which each sample was withdrawn was also recorded. Blood samples were stored in an ice-bath immediately after withdrawal up until centrifugation at 2800 x *g* for 10 minutes at 4 ºC, which was done within 30 minutes. Duplicate aliquots of harvested plasma were stored in polypropylene tubes at -80 ± 10 ºC until transfer to the analytical laboratory, where the samples were stored at -10 ± 2 ºC. The tubes were labelled with the study number, phase number, subject number, sample number and sampling time.

From Day 3, each subject started a twice daily regimen (one tablet twice a day) of Sutherlandia SU1™ tablets (Phyto Nova, Cape Town, Western Cape, South Africa). The label of Sutherlandia SU1™ stated that each tablet contained 300 mg of SF plant material. Subjects reported to the study investigator every day between 08h00 and 09h00 and between

20h00 and 21h00 to receive these doses with a 240 ml glass of water. A hand and mouth check was carried out to confirm that these doses were taken. On Day 14, the subjects checked into the clinic for screening as described for Day 0. On Day 15 (start of Phase II), each subject received a single oral dose of ATV (2 X 200 mg capsules) and a dose (1 X 300mg tablet) of Sutherlandia SU1™, 30 minutes after a light meal. The rest of Phase II was conducted according to the same procedures described for Phase I. Dropouts at any time during the study were not replaced.

8.2.2.4 Analysis of Plasma Samples

Plasma samples were analysed according to the validated HPLC-UV method described in Chapter 4. The longest period over which stability of ATV in plasma samples was demonstrated, was 2 months (See Chapter 4, Table 4.11), therefore all plasma samples were analysed within this time period after collection. Six analytical batches, each consisting of the Phase I and Phase II samples of two subjects per day, 11 calibration standards in triplicate, which included blank and zero samples and four sets of QC samples at three different concentrations (0.3, 1.5 and 7.5 μg/ml) were analysed. Two sets of QC samples were analysed, one set at the beginning and the other at the end of each run. Another set of QC samples was analysed between the calibration standards and one of the subject samples, whilst the last set was analysed immediately prior to the analysis of the second subject's samples.

The acceptance criteria for the calibration plots of each analytical batch was that the LLOQ should be 80–120% of the nominal concentration and at least five other non-zero calibration standards, including the highest concentration which should be 85–115% of the nominal concentration. Similarly, the latter also applied to at least eight of the twelve QC samples. However, not more than two of the QC samples which did not meet this criterion could be at the same concentration. Upon acceptance of the calibration plot and QC samples for each analytical run, ATV concentrations of the subject samples were determined, and a mean ATV concentration-time profile, as well as individual ATV concentration-time profiles for each subject, was constructed.

Subject samples were re-assayed only if aberrant concentration values for ATV were obtained that were possibly attributable to analytical or PK deviances.

8.2.2.5 Non-compartmental Analysis

The PK parameters of ATV before and after co-administration with SF were determined by non-compartmental analyses. Exposure measures, $AUC_{0\text{-}24}$ and C_{max} were the primary PK parameters used to evaluate whether multiple dosing of SF alters the single-dose PK of ATV. Other parameters which were monitored include $t_{½}$, t_{max}, and k_{el}. These analyses were all conducted using the SAS® software (SAS Institute Inc, Cary, North Carolina, USA).

8.2.2.6 Statistical Analysis

Equiv Test™ (Statcon, Witzenhausen, Germany) was used to construct 90% CIs about the equivalence parameter, geometric mean ratio (difference of means on natural log scale) for $AUC_{0\text{-}24hr}$ and C_{max}. The ratio was computed as Phase II/Phase I *viz* ATV + SF/ATV alone. A clinically significant interaction was concluded if the 90% CIs for $AUC_{0\text{-}24hr}$ and C_{max} were found to be outside the limits of 0.8–1.25.

If carry-over of ATV was detected in the plasma of pre-dose samples collected during Phase II, the data from that subject were only included in the statistical analysis if they represented less than 5% of the C_{max} value [624].

8.2.3 Results and Discussion

The study was completed without any major protocol deviations. There were no subject dropouts and no adverse events were reported. The bioanalytical summary record in Annexure II shows that all six analytical runs were valid, based on the acceptance criteria for the calibration standards (see Table A4) and QC samples (see Table A5) described in section 8.2.2.4.

Plasma concentrations (Table A6), PK parameters determined by non-compartmental analysis (Table A7) and concentration-time profiles (Figure A1) for both phases of each subject are shown in Annexure III. From the mean PK parameters (Table 8.4) presented as mean ATV plasma concentration-time profiles (Figure 8.1) for Phase I (ATV alone) and Phase II (ATV + SF), it is evident that the two profiles were superimposable for the first 2 hours post-dose. From 2 to 4 hours post-dose, the rate and extent of ATV absorption appears to be reduced when ATV was co-administered with SF in comparison to ATV administered alone. From 4 to 24 hours post-dose, a similar rate of elimination was observed between the two phases.

The statistical analysis (Table 8.4) revealed that for both C_{max} and $AUC_{0\text{-}24\ hours}$, the geometric mean ratio (point estimate) and the lower limit of the 90% CI fell well below the lower limit of the "no-effect" boundary of 0.8–1.25, therefore the results of this *in vivo* study suggest that a two-week regimen of Sutherlandia su1™ tablets significantly reduced the bioavailability of a single-dose of ATV in a cohort of 12 healthy male subjects. This alludes to enhanced P-gp transport and/or CYP3A4/5 metabolism of ATV elicited by SF as the potential mechanism of the observed effect, which may in turn be facilitated by an elevation in the activities and/or the expression of P-gp and CYP3A4/5 in the small intestines and/or livers of some of the subjects. Individuals in whom the activity of (S) metabolising enzymes are low (shown by higher C_{max} and AUC of (S) compared to others, when (S) administered alone) are more susceptible to increased expression (induction) of the enzyme [611]. It is likely that the subjects in this study, in whom this phenomenon is manifested, are CYP3A5 non-expressors who are only reliant on CYP3A4 for metabolism. The *in vivo* inhibitory effects of SF on ATV metabolism, is more likely to manifest in subjects with high activity of (S) metabolising enzymes [611], in this case, likely the CYP3A5 expressors. This effect may be masked in the results of this study, since it may be that the cohort of subjects used in the study had more CYP3A5 non-expressors than CYP3A5 expressors. Further investigations into the underlying pharmacogenetics of this potential ARV-ATM interaction may thus be useful for determining which individuals may be particularly susceptible to clinically significant changes in the bioavailability of ATV (whether lower or higher) when co-administered with SF.

It is well known that the PK of drugs metabolised by the enzymes which exhibit polymorphism, such as ATV, is confounded by large inter-individual variability [611]. At steady state, the variability of the $AUC_{0\text{-}t}$ and C_{max} of ATV was relatively variable in 14 healthy subjects with values of 28.2 and 32.3% respectively (calculated from data in Chapter 2, Table 2.2). The single-dose (as opposed to steady state) CV% for C_{max} of ATV in 12 healthy subjects in this study was comparable at 30.3%, whilst that of the $AUC_{0\text{-}t}$ was considerably higher at 50.3%. However, in a study of 32 healthy subjects to whom, as in the present study, a single-dose of ATV was administered after a light meal, the $AUC_{0\text{-}t}$ was more comparable at 37% (see Chapter 2, section 2.7.3.1). C_{max} is a continuous variable; however, in a clinical study, blood samples are drawn and plasma concentrations determined at discrete time points. It is thus expected that C_{max} would exhibit a higher variability than AUC. On the other hand, AUC is dependent on drug clearance, and may thus be particularly

susceptible to large inter-individual variability, in comparison to C_{max}, if the drug is metabolised by enzymes which demonstrate polymorphism. This may partly explain the anomaly of a higher variability for the AUC than the C_{max} data in the present study, where ATV is metabolised by CYP3A5, which exhibits polymorphism. The cohort of subjects used in the present study may have exhibited greater heterogeneity with respect to such genetic polymorphisms than those in the studies mentioned above, which may at least in part, account for differences in AUC_{0-t} variability between the two studies. Moreover, multiple-dose studies have been documented to reduce variability in comparison to single-dose studies [625], which may further explain the higher CV % for AUC_{0-t} observed in this study in comparison to the one study mentioned above in which multiple dosing of ATV was undertaken.

It is not known whether the reduction in bioavailability of ATV by SF was due to altered activity or altered expression of the relevant transporter (P-gp) and CYP450 isoenzymes (CYP3A4/5). Protein expression via induction may only occur after chronic rather than acute administration of a xeniobiotic (See Chapter 1, section 1.3.7.8.1), whilst modulation of the activity of the transporters and enzymes may manifest even after acute administration of the potential interacting agent. A study in which a single dose/single dose regimen combination of ATV and SF is used may therefore determine the degree to which altered enzyme or transporter activity may have contributed, if at all, to the change in oral bioavailability of ATV observed in the present study, where SF was dosed chronically. Interestingly, as previously mentioned (see Chapter 1, section 1.3.7.9), in rats, a single dose of SF (12 mg/kg) had no significant effect on the PK parameters of a single dose of nevirapine, which may indicate that, *in vivo*, altered enzyme or transporter activity by SF may not have a major role to play in reducing the bioavailability of this NNRTI. However, ATV is a substrate of both P-gp and CYP3A4 (see Chapter 1, Table 1.6), whereas, nevirapine is only a substrate of CYP3A4. This indicates that modulation of the bioavailability of ATV by SF through altered transporter or enzyme activity may occur through both P-gp and CYP3A4 and may, therefore, not necessarily exhibit the same effect as observed for nevirapine. Moreover, as previously mentioned, inter-species differences prevent reliable correlations regarding *in vivo* drug-drug interactions from being drawn.

As previously mentioned, from 4 to 24 hours post-dose, a similar rate of elimination was observed between the two phases. This may indicate that SF did not alter post-absorption metabolism or transport pathways of ATV which ocurrs as part of the elimination process in the liver and that the observed decrease in bioavailability of ATV was more likely due to a reduction in the transport and metabolism of ATV during the absorption process in the small intestine. This may imply that the increase in activity and/or expression of P-gp and/or CYP3A4/5 in the small intestines was greater than that in the livers of susceptible subjects. To exert an effect in the liver, the "active" phytochemical constituents of SF must be absorbed across the intestinal epithelium into the systemic circulation. The absorption of at least one of the SF constituents, namely Sutherlandioside B, may be impeded since *in vitro* studies demonstrated that it is subject to P-gp-mediated efflux in Caco-2 cells (see Chapter 3, section 3.8.3.1). Similarly, the absorption of other SF constituents may also be hindered; therefore the concentrations of these which reach the liver may not be sufficiently high to exert the effect.

The clinical relevance of the potential interaction between SF and ATV is difficult to predict since only a single dose of ATV was evaluated, yet clinically, ATV is dosed chronically. It is therefore not known how SF may affect the steady state PK of ATV and thus whether subtherapeutic levels of ATV may result. The bioavailability of ATV is reduced in HIV-patients in comparison to healthy subjects (see Chapter 2, section 2.7.3); therefore the effect may be more pronounced and severe in these patients. On the other hand, the effects of SF may also be diminished if ritonavir, a CYP3A4 inhibitor, is co-administered with ATV as a booster in ARV-experienced patients (see Chapter 2, section 2.7.1). Moreover, other covariates such as co-morbidities and co-medication, which also influence enzyme and/or transporter activity or expression (see Chapter 1, section 1.3.7.10), were not considered.

Interestingly, a significant decrease in the steady state C_{max} and AUC of other PIs, indinavir and saquinavir, has been demonstrated when these were co-administered with the CAMs, St John's Wort (indinavir) ,vitamin C (indinavir) and garlic (saquinavir) to ≤10 healthy subjects, also in one-sequence crossover studies (see Chapter 1, Table 1.8). These studies in the literature, together with the findings of this study highlight the potential susceptibility of the PIs to PK interactions which result in reduced bioavailability. The need for health care providers to be aware of the CAMs and/or TCAMs which patients may be using is therefore

emphasised so that (i) preclinical and clinical data on the potential for PK interactions between specific PIs and CAMs or TCAMs known to be used concomitantly may be generated and (ii) so that patients may be advised accordingly to prevent clinically proven interactions between CAMs or TCAMs and the PIs from occurring.

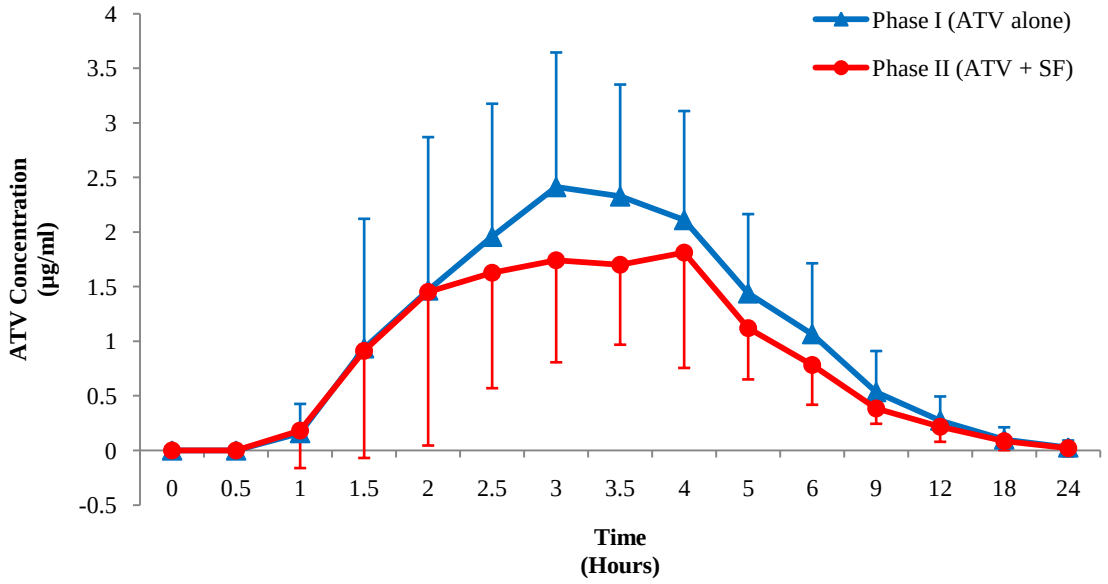


Figure 8.1: Comparison of ATV plasma concentration-time profiles for Phase I (ATV alone) and Phase II (ATV + SF). Each point represents the mean ± SD; n = 12.

Table 8.4: Non-compartmental and statistical analysis of PK parameters of ATV

Pharmacokinetic Parameter	Phase I (ATV alone) Mean [CV %]	Phase II (ATV + SF) Mean [CV %]	Phase II/Phase I Geometric Mean Ratio [90% CI]
AUC_{0-24} (µg/ml.hour)	13.0 [50.3]	10.0 [38.6]	0.801 [0.634–1.01]
C_{max} (µg/ml)	3.17 [30.3]	2.59 [43.6]	0.783 [0.609–1.00]
T_{max} (hours)	2.71 [26.7]	2.67 [33.3]	
$T_{½}$ (hours)	3.77 [39.5]	3.82 [35.1]	
K_{el} ($hour^{-1}$)	0.205 [31.2]	0.200 [30.9]	

8.2.4 Conclusions

The *in vitro* studies conducted in this thesis have alluded to the potential for traditional aqueous decoctions as well as commercial preparations of SF to possibly affect the absorption and/or metabolism and PK in general of ATV by modulation of the functional activity of P-gp and CYP3A4 in the human small intestine and liver. Other researchers demonstrated the potential for SF extracts to activate PXR *in vitro* and to increase mRNA expression of CYP3A4 in both the liver and small intestine in rats *in vivo.* It was therefore considered plausible that SF may alter the bioavailability of ATV. An ATV single dose/ SF multiple dose, one sequence crossover drug-drug interaction study in healthy male subjects was therefore conducted.

The statistical analysis showed that the 90% CIs around the geometric mean ratios for both C_{max} and $AUC_{0\text{-}24\ hours}$, fell well below the lower limit of the "no-effect" boundary of 0.8–1.25, indicating that a two-week regimen of Sutherlandia su1™ tablets significantly reduced the bioavailability of a single-dose of ATV in a cohort of 12 healthy male subjects.

CYP3A5 non-expressors rely solely on CYP3A4 for ATV metabolism and therefore exhibit lower ATV metabolism than CYP3A5 expressors. These individuals may therefore be more susceptible to interactions with SF mediated through CYP3A4. Further investigations into the underlying pharmacogenetics of this potential ARV-ATM interaction may thus be useful for determining which individuals may be particularly susceptible to clinically significant changes in the bioavailability of ATV (whether lower or higher) when co-administered with SF.

From 4 to 24 hours post-dose, a similar rate of elimination was observed between the two phases, which may indicate that SF did not alter post-absorption metabolism or transport pathways of ATV, which may in turn suggest that the increase in activity and/or expression of P-gp and/or CYP3A4/5 in the small intestines was greater than in the livers of susceptible subjects.

The clinical relevance of the potential interaction between SF and ATV is difficult to predict since only a single dose of ATV was evaluated, yet clinically, ATV is dosed chronically.

Moreover, several other covariates such as comorbidities and comedication were not considered.

Other CAM- or TCAM-PI interaction studies, as well as this study underscore the potential susceptibility of the PIs to PK interactions which result in reduced bioavailability. The necessity for health care professionals to be aware of the concomitant intake of specific PIs and CAMs or TCAMs was also emphasised in order for potential interactions to be identified or known interactions to be avoided.

CHAPTER 9
CONCLUSIONS

Numerous reports and publications have provided evidence which suggests that ATMs, such as SF, have the potential to interact with the PI class of ARVs and affect the bioavailability of the PIs, thereby impacting on their safety and efficacy. This research therefore focussed on determining whether such an interaction may occur between SF and ATV, a PI recently registered in South Africa, where the concomitant use of ARVs and ATMs is a common phenomenon. A systematic approach based on the drug interaction screening of new chemical entities was used, whereby *in silico* and *in vitro* screening tools were first applied to justify the need to conduct a more expensive, yet more clinically relevant *in vivo* study in humans.

Prior to investigating a potential interaction between ATV and SF *in vitro* and *in vivo*, it was necessary to develop an HPLC-UV method for the bioanalysis of ATV in human plasma and liver microsomes. A simple "dilute and shoot" approach was effected for the analysis of microsome samples, whilst a liquid-liquid extraction procedure, adapted from a previously published paper was used for the plasma samples to improve efficiency by minimising the volume of solvents used. In both matrices, the use of a narrow bore column with a low flow rate of 0.3 ml/min facilitated the use of reduced volumes of solvents, whilst resulting in a short run time of 10 minutes. A buffered mobile phase, *viz* acetonitrile-buffer (10 mM, pH 3) (45: 55, v/v), as opposed to a simpler mobile phase of acetonitrile-water (50: 50, v/v) was required to eliminate co-eluting interferents. The methods were validated according to the US FDA guidelines for bioanalytical methods to ensure that they complied with requisite reproducibility, accuracy, recovery and linear response. Plasma samples were also found to have adequate stability to withstand the storage conditions likely to be encountered prior to analysis.

Molecular docking studies were conducted to compare the binding modes and affinities of ATV and two major SF constituents with the efflux transporter, P-glycoprotein (P-gp) and the CYP450 isoenzyme, CYP3A4 to determine the potential for these phytochemicals to modulate the binding of ATV to these two proteins, which are mediators of absorption and metabolism. The findings of *in silico* molecular docking studies indicated that Sutherlandioside B and Sutherlandin C were not likely to competitively inhibit ATV binding

with P-gp. Activation of ATV P-gp transport by Sutherlandioside B or Sutherlandin C via the verapamil binding site was found not to be likely, although such modulation at other sites could not be ruled out. Sutherlandin C exhibited similar binding modes to the known non-competitive inhibitors, QZ59-RRR or QZ59-SSS, but had a relatively poor binding affinity for P-gp, which may reduce the efficiency of any P-gp inhibitory potential that the flavonol glycoside may possess. Analogous to activation, non-competitive inhibition by Sutherlandioside B or Sutherlandin C at other binding sites could not be precluded. In contrast, the evidence for modulation of CYP3A4-mediated metabolism of ATV was more compelling. Sutherlandioside B and Sutherlandin C shared similar binding modes with conformations of ATV which were appropriately positioned to allow for CYP3A4 metabolism of ATV to three of five potential ATV metabolites. However, the binding affinities of both phytochemical constituents were lower than the binding affinities of all three of these conformations of ATV, therefore, only weak competitive inhibition, if any, of ATV by the SF constituents, particularly Sutherlandioside B was likely to occur. Interaction with amino acid residues involved in the co-operative function of CYP3A4 alluded to the potential ability of Sutherlandioside B and Sutherlandin C to be effectors of the activation or non-competitive inhibition of the CYP3A4-mediated metabolism of ATV. In summary therefore, the SF constituents, Sutherlandioside B and Sutherlandin C, may have the potential to modulate P-gp-mediated ATV absorption and/or CYP3A4-mediated ATV metabolism.

A qualitative HPLC-UV analysis was used to confirm the presence of triterpenoid glycosides and flavonol glycosides in the aqueous and methanolic extracts of SF using crude triterpenoid and flavonol glycoside fractions from SF as indicators. The flavonol glycosides were present in relativly high quantities in the aqueous extract, but comparatively lower in the methanolic extract. The triterpenoid glycosides were present in low quantities in both extracts, although lower in the aqueous than in the methanolic medium. This analysis also revealed that the triterpenoid and flavonol glycoside fractions contained unknown compounds in addition to the abundant triterpenoid and flavonol glycosides. These extracts and crude fractions were used in many of the *in vitro* experiments which were conducted, therefore this analysis allowed for postulations to be drawn on the possible phytochemical constituents in SF extracts which may contribute to any effects observed in these studies.

In Caco-2 cells, an *in vitro* model of human intestinal absorption, the aqueous extract of SF and D-pinitol significantly reduced ATV accumulation, suggesting decreased ATV absorption, whilst the triterpenoid glycoside fraction increased ATV accumulation, implying the opposite. Oral administration of traditional preparations (i.e: aqueous decoctions) of SF may thus reduce the bioavailability of ATV, whilst the impact following the administration of commercial dosage forms of SF on ATV absorption and thus bioavailability is dependent on whether the effect by constituents present in the aqueous extract or those present in the triterpenoid glycoside fraction prevails. The call for an *in vivo* drug-drug interaction study to unravel the true potential for SF to alter the bioavailability of ATV was therefore justified.

MDCKII cells which overexpress the human efflux transporter P-gp were used to determine whether the effects of the SF extracts and components on ATV accumulation in Caco-2 cells were P-gp-mediated. In order to evaluate human P-gp modulation independently of canine transporters present in the MDCKII cells, the results of experiments undertaken in MDCKII-MDR1 and MDCKII-WT cells were quantitatively compared. The SF methanolic and aqueous extracts, as well as the flavonol glycoside fraction significantly reduced ATV accumulation in MDCKII-MDR1, which signified that P-gp-mediated ATV transport was enhanced probably by positive co-operativity. Conversely, the triterpenoid glycoside fraction increased ATV accumulation, which alluded to competitive or non-competitive inhibition of P-gp-mediated ATV transport. Therefore, although the methanolic extract, aqueous extract and flavonol glycoside fraction of SF enhanced P-gp-mediated transport, only the aqueous extract exhibited the same effect in the Caco-2 cells, suggesting that the effect of the latter was P-gp-mediated. A correlation was likewise demonstrated between the Caco-2 and MDCKII-MDR1 cells for the results of the triterpenoid glycoside fraction. The observed effects of the methanolic extract and flavonol glycoside fraction in the MDCKII-MDR1 cells may have been offset by an opposing effect, such as inhibition of another ATV efflux transporter, *viz* MRP2, in the Caco-2 cells, where no net change in ATV accumulation was observed. Modulation of the P-gp-mediated transport of ATV by constituents of the methanolic extract and flavonol glycoside fraction may thus be of no consequence in human intestinal absorption.

Human liver microsomes were used as an *in vitro* model of human hepatic metabolism. The aqueous and methanolic extracts inhibited ATV metabolism, whilst the triterpenoid glycoside

fraction had a converse effect. These findings suggested that oral administration of traditional preparations of SF in the form of an aqueous decoction may have the potential to inhibit ATV metabolism and thereby increase the bioavailability of ATV, whilst commercial dosage forms of SF such as Sutherlandia Su1™ tablets (Phyto Nova) may exhibit either inhibition or elevation of ATV metabolism depending on which effect of the "active constituents" predominates. The need for an *in vivo* drug-drug interaction study to determine the true potential for SF to alter the bioavailability of ATV was thus once again highlighted.

To determine whether SF extracts and individual or combinations of sutherlandiosides and sutherlandins influence CYP3A4-mediated metabolism of ATV, experiments were conducted in Control and CYP3A4-transfected insect cell Supersomes™. A qualitative HPLC-UV analysis was first initiated to confirm the presence of the sutherlandiosides and sutherlandins in the triterpenoid and flavonol glycoside fractions respectively to draw further conclusions on the presence of these specific compounds in the aqueous and methanolic extracts of SF. The findings for the SF extracts, combinations of Sutherlandins A and B and Sutherlandins C and D correlated to those of the SF extracts and the flavonol glycoside fraction in the human liver microsomes, implying that where statistically significant differences were observed (aqueous and methanolic extracts), these were CYP3A4-mediated. It was thus concluded that CYP3A5 non-expressors, that rely entirely on CYP3A4 for ATV metabolism, may be more susceptible to changes in ATV bioavailability by SF, than CYP3A5 expressors, if the effect is observed *in vivo*. Sutherlandiosides A and B showed no effect, whilst CYP3A4-mediated inhibition of ATV metabolism was exhibited by a combination of Sutherlandioside C and D, which may have contributed to the effect observed in the aqueous and methanolic extracts, which were shown to likely contain these compounds, although in low quantities. This was contrary to the finding of activation of ATV metabolism by the triterpenoid glycoside fraction which contained the sutherlandiosides, in higher quantities, in the experiment conducted in the human liver microsomes. The discrepancy was attributed to potential concentration-dependent differences in effector responses often observed in CYP3A4. Another postulation was that enhanced CYP3A4 activity was possibly induced by the additional unknown compounds contained in the triterpenoid fraction, which may or may not have included triterpenoid glycosides, other than the four known sutherlandiosides. This effect may have predominated, such that any potential inhibitory effect of Sutherlandioside C and/or D may have been masked.

In light of the findings of the *in vitro* experiments which formed part of this research, as well as those of others in the literature, it was considered plausible that SF may alter the PK of ATV *in vivo*. An ATV single dose/ SF multiple dose, one sequence crossover drug-drug interaction study in healthy male volunteers was therefore conducted. The statistical analysis showed that the 90 % CIs around the geometric mean ratios (ATV + SF/ATV alone) for both C_{max} and $AUC_{0\text{-}24\ hours}$, fell below the lower limit of the "no-effect" boundary of 0.8 – 1.25, implying that a two-week regimen of Sutherlandia su1™ tablets significantly reduced the bioavailability of a single-dose of ATV in a cohort of 12 healthy male subjects. Since the rate of elimination between Phase I (ATV alone) and Phase II (ATV + SF) were similar, it was suggested that post-absorption transport and metabolism of ATV, which ocurrs as part of the elimination process in the liver, was not significantly affected by SF and that the observed decrease in bioavailability of ATV was more likely due to a reduction in the transport and metabolism of ATV as part of absorption process in the small intestine.

It may thus be concluded that this research has provided compelling evidence that SF has the potential to reduce the bioavailability of ATV in HIV/AIDs patients who practise concomitant intake of this ATM and PI, thereby compromising the efficacy of the latter, which may lead to drug resistance and/or treatment failure. These findings together with that of other CAM- or TCAM-PI interaction studies emphasise the susceptibility of the PIs to PK interactions with ATMs, CAMs and TCAMs.

ANNEXURE I
RESEARCH PROTOCOL

A SINGLE DOSE/MULTIPLE DOSE, TWO PHASE, ONE-SEQUENCE CROSSOVER DRUG INTERACTION STUDY OF THE EFFECT OF *Sutherlandia frutescens* ON THE PHARMACOKINETICS OF ATAZANAVIR IN HEALTHY MALE SUBJECTS

STUDY NUMBER: ACM_01_2009
PROTOCOL: FINAL VERSION
DECEMBER 2009

Reference Product	**Reyataz ® 200 mg capsules** (Atazanavir sulphate equivalent to atazanavir base 200 mg) Bristol Myers Squibb South Africa
Co-Administered Product	**Sutherlandia SU1™** (Sutherlandia frutescens 300 mg) tablets Phyto Nova Cape Town, South Africa
Site	**Faculty of Pharmacy** **Rhodes University** **Grahamstown** **6140** **South Africa**

FINAL APPROVAL OF PROTOCOL

Study Number	**ACM_01_2009**
Study Title	**A single dose/multiple dose, two phase, one-sequence crossover drug interaction study of the effect of *Sutherlandia frutescens* on the pharmacokinetics of atazanavir in healthy male volunteers.**
Reference Product	**Reyataz ® 200 mg capsules** (Atazanavir sulphate equivalent to atazanavir base 200mg) Bristol Myers Squibb South Africa
Co-Administered Product	**Sutherlandia SU1™** Sutherlandia frutescens 300 mg tablets Phyto Nova Cape Town, South Africa

……. **……**
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1 BACKGROUND INFORMATION

1.1 HIV/AIDS

The World Health Organisation (WHO) estimates that there were 33 million people worldwide infected with the Human Immunodeficiency virus (HIV), in 2007[1]. The report concluded that although the epidemic has stabilised, the prevalence and mortality rates are still unacceptably high[1]. This indicates that the targets of campaigns for the prevention and management of the disease have not yet been achieved, especially in Southern Africa where 67% of the world's HIV infected population live[1].

1.2 MANAGEMENT OF HIV/AIDS

Since 1996, highly active antiretroviral therapy (HAART) has been the cornerstone of the management of HIV/AIDS (acquired immunodeficiency syndrome)[2]. Clinical trials have shown that, even though the virus cannot be totally eradicated, lifelong HAART drastically decreases the mortality rate of the disease [2]. Prior to 2004, most people infected with HIV in South Africa could not afford the antiretrovirals (ARVs) which form the backbone of HAART[3]. In response to this crisis, the South African National Department of Health and South African Development Community encouraged the use of African traditional medicines (ATMs)[4], which are more accessible. These include *Hypoxis hemerocallidea* and *Sutherlandia frutescens*, amongst others, used to boost the immune system and thereby assist in prolonging the progression of HIV to AIDS[4]. However, the clinical safety and efficacy of these ATMs are largely unstudied and therefore, unknown. The fact that marketing and sales of such products are generally unregulated and the lack of control by the South African Medicines Control Council (MCC) have made it easy for the commercial exploitation of conventional dosage forms of ATMs, based on invalid medical claims. In addition, great advantage has been taken of the use of intellectual property of indigenous people who have traditionally been using such medicinal plants for various ailments over the years.

The rollout of HAART by public sector health institutions in 2004 and the production of generic ARVs have increased access to HAART for impoverished patients in South Africa[3 5]. However, HIV/AIDS patients are known to take a wide range of herbs in addition to their conventional medicines (ARVs) [6 7 8], either because they still consult their traditional healers or because they have been convinced by the hype created by the marketing campaigns of the sellers of commercialised herbal products. Therefore, despite access to ARVs, HIV/AIDS patients in South Africa may still be using ATMs particularly to treat HIV/AIDS-related problems.

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1.3 ATM-ARV INTERACTIONS

Many ARV medications, such as the HIV protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) are predominantly metabolised through the cytochrome P450 3A4 (CYP3A4) oxidative metabolic pathway[9]. Furthermore, PIs are also manipulated by efflux transporters, such as P-glycoprotein (P-gp)[10]. The metabolism facilitates elimination of xenobiotics[9], whilst P-gp mediates the transport of drugs from the enterocytes into the intestinal lumen, thereby impeding absorption[10]. Both these processes have the potential to limit bioavailability of orally administered drugs[11]. Herbal medications can significantly affect plasma levels of ARV medications through inhibition or induction of CYP3A4 metabolism and drug efflux in the liver and small intestine [9]. Inhibition increases absorption and reduces metabolism, thereby enhancing plasma levels. This in turn may potentiate the adverse effects of the ARVs, possibly leading to toxicity. Induction occurs if a xenobiotic has the ability to activate the orphan nuclear receptor, pregnane X receptor (PXR), a transcription factor which regulates the transcription of various genes, including those of CYP450 enzymes and P-gp[9]. Through the generation of more CYP3A4 and P-gp, metabolism and drug efflux is enhanced, decreasing plasma levels. If sub-therapeutic levels result, resistance to the ARV may develop, and ultimately the patient will have to use an alternative HAART regimen to prevent progression of the disease.

1.4 THE POTENTIAL FOR *S. frutescens* TO CAUSE PHARMACOKINETIC INTERACTIONS

The flowering shrub, *S. frutescens*, a member of the Fabaceae family, is a popular plant used as an ATM. It is indigenous to Southern Africa, where it has enjoyed a long history of use[12]. Lately, the plant has drawn much attention due to anecdotal reports of HIV/AIDS patients who use it, which purport a significant improvement in quality of life. This is thought to be as a result of reduced wasting of muscle (cachexia), which is associated with the syndrome[13].

Preliminary studies have identified the potential for *S. frutescens* to cause early inhibition of ARV drug metabolism and efflux transport followed by induction or decreased drug exposure with more prolonged therapy[14 15]. These findings showed significant inhibition of CYP3A4 activity at an initial concentration of 100 mg/ml using extracts of *S. frutescens* from bulk ground botanical and capsulated material, with almost complete inhibition seen with the methanol extract. Analysis of adenosine triphosphate activity indicated that *S. frutescens* showed moderate activity of the efflux transporter, P-gp. Moreover, in a PXR assay, exposure to this herb resulted in significant activation of PXR in a dose-dependent manner ($p<0.01$). The results suggest that this African herb has the potential to substantially influence drug transport and metabolism, which raises the possibility of an interaction of *S. frutescens* and PIs, which are substrates of both CYP3A4 and P-gp. Through inhibition of CYP3A4 and P-gp, S. *frutescens* may induce toxicity of a PI, when it is taken acutely. On the other hand,

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[9] Lee LS, Andrade ASA, Flexner C. *Clinical Infectious Diseases* 2006; **43**(8):1052-1059.
[10] Kim RB, Fromm MF, Wandel C, Leake B, Wood AJJ, Roden DM, *et al. Journal of Clinical Investigation* 1998; **101**(2):289-294.
[11] Suzuki H, Sugiyama Y. *European Journal of Pharmaceutical Sciences* 2000; **12**(1):3-12.
[12] van Wyk BE, Albrecht C. *Journal of Ethnopharmacology* 2008; **119**(3):620-629.
[13] van Wyk BE. *Journal of Ethnopharmacology* 2008; **119**(3):342-355.
[14] Mills E, Foster BC, van Heeswijk R, Phillips E, Wilson K, Leonard B, *et al. Aids* 2005; **19**(1):95-97.
[15] Mills E, Cooper C, Seely D, Kanfer I. *Nutr J* 2005; **4**:19.

chronic use of the plant may lead to sub-therapeutic plasma levels by the induction of P-gp and CYP3A4, through enhanced PXR activity. This in turn, increases the risk of drug resistance and treatment failure. These results highlight the extreme caution required in introducing herbal medications into the routine care of HIV patients in any setting and underscore the need for appropriately designed pharmacokinetic studies to unveil the true drug interaction potential of *S. frutescens* with ARVs, especially the PIs.

Recently, we investigated whether methanolic and aqueous extracts as well as various components of *S. frutescens* have the ability to alter the accumulation of atazanavir (ATV), a PI recently registered for use in South Africa, in Caco-2 cells. The latter consists of human intestinal epithelium known to express several influx and efflux transporters such as P-gp and which is used as a model i*n vitro* system to study drug interactions. Only the triterpenoid fraction isolated from methanolic extracts of the plant significantly increased ATV accumulation after a 1 hr co-incubation with ATV. Traditional aqueous decoctions of *S. frutescens* are unlikely to contain substantial quantities of triterpenoids since these compounds are not water-soluble. Hence, it is unlikely that S. *frutescens* will influence ATV accumulation/efflux by enterocytes following administration of aqueous decoctions of the plant. However, this does not rule out the possibility that the dried plant material commercially incorporated into dosage forms (tablets/capsules) may contain triterpenoids at a sufficient concentration to elicit the aforementioned response. Implications of all these results should be of concern to both clinicians and regulatory authorities alike. Appropriate clinical studies are necessary to accurately determine the clinical relevance of these findings.

1.5 ATAZANAVIR

1.5.1 Introduction

ATV belongs to the HIV PI class of ARVs which have had an important role to play in lowering morbidity and mortality of HIV/AIDS[16]. ATV, which was recently registered for use in South Africa, is one of the newer PIs which have several advantages over the older ones, such as lopinavir, indinavir, ritonavir and saquinavir. The distinguishing property of ATV, which may see it rapidly replace use of the other PIs in South Africa, is that it demonstrates non-linear pharmacokinetics[16], which effectively means that it has a longer half-life. It was therefore the first PI approved for once daily administration[16]. This reduction in the frequency of dosing may drastically improve patient compliance. Furthermore, in some patients, who are HAART-naïve, the co-administration of ritonavir, also a PI, as a booster may be avoided, thus reducing the PI-related adverse effects. These include gastrointestinal problems, hyperlipidaemia and hyperbilribinaemia[16].

[16] Harrison TS, Scott LJ. *Drugs* 2005; **65**(16):2309-2336.

1.5.2 Molecular Structure

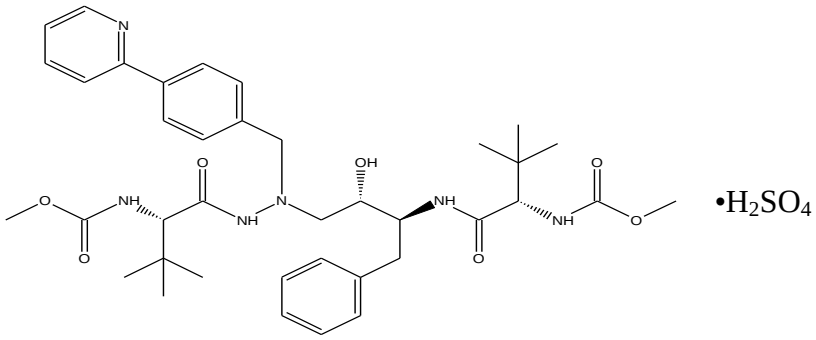

Figure A1: Atazanavir sulphate

1.5.3 Mechanism of Action

ATV is an azapeptide PI, which selectively inhibits the proteases responsible for the virus-specific processing of viral Gag and Gag-Pol polyproteins in HIV-l infected cells, thus preventing formation of mature virions[17].

1.5.4 Pharmacokinetics

1.5.4.1 Absorption[17]

Single dose data were not available in literature. The steady state peak ATV plasma concentration of ~5000 ng/ml (geometric mean) was attained after 2.5 hours (median) after the administration of 400 mg ATV to 14 HIV negative volunteers in the fed state, with an AUC of ~28000 ng·h/ml (geometric mean).

The oral bioavailability of ATV in the fasted state is low at 60-68% and the coefficient of variation of 26% and 28% for C_{max} and AUC respectively indicates relatively large inter-individual variability. However, administration of ATV with food enhances bioavailability and reduces pharmacokinetic variability. Administration of a single 400 mg dose with a light meal (357 kcal, 8.2 g fat, 10.6 g protein) resulted in a 70% increase in AUC and 57% increase in C_{max}. The coefficients of variation for both measures of systemic exposure were reduced by ~50%.

1.5.4.2 Distribution, Metabolism and Elimination

ATV is highly protein bound (86%) in serum[18]. Similar to other PIs, *in vitro* studies using human liver microsomes suggested that ATV is metabolised by CYP3A4 in the liver. The primary and secondary routes of elimination of ATV and its metabolites are biliary and urinary excretion, respectively[17]. The elimination half-life (arithmetic mean) is ~7 hours in healthy volunteers (n = 214).

1.5.5 Interactions

ATV has been shown to inhibit CYP3A4 in human liver microsomes[18] and to a lesser extent CYP1A2, CYP2C9[19], CYP2C8 and the phase II glucuronidation enzyme, uridine diphosphate

[17] Bristol-Myers Squibb Company. Reyataz® package insert. 2006.
[18] Goldsmith DR, Perry CM. Atazanavir. *Drugs* 2003; **63**(16):1679-1693.
[19] Busti AJ, Hall RG, Margolis DM. *Pharmacotherapy* 2004; **24**(12):1732-1747.

glucuronosyltransferase 1A1 (UGT1A1)[17], therefore any drugs metabolised by any of these enzymes may have increased plasma concentrations which could in turn prolong its therapeutic and/or adverse effects.

Furthermore, as previously mentioned, ATV is a substrate of CYP3A4, therefore, drugs that induce this CYP450 enzyme may decrease ATV plasma concentrations, thereby reducing its therapeutic effect.

ATV solubility decreases as pH increases, thus co-administration with agents which increase pH, such as proton-pump inhibitors, antacids, buffered medications, or H_2-receptor antagonists could result in reduced plasma concentrations of ATV[17].

Some of the ARVs that have been specifically flagged include efavirenz and nevirapine, CYP3A4 enzyme inducers, as well as tenofovir and buffered formulations of didanosine[17].

The mechanism of the interaction between ATV and tenofovir is unknown. However, it is unlikely that is related to metabolism since the latter is not a substrate, inhibitor or inducer of CYP enzymes[20].

Treatment of HIV appears to require the use of various non-ARV agents. The list of these drugs is exhaustive and continues to grow. Some examples of such agents which are known to interact pharmacokinetically with ATV include rifampicin and St. John's Wort (CYP3A4 inducers) and irinotecan (a UGT1A1 substrate)[17].

1.5.6 Dosage[17]

The recommended dosage of ATV depends on the treatment history of the patient and the use of co-administered drugs.

In therapy-naïve patients, the recommended ATV dose is 400mg once daily with food. Food is required to improve the oral bioavailability of ATV, as indicated in section 1.5.4.1. If tenofavir, efavirenz or an H_2 receptor antagonist is co-administered, 300 mg ATV with ritonavir 100 mg, as a single dose, with food is suggested. As mentioned in section 1.5.5, the above co-administered drugs reduce plasma concentrations of ATV. Ritonavir is co-administered with ATV to counter this effect as it is a potent inhibitor of CYP3A4 activity. This property of ritonavir allows for reduced metabolism of ATV, thereby raising ATV plasma levels as compared to that obtained in the absence of ritonavir.

Therapy-experienced patients, who have experienced virologic failure on at least one other PI-containing HAART regimen should receive the latter regimen (ATV with ritonavir)[17]. This is based on the results of a clinical study which showed that although ATV with ritonavir is therapeutically equivalent to lopinavir with ritonavir, ATV without ritonavir is inferior in comparison to lopinavir with ritonavir.

[20]Kearney BP, Flaherty JF, Shah J. *Clinical Pharmacokinetics* 2004; **43**(9):595-612.

1.5.7 Adverse effects

The most common adverse effects (≥2%) of ATV are nausea, jaundice/scleral icterus, rash, headache, abdominal pain, vomiting, insomnia, peripheral neurologic symptoms, dizziness, myalgia, diarrhoea, depression, and fever[17]. In general, the adverse effects usually associated with PIs are less severe with ATV. These include gastrointestinal problems, hyperglycaemia, hyperlipidaemia and hyperbilribinaemia.

Other potentially serious, although rare, adverse effects include cardiac conduction abnormalities, hepatotoxicity and nephrolithiasis (formation of kidney stones)[17].

1.5.8 Contra-indications[17]

ATV should not be administered to patients who have previously shown clinically significant hypersensitivity to this compound. It is also contraindicated when co-administered with drugs that are highly dependent on CYP3A4 or UGT1A1 for clearance, and for which elevated plasma concentrations are associated with serious and /or life-threatening events.

1.6 *Sutherlandia frutescens*

1.6.1 Common names[12]

Sutherlandia, cancer bush, balloon pea, turkey flower (English.); kankerbos, gansies, grootgansies, wildekeur(tjie), keurtjie, rooikeurtjie, kalkoen(tjie)bos, kalkoenblom, belbos, kalkoenbelletjie, klapperbos, jantjie-bêrend, bitterbos, wildekeurtjie, eendjies, hoenderbelletjie (Afrikaans.); musa-pelo, musa-pelo-oa-nōka, motlepelo (Sesotho); phetola (Setswana); insiswa, unwele (isiZulu, isiXhosa).

1.6.2 Constituents

S. frutescens is purported to contain free amino acids, the non-protein amino acid, L-canavanine, a sugar, D-pinitol, the neurotransmitter, γ-amino butyric acid (L-GABA), flavonol glycosides and triterpenoid saponins, all of which have been individually or collectively linked to various therapeutic effects[12 13]. The phytochemical content of the plant is very variable, both genetically and geographically[12]. This manifests as inconsistent quantities of some of these components[12]. For example, the reported quantity of L-canavanine ranges from 0.42-14.5 mg per g, whilst up to 14mg/g dry weight of D-pinitol has been found in the leaves[12].

1.6.3 Uses

There are a wide range of conditions for which *S. frutescens* has been traditionally used. These include, but are not limited to: fever, poor appetite, unspecified wasting, indigestion, gastritis, oesophagitis, peptic ulcer, dysentery, prevention and treatment of cancer (cancer tonic), diabetes, common colds, influenza, cough, asthma, chronic bronchitis, kidney and liver conditions, rheumatism, heart failure, urinary tract infections, and stress and anxiety[13]. As previously mentioned, S. *frutescens* has recently gained popularity due to anecdotal reports of the improvement in the quality of life of HIV/AIDS patients who have used the herb.

1.6.4 Pharmacology

Several *in vitro* and pre-clinical *in vivo* studies allude to *S. frutescens* having antibacterial[21], antioxidant[21 22], anti-inflammatory[23], anti-diabetic[24], anti-convulsant[25], anti-cancer[26] and anti-stress[27] properties. However, to date, no clinical trials have been conducted to confirm biological activity or to determine whether any effects are clinically significant.

1.6.5 Pharmacokinetics

Some of the pharmacokinetic parameters of L-canavanine have been determined in rats. The bioavailability of an oral dose (2.0 g/kg) was 43%, and only 1% was eliminated in the urine[28]. Furthermore, 21% of the administered L-canavanine remained in the gastrointestinal tract 24 hr after the oral dose[28]. However, there are no reports on the metabolism of L-canavanine nor on the pharmacokinetics, in general, of any of the other suggested components of *S. frutescens*.

1.6.6 Potential for Adverse Effects

Seier and co-workers have suggested that at daily doses up to 9X the recommended dose of 9mg/kg, *S. frutescens* leaf powder was not associated with toxic or other side effects in Vervet monkeys. The statistically significant differences found in physical, haematological, biochemical and physiological parameters, between the control group and treatment groups were not considered clinically significant[29].

S. *frutescens* leaf powder has[30] been considered safe in healthy adults at the recommended dose, based on a randomised, double-blind, placebo-controlled trial[30]. The treatment group on the recommended regimen of one capsule (400mg) twice daily for 3 months had no significant differences in general adverse effects, or physical, vital, blood, and biomarker indices when compared to the placebo group. Participants in the treatment group experienced improved appetite. Furthermore, they had a lower respiration rate and a higher platelet count, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, total protein and albumin than the placebo group. However, all these vital, haematological and biochemical endpoints remained within the normal physiological range and were therefore not considered clinically relevant. Furthermore, despite the presence of 600 µg of L-canavanine per capsule, this non-protein amino acid, which has the potential for human toxicity, was not detected in the plasma of the study participants.

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[21] Katerere DR, Eloff JN. *Phytotherapy Research* 2005; **19**(9):779-781.
[22] Fernandes AC, Cromarty AD, Albrecht C, van Rensburg CEJ. *Journal of Ethnopharmacology* 2004; **95**(1):1-5.
[23] Kundu JK, Mossanda KS, Na HK, Surh YJ. C*ancer Letters* 2005; **218**(1):21-31.
[24] Chadwick WA, Roux S, de Venter MV, Louw J, Oelofsen W. *Journal of Ethnopharmacology* 2007; **109**(1):121-127.
[25] Ojewole JAO. *Brain Research Bulletin* 2008; **75**(1):126-132.
[26] Chinkwo KA. *Journal of Ethnopharmacology* 2005; **98**(1-2):163-170.
[27] Prevoo D, Smith C, Swart P, Swart AC. *Endocrine Research* 2004; **30**(4):745-751.
[28] Thomas DA, Rosenthal GA. *Toxicology and Applied Pharmacology* 1987; **91**(3):395-405.
[29] Seier JV, Mdhuli M, Dhansay MA, Loza J, Laubscher R. A toxicity study of Sutherlandia leaf powder (*Sutherlandia microphylla*) consumption. Final report by the Medicines Control Council of South Africa 2009.
[30] Johnson Q, Syce J, Nell H, Rudeen K, Folk WR. *Plos Clinical Trials* 2007; **2**(4).

2 OBJECTIVE

The objective of this study is to assess the effect of *S. frutescens* on the pharmacokinetics of ATV by comparing the single-dose pharmacokinetics of ATV before and after administration of the recommended daily dose of *S. frutescens* for a period of two weeks.

3 STUDY DESIGN

A clinical drug-drug interaction study can be a randomised crossover, a one-sequence crossover or a parallel design with various possible dosing regimen combinations of single dose and/or multiple dose[31]. This study will have a one-sequence crossover, two phase design with a single dose/multiple dose regimen combination for ATV and *S. frutescens* respectively. The multiple dosing of *S. frutescens* allows for both induction and inhibitory effects on ATV to be assessed. Although clinically, ATV is dosed chronically, this investigation into a possible drug-drug interaction is a preliminary (pilot) study; therefore, a single dose of ATV will be used.

On Day 1 of the study (start of Phase I), all subjects will receive a single oral dose of ATV (2 X 200 mg capsules) after which blood samples will be taken at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 9, 12, 18, 24 hours post-dose. From Day 3, all subjects will take an oral regimen (one tablet twice a day) of Sutherlandia SU1™, for 12 days. To ensure uniformity of content, this dose of 9mg/kg body weight will be standardised according to flavonol glycoside and triterpenoid glycoside content. On Day 15 (start of Phase II), all subjects will receive a single oral dose of ATV (2 X 200 mg capsules) and a dose (1 X 300mg tablet) of Sutherlandia SU1™, about 30 minutes after a light meal. Blood samples will then be taken at the same time intervals and for the same time period as for Phase I.

Plasma will be harvested from the blood samples and assayed for ATV using a validated high performance liquid chromatography method with ultraviolet detection. The data will be used to determine the pharmacokinetics of ATV, on which statistical analyses will be performed to determine whether there is a clinically significant pharmacokinetic interaction between ATV and *S. frutescens*.

4 STUDY POPULATION

Healthy, non-smoking male volunteers between the ages of 18 and 55 will be used in this preliminary (pilot) study to investigate the possibility of any pharmacokinetic interactions.

4.1 Number of subjects

Based on the variability of pharmacokinetic data of ATV, to obtain a power of 80% for this study, at least 24 subjects would be required. However, being a pilot study, only 12 subjects will be enrolled. Dropouts will not be replaced. All subjects who complete the study will be included in the statistical analysis.

If needed, an add-on study of up to 12 subjects will be conducted to obtain a degree of variability of less than 30% if possible. If no further study is required, preliminary statistical analysis will be finalised and presented in the final report. If an add-on study is conducted,

[31] FDA Guidance for Indstry: In vivo drug metabolism/drug interaction studies-study design, data analysis, and recommendations for dosing and labeling 1999.

data for all evaluable subjects from both Group 1 and Group 2 will be used in the preparation of the final report. Homogeneity testing will be conducted between the two groups.

4.2 Inclusion Criteria

Subjects should be mentally competent and able to give informed consent by signing the informed consent form. They should be available for the entire study period and willing to adhere to the protocol requirements.

Subjects should be males between the ages of 18 and 55 years.
Subjects should have a body mass index (BMI) of between 19 and 30.

Subjects should be non-smokers who have not smoked for at least two months.

The medical history of the subject should be satisfactory. Any abnormality is only acceptable if the Study Physician considers it to be clinically insignificant.

The physical examination of the subject at the pre-study medical screening should be satisfactory. Any abnormality is only acceptable if the Study Physician considers it to be clinically insignificant.

The laboratory test values of the subject should be within the laboratory's stated normal range. Any abnormality is only acceptable if the Study Physician considers it to be clinically insignificant.

4.3 Exclusion Criteria

Subjects should not have any history of hypersensitivity or idiosyncratic reaction to ATV or *S. frutescens.*

Subjects should not have any history of cardiovascular dysfunction or disease.

Subjects should not have any history of liver dysfunction or disease.

Subjects should not have any history of anaemia or cytopaenia.

Subjects should not have any history of renal dysfunction or disease.

Subjects should not have any history of adrenal or pituitary insufficiency.

Subjects should not have any history of chronic asthma, bronchitis or other bronchospastic conditions.

Subjects should not have any history of epilepsy or other convulsive disorders.

Subjects should not have any history of psychiatric disorders.

Subjects should not have any history or other condition which the Study Physician regards as clinically significant (including fainting upon blood sampling).

Subjects should not have any history of drug or alcohol abuse. They should not have smoked tobacco within two months of the start of the study.

Subjects should not have received treatment with any drug known to have a well-defined potential for toxicity to one of the major organs, especially the kidneys or the liver, within three months of the start of the study.

Subjects should not have received treatment with any drug known to modify renal excretion of other drugs (e.g. probenecid) within one month of the start of the study.

Subjects should not be on a restricted or abnormal diet for longer than one week within one month of the start of the study.

Subjects should not be on maintenance therapy or on chronic medication.

Subjects should not have had a major illness considered to be clinically significant by the Study Physician within three months of the start of the study.

Subjects should not have participated in another clinical study or donated one pint or more of blood within one month of the start of the study.

Subjects should not test positive for Hepatitis B, Hepatitis C or HIV.

Subjects should not receive treatment with any prescription drug within one week of the start of the study, unless the drug is considered to be clinically insignificant by the Study Physician.

Subjects should not receive treatment with any over-the-counter drug within one week of the start of the study, unless the drug is considered to be clinically insignificant by the Study Physician.

Subjects should not consume alcohol or any other agents which have the potential to inhibit or induce cytochrome P450 enzymes and drug efflux transporters (e.g. grapefruit juice) within 96 hours of the start of the study.

Subjects' urine should not test positive for any drug of abuse at the pre-study medical screening.

4.4 Concomitant Medication and Subject Restrictions

4.4.1 Medication

No prescription medication or over-the-counter medication (cold preparations, antacids, vitamins, complementary medicines or natural products used for therapeutic benefits) will be allowed for at least one week prior to the start of the study. Any medication taken between the pre-study medical screening and the start of the study will be assessed for its effect on the study and subjects may be excluded if necessary.

With the exception of the study drugs, no medication may be taken by subjects for the duration of the study.

4.4.2 Alcohol

No alcohol may be taken by subjects from 96 hours prior to taking the ATV dose until the last sample of each phase has been taken and after Phase II until the post-study clinical investigations have been completed.

4.4.3 Caffeine

No caffeine-containing beverages and foods (e.g. tea, coffee, cola drinks, chocolate) may be taken by subjects from 48 hours prior to taking the ATV dose until the last blood sample of each phase has been drawn.

4.4.4 Exercise

No strenuous physical activity may be undertaken by subjects from 24 hours before the ATV dose in each phase until the last blood sample of Phase I has been drawn and until the post-study clinical investigations after Phase II have been completed.

4.4.5 Smoking

Subjects should be non-smokers or smokers who have refrained from smoking for at least two months prior to the start of the study. They should refrain from smoking until the last blood sample has been drawn at the end of Phase II.

4.4.6 Grapefruit

No grapefruit juice may be consumed from 48 hours before taking the ATV dose until the last blood sample has been drawn at the end of each phase.

Subjects will be informed of the above restrictions and each subject will be specifically questioned on these points prior to ATV administration. Any deviations from the restrictions which are made known to the investigators either voluntarily or on questioning will be recorded on the appropriate Case Report Form (CRF). A decision as to whether the affected subject should continue with the study will be taken by the Principal Investigator in consultation with the Study Physician/Co-Investigator.

4.4.7 Food and Fluid Intake

To minimise inter-individual variation, the food and fluid intake of the subjects will be standardised for the duration of the clinic stay in both phases.

4.4.8 Criteria for removal of a subject from the study

Any subject may withdraw from the study voluntarily for any reason. In addition, any subject may be withdrawn from the study at any time if:

A subject experiences vomiting or diarrhoea within 24 hours of taking the ATV dose in either phase of the study.

A subject suffers from an illness or injury during the study and it is considered clinically significant by the Study Physician.

A subject experiences any adverse event or signs of toxicity which are/is considered clinically significant by the Study Physician.

A subject fails to comply with any of the study requirements and it is considered clinically significant by the Study Physician.

It is determined that the subject did not follow pre-study directions regarding alcohol and drug use etc.

A post study medical examination will be conducted on any subject who withdraws from the study, as well as any follow up interventions where necessary. Reasons for a subject's withdrawal will be documented in the subject's CRF and in the final study report. Blood concentration data of a subject who withdraws due to an adverse drug event will be presented in the final report. However, the data will not be presented for a subject who withdraws voluntarily or is removed from the study due to reasons other than an adverse event.

5. STUDY PROCEDURE

5.1 Pre- and Post-Study Medical Screening

Pre- and post-study evaluations will be conducted as listed in the table below. Pre-study screening will be conducted not more than 30 days prior to the start of Phase I and post-study screening will be conducted within 48 hours after the last blood sample has been withdrawn in Phase II.

Table A1: Pre- and post-study medical screening

Screening Test	***Pre-Study***	***Post-Study***
Medical History	Yes	Yes
Physical Examination	Yes	No
Haematology	Yes	Yes
Blood Chemistry	Yes	Yes
Urinalysis	Yes	Yes
Serology	Yes	Yes
Drugs of abuse	Yes	No

5.1.1 Pre-study Medical Screening

5.1.1.1 Medical History:

Demographic data (date of birth, age, sex, race), emotional (psychiatric), CNS, ears-eyes-nose-throat, cardiovascular, respiratory, hepatic, gastrointestinal, renal, genitourinary, endocrine, metabolic, musculoskeletal, skin (dermatological), connective tissue and blood-lymphatics illnesses, allergies, blood donation, alcohol consumption, smoking habits, dietary habits and sporting commitments.

5.1.1.2 Physical

Demographic data (height, weight, BMI), vital signs (BP, pulse, oral temperature), skin, head-neck, thyroid, eyes, ears-nose-throat, chest, lungs, heart, 10-Lead ECG, neurological, musculoskeletal, abdomen, nutritional status, JACCOL (jaundice, anaemia, cyanosis, clubbing, oedema, lymphadenopathy).

5.1.1.3 Haematology

Haemoglobin, total and differential red cell count, haematocrit, total and differential white cell count, platelet count and sedimentation rate.

5.1.1.4 Blood Chemistry
Sodium, potassium, chloride, urea, urate, creatinine, cholesterol, random glucose, total protein, albumin, total and conjugated bilirubin, alkaline phosphatase, GGT (glutamyl transpeptidase), ALT (alanine transaminase) and AST (aspartate transaminase).

5.1.1.5 Urinalysis
Appearance, microscopic examination if positive for sediment, glucose, ketones, blood protein, nitrite, specific gravity, pH, leucocyte esterase, bilirubin and urobilinogen.

5.1.1.6 Serology
Hepatitis: B S-antigen and C, HIV.

5.1.1.7 Drugs-of-Abuse
Urine samples from each subject will be tested for amphetamines, barbiturates, benzodiazepines, cocaine, methamphetamine, morphine, phencyclidine, tetrahydrocannabinol (THC) and cannabinoids, tricyclic antidepressants (TCA) and ethyl alcohol using test kits.

5.1.2 Post-Study Screening

5.1.2.1 Medical History
Since start of Phase 1.

5.1.2.2 Haematology
Haemoglobin, total and differential red cell count, haematocrit, total and differential white cell count, platelet count and sedimentation rate.

5.1.2.3 Blood Chemistry
Urea, urate, alkaline phosphatase, GGT (glutamyl transpeptidase), ALT (alanine transaminase), AST (aspartate transaminase), total and conjugated bilirubin.

5.1.2.4 Urinalysis
Appearance, microscopic examination if positive for sediment, glucose, ketones, blood, protein, nitrite, specific gravity, pH, leucocyte esterase, bilirubin and urobilinogen.

5.1.2.5 Serology
HIV 6 months after end of study.

5.1.3 Post-study abnormalities
If any study related abnormalities are observed at the post-study medical screening, appropriate follow-up action will be taken and re-examination and re-testing conducted until the abnormality returns to normal or until the Principal Investigator/Study Physician considers the abnormality as clinically insignificant. Clinically significant abnormal laboratory values will be reported as an adverse event.

5.2 Rationale for HIV Screening
The subject will undergo the standard pre- and post-counselling for an HIV test which will be conducted prior to the study. Subjects who are HIV-positive will be excluded from participation. This is necessary to prevent HIV-positive persons being exposed to single doses of ATV, which could result in HIV resistance to ATV and significantly compromise the individual's future treatment.

The possibility exists that subjects enrolled into the study may have been infected with HIV at the time of screening, but were in the "window period" during which the HIV is not detected. In addition, subjects could become infected during the study. The duration of the window period varies from subject to subject and on the type and sensitivity of HIV test conducted, and may be from ten days to six months. For the ELISA HIV screening tests which will be used for this study, it is generally accepted that this period is a maximum of six months. Screening at the end of the maximum window period post-study will ensure that any subject who was infected with HIV at the time of the study will be detected, although it should be noted that an HIV-positive result at this time does not necessarily mean that the subject was infected during the study. All subjects who take ATV during the course of the study will therefore be screened for HIV six months post-study. In these cases subjects infected with HIV would be inadvertently exposed to ATV. Appropriate modification of anti-HIV treatment would then be necessary. These subjects will therefore be advised to inform their own medical practitioner that they have received two single doses of ATV as this may affect their future ARV therapy.

5.3 Clinic Check-in and Confinement

Subjects will undergo a brief medical examination, inclusion and exclusion criteria check, study restriction check, and urine screening for the specified drugs of abuse.

Subjects accepted into the study will remain in the clinic from check-in until the 24 hour blood sample has been withdrawn. Subjects will then be allowed to leave the clinic provided that there are no significant symptoms or adverse events present.

5.4 Preparation for Dosing and Dosing Instructions

On Day 1 of the study (start of Phase I), all subjects will receive a single oral dose of ATV (2 X 200 mg capsules) half an hour after a light meal, after which blood samples will be taken at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 9, 12, 18, 24 hours post-dose. From Day 3, all subjects will take an oral regimen of Sutherlandia SU1™ (1 X 300 mg tablet twice a day) for 12 days. To ensure uniformity of content, this dose will be standardised according to flavonoid glycoside and triterpenoid glycoside content. On Day 15 (start of Phase II), all subjects will receive a single oral dose of ATV (2 X 200 mg capsules) and a dose (1 X 300 mg tablet) of Sutherlandia SU1™, with a light meal. Blood samples will then be taken at the same time intervals and for the same time period as for Phase I.

Prior to each subject's scheduled dosing time an indwelling cannula will be inserted into an arm vein and a pre-dose blood sample withdrawn. Vital signs (blood pressure and pulse) will be monitored. At the scheduled dosing time for each phase, subjects will receive an oral dose (400 mg) of ATV. Each dose will be taken with 240 ml of tap water at room temperature, before the light meal. A mouth and hand check will be conducted to confirm that the dose has been ingested.

5.5 Posture and Physical Activity

Subjects will be seated for the first four hours after drug administration and will not be permitted to lie down or sleep until four hours post-dose, unless this is necessary due to an adverse event. Subjects will be required to adhere to restrictions on physical activity until the last blood sample has been withdrawn for each phase.

5.6 Blood Sampling, Processing and Storage

Blood samples will be collected for 36 hours after dosing into 10 ml syringes via an intravenous cannula then transferred directly into 10 ml Vacutainer® tubes containing K-EDTA. Subsequent samples will be collected by repeat venipuncture. Samples (10 ml) will be withdrawn at the time of cannulation (pre-dose, 0.0) then at the following time points:
0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 9, 12, 18, 24 hours after dosing.

A total of 15 samples will be collected from each subject for each phase.

The clinic clock time of the blood samples will be recorded and reported for each subject. Any deviation from the sampling time schedule will be recorded and reported for each subject.The total amount of blood collected from each subject over the entire study period, including blood samples withdrawn for pre- and post-study screening tests, will not be greater than 400 ml.

Blood samples will be stored in an ice-bath immediately after being collected until centrifugation commences. Centrifugation at 2800 rpm for 10 minutes at 4 ºC will commence within 30 minutes of the sampling time. Duplicate aliquots of harvested plasma will be stored in polypropylene tubes at -80 ± 10 ºC until transfer to the analytical facility. The tubes will be labelled with study number, phase number, subject number, sample number and sampling time.

5.7 Refreshments and Meals

Food and fluid intake will be standardised from the time subjects check in at the clinic until the 24 hour post-dose sample.

Water will be allowed *ad libitum* until 7h00 on the morning of the dose (1.5 hours before the dose). The dose (2 X 200 mg ATV capsules) will be given with 240 ml water. Water will be permitted *ad libitum* four hours post-dose. The time and content of all meals will be as indicated in the table below.

Table A2: Summary of schedule and content of meals

No. of hours pre- or post-dose	***Meal***	***Menu***
≥10 hours pre-dose	Light snack	Cake and xanthine-free soda
0.5 hours pre-dose	Balanced breakfast (light meal)	Deboned and skinned chicken breast burger, roll (no butter or margarine), tomato, lettuce, egg, flavoured water.
4 hours post-dose	Lunch	Chicken pie, rice vegetables, salad, fruit salad, custard, flavoured water.
8 hours post-dose	Snack	Cake and xanthine-free soda.

12 hours post-dose	Dinner	Pizza, salad, fruit and xanthine-free soda.
24 hours post-dose	Breakfast	Cereal, yoghurt, toast, egg and juice.

5.8 Subject Monitoring

The Study Physician will remain at the clinic from before the commencement of dosing until 4 hours after the first dose has been administered, and be contactable by phone for the duration of the study.

Blood pressure and pulse will be checked just prior to dosing, then after sampling at 4 and 8 hours post-dose. Additional readings will be taken if necessary.

Subjects will be asked open-ended questions about their health during the blood sample withdrawal.

Clinic nursing staff will ensure that subjects adhere to the restrictions regarding posture, movement and the consumption of food and beverages while at the clinic.

6 ETHICAL AND REGULATORY REQUIREMENTS

6.1 Ethical and Institutional Review

Approval by the Faculty of Pharmacy Departmental Ethics Committee at Rhodes University will be obtained before the study commences. A letter of approval and a list of committee members and their qualifications will be provided to the funding body. The original signed letter of ethical approval will be retained.

The study will be conducted in accordance with the recommendations of the guidelines as set out in the Declaration of Helsinki (1964) and its amendments of Tokyo (1975), Venice (1983), Hong Kong (1989), Somerset West (1996) and Edinburgh (2000), according to International Conference on Harmonisation (ICH) Good Clinical Practice (GCP) guidelines and in compliance with the Biopharmaceutics Research Institute's (BRI) Standard Operating Procedures (SOPs) and South African regulatory authority requirements.

6.2 Written Informed Consent

Preceding the study, the nature, purpose and risk of participating in the study will be explained to all volunteers. If volunteers desire, they will be given time to consider the information and any questions that they might have will be answered. The nature of the insurance cover will also be explained. They will also be informed that they may withdraw from the study at any time without penalty to themselves (other than a reduced remuneration) but that they must be committed to completing the study prior to their enrolment. Subjects will receive written, detailed instructions concerning the study performance and restrictions.

6.3 Case Report Forms

All CRFs will be quality assured and all major events such as final acceptance of a subject, adverse events and final release from the study will be signed by the Principal Investigator and a copy included in the final report if requested.

6.4 Quality Assurance

Designated personnel are responsible for maintaining quality assurance and quality control systems with written SOPs to ensure that the study is conducted and that data are generated, documented and reported in compliance with the study protocol, GCP and applicable regulatory requirements.

6.5 Record Retention

All source documents, study reports and other study documentation will be archived and retained for a period of 10 years after the completion of the study or for a longer period if required.

6.6 Insurance

Adequate insurance cover will be provided. Insurance is in accordance with The Association of British Pharmaceutical Industry (ABPI) guidelines and documentation of the policy will be made available for scrutiny by the funder and volunteers at their request. The funder will not be liable in the event of negligence on the part of study subjects, investigators, employees and personnel. Insurance compensation is only payable in cases where a physical disability arises as a direct result of participation in the study. Compensation is determined by the nature and severity of the physical disability as assessed by qualified medical practitioners and according to a standardised scale. A maximum of R500 000 can be paid to a subject. Medical expenses up to R100 000 which result directly from participation in the study are covered by insurance.

6.7 Termination of the Study

The study may be terminated if considered to be in the interests of subject welfare following consultation with the Principal Investigator/Supervisor/Study Physician. The Principal Investigator/Supervisor/Study Physician may terminate the study at any time for scientific or corporate reasons. If the study is prematurely terminated or suspended for any reason, the Principal Investigator will promptly inform the subjects, take appropriate steps as deemed necessary under the circumstances to assure the subjects and where applicable follow up with therapy and inform the regulatory authorities.

6.8 Adherence to Protocol

Excluding an emergency situation in which proper treatment is required for the protection, safety and well-being of the study subjects, the study will be conducted as described in the approved protocol and performed according to ICH and GCP guidelines. Any deviation from the protocol will be recorded and explained.

Should amendments to the protocol be required, the amendments will be documented and signed by the Principal Investigator. If a protocol amendment has an impact on the safety of subjects, such as a change in dosing regimen or additional blood withdrawals, the amendment will be resubmitted to the Institutional Review Board for approval.

6.9 Blinding

The study will be an open-label study and will not be blinded in any way.

6.10 Drug Accountability

Sufficient quantities of the reference and co-administered products will be provided for the study. The quantity as well as any batch numbers and expiry dates of both products received

will be recorded in the appropriate register. Dispensing and administration of test and reference products will also be recorded.

Unused products will be stored for two years after the submission of the final report after which they will be disposed of appropriately.

6.11 Adverse Events/Adverse Drug Reactions:

Subjects will be questioned on their health status at check-in for each phase of the study, during the study and before leaving the clinic at the end of each phase. During the study, open-ended questions will be asked. If any adverse events (AEs) are reported, the Study Physician will monitor the AE, initiate appropriate treatment if required and decide whether or not to withdraw the subject from the study. Signs and symptoms of any adverse events which occur during the study will be fully documented in the appropriate CRF.
AE (which include illnesses, subjective and objective signs and symptoms that have appeared or worsened during the course of the study and significant shifts in laboratory values or vital signs) will be assessed by the study physician during and after each phase of the study to determine whether or not they are related to the investigational products, i.e., an adverse drug reaction (ADR) to the study procedure or other. The outcome of this assessment will be recorded in the appropriate CRF.

AEs classified as serious will be reported to the Principal Investigator and the Departmental Ethics Committee.

AEs classified as serious and unexpected will be subject to expedited reporting as detailed in the ICH E2A and E2B guidelines on Clinical Safety Data Management and Data Elements for Transmission of Individual CRFs respectively. The Principal Investigator will inform the funder of any serious or unexpected AEs occurring during the study. A serious AE initial report form and any relevant follow-up information will be sent to the funder, who in turn should forward the relevant information to the Departmental Ethics Committee and MCC. A serious AE will be reported within 24 hours, whilst unexpected AEs will be reported without undue delay.

Samples obtained from a subject who withdraws due to an AE will be assayed for ATV. The withdrawn volunteer's plasma level data will be provided in the final report but will not be included in statistical analysis. If the volunteer is withdrawn due to pharmacokinetic reasons i.e., non- or mal-absorption of the drug for reasons such as vomiting, diarrhoea or a drug dosing problem, or, if the volunteer withdraws voluntarily for personal reasons, plasma level data are not required and samples collected therefrom will not be analysed.

7. SAMPLE STORAGE AND ANALYSIS

7.1 Sample Storage

Study plasma samples will be stored at -80 ± 10 ºC immediately after harvesting. These samples will be transferred to the analytical facility in dry ice and maintained at a temperature of -10 ± 2 ºC until analysis.

7.2 Analytical Facility

Analysis of the samples will be undertaken in the laboratories of the Faculty of Pharmacy, Rhodes University.

7.3 Analytical Method

Concentrations of ATV in plasma will be measured by a validated, sensitive and selective HPLC-UV method.

Briefly, following arrival in the analytical laboratory, samples will be thawed and the analytes extracted from the plasma samples (400 µl) by liquid-liquid extraction with 800 µl of hexane:ethyl actetate (50:50) after addition of 25 µl diazepam (10 µg/ml) (internal standard) in acetonitrile:10 mM Ammonium formate/formic acid buffer at pH 3 (45:55) and 400 µl of sodium carbonate (2 M). The organic extract will be evaporated to dryness under a gentle stream of nitrogen and the residue will be re-constituted in 100 µl mobile phase, which will consist of acetonitrile:10 mM Ammonium formate/formic acid buffer at pH 3 (45:55). An aliquot (20 µl) will be injected for analysis by HPLC. Chromatographic separation will be performed on a Phenomenex® Luna 5 µm (150x 2 mm) column with isocratic elution using a mixture of acetonitrile and water (50:50). UV detection of ATV and diazepam will be carried out at 210 nm. A calibration curve (200 ng/ml to 10 000 ng/ml) for ATV will be constructed and fitted to a linear regression model. Concentrations above 10 000 ng/ml will be re-analyzed after dilution with drug-free human plasma

7.4 Quality Control Samples

Quality Control Samples at low (300 ng/ml), medium (1500 ng/ml) and high concentrations (7500 ng/ml) will be prepared and analysed with study samples for each analytical run.

7.5 Aberrant Values and Retested Samples

Unacceptable values attributable to analytical or pharmacokinetic reasons will be re-assayed. Final concentrations will be chosen according to the analytical site's SOPs. All cases of re-assay will be reported in the final report.

8. PHARMACOKINETICS AND STATISTICS

8.1 Pharmacokinetic Analysis

ATV plasma concentration vs time profiles will be constructed and as recommended[31], the exposure measures and PK parameters tabulated below will be derived therefrom:

Table A3: Exposure measures and PK parameters

Exposure measure/ Pharmacokinetic Parameter	***Definition and Calculation***
C_{max}	The maximum concentration of ATV in the plasma of a subject.
C_{last}	The last measurable concentration in the plasma of a subject.
T_{max}	The sample time at which the C_{max} was attained. If this occurs at more than one sample time, it is identified as the first sample time with that value.
T_{last}	The time at which the C_{last} is attained.
T_{lin}	The sample time at which log linear elimination begins.
AUC_{0-T}	The cumulative area under the plasma concentration vs time curve from the time zero to T_{last}.
$AUC_{0-\infty}$	The area under the extrapolated area under the plasma

	concentration vs time curve from time zero to infinity, calculated as the sum of the AUC_{0-T} and C_{last}/k_{el}.
$AUC_{0-T/0-\infty}$	The ratio of AUC_{0-T} to $AUC_{0-\infty}$ as a measure of the extent to which elimination of the drug was followed.
K_{el}	The apparent first-order terminal elimination rate constant calculated by linear regression of the terminal linear portion of a semi-logarithmic plot of plasma concentration vs time, using at least the last three measurable concentrations.
$T_{1/2el}$	Mean elimination half life.

K_{el}, $T_{1/2el}$, $AUC_{0-\infty}$, $AUC_{0-T/0-\infty}$ and T_{lin} will not be calculated for plasma concentration vs time profiles that do not exhibit a terminal log-linear phase.

8.2 Statistical Analysis

Descriptive statistical parameters namely, the arithmetic mean, minimum, maximum, standard deviation (SD) and coefficient of variation (CV %) will be calculated for the demographic variables of age, height, weight and BMI. These statistical parameters will also be calculated for the plasma concentration at each individual sample time point, as well as for the pharmacokinetic parameters in the table above.

To determine the clinical significance of any increase or decrease in systemic exposure to ATV when co-administered with *S. frutescens*, the relevant pharmacokinetic measures of systemic exposure to ATV from Phase I and Phase II need to be compared[31].

The statistical approach which will be used is Average Bioequivalence, which is based on the two one sided *t*-test procedure[32]. The principles behind these two hypothesis tests are the same; however, to ensure that the outcome of the statistics is clinically relevant, CIs are constructed, instead of using tests of significance[31]. Briefly, the C_{max}, AUC_{0-T} and $AUC_{0-\infty}$ data from each subject is ln-transformed and the ratio of Phase I to Phase II for each subject is calculated[32]. A 90% CI for the geometric mean of the ratios is constructed[31 32]. An interaction will be concluded if the CIs of all the systemic exposure measures are found to be outside the limit for bioequivalence, which is 0.8-1.25[32]. If the results for the systemic exposure measures are not in agreement, then the one which relates more closely to the clinical outcome of ATV will be considered more valid[31].

9. REPORTS

A full report on the clinical, analytical and statistical sections of the study will be prepared.

[32] Guidance for Industry: Statistical Approaches to Establishing Bioequivalence 2001.

ANNEXURE II
BIOANALYTICAL SUMMARY RECORD

Table A4: Accuracy of calibration standards for each analytical batch

Calibration Standard (µg/ml)	Accuracy (%)					
	Analytical Batch 1	Analytical Batch 2	Analytical Batch 3	Analytical Batch 4	Analytical Batch 5	Analytical Batch 6
0.1	106	99.9	98.9	104	108	101
0.2	90.6	98.7	103	101	90.4	93.4
0.4	95.9	102	105	91.2	86.3	104
0.8	96.4	106	89.5	102	96.5	100
1	99.3	95.3	106	96.0	103	100
2	105	110	99.3	101	109	103
4	107	94.5	109	115	107	103
8	104	97.0	99.8	96.2	108	102
10	102	101	95.6	101	107	95.3

Table A5: Accuracy of QC samples for each analytical batch

QC **Concentration** (µg/ml)	QC set	Accuracy (%)					
		Analytical Batch 1	Analytical Batch 2	Analytical Batch 3	Analytical Batch 4	Analytical Batch 5	Analytical Batch 6
0.3	**A**	91.0	109	89.8	101	93.2	108
	B	85.0	90.6	92.1	85.6	84.8	90.6
	C	94.6	113	101	105	113	115
	D	94.9	90.8	105	101	111	90.8
1.5	**A**	100	106	95.1	104	90.4	104
	B	104	104	97.9	87.5	89.7	100
	C	108	100	111	105	107	90.0
	D	94.9	96.3	115	105	105	104
7.5	**A**	106	89.8	99.0	102	96.7	106
	B	102	97.3	102	106	102	86.3
	C	110	115	112	110	109	97.8
	D	103	99.1	103	111	111	99.4

ANNEXURE III
NON-COMPARTMENTAL ANALYSIS

Table A6: Plasma concentrations of each subject for Phase I and Phase II

Subject Number	Phase Number *	Time (Hours)														
		0	**0.5**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**5**	**6**	**9**	**12**	**18**	**24**
		ATV plasma concentration (µg/ml)														
1	**1**	0.00	0.00	0.114	2.84	3.33	3.88	2.36	2.03	1.80	1.18	0.922	0.542	0.297	0.117	0.00
1	**2**	0.00	0.00	0.356	3.13	3.89	3.04	2.70	2.42	2.44	1.39	1.07	0.608	0.272	0.136	0.00
2	**1**	0.00	0.00	0.262	1.23	2.96	2.98	3.67	3.14	1.96	1.11	0.682	0.497	0.311	0.00	0.00
2	**2**	0.00	0.00	0.00	0.103	0.619	1.57	3.15	2.88	2.74	1.66	1.21	0.564	0.403	0.146	0.00
3	**1**	0.00	0.00	0.00	0.00	0.166	1.63	2.85	1.87	1.41	0.985	0.605	0.252	0.196	0.00	0.00
3	**2**	0.00	0.00	0.00	0.106	0.147	1.40	1.61	1.49	1.28	0.802	0.515	0.237	0.122	0.00	0.00
4	**1**	0.00	0.00	0.00	0.686	1.80	1.75	1.41	1.05	1.12	0.678	0.422	0.194	0.00	0.00	0.00
4	**2**	0.00	0.00	0.00	0.177	1.09	1.92	1.59	1.09	0.897	0.805	0.312	0.238	0.00	0.00	0.00
5	**1**	0.00	0.00	0.00	0.709	2.54	1.85	1.52	1.38	1.23	0.869	0.574	0.301	0.161	0.00	0.00
5	**2**	0.00	0.00	0.370	1.22	1.87	1.53	1.40	1.05	0.940	0.578	0.465	0.405	0.317	0.151	0.00
6	**1**	0.00	0.00	0.00	0.00	0.688	3.86	5.31	4.26	4.14	2.38	2.58	1.59	0.847	0.350	0.150
6	**2**	0.00	0.00	0.00	0.240	0.344	0.378	0.509	0.724	1.26	1.05	0.894	0.334	0.447	0.00	0.00
7	**1**	0.00	0.00	0.654	3.16	3.71	3.09	2.58	2.54	2.24	1.84	1.34	0.580	0.315	0.148	0.00
7	**2**	0.00	0.00	0.00	1.01	1.50	2.69	2.70	2.32	2.33	1.81	1.25	0.560	0.234	0.127	0.00
8	**1**	0.00	0.00	0.00	0.00	0.00	0.851	3.32	3.18	3.15	1.66	1.14	0.450	0.284	0.133	0.00
8	**2**	0.00	0.00	0.00	1.24	4.51	3.63	2.68	2.18	2.07	0.998	0.667	0.360	0.202	0.118	0.00
9	**1**	0.00	0.00	0.00	0.00	0.00	1.24	2.04	1.74	1.36	0.868	0.632	0.309	0.142	0.00	0.00
9	**2**	0.00	0.00	0.00	0.141	0.867	1.30	2.19	2.23	1.95	1.36	0.862	0.372	0.235	0.00	0.00
10	**1**	0.00	0.00	0.758	2.33	1.90	1.44	1.32	1.02	0.965	0.679	0.527	0.265	0.00	0.00	0.00
10	**2**	0.00	0.00	0.181	2.08	1.39	0.979	0.926	0.827	0.730	0.584	0.501	0.310	0.187	0.00	0.00
11	**1**	0.00	0.00	0.0913	0.254	0.350	0.453	1.12	3.54	2.94	2.20	1.72	0.744	0.385	0.247	0.181
11	**2**	0.00	0.00	0.00	0.00	0.00	0.00	0.356	2.11	4.32	1.79	1.30	0.458	0.183	0.231	0.260
12	**1**	0.00	0.00	0.00	0.00	0.129	0.451	1.47	2.18	3.03	2.83	1.65	0.694	0.351	0.118	0.00
12	**2**	0.00	0.00	1.17	1.50	1.19	1.11	1.10	1.09	0.802	0.610	0.341	0.174	0.00	0.00	0.00

* Phase I = ATV alone; Phase II = ATV + SF

Table A7: Plasma concentrations of each subject for Phase I and Phase II

Subject Number	Phase Number *	PK Parameter				
		Cmax (µg/ml)	Tmax (Hours)	$AUC_{0\text{-}24\ hour}$ (µg/ml.hour)	K_{el} ($Hour^{-1}$)	$T_{½}$ (Hours)
1	**1**	3.88	2.50	15.0	0.173	4.02
1	**2**	3.89	2.00	16.6	0.174	3.99
2	**1**	3.67	3.00	14.2	0.167	4.16
2	**2**	3.16	3.00	14.2	0.170	4.07
3	**1**	2.86	3.00	7.56	0.228	3.04
3	**2**	1.61	3.00	6.06	0.262	2.64
4	**1**	1.80	2.00	6.00	0.340	2.04
4	**2**	1.92	2.50	5.39	0.277	2.51
5	**1**	2.54	2.00	8.09	0.233	2.98
5	**2**	1.87	2.00	9.02	0.0961	7.21
6	**1**	5.31	3.00	28.8	0.155	4.49
6	**2**	1.26	4.00	8.19	0.167	4.15
7	**1**	3.71	2.00	17.7	0.179	3.88
7	**2**	2.70	3.00	14.3	0.189	3.67
8	**1**	3.32	3.00	13.0	0.142	4.05
8	**2**	4.51	2.00	13.4	0.1709	4.87
9	**1**	2.04	3.00	6.80	0.255	2.72
9	**2**	2.23	3.50	9.39	0.247	2.81
10	**1**	2.33	1.50	7.23	0.252	2.75
10	**2**	2.08	1.50	6.54	0.163	4.26
11	**1**	3.55	3.50	16.7	0.0879	7.88
11	**2**	4.32	4.00	11.3	0.203	3.42
12	**1**	3.03	4.00	14.5	0.216	3.21
12	**2**	1.50	1.50	5.73	0.308	2.25

* Phase I = ATV alone; Phase II = ATV + SF

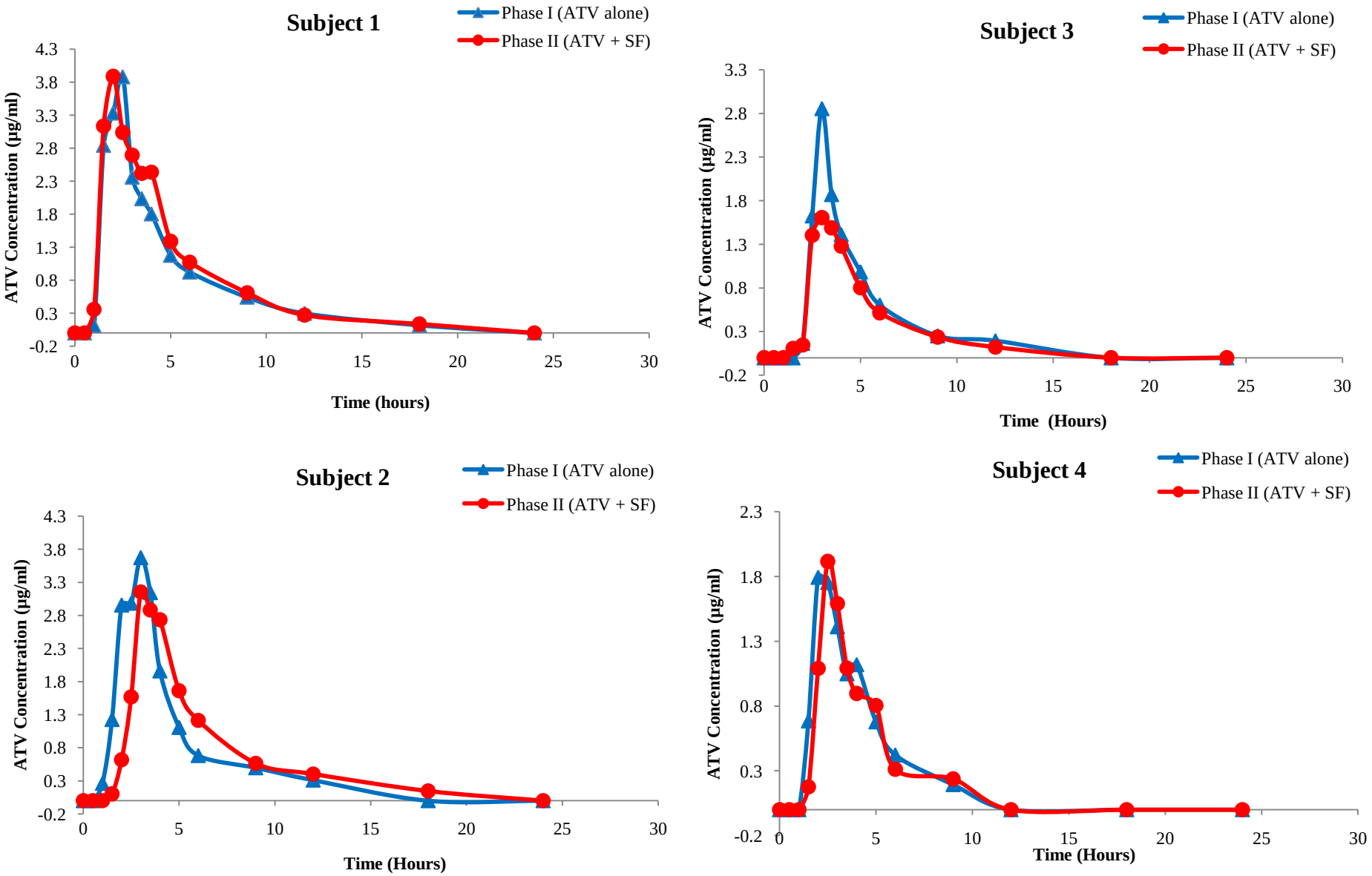

Figure A2: ATV plasma concentration-time profiles for Subjects 1−4

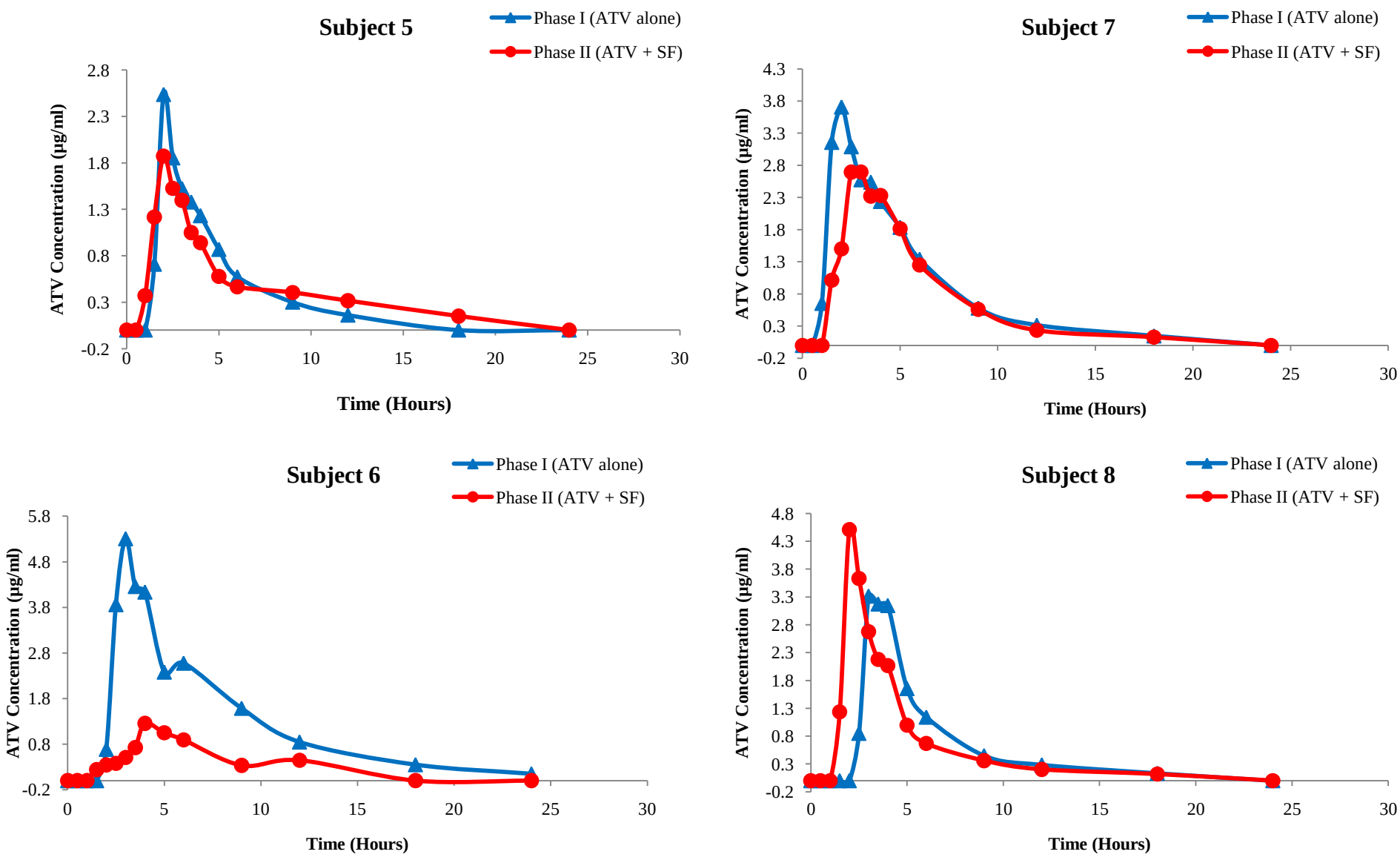

Figure A3: ATV plasma concentration-time profiles for Subjects 5–8

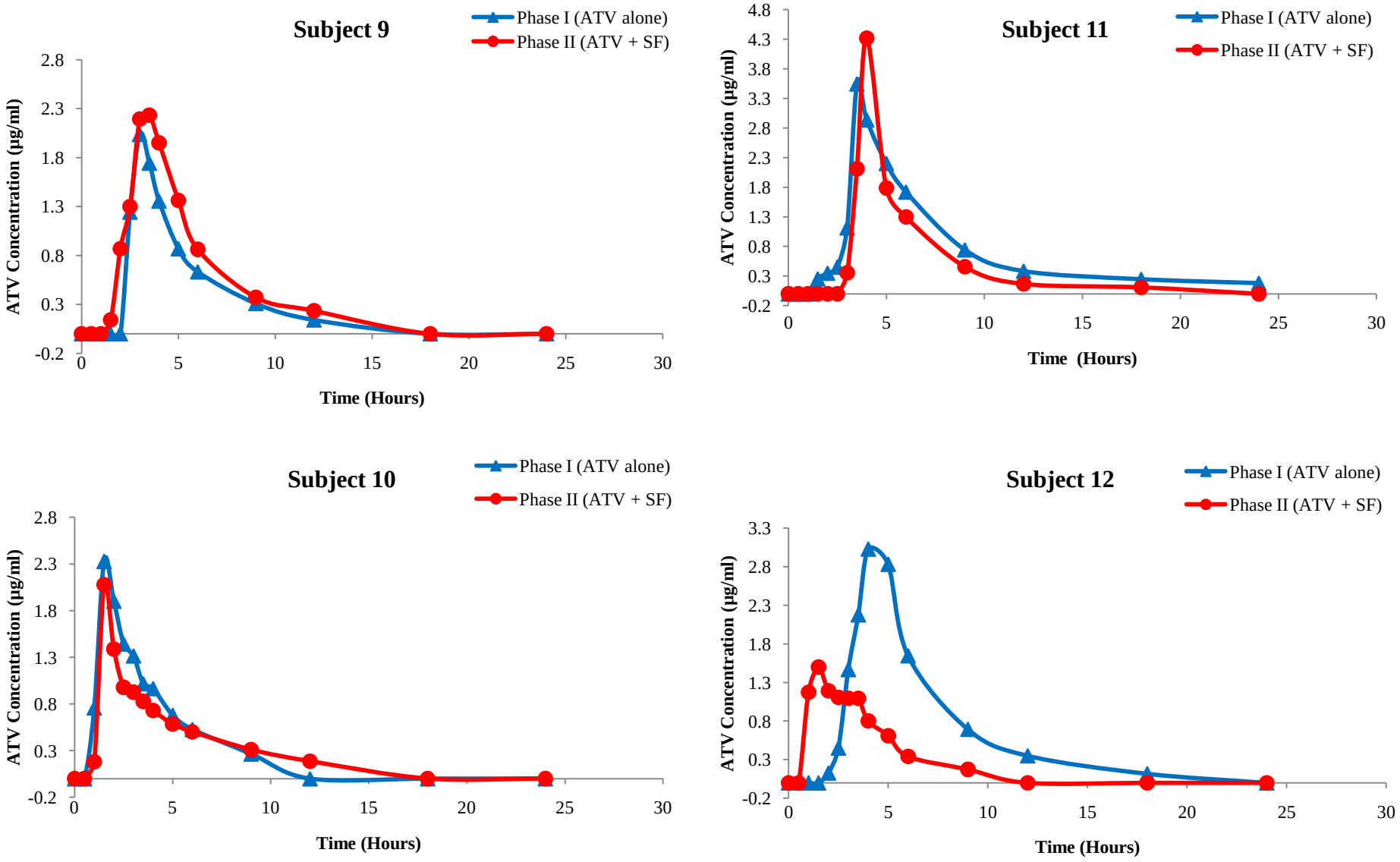

Figure A4: ATV plasma concentration-time profiles for Subjects 9−12

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