

Impact of selected herbal products on intestinal epithelial permeation and metabolism of indinavir

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

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November 2014

Invictus

*Out of the dark night that covers me,
Black as the pit from pole to pole
I thank whatever gods may be
For my unconquerable soul.*

*In the fell clutch of circumstance
I have not winced nor cried aloud.
Under the bludgeoning of chance
My head is bloody, but unbowed.*

*Beyond this place of wrath and tears
Looms but the Horror of the shade,
And yet the menace of the years
Finds and shall find me unafraid.*

*It matters not how strait the gate,
How charged with punishments the scroll,
I am the master of my fate:
I am the captain of my soul.*

-William Ernest Henley-

ACKNOWLEDGEMENTS

This dissertation is presented to you not only on my own accord; it would not have been possible without the aid of various contributors. The following individuals should be acknowledged:

- My heavenly Father, without your precious gifts that you bestowed onto me, this would never have been possible. I have never during all this time wanted for anything, all that I needed came in abundance from You!
- They say that family isn't always blood. It's the people in your life who want you in theirs. The ones who accept you for who you are. The ones who would do anything to see you smile, and succeed, and who love you no matter what. Steve and Theresa Engelbrecht, thank you for taking me in and loving me as your own, without your love, guidance and constant encouragement all this would never have been possible. Words will never be enough. Mea Engelbrecht for your constant encouragement thank you, I love you as my own.
- Megan Nagel, Marisa De Wet and Trizel du Toit, I can never thank you enough, you are the best friends any girl could ask for. Megan and Marisa, thank you for every little thing you do for me, I do not deserve friends like you, you cared for me, put up with my whirlwind emotions at times and kept on encouraging me to the very end. Trizel, my partner in crime, early mornings, late nights and that labdancer! Thank you for your support and friendship!
- My study supervisors Prof Sias Hamman, Prof Jan Steenekamp and Dr Joe Viljoen. Prof Sias, thank you for your patience and guidance for allowing me to storm into your office for anything and everything. I have learned so much from you and have the utmost respect for you. Prof Jan, I have learned a great deal from you over the last two years, helping me grow not only as a student but as a person. You are my "go to guy" for when the world gets too much, thank you for your wisdom and encouragements. Dr Joe Viljoen, thank you for not only your academic input, but also your friendship.
- Dr Chrisna Gouws, my mentor and friend, you took a very insecure undergraduate student and turned her into an "almost scientist". Thank you for all the effort in regards to the cell culturing, transport and metabolism studies. You are a saint; none of this would have been possible without your help.
- Dr Dewald Steyn, thank you for your input on your subject matter.

- Tannie Mariëtta Fourie, no student will ever survive doing an M.Sc or Ph.D without you around, you are like a mother to us all, thank you for the person that you are and all your help.
- Tannie Anriëtte Pretorius, thank you for your friendship, but also your patience and guidance in regards to the reference list and help with finding articles.
- Prof Jan du Preeze, thank you for all your help and support and patience in regards to the HPLC analysis of the transport samples.
- Dr Lubbe Wiessner and staff at the Cape Town bioanalytical services for the analysis of the metabolism samples and the development of the LC/MS/MS method.
- The North-West University for the masters and institutional bursary.

ABSTRACT

Patients on anti-retroviral (ARV) drug treatment are sometimes simultaneously taking other prescribed drugs and/or over-the-counter drugs and/or herbal remedies. Pharmacokinetic drug-drug or herb-drug interactions can occur in these patients, which might be synergistic or antagonistic in nature leading to increased or decreased bioavailability of the ARV. Consequences of bioavailability changes may either be adverse effects due to increased plasma levels, or lack of pharmacological responses due to decreased plasma levels. The aim of this study is to determine if pharmacokinetic interactions exist between selected commercially available herbal products, namely Linctagon Forte[®], Viral Choice[®] and Canova[®] and the ARV, indinavir, in terms of transport and metabolism in cell culture models.

Bi-directional transport of indinavir was evaluated across Caco-2 cell monolayers in four experimental groups, namely indinavir alone (200 μ M, negative control group), indinavir in combination with Linctagon Forte[®], indinavir in combination with Viral Choice[®] and indinavir in combination with Canova[®] at three different concentrations. Verapamil (100 μ M), a known P-gp inhibitor, was combined with indinavir in the positive control group. Samples obtained from the transport studies were analysed by means of a validated high performance liquid chromatography (HPLC) method. The apparent permeability coefficient (P_{app}) values were calculated from the transport results in both directions and the efflux ratio (ER) values were calculated from these P_{app} values. The metabolism of indinavir was determined in LS180 cells in the same groups as mentioned for the transport study but with ketoconazole (40 μ M), a known CYP3A4 inhibitor, as the positive control group. Indinavir and its predominant metabolite (M6) were analysed in the metabolism samples by means of liquid chromatography linked to mass spectroscopy (LC/MS/MS) to determine the effect of the herbal products on the biotransformation of indinavir.

The BL-AP transport of indinavir increased in a concentration dependent way in the presence of Linctagon Forte[®] and Viral Choice[®] when compared to that of indinavir alone (control group). Canova[®] only slightly affected the efflux of indinavir compared to that of the control group. Noticeable increases in the efflux ratio values of indinavir were found for Linctagon Forte[®] and Viral Choice[®], whilst the effect of Canova[®] on the efflux ratio value was negligible.

There was a pronounced inhibition of the metabolism of indinavir in LS180 cells over the entire concentration range for all the herbal products investigated in this study. These *in vitro* pharmacokinetic interactions indicate the selected herbal products may affect indinavir's

bioavailability, but the clinical significance needs to be confirmed with *in vivo* studies before final conclusions can be made.

Key words: Herb-drug interactions, efflux, P-glycoprotein, CYP3A4, Caco-2, LS180, metabolism

UITTREKSEL

Pasiënte wat anti-retrovirale (ARV) geneesmiddels neem, maak ook soms gelyktydig gebruik van ander voorgeskrewe geneesmiddels en/of oor-die-toonbank geneesmiddels en/of kruie geneesmiddels. Farmakokinetiese geneesmiddel-geneesmiddel of kruie-geneesmiddel interaksies kan voorkom in hierdie pasiënte, wat hetsy sinergisties of antagonisties van aard kan wees en mag lei tot verhoogde of verlaagde biobeskikbaarheid van die ARV. Die nagevolge van biobeskikbaarheidsveranderings mag moontlike ongunstige nuwe-effekte wees weens verhoogde bloed plasmavlakke of 'n afwesigheid van 'n farmakologiese reaksie weens verlaagde bloed plasmavlakke. Die doel van die studie was om te bepaal of daar enige farmakokinetiese interaksies bestaan tussen geselekteerde kommersiële beskikbare kruieprodukte, naamlik Linctagon Forte[®], Viral Choice[®] en Canova[®] met die ARV, indinavir, in terme van transport en die metabolisme in selkultuurmodelle.

Die twee-rigting transport van indinavir was ge-evalueer oor Caco-2 selmonolae in vier eksperimentele groepe naamlik indinavir alleen (200 μ M, negatiewe kontrole groep), indinavir in kombinasie met Linctagon Forte[®], indinavir in kombinasie met Viral Choice[®] en indinavir in kombinasie met Canova[®] by drie verskillende konsentrasies. Verapamil (100 μ M), 'n bekende P-gp inhibeerder, was gekombineer met indinavir as die positiewe kontrole groep. Monsters verkry vanaf die transport studies was geanaliseer deur middel van 'n gevaludeerde hoedruk vloeistofchromatografiese (HDVC) metode. Die deurlaatbaarheidskoeffisient (P_{app}) waardes was bereken vanaf die transport resultate vir beide rigtings en die effluksverhouding (EV) was bereken vanaf die (P_{app}) waardes. Die metabolisme van indinavir was bepaal in die LS180 sellyn deur gebruik te maak van dieselfde groepe soos genoem vir die transport studie maar met ketoconazole (40 μ M), 'n bekende CYP3A4 inhibeerder, as die positiewe kontrole groep. Indinavir en sy mees prominente metaboliet (M6) was geanaliseer in die metabolisme monsters deur middel van vloeistofchromatografie gekoppel aan 'n massaspektrometer (VC/MS/MS) om te bepaal wat die effek van die kruieprodukte was op die biotransformasie van indinavir.

Die basolaterale-na-apikale (BL-AP) transport van indinavir het in 'n konsentrasie afhanklike wyse verhoog in die teenwoordigheid van Linctagon Forte[®] en Viral Choice[®] in vergelyking met die van indinavir alleen (kontrole groep). Canova[®] het slegs 'n effense effek op die effluks van indinavir gehad in vergelyking met die kontrole groep. Daar is 'n kenmerklike toename in die effluksverhoudings van indinavir vir Linctagon Forte[®] and Viral Choice[®], terwyl die effek van Canova[®] op die effluksverhouding amper weglaatbaar was. Daar was definitiewe inhibisie in die metabolisme van indinavir in die LS180 selle oor die hele

konsentrasie reeks vir al die kruieprodukte geondersoek gedurende die studie. Hierdie *in vitro* farmakokinetiese interaksies dui op 'n moontlikheid dat die geselekteerde kruieprodukte indinavir se biobeskikbaarheid mag affekteer, maar die kliniese betekenisvolheid hiervan behoort bevestig te word deur *in vivo* studies voor enige finale gevolgtrekkings gemaak kan word.

Sleutel woorde: Kruie-geneesmiddel interaksies, efflux, P-glikoproteïen, CYP3A4, Caco-2, LS180, metabolisme

CONGRESS PROCEEDINGS & ARTICLES

1.1 Congress proceedings

Impact of selected herbal products on the *in vitro* transport and metabolism of indinavir. Presented at the 35th Conference of the Academy of Pharmaceutical Sciences held from 12–14 September 2014 at the Summerstrand Hotel in Port Elizabeth hosted by the Department of Pharmacy at the Nelson Mandela Metropolitan University. (See Appendix A)

1.2 Articles

Calitz, C., Steenekamp, J.H., Steyn, J.D., Gouws, C., Viljoen, J.M. & Hamman, J.H. 2014. Impact of traditional African medicine on drug metabolism and transport. *Expert Opinion on Drug Metabolism & Toxicology*, 7:991-1003. (See Appendix A)

Calitz, C., Gouws, C., Viljoen, J., Steenekamp, J.H., Wiesner, L. & Hamman, J.H. 2014. Interactions of herbal products with indinavir transport and metabolism. Ready for submission. (See Appendix A)

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LIST OF ABBREVIATIONS

%RSD	Relative standard deviation
ABC	ATP-binding cassette
ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
AP-BL	Apical to basolateral
ARV	Anti-retroviral
<i>A. sativum</i>	<i>Allium sativum</i>
ATP	Adenosine triphosphate
BaCO ₃	Barium carbonate
BL-AP	Basolateral to apical
<i>C. ipecacuanha</i>	<i>Carapichea ipecacuanha</i>
Caco-2	Human Caucasian colon adenocarcinoma cell lines
CaCO ₃	Calcium carbonate
CAM	Complementary and alternative medicines
CAR	Constitutive androstane receptor
cDNA	Complimentary deoxyribonucleic acid
CYP	Cytochrome P450
ddH ₂ O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
<i>E. angustifolia</i>	<i>Echinacea angustifolia</i>
<i>E. pallid</i>	<i>Echinacea pallid</i>
<i>E. purpurea</i>	<i>Echinacea purpurea</i>
ECACC	European Collection of Cell Cultures
EDTA	Typsin-Versene
Fe ²⁺	Ferrous

Fe ³⁺	Ferric
GI	Gastrointestinal tract
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
<i>L. clavatum</i>	<i>Lycopodium clavatum</i>
<i>L. muta</i>	<i>Lachesis muta</i>
LC/MS/MS	Liquid chromatography mass spectrometry
LS180	Human Caucasian colon adenocarcinoma cell lines
<i>MDR1/ABCB1</i>	Multiple drug resistance
MFOs	Mixed-function oxidases
mRNA	Messenger ribonucleic acid
MRPs	Multiple resistance proteins
NADH	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NBDs	Nucleotide binding domains
NEAA	Non-essential amino acids
NH ₄ HCO ₂	Ammonium formate
<i>P. sidoides</i>	<i>Pelargonium sidoides</i>
P _{app}	Apparent permeability coefficient
PBS	Phosphate buffered saline
P-gp	P-glycoprotein
P _i	Phosphate
PXR	Pregnane X receptor
Rh-123	Rhodamine-123
RXR	Retinoid-X-receptor
SD	Standard deviation
SNP	Single nucleotide polymorphism

<i>T. occidentalis</i>	<i>Thuya occidentalis</i>
TEER	Transepithelial electrical resistance
TM	Trans-membrane
UV	Ultra violet
<i>V. album</i>	<i>Veratrum album</i>

CHAPTER 1

INTRODUCTION

1.1 Background

The use of natural plant products dates back centuries to the Assyrians, Egyptians and Mesopotamians, 2600 BC (Ji *et al.*, 2009:194; Silverman, 2004:1). In South Africa, the consultation of traditional healers and use of traditional herbal remedies still remains popular (Cordier & Steenkamp, 2011:53; Fenell *et al.*, 2004: 205; Taylor *et al.*, 2001:25) Ras and colleagues (1983:140-142) reported the first two known cases of human immunodeficiency virus (HIV) infections, associated with acquired immunodeficiency syndrome (AIDS), in South Africa and by 2013 a staggering 5.24 million people were HIV infected, with 55% of these patients receiving anti-retroviral (ARV) treatment (Mc Neil, 2012; Statistics SA, 2013). Patients infected with HIV and receiving ARV treatment are, sometimes, simultaneously taking other prescribed drugs as well as traditional and herbal medicines without informing their health care provider (Cohen *et al.*, 2002:42,45).

Any herbal product or natural plant product is usually composed of, not only one, but a complex mixture of various phytochemicals, each exerting potential pharmacological effects to a certain extent. Unfortunately, the phytochemicals in natural products may exhibit interactions with drugs such as ARVs when taken simultaneously by the same patient (Pal & Mitra., 2006:2132) and concurrent intake could result in pharmacokinetic and/or pharmacodynamic interactions (Tarirai *et al.*, 2010:1517). Pharmacokinetic interactions occur by means of induction and/or inhibition of intestinal efflux proteins such as p-glycoprotein (P-gp) or multiple resistance proteins (MRPs), as well as modulation of intestinal and hepatic metabolising enzymes, specifically the cytochrome P450 super family (Pal & Mitra., 2006:2132).

P-gp, an ATP-binding cassette (ABC) protein, is a drug transporter located in the plasma membrane of cells and is actively involved in the efflux of drug and drug conjugates from cells. This protein is highly expressed on the apical membranes of epithelial cells in the colon, small intestine, pancreatic and hepatic bile ducts, as well as the kidney proximal tubule (Sharom, 2008:106,108). The physiological role of efflux pumps in cells is to offer protection against potential toxicity of various endogenous and exogenous compounds. P-gp mediates the efflux transport of various structural dissimilar compounds including, but not limited to, HIV-protease inhibitors, antihistamines, immunosuppressive agents, analgesics and H₂-receptor antagonists. When P-gp extrudes drug molecules from the

epithelium back into the intestinal lumen, it may lead to reduced absorption and consequently poor oral bioavailability (Sharom, 2008:106).

The enzyme detoxification system is mostly located in the liver and secondarily in the intestinal mucosal wall and is responsible for ridding the body of harmful lipophilic compounds. It consists of a two phased system working in conjunction with one another to produce a more water soluble compound (Liska, 1998:188). Cytochrome P450 (CYP) enzymes facilitate the first degradation onslaught to xenobiotic compounds in phase I reactions by means of oxidation, reduction and hydrolysis reactions (Liska, 1998:189). The CYP enzyme system accounts for 30% of the hepatic metabolism activity as well as more than 70% of the intestinal metabolism activity. The most abundant isoform of the CYP family is CYP3A4, which is responsible for metabolising up to 70% of all administered drugs (Hellum & Nilsen 2008:466; Pal & Mitra., 2006:2136). CYP phase I enzymes are responsible for the production of hydroxyl radicals, or any other reactive group, with the aid of oxygen and the cofactor, NADH. The process involves production of reactive oxygen intermediates capable of secondary tissue damage if not otherwise properly metabolised by phase II of the enzyme detoxification system (Liska, 1998:189). Phase II metabolism is primarily known as the conjugation phase. The reactive oxygen intermediate compound produced during phase I is biotransformed to one which is more water soluble by means of sulfation, glucuronidation, glutathionine conjugation, acetylation or amino acid conjugation; the product is more water soluble allowing excretion via urine or bile (Liska, 1998:190).

Patients with HIV/AIDS on ARV treatment often use supplements and herbal products in addition to their allopathic medicines. Therefore, commercially available herbal products, often used by patients as immune system boosters, were selected for this project. Herbal remedies such as Canova[®], Linctagon Forte[®] and Viral Choice[®] are composed of extracts from various herbs which may exert possible interactions with the metabolism and transport of the ARV drug, indinavir.

Canova[®] is a homeopathic product composed of various extracts from *Aconitum napellus* (Ranunculaceae), *Arsenicum album* (arsenic trioxide), *Bryonia alba* (Cucurbitaceae), *Apis mellifica* (Apidae), *Lycopodium clavatum* (Lycopodiaceae), *Pulsatilla nigricans* (Ranunculaceae), *Asa foetida*, *Rhus toxicodendrum*, *Barita carbônica*, *Ricinus communis* (Euphorbiaceae), *Silicea*, *Calcarea carbônica*, *Conium maculatum* (Apiaceae), *Veratrum album* (Liliaceae), *Carapichea ipecacuanha* (Rubiaceae), *Lachesis muta* (Viperidae) and *Thuja occidentalis* (Cupressaceae). Canova[®] is known as an immune response modifier directed at macrophages which result in a decrease in their production together with the

release of cytokine TNF α in cancer and AIDS patients (De Oliveira *et al.*, 2008:3; Takahachi *et al.*, 2007:350).

Linctagon Forte[®] is a natural product used to prevent and treat colds, flu and respiratory tract infections. It contains extracts of *Pelargonium sidoides* as well as the natural occurring compounds quercetin and bromelain. Viral Choice[®], also a natural product, contains a variety of minerals, vitamins, amino acids, trace elements, phytosterols as well as *Echinacea purpurea* and *Allium sativum* extracts.

1.2 Research problem

The incidence of pharmacokinetic herb-drug interactions is a realistic outcome in patients simultaneously taking natural herbal products and ARVs. These pharmacokinetic interactions might be synergistic or antagonistic in nature, leading to increased or decreased bioavailability of the ARV. Consequences of bioavailability changes may either be adverse effects due to increased plasma levels, or lack of pharmacological responses due to decreased plasma levels. The research problem to be investigated in this project is to determine if pharmacokinetic interactions exist between selected commercially available herbal products and indinavir in terms of transport and metabolism in intestinal epithelial cell cultures.

1.3 Aim and objectives

The aim of this research project is to investigate the effect of selected herbal medicinal products, namely Linctagon Forte[®], Viral Choice[®] and Canova[®] on the *in vitro* transport and metabolism of indinavir in intestinal epithelial cells.

To achieve this aim, the following objectives were set:

- To modify and validate a high performance liquid chromatographic (HPLC) analysis method for indinavir for the *in vitro* transport study.
- To modify and validate an analysis method by means of a liquid chromatography linked to a mass spectrometer (LC/MS/MS) for indinavir in the presence of its predominant metabolite (i.e. M6) for the *in vitro* metabolism study.
- To grow and seed out Caco-2 and LS180 cells as *in vitro* models for the transport and metabolism studies, respectively.
- To evaluate the effects of the selected herbal products at an extreme range of concentrations on the bi-directional transport of indinavir across Caco-2 cell monolayers.

- To evaluate the effects of the selected herbal products in different concentrations on the *in vitro* metabolism of indinavir in the LS180 cell line.

1.4 Structure of dissertation

This dissertation begins with an introductory chapter (Chapter 1), which provides a motivation and justification for the research project as well as an outline of the aim and objectives. This is followed by a literature overview in Chapter 2 focusing on mechanisms of pharmacokinetic interactions, such as P-glycoprotein related efflux modulation, as well as changes in metabolism of drugs by co-administered compounds. The scientific methods used during the different *in vitro* studies are described in Chapter 3 and the results obtained from these experiments are presented and discussed in Chapter 4. Finally, Chapter 5 draws final conclusions from the results displayed in the dissertation and offers recommendations for future studies.

CHAPTER 2

LITERATURE REVIEW ON HERB-DRUG PHARMACOKINETIC INTERACTIONS

2.1 INTRODUCTION

The use of herbal medicines has a long history and dates back as far as the Assyrians in 1900 to 400 BC. Historically, the Egyptian, Chinese, African, Indian as well as Native American cultures have employed herbal remedies for treatment of ailments (Ji *et al.*, 2009:194; Ehrlich, 2013; Silverman, 2004:1). Advances made in chemical and pharmaceutical sciences over the last few decades made the extraction and modification of active ingredients found in plants possible, which resulted in a decline in the use of herbal medicines in some instances (Ji *et al.*, 2009:194; Ehrlich, 2013). However, some traditional herbal medicines maintained popularity, whilst the use of herbal medicines in general has recently started to grow dramatically in the Western world (Liu *et al.*, 2011:835).

It is estimated that between 60 and 90% of Africa's population still make use of traditional healers and medicine to meet their health care needs (Fenell *et al.*, 2004:205; Taylor *et al.*, 2001:25). Africa is the continent most severely affected by the human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS), hosting 22.4 million people living with HIV/AIDS. As many as 5.28 million people were infected with HIV in South-Africa alone in 2013 and 55% of these patients are receiving anti-retroviral (ARV) treatment, of which 84% are also making use of traditional medicines (Mc Neil, 2012, Statistics SA, 2013, Unge *et al.*, 2011:851). In South Africa, approximately 60% of the population consult traditional healers, of whom there are an estimated 200 000 (Taylor *et al.*, 2001:25). Traditional healers are predominantly located and consulted in rural areas where there is a general lack of health care providers and/or a higher preference for traditional healers. An astonishing 80% of the population in KwaZulu Natal Province, South Africa, indicated they would rather consult a traditional healer than a registered health care practitioner (Taylor *et al.*, 2001:25).

Various reasons exist for the use and popularity of traditional medicines. As mentioned before, the general public has greater access to traditional medicines in certain developing countries, which offer affordability and these treatment practices often correspond to the patient's ideology, religious and even cultural beliefs (Fenell *et al.*, 2004: 205; Taylor *et al.*, 2001:25; Wachtel-Galor & Benzie, 2011). It is also generally believed by many patients that due to the natural origin of herbal medicines, they are safer with less adverse effects than

allopathic medicines (Wachtel-Galor & Benzie, 2011). There are currently no regulations to control the prescription and usage of traditional medicines in South Africa, which may increase mis-administration. Scientific knowledge regarding the genotoxicity during prolonged use of most herbal medicines is limited (Fenell *et al.*, 2001:205). The use of commercially available complementary and alternative medicines (CAM) is also on the rise in the higher income groups, for reasons which include ease of access, the belief it is non-toxic and effective alleviation of symptoms associated with some diseases (Littlewood & Vanable, 2008:1004).

However CAM, as well as traditional herbal remedies, may cause interferences with the effectiveness of anti-retroviral (ARV) drugs (Cordier & Steenkamp, 2011:53; Littlewood & Vanable, 2008:1004), due to the fact herbal medicines are generally composed of a complex mixture of a variety of phytoconstituents. These phytoconstituents each potentially possesses the ability to cause pharmacodynamic and/or pharmacokinetic interactions when administered with other prescribed drugs (Liu *et al.*, 2011: 835; Pal & Mitra., 2006:2132; Tarirai *et al.*, 2010:1517). Pharmacokinetic interactions involve interferences with a drug's absorption and disposition in the body and can often be associated with modulation of the active transporter, P-glycoprotein, and the cytochrome P450 enzyme family, or both (Darby *et al.*, 2011:722).

2.2 PHARMACOKINETIC INTERACTIONS INVOLVING P-GLYCOPROTEIN AND CYTOCHROME P450

Although various routes of administration are available, the oral route still remains the most preferred route of drug administration (Gavhane & Yadav, 2012:1). For a drug to be therapeutically effective, it is required to be pharmacologically active and have sufficient intestinal permeability to reach the target tissue or organ intact at the required concentration (Shargel *et al.*, 2006:453,459). After oral administration, the drug molecules undergo absorption, distribution, metabolism and excretion as illustrated in Figure 2.1.

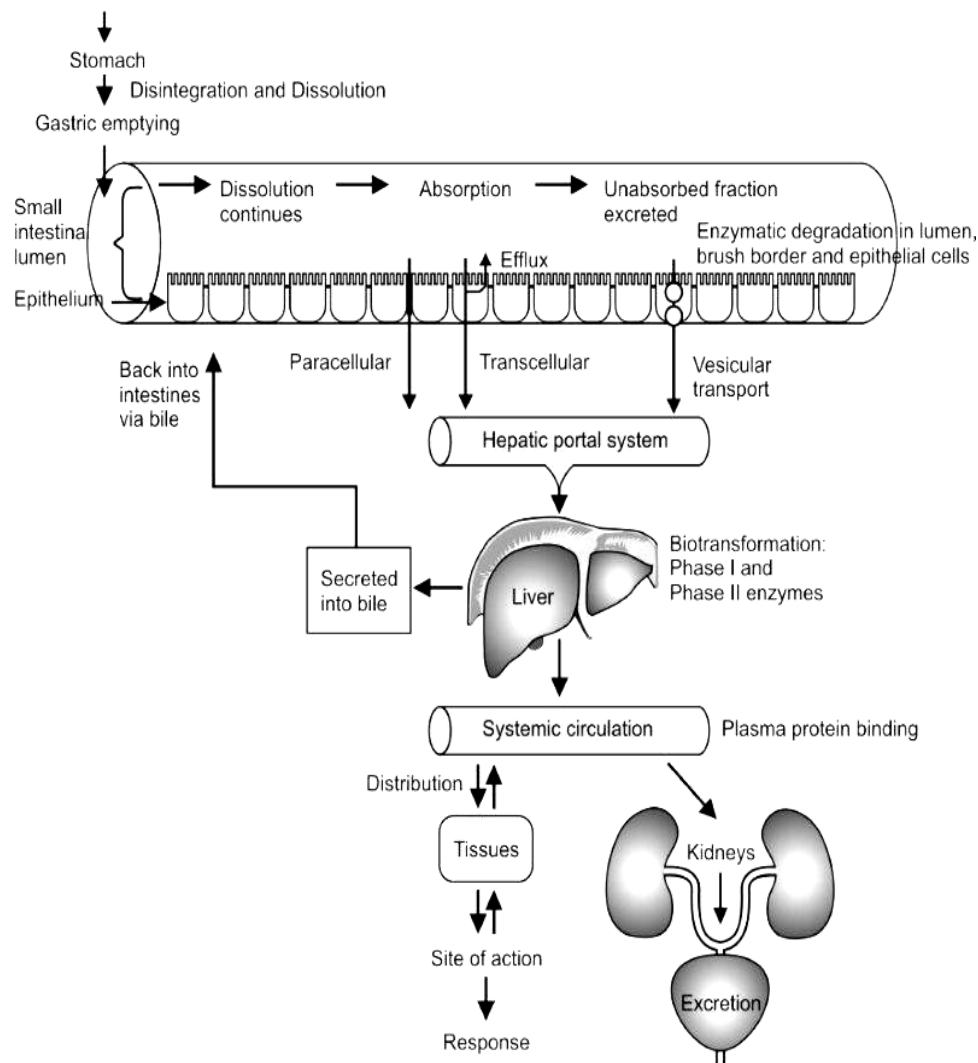


Figure 2.1: Pharmacokinetics showing absorption, distribution, metabolism and elimination of administered drugs (Hamman, 2007:64)

The stomach and small intestine serves as the major site for drug absorption. The extent of drug absorption is influenced by various physiological as well as biochemical mechanisms (Daugherty & Mersny, 1999:144). These biochemical and physiological mechanisms include enzymatic activity and efflux pathways found in the brush border of the intestinal lumen epithelial cells, influencing the subsequent distribution and bioavailability of the administered drug (Daugherty & Mersny, 1999:144; Engman, 2003:7; Hamman, 2007:64). Following absorption the administered drug is distributed by means of the hepatic portal system, subjecting it to the liver and the liver enzyme detoxification system, which is a two phased system responsible for the metabolism of various orally administered drugs (Hamman, 2007:64, Liska 1998:189). The remaining drug now enters the systemic circulation where it is transported to the target site resulting in a pharmacological response (Hamman, 2007:64).

2.3 EFFLUX OF DRUGS

Efflux refers to the counter-transport of drugs by efflux proteins, which are active transporters responsible for the expulsion of drugs back into the lumen of the gastrointestinal tract after uptake into the epithelial cells (Chan *et al.*, 2004:26). This efflux mechanism is crucial to the health of the organism as it limits both absorption and accumulation of harmful exogenous substances. Conversely, it is also known for its detrimental effects on the treatment of tumour cells by lowering cellular concentrations of anti-cancer drugs and actively contributing to multi drug resistance (Chan *et al.*, 2004:25). One of the most important and prevalent efflux transporters is P-glycoprotein (P-gp) (Yang, 2013:2).

2.3.1 P-Glycoprotein (P-gp)

Juliano and Ling were responsible for the elucidation of P-gp when they noticed the appearance of a membrane protein in drug-resistant ovary cells of Chinese hamsters (Chan *et al.*, 2004:25; Lin & Yamazaki., 2003:60). P-gp forms part of the ATP-binding cassette (ABC) superfamily of transporter proteins and was one of the first active counter-transporters to be described (Ambudkar *et al.*, 2003:7469; Russel, 2010:28). Various ABC transporters have since been identified which are subdivided into seven classes based on gene sequence similarities (Ambudkar *et al.*, 2003:7469). These ABC transporters are found in a multitude of organs where they attribute to the active transport of a wide-ranging set of substrates (Russel, 2010:28). The P-gp protein, encoded by the multiple drug resistance (*MDR1/ABCB1*) gene, is present on the apical side of the brush border membrane of intestinal enterocytes present in the gastrointestinal (GI) tract, where its expression in the jejunum is the highest followed by ileum and then the colon (Giacomini & Sugiyama., 2006:66,67; Russel, 2010:32,38; Yang, 2013:2). P-gp is also widely expressed in the canicular membrane of hepatocytes in the liver, proximal tubule of the kidneys, blood brain barrier, blood-testis barrier, blood-ovarian barrier and the placenta (Giacomini & Sugiyama, 2006:66,67; Marchetti *et al.*, 2007:928; Russel, 2010:32,38; Yang, 2013:2).

2.3.1.1 Structure of P-Glycoprotein

Functional ABC proteins are composed of at least four core domains; two membrane-bound domains forming the permeation pathway for substrate transport and two nucleotide binding domains (NBDs) responsible for the hydrolysis of adenosine triphosphate (ATP) (Sharom, 2008:105). The P-gp transmembrane protein comprises a single polypeptide of 170- 190 kDa in length, which consists of two homologous and two symmetrical cassettes as illustrated in Figure 2.2. These cassettes contain six transmembrane domains with an α -helix structure each separated by six extracellular hydrophobic loops of which one is

glycosylated and two NBDs are found on the membrane's cytoplasmic face (Lin & Yamazaki., 2003:61; Rosenberg *et al.*, 1997:10685,10693; Sharom 2008:106). The two cassettes are separated by an intracellular flexible linker polypeptide loop with an ATP-binding motif. They interact cooperatively and function as a single unit. The flexible linker region assures the proper interaction of the cassettes due to its role in ATPase and transport activities (Lin & Yamazaki, 2003:61).

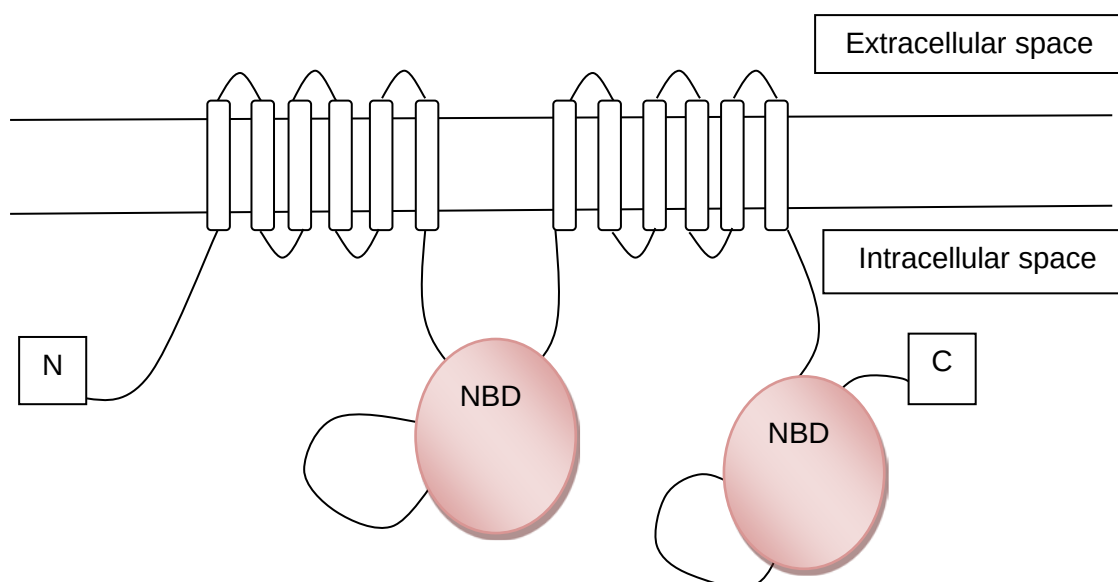


Figure 2.2: P-gp (MDR1) transmembrane arrangement where the N denotes the nitrogen-terminus of the amino acid chain and the C denotes the carboxylic terminus of the amino acid chain, NBD denotes nucleotide binding domain (Adapted from Chan *et al.*, 2004:28)

Three dimensionally, P-gp occupies a surface area of approximately 60 nm^2 . On the extracellular side of the lipid bilayer, P-gp appears cylindrical with a 10 nm diameter and a maximum height of 8 nm of which 4 nm is imbedded into the lipid bilayer. The 12 transmembrane domains form an aqueous annulated structure of approximately 5 nm in diameter, open on the extracellular space and closed to the intracellular space, thus creating an aqueous chamber within the membrane. Embedded in the lipid bilayer, an opening to P-gp can be found which corresponds with the “flippase” model of drug substrate translocation (Rosenberg *et al.*, 1997:10693). A schematic illustration of the three dimensional structure of P-gp is given in Figure 2.3 below.

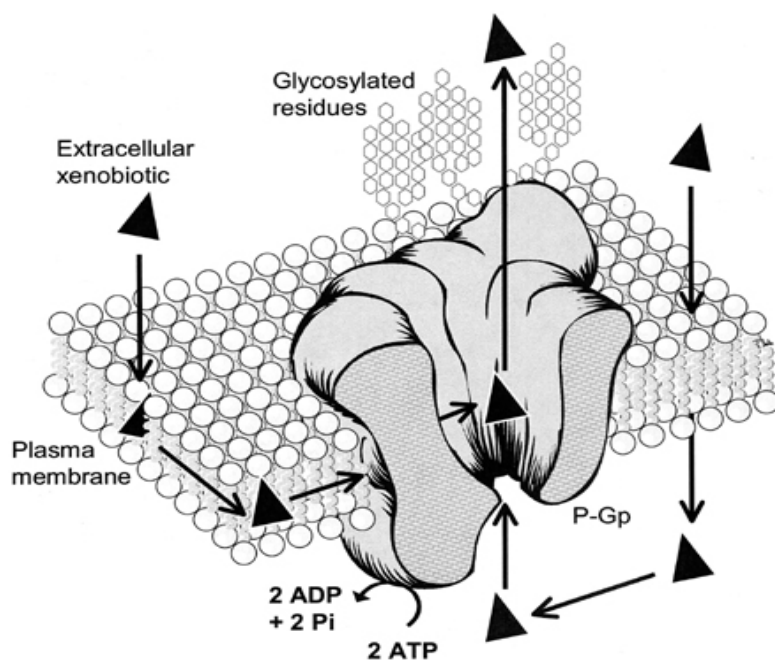


Figure 2.3: Schematic illustration of the three dimensional structure of P-gp (Marzolini *et al.*, 2004:17)

2.3.1.2 *P-gp mechanism for the transport model*

Various mechanistic models have been proposed to elucidate drug transport mechanisms of P-gp, which are illustrated in Figure 2.4.

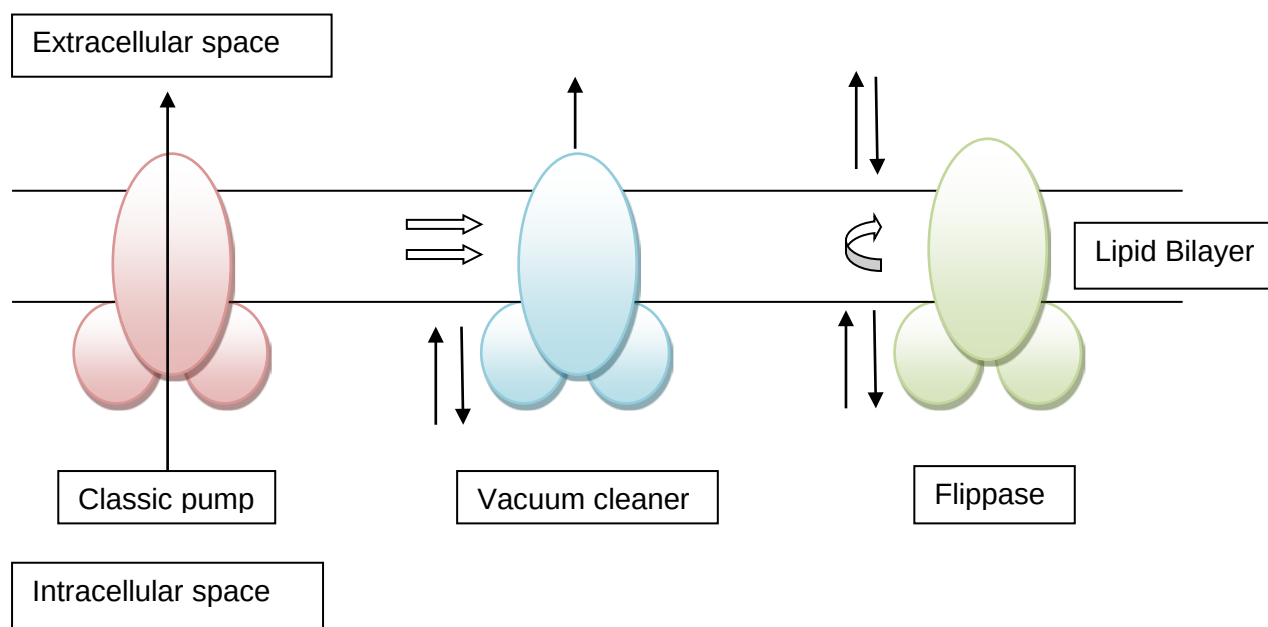


Figure 2.4: Schematic illustration of the classical pump, vacuum cleaner and 'flippase' models as potential mechanisms of action of P-gp (adapted from Sharom, 2008:115)

The initial model viewed P-gp as an ion channel protein, which acts as an aqueous transmembrane pore that transports polar substances from the intracellular space to the extracellular space. The second model, known as the vacuum cleaner, views P-gp as an extruder, which moves drug molecules that are partitioned into the cell membrane directly back into the extracellular space before these molecules reach the cytoplasm (Lin & Yamazaki, 2003:62; Sharom, 2008:115). The third model views P-gp as a 'flippase', where an interaction occurs between lipophilic drug molecules and the lipid bilayer membrane rather than direct interaction with the P-gp transporter. Upon interspersal of the lipid soluble drugs into the inner leaflet of the bilayer, the drug is flipped by the P-gp transporter into the outer leaflet of the bilayer (Higgins & Gottesman, 1992:18-20; Lin & Yamazaki, 2003:62; Sharom, 2008:114).

2.3.1.3 Substrate specificity and drug binding sites of P-gp

P-gp is known for the active transport of various structural dissimilar compounds, thus showing a broad range of substrate specificity. P-gp substrates range from small molecules to large peptides (i.e. approximately 200 - 4 000 Da in size) with a preference to hydrophobic, cationic or amphiphatic molecules with a planar ring system (Estudante *et al.*, 2013:1342; Russel, 2010:38; Yang, 2013:2). P-gp is also known for its capability to transport neutral and hydrophilic compounds as well as negatively charged carboxylic groups (Estudante *et al.*, 2013:1343). Table 2.1 offers an indication of the various drugs that are substrates of P-gp.

Table 2.1: Examples of drugs from different classes that are substrates of P-gp (Adapted from, Amin, 2013:29; Fong *et al.*, 2011:9; Hayeshi *et al.*, 2006:74,75; Lee *et al.*, 1998:3598; Levin, 2012:40; Mechetner & Roninson, 1992:5827; Meijerman *et al.*, 2006:745; Sharom, 2008:107; Srivalli & Lakshmi, 2012:355; Wessler, 2013; Zha *et al.*, 2013:e54349)

Anticancer drugs	Vinblastine	• Substrate
	Doxorubicin	• Substrate
	Paclitaxel	• Substrate
	Epitoposide	• Substrate
	Ttopotecan	• Substrate

	Bisantrene	• Substrate
HIV protease inhibitors	Indinavir	• Substrate • Inhibitor
	Ritonavir	• Substrate • Inhibitor
	Saquinavir	• Substrate
	Nelfinavir	• Substrate
Analgesics	Morphine	• Substrate
Antihistamines	Terfenadine	• Substrate
	Fexofenadine	• Substrate
H₂-receptor antagonists	Cimetidine	• Substrate
Immunosuppressive agents	Cyclosporine A	• Substrate • Inhibitor
	Tacrolimus (FK506)	• Substrate
Antiarrhythmics	Quinidine	• Substrate • Inhibitor • Inducer
	Amiodarone	• Substrate • Inhibitor
	Propafenone	• Substrate • Inhibitor
Antiepileptics	Felbamate	• Substrate
	Topiramate	• Substrate
Fluorescent compounds	Calcein-AM	• Substrate
	Hoechst 33342	• Substrate
	Rhodamine 123	• Substrate
HMG-CoA reductase inhibitors	Lovastatin	• Substrate

	Atorvastatin	• Substrate • Inhibitor
Antiemetics	Ondansetron	• Substrate
Tyrosine kinase inhibitors	Imatinib mesylate	• Substrate
	Gefitinib	• Substrate
Cardiac glycosides	Digoxin	• Substrate
Anthelmintics	Ivermectin	• Substrate
Calcium-channel blockers	Verapamil	• Substrate • Inhibitor
	Nifedipine	• Substrate
Calmodulin antagonists	Trifluoperazine	• Substrate
	Chlorpromazine	• Substrate • Inhibitor
	Trans-flupentixol	• Substrate
Antihypertensives	Reserpine	• Substrate • Inhibitor
	Propanolol	• Substrate • Inhibitor
Antibiotics	Erythromycin	• Substrate
	Ketoconazole	• Substrate
Steroids	Corticosterone	• Substrate
	Progesterone	• Substrate • Inhibitor
	Cortisol	• Substrate
Pesticides	Methylparathion	• Substrate
	Endosulfan	• Substrate

Natural Products	Curcuminoids	• Substrate • Inhibitor
	Colchicine	• Substrate
Antialcoholism drug	Disulfiram	• Substrate
Psychotropics	Amitriptyline	• Substrate • Inhibitor
	Carbamazepine	• Substrate • Inhibitor
	Sertraline	• Substrate • Inhibitor

The drug-binding pocket of P-gp, located in the membrane leaflet on the cytoplasmic side, comprises the trans-membrane (TM) regions. These funnel shaped drug binding pockets are 6000 Å wide, which are narrower on the cytoplasmic side with the potential to accommodate two drug molecules simultaneously (Gottesman *et al.*, 2009:546, Sharom, 2008:110). Drug substrates enter the drug binding sites by means of “gates” formed by the cytoplasmic ends of different TM helices, which are in close proximity to each other (Gottesman *et al.*, 2009:546; Sharom, 2008:110). The ability of P-gp to transport various dissimilar chemical compounds is attributed to the flexibility of these binding sites. Drug molecules interact with different subsets of the residues in the binding pockets in an induced-fit fashion. Various drug binding sites forming mini-pockets exist, which sterically or allosterically interact with each other in a very complex way, leading to either the stimulation or inhibition of transport of a second drug molecule. It is presumed the P-gp transporter is poly-specific rather than non-specific (Aller *et al.*, 2009:1720; Sharom, 2008:110-113). The number of hydrogen bonds possible between the drug molecule and the binding site of P-gp as well as the binding strength determines the affinity of the drug for P-gp. The TM region of P-gp is rich in amino acid side chains, especially tryptophan, which play an important role during binding of substrates containing aromatic rings (Sharom, 2008:113). It is proposed that the surface area, together with the amphiphilic characteristics of chemical compounds, may play a role in P-gp activity (Lin & Yamazaki, 2003:64). It is furthermore important to note that the lack of structure-activity relationship between P-gp and its substrates may be attributed to the structural complexity of this efflux transporter protein (Lin & Yamazaki, 2003:64).

2.3.1.4 ATP dependence of P-gp related transport

For a drug to be transported by P-gp, binding of ATP to specific binding sites on the P-gp protein, as well as the subsequent hydrolysis thereof, are crucial for active transport to take place (Lin & Yamazaki, 2003:62, 63). The two ATP-binding domains of P-gp, also known as the nucleotide binding domains (NBDs), can be found on the intracellular side of the cell membrane. These ATP binding domains consist of three regions known as the Walker A and B motif; characteristic of proteins that bind ATP or GTP; and the Signature C-motif which is exclusive to the ABC superfamily (Lin & Yamazaki, 2003:62, 63; Sharom, 2006:981).

The transport of substrates by P-gp is a process involving two unified cycles. The first cycle involves the hydrolysis of ATP, which is the catalytic reaction that drives the transport process. During the second cycle or substrate transport cycle, the drug substrate is actively displaced from the cytoplasmic side of the membrane into the extracellular space. However, the details regarding these two interconnected cycles as well as the coupling mechanism remains unresolved (Sharom, 2008:113).

During the catalytic cycle, the drug substrate as well as ATP bind with low affinity to the NBD of P-gp, a step that does not require energetic input. The rate-limiting step is the hydrolysis of ATP to adenosine diphosphate (ADP) and phosphate (P_i). This is a very important step in the transport process as it is a prerequisite for reducing the affinity of substrate binding, but not nucleotide binding due to the conformational changes occurring during the release of ADP. The NBD reloads with ATP and a second ATP hydrolysis event is initiated. Substrate binding is not possible during this stage. With the release of ADP and P_i , the catalytic cycle is complete and P-gp returns to its original state with the possibility to initiate the next cycle (Ambudkar *et al.*, 2003:7480-7481; Sharom, 2008:113).

The drug substrate is therefore transported by entering the binding pocket of P-gp on the cytoplasmic side of the membrane, after which it is moved either by the vacuum cleaner model or flippase model. Conformational changes now occur, driven by binding and hydrolysis of ATP as described in the catalytic cycle. Following the protein conformational changes, the drug substrate is then released in either the opposing membrane leaflet or the extracellular space (Lin & Yamazaki, 2003:62; Sharom, 2008:114).

2.3.1.5 Induction and Inhibition of P-gp

P-gp is susceptible to inhibition, activation as well as induction, which may alter the pharmacokinetics of co-administered drug substrates by elevation or reduction of

intracellular drug concentrations (Izzo, 2004:3; Marchetti *et al.*, 2007:930). Various inhibitors and inducers of P-gp have been identified as indicated in Table 2.1.

Inhibition of P-gp may either be competitive or non-competitive. Competitive inhibition occurs when two drug substrates compete for the same drug-binding site on P-gp. Non-competitive inhibition, conversely, occurs when one of the drug substrates inhibits the ATP hydrolysis cycle and thereby changes the conformation of the binding site by means of an allosteric mechanism (Marchetti *et al.*, 2007:930). Inhibition, especially competitive inhibition, occurs within a short time frame (Pal & Mitra, 2006:2136). One of the best known inhibitors of P-gp efflux is grapefruit juice. Co-administration of grapefruit juice with cyclosporine, an immunosuppressant agent that prevents organ rejection during organ transplants, results in a dramatic increase in the bioavailability of cyclosporine (Colombo *et al.*, 2014:2).

Induction of P-gp results from up-regulation of the *ABCB1/MDR1* gene by means of the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) acting as sensors for drug substrates. The cellular uptake of drug substrates results in the activation of CAR. Activation causes both CAR and PXR to dimerise with the retinoid-X-receptor (RXR), forming a heterodimer that binds to the response elements located on the *ABCB1/MDR1* gene. This prompts the transcription of the specific gene causing an increase in messenger ribonucleic acid (mRNA) for protein formation (Pal & Mitra., 2006:2140). Induction is time dependent, which results in a reduction of the intact drug reaching the systemic circulation, leading to an active decrease in oral bioavailability and efficacy of co-administered drugs (Pal & Mitra, 2006:2136). An example is the co-administration of St John's Wort, a known inducer of P-gp, with cyclosporine, a known P-gp substrate, resulting in a decrease in cyclosporine plasma concentrations (Colombo *et al.*, 2014: 2).

2.3.1.6 Physiological function of P-gp and genetic polymorphism

Physiologically, P-gp plays a detoxifying role in the body and offers protection against the onslaught of toxic xenobiotics as well as their metabolites. The body is protected through the effective extrusion of toxic xenobiotics and accompanying metabolites back into the intestinal lumen as well as other excretory organs, preventing accumulation in vital organs such as the brain and offering protection to the developing foetus (Marchetti *et al.*, 2007:928). P-gp affects the rate at which drug substrates are absorbed, distributed and eliminated. Efflux of drugs by P-gp is therefore an important factor to consider during drug design (Sharom, 2008:117). The role of P-gp in the development of drug resistance during

cancer treatment is also widely known and an important factor to take into consideration for optimised treatment (Ambudkar *et al.*, 2003:7469; Lin & Yamazaki, 2003:60).

Inter-individual genetic variability attributes to the problem of variable pharmacokinetics during pharmacotherapy. Various genetic polymorphisms of P-gp have been reported in both humans and animals. Genetic polymorphism in the ABC drug efflux pump family contributes to the impact of efflux on drug bioavailability (Chan *et al.*, 2004:42; Sharom, 2008:117). A point mutation affecting a percentage of the population is considered a single nucleotide polymorphism (SNP), which may either be synonymous, silent or non-synonymous, resulting in a change to the coding sequence. SNPs may lead to variances in protein expression and transport functionality affecting the absorption, distribution and elimination of drugs, as well as plasma concentrations (Sharom, 2008:117).

Cancer cells in humans presented signs of genetic polymorphism for P-gp during *in vitro* studies until the first reports from Hoffmeyer *et al.* (2000:3477) gave insight into *in vivo* polymorphisms by identifying several different MDR1 SNPs (Lin & Yamazaki, 2003:65; Hoffmeyer *et al.*, 2000:3477). It is estimated that 105 variants in the ABCB1 gene exists, varying in occurrence amongst different ethnic groups. However, many of these variants involve introns or non-coding regions and do not have an effect on the amino acid sequence of P-gp (Marchetti *et al.*, 2007:928).

Three of the most frequent SNPs of P-gp are C1236T in exon 12, G2677T/A in exon 21 and C3435T in exon 26 (Estudante *et al.*, 2013:1344). One of the most common SNPs, namely C3435T, is located at exon 26 and was 24% TT homozygous, which resulted in two-fold reduction of P-gp in subjects which were TT homozygous in contrast to the CC homozygotes (Chan *et al.*, 2004:42; Drescher *et al.*, 2002:527). C3435T, when expressed, is associated with alteration of the tertiary structure of P-gp, affecting its interaction with drugs and modulators (Sharom, 2008: 118). This SNP is associated with a high frequency in European/American subjects and was also found to be in association with two other SNPs, namely G2677T in exon 21 and C1236T in exon 12 (Chan *et al.*, 2004:42). The G2677T/A/C SNP is non-synonymous resulting in amino acid modification A893S, A893P and A983T altering substrate specificity as well as ATPase kinetic properties of P-gp (Sharom, 2008:118).

The frequency of the C3435T polymorphism at exon 26 was studied to identify both ethnic and inter-ethnic differences associated with MDR1. During this study, 1280 subjects were picked from 10 ethnic groups for evaluation. African populations comprised Ghanaians, Kenyans, African-Americans and Sudanese who had frequencies ranging from 73% to 84%, whereas the Caucasian/Asian populations comprised of British Caucasians, Portuguese,

southwest Asians, Chinese, Filipinos and Saudis presented with lower frequencies between 34% and 55%. This would suggest a possible increase in the expression of P-gp in African populations, correlating with their subjection to various environmental influences and corresponding large population size (Cordier & Steenkamp, 2011:54; Lin & Yamazaki, 2003:66).

2.4 METABOLISM

The principal site of metabolism for many drugs is the liver, however, other secondary organs and tissues may also be involved including the skin, lungs, mucosal cells of the gastrointestinal tract and also the microbiological flora of both the ileum and large intestine (Shargel *et al.*, 2005:303). Various chemical reactions in the liver are responsible for the metabolism or biotransformation of drugs, which include oxidation, reduction, hydrolysis and conjugation. Phase I reactions also known as asynthetic reactions involve oxidation, reduction and hydrolysis, which are responsible for biotransformation of most non-polar drugs into reactive oxygen intermediates. These reactive oxygen intermediates are further biotransformed by phase II conjugation reactions, also known as synthetic reactions, which result in a more polar compound with increased water solubility allowing rapid excretion through the bile and urine (Liska, 1998:190; Shargel *et al.*, 2005:320).

2.4.1 Cytochrome P450 (CYP) isoenzyme family

The mixed-function oxidases (MFOs) enzyme system is responsible for oxidation and reduction reactions of various chemically diverse endogenous and exogenous substances during drug biotransformation (Sheweita, 2000:107). These structural enzymes, found in the endoplasmic reticulum of hepatic parenchyma cells, comprise an electron-transport system dependent on molecular oxygen, reduced nicotinamide adenine dinucleotide phosphate (NADPH), cytochrome P450 (CYP), NADPH-cytochrome P450 reductase and also phospholipids. The phospholipids play an important role in the binding of the drug molecules to the CYP and are involved in the coupling of the NADPH-cytochrome P450 reductase to CYP (Shargel *et al.*, 2005:319).

Central to the MFO enzymes is the cytochrome P450 isoenzyme family (Sheweita, 2000:107). CYP enzymes are closely related isoenzymes, differing only in their amino acid sequence and drug specificity. CYP forms part of a haeme containing class of oxygenases with an iron protoporphyrin IX prosthetic group. This class of oxygenases is not only responsible for the metabolism of drugs and xenobiotics by means of oxidation and reduction, but also for the synthesis of steroid hormones, fat soluble vitamin (A, D, E & K)

metabolism and the conversion of polyunsaturated fatty acids into biologically active molecules (Denisov *et al.*, 2005:2253).

2.4.2 CYP families, subfamilies and nomenclature

A multitude of CYP enzymes exist for which approximately 4 000 CYP genes have been identified. This superfamily of enzymes can be found not only in humans, but in a diversity of life forms (Danielson, 2002:562; Denisov *et al.*, 2005:2253). The various family names are each assigned an Arabic numeral and the subfamily is assigned a capital letter followed by another numeral, indicating the order in which they were discovered (Danielson, 2005:562).

Families are grouped according to amino acid sequence, if the sequence has a 40% similarity it is placed within a certain family. Accordingly, a 55% similarity results in the protein being placed in the same subfamily. Allelic variations occur when there is greater than 97% similarity (Danielson, 2002:562). Humans encode for a total of 57 CYP genes, as well as 27 pseudo-genes, organised into 18 families further divided into 43 subfamilies. Members of the CYP1, CYP2 and CYP3 families are of significant importance during biotransformation of drugs (Danielson, 2002:572).

2.4.3 CYP biochemistry

CYP serves as an important component of the electron transfer system found in the endoplasmic reticulum. It binds not only to oxygen, but acts as a substrate binding locus for drugs as well as endogenous substances, which is done in combination with the flavoprotein reductase and NADPH (Shargel *et al.*, 2005:365; Sheweita, 2000:111). Substrate metabolism by CYP produces an oxidised compound. The following figure illustrates the electron flow pathway and drug substrate binding of CYP during biotransformation of xenobiotics.

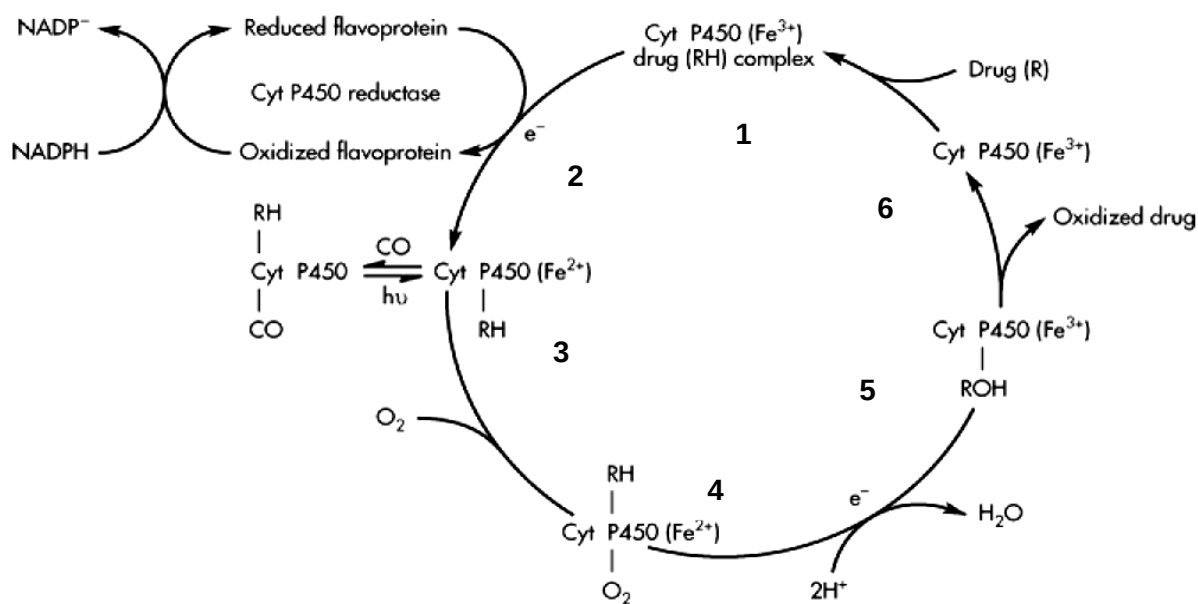


Figure 2.5: Schematic illustration of the electron flow pathway of cytochrome P450 during metabolism of a drug molecule (Darby *et al.*, 2011:723; Shargel *et al.*, 2005:365)

From Figure 2.5, it is clear the cycle starts with the binding of the drug substrate to the apoprotein moiety of the ferric (Fe³⁺) haemoprotein forming a ferric substrate complex (indicated by “1” in Figure 2.5). The ferric substrate complex is then subjected to electron reduction from NADPH cytochrome P450 reductase (indicated by “2” in Figure 2.5) which converts the haeme iron to a ferrous (Fe²⁺) state (indicated by “3” in Figure 2.5). The ferrous (Fe²⁺) complex reacts with molecular oxygen producing an oxygenated haemoprotein substrate complex (indicated by “4” in Figure 2.5) that undergoes additional electron reduction. The complex undergoes intermolecular rearrangement with the addition of molecular oxygen to the substrate, which produces a reactive oxygen intermediate (indicated by “5” in Figure 2.5). The haeme complex dissociates and thereby frees the enzyme and oxidised drug molecule (indicated by “6” in Figure 2.5) which enters into phase II conjugation reactions (Shargel *et al.*, 2005:365; Sheweita, 2000:111).

CYP is responsible for multitude metabolic reactions, including N-, S- and O-dealkylation, aromatic hydroxylation, N-and S-oxidation, deamination, desulfuration, epoxidation, peroxidation and dehalogenation, to name but a few (Shargel *et al.*, 2005:367). These chemical reactions result in the production of reactive oxygen intermediates, which are capable of secondary tissue damage, if not properly metabolised by phase II of the enzyme detoxification system (Liska, 1998:189).

2.4.4 Pre-systemic metabolism

Drugs administered by means of the oral route are susceptible to pre-systemic metabolism (Shargel, 2005:333; Thummel, 2007:3173). Pre-systemic metabolism refers to the metabolism of the drug before it reaches the general circulation, which takes into account the contributions of the small intestine and the liver (Doherty & Charman, 2002:236). Drugs that are orally administered are obligated to first pass through the gastrointestinal tract epithelium (as shown in Figure 2.6), then transported to the hepatic portal vein by means of the mesenteric vessel. Orally administered drugs are therefore exposed to intestinal epithelium and liver metabolism during this “first pass” movement before entering the systemic circulation (Shargel *et al.*, 2005:333; Thelen & Dressman, 2009:541). Drugs subjected to metabolism by the liver, as well as the enterocytes of the small intestine, may present with poor systemic bioavailability (Shargel *et al.*, 2005:333).

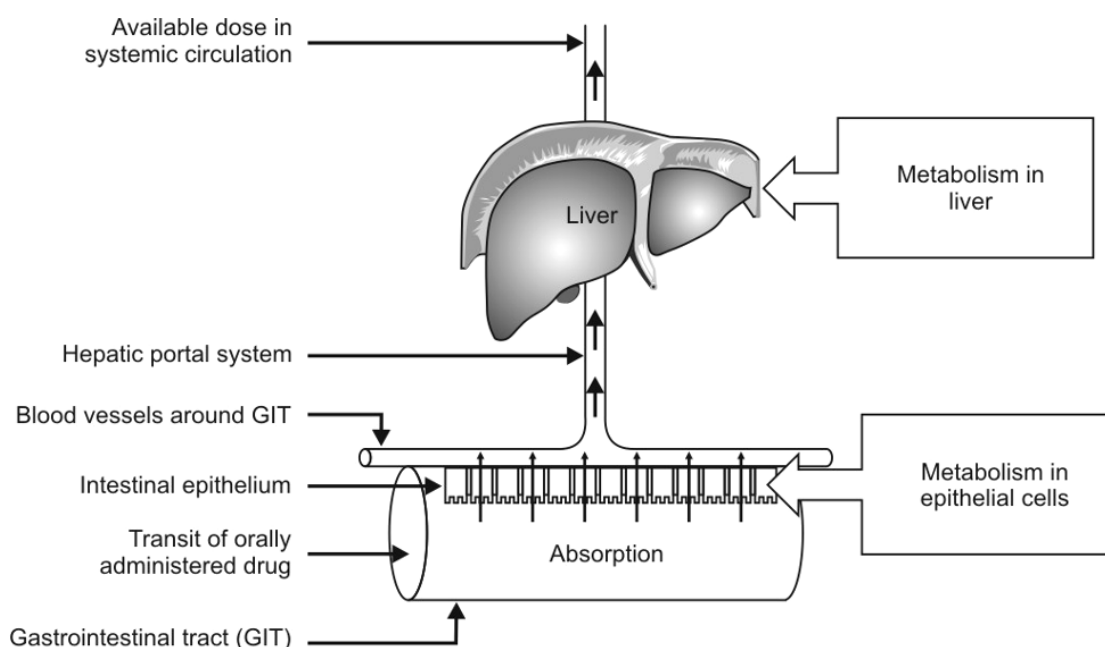


Figure 2.6: First pass metabolism of orally administered drugs (Hamman, 2007:64)

Bioavailability of a drug is determined by the product of the following (Thelen & Dressman, 2009: 541; Zhang & Bennet, 2001: 159):

- fraction of the absorbed dosage (F_a),
- fraction of the dosage absorbed that passed through the gastrointestinal tract epithelium into the hepatic portal vein unchanged (F_g),
- fraction that is not metabolised by the liver (F_h).

2.4.5 Distribution of CYP

The CYP enzyme system accounts for 30% of the hepatic metabolism activity and more than 70% of the intestinal metabolism activity (Hellum & Nilsen 2007:466; Pal & Mitra., 2006:2136). A substantial amount of the enzymes involved in Phases I and II reactions occurring in the liver, have also been detected in the intestinal epithelial cells of the gastrointestinal tract. These include first and foremost the CYP enzyme system, but also other metabolic enzymes such as sulfotransferase, epoxide hydrolase, alcohol dehydrogenase, glutathione S-transferase, sulfotransferase, acetyl transferase and uridine diphosphate glucuronosyltransferase (Thelen & dressman, 2009: 541).

In addition to the liver and intestinal mucosa, the CYP enzyme system can also be found in the kidney, brain, lungs, skin as well as the olfactory mucosa (Paine *et al.*, 2006:880; Shargel *et al.*, 2005:316). Of these tissues, the mucosa of the gastrointestinal tract remains the most significant extra-hepatic site for CYP metabolism (Paine *et al.*, 2006:880). The Figure 2.7 illustrates the distribution of CYP isoforms in the liver and intestinal mucosa.

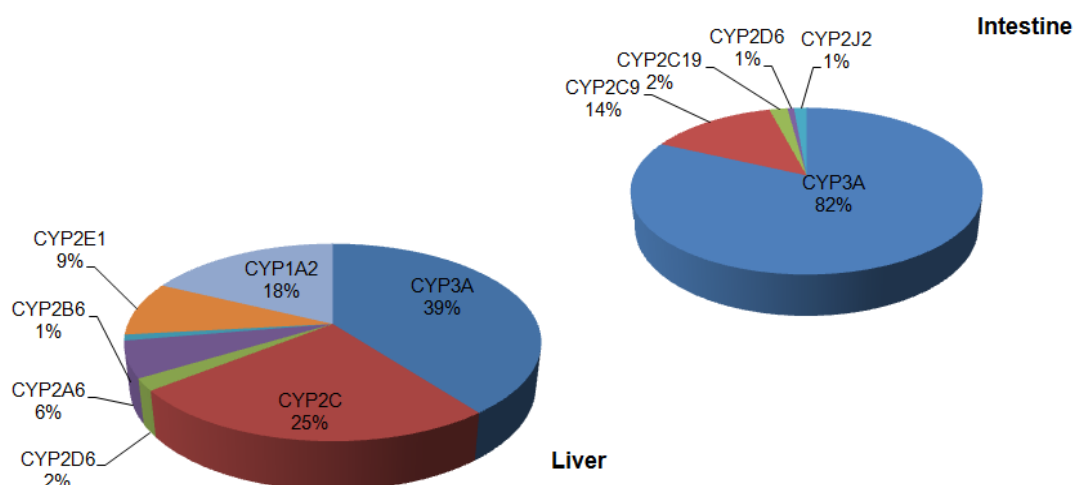


Figure 2.7: Distribution of CYP isoforms in the liver and intestinal epithelium (Paine *et al.*, 2006:884)

2.4.5.1 Intestinal CYP

Since the pathway of drug transport across the intestinal epithelium will play an important role in its metabolism, it will briefly be described here. Drug absorption from the gastrointestinal tract can occur by one of two pathways, namely the transcellular pathway through the epithelial cells, or the paracellular pathway through the intercellular spaces between epithelial cells (Thelen & Dressman, 2008:543).

Figure 2.8 schematically illustrates the pathways and mechanisms by which drugs can be absorbed from the gastrointestinal tract.

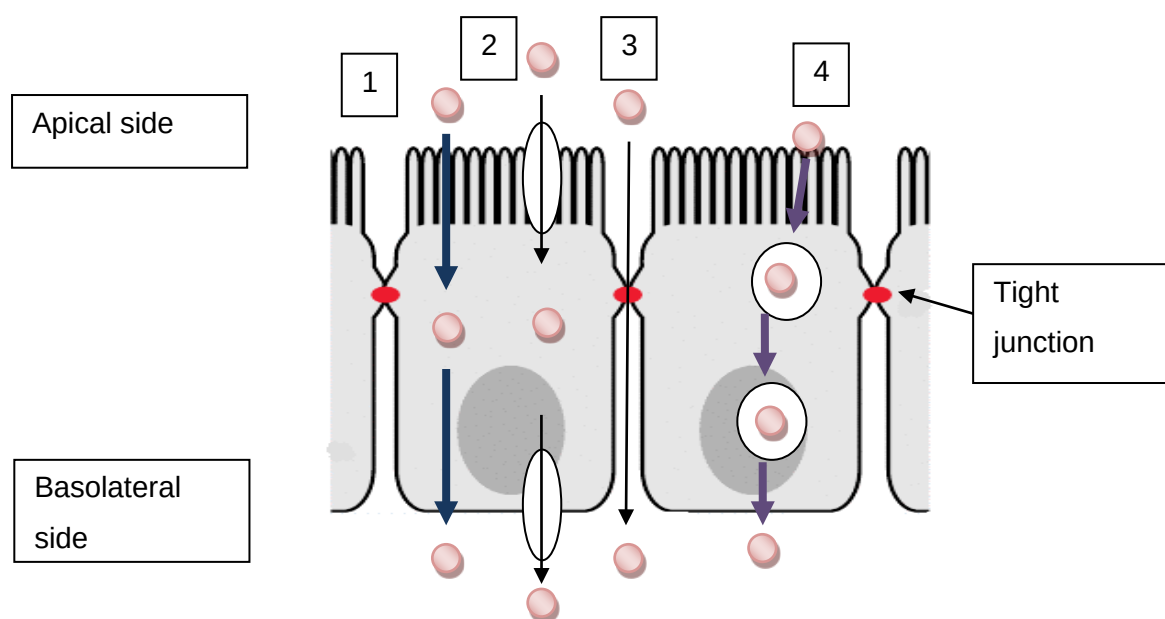


Figure 2.8: Schematic illustration of the pathways by which drugs cross the intestinal epithelium: Passive transcellular diffusion (1), carrier-mediated transport (2), passive paracellular diffusion (3) and endocytosis (4) (adapted from Thelen & Dressman, 2009:543)

The pathway by which drugs are absorbed across the intestinal epithelium is of particular significance with respect to pre-systemic metabolism in the intestinal mucosa, because drugs absorbed by the paracellular pathway will not be influenced by intracellular CYP enzymes (Thelen & Dressman, 2009:543). The blood flow rate through the small intestine is also of importance since the mucosal blood flow determines the intracellular residence time and the effective duration of exposure of drug molecules to the intracellular CYP enzymes (Thelen & Dressman, 2009:543).

The most abundant CYP enzyme expressed in the intestines is CYP3A. This is expressed by a single cell type found in the mucosal lining, the mature absorptive columnar epithelial cells also known as enterocytes (Paine *et al.*, 2006:880). The amount of CYP3A expressed is not uniform and decreases as one moves through the GI tract, with the highest expression in the proximal region declining towards the distal ileum (Paine *et al.*, 2006:881; Thelen & Dressman, 2009:544). Expression of CYP in the villi of the small intestine also varies, where the highest concentration of CYP enzymes can be found in mature enterocytes which line the tip of the villi. A lower concentration is expressed in the goblet cells as well as the epithelial cells of the crypts found between the villi, which is not surprising as the villi are the primary site of absorption (Thelen & Dressman, 2009: 544).

The most abundant CYP subfamily found in the intestinal epithelium is CYP3A, comprising CYPs 3A3, 3A4, 3A5, 3A7 and 3A43, of which CYP3A4 is the most abundant. This is followed by the CYP2C subfamily, consisting of CYPs 2C8, 2C9, 2C18 and 2C19, as well as CYP2J2, CYP2D6, CYP1A1/2 and CYP2E1 (Paine *et al.*, 2006:881; Thelen & Dressman, 2009:545). The CYP3A family accounts for approximately 80% of the total CYP content found in the intestines, however, large inter-individual variability in the expression and therefore, abundance of CYP3A have been noted (Thelen & Dressman, 2009:545). Although expressed at lower levels in the intestine, the CYP3A subfamily contributes to higher metabolism of certain drug substrates in the intestines when compared to that of the liver (Galetin & Houston, 2006:1220; Thelen & Dressman, 2009:545).

2.4.5.2 Liver CYP

The liver, due to its relatively high expression of the CYP enzyme family, serves as the most important site for drug metabolism and biotransformation (Liska, 1998:189, Paine *et al.*, 2009:880; Shargel *et al.*, 2005:316). The liver consists of basic units known as liver lobules, containing parenchymal cells with intricate blood and lymph connections. Blood is supplied to the liver from both the hepatic artery and the hepatic portal vein and leaves by means of the hepatic vein, emptying into the vena cava (Shargel *et al.*, 2005:316). Metabolism in the liver is dependent on two factors, blood flow and site of blood flow. Metabolising enzymes are not uniformly dispersed as within the small intestine and therefore blood flow influences which enzymes are reached as well as the fraction of the drug that reaches the enzymes (Shargel *et al.*, 2005:316). The most abundant CYP3A subfamilies are 3A4 and 3A5, which represent 40% of the total CYP content in the liver, second most abundant is the CYP2C subfamily (18% of the total CYP content), comprising of 2C8, 2C9, 2C18 and 2C19, followed by CYP1A2 (13%), CYP2E1 (7%), CYP2A6 (4%), CYP2D6 with less than 2% and CYP2B6 with less than 1% of the total CYP content (Paine *et al.*, 2006:880).

2.4.5.2.1 CYP induction

Induction of CYP refers to the increase in enzyme expression due to a chemical stimulus of the gene (Pelkonen, 2009:126). During induction of CYP3A4, the pregnane X receptor (PXR), as well as the constitutive androstane receptor (CAR), are activated. PXR and CAR can be activated via two mechanisms, either by the triggering of the cytoplasmic translocation of CAR, thus endorsing the release of certain unknown proteins, or the direct activation of PXR in the nucleus. Activation causes both CAR and PXR to homo-dimerise with the retinoid-X-receptor (RXR), forming a heterodimer that binds with the response elements located on target genes. This prompts an increase in the transcription of

the specific gene, thus, increasing messenger ribonucleic acid (mRNA), which in turn results in increased enzyme production and consequently an increase in the enzyme activity (Pal & Mitra, 2006:2136-2140; Pelkonen, 2009:133; Wilkinson, 2005:2216).

Induction of the CYP enzyme system may result in a decrease in the plasma concentration of an orally administered drug, thus, effectively reducing the bioavailability and efficacy of the drug (Bibi, 2008:4; Pelkonen, 2009:127; Wilkinson, 2005:2215). Conversely, an increase in CYP expression may result in enhanced pharmacodynamic effects of pro-drugs which require metabolic activation (Pelkonen, 2009:127). The time course of CYP induction is dependent on both the half-life of the drug as well as the enzymatic turnover. The known CYP inducer, Rifampicin, induces CYP within 24 h due to its short half-life compared to Phenobarbital, with a half-life of 3 to 5 days, which requires a week for induction of CYP to become apparent (Bibi, 2008:4, Wilkinson, 2005:2216). This up-regulation of CYP enzymes by certain drug modulators may have a detrimental effect on co-administered drug substrates in terms of their bioavailability (Wilkinson, 2005:2216).

2.4.5.2.2 CYP inhibition

Inhibition of the CYP enzyme system results in the increased bioavailability of orally administered drugs that are CYP substrates, causing in some instances, heightened adverse effects and even possible drug toxicity (Wilkinson, 2005:2214). Inhibition is possible by means of one of three mechanisms namely competitive, non-competitive and mechanism-based inhibition (Bibi, 2008:3).

Competitive inhibition

During reversible competitive inhibition, the inhibitor competes with the drug substrate for the same binding site located on the enzyme (Palmer & Bonner, 2007:126). Competitive inhibition of metabolic enzymes is, for example, observed during the co-administration of the beta-adrenoceptor blocking agent, metoprolol, and profenone resulting in a two-fold decrease in the biotransformation of metoprolol with increased blood levels. Competitive inhibition of enzymatic metabolism is also observed during the co-administration of fluoxetine and desipramine, as well as diltiazem and cyclosporine (Bibi, 2008:3, Wilkinson, 2007:2214).

Non-competitive inhibition

In reversible non-competitive inhibition, the substrate as well as the inhibitor binds at different binding sites on the enzyme, which produces a dead-end complex. Dissociation of the inhibitor molecule is mandatory before reaction between the enzyme and substrate can commence (Palmer & Bonner, 2007:127-136). Non-competitive inhibition occurs when

cimetidine is co-administered with other drug substrates. This is attributed to the fact that cimetidine forms a very stable CYP-substrate complex when it binds to the CYP enzyme, preventing the metabolism of other co-administered drug substrates by that specific enzyme (Bibi, 2008:4).

Mechanism-based inhibition

Mechanism-based inhibition refers to binding of a drug substrate resulting in the formation of metabolic intermediates which deactivate the enzyme, forming tight irreversible bonds with the binding site (Zhou *et al.*, 2005:3). Mechanism-based inhibition occurs during the co-administration of terfenadine and erythromycin or any other inhibitor such as ketoconazole, diltiazem or verapamil. This specific example of enzymatic inhibition may result in prolonged electrocardiographic intervals attributed to prolonged cardiac repolarisation, which may result in cardiac dysrhythmia, better known as *torsades de pointes*, which could lead to sudden death (Bibi, 2008:3; Wilkinson, 2005:2214). The cause of *torsades de pointes* is attributed to the formation of a complex between a nitro compound, usually a metabolite, demethylated by the CYP enzyme system and the CYP enzyme forming an irreversible metabolite intermediate complex (Bibi, 2008:3).

2.4.6 Inter-individual variability of CYP

Genetic variations found in drug metabolising enzymes are known to contribute to differences in drug responses due to altered plasma concentration levels found in individuals. These inter-individual differences may have clinically significant consequences (Al Omari & Murry, 2007:206). The environment, physiological status, disease state, as well as genetic polymorphisms are known contributors in the variation of CYP enzyme activity, which affect the bioavailability and adverse effects of certain drugs (Al Omari & Murry 2007:207). Each of the CYP enzymes are encoded by a specific gene and genetic alleles are inherited from each parent. These alleles are described as “wild type” (occurring predominantly) or “variant”. When a “wild type” allele is replaced by a “variant”, polymorphism occurs (Lynch, 2007:391). In terms of genetic variability, four metabolic classes exist in which these individuals can be grouped. An individual receiving two “variant” alleles has a lack of functional enzyme and is referred to as a “poor metaboliser”. “Intermediate metabolisers” are individually heterogeneous in terms of alleles, receiving both the “wild type” and “variant” alleles. “Extensive metabolisers” have received two “wild type” alleles and “ultra-rapid metabolisers” received multiple copies of the “wild type” alleles (Ingelman-Sundberg, 2004:193; Lynch, 2007:391).

There are 57 genes in the human genome known to encode the various CYP genes of the CYP enzyme family (Danielson, 2002:572). Genes found in the CYP1, CYP2 and CYP3 sub-families are all polymorphic and responsible for the metabolism of a vast amount of clinically used drugs (Ingelman-Sundberg 2004:193; Zanger & Schwab 2013:104). It is estimated that 90% of drug metabolism in humans is attributed to the following CYP enzymes: CYP1A2, 2C9, 2C19, 2D6, 3A4 and 3A5. CYP3A contributes approximately 50% and CYP2D6 between 20% and 30% to the total metabolism of drugs (Cordier & Steenkamp, 2011:53). There are, however, differences in the functional importance of the variant alleles as well as differences in ethnic groups in terms of distribution frequencies. The genetic variation of selected CYP isoenzymes will be discussed below.

CYP2C9

The CYP2C9 enzyme is responsible for the catalytic activity against the anti-coagulant drugs, arbuturate sedatives, anti-diabetic agents and anti-convulsants (Danielson, 2002:580). Thirty allelic variants have been identified for CYP2C9 and of these the alleles, *CYP2C9*2*, *CYP2C9*3*, *CYP2C9*5* and *CYP2C9*11* have the most prominent decreasing effects on the enzymatic function (Ali *et al.*, 2009:110; Cordier & Steenkamp, 2011:55). The frequency of *CYP2C9*2*, *CYP2C9*5* and *CYP2C9*11* alleles are very low in Caucasians (0.25%) and Asians (<0.25%), occurring predominantly in Africans (Cordier & Steenkamp, 2011:55; Danielson, 2002:580).

CYP2D6

Major CYP2D6 substrates include β -adrenergic blocking agents, neuroleptics, anti-depressants, anti-arrhythmics and narcotic analgesics (Danielson, 2002:580; Ingelman-Sundberg, 2005:7). It is estimated there are more than 80 known polymorphic CYP2D6 alleles for which Caucasians and Asians present with a 7% and 50% frequency, respectively. The following are of importance: *CYP2D6*2*, *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6*, *CYP2D6*9*, *CYP2D6*10*, *CYP2D6*17*, *CYP2D6*29* and *CYP2D6*41* (Cordier & Steenkamp, 2011:55; Ingelman-Sundberg, 2005:8). *CYP2D6*3*, *CYP2D6*4*, *CYP2D6*5*, *CYP2D6*6* and *CYP2D6*4xN* result in inactivity of the enzyme and are found in high frequencies in Western, Southern and Northern Africa. *CYP2D6*9*, *CYP2D6*10*, *CYP2D6*17*, *CYP2D6*29* and *CYP2D6*41* result in decreased enzyme activity prevalent in Eastern and Western populations. *CYP2D6*1xN*, *CYP2D6*2xN*, *CYP2D6*10xN* and *CYP2D6*41xN* result in increased enzyme activity with a high frequency of 21-29% in Northern Africa, decreasing in frequency from Central to Southern Africa (Cordier & Steenkamp, 2011:55; Danielson, 2002:581). *CYP2D6*4* and *CYP2D6*41* are more prevalent amongst the Caucasians, with *CYP2D6*17* and *CYP2D6*29* amongst Africans. Of

note is the high frequency of *CYP2D6*5* in the coloured South African population (Cordier & Steenkamp, 2011:55).

CYP3A4/5

CYP3A4 and *3A5* overlap in substrate specificity and are responsible for metabolism of a broad range of drugs such as antibiotics, anti-arrhythmics, benzodiazepine sedatives, immunomodulating agents, antiretrovirals, antihistamines and calcium channel blockers, to name but a few (Danielson, 2002:583). Up to 20 allele variants have been identified for *CYP3A4* namely *CYP3A4*2*, *CYP3A4*3*, *CYP3A4*4*, *CYP3A4*5*, *CYP3A4*6*, *CYP3A4*7*, *CYP3A4*8*, *CYP3A4*9*, *CYP3A4*10*, *CYP3A4*11*, *CYP3A4*11b*, *CYP3A4*11c*, *CYP3A4*12*, *CYP3A4*13*, *CYP3A4*14*, *CYP3A4*15*, *CYP3A4*16*, *CYP3A4*16b*, *CYP3A4*17*, *CYP3A4*18*, *CYP3A4*18b* and *CYP3A4*19* (Danielson, 2002:583, Keshava *et al.*, 2004:828). *CYP3A4*8*, *CYP3A4*11*, *CYP3A4*11b*, *CYP3A4*13*, and *CYP3A4*17* are known to decrease enzyme activity, whereas *CYP3A4*20* causes no activity change in the enzyme and *CYP3A4*18* results in an increased activity. Overall, there is a very low frequency of the allelic variants in the gene expression of this enzyme. Inter-individual variation observed in *CYP3A4* may be attributed to genes encoding PXR and CAR, which are involved in the posttranslational regulation of *CYP3A4* (Božina *et al.*, 2009:232).

Allelic variations in the *CYP3A5* gene include *CYP3A5*1B*, *CYP3A5*1C*, *CYP3A5*2*, *CYP3A5*3*, *CYP3A5*5*, *CYP3A5*6*, *CYP3A5*7* and *CYP3A5*11* (Cordier & Steenkamp, 2011:55; Roy *et al.*, 2005:886). The expression of the *CYP3A5*3* and *CYP3A5*6* variations reduces the synthesis of the functional *CYP3A5* enzyme, thus effectively reducing its drug substrate clearance (Božina *et al.*, 2009:232; Roy *et al.*, 2005:886). *CYP3A5*3* is highly expressed in most ethnic populations, for example between 70 to 90% in Caucasians, 75% in Asians and 50 to 75% in African-Americans and Zimbabweans, whereas *CYP3A5*6* is only expressed in low frequencies amongst African-Americans and Zimbabweans (Roy *et al.*, 2005:886). The *CYP3A5*1B* and *CYP3A5*1C* variants result in an increase in the clearance of drug substrates which may influence efficacy. Both allelic variants are expressed in very low frequencies in Caucasians, whilst being highly expressed in African-Americans (Božina *et al.*, 2009:232; Cordier & Steenkamp, 2011:55; Roy *et al.*, 2005:886). The *CYP3A5*11* variation is extremely rare, occurring predominantly in Caucasians, resulting in a decrease of the catabolic activity of *CYP3A5* (Cordier & Steenkamp, 2011:55).

2.5 INTERPLAY BETWEEN CYTOCHROME P450 AND P-GLYCOPROTEIN DURING HERB-DRUG INTERACTIONS

It is apparent that CYP3A4 and P-gp are two of the major determining factors in oral bioavailability of drug substrates (Van Waterschoot & Schinkel, 2011:391). Not only is there a possibility that administered drugs may present with overlapping substrate specificity for both CYP3A4 and P-gp, but the strategic co-localisation in both the enterocytes and hepatocytes further attributes to possible interplay (Van Waterschoot & Schinkel, 2011:391; Zhang & Benet, 2001:163,164). The latter may result in a possible synergistic effect, specifically in the small intestine as P-gp efflux prolongs exposure to CYP3A4 during the repetitive counter transport of substrates (Zhang & Benet, 2001:163,164). This synergistic reaction limits the amount of intact drug that will reach the systemic circulation (Zhang & Benet, 2001:164). Figure 2.9 schematically illustrates the interplay between P-gp and CYP mediated metabolism.

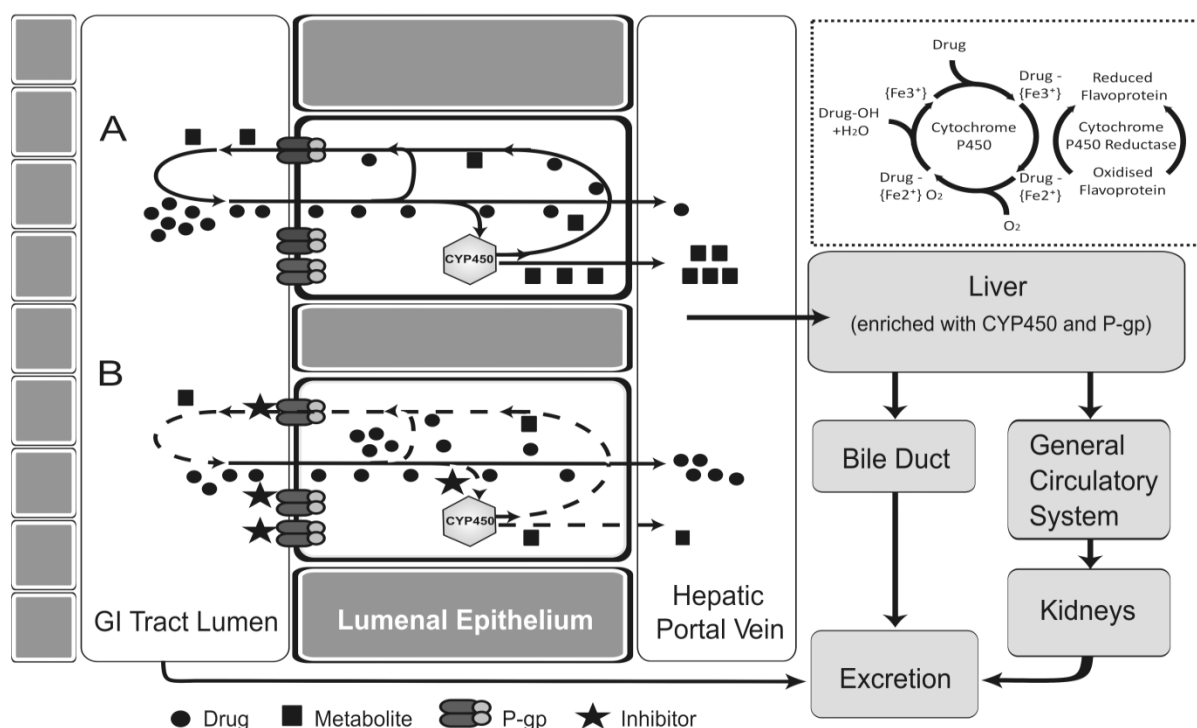


Figure 2.9: Schematic illustration of the interplay between P-glycoprotein and cytochrome P450 metabolism (Darby *et al.*, 2011:723)

During the co-administration of two or more drugs which are substrates for CYP3A4 and P-gp, the possibility of drug-drug interactions arises. These interactions may be caused by the induction or inhibition of CYP3A4 and P-gp, respectively. A sub-therapeutic drug plasma concentration is the result of induction of both P-gp and CYP3A4, with a resulting reduction in drug bioavailability and efficacy. Conversely, the inhibition of both P-gp and CYP3A4

result in increased bioavailability and adverse effects exceptionally harmful during the administration of drugs with narrow therapeutic indexes (Pal & Mitra, 2006:2136).

There are various known herb-drug pharmacokinetic interactions. In a recently published review article by Calitz *et al.* (2014), the herb-drug interactions involving various traditional African herbs were evaluated and discussed. Since the candidate has been involved in writing this review article, it will not be repeated in the literature review chapter, but instead it is included in Appendix A.

2.6 INDINAVIR (USED AS MODEL COMPOUND IN THE TRANSPORT AND METABOLISM STUDIES)

Indinavir is an antiretroviral drug, more specifically, an HIV protease inhibitor and is also a substrate for both P-gp and CYP 3A4 (Hochman *et al.*, 2000:315,316; Lin, 1999:34). Indinavir is a class II drug according to the Biopharmaceutics classification system (BCS), thus having low solubility and high permeability (Wu & Bennet, 2005:12, 21). It is commercially available as Crixivan[®] capsules (indinavir sulfate), with a molecular chemical formula of $C_{36}H_{47}N_5O_4 \cdot H_2SO_4$ (chemical structure is shown in Figure 2.10) and a molecular weight of 711.88 g/mol (see package insert in Appendix B).

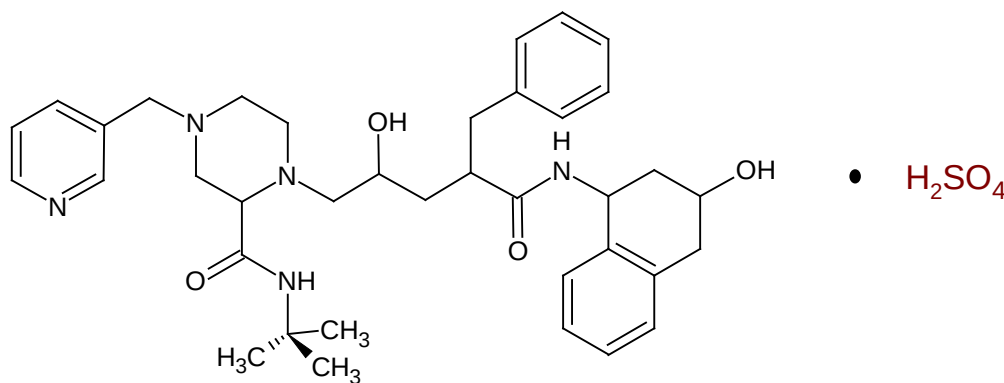


Figure 2.10: Chemical structure of indinavir sulphate

During the hepatic and intestinal metabolism of indinavir, seven prominent metabolites are produced namely M1, M2, M3, M4a, M4b, M5 and M6 (Chiba *et al.*, 1997:1190). Metabolite M1 is a quaternised ammonium conjugate, which is produced by glucuronidation at the pyridine nitrogen; M2 and M4a by pyridine N-oxidation; M4b by means of *para*-hydroxylation of the phenyl methyl group; metabolites M2, M3 and M5 are formed during the 3'-hydroxylation of the indan moiety; M5 and M6 by means of N-depyromethylation (Chiba *et al.*, 1997:1190; Lin, 1999:41). The N-dealkylation biotransformation pathway serves as the

most important during metabolism of indinavir as more than 50% thereof is metabolised to the N-dealkylated metabolites, M5 and M6 (Chiba *et al.*, 1997:1190).

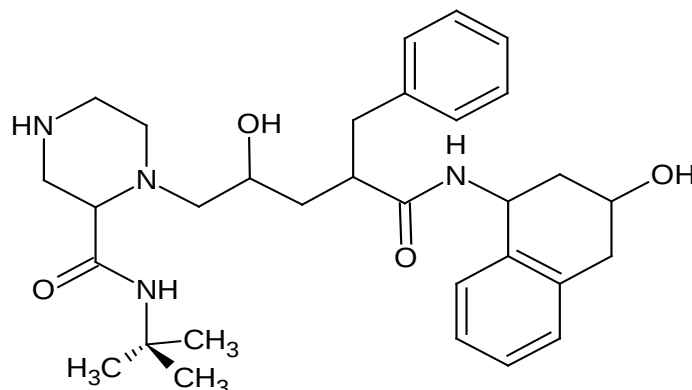


Figure 2.11: Chemical structure of the N-dealkylated metabolite M6 of indinavir

Hormonal contraceptives, anti-tuberculosis drugs (e.g. rifampicin) and anti-convulsants (e.g. phenobarbital, phenytoin and carbamazepine) are examples of drugs that have interactions with indinavir during co-administration (Cohen *et al.*, 2002:45).

2.7 OVERVIEW OF SELECTED COMMERCIAL HERBAL PRODUCTS USED IN THE TRANSPORT AND METABOLISM STUDIES

HIV/AIDS patients on ARV treatment often use supplements and herbal products in addition to their allopathic medicines, therefore, herbal products commercially available and often used by patients as immune system boosters were selected for this project. Herbal remedies such as Canova[®], Linctagon Forte[®] and Viral choice[®] are composed of extracts from various herbs that each may exert possible interactions with the metabolism and transport of indinavir.

2.7.1 Canova[®]

Canova[®] is a homeopathic product composed of extracts from *Aconitum napellus* (Ranunculaceae), *Arsenicum album* (arsenic trioxide), *Bryonia alba* (Cucurbitaceae), *Apis mellifica* (Apidae), *Lycopodium clavatum* (Lycopodiaceae), *Pulsatilla nigricans* (Ranunculaceae), *Asa foetida*, *Rhus toxicodendrum*, *Barita carbônica*, *Ricinus communis* (Euphorbiaceae), *Silicea*, *Calcarea carbônica*, *Conium maculatum* (Apiaceae), *Veratrum album* (Liliaceae), *Carapichea ipecacuanha* (Rubiaceae), *Lachesis muta* (Viperidae) and *Thuya occidentalis* (Cupressaceae) (see package insert of Canova[®] in Appendix C). Canova[®] is known as an immune response modifier directed at macrophages that result in an increase in their production, together with the release of cytokine TNF α in cancer and

AIDS patients having an anti-inflammatory effect (De Oliveira *et al.*, 2008:3; Takahachi *et al.*, 2007:350). A picture of the commercially available Canova® product is shown in Figure 2.12.



Figure 2.12: Picture of the Canova® drops product used in this study (www.immunesupplementreview.com, 2014)

Aconitum napellus (Ranunculaceae) is a toxic plant originally used by ancient tribes to produce poisonous arrows and spears. Active as well as toxic constituents include aconitines, benzoyleaconines and aconines that influence the voltage-dependant sodium channels, resulting in cardiotoxicity and neurotoxicity (Fujita *et al.*, 2007:132; Yang *et al.*, 2013a:561). Aconite has been in use since 1363 AD as treatment for fever, diarrhoea, indigestion, coughs and as an aphrodisiac (Rastogi, 2011:234,235). Aconite has also been employed as an analgesic, cardiotonic and anti-inflammatory agent (Yang *et al.*, 2013a:561). During a study covering *in vitro* (Caco-2 and MDCKII-MDR1 cells), *in situ* (single-pass intestinal perfusion), *in vivo* (Sprague-Dawley rats) and *in silico* (molecular modelling) methods by Yang *et al.* (2013a), it was determined that aconitum is significantly transported by P-gp (Yang *et al.*, 2013b:199). Tang *et al.* (2011) investigated the involvement of various CYP isoenzymes in the metabolism of aconitine by means of human liver microsomes, as well as recombinant CYP enzymes. It was determined that CYP3A4 and CYP3A5 were responsible for the metabolism of aconitine, whereas CYP2C8, 2C9, 2D6 and 2C19 played a lesser role in the metabolism (Tang *et al.*, 2011:53). Both studies suggested that co-administration of aconitine together with other P-gp and CYP3A4/5 substrates may result in possible pharmacokinetic herb-drug interactions.

Bryonia alba (Cucurbitaceae) dates back to ancient Greece and had both medicinal and cosmetic uses. As homeopathic medicine it is used as an anti-inflammatory agent. There is currently no data available with regards to the effect of *B. alba* on CYP3A4 and P-gp.

Lycopodium clavatum (Lycopodiaceae) is used as a pollen paste for the treatment of cuts and wounds (Uprety *et al.*, 2010:7). In the Cree nations it is known as “Pashtanhoagin” and used as an anti-diabetic (Tam *et al.*, 2011:15). During a study using a cell free model by

Tam *et al.* (2011), the effect of *L. clavatum* on the NADPH-dependant inhibition of CYP3A4 was examined. It was found that *L. clavatum* significantly influenced both the NADPH and time dependent inhibition of CYP3A4 when combined with testosterone. This suggests that *L. clavatum*, like grapefruit juice, may possibly result in mechanism-based inactivation of CYP3A4 (Tam *et al.*, 2011:14,19,20).

Pulsatilla nigricans (Ranunculaceae), also known as the wild flower or meadow anemone, is traditionally a Chinese herbal remedy used for the treatment of dysentery, diarrhoea, wounds and trauma. As homeopathic treatment, it is used as treatment for shock, anger, grief, bronchitis, chickenpox, digestive troubles and headaches (Souter, 2000; Wurges, 2005). Investigation of pharmacokinetic and pharmacological interactions during co-administration with other drugs has not yet been explored.

Asafoetida is the gum resin obtained from *Ferula asafoetida* (Apiacea) (Mahendra & Bisht, 2012:141). This gum resin, also known as devil's dung or "duiwelsdrek" due to its foul smell and nauseating taste, is used to give a distinctive taste to Worcestershire sauce. Medicinally, it is used as anti-spasmodic, diuretic, analgesic, anti-flatulence, vermifuge and treatment of asthma (Mahendra & Bisht, 2012:142; Milad & Mehrdad, 2011:2). Active constituents include sulphur containing compounds and various coumarins (Milad & Mehrdad, 2011:3). In a recent study by Al-Jenoobi *et al.* (2014), the effect of asafoetida resin on the modulation of CYP2D6 and CYP3A4 was evaluated *in vitro*, in human liver microsomes, as well as in a clinical *in vivo* study. The *in vitro* study suggested that asafoetida is responsible for the inhibition of CYP2D6, but a concentration dependent activation of CYP3A4. However, the *in vivo* study suggested that asoefetida significantly inhibits CYP3A4 activity with little to no inhibitory effect on CYP2D6 (Al-Jenoobi *et al.*, 2014:4,5).

Rhus toxicodendrum or poison ivy is the name used for the *Toxicodendron pubescens* (Anacardiaceae) plant (Dos Santos *et al.*, 2007:95). Traditional uses include the treatment of herpetic eruptions and use as an allergen, while current medicinal uses include treatment of oedema (Dos Santos *et al.*, 2007:100).

Ricinus communis (Euphorbiaceae), known as the castor plant, has been widely employed as a purgative and laxative. Pharmacological activities include anti-oxidant activity, anti-nociceptive activity, anti-asthmatic activity and anti-diabetic activity (Jena & Gupta, 2012:26-27). It was reported during an *in silico* study involving HerboChip[®] followed by fluorometric enzyme inhibition studies, that *Ricinus communis* may have a significant effect on the inhibition of CYP3A4 (Calitz *et al.*, 2014:5).

Conium maculatum (Apiaceae), also known as hemlock or poisonous hemlock, was historically used in Greece as a lethal poison administered to criminals. Medicinal uses include the treatment of herpes, erysipelas and breast tumours. However, due to extreme toxic effects, specifically on the central nervous system and very narrow therapeutic range, the medicinal uses are limited (Vetter, 2004:1374,1379). As a homeopathic treatment, hemlock is prescribed for nervous disorder, cancerous tumours and dizziness (Herbs2000.com, 2014).

Veratrum album (Liliaceae), also known as the false helleborine, has been in use since 377 BC where the famous Hippocrates employed it as an emetic (Ji *et al.*, 2009:194,195). It is a highly toxic plant resulting in abdominal pain accompanied by nausea and vomiting after ingestion, symptoms progress into cardiovascular effects which may ultimately result in death (Grobosch *et al.*, 2008:768). Active constituents include the alkaloids protoveratrine A, protoveratrine B, jervine, veratride and cevadine (Grobosch *et al.*, 2008:769). As a homeopathic treatment, *V. album* is prescribed for use after minor surgery relieving constipation and intestinal cramping. It is also useful as treatment for shock and trauma (elixirs.com, 2014).

Carapichea ipecacuanha (Rubiaceae), or ipecac, is traditionally used as an emetic and expectorant. Current studies on the pharmacological activities deemed *C. ipecacuanha* effective as treatment for dysentery, bronchitis, blood disorders, leukemia, cancer and induction of vomiting, etc. (Junior *et al.*, 2012:110). The active constituents in *C. ipecacuanha* are, amongst others, cephaeline and emetine. It was found that both these active constituents are substrates for CYP2D6 and CYP3A4. In an *in vitro* study involving human liver microsomes, both cephaeline and emetine resulted in low inhibitory action against CYP2D6 as well as CYP3A4 (Asano *et al.*, 2001:682).

Thuja occidentalis (Cupressaceae), or *Arbor vitae*, was traditionally used as contraceptive and abortifacient (EMEA, 1999:2). Pharmacological properties include *in vitro* HIV-1 activity, antibody production and cytokine induction with *in vivo* increase in antibody response as well as cytokine induction (Naser *et al.*, 2005:69-73). The essential oil from *T. occidentalis* contains the active ingredient α -thujone, which is a known substrate for CYP2A6 and CYP3A4. The latter suggests that co-administration with other CYP2A6 and CYP3A4 substrates may result in pharmacokinetic interactions (Pelkonen *et al.*, 2013:102,104).

The medicinal use of animals or products derived from organs and various body parts dates back to ancient time and is still in use today (Alves *et al.*, 2007:455). Snakes, for example, *Lachesis muta* (Viperidae), also known as the bushmaster, are notorious for their medicinal properties. The fat of *L. muta* is used as an ointment in the treatment of tumours and

furuncles, respectively (Alves *et al.*, 2007:461). The female honey bee, *Apis mellifica* (Apidae), is used in homeopathic remedies by crushing the bee as a whole which is then repeatedly diluted with lactose powder, distilled water or ethanol until no cellular remnants remain. The dilution can then be purchased as a tincture, gel or pellets. *Apis mellifica* is used in the treatment of bites, stings, pharyngitis, glomerulonephritis, headaches, influenza, allergies, arthritis, infertility and nephritic syndrome (wiseGeek, 2014).

Calcarea carbônica, or impure calcium carbonate (CaCO_3), is prescribed in homeotherapy as a so called “constitutional” for people suffering from cold with an inability to keep warm (Frey, 2005). Barita carbônica, or barium carbonate (BaCO_3), is used for the treatment of a variety of symptoms involved in physical, emotional and mental impairments (wiseGeek, 2014). Silicea, better known as silica, is used in homeotherapy as “mood” treatment for people lacking assertiveness, indecisiveness and confidence, as well as people suffering from exhaustion (wiseGeek, 2014). Arsenicum album (arsenic trioxide), the trace element of arsenic although toxic, has been used as a homeopathic treatment in a diluted form for the treatment of for example digestive disorders, anxiety, depression and obsessive compulsive disorder (webMD, 2014).

2.7.2 Linctagon Forte®

Linctagon Forte® is a natural product used to prevent and treat colds, flu and respiratory tract infections. It contains extracts of *Pelargonium sidoides*, as well as the natural occurring compounds quercetin and bromelain (See package insert Appendix D). A picture of the commercially available Linctagon Forte® product is shown in Figure 2.12.



Figure 2.13: Picture of the Linctagon Forte® tablet product used in this study (www.nativia.co.za, 2014)

Pelargonium sidoides (Geraniaceae) is a traditional South African herb, also available in the commercial herbal product Umckaloabo[®], of which the roots are used as treatment for diarrhoea, dysentery, colds, as well as improvement of upper respiratory tract viral infections and tuberculosis (Brendler & van Wyk, 2008:421,423, 428, 429; Schnitzler *et al.*, 2008:1114; Tahan & Yaman, 2012:2-3). The potential active constituents of *P. sidoides* are gallic acid and its methyl ester, (+)-catechin, as well as coumarins (Kayser *et al.*, 2001:123). The efficacy and the immunomodulating characteristics of *P. sidoides* can be attributed to the active constituents', gallic acid and its methyl ester (Kayser *et al.*, 2001:125). Currently, there are no known interactions between co-administered drugs and *P. sidoides* extract containing products with respect to P-gp and CYP3A4. However, due to synergism with anti-coagulants and anti-platelet agents (*in vitro*), as well as hepatotoxic agents and laxatives, *P. sidoides* extracts may theoretically increase the risk of bleeding, cause hepatotoxicity and have possible laxative effects. The inhibition of immunosuppressants *in vitro* has also been reported (Posadski *et al.*, 2012:610).

Quercetin [3,3',4,5,7-pentahydroxyflavone] is a natural occurring polyphenolic flavonoid present in apples, onions, tomatoes, broccoli and berries (Borska *et al.*, 2012:3375; Harwood *et al.*, 2007:2180; Russo *et al.*, 2012:7). Flavonols are known to exhibit a variety of biological and pharmacological effects such as anti-inflammatory, anti-oxidant, anti-carcinogenic, bacteriostatic and cardioprotective activities (Borska *et al.*, 2012:3375; Harwood *et al.*, 2007:2181). During an *in vivo* study on male Sprague-Dawley rats by Choi *et al.* (2011), it was found that quercetin has an inhibitory action on CYP3A4 in a concentration-dependent manner. It was also determined by means of rhodamine-123 (Rh-123) transport studies that P-gp activity is significantly inhibited by quercetin (Choi *et al.*, 2011:609). In another study by Borska *et al.* (2012), the cell line EPG85-257RDB (RDB-cells) exposed to 12 μ M quercetin, showed a significant reduction in the expression of P-gp. It is also believed that quercetin may have a possible modulating role in the MDR gene on a deoxyribonucleic acid (DNA) or mRNA level (Borska *et al.*, 2012:3382).

Bromelain is the aqueous extract of the leaves, stems and fruit of the pineapple plant (*Ananas comosus* Merr. mainly var. cayenne, Bromeliaceae) and forms part of a group of related proteolytic enzymes. It is used for systemic treatment of inflammatory and blood-coagulation related diseases (Chobotova *et al.*, 2010:148; Lotz-Winter, 1989:249; Maurer, 2001:1234). Active constituents include closely related proteinases attributing to bromelain's fibrinolytic, as well as anti-thrombotic effects; both observed *in vitro* and *in vivo* (Pavan *et al.*, 2012:1). Bromelain has been shown to have immune-modulatory effects acting as an anti-cancer therapeutic agent and is also used as treatment for sinusitis and bronchitis (Chobotova *et al.*, 2010:152 -154; Pavan *et al.*, 2012:1). Other therapeutic benefits include its ability to

enhance the absorption of drugs (Pavan *et al.*, 2012:1). Bromelain is absorbed by means of a self-enhanced paracellular route without degradation and maintaining biological activity (Bock *et al.*, 1998:1399; Pavan *et al.*, 2012:1).

2.7.3 Viral Choice®

Viral Choice® is a natural product containing a variety of minerals, vitamins, amino acids, trace elements, phytosterols, as well as *Echinacea purpurea* and *Allium sativum* extracts (see package insert in Appendix E). A picture of the Viral Choice® capsule product used in this study is shown in Figure 2.14.

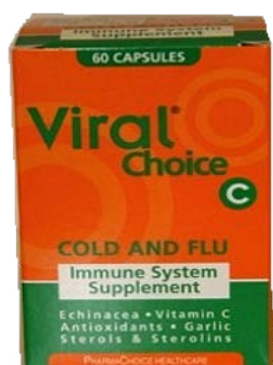


Figure 2.14: Picture of the Viral Choice® capsule product used in this study (www.ciao.co.uk, 2014)

Echinacea spp forms part of the Asteraceae family and extracts for medicinal purposes are prepared from three species: *E. purpurea*, *E. angustifolia* and *E. pallid* (Barrett, 2003:66-67; Gorski *et al.*, 2003:90; Hansen & Nilsen 2009:86). Only the aboveground parts of *E. purpurea* and roots of *E. pallid* have proven effective and suitable for oral treatment of certain conditions (Gorski *et al.*, 2003: 90). Common uses of *E. purpurea* include treatment of the common cold, mainly due to its proposed immune modulation, treatment of influenza, bronchitis, inflammation and it also has anti-fungal effects (Barrett, 2003:67; Gorski *et al.*, 2003:89). The active constituents of *E. purpurea* include polysaccharides, inulin, caffeic acids and derivatives of cichoric acid and echinacoside, alkaloids, alkylamides such as echinacein and polyacetylenes (Goldhaber-Fiebert & Kemper, 1999:4; Wills *et al.*, 2000:54). In an *in vitro* study by Budzinski *et al.* (2000), it was found that extracts of the root and aerial portions of *E. purpurea* and the roots of *E. angustifolia* had an inhibitory effect on cDNA expressing CYP3A4. This was confirmed by an *in vivo* study conducted by Gorski *et al.* (2004), during which 400 mg of *E. purpurea* root was administered 4 times daily for 8 days. It was found that the clearance of caffeine and tolbutamide serving as *in vivo* probes for CYP1A2 and CYP2C9 metabolism was significantly reduced. Clearance of midazolam, the

in vivo probe for hepatic CYP3A, was also significantly increased, which was consistent with previous *in vitro* findings (Gorski *et al.*, 2004: 96). An *in vitro* study by Hansen and Nilsen (2009) provided insight into the interaction of a 20% commercial *E. purpurea* ethanol extract with digoxin during P-gp mediated transport in the Caco-2 cell line. It was concluded that *E. purpurea* extract in a concentration range between 0.0064 mg/ml and 6.36 mg/ml may have a biphasic effect presenting low allosteric activation and possible inhibition at low and high concentrations, respectively (Hansen & Nilsen, 2009:87-90).

Allium sativum (Liliaceae), known as garlic, has dietary and medicinal uses (Banerjee *et al.*, 2003:97). The bulbous root of the garlic plant is used for medical purposes as a fresh plant, dehydrated, or in a steam distilled oil form. It is used as an immune system modulator, anti-microbial, anti-cancer, anti-atherosclerotic and lipid lowering agent (Banerjee *et al.*, 2003:97; Tattelman, 2005:103). Active constituents include high concentrates of thiosulfinates which are sulphur containing compounds (e.g. allicin) (Tattelman, 2005:103). Other active constituents includes diallyl disulphide, methyl-allyl thiosulfinates, 1-propenyl allyl thiosulfinates, L-glutamyl-S-alkyl-L-cysteine, allin, S-allyl cysteine, S-allyl mercaptocysteine, diallyl trisulphide, allyl methyl trisulfide, allyl methyl disulphide, diallyl tetrasulphide, allyl methyl tetrasulphide, dimethyl trisulphide, diallyl sulphide, 2-vinyl-4-H-1,3-dithiin, 3-vinyl-4-H-1,2-dithiin, E-ajoene, Z-ajoene and allylyl mercaptan. However, active constituents vary with different garlic preparations resulting in varying effects on the CYP enzyme system (Banerjee *et al.*, 2003:98; Colalto *et al.*, 2010:210).

2.8 SUMMARY

From the literature overview given in this chapter it is apparent that pharmacokinetic herb-drug interactions have high potential to influence the bioavailability and efficacy of not only anti-retroviral drugs, but also various other orally administered drugs. Factors to take into consideration include induction and inhibition of both efflux and metabolic pathways of orally administered drugs, as well as polymorphic inter-individual variability. Various herbs and drug substrates of both P-gp and CYP3A4 have been studied with regard to their influence on these pathways. However, the unregulated use of herbal supplementation as alternative medicine remains as popular as ever; even with limited knowledge regarding safety during prolonged used. The latter urges the investigation into possible pharmacokinetic interactions that may result during the concomitant usage of these herbal supplements and other prescribed drugs.

CHAPTER 3

MATERIALS AND METHODS

3.1 INTRODUCTION

Various methods have been developed providing insight into the mechanisms involved in drug transport and metabolism, which can be subdivided into three categories namely *in vivo*, *in situ* and *in vitro* models (Le Ferrec *et al.*, 2001:653). In this study, *in vitro* models were used, which are techniques employed throughout the pharmaceutical industry for pre-clinical screening tests (Balimane & Chong, 2005:336). *In vitro* models that can be used for drug transport studies include Ussing chambers, everted intestinal sac systems, cell based systems and artificial lipid-based systems (Alqahtani *et al.*, 2013:4; Balimane & Chong, 2005:336). *In vitro* models which can be used for metabolism studies include primary hepatocyte models, cell cultures, microsomes and precision-cut liver slices (Alqahtani *et al.*, 2013:4). The two human Caucasian colon adenocarcinoma cell lines, Caco-2 and LS180, were employed in this study for bi-directional transport and metabolism studies, respectively.

The Caco-2 cell line is one of the best characterised and most commonly used *in vitro* models for drug transport studies during the drug discovery and development process. It offers a simple model to screen the influence of P-gp on drug absorption and has been extensively studied as an *in vitro* model (Gan & Thakker, 1997:89; Van de Kerkhof *et al.*, 2007:667). Despite their colonic origin, Caco-2 cells are enterocyte-like cells with villi on the apical membrane. Being of human origin, they present with morphological and functional characteristics similar to that of their *in vivo* counterparts. They form tight junctions and express various brush border enzymes, certain CYP enzymes as well as phase II enzymes at 21 days after spontaneous differentiation (Artursson *et al.*, 2001:29; Travelin *et al.*, 2002:234,235).

The Caco-2 cell line has certain limitations, such as the relatively low expression of CYP3A4, lack of expression of PXR and it requires relatively long culture periods (i.e. 21 days). The LS180 cell line is not as extensively characterised as the Caco-2 cell line, but was chosen as the *in vitro* model for the metabolism experiments in this study because it expresses CYP enzymes to a higher extent (Brandon *et al.*, 2006:7; Gupta *et al.*, 2008:1178; Van de Kerkhof *et al.*, 2007:667).

3.2 HIGH PRESSURE LIQUID CHROMATOGRAPHY ANALYTICAL METHOD FOR INDINAVIR

A previously validated high performance liquid chromatography (HPLC) analysis method for indinavir was employed (Roos, 2012:44-57). To ensure the method was applicable and suitable for the current study, the linearity and repeatability were confirmed, as well as the specificity of the method in combination with the commercial herbal products.

3.2.1 Chromatographic conditions

The indinavir analysis was performed on an HP1100n chromatograph equipped with a UV detector and Chemstation Rev.A.10.01 Agilent® Technologies data acquisition and analysis software (Hewlett-Packard Paulo Alto, California, USA). The separation of the indinavir was performed on a Venusil XBP C18(2) column, 4.6 x 150 mm, 5 µm, 100 Å (Agela technologies, Wilmington, Delaware, USA) using an acetonitrile solvent adjusted to pH 7, with 0.1% ammonium formate (NH₄HCO₂) as the mobile phase in isocratic elution mode with a flow rate of 1 ml/min and an injection volume of 100 µL. The UV detector was set at 210 nm and the retention time for indinavir was 4.6 min.

3.2.2 Linearity

Linearity is defined as the proportional relationship of response versus the concentration of the analyte over a working range (Shabir, 2004:30). A standard solution was prepared by dissolving 24.93 mg indinavir in 100 ml Dulbecco's Modified Eagle's Medium (DMEM), which was further diluted to 0.2493 µg/ml and 2.493 µg/ml. Different volumes (i.e. 2.5, 5, 10, 20, 30, 40 and 50 µl) of the original and diluted solutions were withdrawn and transferred to vials in duplicate, before being placed into the auto sampler of the chromatograph. The correlation coefficient as well as the y-intercept of the linear regression curve provides an indication of the acceptability of the linearity. A regression coefficient (R^2) value higher than 0.998 indicates an acceptable fit (Shabir, 2004:31; Singh, 2013:29).

3.2.3 Repeatability

Repeatability is performed to determine the intra-day precision of the method under the same conditions over a relatively short time frame (i.e. within the same day) (Shabir, 2004:33). A maximum of six measurements, at concentration 24.93 µg/ml, were determined. Repeatability is reported by standard deviation, as well as relative standard deviation which should be less than 1% and vary little, remaining constant between replicates (Shabir, 2004:33; Singh, 2013:30).

3.2.4 Specificity

Specificity of the HPLC method refers to the ability to unambiguously measure a specific analyte in the presence of other components present during the analysis (Singh, 2013:30). The selected herbal products investigated in this study (i.e. Linctagon Forte[®], Viral Choice[®] and Canova[®]), in combination with indinavir (Crixivan[®]), were analysed to ensure the accurate determination of indinavir by means of HPLC. The prescribed dosage of each product as directed by the package inserts (Appendix B-E) were ground, with a mortar and pestle, to a fine powder (where applicable) and dispersed in 100 ml distilled water (ddH₂O). Each solution was stirred with a magnetic stirrer for 1 h. The solutions were filtered using a syringe filter (0.4 µm pore size) and a 10 ml syringe. The filtrate of each product in combination with indinavir was placed in separate HPLC vials and evaluated by means of HPLC (as previously described for indinavir analysis in section 3.2.4.1) with the detector set at two wavelengths namely, 210 nm and 270 nm.

3.3 LIQUID CHROMATOGRAPHY MASS SPECTROMETRY ANALYTICAL METHOD FOR INDINAVIR METABOLITE ANALYSIS

A liquid chromatography mass spectrometry (LC/MS/MS) method was developed and employed for the analysis of indinavir metabolites in the samples obtained from the *in vitro* metabolism experiments.

Liquid chromatography was performed on an SB-Phenyl (2.1 x 100 mm, 1.8 µm) column using an Agilent 1100 series HPLC instrument. The mobile phase consisted of a mixture of phase A and B at 40:60 v/v ratio, where phase A was 0.1% w/v formic acid in water and phase B 0.1% w/v formic acid in acetonitrile. The mobile phase was delivered at a constant flow rate (180 µl/min) with an injection volume of 5 µl, while the column was kept in a compartment at 40°C.

Quantitative analysis of indinavir, M6 ((2R)-1-[(2S,4R)-4-benzyl-2-hydroxy-4-[[[(1R,2S)-2-hydroxy-2,3-dihydro-1H-inden-1-yl]-C-hydroxycarbonimidoyl]butyl]-N-tert-butylpiperazine-2-carboximidic acid) (indinavir metabolite) and the internal standard (indinavir-d6) was performed on an AB Sciex API 3200 mass spectrometer (ESI in the positive ion mode) and the settings on the apparatus are summarised in Tables 1 and 2. The mass spectrometer was operated at unit resolution in the multiple reactions monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 614.4, m/z 523.4 and m/z 620.5 to the product ions at m/z 421.3, m/z 273.1 and m/z 421.2 for indinavir, M6 and the internal standard, respectively.

Table 3.1: Ionisation source settings for the LC/MS/MS analysis of indinavir metabolite

Electro Spray Ionisation Settings	Value
Nebulizer gas (Gas 1) (arbitrary unit)	30
Turbo gas (Gas 2) (arbitrary unit)	40
CUR (curtain gas) (arbitrary unit)	15
CAD (collision gas) (arbitrary unit)	3
TEM (source temperature) (°C)	500
IS (Ion Spray Voltage) (V)	3500

Table 3.2: MS/MS detector settings for the analysis of indinavir, metabolite and internal standard.

MS/MS Settings	Indinavir	M6	ISTD
Protonated molecular mass (<i>m/z</i>)	614	523	620
Product ion molecular mass (<i>m/z</i>)	421	273	421
Dwell time (ms)	150	150	150
DP (declustering potential) (V)	45	40	40
EP (entrance potential) (V)	12	9	12
CEP(collision cell entrance potential) (V)	35	40	25
CE (collision energy) (eV)	50	45	45
CXP (collision cell exit potential) (V)	11	11	8
Scan Type	MRM	MRM	MRM
Polarity	Positive	Positive	Positive
Pause time	5ms	5ms	5ms

3.4 CHEMICAL FINGERPRINTING OF HERBAL PRODUCTS

The selected herbal products investigated in this study (i.e. Linctagon Forte[®], Viral Choice[®] and Canova[®]) were chemically fingerprinted by means of HPLC. The prescribed dosage of each product, as directed by the package inserts, were ground with a mortar and pestle to a

fine powder (where applicable) and dispersed in 100 ml ddH₂O. Each solution was stirred with a magnetic stirrer for 1 h. The solutions were filtered using a syringe filter (0.4 µm pore size) and a 10 ml syringe. The filtrate of each product was placed in separate HPLC vials and evaluated by means of HPLC (as previously described for indinavir analysis in section 3.2.4.1) with the detector set at two wavelengths namely, 210 nm and 270 nm.

3.5 SAMPLE PREPARATION FOR TRANSPORT AND METABOLISM STUDIES

3.5.1 Negative control group for both transport and metabolism studies

The negative control group consisted of a 200 µM indinavir solution for both the transport and metabolism studies, which was prepared by dissolving two Crixivan[®] capsules in 100 ml ddH₂O (thus 800 mg indinavir in 100 ml). To obtain an indinavir solution with a concentration of 200 µM, 444 µl of the indinavir solution was added to 49.556 ml DMEM. For the combination with the positive control and the sample preparations during the experiments, 444 µl indinavir solution was added to 25.556 ml DMEM to maintain a 200 µM indinavir solution.

3.5.2 Positive control group for the transport studies

As a positive control group for the transport studies, a 100 µM verapamil (a known inhibitor of P-g) solution was prepared by dissolving 2.25 mg verapamil in 25 ml DMEM. To obtain final concentrations of 100 µM verapamil and 200 µM indinavir in 2.5 ml media, which was administered to the cells during bi-directional transport studies, 1.25 ml 200 µM verapamil solution was combined with 1.25 ml 400 µM indinavir solution.

3.5.3 Positive control group for the metabolism studies

The positive control group for the metabolism studies consisted of a 40 µM ketoconazole, a known inhibitor of CYP3A4 solution. The ketoconazole solution was prepared by dissolving 1 mg ketoconazole in 940.32 µl methanol. A volume of 50 µl of this solution was administered to the cells in combination with 2.5 ml of the 200 µM indinavir solution, to obtain final concentrations of 40 µM ketoconazole and 200 µM indinavir.

3.5.4 Herbal product test solution for transport and metabolism studies

For the bi-directional transport and metabolism inhibition studies, a concentration range for each of the herbal products were selected, based on a series of different potential distribution volumes to cover an extreme range of possible *in vivo* concentrations at different

sites as described before (Hellum *et al.*, 2007:26). The highest concentration consisted of a prescribed dose of the herbal product diluted in 500 ml, the medium concentration represented a prescribed dose of the herbal product diluted in 5 L and the lowest concentration represented a prescribed dose of the herbal product diluted in 50 L. All herbal preparations were freshly prepared prior to the transport and metabolism experiments.

The prescribed dose of each herbal product, as directed by the package insert, was ground (where applicable) using a mortar and pestle and dissolved in 100 ml ddH₂O. The herbal product solutions were stirred with a magnetic stirrer for 1 h, after which they were diluted to 500 ml, 5 L and 50 L. The ingredient with the highest concentration in each selected herbal product was used to calculate the concentration of the dilutions, for example in Linctagon Forte[®], the following calculations were done:

A single Linctagon Forte[®] tablet comprises (See package insert Appendix D):

<i>Pelargonium sidoides</i>	250 mg
Quercetin	60 mg
Bromelain	40 mg

Pelargonium sidoides extract is the ingredient with the highest concentration in Linctagon Forte[®]. The directed dosage of Linctagon Forte[®] is one tablet four times daily. One tablet dissolved in 100 ml ddH₂O would result in a *Pelargonium sidoides* extract concentration of 250 mg/100 ml, which is equal to 2.5 mg extract (C₁).

If one tablet is dissolved in 500 ml (to produce the highest concentration tested in this study), then 250 mg *Pelargonium sidoides* extract will be present in 500 ml, which is equal to 0.5 mg/ml (C₂). To obtain 0.5 mg/ml in 50 ml (V₂), the following equation is used to calculate the volume needed from the original solution to be diluted to 50 ml:

$$C_1 \times V_1 = C_2 \times V_2 \quad \text{Eq (1)}$$

Where C₁ and C₂ represent the initial and final concentrations, respectively and V₁ and V₂ represent the initial and final volumes, respectively.

Thus:

$$\begin{aligned}C_1 \times V_1 &= C_2 \times V_2 \\2.5 \text{ mg/ml} \times V_1 &= 0.5 \text{ mg/ml} \times 50 \text{ ml} \\V_1 &= \frac{0.5 \text{ mg/ml} \times 50 \text{ ml}}{2.5 \text{ mg/ml}} \\V_1 &= 10 \text{ ml of the initial concentration.}\end{aligned}$$

A volume of 10 ml of the initial solution was therefore diluted to 50 ml with DMEM to reach a final concentration of 0.5 mg/ml of *Pelargonium sidoides* extract. All other calculations can be found in Appendix F.

3.6 BI-DIRECTIONAL TRANSPORT STUDIES

3.6.1 Culturing of the Caco-2 cells

The Caco-2 cell line was used as the *in vitro* transport model, which was obtained from the European Collection of Cell Cultures (ECACC), by Sigma Aldrich, Johannesburg, South Africa. The cells were subsequently cultured in high-glucose DMEM (Separations, Randburg, South Africa) supplemented with 10% foetal bovine serum (The Scientific Group, Johannesburg, South Africa), 1% non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% penicillin/streptomycin (Separations, Johannesburg, South Africa), 1% 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa) and 1% amphotericin B (250 µg/ml) (The Scientific Group, Randburg, South Africa).

The Caco-2 cells were cultured at 37°C with 5% carbon dioxide and 95% humidified air in a Galaxy 170R incubator (Eppendorf Company, Stevenage, UK). The growth medium was exchanged every second day. Prior to exchange of the growth medium, the cells were inspected by means of a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) to estimate the percentage confluence as well as ensuring the absence of any contamination. The growth medium was decanted and removed from the cell culture flask and replaced with 10 ml pre-warmed growth medium using a serological pipette and pipettor, under sterile conditions, inside a laminar flow hood.

3.6.2 Sub-culturing of the Caco-2 cells

Upon reaching 50% confluency, the Caco-2 cells were sub-cultured by means of trypsinisation. Growth medium and phosphate buffered saline (PBS) (Sigma Aldrich, Johannesburg, South Africa) solution were pre-warmed to 37°C in a circulating water bath. The growth medium was decanted and removed from the cell culturing flask and the cells

were rinsed twice with 10 ml PBS using a pipettor and serological pipettes, under sterile conditions, in a laminar flow hood. A volume of 3 ml Trypsin-Versene (EDTA) mixture (Whitehead scientific, Cape Town, South Africa) was added to the cell culturing flask and the mixture was evenly distributed on the cell layer. The flask was placed in the CO₂ incubator for 3 to 5 min at 37°C. To ensure detachment of the cells, the flask was gently shaken every 2 min and detachment of the cells was determined with the aid of a light microscope. Upon complete detachment of the cells, 6 ml pre-warmed growth medium was added to the trypsin mixture to inactivate its action. The cell suspension was gently agitated by a pipette to ensure all cells were rinsed from the bottom of the flask and then divided to a ratio of 1:4, whereafter 10 ml pre-warmed growth medium was added to each flask. The flasks were returned to the CO₂ incubator to grow the cells under normal culturing conditions.

3.6.3 Seeding of Caco-2 cells onto Transwell® membrane filters

Caco-2 cells were seeded onto Transwell® 6-well membrane filters (Corning Costar® Corporation, Tewksbury, USA) with a pore diameter of 0.4 µm and surface area of 4.67 cm². A cell suspension was obtained by means of trypsinisation with Trypsin-Versene as previously described. After detachment of the cells, 6 ml pre-warmed growth medium was added to the flask and the cell suspension was agitated extensively with a pipette to ensure complete detachment and de-agglomeration of the cells to form a suspension consisting of single cells. This single cell suspension was transferred to a 50 ml tube. A Pasteur pipette was used to agitate the cell suspension to ensure a homogenous distribution of the cells. Cells in the suspension were counted by means of a haemocytometer after addition of Trypan blue. In order to count the cells, 10 µl cell suspension, 15 µl PBS and 25 µl Trypan blue (Sigma Aldrich, Johannesburg, South-Africa) were mixed in a 2.5 ml Eppendorf tube using a pipette and left for 3 min, thereafter 10 µl of the mixture was placed on either side of the cover slip of the haemocytometer. Using a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan), cells were counted on 5 of the 9 squares on the grid of the haemocytometer on each side. The average number of cells per square was calculated; this number was then multiplied with the dilution factor (5×10^4) which established the total number of cells per ml in the cell suspension. The cell suspension was diluted to a concentration of 20 000 cells per ml using equation 1.

Seeding of the cells onto Transwell® filter membranes occurred under sterile conditions in a laminar flow hood by pipetting 2.5 ml of the final cell suspension into each apical chamber of the membrane filter plate wells. The Caco-2 cells were grown for 21 to 24 days on the membrane filters to produce intact epithelial cell monolayers, whilst the growth medium was replaced every second day under sterile conditions.

3.6.4 *In vitro* bi-directional transport studies

Prior to commencement of the transport experiments, the transepithelial electrical resistance (TEER) of each cell monolayer was measured with a Millcell ERS II meter (Millipore, Billerica, Massachusetts, USA) as an indication of the integrity thereof. A TEER reading higher than 250 Ω (which is equivalent to 1167.5 Ω/cm^2) was required before the transport studies could be conducted.

3.6.4.1 *Transport in the apical to basolateral direction*

For the transport studies in the apical to basolateral (AP-BL) direction, the growth medium was removed from the basolateral chamber and replaced with 2.5 ml pre-heated DMEM buffered with HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (The Scientific group, Biochrom, Randburg, South Africa) and incubated for 30 min at 37°C. The growth medium was removed from the apical chamber and replaced with 2.5 ml of each of the test solutions (i.e. each selected herbal product at three different concentrations in the presence of 200 μM indinavir). As mentioned before, the negative control group consisted of indinavir alone in solution (200 μM), whereas indinavir combined with verapamil (100 μM) formed the positive control. Samples (200 μl) were withdrawn from the basolateral side at pre-determined time intervals of 20, 40, 60, 80, 100 and 120 min, which were each replaced with 200 μl of the pre-heated DMEM buffered with HEPES. At the end of the transport study, the TEER was again measured to confirm the integrity of the cell monolayer was still intact after exposure of the cells to the test solutions. Samples were analysed by means of HPLC to determine the indinavir concentration.

3.6.4.2 *Transport in the basolateral to apical direction*

For the transport studies in the basolateral to apical (BL-AP) direction, the growth medium was removed from the apical chamber and replaced with 2.5 ml pre-heated DMEM (37°C) and incubated for 30 min at 37°C. The growth media was removed from the basolateral chamber and replaced with 2.5 ml of each of the test solutions (i.e. each selected herbal product at three different concentrations in the presence of 200 μM indinavir). Indinavir alone was employed as a negative control, with verapamil (100 μM), a known P-gp inhibitor, in combination with indinavir as a positive control. Samples (200 μl) were withdrawn from the apical side at pre-determined time intervals of 20, 40, 60, 80, 100 and 120 min, which were each replaced with 200 μl pre-heated DMEM (37°C). At the end of the transport study, the TEER was again measured to confirm the integrity of the cell monolayer over the time period of the transport study. Samples were analysed by means of the HPLC method.

3.7 IN VITRO METABOLISM STUDIES

3.7.1 Culturing of the LS180 cells

The LS180 cell line was used as the *in vitro* model for metabolism, which was obtained from the European Collection of Cell Cultures (ECACC) by Sigma Aldrich, (Johannesburg, South Africa). The cells were subsequently cultured in high-glucose DMEM (Separations, Randburg, South Africa) supplemented with 10% foetal bovine serum (The Scientific Group, Randburg, South Africa), 1% non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% penicillin/streptomycin (Separations, Thermo Scientific), 1% 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa) and 1% amphotericin B (250 µg/ml) (The Scientific Group, Randburg, South Africa).

The LS 180 cells were cultured at 37°C with 5% carbon dioxide and 95% humidified air in a Galaxy 170R incubator (Eppendorf Company, Stevenage, UK). Growth medium was exchanged every second day. Prior to the growth medium exchange, the cells were inspected by means of a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) to estimate the percentage confluence as well as ensuring the absence of any contamination. The growth medium was decanted, removed and then replaced with 10 ml pre-warmed growth medium using a serological pipette and pipettor under sterile conditions inside a laminar flow hood.

3.7.2 Sub-culturing of the LS180 cells

Upon reaching 50% confluency, the LS180 cells were sub-cultured by means of scraping. The growth media and PBS (Sigma Aldrich, Johannesburg, South Africa) solution were pre-warmed to 37°C in a circulating water bath. The growth medium was decanted and removed from the cell culturing flask; 6 ml pre-warmed growth medium was added and the cells were scraped using a cell scraper under sterile conditions in a laminar flow hood. Using a serological pipette and pipettor, the cell suspension was gently agitated by a pipette ensuring all the cells were rinsed from the sides of the flask. The cell suspension was divided to a ratio of 1:4 and 10 ml pre-warmed growth medium was added to each flask. The flasks were returned to the CO₂ incubator to grow the cells under normal culturing conditions.

3.7.3 Seeding of LS180 cells onto 6-well plates

The LS180 cells were seeded onto 6-well plates (Corning Costar Corporation, Tewksbury, USA). A cell suspension was obtained by means of scraping the cells from the culturing

flask; the cell suspension was extensively agitated with a pipette to ensure a homogeneous suspension consisting of single cells. The cell suspension was transferred to a 50 ml tube and a Pasteur pipette was used to agitate the cell suspension for homogenous distribution of the cells in the suspension. Cells in the suspensions were counted using a haemocytometer after addition of Trypan blue. A volume of 10 μ l cell suspension, 15 μ l PBS and 25 μ l Trypan blue (Sigma Aldrich, Johannesburg, South-Africa) were mixed in a 2.5 ml Eppendorf tube using a pipette and left for 3 min. Thereafter 10 μ l of the mixture was placed on either side of the cover slip of the haemocytometer. Using a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) cells were counted on 5 of the 9 squares on the grid of the haemocytometer on each side. The average number of cells per square was calculated; this number was then multiplied with the dilution factor (5×10^4) which established the total number of cells per ml in the cell suspension. The cell suspension was diluted to a concentration of 50 000 cells per ml using equation 1.

Seeding of the cells onto the 6-well plates occurred under sterile conditions in a laminar flow hood by pipetting 2.5 ml of the final cell suspension into each chamber of the 6 well plates. The seeded LS180 cells were left to adhere to the culture treated bottom surfaces of the wells and cultured for 24 h prior to commencement of the metabolism inhibition studies.

3.7.4 Metabolism inhibition studies

The growth medium was removed from the LS180 cells and replaced with 2.5 ml test and control solutions (i.e. each selected herbal product at three different concentrations in the presence of 200 μ M indinavir as well as 40 μ M ketoconazole and indinavir solution as positive control), which were then incubated for 4 h at normal culturing conditions. The test/control solutions were removed and the cells were washed with 1 ml PBS (Sigma-Aldrich, Johannesburg, South Africa). The cells were harvested by gently scraping each well in order to detach all the cells and transferring them to labelled micro-centrifuge tubes. The cell suspension was removed and placed in 2.5 ml centrifugal tubes on ice, which were centrifuged in a Sigma Refrigerated Centrifuge 3-16KL (Wirsam Scientific, Johannesburg, South Africa) for 5 min at 300 x g (1495 rpm) to ensure lysis of the cell membranes. After centrifugation, the supernatant was removed and the cell pellets were re-suspended in 200 μ l ice-cold methanol, vortexed for 40 s and kept at -80°C until dried. Before drying, the cell suspension was thawed and centrifuged at 10 000 x g (9458 rpm) for 10 min to remove proteins and cell debris. The supernatant was removed and placed in 2.5 ml micro-centrifuge tubes, which were then dried under nitrogen. Prior to LC/MS/MS analysis, the samples were reconstituted in 100 μ l 0.1% formic acid in water and vortex mixed for 1 min.

3.8 DATA ANALYSIS

3.8.1 Indinavir transport data

The apparent permeability coefficient ($P_{app} \times 10^{-6}$ cm/s) values for indinavir transport in each direction across the Caco-2 cell monolayers were calculated using equation 2 (Tarirai *et al.*, 2012:257). The P_{app} values represent the diffusion rate of the compound normalised for surface area of the cell monolayers and drug concentration on the donor side.

$$P_{app} = \frac{dQ}{dt} \left(\frac{1}{A \cdot C_0 \cdot 60} \right) \quad \text{Eq (2)}$$

Where dQ/dt (cm/s) represents the increase in the amount of drug present in the acceptor compartment over time, A is the effective surface area of the cell monolayer in cm^2 that is exposed to the test solutions and C_0 ($\mu\text{g/ml}$) is the initial drug concentration present in the donor chamber.

Efflux ratio (ER) indicates any asymmetry in the directional transport of indinavir in combination with the various herbal products. The ER of indinavir transport was calculated by means of Equation 3 (Tarirai *et al.*, 2012:257).

$$ER = \frac{P_{app}(\text{BL-AP})}{P_{app}(\text{AP-BL})} \quad \text{Eq (3)}$$

Where AP-BL is the transport of indinavir in the apical to basolateral direction and BL-AP indicates the transport of indinavir in the basolateral to apical direction.

3.8.2 Indinavir metabolism data

The ratio of the concentration of indinavir metabolite (i.e. M6) to that of indinavir was calculated to indicate any modulation of indinavir metabolism in the LS180 cells by the herbal products. Concentration ratios of metabolite to parent drug (i.e. indinavir) were used to determine if inhibition occurred. CYP3A4 and CYP2B6 are responsible for the oxidative metabolism of indinavir during phase I of enzyme detoxification resulting in the production of six metabolites. During inhibition of CYP3A4 and CYP2B6, as a cause of combined herb and indinavir usage, there should be a decline in the concentration of these metabolites in the presence of the parent drug.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

The possibility of herb-drug pharmacokinetic interactions occurring during co-administration of commercially available herbal products (i.e. Linctagon Forte[®], Viral Choice[®] and Canova[®]) and indinavir was investigated. *In vitro* cell culture models (i.e. Caco-2 and LS180) were employed to study the possible modulation of the herbal products on the transport and metabolism of indinavir, specifically with respect to inhibition of P-glycoprotein (P-gp) associated efflux and CYP3A4 metabolism.

To ensure the effect of the experimental groups on the bi-directional transport and metabolism of indinavir was not due to chance interferences, control groups were included. An indinavir solution served as the negative control group for both the transport and metabolism studies, whereas verapamil (100 μ M), a known inhibitor of P-gp, served as the positive control group for the bi-directional transport. Ketoconazole, a known inhibitor of CYP3A4, served as the positive control group for the metabolism inhibition studies.

The effect of commercially available herbal products (i.e. Linctagon Forte[®], Viral Choice[®] and Canova[®]) on indinavir transport and metabolism was evaluated at extreme concentration ranges to cover all potential concentrations that may occur *in vivo*. The amount of indinavir transported in both the apical to basolateral and the basolateral to apical directions across Caco-2 cell monolayers in the absence and presence of the selected herbal products was measured in 6-well Transwell[®] plates. Production of the indinavir metabolite, M6, in LS180 cells in the absence and presence of the selected herbal products was measured during the metabolism inhibition study.

A previously validated HPLC method was used to analyse the indinavir concentration in the transport samples. Indinavir and M6 concentrations in the metabolism study samples were determined by means of a validated LCMS/MS method. The apparent permeability coefficient (P_{app}) values and efflux ratios were calculated for the bi-directional transport and the M6/indinavir ratios were calculated for the metabolism experiments.

4.2 High performance liquid chromatography analytical method

Although a previously validated HPLC analytical method was used, the linearity, repeatability and specificity were confirmed.

4.2.1 Linearity

Linearity is defined as the proportional relationship of response versus the concentration of the analyte over a working range (Shabir, 2004:30). An R squared (R^2) value higher than 0.998 is indicative of an acceptable fit. From the linear calibration graph (Figure 4.1) an R^2 value of 0.999 was obtained. It can therefore be concluded that the HPLC method complies with the criteria for linearity.

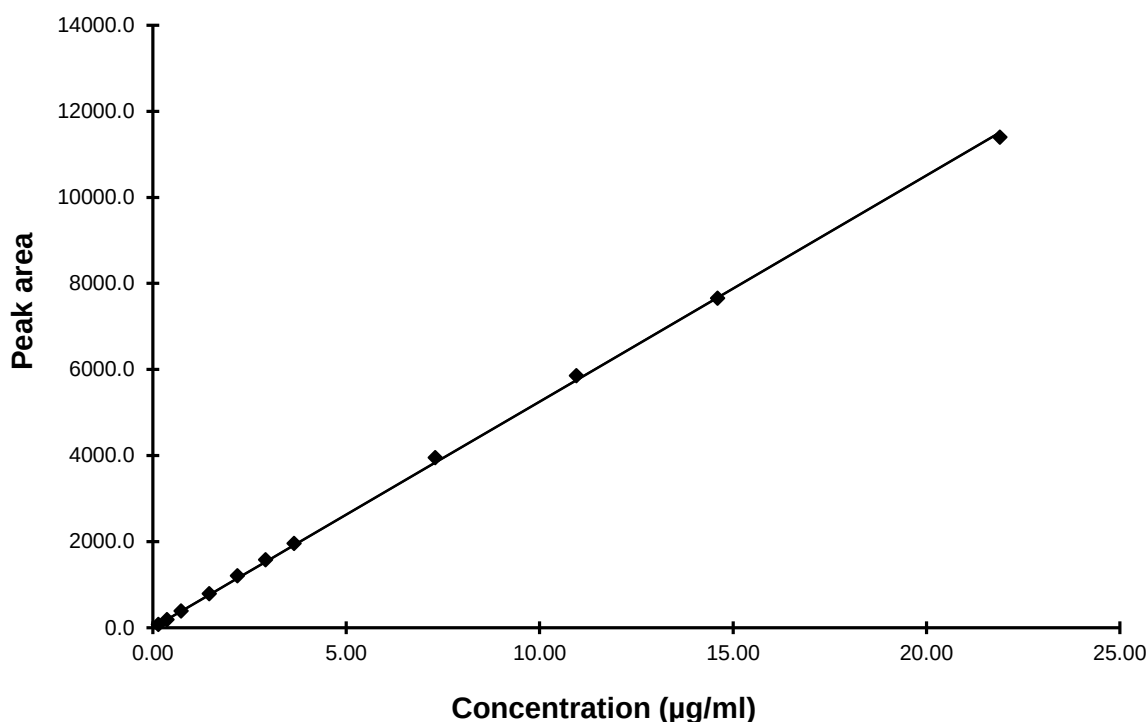


Figure 4.1: Calibration curve for indinavir where peak area is plotted as a function of concentration

4.2.2 Repeatability

Repeatability is performed to determine the intra-day precision of the method under the same conditions over a relatively short time frame (Shabir, 2004:33). A standard deviation (SD) and relative standard deviation (%RSD) of less than 1% is required for a method to be repeatable. The HPLC method used for indinavir analysis satisfies this criterion with a relative standard deviation of 0.3%, as seen in Table 4.1.

Table 4.1: Repeatability data for indinavir analysis

Peak area	Retention times (min)
1512	5.786
1515	5.777
1513	5.791
1521	5.794
1522	5.799
1523	5.811

Mean	1518	5.793
SD	4.56	0.011
%RSD	0.30	0.182

4.2.3 Specificity

The chromatograms in Figures 4.2 to 4.5 illustrate the specificity of the method to detect indinavir in the presence of other chemicals from Crixivan[®] and from each herbal product.

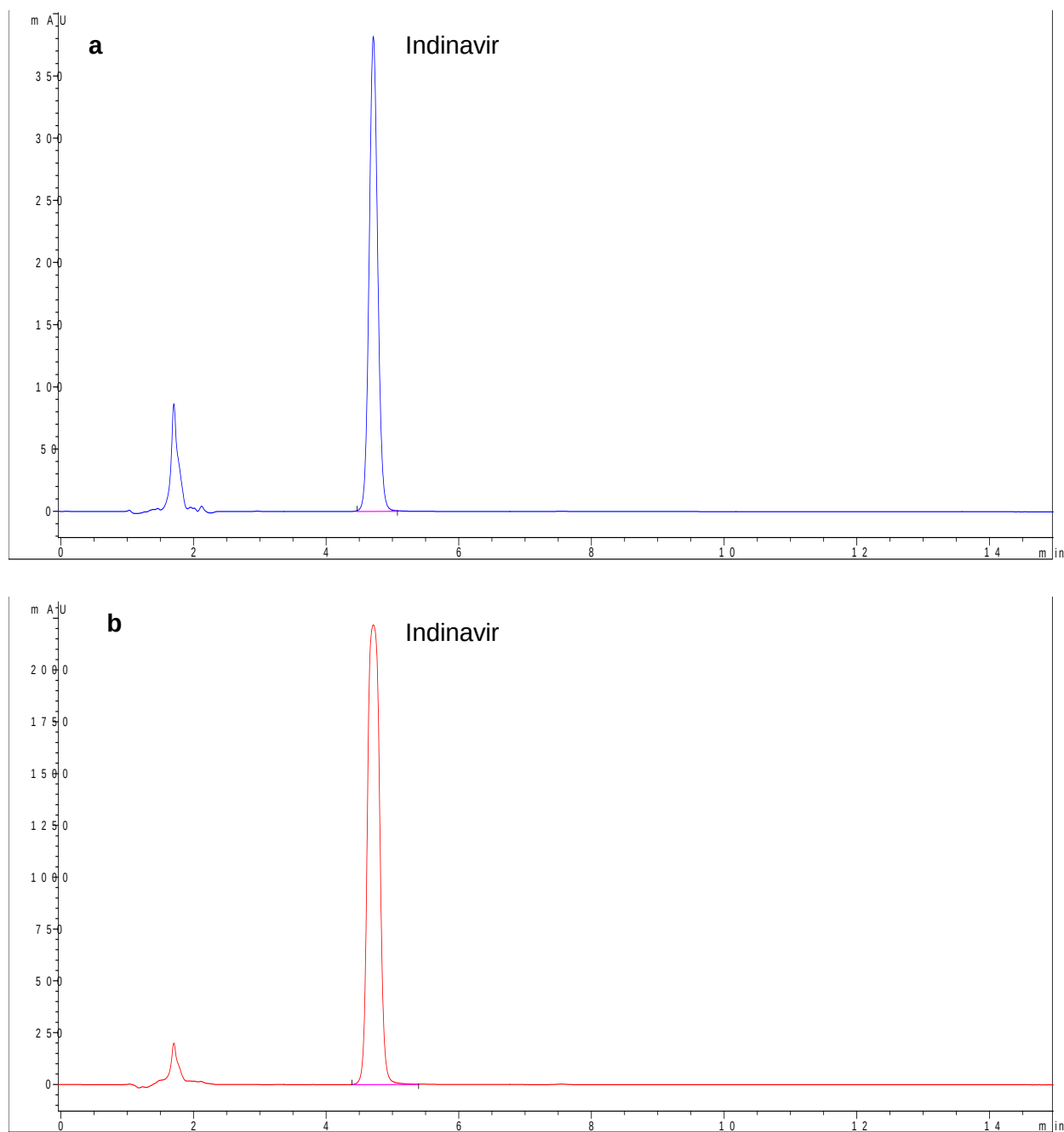


Figure 4.2: Chromatographic profiles of indinavir sulphate in the presence of Crixivan[®] excipients at a wavelength of (a) 270 nm and (b) 210 nm

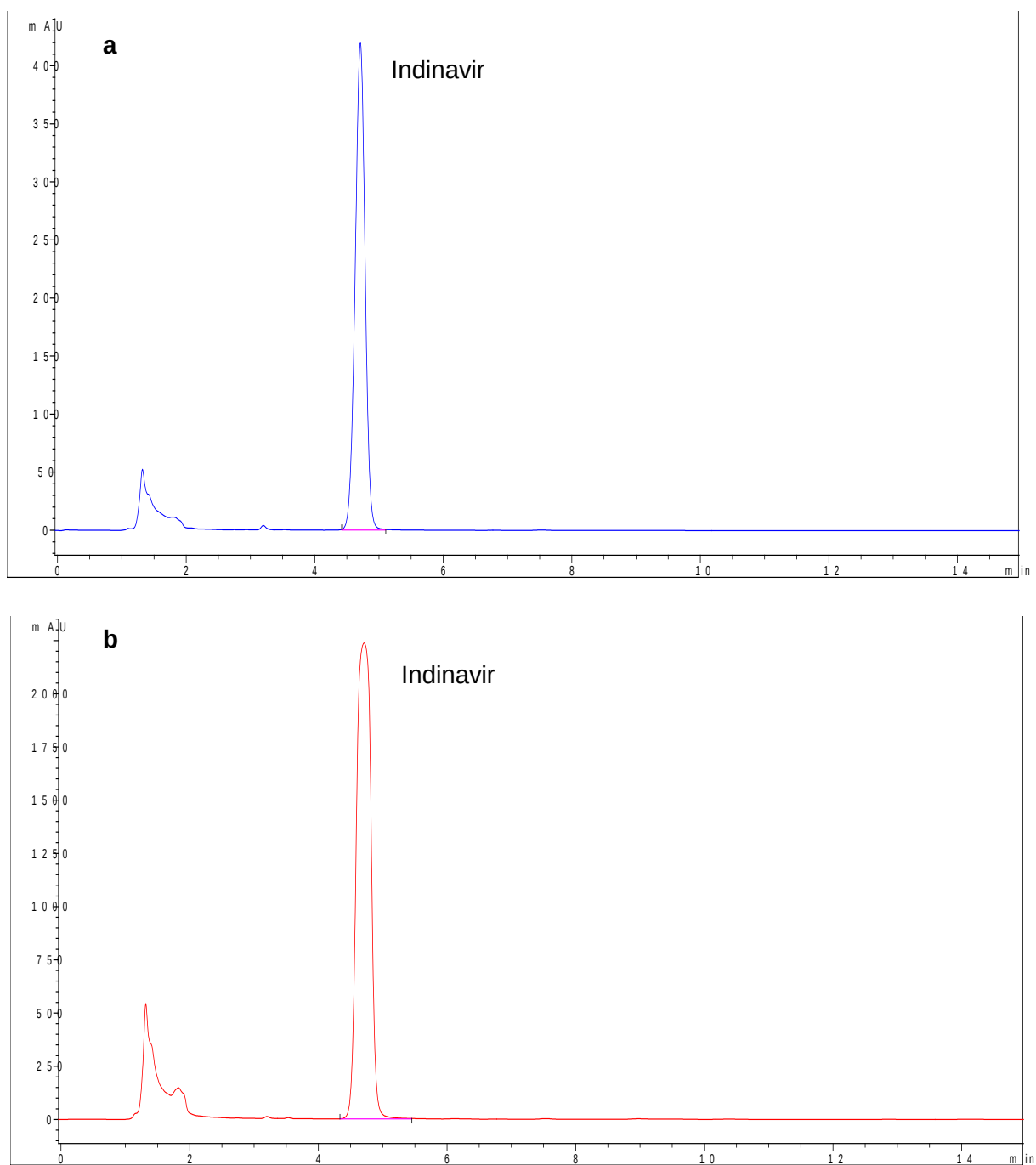


Figure 4.3: Chromatographic profiles of Crixivan[®] indinavir sulphate in the presence of Linctagon Forte[®] at wavelengths of (a) 270 nm and (b) 210 nm

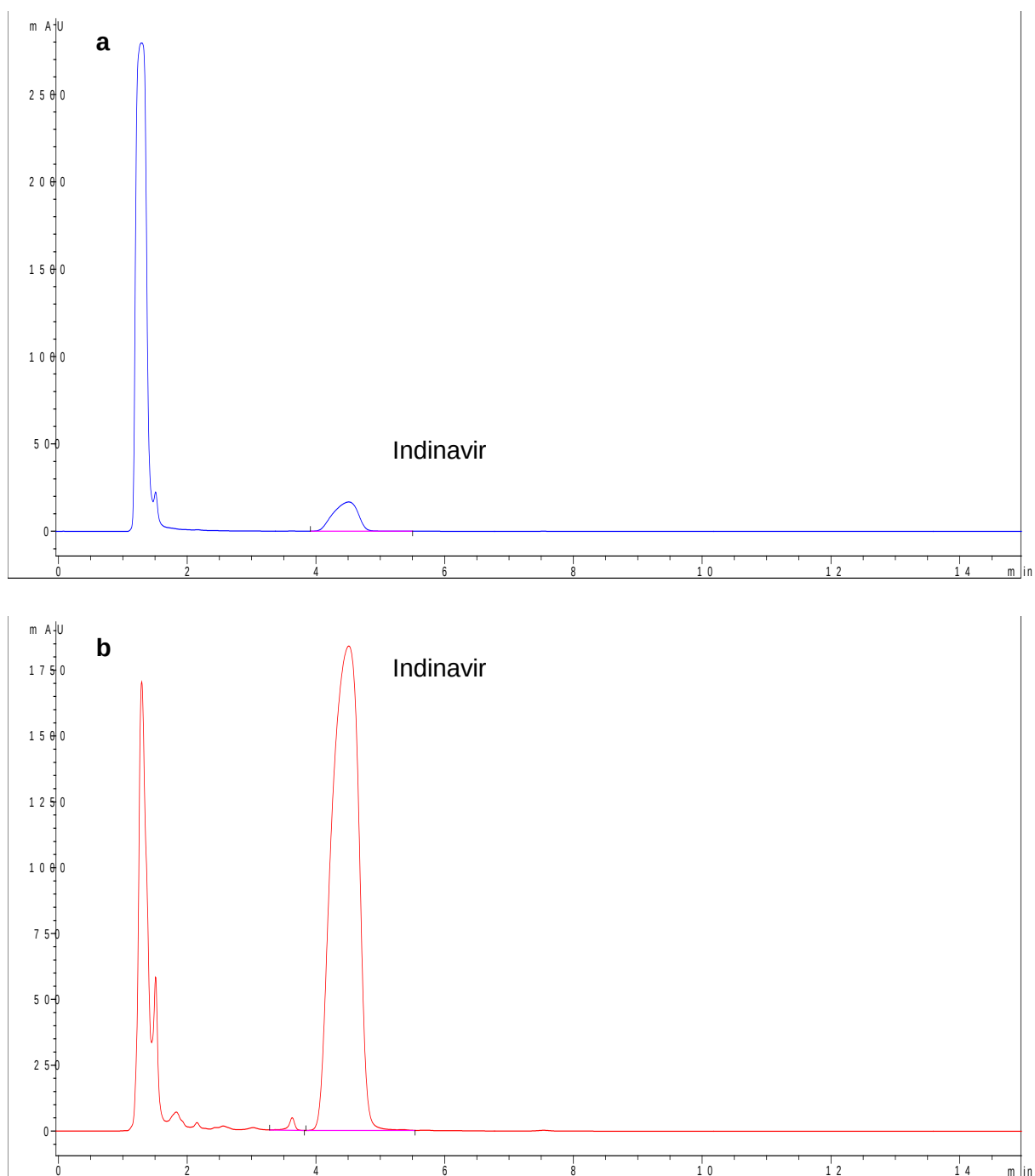


Figure 4.4: Chromatographic profiles for Crixivan® indinavir sulphate in combination with Viral Choice® at wavelengths (a) 270 nm and (b) 210 nm

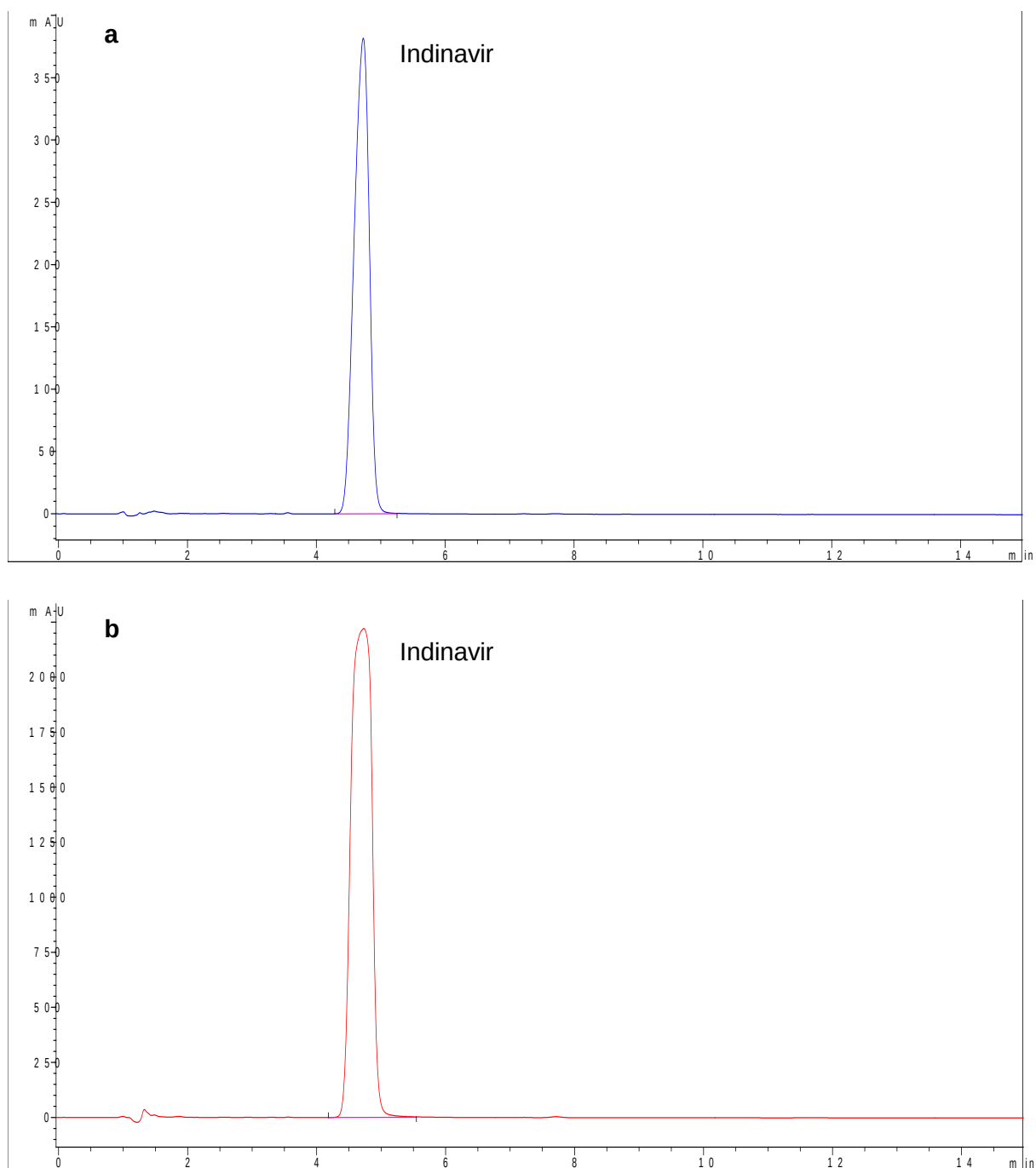


Figure 4.5: Chromatographic profiles for Crixivan[®] indinavir sulphate in combination with Canova[®] at wavelengths (a) 270 nm and (b) 210 nm

4.3 Chemical fingerprinting of herbal products

Figures 4.6 to 4.8 illustrate the HPLC chromatographs of the selected herbal products at wavelengths 270 nm and 210 nm, respectively.

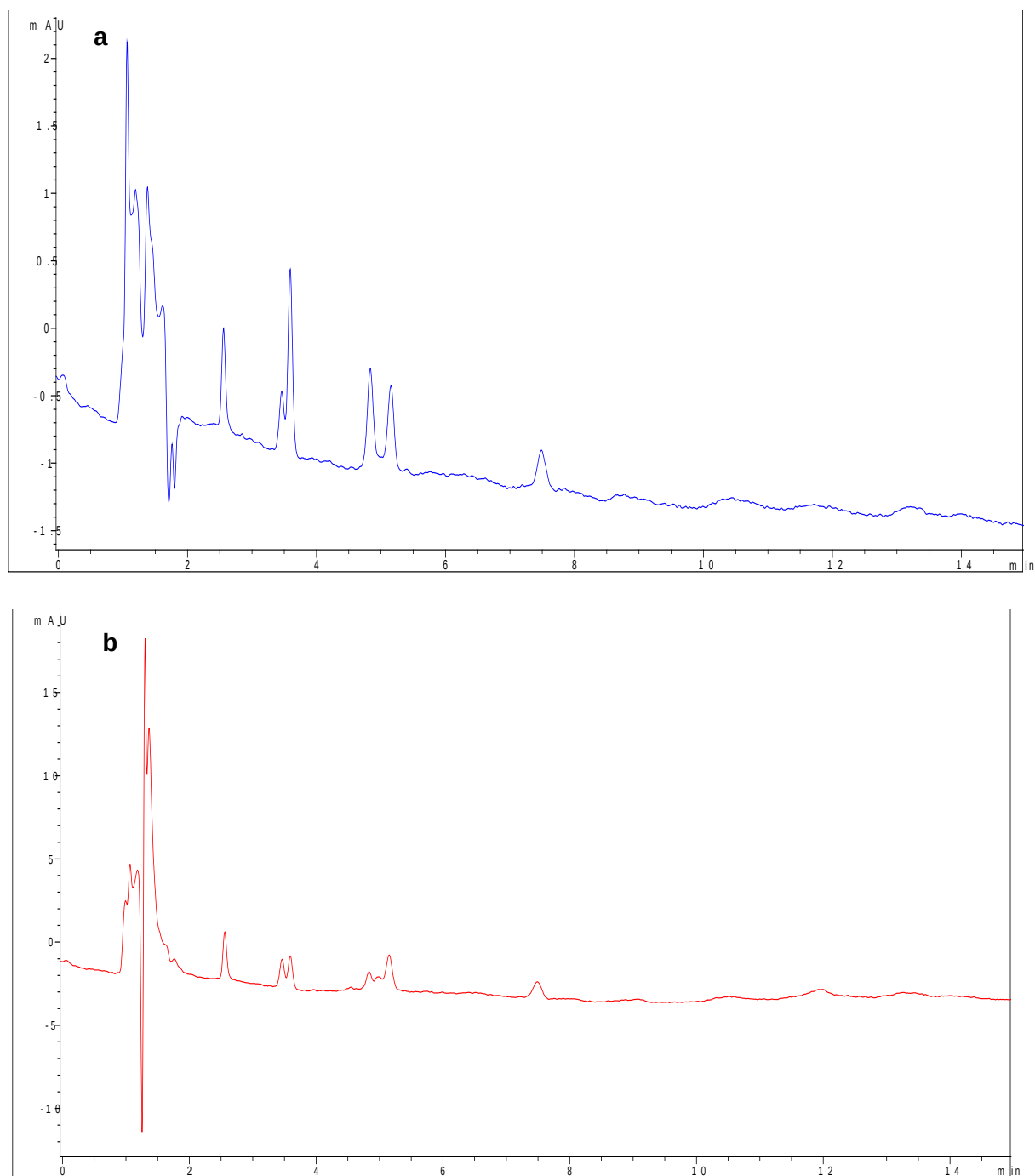


Figure 4.6: Chromatographic profiles for Canova[®] solutions at wavelengths (a) 270 nm and (b) 210 nm

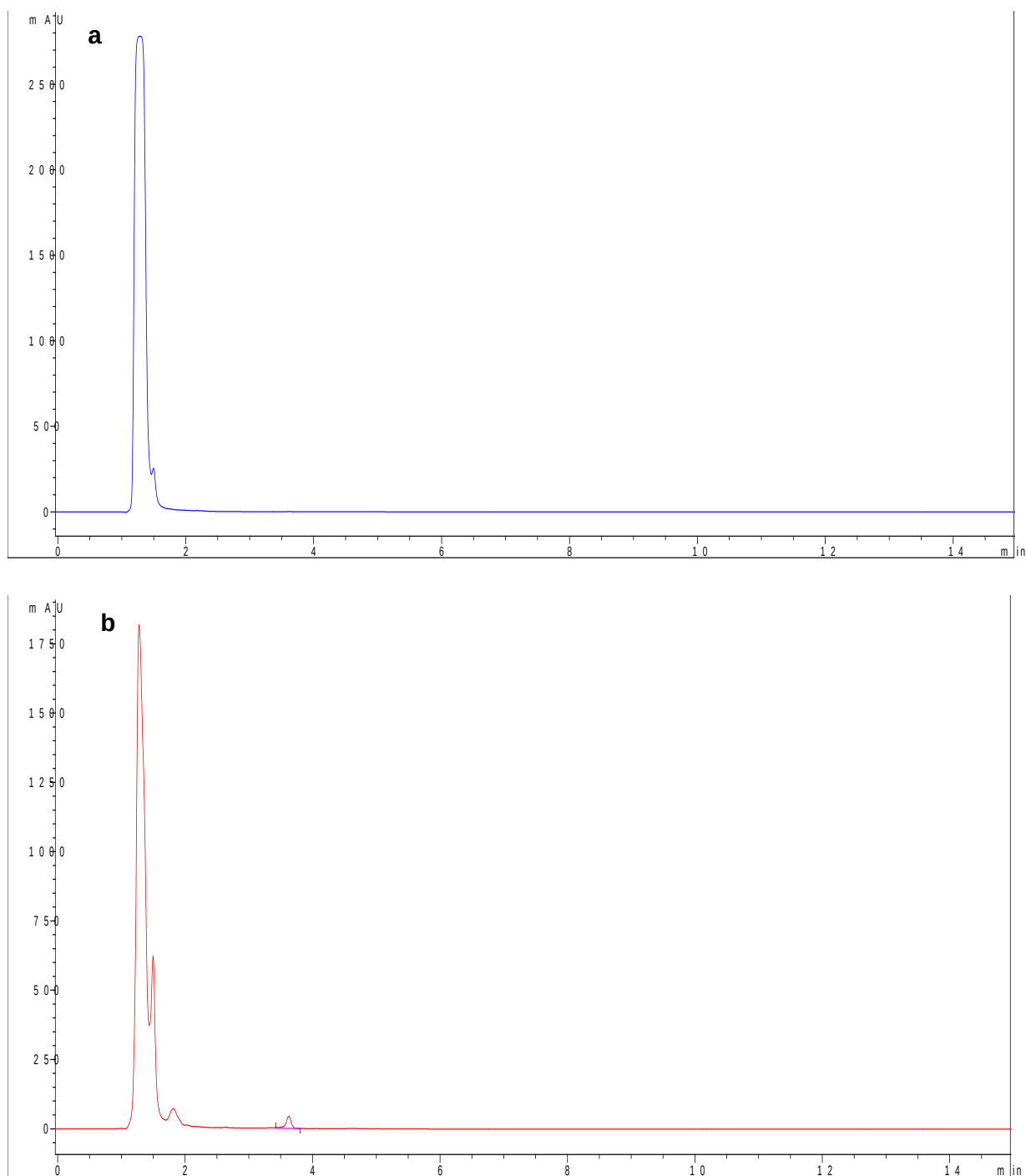


Figure 4.7: Chromatographic profiles for Viral Choice[®] solutions at wavelengths (a) 270 nm and (b) 210 nm

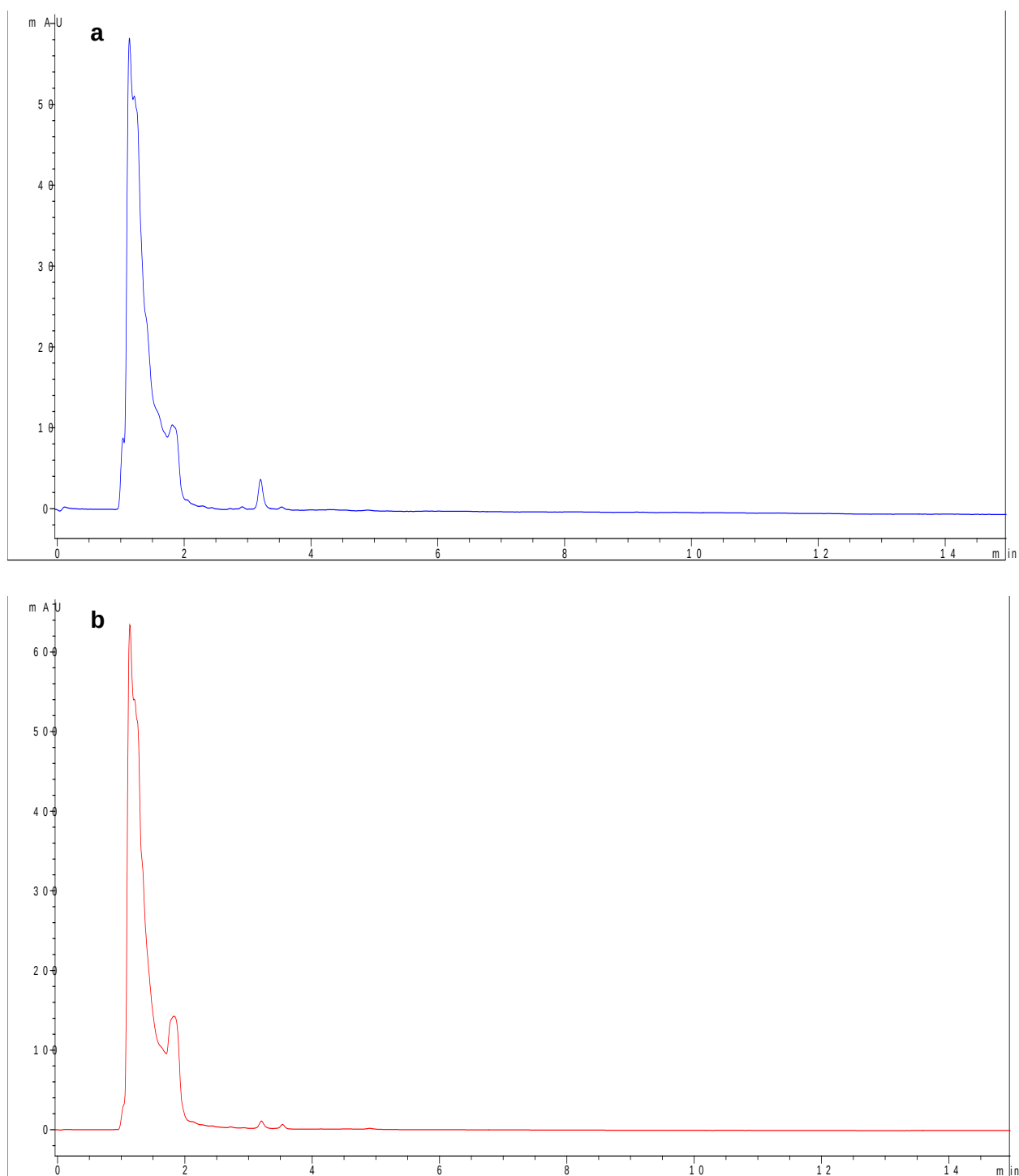


Figure 4.8: Chromatographic profiles for Linctagon Forte® solutions at wavelengths (a) 270 nm and (b) 210 nm

4.4 Bi-directional transport and the effect on P-glycoprotein

4.4.1 Bi-directional transport of indinavir in the presence of Linctagon Forte®

The bi-directional transport of indinavir (200 µM) alone, indinavir (200 µM) in combination with verapamil (100 µM), as well as in combination with three concentrations of Linctagon Forte® was evaluated by means of Caco-2 cell monolayers on 6-well Transwell® plates. For the negative control group, there was only a 13% decrease in the TEER values over the duration of the transport study (Table 4.2). This relatively low decrease in TEER of the Caco-2 cell monolayer suggested the Caco-2 cell model was suitable for the duration of the transport study. Higher reductions in TEER values for the other groups could be explained by the modulation effects of phytochemicals in the herbal products on the tight junctions between adjacent epithelial cells. A TEER value at the beginning, before commencement of the transport study, higher than 250 Ω (i.e. equal to 1167.5 Ω/cm²) served as an indication of the confluency of the cell monolayer. As can be seen in Table 4.2, the average TEER values were all above this prescribed value.

Table 4.2: Average TEER values at the beginning (0 min) and end (120 min) of the bi-directional transport study of indinavir in the presence of Linctagon Forte® across Caco-2 cell monolayers

Experimental groups	Average TEER value (Ω) (n=3)			
	AP-BL direction		BL-AP direction	
	0 min	120 min	0 min	120 min
Negative control (indinavir)	347 ± 17.74	298.8 ± 14.07	319 ± 22.78	250.3 ± 6.05
Positive control (indinavir + verapamil)	335.5 ± 21.62	313.6 ± 9.50	385.1 ± 9.50	335.6 ± 7.05
Low concentration	370 ± 19.00	282.3 ± 8.51	369.3 ± 7.23	291.3 ± 7.23
Medium concentration	323.6 ± 14.57	277.6 ± 8.08	331.6 ± 9.07	291.6 ± 2.08
High concentration	337.3 ± 16.77	252 ± 8.89	313.3 ± 12.10	336.6 ± 13.43

The percentage indinavir transport in both directions, apical to basolateral (AP-BL) as well as basolateral to apical (BL-AP), was calculated and presented as a function of time in Figures 4.9 and 4.10, respectively. The percentage transport indicates the percentage indinavir crossing the cell monolayer in relation to the concentration of indinavir administered during the transport study. A higher percentage transport in the BL-AP direction compared to the AP-BL direction indicates active efflux of the compound occurred.

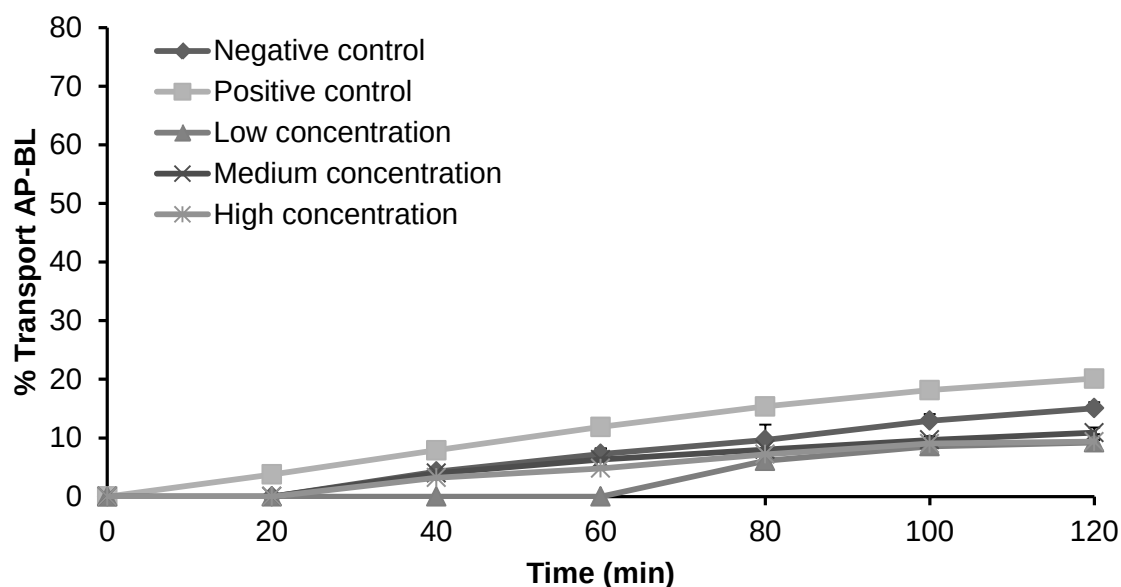


Figure 4.9: Percentage transport of indinavir in the apical to basolateral (AP-BL) direction as a function of time across Caco-2 cell monolayers (n=3) (error bars represent SD)

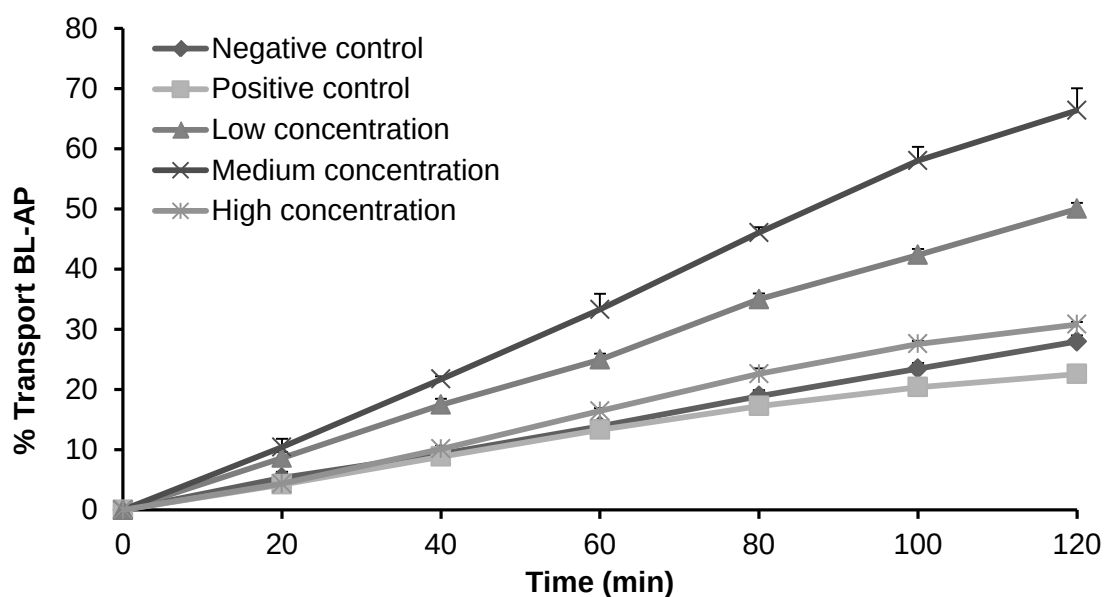


Figure 4.10: Percentage transport of indinavir in the basolateral to apical (BL-AP) direction as a function of time across Caco-2 cell monolayers (n=3) (error bars represent SD)

From Figures 4.9 and 4.10 it can be seen that verapamil, a known inhibitor of P-gp (i.e. positive control group), increased the uptake of indinavir in the AP-BL direction, whereas it decreased the efflux of indinavir in the BL-AP direction. The overall uptake of indinavir (i.e. transport in the AP-BL direction) decreased for all the concentrations of Lingtagon Forte[®] when combined with indinavir, as indicated in Figure 4.9. An overall increase in the efflux of indinavir is noted for all the concentrations of Lingtagon Forte[®] when combined with indinavir (Figure 4.10). The medium concentration produced the most pronounced effect, followed by the low and high concentrations respectively, when compared to both the positive and negative control groups.

Figure 4.11 presents the P_{app} values for each of the experimental groups of Linctagon Forte® in combination with indinavir.

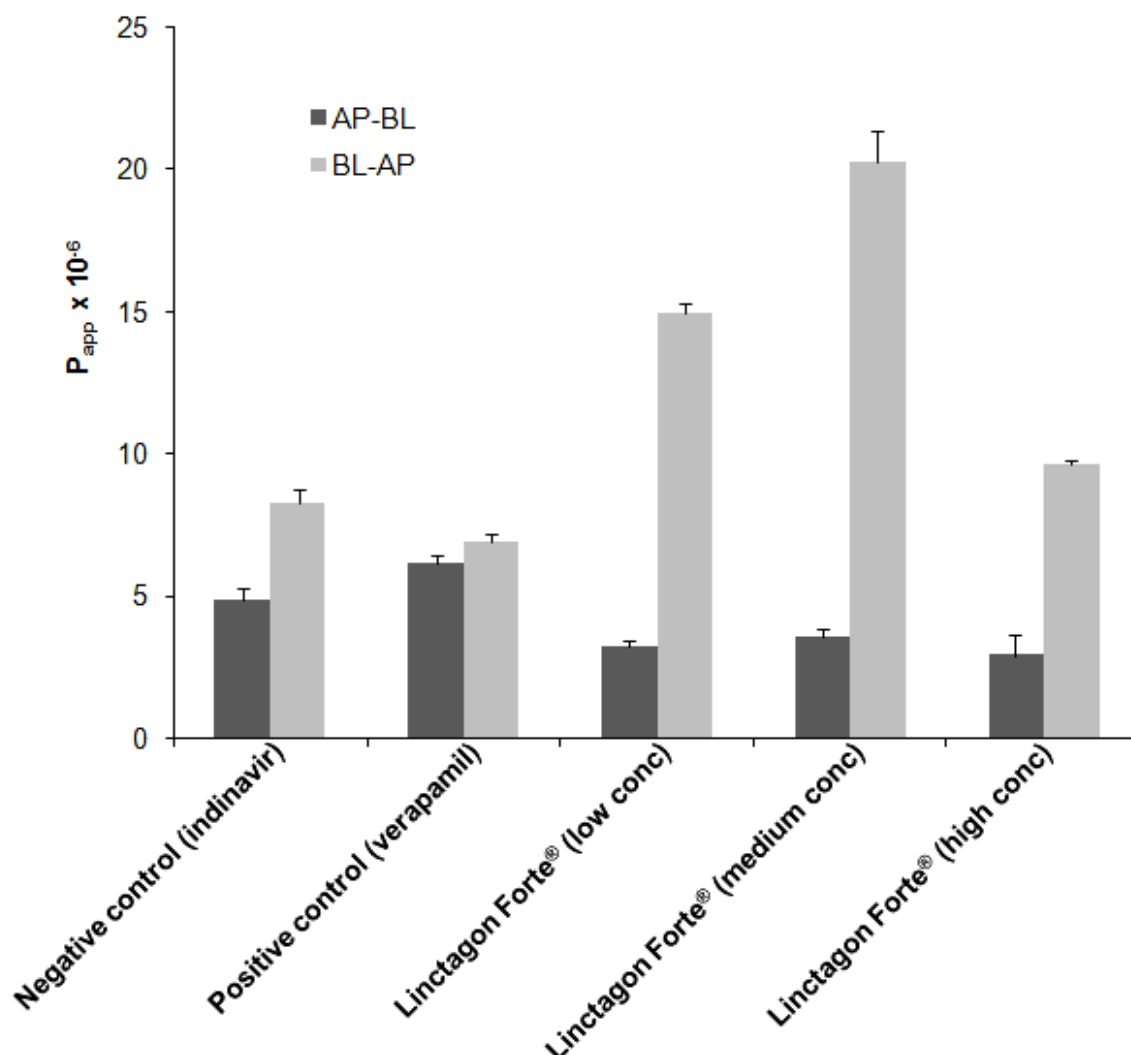


Figure 4.11: Bi-directional P_{app} values for indinavir in the presence of three concentrations of Linctagon Forte® as well as for the negative control group (indinavir alone) and positive control group (indinavir with verapamil) (n=3) (error bars represent SD)

The P_{app} values represent the diffusion rate of the compound normalised for surface area of the cell monolayers and drug concentration on the donor side (Tarirai *et al.*, 2012:257). From Figure 4.11, it is clear Linctagon Forte® pronouncedly increased the BL-AP diffusion rate (i.e. efflux) of indinavir and concurrently decreased the AP-BL diffusion rate (i.e. uptake) compared to that of the negative control group (indinavir alone). The efflux activation effect was concentration dependent for the first two concentrations of Linctagon Forte®, but the effect was lower for the highest concentration. The latter corresponds well with observations made for the percentage transport in both the AP-BL and BL-AP directions. This “bell shape” dose dependent curve may implicate a saturation effect above a threshold

concentration. An increase in efflux, as a pharmacokinetic interaction, may cause a reduction in the bioavailability of indinavir when simultaneously taken with Linctagon Forte® from the results obtained. This acute effect of Linctagon Forte® on indinavir transport across intestinal epithelial cells may be explained by potential activation of the P-gp efflux transporter however, the precise mechanism of action should be investigated further in conjunction with the potential enhancement of gene expression over longer term exposure.

Linctagon Forte® is composed of *Pelargonium sidoides* (Geraniaceae), quercetin and bromelain. Currently, there are no known interactions between co-administered drugs and *P. sidoides* extract containing products with respect to P-gp (Ulbricht *et al.*, 2010:723). The quercetin component has in the past shown inhibitory action of P-gp activity. It had been determined, by means of rhodamine-123 (Rh-123) transport studies, that P-gp activity is significantly inhibited by quercetin (Choi *et al.*, 2011:609). In another study by Borska *et al.* (2012), the cell line EPG85-257RDB (RDB-cells) exposed to 12 µM quercetin showed a significant reduction in the expression of P-gp. The bromelain component, however, is absorbed by means of a self-enhanced paracellular route without degradation and maintains biological activity (Bock *et al.*, 1998:1399; Pavan *et al.*, 2012:1) and therefore, may not have an effect on the P-gp transporter. This suggests the overall increase in efflux observed for Linctagon Forte® may potentially be attributed to the high concentration of *P. sidoides* extract present within the product.

4.4.2 Bi-directional transport of indinavir in the presence of Viral Choice®

The bi-directional transport of indinavir (200 µM) alone (negative control group), indinavir (200 µM) in combination with verapamil (100 µM, positive control group), as well in combination with three extreme concentrations of Viral choice® was evaluated by means of Caco-2 cell monolayers on 6-well Transwell® plates. The TEER was measured at the beginning as well as at the end of the transport study. For the negative control group, (Table 4.3) there was only a 13% decrease in the TEER value over the duration of the transport study. This suggested the Caco-2 cells used in this study provided a suitable model that stayed intact over the duration of the transport study. It is clear from Table 4.4, that the minimum initial TEER value of 250 Ω (i.e. equal to 1167.5 Ω/cm²) was achieved for each experimental group.

Table 4.3: Average TEER value at the beginning (0 min) and end (120 min) of the bi-directional transport study of indinavir in the presence of Viral Choice® across Caco-2 cell monolayers

Experimental groups	Average TEER value (Ω) (n=3)			
	AP-BL direction		BL-AP direction	
	0 min	120 min	0 min	120 min
Negative control (indinavir)	347 $\pm$ 17.74	298.8 $\pm$ 14.07	319 $\pm$ 22.78	250.3 $\pm$ 6.05
Positive control (indinavir + verapamil)	335.5 $\pm$ 21.62	313.6 $\pm$ 9.50	385.1 $\pm$ 9.50	335.6 $\pm$ 7.05
Low concentration	422.3 $\pm$ 6.35	352 $\pm$ 10.58	483 $\pm$ 10.58	420.6 $\pm$ 8.08
Medium concentration	506.6 $\pm$ 20.55	409.6 $\pm$ 28.10	499.6 $\pm$ 9.07	345.6 $\pm$ 57.55
High concentration	479 $\pm$ 8.19	407.6 $\pm$ 33.26	495 $\pm$ 2.00	378 $\pm$ 19.97

The percentage transport in both directions (i.e. AP-BL and BL-AP) are presented in Figures 4.12 and 4.13, respectively.

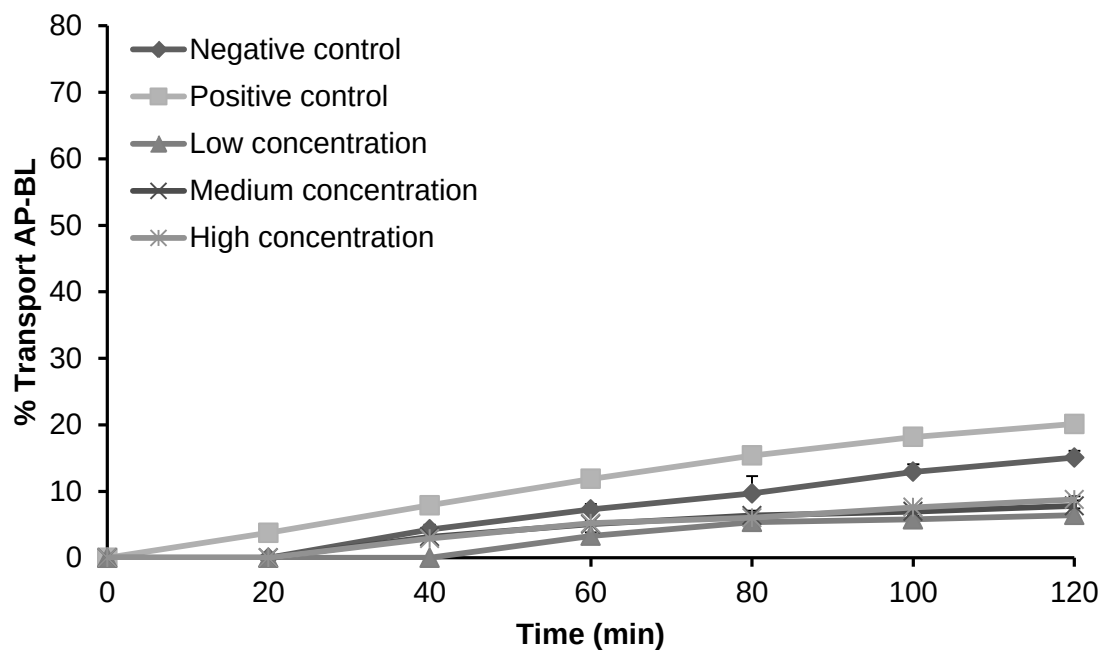


Figure 4.12: Percentage transport of indinavir in the apical to basolateral (AP-BL) direction as a function of time across Caco-2 cell monolayers (n=3) (error bars represent SD)

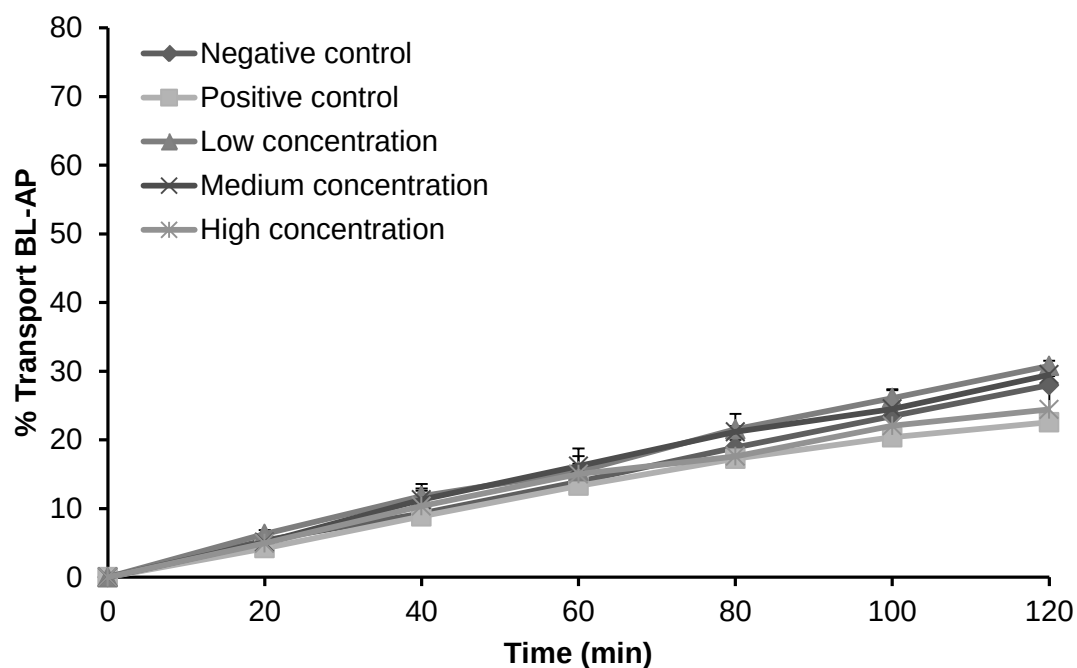


Figure 4.13: Percentage transport of indinavir in the basolateral to apical (BL-AP) direction as a function of time across Caco-2 cell monolayers (n=3) (error bars represent SD)

From Figures 4.12 and 4.13, it is clear verapamil increased the uptake of indinavir (i.e. transport in the AP-BL direction), whereas it decreased the efflux (i.e. transport in the BL-AP direction) of indinavir. There was an overall decrease in the percentage uptake of indinavir in the presence of Viral Choice[®] for the entire concentration range compared to the negative control group as can be seen in Figure 4.12. The low and medium concentrations showed percentage efflux similar to that of the negative control group (i.e. indinavir alone); the highest concentration showed a decrease in the efflux compared to the negative control group, almost comparable to the positive control group (i.e. indinavir and verapamil combination).

Figure 4.14 is a visual representation of the average P_{app} values calculated from the transport of indinavir when combined with Viral Choice®.

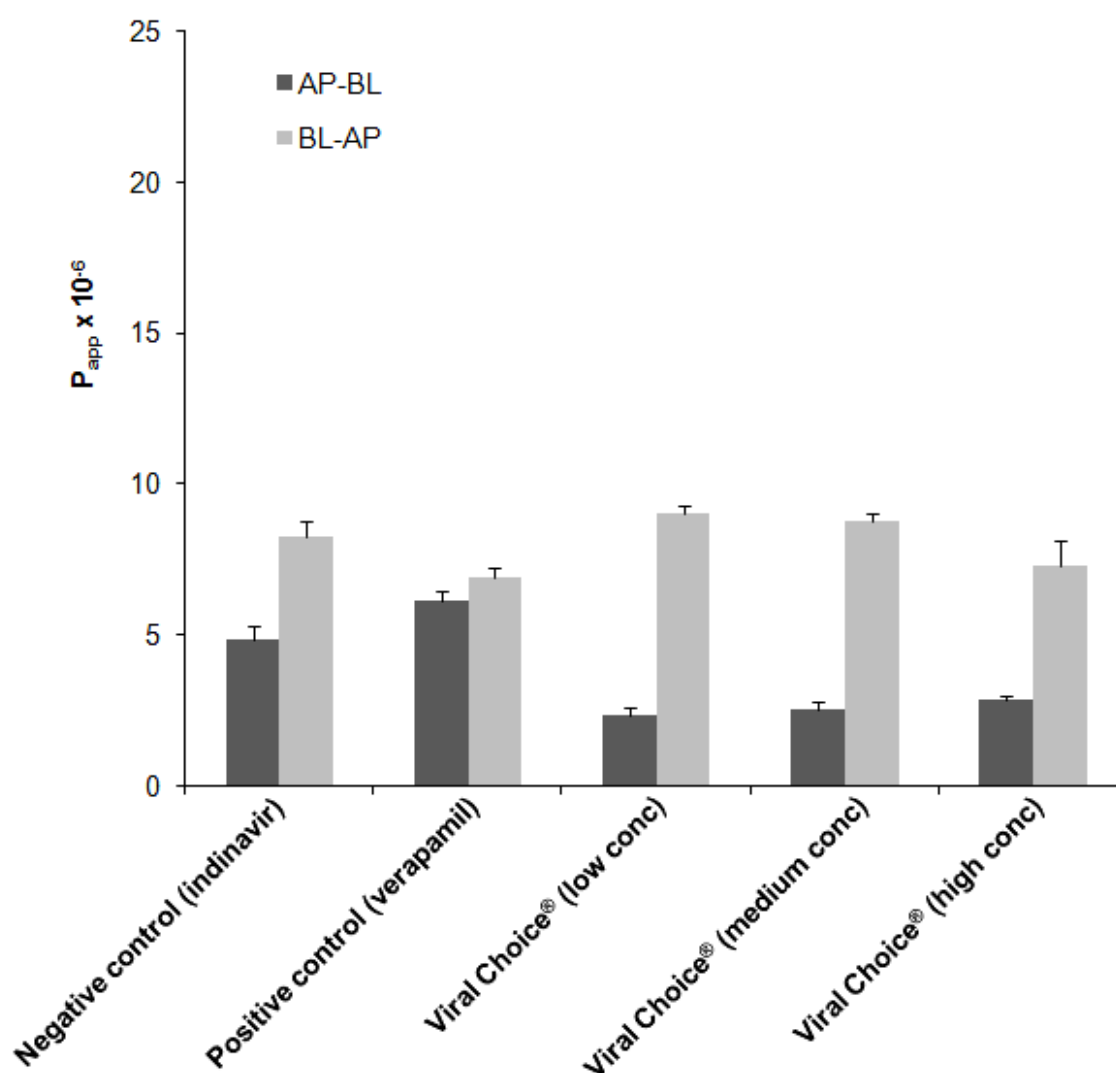


Figure 4.14: Bi-directional P_{app} values for indinavir in the presence of three concentrations of Viral Choice® as well as for the negative control group (i.e. indinavir alone) and positive control group (i.e. indinavir with verapamil) (n=3) (error bars represent SD)

Figure 4.14 confirms the decrease in the uptake of indinavir from the AP-BL direction during the combined application of Viral Choice® for the entire concentration range (i.e. low, medium and high concentrations) when compared to the negative control group. Efflux of indinavir in the BL-AP direction for the low and medium concentration of Viral Choice® increased to a certain extent. However, at the highest Viral Choice® concentration, there is a slight decrease in the indinavir efflux from the BL-AP direction when compared to the negative control group, but not to the same extent as the positive control group. Also the decrease in uptake corresponds with the increase in efflux. This trend corresponds to that

observed in a study by Hansen and Nilsen (2009:86-91), in which the effect of *Echinacea purpurea* was evaluated on P-gp transport in Caco-2 monolayers. Viral Choice[®] is composed of a variety of minerals, vitamins, amino acids, trace elements, phytosterols as well as *Echinacea purpurea* and *Allium sativum* extracts; of these *Echinacea purpurea* is present in the highest concentration at 80 mg per capsule (See Package insert Appendix E).

4.4.3 Bi-directional transport of indinavir in the presence of Canova[®]

The bi-directional transport of indinavir (200 μ M) alone, indinavir (200 μ M) in combination with verapamil (100 μ M) as well as in combination with three extreme concentrations of Canova[®] was evaluated by means of Caco-2 cell monolayers on 6-well Transwell[®] plates. The TEER was measured at the beginning as well as at the end of the transport study. Focusing on the negative control group in Table 4.4, there was only a 13% drop in the TEER measurements over the duration of the transport study. This suggested the measurement of the TEER values, as criteria for monolayer integrity over the duration of the transport study, was a suitable model. A TEER measurement higher than 250 Ω serves as an indication of the integrity of the cell monolayer. As can be seen from Table 4.4, showing the average TEER measurement, this criterion was met for each experimental group, thus, the results obtained can be viewed as a true reflection of the inherent actions of the experimental groups on the bi-directional transport.

Table 4.4: Average TEER values at the beginning (0 min) and end (120 min) of the bi-directional transport of indinavir in the presence of Canova[®] across Caco-2 cell monolayers

Experimental groups	Average TEER values (Ω) (n=3)			
	AP-BL direction		BL-AP direction	
	0 min	120 min	0 min	120 min
Negative control (indinavir)	347 $\pm$ 17.74	298.8 $\pm$ 14.07	319 $\pm$ 22.78	250.3 $\pm$ 6.05
Positive control (indinavir + verapamil)	335.5 $\pm$ 21.62	313.6 $\pm$ 9.50	385.1 $\pm$ 9.50	335.6 $\pm$ 7.05
Low concentration	437.6 $\pm$ 23.86	323.6 $\pm$ 24.09	415.3 $\pm$ 5.03	355.3 $\pm$ 10.50
Medium concentration	501.33 $\pm$ 8.08	446.3 $\pm$ 13.58	400.3 $\pm$ 9.07	350.6 $\pm$ 4.51
High concentration	533.3 $\pm$ 10.50	397.6 $\pm$ 3.21	404.6 $\pm$ 11.15	414.6 $\pm$ 10.79

The percentage transport of indinavir in both directions in the presence of Canova[®] was calculated and is presented in Figures 4.15 and 4.16, respectively.

Figure 4.15 shows there was a concentration dependent (i.e. from lowest to highest concentration) increase in the percentage uptake of indinavir in the presence of Canova[®]. The percentage uptake of indinavir in the presence of the lowest concentration Canova[®] was lower than that of both the positive and negative control groups, whereas it was slightly elevated in the presence of the medium and high Canova[®] concentrations in comparison to the negative control group (i.e. indinavir). The same trend, to some extent, was observed for the percentage efflux of indinavir (Figure 4.16). There was a slight concentration dependent increase in the efflux of indinavir for the entire concentration range; nevertheless, when compared to the negative control group, this increase for the medium and high concentrations of Canova[®] was almost negligible. The lowest concentration of Canova[®], however, decreased the percentage efflux of indinavir to values lower than those of the positive control group (i.e. verapamil and indinavir).

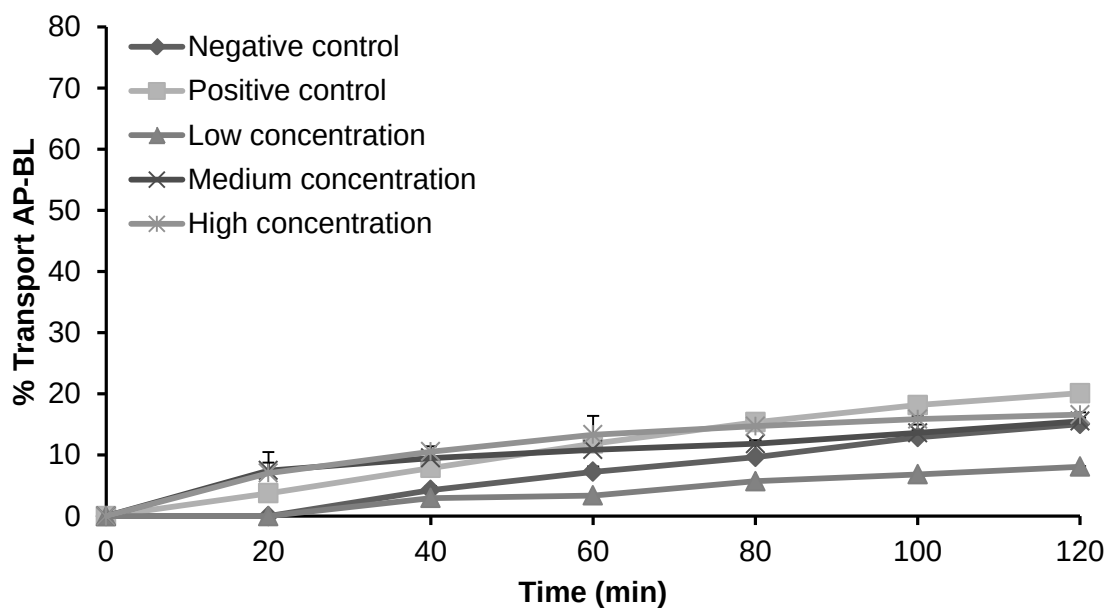


Figure 4.15: Percentage transport of indinavir in the apical to basolateral (AP-BL) direction as a function of time across Caco-2 cell monolayers (n=3) (error bars represent SD)

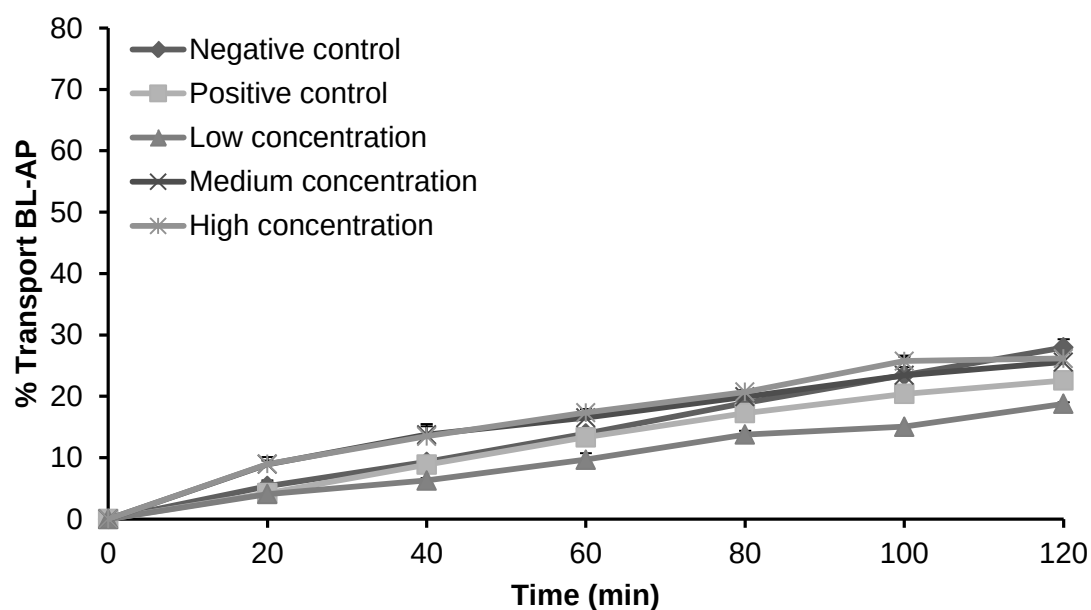


Figure 4.16: Percentage transport of indinavir in the basolateral to apical (BL-AP) direction as a function of time across Caco-2 cell monolayers (n=3) (error bars represent SD)

Figure 4.17 shows the average P_{app} values calculated for indinavir when combined with Canova®.

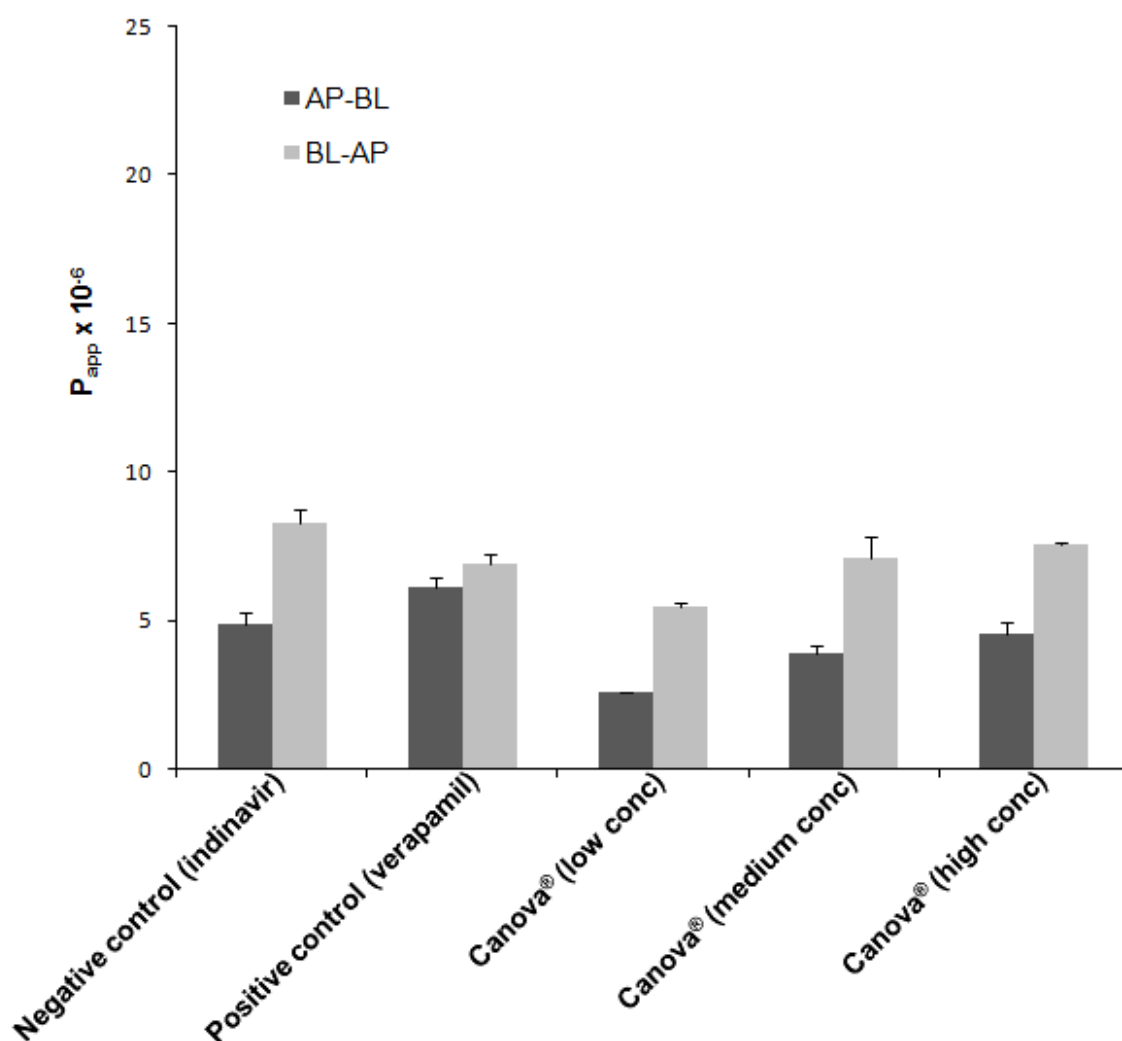


Figure 4.17: Bi-directional P_{app} values for indinavir in the presence of three concentrations of Canova® as well as for the negative control group (indinavir alone) and positive control group (indinavir with verapamil) (n=3) (error bars represent SD)

The combination of Canova® with indinavir increased the uptake of indinavir in a concentration dependent manner (i.e. from low to high concentration) when compared to each other, but it was generally lower than that of the negative control group. Efflux of indinavir also increased in a concentration dependent manner when compared to each other. In general, it appeared as though Canova® had a negligible effect on the bi-directional transport of indinavir.

Canova® is a highly diluted homeopathic remedy, which is composed of extracts from different plants (see package insert of Canova® in Appendix C), and of these

phytoconstituents, only *Aconitum napellus* has been identified as a potential substrate for P-gp (Yang *et al.*, 2013b:197).

4.5 Transport efflux ratio (ER) values

The ER values indicate asymmetry in the directional transport of indinavir in combination with the various herbal products. When efflux of a drug from the epithelium is increased, lower uptake transport of the drug is expected across the epithelium, because the molecules are actively pumped from within the cells back to the apical side. An increase in efflux, as a pharmacokinetic interaction, may therefore cause a reduction in the bioavailability of the drug when simultaneously taken with the inducer. Conversely, a decrease in the efflux, as caused by an inhibitor, may result in increased bioavailability of the drug as more drug will now reach the systemic circulation.

Figure 4.18 offers a graphic representation of the various ER values observed for each selected herbal product at an extreme range of concentrations (i.e. low, medium and high) in combination with indinavir.

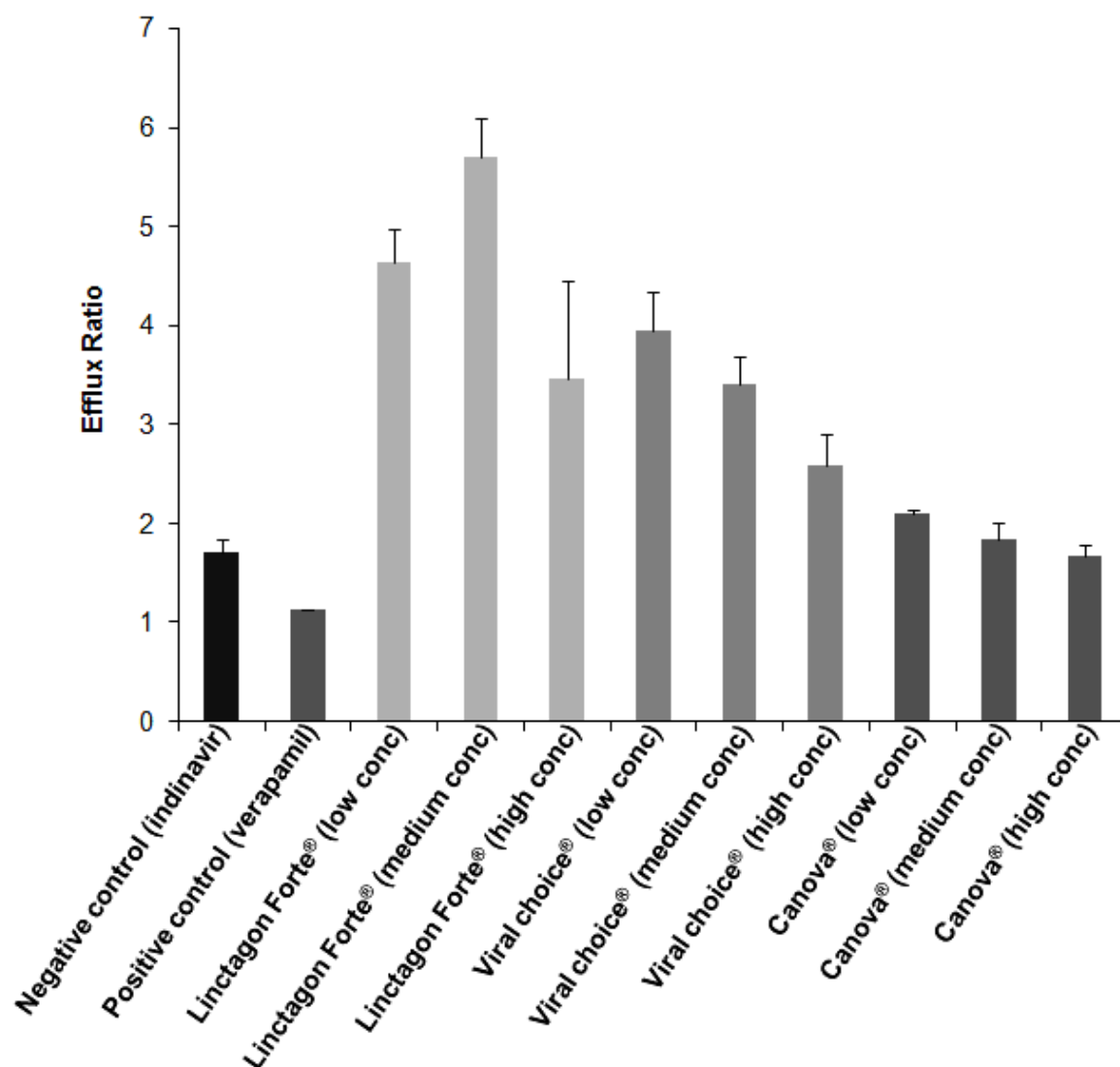


Figure 4.18: Efflux ratio (ER) values for indinavir in the presence of Linctagon Forte®, Viral Choice® and Canova®, including the negative control group (indinavir alone) and positive control group (indinavir with verapamil) (n=3) (error bars represent SD)

From Figure 4.18, it is noticeable that a pronounced increase in efflux occurred for the entire concentration range (i.e. low, medium and high concentrations) of Linctagon Forte® in combination with indinavir, when compared to both the negative and positive control group. One again, we notice a concentration dependent activation of efflux for the lower two concentrations of Linctagon Forte®, with a lower effect noticed for the highest concentration tested, which is indicative of a possible saturation effect.

During the co-application of Viral Choice®, there was a definite increase in efflux for the entire concentration range (i.e. low, medium and high concentrations) when compared to both the negative and positive control. The increase in the extent of efflux appears to be

concentration dependent. The data shows that Viral Choice[®] interacts with the P-gp mediated transport of indinavir, especially during efflux from the BL-AP direction, suggesting a biphasic effect with low allosteric activation at low concentrations and low inhibition at higher concentrations. This is similar to the trends observed for *Echinacea pururea* during an *in vitro* study by Hansen and Nilsen (2009:87-90).

The combination of Canova[®] with indinavir slightly increased the ER values of indinavir in a concentration dependent manner (i.e. from low to high concentration) when compared to the negative control group. However, when the highest concentration was compared to that of the negative control group, an increase in the efflux of indinavir was hardly detected. Generally, it appears as though Canova[®] had a negligible effect on the ER value of indinavir.

4.6 Metabolism inhibition studies

The concentration ratio of indinavir metabolite (i.e. M6) to that of indinavir was calculated in order to indicate any modulation of indinavir metabolism in the LS180 cells by the herbal products. The concentration ratio of metabolite to parent drug (i.e. indinavir) provides an indication of the extent of inhibition which occurred. Inhibition of CYP3A4 by drug and herbal substrates may potentiate the occurrence of adverse effects of orally administered drugs that are substrates of this enzyme due to the subsequent increase in bioavailability thereof.

Figure 4.19 provides a graphic representation of the results obtained during the metabolism inhibition experiment conducted during the combination of three commercially available herbal products (i.e. Linctagon Forte[®], Viral Choice[®] and Canova[®]), at an extreme range of concentrations (i.e. low, medium and high), with indinavir. Indinavir alone (200 µM) served as the negative control group and ketoconazole (40 µM), in combination with indinavir, (200 µM) served as the positive control group. Ketoconazole is a known substrate and inhibitor of CYP3A4, thus, decreasing the concentration of the most prominent indinavir metabolite (M6).

It is apparent from Figure 4.19, that there was a marked decrease in the concentration ratio of M6 to indinavir in the positive control group (indinavir with ketoconazole) compared to that of the negative control group (indinavir alone). The latter validates the model and the results obtained can now be viewed as a true reflection of the inherent actions of the experimental groups on the metabolism inhibition of indinavir.

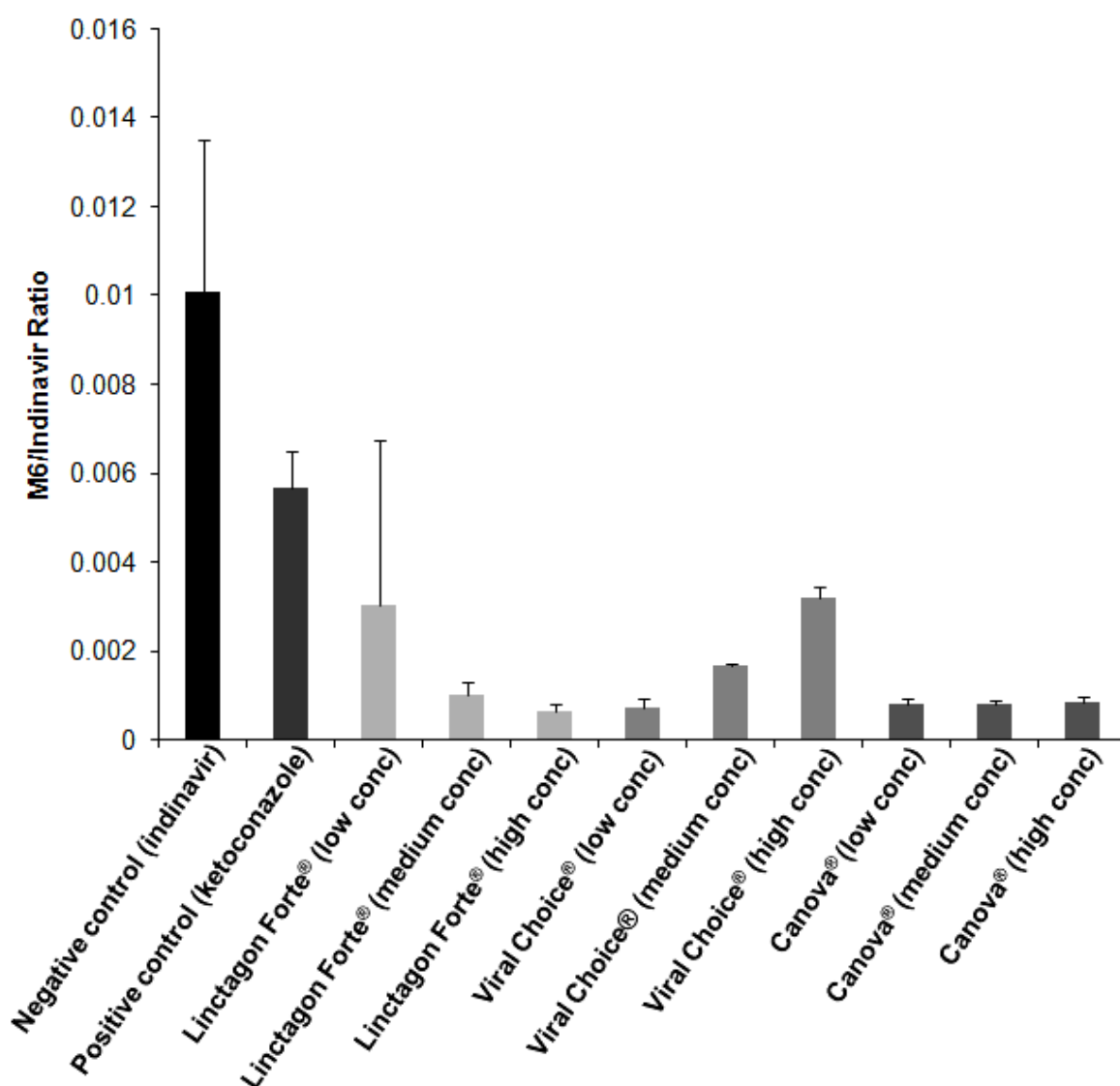


Figure 4.19: M6/indinavir concentration ratios for indinavir in the presence of Linctagon Forte®, Viral Choice® and Canova®, including the negative control group (indinavir alone) and positive control group (indinavir with ketoconazole) (n=3) (error bars represent SD)

Linctagon Forte® resulted in a concentration dependent inhibition of indinavir metabolism, most probably CYP3A4. A ± 9 fold increase in metabolism inhibition was observed with the highest concentration Linctagon Forte® when compared to the positive control group. This was consistent with the previous finding of at least one active constituent of Linctagon Forte®, namely quercetin. During an *in vivo* study on male Sprague-Dawley rats, by Choi *et al.* (2011), quercetin was capable of producing an inhibitory action on CYP3A4 in a concentration-dependent manner. The metabolism results therefore suggest a possible increase in the bioavailability of indinavir when given in combination with Linctagon Forte®.

Viral Choice[®] exhibited an inverse concentration dependent inhibition of indinavir metabolism in LS180 cells possibly due to a saturation effect. A ± 8 fold increase in inhibition was observed with the lowest concentration of Viral Choice[®] when compared to the positive control. It has previously been found that *Echinacea purpurea*, one of the major active constituents of Viral Choice[®], inhibited CYP3A4. In an *in vitro* study by Budzinski *et al.* (2000), extracts of the root and aerial portions of *E. purpurea* had an inhibitory effect on complementary deoxyribonucleic acid (cDNA) expressing CYP3A4. This was confirmed by an *in vivo* study conducted by Gorski *et al.* (2004), during which 400 mg of *E. purpurea* root was administered 4 times daily for 8 days. They found the clearance of caffeine and tolbutamide, serving as *in vivo* probes for CYP1A2 and CYP2C9 metabolism, was significantly reduced. Clearance of midazolam, the *in vivo* probe for hepatic CYP3A, was also significantly increased, which was consistent with preceding *in vitro* findings (Gorski *et al.*, 2004: 96). *Allium sativum* (Liliaceae), another active constituent of Viral Choice[®] includes high concentrates of thiosulfinates, which are sulphur containing compounds (e.g. allicin) (Tattelman, 2005:103). However, active constituents within *A. sativum* vary with different garlic preparations resulting in varying effects on the CYP enzyme system (i.e. some found inhibitory and some inducing effects on this enzyme system) (Banerjee *et al.*, 2003:98; Colalto *et al.*, 2010:210). The metabolism results presented in Figure 4.19 suggest the co-administration of Viral Choice[®] will result in an increase in bioavailability of indinavir due to a reduction in the metabolism of indinavir.

The combination of Canova[®] across the whole concentration range markedly inhibited CYP3A4 related metabolism of indinavir. A ± 7 fold increase in inhibition was observed for the entire concentration range of Canova[®] when compared to the positive control. The homeopathic remedy Canova[®] is composed of various phytoconstituents, of which several had previously shown interactions with CYP3A4, including *Aconitum napellus*, *Lycopodium clavatum*, *Ferula asafoetida*, *Ricinus communis*, *Carapichea ipecacuanha* and *Thuya occidentalis*.

Tang *et al.* (2011) investigated the involvement of various CYP isoenzymes in the metabolism of *Aconitum napellus* by means of human liver microsomes as well as recombinant CYP enzymes. It was determined that CYP3A4 and CYP3A5 were responsible for the metabolism of aconite, whereas CYP2C8, 2C9, 2D6 and 2C19 played a slight role in the metabolism (Tang *et al.*, 2011:53). Both studies suggest co-administration of aconitine with other P-gp and CYP3A4/5 substrates may result in possible pharmacokinetic herb-drug interactions.

During a study using a cell free model by Tam *et al.* (2011), the effect of *L. clavatum* on the NADPH-dependant inhibition of CYP3A4 was examined. They found *L. clavatum* significantly influenced both the NADPH and time dependent inhibition of CYP3A4 when combined with testosterone. This suggests *L. clavatum*, similar to grapefruit juice, may possibly result in mechanism-based inactivation of CYP3A4 (Tam *et al.*, 2011:14,19, 20).

Asafoetida, is the gum resin obtained from *Ferula asafoetida* (Apiacea) (Mahendra & Bisht, 2012:141). In a recent study by Al-Jenoobi *et al.* (2014), the effect of asafoetida resin on the modulation of CYP2D6 and CYP3A4 was evaluated *in vitro*, in human liver microsomes and in a clinical *in vivo* study. The *in vitro* study suggested that asafoetida was responsible for the inhibition of CYP2D6, but a concentration dependent activation of CYP3A4 was further observed. However, the *in vivo* study suggested asafoetida significantly inhibits CYP3A4 activity with little to no inhibitory effect on CYP2D6 (Al-Jenoobi *et al.*, 2014:4, 5).

In a study regarding *Ricinus communis*, it was reported during an *in silico* study, involving HerboChip® followed by fluorometric enzyme inhibition studies, that *R. communis* may have a significant effect on the inhibition of CYP3A4 (Calitz *et al.*, 2014:5).

The active constituents in *Carapichea ipecacuanha* are cephaeline and emetine amongst others. Both these active constituents were found to be substrates for CYP2D6 and CYP3A4. In an *in vitro* study involving human liver microsomes, both cephaeline and emetine, resulted in low inhibitory action against CYP2D6 and CYP3A4 (Asano *et al.*, 2001:682).

The essential oil from *Thuja occidentalis* contains the active ingredient α -thujone, which is a known substrate for CYP2A6 and CYP3A4. The latter suggests co-administration with other CYP2A6 and CYP3A4 substrates may result in pharmacokinetic interactions (Pelkonen *et al.*, 2013:102,104).

From these published studies, together with the results obtained from the current metabolism inhibition study on Canova®, it is possible these active constituents may have had a combined effect producing the pronounced overall inhibition of CYP3A4 during the co-administration with indinavir. The latter may potentially result in a possible increase of indinavir bioavailability, during the co-administration.

4.7 Conclusion

An increase in efflux as the mechanism of pharmacokinetic interaction may cause a reduction in the bioavailability of indinavir when simultaneously taken with Linctagon Forte[®], which was shown to a lesser extent in Viral Choice[®] with very little effect on Canova[®]. All three commercial products depicted *in vitro* inhibition of CYP. However, these pharmacokinetic interactions need to be confirmed with *in vivo* tests to be conclusive in terms of clinical significance.

CHAPTER 5

FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 Final conclusions

The phytochemical complexity of herbal remedies potentiates the possible pharmacological effects, resulting in pharmacokinetic or pharmacodynamic interactions with other drugs. These pharmacokinetic interactions caused by herbal products may be due to the induction and/or inhibition of intestinal efflux proteins (e.g. P-gp), as well as intestinal and hepatic metabolising enzymes (e.g. CYP3A4). When the efflux of a drug from the intestinal epithelium is increased, lower uptake of the drug is expected across the epithelium due to more molecules being actively pumped from within the epithelial cells back to the lumen. During the inhibition of CYP3A4, there is a decline in the amount of drug substrate being metabolised, resulting in an increased bioavailability with potential adverse effects or even toxicity.

The results from this study indicated Linctagon Forte® and to a lesser extent Viral Choice®, are capable of increasing efflux of P-gp substrates such as indinavir. This pharmacokinetic interaction may cause a reduction in the bioavailability of indinavir when simultaneously taken with these herbal products. This acute effect of Linctagon Forte® on indinavir transport across intestinal epithelial cells may be explained by potential activation of active transporters such as P-gp. However, the precise mechanism of action should be investigated further, as well as the potential enhancement of gene expression over longer term exposure. The data showed that Viral Choice® interacts with the P-gp mediated efflux transport of indinavir, suggesting a biphasic effect with low allosteric activation at low concentrations and low inhibition at higher concentrations. Canova®, conversely, did not appear to affect the efflux of indinavir, this may possibly be attributed to the highly diluted nature of Canova®.

There is no *in vivo* data currently available on the effects of Linctagon Forte®, Viral Choice® or Canova® on systemic delivery of drugs, but this *in vitro* study suggests that a clinical significant effect on the bioavailability of indinavir cannot be excluded.

The complete range of selected commercial products (Linctagon Forte®, Viral Choice® and Canova®) investigated in this study showed a definite effect on the extent of indinavir metabolism by using an *in vitro* model. The results suggested possible prolonged exposure to indinavir as well as increased bioavailability when co-administered with any one of the commercial products.

However, it is important for this *in vitro* study to be followed up with an *in vivo* pharmacokinetic study to be conclusive in terms of any clinically relevant bioavailability changes of indinavir by Linctagon Forte[®], Viral Choice[®] and Canova[®], as observed during this *in vitro* study.

5.2 Future recommendations

In addition to the recommended *in vivo* pharmacokinetic study, it is suggested that each of the active constituents in the selected commercial herbal products be extracted to evaluate each constituent individually. The study was based on dosages as directed during a preventative period, as in the case of Viral Choice[®], however, during a short-term boost the dosage of Viral Choice[®] is increased for a period of ten days. This suggests the possibility of the potentiating effects observed during this study and further investigations should be considered during prolonged exposure times for both *in vitro* and *in vivo* studies. This study only focused on the inhibition of indinavir metabolism in LS180 cells, but the effects on both the induction and inhibition of other models such as microsomes containing different CYP isoenzymes should also be considered for investigation.

Not only should these effects be evaluated on the protease inhibitor indinavir, but also on other ARV drugs that are substrates for P-gp, CYP3A4 and CYP2B6. This will allow identification of other possible herb-drug interactions, as these may have detrimental effects on the combined treatment regimens of HIV/AIDS infected patients. Inter-individual variability with regard to single nucleotide polymorphisms (SNPs) and polymorphisms of P-gp, CYP3A4 and CYP2B6, respectively, in various ethnic groups, could also be investigated to determine how these factors influence the bi-directional transport and metabolism of indinavir and other ARVs during the co-administration of these commercially available herbal products.

There are a myriad of commercially available herbal products which need to be screened for possible pharmacokinetic interactions to broaden our knowledge with regard to this subject as it will improve the overall treatment and wellbeing of HIV/AIDS infected patients.

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APPENDIX A

CONGRESS PROCEEDINGS & ARTICLES

Impact of selected herbal products on the *in vitro* transport and metabolism of indinavir

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Purpose: Patients infected with the human immunodeficiency virus (HIV) whom receive anti-retroviral (ARV) drug treatment are sometimes also taking herbal remedies. Pharmacokinetic herb-drug interactions are a realistic outcome in these patients. The aim of this study was to investigate the effect of selected herbal products, namely Linctagon C[®], Viral Choice[®] and Canova[®] on the *in vitro* transport and metabolism of indinavir in cell culture models.

Methods: Bi-directional transport of indinavir was conducted across Caco-2 cell monolayers in the absence (control group) and presence of three selected herbal products namely Linctagon C[®], Viral Choice[®] and Canova[®]. The apparent permeability coefficient (P_{app}) and efflux ratio (ER) values were calculated from the transport results. The metabolism of indinavir was determined in LS180 cells in the absence (control group) and presence of the four selected herbal products (experimental groups).

Results: The efflux of indinavir across Caco-2 cells increased in the presence of some of the herbal products in a concentration dependent way. However, one of the herbal products caused a reduction in the efflux of indinavir. All the selected herbal products promoted indinavir metabolism in LS180 cells.

5. Conclusions

Some of the herbal products investigated promoted indinavir efflux across Caco-2 intestinal epithelial cells and increased metabolism of indinavir in LS180 cells. This may negatively affect indinavir's bioavailability, but this needs to be confirmed with *in vivo* studies before conclusions regarding the clinical significance of this effect can be made. The product that reduced indinavir efflux may enhance its metabolism.



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Academy of Pharmaceutical Sciences held
from 12 – 14 September at the
Summerstrand Hotel in
Port Elizabeth

Proudly hosted by the Department of Pharmacy at the
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EXPERT OPINION

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Impact of traditional African medicine on drug metabolism and transport

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Introduction: Africa is a continent of rich plant biodiversity with many indigenous plants having a long history of being used for medicinal purposes. A considerable number of patients consult traditional healers in African countries for their primary health-care needs. As Western medicines become more available through governmental programmes to treat diseases such as infections with HIV/AIDS, patients are faced with an increased potential of herb-drug interactions.

Areas covered: Several medicinal herbs indigenous to Africa are discussed in terms of their effects on pharmacokinetics of allopathic drugs through modulation of enzymes and active transporters. Clinically relevant herb-drug interactions obtained from *in vivo* studies are discussed, with data from *in vitro* studies also included to ensure a complete review.

Expert opinion: Traditional herbal medicines are often used under a false sense of security because of the perception that it is safe due to its natural origin. The potential for interactions between herbal and allopathic drugs is often neglected. Data on clinically relevant herb-drug interactions from clinical trials can be used to educate health-care workers and patients, contributing to improved therapeutic outcomes.

Keywords: efflux, herb-drug interaction, metabolism, pharmacokinetics, traditional African medicines

Expert Opin. Drug Metab. Toxicol. [Early Online]

1. Introduction

The use of traditional herbal medicines remains popular in Africa as an alternative treatment with approximately 90% of the population using herbs as part of their primary health-care needs [1-4]. Many aspects such as a shortage of health-care facilities and a lack of trained health-care professionals in sub-Saharan African countries contribute to the attractiveness of using traditional herbal medicines [2,5]. The high number of people that consult traditional healers in developing countries can further be attributed to factors such as affordability, cultural acceptability, better access in rural areas and the idea that this treatment is more natural and therefore safer [2,4]. Africa is known for its rich plant biodiversity with exceptional concentrations of endemic plant species. Over 5000 plants are known to be used for medicinal purposes in Africa, but only a fraction has been properly studied [4,6,7].

There is a general lack of scientific data on the safety and efficacy of many African herbal treatments offered by traditional healers and the formulation thereof is often unknown or kept secret [3,4]. The regulation of natural products varies considerably between countries, which determines the quality of the herbal products and standardisation of their production [8]. It is well known that products prepared from the same plant can vary considerably in chemical composition due to the part of the

Article highlights.

- Use of traditional herbal medicines is still playing an important role in the primary health care of a high number of people in African countries.
- Combined use of traditional herbal medicines with allopathic drugs may result in pharmacokinetic interactions such as modulation of enzymatic metabolism and changes in active transporter activities.
- Some medicinal herbs indigenous to Africa have been found to interfere with the pharmacokinetics of allopathic (conventional) medicines, which have mostly been indicated by *in vitro* screening assays or preclinical *in vivo* studies in animals.
- The clinical significance of many of these herb–drug pharmacokinetic interactions cannot be deduced from the *in vitro* results and more clinical studies are needed in humans to be conclusive.

This box summarises key points contained in the article.

plant that was used, the geographical location where it was sourced, the manufacturing process followed and the dosage form in which it was formulated [9]. Furthermore, herbal or natural plant products are usually composed of a complex mixture of various phytochemicals, each exerting potential pharmacological effects to a certain extent. These phytochemicals may cause pharmacodynamic or pharmacokinetic interactions with allopathic medicines when taken simultaneously by a patient [10]. To this extent, even herbal products that are safe when used on their own may pose a risk when taken in combination with synthetic pharmaceutical agents due to the potential of herb–drug interactions [11].

Pharmacokinetic interactions occur primarily by means of modulation of intestinal and hepatic metabolising enzymes, but it can also occur by modulation of intestinal transporters including efflux by p-glycoprotein (P-gp), a member of the ATP-binding cassette sub-family B member 1 (ABCB1), or other multiple resistance proteins [10]. The CYP system is a super family of enzymes consisting of several isoforms that play an integral role in the biotransformation of drugs. One of the most abundant isoforms of the CYP enzyme system in the human liver and intestinal epithelium is CYP3A4, which is capable of catalysing the oxidation of 43% of commercial drugs. Clinically relevant drug interactions can occur due to inhibition of these enzymes through mechanism-based inhibition (irreversible inactivation of the CYP enzyme) or competitive inhibition (reversible inactivation of the CYP enzyme), but interactions may also include induction of enzymes to enhance drug metabolism [12]. P-gp is primarily found in the epithelial cells of the gastrointestinal tract, pancreatic and bile ducts, kidney proximal tubules and adrenal gland, while it is also present in the endothelium of the blood–brain barrier. The physiological function of P-gp is believed to be the protection of organs against toxic compounds. Since P-gp actively transports compounds with a

large variation in chemical structures, many drugs and metabolites thereof are extruded (e.g., from the blood into the lumen of the gastrointestinal tract or from the hepatocytes into the bile ducts, etc.). P-gp is, therefore, responsible for a decrease in bioavailability and retention time of drugs in the body [13].

This review focuses on African herbal medicines that have been associated with modulation of drug metabolism, but will also refer to other pharmacokinetic interactions such as modulation of active transporters where applicable. For the sake of completeness and due to scarcity of *in vivo* data, information obtained from *in vitro* studies is also included.

2. *Aloe ferox*

Aloe ferox Miller or Cape Aloe is a single-stem aloe that can reach a height of approximately 10 m [7]. *A. ferox* is widely distributed throughout South Africa, but a concentrated population is found in the south-eastern regions. Traditionally, extracts from different parts of this plant were used as a laxative, for treatment of burns, sores, insect bites, toothaches, sinusitis, stomach pains, conjunctivitis and arthritis, while boiled leaves and roots were used for hypertension and stress. The active components aloeresin A, aloecin and aloin are found in the external green rind of the leaves. Other compounds isolated from *A. ferox* include chromones, anthraquinones, anthrones and other phenolic compounds [7].

Although no information could be found on the effects of *A. ferox* on drug metabolism, an *in vitro* study investigated the influence of gel, whole leaf and precipitated polysaccharide materials from *A. ferox* gel on the bidirectional transport of cimetidine (a known substrate for various efflux transporters including P-gp) across excised rat intestinal tissue mounted in Sweetana-Grass diffusion chambers. In this study, the gel and whole leaf materials inhibited the efflux of cimetidine with a potential to change the pharmacokinetics of drugs that are substrates for efflux transporters. However, the clinical relevance of this interaction needs to be confirmed with *in vivo* studies before final conclusions can be drawn [14].

3. *Asphalatus linearis*

Asphalatus linearis (Brum.f) R. Dahlger (Fabaceae), better known as ‘Rooibos’, forms part of the fynbos family indigenous to South-Africa endemic to the Western Cape region where it is harvested during the summer months. It is enjoyed as a recreational beverage, but has various medicinal uses such as cancer prevention, treatment of insomnia, eczema, infantile colic, anxiety, asthma and dyspepsia [15,16]. Tea prepared from *A. linearis* known as Rooibos tea is known to be caffeine-free, low in tannins but rich in antioxidants [17]. Twigs and leaves of *A. linearis* are traditionally used in the fermented or oxidised form [7,18]. The dihydrochalcone C-glucosides, asphalathin and nothofagin, eriodictyol-C-glycoside isomers, aspagenin and luteolin are major flavonoids found in the

Rooibos plant [16]. Other compounds include various flavones, flavanones and flavonols [7]. These active ingredients contribute to the biological activity of Rooibos. Moderate anti-mutagenic activity is associated with aspalathin and nothofagin, and mutagenic, co-mutagenic as well as pro-mutagenic and anti-mutagenic activities have been displayed by the flavonoids quercetin and isoquercetin [7]. The *in vivo* lipid profile and the redox status are significantly improved with the consumption of Rooibos tea for 6 weeks, by decreasing low-density lipoprotein-cholesterol and triacylglycerol levels and increasing high-density lipoprotein-cholesterol levels, thus adding protection against cardiovascular disease to its various benefits [19].

Rooibos tea (tea prepared from *A. linearis*) is responsible for the inhibition of CYP21 and CYP17 that affect the glucocorticoid and androgen biosynthesis, respectively, in an *in vitro* ovine adrenal microsome model [20]. Jang *et al.* [21] found a 50% inhibition of CYP2C11 in Sprague-Dawley rats when treated with 2 g/kg of Rooibos plant material in water for 3 days. It has also been established that Sprague-Dawley rats exposed to Rooibos tea during a 2-week time period resulted in a significant decrease in the plasma concentrations of midazolam with an increase in the expression of intestinal CYP3A [3]. Cytosolic S-transferase- α activity and microsomal UDP-glucuronosyl transferase activity have been significantly enhanced by Rooibos in male Fisher 344 rats affecting the metabolism of drugs and promutagens [3].

4. *Cyclopia* spp.

Cyclopia spp. Vent. (Fabaceae) also known as honeybush, bushtea, 'honigtee', 'heuningtee', 'heuningbostee', 'bergtee', 'boertee', 'bossietee' (Afrikaans) is a shrub indigenous to South-Africa found in areas of the Eastern and Western Cape provinces [15,22-24]. Various *Cyclopia* species are used for tea preparation, namely *C. vogelii* later renamed *C. subternata*, *C. latifolia*, *C. intermedia*, *C. sessilifolia*, *C. longifolia*, *C. bowieana*, *C. burtonii*, *C. buxifolia*, *C. maculata*, *C. meyeriana*, *C. plicata*, *C. pubescens* and *C. genistoides* [22]. Traditionally, it was not only enjoyed as a recreational beverage or tea but a decoction of the leaves and flowers were used as an expectorant in pulmonary tuberculosis [22]. The major active compounds present in all the *Cyclopia* species are mangiferin and isomangiferin and the flavone hesperidin [22-24]. Other compounds include various flavanones, flavones, isoflavones, flavonols and coumestan [22]. Various *in vitro* as well as *in vivo* (animal models) investigations were conducted on mangiferin and it was found to have immunomodulating, lipolytic and antioxidant activities, but also chemopreventive and anti-diabetic actions [24].

Mangifera indica, commonly known as the mango tree and a constituent in the phytodrug Vimang[®], is composed of various polyphenols, phenolic esters, flavan-3-ols and xanthones, one of which is mangiferin, also found in *Cyclopia* spp. [25,26]. An *in vitro* investigation by Rodeiro *et al.* [25], on the effects of

M. indica extracts on CYP metabolism in primary culture rat hepatocytes, revealed the inhibition of CYP1A1 and the induction of CYP2B1 with no appreciable effect on CYP2E1, CYP2C, CYP2D1 and CYP3A1. However, in 2009, Rodeiro *et al.* [27] investigated the potential inhibitory effects of mangiferin on CYP3A4, CYP2D6, CYP1A1 and CYP1A2 in human liver microsomes. Only CYP3A4 activity appeared to be inhibited with no significant effects on both CYP1A1 and CYP1A2, contradicting the inhibition of CYP1A1 and induction of CYP2B1 found in the previous study on rat hepatocytes [27]. The study on rat hepatocytes showed that an extract of *M. indica* had no effect on CYP3A1. However, in a study using human liver microsomes on mangiferin showed significant inhibition of CYP3A4 [27]. Chieli *et al.* [26] then conducted a study of the *in vitro* effects on ABCB1/P-gp activity of *M. indica* and the mangiferin metabolite, norathyriol, in the human kidney (HK-2) and Caco-2 cell lines, with the use of a Calcein-AM as well as the Rhodamine-123 accumulation assays. Chieli *et al.* [26] found that mangiferin and norathyriol significantly inhibited P-gp activity. Also, Mertens-Talcott *et al.* [28] found that the flavonoids naringenin as well as naringin (flavonoids that are present in *Cyclopia* spp.) inhibited P-gp, thus affecting drug bioavailability. Based on these findings, it can be accepted that *Cyclopia* spp., which contain mangiferin, may have similar CYP3A4 and P-gp inhibitory activities; however, it should be confirmed before final conclusions can be made.

5. *Harpagophytum* spp.

Two species in the genus *Harpagophytum* de Candolle ex Meissner (Pedaliaceae) are commercially used, namely *Harpagophytum procumbens* and *H. zeyheri*. Common names for these plants include Devil's Claw, teufelskralle, grapple plant, kanako, woodspider and duiwelsklou (Afrikaans). *H. procumbens* consists of two subspecies and *H. zeyheri* consists of three subspecies. *Harpagophytum procumbens* subspecies *transvaalense* Ihlenfeldt and *H. Hartmann* are restricted to the Limpopo province of South Africa, while *H. procumbens* (Burch) de Candolle ex Meissner occurs in the northern parts of South Africa, Namibia and Botswana. *Harpagophytum zeyheri* subspecies *schijffii* occurs in a small region in the north-eastern parts of South Africa, *H. zeyheri* subspecies *sublobatum* occurs in Angola, Botswana, Zimbabwe, Zambia and Namibia, whereas *H. zeyheri* subspecies *zeyheri* occurs in the north-eastern region of South Africa [29].

Devil's Claw (*H. procumbens* and *H. zeyheri*) is primarily used for the treatment of rheumatism and arthritis due to its analgesic and anti-inflammatory properties. However, other uses include treatment of cardiovascular disease, adult-onset type-2 diabetes, gastrointestinal disturbances such as dyspepsia, and ulcers and boils [29-31]. The biological activity of Devil's Claw is primarily attributed to the iridoid glycoside harpagoside. Other glycosides including harpagide and procumbide have also been isolated. Devil's Claw preparations

are usually standardised with respect to the harpagoside content [31–33]. The effectiveness of Devil's Claw preparations cannot be transferred from one product to another. However, it seems that products that contain at least 50 mg of harpagoside as daily dosage appear to be more effective compared to products containing lower quantities of harpagoside [34].

With regard to safety and toxicity, it appears that Devil's Claw (*H. procumbens* and *H. zeyheri*) is well tolerated as no serious adverse effects have been reported to date. Most side effects are described as gastrointestinal disturbances including bloating [35]. Furthermore, current evidence supports the relative safety of Devil's Claw to that of NSAIDs [31].

Most interactions of Devil's Claw (*H. procumbens* and *H. zeyheri*) with conventional medicines are mediated through modulation of CYP isoenzymes and/or modulation of multi-drug transporters such as multi-drug resistance-1/P-glycoprotein (MDR-1/P-gp) [31]. Unger and Frank [36] showed that Devil's Claw (*H. procumbens*) extracts inhibited six different CYP enzymes (i.e., CYP1A2, 2C8, 2C9, 2C19, 2D6 and 3A4) *in vitro*. In another study, employing a HK-2 proximal tubule cell line, it has been shown that three different commercial Devil's Claw (*H. procumbens*) preparations were able to inhibit the activity of P-gp, although to a different extent. However, pure harpagoside was almost unable to inhibit P-gp activity, but modulated the expression of P-gp. Further studies are therefore required to identify the components in Devil's Claw (*H. procumbens*) that modulate the activity and expression of P-gp as well as the clinical implications thereof [33].

6. Hypoxis hemerocallidea

Hypoxis hemerocallidea Fisch. & C.A. Mey (Hypoxidaceae) also known as African potato is a plant originally from Southern Africa, but is now also cultivated in South America, Australia and Asia [3]. It is widely used in Southern Africa, especially as an immunostimulant by HIV/AIDS patients receiving anti-retroviral (ARV) therapy [37–40]. This traditional African medicinal plant is also used for various other conditions including diabetes, urinary diseases, tumours, internal parasites, headaches, cardiac diseases, immune-related illnesses and prostate hypertrophy [3,7,38,41]. It is claimed that therapeutic effects can be obtained with 2400 mg raw plant material daily, which can be ingested by eating the root or preparing an aqueous decoction of the root or the corms [38,41]. The main active constituent is hypoxoside, which is a nor-lignan di-glucoside present in the corms that is converted to the biologically active rooperol in the gastrointestinal tract, while other active compounds include sterols, sterolins, stanols and stigmasterols [3,7,38,41]. Extracts of *H. hemerocallidea* are commercially available, for example, 'Harzol®' for the treatment of prostate adenoma [38].

The safety of concomitant administration of *H. hemerocallidea* and conventional medicines has not been fully determined, but several studies have investigated its potential

interaction with the transport and metabolism of prescription drugs. Mills *et al.* [41] used an *in vitro* microsome fluorometric microtitre plate assay to assess the effect of water and methanol extracts of two ground and a capsulated *H. hemerocallidea* preparation on CYP3A4 activity. The water extracts (100 mg/ml) inhibited CYP3A4 activity significantly and the methanol extracts (100 mg/ml) showed even higher inhibition. It was determined that *H. hemerocallidea* raw material as well as commercial products containing *H. hemerocallidea* inhibited CYP3A4, CYP3A5 and CYP19, which was concentration dependent. An aqueous extract of *H. hemerocallidea* resulted in 20 – 45% inhibition of the above-mentioned CYP enzymes. A commercial product containing 70 mg *H. hemerocallidea* extract inhibited these enzymes 62.29, 73.17 and 85.51%, respectively, at a concentration of 10 mg/ml of the product. The aqueous extract of *H. hemerocallidea* showed induction of the P-gp transporter (38%) at 24 h after administration. Interestingly, it was shown that CYP inhibition did not correlate with the hypoxoside content of the preparations [38].

The effect of a water extract of *H. hemerocallidea* corms on the transport of nevirapine, a non-nucleoside reverse transcriptase inhibitor (NNRTI) predominantly metabolised by CYP3A4 and CYP2B6, was investigated in an *in vitro* study using the Caco-2 cell model. It was found that the *H. hemerocallidea* extract significantly reduced the efflux of nevirapine, which suggested that it may lead to increased bioavailability of the drug when taken simultaneously [42,43]. The effects of an aqueous extract of *H. hemerocallidea* on the *in vivo* pharmacokinetics of efavirenz, an NNRTI effective against HIV-1 infection, were studied in human subjects. Efavirenz is mainly metabolised by CYP2B6 and to a lesser extent by CYP3A4, but this study did not find any interference in the pharmacokinetics of efavirenz by the co-administration of the *H. hemerocallidea* extract [39]. A recent clinical study investigated the effects of *H. hemerocallidea* on the pharmacokinetics of the ARVs, lopinavir and ritonavir (CYP3A4 substrates), in 16 healthy adults. In the study, 400 mg lopinavir and 100 mg ritonavir were administered twice daily for 14 days followed by 7 days of simultaneous *H. hemerocallidea* dosing, which consisted of four capsules containing 320 mg hypoxoside each morning. The study did not find any clinically significant changes in the pharmacokinetics of the ARVs [44].

The effects of an *H. hemerocallidea* corm extract were investigated on nine CYP enzymes in human liver microsomes as well as on some transporter proteins in cell cultures [40]. Within the concentration range tested (i.e., 0.39 – 100 µg/ml *H. hemerocallidea* extract), CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9 and CYP3A4/5 were inhibited in a concentration-dependent manner, while CYP2C19, CYP2D6 and CYP2E1 were not significantly inhibited. The P-gp efflux transporter protein also did not appear to be inhibited (500 µg/ml *H. hemerocallidea*).

When *in vitro* findings are compared with the results from the *in vivo* herb-drug interaction studies so far undertaken

with *H. hemerocallidea*, it is clear that further investigations are needed to establish to which extent this herbal medicine will affect pharmacokinetics of drugs that are substrates for efflux transporters and CYP enzymes.

7. *Polygala fruticosa*

Polygala fruticosa P.J. Bergius, family Polygalaceae, better known as butterfly bush (English) or ertjiebos (Afrikaans) or ithethe (Zulu), is a shrub indigenous to South-Africa found in areas of the south-western Cape and as far as KwaZulu-Natal [45-47]. Decoctions prepared from the roots and leaves are used for the treatment of various ailments such as tuberculosis, blood purification and poor circulation, intestinal ulcers, sinusitis and gonorrhoea [46,48]. It has also been traditionally employed to facilitate childbirth [46]. Van Vuuren and Naidoo [45] found no significant antimicrobial activity for *P. fruticosa* on *Neisseria gonorrhoeae*, although it did prove significant antimicrobial activity against *Ureaplasma urealyticum* and *Gardnella vaginalis*. Mukinda and Eagles [46] found *P. fruticosa* to be non-toxic in low dosages with only minimal toxicity potential when administered acutely and chronically. *Polygala fruticosa* contains the active ingredients frutinone A, frutinone B and frutinone C, of which frutinone A has proven to be the most active in terms of antifungal activity, and methoxy and hydroxyl substitutions differentiate these active constituents from each other and are associated with the lack of activity seen in frutinone B and C [49]. Thelingwani *et al.* [49] conducted a study on the *in vitro* reaction phenotyping and inhibition in human liver microsomes to determine the effects of frutinone A on CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 enzymes. Moderate inhibitory effects of Frutinone A were noted on CYP2C9, CYP2C19, CYP2D6 and CYP3A4, while it proved to be a potent inhibitor of CYP1A2. However, the clinical significance of this inhibitory effect is not yet known on drug disposition and further *in vitro* as well as clinical studies are needed to confirm these possible interactions.

8. *Ricinus communis*

Ricinus communis (Euphorbiaceae) is also known as the Castor oil plant, Castor bean, Ricin or Lepo and is originally native to north-eastern Africa and the Middle East, but it has now become a weed in almost every area in the world with a tropical or subtropical climate. It is an evergreen semi-woody shrub that can reach heights of up to 5 m. The seeds contain ricin, the main toxic protein, whereas seed oil contains ricinine and toxalbumin ricin, which are considered purgative. Human poisoning starts within a few hours of ingestion and children are most sensitive as a single seed can be fatal in a child [50,51].

Oil from *R. communis* seeds known as castor oil is an ingredient in many traditional remedies used for treatment of a variety of ailments, which include amongst others arthritis,

asthma, boils, burns, cancer, carbuncles, colds, colic, convulsions, dermatitis, headache, inflammation, rheumatism, seborrhoea, stomach-ache, swellings, toothaches and wounds. It is traditionally used in the Machakos district of Kenya to induce or enhance labour, to manage delayed labour, for post-partum haemorrhage and retained placenta [51-56].

Lee *et al.* [57] screened 49 herbal species for their interactions with CYP3A4. Only 14 of the species screened by HerboCHip[®] exhibited positive interactions with CYP3A4. Seven of the species depicted non-specific binding profiles, whereas the other seven displayed solid responses of which *R. communis* was one. The initial screening process was followed by fluorometric enzyme inhibition assays. They found that *R. communis* significantly inhibited CYP3A4 and the inhibition assay depicted a maximum inhibition ratio of 0.30 (inhibition ratio = [no inhibition - sample]/[no inhibition - blank]). However, these *in vitro* findings need to be followed up by clinical studies to be conclusive on the effects that *R. communis* has on the pharmacokinetics of conventional medicines in humans.

9. *Sutherlandia frutescens*

Sutherlandia frutescens (Fabaceae), also referred to as cancer bush, is a flowering shrub indigenous to South Africa. It has red flowers and bulbous pods, which are commonly used as a traditional medicine on its own or as an ingredient in various traditional medicinal mixtures. The isiZulu, isiXosa, isiSotho and Khoi-San people traditionally prepared aqueous decoctions of this plant to treat a variety of symptoms and illnesses such as stress, anxiety, pain, fever, gastrointestinal cramps, cancer, diabetes, heartburn and chicken pox [23,58]. It was also previously stated that its use may have beneficial effects such as weight gain, improved T-helper cells or CD4 counts, viral load reduction, improved appetite and general mood improvements when administered to HIV/AIDS patients [59,60]. The recommended therapeutic dose of *S. frutescens* leaf powder in humans is 9 mg/kg/day. It is commercially available in capsules containing 300 mg dry leaf powder each and it is usually taken twice daily [58,61]. The ethnomedicinal properties of *S. frutescens* may be attributed to the presence of a variety of biologically active compounds such as L-canavanine, pinitol, γ -aminobutyric acid, flavonoids and triterpenoid glucosides [62,63].

The use of extracts or medicinal products of *S. frutescens* in combination with prescription drugs was reported to exhibit potential herb-drug interactions. These interactions may have important implications on the absorption and metabolism and thus the overall oral bioavailability of co-administered drugs [41].

Mills *et al.* [60] demonstrated *in vitro* effects of *S. frutescens* extracts on CYP3A4 and P-gp in a microsome-based fluorometric microtitre plate assay. The results showed a massive 96% inhibition of CYP3A4 with methanol extracts and to a lesser degree with water extracts. The P-gp activity was only

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moderately affected by the presence of either water or methanol extracts of *S. frutescens*. The activity strength of both *S. frutescens* extracts was between 19 – 31% relative to that of the known P-gp inhibitor verapamil. Müller *et al.* [64] showed that various phytochemical components in aqueous extracts of *S. frutescens* had a potentially negative effect on the accumulation of atazanavir in Caco-2 cells. The reduced accumulation was attributed to the presence of sutherlandiosides and/or sutherlandins in the triterpenoid and flavonol glycoside fractions of *S. frutescens*. It was speculated that the mechanism of action of these phytoconstituents to limit accumulation of atazanavir in Caco-2 cells might be the inhibition in the activity of influx transporters or induction of efflux transporters such as P-gp. It was suggested that the absorption and bioavailability of atazanavir may be limited due to one or both of these mechanisms. However, it was also found that both aqueous and methanolic extracts of *S. frutescens* inhibited atazanavir metabolism in human liver microsomes, suggesting a potential enhanced bioavailability [64].

In another study [65], aqueous extracts of *S. frutescens* were administered to male Sprague-Dawley rats to investigate its effect on the pharmacokinetic parameters of nevirapine. Nevirapine was chosen as a model drug because it is a known substrate for rat CYP3A2. Based on the results it was evident that a single administration (referred to as short-term exposure) of *S. frutescens* did not have a statistically significant effect on the plasma concentration of nevirapine. However, exposure for a period of 5 days (referred to as chronic exposure) did yield a statistically significant ($p < 0.01$) decrease in the AUC and C_{max} values of nevirapine, which would suggest a possible induction of CYP3A2 in rats [65]. Prevoo *et al.* [62] reported that aqueous, methanolic and chloroform extracts of *S. frutescens* were able to inhibit CYP17, CYP21 and CYP11B1 to varying degrees.

From the results of a limited number of studies available in the literature as described above, it is evident that *S. frutescens* may have important effects on the pharmacokinetics of certain drugs, especially when used over longer periods. However, more data from clinical trials are needed to be conclusive regarding interactions of *S. frutescens* on drug pharmacokinetics in humans.

10. Miscellaneous West-African medicinal plants

Several medicinal plants from West-Africa have been screened for *in vitro* interactions with CYP enzymes as summarised in Table 1.

11. Conclusion

Traditional herbal medicines continue to play an important role in the treatment of patients in Africa, while government programs make conventional medicines more accessible to patients in rural areas. The combined use of traditional herbal

medicines with conventional medicines poses risks of pharmacokinetic interactions that may affect the therapeutic outcome in certain cases. Pharmacokinetic interactions occur mostly due to modulation of enzymes but may also occur due to changes in active transporters. Research has highlighted some pharmacokinetic interactions between African herbs and allopathic drugs, but more information is needed to demonstrate clinically significant interactions.

12. Expert opinion

The interest in herbal medicines is growing vastly worldwide, while the use of traditional medicines is common practice and has been maintained over centuries in some countries. The high incidence of HIV/AIDS infections in sub-Saharan African countries led to the rollout of governmental treatment campaigns such as provision of free ARV medicines to people living in rural areas where traditional medicines usage is common practice. This situation lends itself to potentially high rates of herb–drug interactions. Some patients use herbal remedies with a false sense of security because of the perception that medicinal products of natural origin are harmless and they often self-medicate in combination with prescription drugs without realising that it may impact on the therapeutic outcome. Furthermore, patients are generally unwilling to declare their use of herbal medicines to medical practitioners or to report serious adverse effects as a result of combining herbal products with allopathic medicines. The true prevalence of herb–drug interactions is therefore not only underestimated, but also difficult to determine accurately. Factors such as lack of meaningful data from adverse reports and clinical trials as well as under-reporting by patients and health-care workers contribute to this situation.

Although ample information is available on well-known herbal products used in the Western world regarding their biopharmaceutical properties and pharmacokinetic interactions with allopathic medicines (e.g., interactions between *Hypericum perforatum* or St. John's wort and drugs such as warfarin), less information is available on this aspect of African traditional medicines. Furthermore, many of the pharmacokinetic interactions of these traditional herbal medicines have only been identified by means of *in vitro* studies and data from well executed clinical trials are scarce. Although the usefulness of data obtained from preclinical studies on herb–drug interactions has been questioned in terms of clinical practice, these studies play an important role to identify potential interactions early on and to clarify the mechanisms of action of these interactions. Furthermore, technological advancements have led to improved *in vitro* models such as three-dimensional cell cultures as well as *in silico* models that mimic the *in vivo* situation much more realistically. Data obtained from these models have become highly predictive and correlate much better with data from clinical studies in humans.

Table 1. Summary of medicinal plants from West-Africa, which indicated potential interactions with metabolic enzymes.

Scientific name	Common name	Active phytochemical compounds	Uses and pharmacological activities	CYP interactions	Ref.
<i>Aframomum melegueta</i> (Roscoe) K. Schum (Zingiberaceae)	Grains of paradise, Alligator pepper, Melegueta pepper, Guinea grains, Guinea pepper	Essential oils: gingerol, 6-shagaol 6-paradol	<i>Traditional use:</i> Abscesses and joint distortions, constipation, diarrhoea, rheumatism and fever <i>Pharmacological activity:</i> Antimicrobial as well as antifungal effects, specifically in the treatment of bilharzias	Inhibition of CYP3A4, CYP3A5 and CYP3A7	[66-70]
<i>Harrisonia abyssinica</i> (Simaroubaceae)	Baingou, Hedja, Mkidori, Mkoromando, Mkusu, Mpapuradoko, Msamburini, Msoma	Limonoids, terpenoids, spiro-tetranortriterpenoid pederin and quassinoid perfoaquassin A, sterols and ketosteroids and chromone derivatives	<i>Traditional use:</i> Fever, coughing, malaria, infertility, dysmenorrhea, gonorrhoea, dysentery, skin diseases, bilharzia infections, intestinal worms, insomnia, dizziness, nausea, vomiting, bubonic plague, inflamed testicles, tuberculosis and induction of abortion <i>Pharmacological activity:</i> Antibacterial, antiparasitodal, antifungal, antiviral and molluscicidal activities	Inhibition of CYP3A4, CYP3A5 and CYP3A7	[70-87]
<i>Jatropha curcas</i> (Euphorbiaceae)	Physic nut, Purging nut, Purgeerroot, Babati, Hab el meluk	Organic acids, saponins, tannins Cyclic triterpenes stigmasterol, flavonoids, sitosterol, curculathyrane A and B, curcussone A-D, diterpenoids jatrophaol and jatrophaolone A and B, phorbol esters	<i>Traditional use:</i> Sciatica, dropsy, paralysis, rheumatism, dysentery, diarrhoea and skin diseases <i>Pharmacological activity:</i> Coagulant and anticoagulant activities, antibacterial, anti-inflammatory activity, pregnancy-terminating effect, anti-diabetic activity, anti-helminthic activity, anti-diarrhoeal activity, anti-ulcer activity and gastroprotective effect, anti-diabetic action	Inhibition of CYP3A4 and CYP3A7	[70,88]
<i>Lippia multiflora</i> Moldenke (Verbenaceae) also known as <i>Lippia adoensis</i> Hochst	Lippia tea, Gambian bush tea, Bush tea, Healer herb	Monoterpene, sesquiterpenes, flavonoids, other compounds such as verbascoside, derhamnosylverbascoside, isoverbascoside, sterols, carotenoids	<i>Traditional use:</i> Hepatic disease, anti-hypertensive, antimalarial and respiratory diseases <i>Pharmacological activity:</i> Antifungal, anti-hypertensive, antiviral, antimalarial	Inhibition of CYP3A4, CYP3A5 and CYP3A7	[70,89-91]

Table 1. Summary of medicinal plants from West-Africa, which indicated potential interactions with metabolic enzymes (continued).

Scientific name	Common name	Active phytochemical compounds	Uses and pharmacological activities	CYP interactions	Ref.
<i>Lonchocarpus sericeus</i> (Poir.) Kunth (Fabaceae)	Angelim	Pyrolidone alkaloids, rotenone, flavonoids, chalcones	<i>Traditional uses:</i> Treatment for convulsions and backache, parasitic skin conditions and eruptions, laxative, cure for biliousness and fever	Inhibition of CYP3A5 and CYP3A7	[70,92,93]
<i>Oxythenanthera abyssinica</i> (A.Rich.) Munro (Poaceae)	Kawu, Goora, Nyantanga, Kewal, Eman, Holy vanda bamboo, Wild bamboo, Hellige vanda bamboes	Steroids, alkaloids, saponins Tannins, cardiac glycosides, flavonoids, phlobatanins, anthraquinones, terpenes	<i>Traditional uses:</i> Dysentery, diabetes, colic, rheumatism, skin diseases, polyuria, oedema and albuminuria <i>Pharmacological activity:</i> Antioxidant activity	Moderate inhibition of CYP3A7 and CYP3A5	[70,94]
<i>Persea americana</i> Miller. (Lauraceae)	Alligator pear, Butter pear, Guatemalan, Avocado, Mandinka, Mparachichim, Mpea, Mwembe	Monoglycosyldiglycerides, diglycosyldiglycerides, phospholipids, carotenoids, chlorophylls	<i>Traditional uses:</i> Menorrhagia, hypertension, stomach ache, bronchitis, diarrhoea, diabetes, jaundice, dysentery, convulsions and epilepsy, promotion of menstruation, spasmodic and abortion, scabies, purulent wounds, lesions of the scalp, rheumatism, neuralgia, toothache, anaemia, exhaustion, hypercholesterolemia, hypertension, gastroduodenal ulcers and pyorrhoia <i>Pharmacological uses:</i> Antioxidant activity, anti-hypercholesterolemic and anti-hyperlipidemic activities, anti-cancer, anti-osteoarthritic, antimicrobial and larvicidal activities, antiviral, anti-inflammatory, hepatoprotective, hypoglycaemic and anti-diabetic activities, anti-convulsant, wound-healing and anti-psoriasis activity	Inhibition of CYP3A4 and CYP3A7 Moderate inhibition of CYP3A5	[70,95]

Table 1. Summary of medicinal plants from West-Africa, which indicated potential interactions with metabolic enzymes (continued).

Scientific name	Common name	Active phytochemical compounds	Uses and pharmacological activities	CYP interactions	Ref.
<i>Phyllanthus ananrus</i> Schum. and Thonn (Euphorbiaceae)	Hinlinew, Bounrou, Bomagua kene, Ahivi, Bhunyaamalaki, Eyin olobe, Kidney stone plant, Nela urika, Black catnip, Gale of wind	Lignans, flavonoids, hydrolysable tannin, alkaloids, triterpenes, sterols and volatile oil	<i>Traditional uses:</i> Jaundice, diarrhoea, dysentery, diabetes, fevers, ulcers, sores, boils, wounds, malaria, venereal diseases, oral or vaginal thrush and urinary tract infections <i>Pharmacological uses:</i> Anti-anesthetic activity, antibacterial, anti-plasmodial and antifungal activities, anti-cancer, anti-diarrhoeal, gastroprotective and anti-ulcer activity, analgesic, anti-inflammatory, anti-allodynic and anti-oedematogenic activities, anti-nociceptive activity, antioxidant, antiviral, hypoglycaemic and hypocholesterolemic activities, diuretic and anti-hypertensive activity, hepatoprotective activity and contraceptive effect	Inhibition of CYP3A4, CYP3A5 and CYP3A7	[70,96,97]
<i>Piper guineense</i> Schum and Thonn. (Piperaceae)	Pepper bush, Ashanti, Ashanti pepper, West African black pepper, Guinea cubebs	Tannins, flavonoids, phlobatannins, saponins, terpenoids, glycosides, steroids	<i>Traditional uses:</i> Rheumatism, bronchitis, arthritis, stomach problems, pulmonary problems and diuretic <i>Pharmacological uses:</i> Hepatoprotective effect, antimicrobial activity by the essential oils and minimal antibacterial effect	Inhibition of CYP3A4, CYP3A5 and CYP3A7	[70,98-101]
<i>Talinum triangulare</i> (Jacq.) Willd (Portulacaceae)	Water leaf, Okigbo	Alkaloids, saponins, tannins, essential oils, cardioglycosides, flavonoids, polyphenols, kaempferol	<i>Traditional uses:</i> Diuretic, gastrointestinal disorders, cardiovascular diseases, polyuria, measles, hepatic ailments and cancer <i>Pharmacological uses:</i> Antioxidant activity and dose-dependent antiulcer properties	Moderate inhibition of CYP3A7	[70,102-104]

Discrepancies between the outcomes of *in vitro* and *in vivo* herb–drug interaction studies as well as between studies from different scientific groups make it difficult to reach final conclusions in some instances. Use of standardised extraction methods, validated *in vitro* models and realistic dosing sizes and durations during *in vivo* studies are some of the factors that need to be addressed in future studies. The concentrations of herbal extracts to be applied during *in vitro* studies as well as type of assay to be used are controversial topics that need careful consideration in each study design to obtain meaningful results with potential extrapolation to the *in vivo* situation.

Traditional herbal medicines continue to play an important role in primary health care of many patients in developing countries and information on how these herbs may impact therapeutic outcomes of co-administered allopathic medicines is of cardinal importance. Patients on drugs with narrow therapeutic indices or drugs that are chronically used (e.g., ARV drugs) are particularly vulnerable to be exposed to potentially hazardous pharmacokinetic interactions. Although many

herb–drug interactions have negative inhibitory effects, it is important to realise that some of these interactions may occur to the benefit of the patient by improving the bioavailability of the co-administered drug or through synergistic pharmacodynamic effects. Awareness of potentially dangerous herb–drug interactions among health-care workers and patients can contribute to improve therapeutic outcomes. A need, therefore, exists for clinical trials on defining clinically relevant interactions between traditional herbal medicines and co-administered allopathic drugs.

Declaration of interest

The financial support of the Medical Research Council (MRC) of South Africa is acknowledged. The authors have no other relevant affiliations or financial involvement with any organisation or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Interactions of herbal products with indinavir transport and metabolism

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Abstract

Patients on anti-retroviral (ARV) drug treatment are sometimes simultaneously taking other prescribed drugs and/or over-the-counter drugs and/or herbal remedies. Pharmacokinetic drug-drug or herb-drug interactions can occur in these patients, which might be synergistic or antagonistic in nature leading to increased or decreased bioavailability of the ARV. Consequences of bioavailability changes may either be adverse effects due to increased plasma levels, or lack of pharmacological responses due to decreased plasma levels. The aim of this study is to determine if pharmacokinetic interactions exist between selected commercially available herbal products, namely Linctagon Forte[®], Viral Choice[®] and Canova[®] and the ARV, indinavir, in terms of transport and metabolism in cell culture models.

Bi-directional transport of indinavir was evaluated across Caco-2 cell monolayers in four experimental groups, namely indinavir alone (200 μ M, negative control group), indinavir in combination with Linctagon Forte[®], indinavir in combination with Viral Choice[®] and indinavir in combination with Canova[®] at three different concentrations. Verapamil (100 μ M), a known P-gp inhibitor, was combined with indinavir in the positive control group. Samples obtained from the transport studies were analysed by means of a validated high performance liquid chromatography (HPLC) method. The apparent permeability coefficient (P_{app}) values were calculated from the transport results in both directions and the efflux ratio (ER) values were calculated from these P_{app} values. The metabolism of indinavir was determined in LS180 cells in the same groups as mentioned for the transport study but with ketoconazole (40 μ M), a known CYP3A4 inhibitor, as the positive control group. Indinavir and its predominant metabolite (M6) were analysed in the metabolism samples by means of liquid chromatography linked to mass spectroscopy (LC/MS/MS) to determine the effect of the herbal products on the biotransformation of indinavir.

The BL-AP transport of indinavir increased in a concentration dependent way in the presence of Linctagon Forte[®] and Viral Choice[®] when compared to that of indinavir alone (control group). Canova[®] only slightly affected the efflux of indinavir compared to that of the control group. Noticeable increases in the efflux ratio values of indinavir were found for

Linctagon Forte® and Viral Choice®, whilst the effect of Canova® on the efflux ratio value was negligible.

There was a pronounced inhibition of the metabolism of indinavir in LS180 cells over the entire concentration range for all the herbal products investigated in this study. These *in vitro* pharmacokinetic interactions indicate the selected herbal products may affect indinavir's bioavailability, but the clinical significance needs to be confirmed with *in vivo* studies before final conclusions can be made.

Key words: Herb-drug interactions, efflux, P-glycoprotein, CYP3A4, Caco-2, LS180, metabolism

1. INTRODUCTION

Human immunodeficiency virus (HIV) infection and acquired immune deficiency syndrome (AIDS) remains one of the biggest health problems that the world and more specifically, sub-Saharan Africa, is facing. Many HIV positive patients are also taking other drugs as well as herbal medicines concurrently with their anti-retroviral (ARV) drugs (Cohen *et al.*, 2002:42,45, Littlewood & Vanable, 2008:1). Herbal remedies usually comprise a complex mixture of phytochemicals, each with the potential of exerting pharmacological effects but may potentially also cause pharmacokinetic or pharmacodynamic interactions with other drugs (Pal & Mitra., 2006:2132; Tarirai *et al.*, 2010:1517).

Pharmacokinetic interactions caused by herbal products involve induction and/or inhibition of intestinal efflux proteins as well as intestinal and hepatic metabolising enzymes (Pal & Mitra., 2006:2132). Of the active efflux transporters involved in herb-drug interactions, P-glycoprotein (P-gp) is of special importance. P-gp is an ATP-binding cassette family transporter, which is highly expressed on the apical membrane of intestinal epithelial cells. Its main physiological function is believed to provide protection against the onslaught of endogenous and exogenous compounds due to its ability to transport various structural dissimilar compounds out of the epithelial cells. This action of P-gp to extrude drug molecules from the epithelium back into the gastro-intestinal lumen may cause reduction in the absorption of a drug and consequently results in decreased oral bioavailability thereof (Sharom, 2008:106, 108). The cytochrome P450 (CYP) enzyme family counts for approximately 30% of the hepatic metabolism activity and 70% of the intestinal wall metabolism activity (Hellum & Nilsen 2007:466; Pal & Mitra., 2006:2136, Liska, 1998:188). Various isoforms of the CYP P450 enzyme family exist, however, the most abundant form namely CYP3A4 is responsible for the metabolism of up to 70% of all drugs. Inhibition of CYP3A4 may result in increased bioavailability of orally administered drugs with potentiation

of adverse effects and possible toxicity, while induction thereof will increase metabolism of administered drugs resulting in reduced oral bioavailability and efficacy of drugs (Pal & Mitra., 2006:2136).

Canova[®] is a homeopathic product composed of diluted extracts from *Aconitum napellus* (Ranunculaceae), *Arsenicum album* (arsenic trioxide), *Bryonia alba* (Cucurbitaceae), *Apis mellifica* (Apidae), *Lycopodium clavatum* (Lycopodiaceae), *Pulsatilla nigricans* (Ranunculaceae), *Asa foetida*, *Rhus toxicodendrum*, *Barita carbônica*, *Ricinus communis* (Euphorbiaceae), *Silicea*, *Calcarea carbônica*, *Conium maculatum* (Apiaceae), *Veratrum album* (Liliaceae), *Carapichea ipecacuanha* (Rubiaceae), *Lachesis muta* (Viperidae) and *Thuya occidentalis* (Cupressaceae). Canova[®] is known as an immune response modifier directed at macrophages that results in a increase in their production together with the release of cytokine TNF α in cancer and AIDS patients (De Oliveira *et al.*, 2008:3; Takahachi *et al.*, 2007:350). Linctagon Forte[®] is a herbal product used to prevent and treat colds, flu and respiratory tract infections. It contains extracts of *Pelargonium sidoides* (Geraniaceae) as well as the natural occurring compounds quercetin and bromelain. *Pelargonium sidoides* is a traditional South African medicinal herb, which is commonly used as treatment for diarrhea, dysentery, colds as well as improvement of upper respiratory tract infections and tuberculosis (also available in the commercial herbal product Umckaloabo[®]) (Brendler & van Wyk, 2008:421,423, 428, 429; Schnitzler *et al.*, 2008:1114; Tahan & Yaman, 2012:2-3). Viral Choice[®] is a natural product containing a variety of minerals, vitamins, amino acids, trace elements, phytosterols as well as extracts of *Echinacea purpurea* (Asteraceae) and *Allium sativum* (Alliaceae). (Barrett, 2003:66-67; Hansen & Nilsen 2009:86; Gorski *et al.*, 2003:90). Common uses of *E. purpurea* includes treatment of the common cold, mainly due to its proposed immune modulation, treatment of influenza, bronchitis, inflammation and it also has anti-fungal effects (Barrett, 2003:67; Gorski *et al.*, 2003:89). *Allium sativum*, better known as garlic, contains high concentrates of thiosulfinates that are sulfur containing compounds specifically allicin, the active substance present in garlic (Tattelman, 2005:103). The bulbous root of the garlic plant is medicinally used either fresh, dehydrated or in a steam distilled oil form as an immune system modulator, anti-microbial and anti-cancer agent, as well as anti-atherosclerotic and lipid lowering agent (Tattelman, 2005:103; Banerjee *et al.*, 2003:97).

Indinavir (Crixivan[®]) is an ARV drug (protease inhibitor) that is a substrate for both P-gp as well as CYP3A4. Pharmacokinetic interactions between indinavir and herbs (e.g. St Johns' Wort, Milk thistle, Gingko, Ginseng, Echinacea and garlic) have been shown to decrease it's bioavailability (Staines, 2011:41). The current study investigated if *in vitro* pharmacokinetic interactions in terms of efflux and metabolism modulation exist between indinavir and three

selected commercially available herbal products (i.e. Canova[®], Linctagon Forte[®] and Viral choice[®]) that are often used by patients for immune boosting effects.

2. METHODS

2.1 Materials

Canova[®] drops, Linctagon Forte[®] tablets Viral choice[®] capsules and Crixivan[®] capsules containing 400 mg indinavir each were purchased from a community pharmacy in Potchefstroom, South Africa. All other chemicals were of analytical grade and growth media were obtained from suppliers as indicated in the text of the methods below.

2.2 *In vitro* transport studies

2.2.1 *Caco-2* cell culturing and seeding on Transwell filter plates

The Caco-2 cell line was used as the *in vitro* transport model, which was obtained from the European Collection of Cell Cultures (ECACC), by Sigma Aldrich, Johannesburg, South Africa. The cells were subsequently cultured in high-glucose DMEM (Separations, Randburg, South Africa) supplemented with 10% foetal bovine serum (The Scientific Group, Johannesburg, South Africa), 1% non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% penicillin/streptomycin (Separations, Johannesburg, South Africa), 1% 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa) and 1% amphotericin B (250 µg/ml) (The Scientific Group, Randburg, South Africa). The Caco-2 cells were cultured at 37°C with 5% carbon dioxide and 95% humidified air in a Galaxy 170R incubator (Eppendorf Company, Stevenage, UK). The growth medium was exchanged every second day.

Prior to the transport studies the caco-2 cells were seeded onto Transwell[®] 6-well membrane filters (Corning Costar[®] Corporation, Tewksbury, USA) with a pore diameter of 0.4 µm and surface area of 4.67 cm². A cell suspension was obtained by means of trypsinisation with Trypsin-Versene (EDTA) mixture (Whitehead scientific, Cape Town, South Africa). Cells in the cell suspension were counted by means of a haemocytometer after addition of Trypan blue (Sigma Aldrich, Johannesburg, South-Africa). Using a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan). The cell suspension was then diluted to a concentration of 20 000 cells per ml, which were seeded onto the membrane filters. Seeding of the cells onto Transwell[®] filter membranes occurred under sterile conditions in a laminar flow hood by pipetting 2.5 ml of the final cell suspension into each apical chamber of the membrane filter plate wells. The Caco-2 cells were grown for 21 to 24 days on the

membrane filters to produce intact epithelial cell monolayers, whilst the growth medium was replaced every second day under sterile conditions.

2.2.2 Herbal product test concentrations

For the transport studies, a concentration range for each of the herbal products were selected based on a series of different potential distribution volumes to cover an extreme range of possible *in vivo* concentrations at different sites as described before (Hellum *et al.*, 2007:26). The highest concentration represented a dose of the herbal product diluted in 500 ml, the medium concentration represented a dose of the herbal product diluted in 5 l and the lowest concentration represented the herbal product diluted in 50 l.

2.2.3 In vitro bi-directional transport studies

Prior to commencement of the transport experiments, the transepithelial electrical resistance (TEER) of each cell monolayer was measured with a Millcell ERS II meter (Millipore, Billerica, Massachusetts, USA) as an indication of the integrity thereof. A TEER reading higher than 250 Ω (which is equivalent to 1167.5 Ω/cm^2) was required before the transport studies could be conducted.

2.2.3.1 Transport in the apical to basolateral direction

For the transport studies in the apical to basolateral (AP-BL) direction, the growth medium was removed from the basolateral chamber and replaced with 2.5 ml pre-heated DMEM buffered with HEPES (2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid) (The Scientific group, Biochrom, Randburg, South Africa) and incubated for 30 min at 37°C. The growth medium was removed from the apical chamber and replaced with 2.5 ml of each of the test solutions (i.e. each selected herbal product at three different concentrations in the presence of 200 μM indinavir). The negative control group consisted of indinavir alone in solution (200 μM), whereas indinavir combined with verapamil (100 μM) formed the positive control. Samples (200 μl) were withdrawn from the basolateral side at pre-determined time intervals of 20, 40, 60, 80, 100 and 120 min, which were each replaced with 200 μl of the pre-heated DMEM buffered with HEPES. At the end of the transport study, the TEER was again measured to confirm the integrity of the cell monolayer was still intact after exposure of the cells to the test solutions. Samples were analysed by means of HPLC to determine the indinavir concentration.

2.2.3.2 Transport in the basolateral to apical direction

For the transport studies in the basolateral to apical (BL-AP) direction, the growth medium was removed from the apical chamber and replaced with 2.5 ml pre-heated DMEM (37°C) and incubated for 30 min at 37°C. The growth media was removed from the basolateral chamber and replaced with 2.5 ml of each of the test solutions (i.e. each selected herbal product at three different concentrations in the presence of 200 µM indinavir). Indinavir alone was employed as a negative control, with verapamil (100 µM), a known P-gp inhibitor, in combination with indinavir as a positive control. Samples (200 µl) were withdrawn from the apical side at pre-determined time intervals of 20, 40, 60, 80, 100 and 120 min, which were each replaced with 200 µl pre-heated DMEM (37°C). At the end of the transport study, the TEER was again measured to confirm the integrity of the cell monolayer over the time period of the transport study. Samples were analysed by means of the HPLC method.

2.3 LS180 cell culturing and seeding out for metabolism inhibition studies

The LS180 cell line was used as the *in vitro* model for metabolism, which was obtained from the European Collection of Cell Cultures (ECACC) by Sigma Aldrich, (Johannesburg, South Africa). The cells were subsequently cultured in high-glucose DMEM (Separations, Randburg, South Africa) supplemented with 10% foetal bovine serum (The Scientific Group, Randburg, South Africa), 1% non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% penicillin/streptomycin (Separations, Thermo Scientific), 1% 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa) and 1% amphotericin B (250 µg/ml) (The Scientific Group, Randburg, South Africa). The LS 180 cells were cultured at 37°C with 5% carbon dioxide and 95% humidified air in a Galaxy 170R incubator (Eppendorf Company, Stevenage, UK).

2.3.1 Seeding of LS180 cells onto 6-well plates

The LS180 cells were seeded onto 6-well plates (Corning Costar Corporation, Tewksbury, USA). A cell suspension was obtained by means of scraping the cells from the culturing flask; the cell suspension was extensively agitated with a pipette to ensure a homogeneous suspension consisting of single cells. Cells in the suspensions were counted using a haemocytometer after addition of Trypan blue (Sigma Aldrich, Johannesburg, South-Africa) using a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan). The cell suspension was diluted to a concentration of 50 000 cells per ml.

Seeding of the cells onto the 6-well plates occurred under sterile conditions in a laminar flow hood by pipetting 2.5 ml of the final cell suspension into each chamber of the 6 well plates.

The seeded LS180 cells were left to adhere to the culture treated bottom surfaces of the wells and cultured for 24 h prior to commencement of the metabolism inhibition studies.

2.3.2 Metabolism inhibition studies

The growth medium was removed from the LS180 cells and replaced with 2.5 ml test and control solutions (i.e. each selected herbal product at three different concentrations in the presence of 200 μ M indinavir as well as 40 μ M ketoconazole and indinavir solution as positive control), which were then incubated for 4 h at normal culturing conditions. The test/control solutions were removed and the cells were washed with 1 ml PBS (Sigma-Aldrich, Johannesburg, South Africa). The cells were harvested by gently scraping each well in order to detach all the cells and transferring them to labelled micro-centrifuge tubes. The cell suspension was removed and placed in 2.5 ml centrifugal tubes on ice, which were centrifuged in a Sigma Refrigerated Centrifuge 3-16KL (Wirsam Scientific, Johannesburg, South Africa) for 5 min at 300 x g (1495 rpm) to ensure lyses of the cell membranes. After centrifugation, the supernatant was removed and the cell pellets were re-suspended in 200 μ l ice-cold methanol, vortexed for 40 s and kept at -80°C until dried. Before drying, the cell suspension was thawed and centrifuged at 10 000 x g (9458 rpm) for 10 min to remove proteins and cell debris. The supernatant was removed and placed in 2.5 ml micro-centrifuge tubes, which were then dried under nitrogen. Prior to LC-MS/MS analysis, the samples were reconstituted in 100 μ l 0.1% formic acid in water and vortex mixed for 1 min.

2.4 Chromatographic analysis

2.4.1 Indinavir content in transport samples and fingerprinting of herbal products

A validated high performance liquid chromatography (HPLC) method was employed on an HP1100n chromatograph equipped with a UV detector and Chemstation Rev.A.10.01 Agilent® Technologies data acquisition and analysis software (Hewlett-Packard Paulo Alto, California, USA).

The analysis of the transport samples for indinavir content and fingerprinting of the herbal products was performed on a Venusil XBP C18(2) column, 4.6 x 150 mm, 5 μ m, 100 Å (Agela technologies, Wilmington, Delaware, USA using an acetonitrile solvent adjusted to pH 7 with 0.1% ammonium formate (NH_4HCO_2) as the mobile phase in isocratic elution mode with a flow rate of 1 mL/min and an injection volume of 100 μ L. The UV detector was set at 210 nm with a retention time at 4.6 min for indinavir. The detection of indinavir was linear over the concentration range of 0.0-24.93 μ g/ml and $R^2=0.999$. The system performance proved well with relative standard deviation (RSD) values of 0.30% for peak area and

0.182% for retention time. Indinavir was stable over a period of 24 h. The method was able to tolerate small changes in the chromatographic conditions and performed well under normal use. It was concluded that the method was suitable to analyse indinavir in the transport samples.

2.4.2 Indinavir and metabolite concentration in metabolism inhibition samples by liquid chromatography linked to mass spectroscopy (LC-MS/MS)

The liquid chromatography was performed on an SB-Phenyl (2.1 x 100 mm, 1.8 μ m) column using an Agilent 1100 series HPLC instrument. The mobile phase consisted of a mixture of phase A and B at 40:60 v/v, where phase A was 0.1 % w/v formic acid in water and phase B was 0.1 % w/v formic acid in acetonitrile. The mobile phase was delivered at a constant flow rate of a 180 μ l/min and injection volume was 5 μ l, while the column was kept in a compartment at 40 °C.

Detection of indinavir, M6 (indinavir metabolite) and the internal standard (indinavir-d6) was performed on an AB Sciex API 3200 mass spectrometer (ESI in the positive ion mode) and the settings on the apparatus are summarised in tables 1 and 2. The mass spectrometer was operated at unit resolution in the multiple reaction monitoring (MRM) mode, monitoring the transition of the protonated molecular ions at m/z 614.4, m/z 523.4 and m/z 620.5 to the product ions at m/z 421.3, m/z 273.1 and m/z 421.2 for indinavir, M6 and the internal standard, respectively.

Table 1: Ionization source setting

Electro Spray Ionisation Settings	Value
Nebulizer gas (Gas 1) (arbitrary unit)	30
Turbo gas (Gas 2) (arbitrary unit)	40
CUR (curtain gas) (arbitrary unit)	15
CAD (collision gas) (arbitrary unit)	3
TEM (source temperature) (°C)	500
IS (Ion Spray Voltage) (V)	3500

Table 2: MS/MS detector setting

MS/MS Settings	Indinavir	M6	ISTD
Protonated molecular mass (<i>m/z</i>)	614	523	620
Product ion molecular mass (<i>m/z</i>)	421	273	421
Dwell time (ms)	150	150	150
DP (declustering potential) (V)	45	40	40
EP (entrance potential) (V)	12	9	12
CEP(collision cell entrance potential) (V)	35	40	25
CE (collision energy) (eV)	50	45	45
CXP (collision cell exit potential) (V)	11	11	8
Scan Type	MRM	MRM	MRM
Polarity	Positive	Positive	Positive
Pause time	5ms	5ms	5ms

2.5 DATA ANALYSIS

2.5.1 Indinavir transport data

The apparent permeability coefficient ($P_{app} \times 10^{-6}$ cm/s) values for indinavir transport in each direction across the Caco-2 cell monolayers were calculated using equation 2 (Tarirai *et al.*, 2012:257). The P_{app} values represent the diffusion rate of the compound normalised for surface area of the cell monolayers and drug concentration on the donor side.

$$P_{app} = \frac{dQ}{dt} \left(\frac{1}{A.C_0.60} \right) \quad \text{Eq (1)}$$

Where dQ/dt represents the increase in the amount of drug present in the acceptor compartment over time, A is the effective surface area of the cell monolayer in cm^2 that is exposed to the test solutions and C_0 ($\mu\text{g/ml}$) is the initial drug concentration present in the donor chamber.

Efflux ratio (ER) indicates any asymmetry in the directional transport of indinavir in combination with the various herbal products. The ER of indinavir transport was calculated by means of Equation 3 (Tarirai *et al.*, 2012:257).

$$ER = \frac{P_{app}(BL-AP)}{P_{app}(AP-BL)} \quad \text{Eq (2)}$$

Where AP-BL is the transport of indinavir in the apical to basolateral direction and BL-AP indicates the transport of indinavir in the basolateral to apical direction.

2.6 Indinavir metabolism data

The ratio of the concentration of indinavir metabolite (i.e. M6) to that of indinavir was calculated to indicate any modulation of indinavir metabolism in the LS180 cells by the herbal products. Concentration ratios of metabolite to parent drug (i.e. indinavir) were used to determine if inhibition occurred. CYP3A4 and CYP2B6 are responsible for the oxidative metabolism of indinavir during phase I of enzyme detoxification resulting in the production of six metabolites. During inhibition of CYP3A4 and CYP2B6, as a cause of combined herb and indinavir usage, there should be a decline in the concentration of these metabolites in the presence of the parent drug.

3 RESULTS

3.1.1 Bi-directional transport of indinavir in the presence of Linctagon Forte®

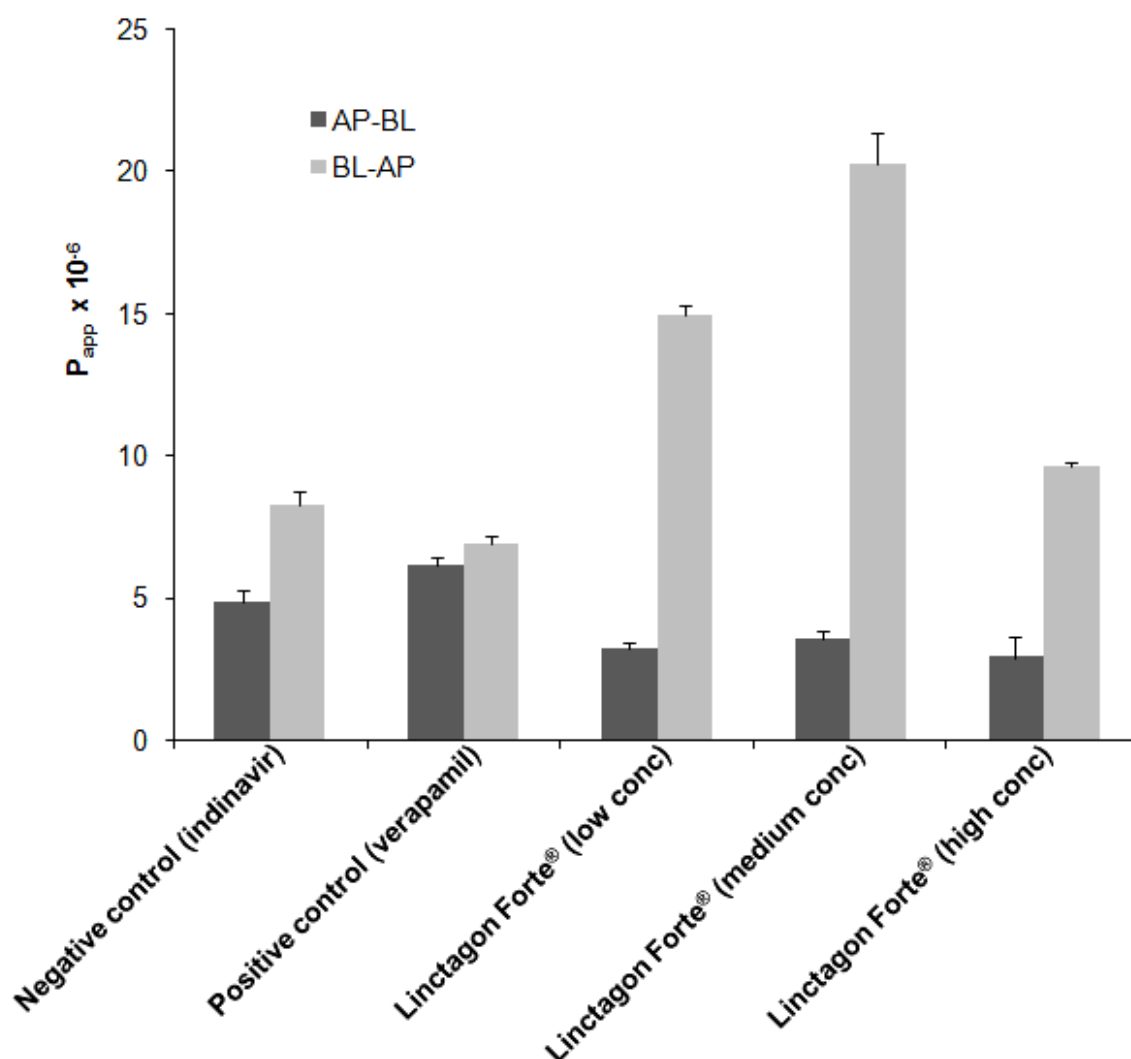


Figure 1: Bi-directional P_{app} values for indinavir in the presence of three concentrations of Linctagon Forte® as well as for the negative control group (indinavir alone) and positive control group (indinavir with verapamil)

The P_{app} values for each of the experimental groups of Linctagon Forte® in combination with indinavir are presented in Figure 1. The P_{app} values represent the diffusion rate of the compound normalised for surface area of the cell monolayers and drug concentration on the donor side (Tarirai *et al.*, 2012:257). From Figure 1 it can be seen that verapamil, a known inhibitor of P-gp (i.e. positive control group), increased the uptake of P-gp in the AP-BL direction, whereas it decreased the efflux of indinavir in the BL-AP direction. The overall uptake of indinavir (i.e. transport in the AP-BL direction) decreased for all the concentrations of Linctagon Forte® when combined with indinavir as indicated in. As expected, an overall

increase in the efflux of indinavir is noted for all the concentrations of Lintagon Forte® when combined with indinavir. The medium concentration produced the most pronounced effect followed by the low and high concentrations, respectively, when compared to both the positive and negative control groups. The efflux activation effect was concentration dependent for the first two concentrations of Lintagon Forte®, but the effect was lower for the highest concentration. This “bell shape” dose dependent curve may implicate a saturation effect above a threshold concentration.

3.1.2 Transport Viral Choice & Indinavir P_{app} values AP-BL and BL-AP

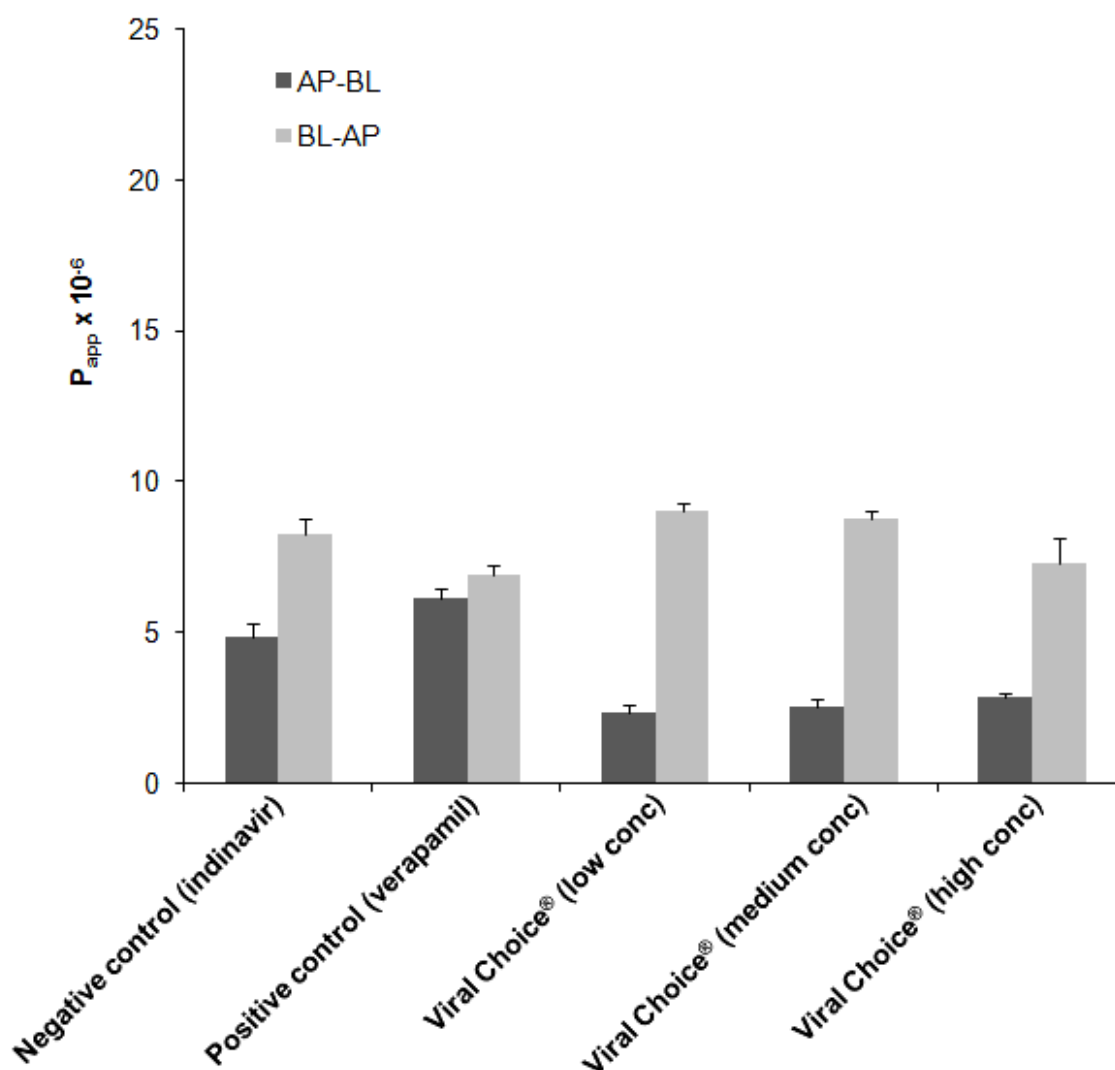


Figure 2: Bi-directional P_{app} values for indinavir in the presence of three concentrations of Viral Choice® as well as for the negative control group (indinavir alone) and positive control group (indinavir with verapamil)

From Figure 2 we notice a decrease in the uptake of indinavir from the AP-BL direction during the combined application of Viral Choice® for the entire concentration range (i.e. low, medium and high concentrations) when compared to the negative control group. Efflux of indinavir in the BL-AP direction for the low and medium concentration of Viral Choice® increased to a certain extent. However, at the highest Viral Choice® concentration, there is a slight decrease in the indinavir efflux from the BL-AP direction when compared to the negative control group; but not to the same extent as the positive control group.

3.2.3 Transport Canova® & Indinavir P_{app} values AP-BL and BL-AP

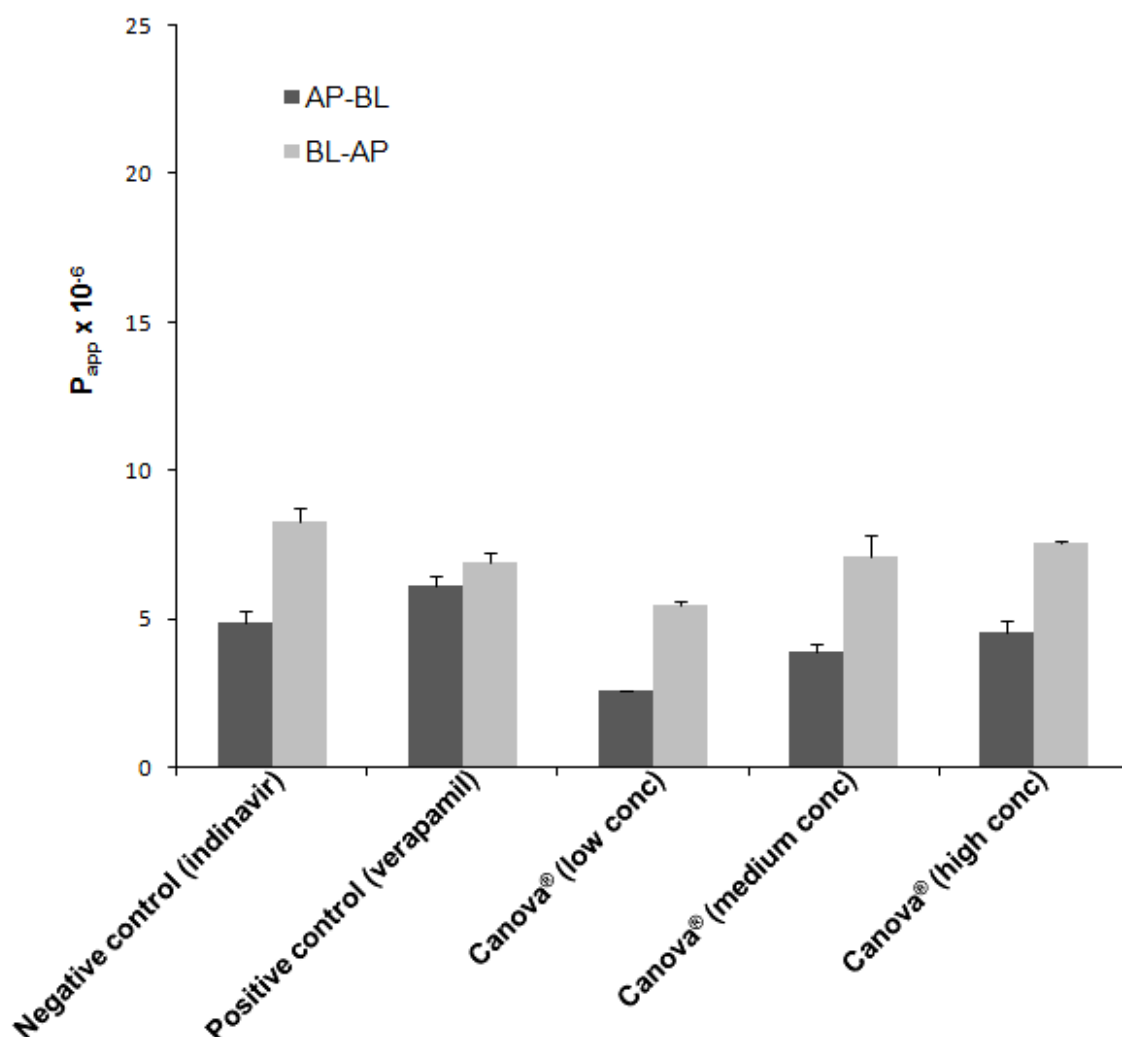


Figure 3: Bi-directional P_{app} values for indinavir in the presence of three concentrations of Canova® as well as for the negative control group (indinavir alone) and positive control group (indinavir with verapamil)

There was a concentration dependent (i.e. from lowest to highest concentration) increase in the percentage uptake of indinavir in the presence of Canova® as seen in Figure 3. The

percentage uptake of indinavir in the presence of the lowest concentration Canova[®] was lower than that of both the positive and negative control groups, whereas it was slightly elevated in the presence of the medium and high Canova[®] concentrations in comparison to the negative control group (i.e. indinavir). The same trend, to some extent, was observed for the percentage efflux of indinavir. There was a slight concentration dependent increase in the efflux of indinavir for the entire concentration range; nonetheless, when compared to the negative control group, this increase for the medium and high concentrations of Canova[®] was almost negligible. The lowest concentration of Canova[®], however, decreased the percentage efflux of indinavir to values lower than that of the positive control group (i.e. verapamil and indinavir). Overall however, it appeared as if Canova[®] had a negligible effect on the bi-directional transport of indinavir.

3.2 Transport efflux ratios (ER) values

Figure 4 offers a graphic representation of the various ER values observed for each selected herbal product at an extreme range of concentrations (i.e. low, medium and high) in combination with indinavir. From Figure 4, it can be noticed that a pronounced increase in efflux occurred for the entire concentration range (i.e. low, medium and high concentrations) of Linctagon Forte[®] in combination with indinavir, when compared to both the negative and positive control group. Again, we notice a concentration dependent activation of efflux for the lower two concentrations of Linctagon Forte[®]; with a lower effect noticed for the highest concentration tested, which is indicative of a possible saturation effect.

During the co-application of Viral Choice[®], there was a definite increase in efflux for the entire concentration range (i.e. low, medium and high concentrations) when compared to both the negative and positive control. The increase in the extent of efflux appears to be concentration dependent.

The combination of Canova[®] with indinavir slightly increased the ER values of indinavir in a concentration dependent manner (i.e. from low to high concentration) when compared to the negative control group. However, when the highest concentration is compared to that of the negative control group, an increase in the efflux of indinavir was hardly detected. Overall, it appears as if Canova[®] had a negligible effect on the ER value of indinavir.

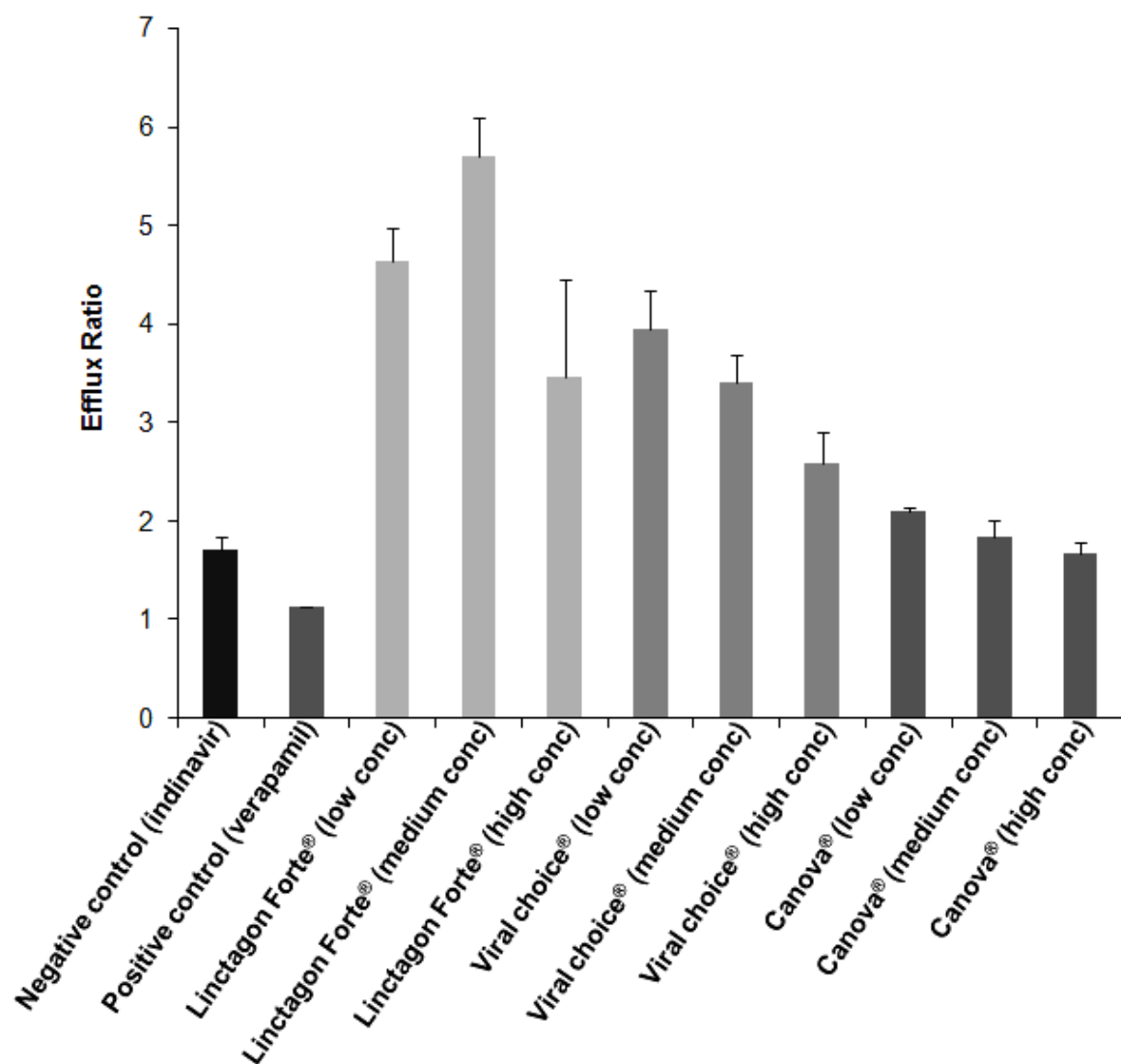


Figure 4: Efflux ratios for indinavir in the presence of Linctagon Forte®, Viral Choice® and Canova® with the negative control group (indinavir alone) and positive control group (indinavir with verapamil)

3.3 Metabolism inhibition

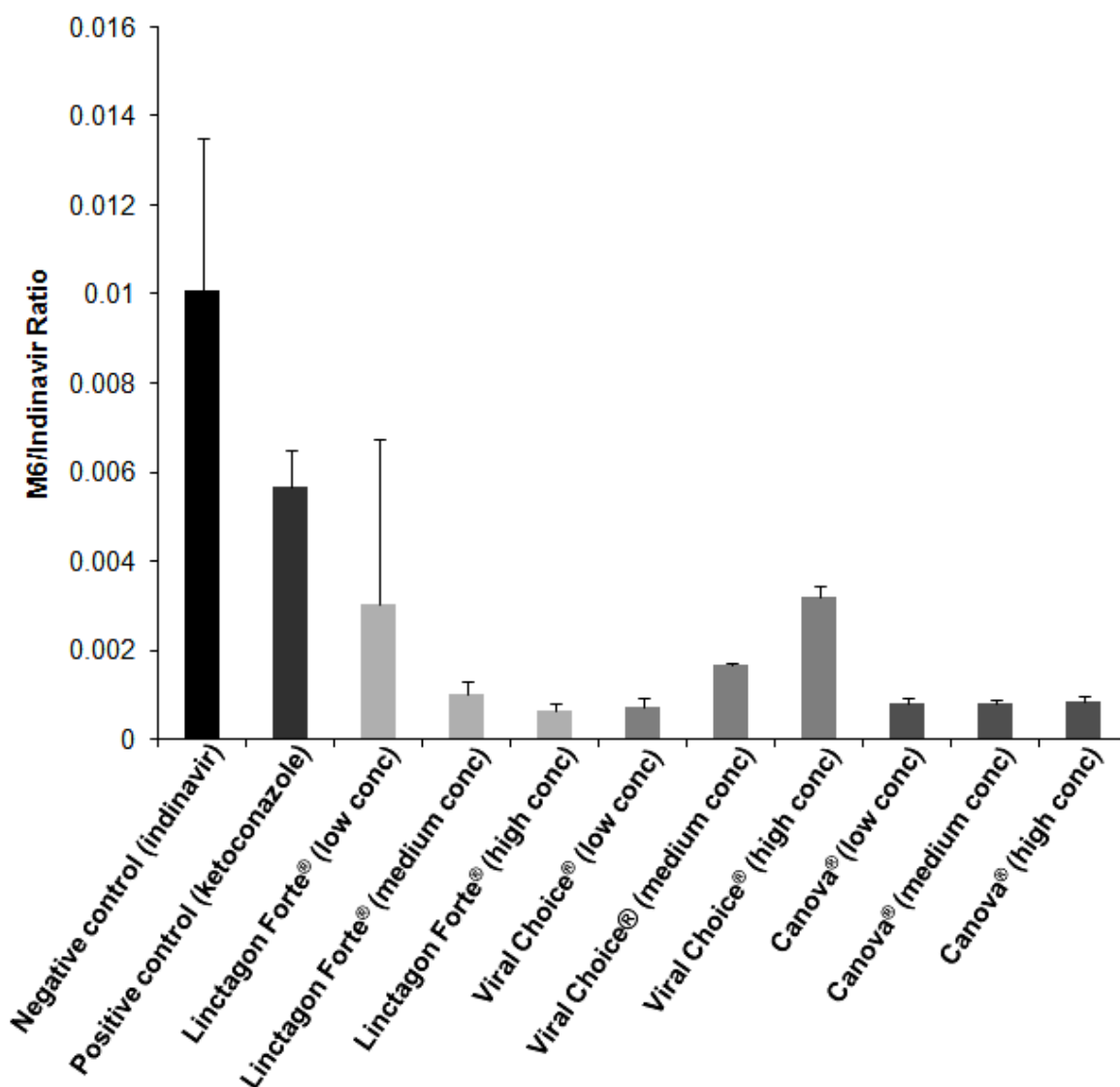


Figure 5: M6/indinavir ratios for indinavir in the presence of Linctagon Forte®, Viral Choice® and Canova® with the negative control group (indinavir alone) and positive control group (indinavir with ketoconazole)

Figure 5 provides a graphic representation of the results obtained during the metabolism inhibition experiment conducted during the combination of three commercially available herbal products (i.e. Linctagon Forte®, Viral Choice® and Canova®) at an extreme range of concentrations (i.e. low, medium and high) with indinavir. Indinavir alone (200 µM) served as the negative control group and ketoconazole (40 µM) in combination with indinavir (200 µM) served as the positive control group. Ketoconazole is a known substrate and inhibitor of CYP3A4, thus, decreasing the concentration of the most prominent indinavir metabolite (M6).

It is apparent from Figure 5 that there was a marked decrease in the concentration ratio of M6 to indinavir in the positive control group (indinavir with ketoconazole) compared to that of the negative control group (indinavir alone). The latter validates the model; and the results obtained can now be viewed as a true reflection of the inherent actions of the experimental groups on the metabolism inhibition of indinavir.

Linctagon Forte® resulted in a concentration dependent inhibition of indinavir metabolism, most probably CYP3A4. A ± 9 fold increase in metabolism inhibition was observed with the highest concentration Linctagon Forte® when compared to the positive control group.

Viral Choice® exhibited an inverse concentration dependent inhibition of indinavir metabolism in LS180 cells. A ± 8 fold increase in inhibition was observed with the lowest concentration of Viral Choice® when compared to the positive control.

The combination of Canova® across the whole concentration range markedly inhibited CYP3A4 related metabolism of indinavir. An overall ± 7 fold increase in inhibition was observed for the entire concentration range of Canova® when compared to the positive control.

4. DISCUSSION AND CONCLUSION

The phytochemical complexity of herbal remedies potentiates the possible pharmacological effects thereof resulting in pharmacokinetic or pharmacodynamic interactions with other drugs. These pharmacokinetic interactions caused by herbal products result in the induction and/or inhibition of intestinal efflux proteins (P-gp) as well as intestinal and hepatic metabolising enzymes (CYP3A4). When efflux of a drug from the epithelium is increased, lower uptake transport of the drug is expected across the epithelium because the molecules are actively pumped from within the cells back to the apical side. During the inhibition of CYP3A4 there is a decline in the amount of drug substrate being metabolised, resulting prolonged exposure of the drug, possible increased bioavailability while potentiating adverse effects or even toxicity. The P_{app} values in both the AP-BL and BL-Ap directions were determined for all the experimental groups. An increase in efflux as pharmacokinetic interaction may cause a reduction in the bioavailability of indinavir when simultaneously taken with Linctagon Forte® from the results obtained. This acute effect of Linctagon Forte® on indinavir transport across intestinal epithelial cells may be explained by potential activation of the P-gp efflux transporter. However, the precise mechanism of action should be investigated further as well as the potential enhancement of gene expression over longer term exposure.

Linctagon Forte® is composed of *Pelargonium sidoides* (Geraniaceae), quercetin and bromelain. Currently, there are no known interactions between co-administered drugs and *P. sidoides* extract containing products with respect to P-gp (Ulbricht *et al.*, 2010:723). The quercetin component has in the past shown inhibitory action of P-gp activity. It had been determined by means of rhodamine-123 (Rh-123) transport studies that P-gp activity is significantly inhibited by quercetin (Choi *et al.*, 2011:609). In another study by Borska *et al.* (2012), the cell line EPG85-257RDB (RDB-cells) exposed to 12 µM quercetin showed a significant reduction in the expression of P-gp. The bromelain component, however, is absorbed by means of a self-enhanced paracellular route without degradation and maintaining biological activity (Bock *et al.*, 1998:1399; Pavan *et al.*, 2012:1). The latter thus, may not have an effect on the P-gp transporter. This suggested that the overall increase in efflux observed for Linctagon Forte® may potentially be attributed to the high concentration of *P. sidoides* extract present within the product.

The trends observed during the calculation of the P_{app} values of Viral Choice® corresponds to that observed in a study by Hansen and Nilsen (2009:86-91) in which the effect of *Echinacea purpurea* was evaluated on P-gp transport in Caco-2 monolayers. Viral Choice® is composed of a variety of minerals, vitamins, amino acids, trace elements, phytosterols as well as *Echinacea purpurea* and *Allium sativum* extracts; of these *Echinacea purpurea* is present in the highest concentration at 80 mg per capsule.

The ER values indicate asymmetry in the directional transport of indinavir in combination with the various herbal products. When efflux of a drug from the epithelium is increased, lower uptake transport of the drug is expected across the epithelium, because the molecules are actively pumped from within the cells back to the apical side. An increase in efflux as pharmacokinetic interaction may therefore cause a reduction in the bioavailability of the drug when simultaneously taken with the inducer. On the other hand, a decrease in the efflux as caused by an inhibitor may result in increased bioavailability of the drug as more drug will now reach the systemic circulation. We notice a concentration dependent activation of efflux for the lower two concentrations of Linctagon Forte®; with a lower effect noticed for the highest concentration tested, which is indicative of a possible saturation effect. The data shows that Viral Choice® interacts with the P-gp mediated transport of indinavir, especially during efflux from the BL-AP direction; suggesting a biphasic effect with low allosteric activation at low concentrations and low inhibition at higher concentrations. This is similar to the trends observed for *Echinacea purpurea* during an *in vitro* study by Hansen and Nilsen (2009:87-90).

The concentration ratio of indinavir metabolite (i.e. M6) to that of indinavir was calculated in order to indicate any modulation of indinavir metabolism in the LS180 cells by the herbal products. The concentration ratio of metabolite to parent drug (i.e. indinavir) provides an indication of the extent of inhibition that occurred. Inhibition of CYP3A4 by drug and herbal substrates may potentiate the occurrence of adverse effects of orally administered drugs that are substrates of this enzyme due to the subsequent increase in bioavailability thereof.

Linctagon Forte[®] resulted in a concentration dependent inhibition of indinavir metabolism. This was consistent with a previous finding of at least one active constituent of Linctagon Forte[®], namely quercetin. During an *in vivo* study on male Sprague-Dawley rats by Choi *et al.* (2011) quercetin was capable of producing an inhibitory action on CYP3A4 in a concentration-dependent manner. The results therefore suggest a possible increase in the bioavailability of indinavir when given in combination with Linctagon Forte[®].

Viral Choice[®] exhibited an inverse concentration dependent inhibition of indinavir. It has previously been found that *Echinacea purpurea*, one of the major active constituents of Viral Choice[®], inhibited CYP3A4. In an *in vitro* study by Budzinski *et al.* (2000), extracts of the root and aerial portions of *E. purpurea* had an inhibitory effect on cDNA expressing CYP3A4. This was confirmed by an *in vivo* study conducted by Gorski *et al.* (2004) during which 400 mg of *E. purpurea* root was administered 4 times daily for 8 days. They found that the clearance of caffeine and tolbutamide serving as *in vivo* probes for CYP1A2 and CYP2C9 metabolism was significantly reduced. Clearance of midazolam, the *in vivo* probe for hepatic CYP3A, was also significantly increased, which was consistent with preceding *in vitro* findings (Gorski *et al.*, 2004: 96). *Allium sativum* (Liliaceae), another active constituent of Viral Choice[®] include high concentrates of thiosulfinates that are sulphur containing compounds (e.g. allicin) (Tattelman, 2005:103). However, active constituents within *A. sativum* vary with different garlic preparations resulting in varying effects on the CYP enzyme system (i.e. some found inhibitory and some inducing effects on this enzyme system) (Banerjee *et al.*, 2003:98; Colalto *et al.*, 2010:210). The results suggest that the co-administration of Viral Choice[®] will result in an increase in bioavailability of indinavir due to a reduction in the metabolism of indinavir.

The combination of Canova[®] across the whole concentration range markedly inhibited the metabolism of indinavir. The homeopathic remedy Canova[®] is composed of various phytoconstituents of which several had previously shown interactions with CYP3A4, including *Aconitum napellus*, *Lycopodium clavatum*, *Ferula asafoetida*, *Ricinus communis*, *Carapichea ipecacuanha* and *Thuya occidentalis*. Tang *et al.* (2011) investigated the involvement of various CYP isoenzymes in the metabolism of *Aconitum napellus* by means

of human liver microsomes as well as recombinant CYP enzymes. It was determined that CYP3A4 and CYP3A5 were responsible for the metabolism of aconite whereas CYP2C8, 2C9, 2D6 and 2C19 played a slight role in the metabolism (Tang *et al.*, 2011:53). Both studies suggest that co-administration of aconite together with other P-gp and CYP3A4/5 substrates may result in possible pharmacokinetic herb-drug interactions. During a study using a cell free model by Tam *et al.* (2011), the effect of *L. clavatum* on the NADPH-dependant inhibition of CYP3A4 was examined. They found that *L. clavatum* significantly influenced both the NADPH and time dependent inhibition of CYP3A4 when combined with testosterone. This suggests that *L. clavatum*, similar to grapefruit juice, may possibly result in mechanism-based inactivation of CYP3A4 (Tam *et al.*, 2011:14,19,20). Asafoetida is the gum resin obtained from *Ferula asafoetida* (Apiacea) (Mahendra & Bisht, 2012:141). In a recent study by Al-Jenoobi *et al.* (2014), the effect of asafoetida resin on the modulation of CYP2D6 and CYP3A4 was evaluated *in vitro*, in human liver microsomes as well as in a clinical *in vivo* study. The *in vitro* study suggested that asafoetida was responsible for the inhibition of CYP2D6, but a concentration dependent activation of CYP3A4 was furthermore observed. However, the *in vivo* study suggested that asafoetida significantly inhibits CYP3A4 activity with little to no inhibitory effect on CYP2D6 (Al-Jenoobi *et al.*, 2014:4,5). In another study regarding *Ricinus communis*, it was reported during an *in silico* study involving HerboChip[®] followed by fluorometric enzyme inhibition studies that *R. communis* may have a significant effect on the inhibition of CYP3A4 (Calitz *et al.*, 2014:5). The active constituents in *Carapichea ipecacuanha* are cephaeline and emetine amongst others. It was found that both these active constituents are substrates for CYP2D6 and CYP3A4. In an *in vitro* study involving human liver microsomes, both cephaeline and emetine, resulted in low inhibitory action against CYP2D6 as well as CYP3A4 (Asano *et al.*, 2001:682). The essential oil from *Thuya occidentalis* contains the active ingredient α -thujone which is a known substrate for CYP2A6 and CYP3A4. The latter suggests that co-administration with other CYP2A6 and CYP3A4 substrates may result in pharmacokinetic interactions (Pelkonen *et al.*, 2013:102,104). From these published studies, together with the results obtained from the current metabolism inhibition study on Canova[®], it is possible that these active constituents may have had a combined effect producing the pronounced overall inhibition of indinavir metabolism during co-administration. The latter may potentially result in a possible increase of indinavir bioavailability, during the co-administration.

An increase in efflux as pharmacokinetic interaction may cause a reduction in the bioavailability of indinavir when simultaneously taken with Linctagon Forte[®] and to a lesser extent Viral Choice[®] as observed from the results obtained. This acute effect of Linctagon Forte[®] on indinavir transport across intestinal epithelial cells may be explained by potential

activation of efflux transport. However, the precise mechanism of action should be investigated further as well as the potential enhancement of gene expression over longer term exposure. The data shows that Viral Choice[®] interacts with the efflux mediated transport of indinavir especially during efflux from the BL-AP direction; suggesting a biphasic effect with low allosteric activation at low concentrations and low inhibition at higher concentrations. Canova[®] did not appear to affect the efflux of indinavir, this may possibly attributed to the highly diluted nature of Canova[®]. No *in vivo* data is currently available on the effects of Linctagon Forte[®], Viral Choice[®] or Canova[®] on systemic P-gp but a possible effect on the bioavailability of indinavir cannot be excluded.

The complete range of commercial products (Linctagon Forte[®], Viral Choice[®] and Canova[®]) selected for this study showed a definite effect on the extent of indinavir metabolism inhibition *in vitro*. The results suggest possible prolonged exposure to indinavir as well as increased bioavailability when co-administered with any one of the commercial products taken over a wide concentration range.

However, it remains that this *in vitro* study needs to be followed up with an *in vivo* pharmacokinetic study to be conclusive in terms of any bioavailability changes of indinavir by Linctagon Forte[®], Viral Choice[®] and Canova[®] as observed during this study.

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APPENDIX B

PACKAGE INSERT CRIXIVAN®



SCHEDULING STATUS S4

PROPRIETARY NAME AND DOSAGE FORM

CRIXIVAN® 200 mg Capsules

CRIXIVAN® 400 mg Capsules

COMPOSITION

CRIXIVAN 200 mg: Each capsule contains 200 mg of indinavir.
CRIXIVAN 400 mg: Each capsule contains 400 mg of indinavir.

CRIXIVAN contains sugar.

PHARMACOLOGICAL CLASSIFICATION

A.20.2.8 Antiviral agents

PHARMACOLOGICAL ACTION

MECHANISM OF ACTION

HIV protease is an enzyme required for the proteolytic cleavage of the viral polyprotein precursors into the individual functional proteins found in infectious HIV. Indinavir binds to the protease active site and inhibits the activity of the enzyme. This inhibition prevents cleavage of the viral polyproteins resulting in the formation of immature non-infectious viral particles. The relationship between *in vitro* susceptibility of HIV to indinavir and inhibition of HIV replication in humans has not been established. The *in vitro* activity of indinavir was assessed in cell lines of lymphoblastic and monocytic origin and in peripheral blood lymphocytes. HIV variants used to infect the different cell types include laboratory-adapted variants, primary clinical isolates and clinical isolates resistant to nucleoside analogues and non-nucleoside inhibitors of the HIV reverse transcriptase. The IC_{50} (95 % inhibitory concentration) of indinavir in these test systems was in the range of 25 to 100 nM. In drug combination studies with the nucleoside analogues zidovudine and didanosine, as well as with an investigational non-nucleoside, indinavir showed synergistic activity in cell culture.

PHARMACOKINETICS

Absorption

Indinavir is rapidly absorbed in the fasted state with a time to peak plasma concentration of 0.8 hours. Over the 200 to 1 000 mg dose range administered in both volunteers and HIV-1 infected patients, there was a slightly greater than dose-proportional increase in plasma concentrations. As a result of the short half-life, only a minimal increase in plasma concentrations occurred after multiple dosing.

Administration of indinavir with a meal high in calories, fat and protein resulted in a blunted and reduced absorption with an approximate 80 % reduction in AUC and an 85 % reduction in C_{max} . Administration with light meals (e.g. dry toast with jelly, apple juice, and coffee with skim milk and sugar or corn flakes, skim milk and sugar) resulted in a 2 to 8 % reduction in AUC and C_{max} . The plasma concentrations six and eight hours after administration of indinavir with these light meals were comparable to the corresponding fasted values at 800 mg every 8 hours.

Distribution

Indinavir was $\pm$ 60 % bound to human plasma proteins.

Biotransformation

Indinavir is metabolised in the liver and excreted mainly in the faeces. *In vitro* studies with human liver microsomes indicated that cytochrome CYP3A4 is the only P450 isozyme that plays a major role in the oxidative metabolism of indinavir. Analysis of plasma and urine samples from subjects who received indinavir, indicated that indinavir metabolites contribute little to the overall *in vivo* protease inhibitory activity.

Elimination

Over the 200 to 1 000 mg dose range, administration in both volunteers and HIV infected patients, there was a slightly greater than dose-proportional increase in urinary recovery of indinavir. Renal clearance (116 ml/min) of indinavir is concentration-independent over the clinical dose range. Less than 20 % of indinavir is excreted unchanged renally. Mean urinary excretion of unchanged medicine following single dose administration in the fasted state was 10.4 % following a 700 mg dose, and 12.0 % following a 1 000 mg dose. Indinavir was rapidly eliminated with a half-life of 1.8 hours.

Characteristics in Patients

Pharmacokinetics of indinavir do not appear to be affected by gender or by race.

Patients with mild-to-moderate hepatic insufficiency and clinical evidence of cirrhosis had evidence of decreased metabolism of indinavir resulting in approximately 60 % higher mean AUC following a single 400 mg dose. The mean half-life of indinavir increased to approximately 2.8 hours. Patients with severe hepatic insufficiency have not been studied.

PHARMACODYNAMICS

The combination of indinavir and one or more nucleoside analogues was associated with somewhat greater median declines in serum viral RNA, and a higher proportion of patients in whom RNA levels fell to below the limit of detection.

Loss of suppression of viral RNA levels occurred in some patients; however, CD4 cell counts were often sustained above pre-treatment levels. When loss of viral RNA suppression occurred, it was typically associated with replacement of circulating susceptible virus with resistant viral variants. Resistance was correlated with the accumulation of mutations in the viral genome that resulted in the expression of amino-acid substitutions in the viral protease enzyme.

At least eleven HIV-1 protease amino-acid residue positions, at which substitutions are associated with resistance, have been identified. No single substitution was capable of engendering measurable resistance to the inhibitor; resistance was mediated by the co-expression of multiple and variable substitutions. In general, higher levels of resistance result from the co-expression of greater numbers of substitutions at the eleven identified positions. Substitutions at these positions appeared to accumulate sequentially, probably as the result of ongoing viral replication.

It should be noted that the decrease in suppression of viral RNA levels was seen more frequently when therapy with indinavir was initiated at doses lower than the recommended oral dose of 2.4 g/day. Therefore, therapy with indinavir should be initiated at the recommended dose to increase suppression of viral replication and therefore inhibit the emergence of resistant virus.

INDICATIONS

CRIXIVAN is indicated for the treatment of HIV infection in adults, in combination with other antiretroviral agents. Clinical studies evaluating patients who received CRIXIVAN in combination with other antiretroviral agents demonstrated a reduced risk of progression to an AIDS-defining illness or death, increased overall survival, a durable reduction in serum viral RNA and a durable increase in CD4 cell counts.

CONTRA-INDICATIONS

CRIXIVAN is contra-indicated in:

1. Patients with clinically significant hypersensitivity to any component of this product.
2. Pregnancy and lactation as safety has not been established.
3. Children under the age of 18 years, as safety and efficacy have not been established.
4. Safety and efficacy in patients with concomitant liver disease (e.g. Hepatitis B) has not been established.
5. Safety and efficacy have not been established in the elderly.
6. Because rifampicin is a potent inducer of P450 3A4 which could markedly diminish plasma concentrations of CRIXIVAN, co-administration with rifampicin is not recommended.

WARNINGS

Nephrolithiasis may occur with CRIXIVAN. If signs and symptoms of nephrolithiasis, including flank pain with or without haematuria (including microscopic haematuria), occur, temporary interruption of therapy (e.g. 1 to 3 days) during the acute episode of nephrolithiasis may be considered. Adequate hydration is recommended in all patients treated with CRIXIVAN (see DOSAGE AND DIRECTIONS FOR USE Nephrolithiasis).

CRIXIVAN should not be administered concurrently with terfenadine, astemizole, cisapride, triazolam, midazolam, pimozide and ergot derivatives because competition for CYP3A4 by indinavir could result in inhibition of the metabolism of these medicines and create the potential for serious and/or life-threatening events (i.e. cardiac arrhythmias, prolonged sedation).

Rifabutin

The co-administration of CRIXIVAN at a dose of 800 mg every 8 hours with rifabutin at a dose of 300 mg once daily for 10 days resulted in a 34 % decrease in AUC and a 25 % decrease in C_{max} of CRIXIVAN. In contrast, the AUC and C_{max} of rifabutin increased by about 173 % and 134 % respectively, vs rifabutin 300 mg once daily alone (see DOSAGE AND DIRECTIONS FOR USE). This increase in rifabutin plasma concentrations is likely related to inhibition of CYP3A4-mediated metabolism of rifabutin by CRIXIVAN. A dosage increase of CRIXIVAN and a dosage reduction of rifabutin are necessary when CRIXIVAN and rifabutin are co-administered.

INTERACTIONS

Specific interaction studies were performed with CRIXIVAN and the following medicines, including: zidovudine, zidovudine/lamivudine, stavudine, trimethoprim/sulfamethoxazole, fluconazole, isoniazid, clarithromycin, cimetidine, methadone or an oral contraceptive (norethindrone/ethinyl estradiol 1/35). No clinically significant interactions were observed with these agents. However, clinically significant interactions with other agents are described below.

Pimozide

Pimozide should not be used together with CRIXIVAN. Inhibition of CYP3A4 by CRIXIVAN could result in elevated plasma concentrations of pimozide which could potentially result in QT prolongation and associated ventricular arrhythmias (see WARNINGS).

Rifampin

Rifampin is a potent inducer of P450 3A4 which markedly diminishes plasma concentrations of CRIXIVAN. Therefore, CRIXIVAN and rifampin should not be co-administered.

Rifabutin

Due to an increase in the plasma concentrations of rifabutin and a decrease in the plasma concentrations of CRIXIVAN, a dosage reduction of rifabutin and a dosage increase of CRIXIVAN are necessary when rifabutin is co-administered with CRIXIVAN (see DOSAGE AND DIRECTIONS FOR USE).

Ketoconazole

Due to an increase in the plasma concentrations of CRIXIVAN, a dosage reduction of CRIXIVAN should be considered when CRIXIVAN and ketoconazole are co-administered.

Itraconazole

Itraconazole is an inhibitor of P450 3A4 that increases plasma concentrations of CRIXIVAN. Therefore a dosage reduction of CRIXIVAN is recommended when CRIXIVAN and itraconazole are co-administered (see DOSAGE AND DIRECTIONS FOR USE).

Delavirdine

Due to an increase in CRIXIVAN plasma concentration (preliminary results), a dosage reduction of CRIXIVAN should be considered when CRIXIVAN and delavirdine are co-administered (see DOSAGE AND DIRECTIONS FOR USE).

Efavirenz

Due to a decrease in plasma concentrations of CRIXIVAN, a dosage increase of CRIXIVAN is recommended when CRIXIVAN and efavirenz are co-administered. No dosage adjustment of efavirenz is necessary when given with indinavir (see DOSAGE AND DIRECTIONS FOR USE).

HMG-CoA Reductase Inhibitors

Concomitant use of CRIXIVAN with lovastatin or simvastatin is not recommended. Caution should be exercised if protease inhibitors, including CRIXIVAN, are also used concurrently with other HMG-CoA reductase inhibitors that are metabolised by the CYP3A4 pathway (e.g. atorvastatin or cerivastatin).

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Side effects occurring less frequently (less than 2 %) of patients receiving CRIVAN in all Phase II/Phase III studies and considered at least possibly related or of unknown relationship to treatment and of at least moderate intensity are listed below by body system:

Body as a Whole/Site Unspecified

Abdominal distention; Chest pain; Chills; Fever; Flank pain; Flu-like illness; Fungal infection; Malaise; Pain; Syncope

Cardiovascular System

Cardiovascular disorder; Palpitations

Digestive System

Acid regurgitation; Anorexia; Aphthous stomatitis; Chelitis; Cholestasis; Constipation; Dry mouth; Dyspepsia; Eructation; Flatulence; Gastritis; Gingivitis; Glossodynia; Gingival haemorrhage; Increased appetite; Infectious gastroenteritis; Jaundice; Liver cirrhosis

Haemic and Lymphatic System

Anaemia; Lymphadenopathy; Spleen disorder

Metabolic/Nutritional/Immune

Food allergy

Musculoskeletal System

Arthralgia; Back pain; Leg pain; Myalgia; Muscle cramps; Muscle weakness; Musculoskeletal pain; Shoulder pain; Stiffness

Nervous System and Psychiatric

Agitation; Anxiety; Anxiety disorder; Bruxism; Decreased mental acuity; Depression; Dizziness; Dream abnormality; Dysaesthesia; Excitement; Fasciculation; Hypaesthesia; Nervousness; Neuralgia; Neurotic disorder; Paraesthesia; Peripheral neuropathy; Sleep disorder; Somnolence; Tremor; Vertigo

Respiratory System

Cough; Dyspnoea; Halitosis; Pharyngeal hyperaemia; Pharyngitis; Pneumonia; Rales/rhonchi; Respiratory failure; Sinus disorder; Sinusitis; Upper respiratory infection

Skin and Skin Appendage

Body odour; Contact dermatitis; Dermatitis; Dry skin; Flushing; Folliculitis; Herpes simplex; Herpes zoster; Night sweats; Pruritus; Seborrhoea; Skin disorder; Skin infection; Sweating; Urticaria

Special Senses

Accommodation disorder; Blurred vision; Eye pain; Eye swelling; Orbital oedema; Taste disorder

Urogenital System

Dysuria; Haematuria; Hydronephrosis; Nocturia; Premenstrual syndrome; Proteinuria; Renal colic; Urinary frequency; Urinary tract infection; Urine abnormality; Urine sediment abnormality; Urolithiasis

Laboratory Test Findings

The most frequently occurring selected laboratory side effects (incidence greater than or equal to 5 %) considered to be possibly, probably, or definitely drug related by the study investigator in the group treated with CRIVAN alone were changes in ALT, AST, indirect serum bilirubin, total serum bilirubin, and urine protein. Only 1 % of patients discontinued treatment due to these laboratory adverse experiences when treated with CRIVAN alone or in combination with other antiretroviral agents.

Isolated asymptomatic hyperbilirubinemia (total bilirubin greater than or equal to 42.75 $\mu\text{mol/L}$), reported predominantly as elevated indirect bilirubin and rarely associated with elevations in ALT, AST, or alkaline phosphatase, has occurred in patients treated with CRIVAN alone or in combination with other antiretroviral agents. Most patients continued treatment with CRIVAN without dosage reduction and bilirubin values gradually declined toward baseline.

Post-Marketed Experience

The following additional laboratory experiences have been reported and the frequencies are unknown: Increased serum triglycerides; Increased serum cholesterol.

SPECIAL PRECAUTIONS

Nephrolithiasis

Nephrolithiasis has occurred with CRIVAN. In some cases, nephrolithiasis has been associated with renal insufficiency or acute renal failure; in the majority of cases, renal insufficiency and acute renal failure were reversible. Signs and symptoms of

nephrolithiasis, including flank pain with or without haematuria (including microscopic haematuria), have been reported in patients receiving the recommended dose of CRIVAN (see **WARNINGS** and **DOSAGE AND DIRECTIONS FOR USE**).

During post-marketing surveillance of patients treated with CRIVAN, rare reports of interstitial nephritis with medullary calcification and cortical atrophy have been observed in patients with asymptomatic severe leukocyturia (greater than 100 cells/high power field). In patients with asymptomatic severe leukocyturia, further evaluation may be warranted.

Acute Haemolytic Anaemia

Acute haemolytic anaemia has been reported which, in some cases, was severe and progressed rapidly. Once a diagnosis is apparent, appropriate measures for the treatment of haemolytic anaemia should be instituted. It may be necessary to discontinue CRIVAN.

Hepatitis

Hepatitis including rare cases of hepatic failure have been reported in patients treated with CRIVAN. Because the majority of these patients had confounding medical conditions and/or were receiving concomitant therapy(ies), a causal relationship between CRIVAN and these events has not been established.

Diabetes and Hyperglycaemia

There have been reports of new onset diabetes mellitus or hyperglycaemia, or exacerbation of pre-existing diabetes mellitus occurring in HIV-infected patients receiving protease inhibitor therapy. Some patients required either initiation or dose adjustments of insulin or oral hypoglycaemic agents for treatment of these events. In some cases diabetic ketoacidosis has occurred.

In majority of cases, treatment with protease inhibitors was continued, while in some cases treatment was either discontinued or interrupted. In some patients, hyperglycaemia persisted after the protease inhibitor was withdrawn, whether or not diabetes was reported at baseline. A causal relationship between protease inhibitor therapy and these events has not been established.

Immune Reconstitution Syndrome

Immune reconstitution syndrome has been reported in patients treated with combination antiretroviral therapy (CART), including CRIVAN. During the initial phase of treatment, a patient whose immune system responds to CART may mount an inflammatory response to indolent or residual opportunistic infections, which may necessitate further evaluation and treatment.

Concomitant use of CRIVAN and HMG-CoA Reductase Inhibitors

(See **INTERACTIONS**)

Concomitant use of CRIVAN with lovastatin or simvastatin is not recommended. Caution should be exercised if CRIVAN is also used concurrently with other HMG-CoA reductase inhibitors that are metabolised by the CYP3A4 pathway. The risk of myopathy, including rhabdomyolysis, may be increased when CRIVAN is used in combination with these medicines.

Fat redistribution

Redistribution/accumulation of body fat including central obesity, dorsocervical fat enlargement (buffalo hump), peripheral wasting, breast enlargement and 'cushingoid appearance' have been frequently observed in patients receiving protease inhibitors. The mechanism and long term consequences of these events are currently unknown. A causal relationship has not been established.

Resistance/Cross-resistance

The potential for HIV cross resistance between protease inhibitors has not been fully explored. Therefore, it is unknown what effect initial protease inhibitor therapy will have on the activity of subsequent protease inhibitors.

Concomitant use of CRIVAN and St. John's wort (*Hypericum perforatum*)

Concomitant use of CRIVAN and St. John's wort (*Hypericum perforatum*) or products containing St. John's wort is not recommended. Co-administration of CRIVAN and St. John's wort has been shown to substantially decrease CRIVAN concentrations and may lead to loss of virologic response and possible resistance to CRIVAN or to the class of protease inhibitors.

Patients with Coexisting Conditions

There have been reports of spontaneous bleeding in patients with haemophilia A and B treated with protease inhibitors. In some patients, additional factor VII was required. In many of the reported cases, treatment with protease inhibitors was continued or restarted. A causal relationship between protease inhibitor therapy and these episodes has not been established (see **SIDE EFFECTS**, **Post-Marketed Experience**).

Patients with hepatic insufficiency due to cirrhosis will require a dosage reduction of CRIVAN due to decreased metabolism of CRIVAN (see **DOSAGE AND DIRECTIONS FOR USE**).

Ingestion of CRIVAN with a meal high in calories, fat, and protein reduces the absorption of CRIVAN. For optimal absorption, CRIVAN should be administered without food but with water 1 hour before or 2 hours after a meal (see **DOSAGE AND DIRECTIONS FOR USE** and **PHARMACOLOGICAL ACTION - PHARMACOKINETICS**, Absorption).

Effects on ability to drive and use machines

There are no data to suggest that CRIVAN affects the ability to drive and use machines.

KNOWN SYMPTOMS OF OVERDOSAGE AND PARTICULARS OF ITS TREATMENT

There have been reports of human overdosage with CRIVAN. The most commonly reported symptoms were gastrointestinal (e.g. nausea, vomiting, diarrhoea) and renal (e.g. nephrolithiasis, flank pain, haematuria). It is not known whether CRIVAN is dialysable by peritoneal or haemodialysis.

IDENTIFICATION

CRIVAN 200 mg is a white semi-translucent capsule with "CRIVAN™ 200 mg" printed in blue ink. CRIVAN 400 mg is a white semi-translucent capsule with "CRIVAN™ 400 mg" printed in green or gold ink.

PRESENTATION

CRIVAN 200 mg is available in plastic bottles containing 360 capsules. CRIVAN 400 mg is available in plastic bottles containing 18, 120 or 180 capsules.

STORAGE INSTRUCTIONS

Store in well-closed containers at room temperature, below 30 °C. Protect from moisture. KEEP OUT OF REACH OF CHILDREN.

REGISTRATION NUMBERS

CRIVAN 200 mg - 31/20.2.8/0186
CRIVAN 400 mg - 31/20.2.8/0187

NAME AND BUSINESS ADDRESS OF THE HOLDER OF THE CERTIFICATE OF REGISTRATION

MSD (Pty) Ltd
16th Road
HALFWAY HOUSE
1685

DATE OF PUBLICATION OF THIS PACKAGE INSERT

10 August 2007

Namibian Only: Crixivan 200 mg	
Registration Number:	04/20.2.8/1443
Scheduling Status:	NS2
Namibian Only: Crixivan 400 mg	
Registration Number:	04/20.2.8/1442
Scheduling Status:	NS2
Botswana Only: Crixivan 200 mg	
License Number:	80T9800146
Scheduling Status:	S2
Botswana Only: Crixivan 400 mg	
License Number:	80T9800326
Scheduling Status:	S2

(WPC-CRX-C-042004, 022005, 072006)

The risk of myopathy, including rhabdomyolysis, may be increased when protease inhibitors, including CRIVAN, are used in combination with these drugs (see **SPECIAL PRECAUTIONS**).

St. John's Wort (*Hypericum perforatum*)

Concomitant use of CRIVAN and St. John's wort (*Hypericum perforatum*) or products containing St. John's wort is not recommended. Co-administration of CRIVAN and St. John's wort has been shown to substantially decrease CRIVAN concentrations and may lead to loss of virologic response and possible resistance to CRIVAN or to the class of protease inhibitors.

Venlafaxine

Venlafaxine decreases CRIVAN plasma concentrations. CRIVAN did not affect the plasma concentrations of venlafaxine and active metabolite O-desmethyl-venlafaxine. The clinical significance of this finding is unknown.

Ritonavir

Ritonavir increases CRIVAN plasma concentration and CRIVAN may affect ritonavir plasma concentration. Currently there are no safety or efficacy data available on the use of this combination in patients.

Sildenafil

Co-administration of CRIVAN with sildenafil is expected to substantially increase sildenafil plasma concentrations and may result in an increase in sildenafil-associated adverse events, including hypotension, visual change and priapism (see the manufacturer's complete prescribing information for sildenafil).

Other

Medicines Metabolized by CYP3A4

Co-administration of CRIVAN, a CYP3A4 inhibitor, with calcium channel blockers, trazodone and other drugs metabolized by CYP3A4, may result in increased plasma concentrations of these drugs which could increase or prolong their therapeutic and adverse effects.

A formal drug interaction study between CRIVAN and didanosine has not been performed. However, a normal (acidic) gastric pH may be necessary for optimum absorption of CRIVAN, whereas acid rapidly degrades didanosine which is formulated with buffering agents to increase pH. CRIVAN and didanosine should be administered at least one hour apart on an empty stomach (consult the manufacturer's prescribing information for didanosine). Antiretroviral activity was unaltered when didanosine was administered three hours after treatment with CRIVAN in one clinical study.

If CRIVAN and didanosine are administered concomitantly, they should be administered at least one hour apart on an empty stomach.

Other drugs that induce CYP3A4 less potently than rifampin, such as phenobarbital, phenytoin, carbamazepine, and dexamethasone should be used cautiously together with indinavir since they could also diminish plasma concentrations of indinavir.

PREGNANCY AND LACTATION

CRIVAN is contra-indicated in pregnancy and lactation as safety has not been established.

DOSAGE AND DIRECTIONS FOR USE

The recommended dosage of CRIVAN is 800 mg (usually given as two 400 mg capsules) orally every 8 hours. Therapy with CRIVAN must be initiated at the recommended dose of 2.4 g/day.

Since CRIVAN must be taken at intervals of 8 hours, a schedule convenient for the patient should be developed.

For optimal absorption, CRIVAN should be administered without food but with water 1 hour before or 2 hours after a meal. Alternatively, CRIVAN may be administered with other liquids such as skim milk, juice, coffee, or tea, or a light meal, e.g. dry toast with jelly, apple juice, and coffee with skim milk and sugar or corn flakes, skim milk and sugar.

To ensure adequate hydration, it is recommended that the patient drink at least 1.5 litres of liquids during the course of 24 hours.

Concomitant Therapy

Rifabutin

Dose reduction of rifabutin to half the standard dose is recommended (consult the manufacturer's prescribing information for rifabutin) and a dose increase of CRIVAN to 1 000 mg every 8 hours are recommended when rifabutin and CRIVAN are co-administered.

Ketoconazole

Due to an increase in plasma concentrations of CRIVAN, a dosage reduction of CRIVAN to 600 mg every 8 hours should be considered when administering ketoconazole concurrently.

Didanosine

If co-prescribed with didanosine (DDI), the two agents should be administered at least one hour apart on an empty stomach.

Itraconazole

Dose reduction of CRIVAN to 600 mg every 8 hours is recommended when administering itraconazole 200 mg twice daily concurrently.

Delavirdine

Dose reduction of CRIVAN to 600 mg every 8 hours should be considered when administering delavirdine 400 mg three times a day.

Efavirenz

Dose increase of CRIVAN to 1 000 mg every 8 hours is recommended when administering efavirenz concurrently.

Patients with Co-existing Conditions

Hepatic Insufficiency due to Cirrhosis

In patients with mild-to-moderate hepatic insufficiency due to cirrhosis, the dosage of CRIVAN should be reduced to 600 mg every 8 hours.

Nephrolithiasis

In addition to adequate hydration, medical management in patients who experience nephrolithiasis may include temporary interruption of therapy (e.g. 1 to 3 days) during the acute episode of nephrolithiasis or discontinuation of therapy.

SIDE EFFECTS AND SPECIAL PRECAUTIONS

SIDE EFFECTS

In clinical trials with CRIVAN, nephrolithiasis, including flank pain with or without haematuria (including microscopic haematuria), has been reported in approximately 9.8 % (25/2 577) of patients receiving CRIVAN at the recommended dose compared to 2.2 % in the control arms. In general these events were not associated with renal dysfunction and resolved with hydration and temporary interruption of therapy (e.g. 1 to 3 days) (see **WARNINGS** and **DOSAGE AND DIRECTIONS FOR USE Nephrolithiasis**).

Asymptomatic hyperbilirubinemia (total bilirubin greater than or equal to 42.75 µmol/litre), reported predominantly as elevated indirect bilirubin, has occurred in approximately 10 % of patients treated with CRIVAN. In less than 1 % this was associated with elevations in ALT or AST.

Hyperbilirubinemia and nephrolithiasis occurred more frequently as doses exceeding 2.4 g/day compared to doses less than or equal to 2.4 g/day.

Post-Marketed Experience

The following additional adverse experiences have been reported in post-marketed experience without regard to causality:

Body as a Whole/Site Unspecified

The following side effects have been reported and the frequencies are unknown:

Abdominal distention; Redistribution/accumulation of body fat in areas such as the back of the neck, breasts, abdomen and retroperitoneum (see **SPECIAL PRECAUTIONS**)

Cardiovascular System

The following side effects have been reported and the frequencies are unknown:

Cardiovascular disorders including myocardial infarction and angina pectoris; Cerebrovascular disorder

Digestive System

The following side effects have been reported and the frequencies are unknown:

Liver function abnormalities; Hepatitis including rare reports of hepatic failure (see **SPECIAL PRECAUTIONS**); Pancreatitis

Haematologic

The following side effects have been reported and the frequencies are unknown:

Increased spontaneous bleeding in patients with haemophilia (see **SPECIAL PRECAUTIONS**); Thrombocytopenia; Anaemia including acute haemolytic anaemia

Endocrine/Metabolic

The following side effects have been reported and the frequencies are unknown:

New onset diabetes mellitus or hyperglycaemia, or exacerbation of pre-existing diabetes mellitus (see **SPECIAL PRECAUTIONS**); Increases in blood glucose and plasma triglycerides have been reported (see **SPECIAL PRECAUTIONS**)

Hypersensitivity

The following side effects have been reported and the frequencies are unknown:

Anaphylactoid reactions; Vasculitis

Nervous system/Psychiatric

The following side effects have been reported and the frequencies are unknown:

Oral paraesthesia

Skin and Skin Appendage

The following side effects have been reported and the frequencies are unknown:

Hyperpigmentation; Alopecia; Urticaria; Rash, including erythema multiforme and Stevens-Johnson syndrome; Ingrown toenails and/or paronychia

Urogenital System

The following side effects have been reported and the frequencies are unknown:

Nephrolithiasis, generally without renal dysfunction (however there have been reports of nephrolithiasis with renal dysfunction); Acute renal failure (see **SPECIAL PRECAUTIONS**); Pyelonephritis; Renal insufficiency; Leukocyturia; Crystalluria; Interstitial nephritis sometimes with indinavir crystal deposits; In some patients the interstitial nephritis did not resolve following discontinuation of CRIVAN

Clinical Trials

Drug-related clinical adverse experiences of moderate or severe intensity in greater than or equal to 2 % of patients treated with CRIVAN alone, CRIVAN in combination with zidovudine, or zidovudine alone are presented below:

ADVERSE EXPERIENCE	CRIVAN Percent (n=198)	CRIVAN Plus Zidovudine Percent (n=198)	Zidovudine Percent (n=198)
Body as a Whole			
Asthenia/fatigue	3.6	9.2	7.7
Abdominal pain	8.7	8.2	5.1
Flank pain	2.6	1.0	0
Malaise	0.5	2.0	1.5
Digestive			
Acid regurgitation	1.0	2.0	0.5
Diarrhoea	4.6	4.1	2.1
Dry mouth	0.5	0	2.1
Nausea	11.7	32.1	14.4
Vomiting	4.1	12.5	4.6
Anorexia	0.5	2.0	3.1
Musculoskeletal System			
Back pain	2.0	1.0	1.5
Nervous System/ Psychiatric			
Dizziness	1.0	3.6	0.5
Headache	5.6	11.7	5.1
Somnolence	1.0	1.5	3.6
Insomnia	3.1	1.5	0
Special Senses			
Taste perversion	2.6	3.6	2.1

In Phase I and II controlled trials, the following side effects were reported significantly more frequently by those randomized to CRIVAN-containing arms than by those randomized to nucleoside analogues: rash, upper respiratory infection, dry skin, pharyngitis, taste perversions.

**PATIENT INFORMATION LEAFLET
INFORMATION FOR THE PATIENT ABOUT**

**CRIVAN®
(INDINAVIR, MSD)**

Please read all of this leaflet carefully before you start taking this medicine, even if you have just refilled your prescription. Some of the information in the previous leaflet may have changed. Keep this leaflet. You may need to read it again. If you have further questions, please ask your doctor or your pharmacist. This medicine has been prescribed for you personally and you should not share your medicine with other people. It may harm them, even if their symptoms are the same as yours.

SCHEDULING STATUS S4

**PROPRIETARY NAME AND DOSAGE FORM
CRIVAN® 200 mg Capsules
(200 mg of indinavir)
CRIVAN® 400 mg Capsules
(400 mg of indinavir)**

WHAT CRIVAN CONTAINS

CRIVAN 200 mg contains 200 mg of indinavir as the active ingredient.
CRIVAN 400 mg contains 400 mg of indinavir as the active ingredient.

In addition, CRIVAN contains the following inactive ingredients: Anhydrous lactose, Magnesium Stearate.

CRIVAN contains sugar.

WHAT CRIVAN IS USED FOR

CRIVAN is a member of a class of medicines called protease inhibitors. It is active against the Human Immunodeficiency Virus (HIV) helping to reduce the amount of virus in the body.

Your doctor has prescribed CRIVAN for you because you have HIV infection. CRIVAN can help reduce your chances of getting illnesses associated with HIV infection and it can help extend your life. CRIVAN can also help lower the amount of HIV in your body (called 'viral load') and raise your CD4 (T) cell count. CRIVAN may not have these effects in all patients.

HIV is a blood borne disease spread by contact with blood or sexual contact with an infected individual.

BEFORE YOU TAKE CRIVAN

Do not take CRIVAN:

1. If you are allergic to indinavir or any of the other ingredients of CRIVAN.
2. If pregnant or breastfeeding as safety has not been established.
3. Children under the age of 18 years, as safety and efficacy have not been established.
4. Antituberculous regimens containing rifampin must be avoided.
5. Safety and efficacy in patients with concomitant liver disease (e.g. Hepatitis B) has not been established.
6. Safety and efficacy have not been established in the elderly.

Take special care with CRIVAN

What should I tell my doctor before I take CRIVAN?
Tell your doctor about any past or present medical problems, including liver disease due to cirrhosis, kidney problems, diabetes, haemophilia, allergies, or high cholesterol and if you are taking cholesterol-lowering medicines called 'statins'.

You should always tell your doctor about all medications you are taking or plan to take, including those obtained without a prescription, herbal products, or dietary supplements.

Pregnancy and Breastfeeding

If you are pregnant or breastfeeding your baby while taking this medicine, please consult your doctor, pharmacist or other healthcare professional for advice.

It is not known whether CRIVAN is harmful to an unborn baby when taken by a pregnant woman. If you are pregnant, you should take CRIVAN only if your doctor decides it is clearly needed.

Tell your doctor if you are breastfeeding. Your doctor will tell you to stop breastfeeding while you are taking CRIVAN.

Driving and using machinery

There is no specific information to suggest that CRIVAN affects your ability to drive and use machinery. However, dizziness and blurred vision have been reported during treatment with CRIVAN. If you experience these you should avoid driving or operating machinery.

Taking other medicines with CRIVAN

CRIVAN may be taken with a number of medications that are commonly used by people with HIV infection. These include zidovudine, didanosine, lamivudine, stavudine, fuzonazole, isoniazid, clarithromycin, cimetidine, trimethoprim/sulfamethoxazole and methadone.

Other medications may be taken with CRIVAN but require dosage adjustment of that medicine or CRIVAN. These include rifabutin, ketoconazole, itraconazole, delavirdine and efavirenz.

Tell your doctor if you are taking calcium channel blockers (medicines to treat hypertension or chest pain).

Tell your doctor if you are taking venlafaxine.

Tell your doctor if you are taking trazodone.

Medications that may not be taken with CRIVAN are terfenadine, midazolam, piroxicam, cisapride, astemizole, triazolam, rifampin and ergot derivatives such as ergotamine tartrate or ergotamine tartrate and caffeine. Consult your doctor before taking CRIVAN with any other medication.

Taking CRIVAN with St. John's wort (*Hypericum perforatum*), an herbal product sold as a dietary supplement, or products containing St. John's wort, is not recommended as it may decrease the effect of CRIVAN or other HIV-related drugs.

If you are taking medicines on a regular basis including complementary or traditional medicines, the use of CRIVAN with these medicines, may cause undesirable interactions. Please consult your doctor, pharmacist or other healthcare professional for advice.

HOW TO TAKE CRIVAN

Always take CRIVAN exactly as your doctor has instructed you. You should check with your doctor or pharmacist if you are unsure.

If you have the impression that the effect of CRIVAN is too strong or too weak, talk to your doctor or pharmacist.

CRIVAN is in capsule form and must be taken orally. Take 800 mg (usually given as two 400 mg capsules) at regular 8-hour intervals. CRIVAN must be taken at intervals of 8 hours for full effectiveness.

CRIVAN should be taken with water 1 hour before or 2 hours after a meal. If water is not preferred, CRIVAN can be taken with skimmed or fat-free milk, juice, coffee, or tea; or a light meal such as dry toast with jam or fruit conserve, juice and coffee with skimmed or fat-free milk and sugar; or corn flakes, skimmed or fat-free milk and sugar. At any other time you can follow your regular diet.

Taking CRIVAN with a meal that is high in calories, fat, and protein reduces your body's ability to absorb the medicine and in turn reduces its effectiveness.

It is important to drink at least 1.5 litres of liquids during each day.

It is very important to take CRIVAN exactly as your doctor prescribes and that you do not stop taking it without first telling your doctor. Taking CRIVAN as described will ensure the full effectiveness of the product.

Do not share medicines prescribed for you with any other person.

If you take more CRIVAN than you should
In the event of over dosage, consult your doctor or pharmacist. If neither is available, contact the nearest hospital or poison control centre. The most commonly reported symptoms were gastrointestinal (e.g. nausea, vomiting, diarrhoea) and renal (e.g. nephrotoxicity, flank pain, haematuria).

If you forget to take CRIVAN
Take CRIVAN 3 times a day at regular 8-hour intervals. However, if you miss a dose, do not take it later in the day. Simply continue to follow your usual schedule. Do not take a double dose to make up for forgotten individual doses.

POSSIBLE SIDE EFFECTS

Any drug may have unintended or undesirable effects, so-called side effects. CRIVAN has been shown to be generally well tolerated.

Side effects occurring frequently in patients receiving CRIVAN:

Digestive system

Diarrhoea

Nausea

Nervous system/Psychiatric

Headache

Side effects occurring less frequently in patients receiving CRIVAN:

Body as a Whole/Site Unspecified

Weakness/fatigue

Digestive system

Dyspepsia

Musculoskeletal system

Severe muscle pain and weakness have occurred in patients taking CRIVAN, together with cholesterol-lowering medicines called 'statins'.

Nervous system and Psychiatric

Dizziness

Skin and Skin Appendage

Dry skin

The following side effects have been reported with an unknown frequency:

Body as a Whole/Site Unspecified

Abdominal pain/swelling
Increased fat appearing in areas such as the neck, breasts, abdomen, and back

Cardiovascular system

Heart problems including heart attack

Stroke

Digestive system

Some patients treated with CRIVAN have had liver problems and in rare cases liver failure
Inflammation of the pancreas

Haemic and Lymphatic system

Some patients treated with CRIVAN have had rapid breakdown of red blood cells (haemolytic anaemia) which in some cases was severe
In some patients with haemophilia increased bleeding has been reported
Low red blood cell count

Endocrine/Metabolic

Diabetes and high blood sugar (hyperglycaemia) has occurred in patients taking protease inhibitors. In some of these patients, this led to ketoacidosis, a serious condition caused by poorly controlled blood sugar. Some patients had diabetes before starting protease inhibitors, others did not. Some patients required adjustments to their diabetes medication. Others needed new diabetes medication
Increased plasma triglycerides

Hypersensitivity

Allergic reactions

Nervous system/Psychiatric

Numbness of the mouth

Skin and Skin Appendage

Change in skin colour

Hair loss

Ingrown toenails with or without infection

Rash

Severe skin reactions

Special Senses

Taste perversion

Urogenital system

There have been kidney stones and in some of these patients this led to more severe kidney problems including kidney failure
Crystals in the urine
Inflammation of the kidneys; Infection of the kidneys; decreased kidney function

Skin and Skin Appendage

Dry skin

Your doctor has a more complete list of side effects.

Tell your doctor promptly about these or any other unusual symptoms. If the condition persists or worsens, seek medical attention.

In addition, tell your doctor if you experience any symptoms that suggest an allergic reaction after taking CRIVAN.

Not all side effects reported for this medicine are included in this leaflet. Should your general health worsen while taking this medicine, please consult your doctor, pharmacist or other healthcare professional for advice.

OTHER CONSIDERATIONS

You should know that CRIVAN is not a cure for HIV infection and that you may continue to develop infections or other illnesses associated with HIV disease. You should, therefore, remain under the care of your doctor while taking CRIVAN.

In some patients with advanced HIV infection (AIDS) and a history of opportunistic infection, signs and symptoms of inflammation from previous infections may occur when combination antiretroviral treatment is started.

The long-term effects of CRIVAN are unknown at this time. Treatment with CRIVAN has not been shown to reduce the risk of transmission of HIV to others through sexual contact or blood contamination.

CRIVAN capsules are sensitive to moisture. At all times, store CRIVAN in the original container with the desiccant. See your doctor for more details.

HOW CAN I LEARN MORE ABOUT CRIVAN?

Not all information about the drug is printed here. If you have any additional questions, ask your doctor or pharmacist who have more detailed information about CRIVAN and HIV infection.

STORING AND DISPOSING OF CRIVAN

How long should I keep my medicine?

Do not use this medicine after the month and year following EXPIRATION on the carton and bottle.
Return all unused medicine to your pharmacist.
Do not dispose of unused medicine in drains or sewerage systems (e.g. toilets).

How should I store CRIVAN?

Store CRIVAN in the well-closed original container at room temperature below 30 °C. Protect from moisture.

Keep all medicines out of reach and sight of children.

PRESENTATION OF CRIVAN

CRIVAN 200 mg is available in plastic bottles containing 360 capsules.

CRIVAN 400 mg is available in plastic bottles containing 18, 120 or 180 capsules.

IDENTIFICATION OF CRIVAN

CRIVAN 200 mg is a white semi-translucent capsule with "CRIVAN" 200 mg printed in blue ink.
CRIVAN 400 mg is a white semi-translucent capsule with "CRIVAN" 400 mg printed in green or gold ink.

REGISTRATION NUMBERS

CRIVAN 200 mg - 31/20.2.8/0186

CRIVAN 400 mg - 31/20.2.8/0187

NAME, BUSINESS ADDRESS AND TELEPHONE

NUMBER OF THE HOLDER OF THE CERTIFICATE

OF REGISTRATION

MSD (Pty) Ltd

167 Road

Halfway House

1685

011 655 3000

DATE OF PUBLICATION OF THE PATIENT INFORMATION

LEAFLET

10 August 2007



(WPPI-CR-C-042004, 022005, 072006)

340215

APPENDIX C

PACKAGE INSERT CANOVA®

SCHEDULING STATUS: N/A

PROPRIETARY NAME (and dosage form):

Canova™ Drops

COMPOSITION:

Canova™ Each ml contains:

Aconitum napellus	DH 20 (0.06 ml)	Lachesis muta	DH 18 (0.06 ml)
Apis mellifica	DH 19 (0.06 ml)	Lycopodium clavatum	DH 20 (0.06 ml)
Arsenicum album	DH 17 (0.06 ml)	Pulsatilla nigricans	DH 13 (0.06 ml)
Asa foetida	DH 20 (0.06 ml)	Rhus toxicodendrum	DH 17 (0.06 ml)
Barita carbónica	DH 20 (0.06 ml)	Ricinus communis	DH 14 (0.06 ml)
Bryonia alba	DH 14 (0.06 ml)	Silicea	DH 18 (0.06 ml)
Calcarea carbónica	DH 20 (0.06 ml)	Thuja occidentalis	DH 16 (0.06 ml)
Conium maculatum	DH 16 (0.06 ml)	Veratrum album	DH 20 (0.06 ml)
Ipecacuanha	DH 13 (0.06 ml)	in a Purified Water base with Ethanol 0.07% v/v. Preservative; Methyl Paraben 0.015 % m/v	

PHARMACOLOGICAL CLASSIFICATION:

A 32.16 Other. Submitted according to the Complementary Listing System. Homeopathic preparation.

PHARMACOLOGICAL ACTION:

Canova™ Drops contains a unique internationally patented blend of homeopathic extracts which act synergistically help to enhance your immune system.

INDICATIONS:

Canova™ Drops has homeopathic activity and help promote, enhance and support your immune system.

CONTRA-INDICATIONS:

Hypersensitivity to any of the ingredients.

WARNINGS:

None known.

INTERACTIONS:

None known.

PREGNANCY AND LACTATION:

Safety during pregnancy and lactation has not been established. Should not be used during pregnancy and lactation, unless recommended by a registered homeopathic practitioner or medical practitioner.

DOSAGE AND DIRECTIONS FOR USE:

- Strike the bottom of the bottle with one hand on the palm of the other 15 times before use, then
- Take 10 drops sub-lingually (under the tongue) 3 times per day, 30 minutes before meals.
- The same dosage can be administered both for paediatric and adult use.
- Do not eat or drink anything for at least 30 minutes before or after taking Canova™ Drops

SIDE EFFECTS AND SPECIAL PRECAUTIONS:

None known.

Special Precautions: Keep medication away from sunlight, TV, Radio, microwaves and other electrical appliances and cell phones.

KNOWN SYMPTOMS OF OVERDOSAGE AND PARTICULARS OF ITS TREATMENT:

None known. Treatment of overdosage is symptomatic and supportive.

IDENTIFICATION:

Canova™ Drops is a clear colourless liquid with no odour and a slight characteristic taste.

PRESENTATION:

Canova™ Drops is presented in amber glass bottles with tamperproof slightly off white dropper caps. The bottles contain 50 ml.

STORAGE INSTRUCTIONS:

Store below 25°C protected from light and moisture.

KEEP OUT OF REACH OF CHILDREN.

THE SHELF-LIFE FOR THE PRODUCT IS 2 YEARS FROM MANUFACTURING DATE

NAME AND ADDRESS OF APPLICANT:

Pharmachem Pharmaceuticals, 365 Soutter Street Pretoria West 0117

DATE OF PUBLICATION OF THIS PACKAGE INSERT:

April 2010



APPENDIX D

PACKAGE INSERT LINCTAGON FORTE[®]

- A. **SCHEDULING STATUS:**
Unscheduled
- B. **PROPRIETARY NAME (AND DOSAGE FORM):**
LINCTAGON FORTE TABLET
- C. **COMPOSITION:**
Each Tablet contains:

Pelargonium sidoides	250mg
Quercetin	60mg
Bromelain	40mg
- D. **PHARMACOLOGICAL CLASSIFICATION:**
Complementary Medicines: Category 8.
Western Herbal Medicine.
- F. **INDICATION:**
Linctagon contains the natural active ingredient Pelargonium sidoides which assists in the treatment of symptoms associated with acute and chronic respiratory tract infections. Quercetin and Bromelain assist in dealing with inflammation and congestion experienced during respiratory tract infections.
- G. **CONTRA-INDICATIONS:**
Avoid simultaneous use with blood thinning medicine like coumarin derivatives.
Do not use during pregnancy and lactation.
- H. **WARNING:**
Keep out of reach of children.
- I. **INTERACTION:**
Can interact with blood thinning medication like coumarin derivatives by enhancing its effect.
- J. **PREGNANCY AND LACTATION:**
Safety not tested during pregnancy and lactation.
- K. **DOSAGE AND DIRECTIONS FOR USE:**
For acute respiratory tract infections, it is advisable to take the recommended dosage for at least 5 days.
- Acute respiratory infection:**
Adults:
Take one tablet four times a day.
- Children between 6 and 12 years old:**
Give half a tablet four times a day.
- Chronic respiratory infection:**
Adults:
Take one tablet twice a day.
- Children between 6 and 12 years old:**
Take half a tablet twice a day.
- L. **SIDE EFFECTS AND SPECIAL PRECAUTIONS:**
No known side effects. If you experience any side effects or sensitivity towards any of the ingredients, please consult your doctor or pharmacist
- M. **KNOWN SYMPTOMS OF OVERDOSE AND TREATMENT:**
No known symptoms.
- N. **IDENTIFICATION:**
Round yellow-brown tablet.
- O. **PRESENTATION:**
30 Tablets in a white plastic securitainer.
- P. **STORAGE INSTRUCTIONS:**
Store below 25 °C. Protect from light and moisture.
- Q. **REGISTRATION / APPLICATION NUMBER:**
(To be allocated)
- R. **NAME AND BUSINESS ADDRESS OF APPLICANT:**
NATIVA (PTY) LTD
Private Bag X 1030, Lyttelton, 0140 Gauteng South Africa
- S. **DATE OF PUBLICATION OF THIS PACKAGE INSERT:**
April 2005.

PPIL010

APPENDIX E

PACKAGE INSERT VIRAL CHOICE®

Product Information Leaflet

ViralChoice® C Capsules



Group
sanofi aventis

Winthrop
pharmaceuticals

XXXXXXXXXX
XXXXXXXXXX
XXXXXXXXXX
XXXXXXXXXX

COMPOSITION

ViralChoice® C contains:

Active ingredient	Amount/ Capsule (3 capsules)	% RDA* in 3 capsules	Role of supplement
Echinacea Ext.	80 (240) mg	--	Herbal extract with a stimulant effect on the immune system, increasing the body's natural resistance to infections, especially colds and flu.
Phytosterols (Plant sterols & sterolins)	25 (75) mg	--	Phytosterols (plant sterols and sterolins) are vitamin-like nutrients found in fresh vegetables and fruit but that are destroyed by cooking and processing of food. They are called 'immunomodulators' as they help restore and stimulate the body's immune system ¹ . Phytosterols also lower cholesterol levels, improve prostate problems in men and have protective anticancer effects on the colon ^{2,3} .
L- Arginine	10 (30) mg	--	Amino acid; enhances immune function; helps recovery from stress and trauma.
L- Methionine	33.3 (100) mg	--	Amino acid; essential for the healthy functioning of the immune system and helps rid the body of toxic substances.
Absorption Enzymes	5 (15) mg	--	Adequate digestion is vital for gastrointestinal and immune system health. Digestive enzymes may be deficient in many people, especially if the diet consists mainly of cooked or processed foods. Partially digested food accumulates in the colon, leading to various gastrointestinal problems. Digestive enzymes are important to maintain and improve digestion and for immune system health. They also increase the absorption of vitamins and minerals.
Garlic	50 (150) mg	--	Increases resistance to colds and flu. Allicin and alliin in garlic have antibacterial, antifungal and antiviral activity, without affecting the body's natural flora.
Vitamin A	333 (1000) RE	100	Needed for night vision, growth of skin, bones and reproductive organs; maintains integrity of mucous membranes; even a mild deficiency of vitamin A may lead to increased susceptibility to infection.
Vitamin B6	3 (9) mg	450	Metabolism of carbohydrates, proteins and fats in the diet; plays a key role in immune system responses.
Vitamin B12	4 (12) µg	100	Needed for healthy blood; deficiency can result in anaemia and poor resistance to infection.
Folic acid	250 (750) µg	375	Needed for healthy blood and the formation of nerve sheaths and various proteins; deficiency reduces certain immune functions.
Vitamin C	150 (450) mg	750	Stress and infection reduce the amount of vitamin C in the body. Vitamin C is needed for many functions in the body. It may also lessen the duration and intensity of a cold or flu infection.
Vitamin D	4 (12) µg	240	Usually needed for strong bones and teeth; deficiency leads to a decreased resistance to infection.
Vitamin E	10 (30) mg	300	Antioxidant; usually needed for proper function of nerves and muscles; stimulates several components of the body's immune system.
Biotin	100 (300) µg	300	Forms part of several enzyme systems and is needed for healthy functioning of the body.
Copper	0.33 (1) mg	--	Trace element – needed in small quantities for healthy functioning of the body.
Iron	2 (6) mg	43	Needed for healthy blood, preventing anaemia and increasing the body's resistance to infection.
Selenium	5 (15) µg	--	Antioxidant and together with vitamin E increases resistance to infection.
Zinc	3 (9) mg	60	Zinc deficiency may lead to a defective immune system.

*South African Recommended Daily Allowance (RDA) for persons 10 years and older.
-- No South African RDA established

Note: Water-soluble vitamins are not stored in the body. Providing more than the RDA of such vitamins has no adverse effects and may in fact be beneficial, especially in people with increased nutritional requirements such as sportsmen and sportswomen or people suffering from, or recovering from, viral infections.

ViralChoice® C does not contain tartrazine, lactose, gluten, yeast, caffeine or salt.

XXXXXXXXXX
XXXXXXXXXX
XXXXXXXXXX
XXXXXXXXXX

ViralChoice® C USES:

ViralChoice® C Capsules contain a combination of echinacea, garlic, phytosterols, vitamins, minerals, trace elements and amino acids. It is a nutrient supplement and is used when the diet alone does not include enough of these substances for optimal health.

ViralChoice® C is used during times of viral infection, in winter to reduce colds and flu infection or when recovering from illness. It helps boost the immune system, reducing the risk of invasion from viruses, bacteria and other harmful microorganisms.

The functioning of the immune system lessens with age, making older people more prone to colds and flu. A strong immune system helps prevent infection or minimizes the severity of infection. ViralChoice® C should be taken at the first sign of a cold or flu.

It may also be taken during winter to reduce the risk of colds or flu. Vitamins, minerals and trace elements do not take the place of a balanced diet.

Your body needs carbohydrates, proteins and fats. Vitamins, minerals and trace elements cannot work without the presence of food.

CONTRA-INDICATIONS AND WARNINGS:

- Do not use this product if you are allergic to any of the ingredients.
- Speak to your doctor if you are pregnant or plan to become pregnant soon or if you are breastfeeding.
- Speak to your doctor if you have immune system problems before you take this product.

DOSAGE AND DIRECTIONS FOR USE:

Take the capsules with half a glass of water, with meals.

Adults & children over 10 years:-

Maintenance dose:

- ONE capsule per day, increasing to TWO capsules per day (one morning; one evening) in people experiencing additional physical and/or mental stress.

Stop ViralChoice® C for one week after every eight weeks of continuous maintenance dosage to maximize immune boosting benefits.

Short-term immune booster:-

At the first sign of a cold or flu infection:

- ONE capsule THREE times a day for 10 days.

Complete the 10-day booster course and then continue with the maintenance dose thereafter.

STORAGE:

Store ViralChoice® C capsules in a cool, dry place below 25 °C. Keep the lid tightly closed when not in use. Keep all medicines out of the reach of children.

IDENTIFICATION:

Orange capsules

NAPPI CODE 30's - 852554-001

NAPPI CODE 60's - 852554-028

PRESENTATION:

Packs of 30, 60 and 120 capsules each in a white, plastic, screw-top container in a unit carton with a package insert.

All products in the Choice range are manufactured to the highest standards and are available in leading pharmacies throughout South Africa. Quality products in the Choice range:-

ArthroChoice™	Helps to preserve and rebuild joint cartilage, as well as helping to provide relief from pain, inflammation and joint stiffness.
ImmunoChoice® Complex	Powerful antioxidant and immune boosting nutrients combined to provide multiple protection for long-term health.
Multi-Vit Choice™	An affordable, comprehensive multivitamin and mineral supplement with Lycopene.
OsteoChoice®	The ultimate calcium supplement containing vitamin D, minerals and other unique bone building nutrients.
PerformaChoice®	Improves sexual, mental and physical energy.
ViralChoice® Effervescent	Long-term immune support for cold and flu management in an effervescent dosage form.
ViralChoice® Capsules	Cold and flu immune system supplement.
ViralChoice® Vegetabs	Cold and flu immune system supplement for vegetarians.
ViralChoice® Junior Syrup	Cold and flu immune system supplement for children.
ViralChoice® Junior Multivitamin	A comprehensive, balanced fruity flavoured vitamin and mineral chewtab supplement for growing children.
VitaChoice® Capsules	Multinutritional supplement for people with busy stressful lifestyles.

NAME AND BUSINESS ADDRESS:

Your natural choice for life from
WINTHROP PHARMACEUTICALS (PTY) LTD
2 Bond Street, Midrand, 1685, South Africa

Toll Free No. 0800 601 228

Facsimile: 011 256 3917

e-mail: customerservice.za@sanofi-aventis.com

website: www.pharmachoice.co.za

Published February 2003

References:-

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Winthrop Pharmaceuticals (Pty) Ltd
2 Bond Street
Midrand 1685
South Africa

VC2-B0609

APPENDIX F

CONCENTRATION CALCULATIONS OF EXPERIMENTAL GROUPS

The prescribed dose of each herbal product as directed by the package insert was grinded (where applicable) by mortar and pestle and dissolved in 100 ml ddH₂O. The herbal product solutions were then stirred with a magnetic stirrer for 1 h, after which they were diluted to 500 ml, 5 l and 50 l. The ingredient with the highest concentration in each selected herbal product was used to calculate the concentration of the dilutions, for example in Linctagon Forte® the following calculations were done:

Linctagon Forte®

A single Linctagon Forte® tablet is composed of (See package insert Appendix D):

<i>Pelargonium sidoides</i>	250 mg
Quercetin	60 mg
Bromelain	40 mg

Pelargonium sidoides extract is the ingredient with the highest concentration in Linctagon Forte®. The directed dosage of Linctagon Forte® is one tablet 4 times daily. One tablet dissolved in 100 ml ddH₂O, would result in a *Pelargonium sidoides* extract concentration of 250 mg/100 ml, which is equal to 2.5 mg/ml extract (C₁).

For the highest concentration:

If one tablet is dissolved in 500 ml (to produce the highest concentration tested in this study), then 250 mg *Pelargonium sidoides* extract will be present in 500 ml, which is equal to 0.5 mg/ml (C₂). To obtain 0.5 mg/ml in 50 ml (V₂), the following equation is used to calculate the volume needed from the original solution to be diluted to 50 ml:

$$C_1 \times V_1 = C_2 \times V_2 \quad \text{Eq (1)}$$

Where C₁ and C₂ represent the initial and final concentrations, respectively and V₁ and V₂ represent the initial and final volumes, respectively.

Thus:

$$\begin{aligned}C_1 \times V_1 &= C_2 \times V_2 \\2.5 \text{ mg/ml} \times V_1 &= 0.5 \text{ mg/ml} \times 50 \text{ ml} \\V_1 &= \frac{0.5 \text{ mg/ml} \times 50 \text{ ml}}{2.5 \text{ mg/ml}} \\V_1 &= 10 \text{ ml of the initial concentration}\end{aligned}$$

A volume of 10 ml of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 0.5mg/ml of *Pelargonium sidoides* extract.

For the medium concentration:

If one tablet is dissolved in 5000 ml (to produce the medium concentration tested in this study), then 250 mg *Pelargonium sidoides* extract will be present in 5000 ml, which is equal to 0.05 mg/ml (C_2). Thus, we needed 0.05 mg/ml in 50 ml (V_2).

Thus using Eq 1:

$$\begin{aligned}C_1 \times V_1 &= C_2 \times V_2 \\2.5 \text{ mg/ml} \times V_1 &= 0.05 \text{ mg/ml} \times 50 \text{ ml} \\V_1 &= \frac{0.05 \text{ mg/ml} \times 50 \text{ ml}}{2.5 \text{ mg/ml}} \\V_1 &= 1 \text{ ml of the initial concentration}\end{aligned}$$

A volume of 1 ml of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 0.05mg/ml of *Pelargonium sidoides* extract.

For the low concentration:

If one tablet is dissolved in 50000 ml (to produce the lowest concentration tested in this study), then 250 mg *Pelargonium sidoides* extract will be present in 50000 ml, which is equal to 0.005 mg/ml (C_2). Thus, we needed 0.005 mg/ml in 50 ml (V_2).

Thus using Eq 1:

$$\begin{aligned}C_1 \times V_1 &= C_2 \times V_2 \\2.5 \text{ mg/ml} \times V_1 &= 0.005 \text{ mg/ml} \times 50 \text{ ml} \\V_1 &= \frac{0.005 \text{ mg/ml} \times 50 \text{ ml}}{2.5 \text{ mg/ml}} \\V_1 &= 100 \text{ } \mu\text{l of the initial concentration}\end{aligned}$$

A volume of 100 µl of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 0.005mg/ml of *Pelargonium sidoides* extract.

Viral Choice®

A single Viral Choice® capsule is composed of (See package insert Appendix E):

<i>Echinacea</i> extract	80 mg
Sterols & sterolins	25 mg
Garlic	50 mg

Echinacea extract is the ingredient with the highest concentration in Viral Choice®. The directed dosage of Viral Choice® is one capsule 3 times daily. One capsule dissolved in 100 ml ddH₂O, would result in an *Echinacea* extract concentration of 80 mg/100 ml, which is equal to 0.8 mg/ml extract (C₁).

For the highest concentration:

If one tablet is dissolved in 500 ml (to produce the highest concentration tested in this study), then 80 mg *Echinacea* extract will be present in 500 ml, which is equal to 0.16 mg/ml (C₂). To obtain 0.16 mg/ml in 50 ml (V₂), equation 1 is used to calculate the volume needed from the original solution to be diluted to 50 ml:

Thus:

$$\begin{aligned}
 C_1 \times V_1 &= C_2 \times V_2 \\
 0.8 \text{ mg/ml} \times V_1 &= 0.16 \text{ mg/ml} \times 50 \text{ ml} \\
 V_1 &= \frac{0.16 \text{ mg/ml} \times 50 \text{ ml}}{0.8 \text{ mg/ml}} \\
 V_1 &= 10 \text{ ml of the initial concentration}
 \end{aligned}$$

A volume of 10 ml of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 0.8 mg/ml of *Echinacea* extract.

For the medium concentration:

If one capsule is dissolved in 5000 ml (to produce the medium concentration tested in this study), then 80 mg *Echinacea* extract will be present in 5000 ml, which is equal to 0.016 mg/ml (C₂). Thus, we needed 0.016 mg/ml in 50 ml (V₂).

Thus using Eq 1:

$$\begin{aligned}C_1 \times V_1 &= C_2 \times V_2 \\0.8 \text{ mg/ml} \times V_1 &= 0.016 \text{ mg/ml} \times 50 \text{ ml} \\V_1 &= \frac{0.016 \text{ mg/ml} \times 50 \text{ ml}}{0.8 \text{ mg/ml}} \\V_1 &= 1 \text{ ml of the initial concentration}\end{aligned}$$

A volume of 1 ml of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 0.016 mg/ml of *Echinacea* extract.

For the low concentration:

If one capsule is dissolved in 50000 ml (to produce the lowest concentration tested in this study), then 80 mg *Echinacea* extract will be present in 50000 ml, which is equal to 0.0016 mg/ml (C_2). Thus, we needed 0.0016 mg/ml in 50 ml (V_2).

Thus using Eq 1:

$$\begin{aligned}C_1 \times V_1 &= C_2 \times V_2 \\0.8 \text{ mg/ml} \times V_1 &= 0.0016 \text{ mg/ml} \times 50 \text{ ml} \\V_1 &= \frac{0.0016 \text{ mg/ml} \times 50 \text{ ml}}{0.8 \text{ mg/ml}} \\V_1 &= 100 \text{ } \mu\text{l of the initial concentration}\end{aligned}$$

A volume of 100 μl of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 0.0016mg/ml of *Echinacea* extract.

Canova[®]

Canova[®] drops contain various phytochemical constituents (See package insert Appendix C). The directed dosage for Canova[®] 15 drops sub-lingually. As the precise concentration of the active constituents could not be determined 10 drops of Canova[®] was weighed six times and the average weight and standard deviation were calculated.

Table F.1: Canova® drops weighed, average and standard deviation

	Weighed amount (mg)
	947.3
	924.2
	876.2
	857.7
	902.2
	907.8
Average	902.56
Standard deviation	32.263

If 10 drops are dissolved in 100 ml ddH₂O, it would result in a concentration of 902.56 mg/100 ml, which is equal to 9.03 mg/ml extract (C₁).

For the highest concentration:

If 10 drops are dissolved in 500 ml (to produce the highest concentration tested in this study), then 902.56 mg of Canova® will be present in 500 ml, which is equal to 1.81 mg/ml (C₂). To obtain 1.81 mg/ml in 50 ml (V₂), equation 1 was used to calculate the volume needed from the original solution to be diluted to 50 ml:

Thus:

$$\begin{aligned}C_1 \times V_1 &= C_2 \times V_2 \\9.03 \text{ mg/ml} \times V_1 &= 1.81 \text{ mg/ml} \times 50 \text{ ml} \\V_1 &= \frac{1.81 \text{ mg/ml} \times 50 \text{ ml}}{9.03 \text{ mg/ml}} \\V_1 &= 10.02 \text{ ml of the initial concentration}\end{aligned}$$

A volume of 10 ml of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 1.81 mg/ml Canova®.

For the medium concentration:

If 10 drops were dissolved in 5000 ml (to produce the medium concentration tested in this study), then 902.56 mg Canova® will be present in 5000 ml, which is equal to 0.181 mg/ml (C₂). To obtain 0.181 mg/ml in 50 ml (V₂), equation 1 was used to calculate the volume needed from the original solution to be diluted to 50 ml:

Thus:

$$C_1 \times V_1 = C_2 \times V_2$$

$$9.03 \text{ mg/ml} \times V_1 = 0.181 \text{ mg/ml} \times 50 \text{ ml}$$

$$V_1 = \frac{0.181 \text{ mg/ml} \times 50 \text{ ml}}{9.03 \text{ mg/ml}}$$

$$V_1 = 1 \text{ ml of the initial concentration}$$

A volume of 1 ml of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 0.181 mg/ml Canova®.

For the low concentration:

If 10 drops were dissolved in 50000 ml (to produce the lowest concentration tested in this study), then 902.56 mg of Canova® will be present in 50000 ml, which is equal to 0.0181 mg/ml (C₂). To obtain 0.0181 mg/ml in 50 ml (V₂), equation 1 was used to calculate the volume needed from the original solution to be diluted to 50 ml:

Thus:

$$C_1 \times V_1 = C_2 \times V_2$$

$$9.03 \text{ mg/ml} \times V_1 = 0.0181 \text{ mg/ml} \times 50 \text{ ml}$$

$$V_1 = \frac{0.0181 \text{ mg/ml} \times 50 \text{ ml}}{9.03 \text{ mg/ml}}$$

$$V_1 = 100 \text{ } \mu\text{l of the initial concentration}$$

A volume of 100 µl of the initial solution was therefore diluted to 50 ml with DMEM to reach the final concentration of 0.181 mg/ml Canova®.

APPENDIX G

EXPERIMENTAL DATA

1. Transepithelial electrical resistance (TEER) values

Table G.1.1: TEER Negative control Indinavir AP-BL

TEER value (Ω)	
0 min	120 min
335	240
329	259
348	275
380	268
343	276
347	275

Table G.1.2: TEER Negative control Indinavir BL-AP

TEER value (Ω)	
0 min	120 min
292	248
331	252
296	239
318	255
325	255
353	252

Table G.1.3: TEER Positive Control Indinavir & Verapamil AP-BL

TEER value (Ω)	
0 min	120 min
299	308
352	309
341	301
321	325
344	315
356	324

Table G.1.4: TEER Positive Control Indinavir & Verapamil BL-AP

TEER value (Ω)	
0 min	120 min
387	338
394	338
397	340
375	324
374	329
384	342

Table G.1.5: TEER Linctagon Forte® AP-BL (Lowest Concentration)

TEER value (Ω)	
0 min	120 min
391	291
365	282
354	274

Table G.1.6: TEER Linctagon Forte® BL-AP (Lowest Concentration)

TEER value (Ω)	
0 min	120 min
374	296
373	295
361	283

Table G.1.7: TEER Linctagon Forte® AP-BL (Medium Concentration)

TEER value (Ω)	
0 min	120 min
330	279
334	285
307	269

Table G.1.8: TEER Linctagon Forte® BL-AP (Medium Concentration)

TEER value (Ω)	
0 min	120 min
340	294
333	291
322	290

Table G.1.9: TEER Linctagon Forte® AP-BL (Highest Concentration)

TEER value (Ω)	
0 min	120 min
346	255
318	242
348	259

Table G.1.10: TEER Linctagon Forte® BL-AP (Highest Concentration)

TEER value (Ω)	
0 min	120 min
327	352
309	327
304	331

Table G.1.11: TEER Viral Choice® AP-BL (Lowest Concentration)

TEER value (Ω)	
0 min	120 min
426	340
426	360
415	356

Table G.1.12: TEER Viral Choice® BL-AP (Lowest Concentration)

TEER value (Ω)	
0 min	120 min
496	412
480	428
500	422

Table G.1.13: TEER Viral Choice® AP-BL (Medium Concentration)

TEER value (Ω)	
0 min	120 min
487	383
505	407
528	439

Table G.1.14: TEER Viral Choice® BL-AP (Medium Concentration)

TEER value (Ω)	
0 min	120 min
490	282
508	361
501	394

Table G.1.15: TEER Viral Choice® AP-BL (Highest Concentration)

TEER value (Ω)	
0 min	120 min
477	370
488	433
472	420

Table G.1.16: TEER Viral Choice® BL-AP (Highest Concentration)

TEER value (Ω)	
0 min	120 min
493	391
497	388
495	355

Table G.1.17: TEER Canova® AP-BL (Lowest Concentration)

TEER value (Ω)	
0 min	120 min
427	335
421	340
465	296

Table G.1.18: TEER Canova® BL-AP (Lowest Concentration)

TEER value (Ω)	
0 min	120 min
420	345
416	355
410	366

Table G.1.19: TEER Canova® AP-BL (Medium Concentration)

TEER value (Ω)	
0 min	120 min
506	459
492	432
506	448

Table G.1.20: TEER Canova® BL-AP (Medium Concentration)

TEER value (Ω)	
0 min	120 min
392	346
399	351
410	355

Table G.1.21: TEER Canova® AP-BL (Highest Concentration)

TEER value (Ω)	
0 min	120 min
523	394
533	400
544	399

Table G.1.22: TEER Canova® BL-AP (Highest Concentration)

TEER value (Ω)	
0 min	120 min
392	407
409	410
413	427

2. Apparent permeability coefficient calculations for experimental and control groups

2.1 P_{app} Calculations Negative Control AP-BL (Indinavir)

Table G.2.1.1 P_{app} Calculations Negative Control AP-BL (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.126143674	4.5E-06
	20	0	0	0	0		
	40	15.83824	15.83824	0.005297	3.720516		
	60	29.76361	31.03067	0.010378	7.289326		
	80	36.35112	38.73221	0.006806	4.780322		
	100	48.38115	51.28924	0.017154	12.04821		
	120	60.67165	64.54214	0.021586	15.16141		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.56888E-05	
0.08							

Table G.2.1.2 P_{app} Calculations Negative Control AP-BL (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.139077032	4.96E-06
	20	0	0	0	0		
	40	15.58445	15.58445	0.005212	3.660899		
	60	28.83761	30.08437	0.010062	7.067033		
	80	42.78687	45.09388	0.015082	10.59287		
	100	50.65781	54.08076	0.018087	12.70396		
	120	60.57326	64.62588	0.021614	15.18108		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.56888E-05	
0.08							

Table G.2.1.3: P_{app} Calculations Negative Control AP-BL (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.148795497	5.31E-06
	20	0	0	0	0		
	40	21.53376	21.53376	0.007202	5.058434		
	60	34.14647	35.86917	0.011996	8.425925		
	80	48.49445	51.22617	0.017133	12.03339		
	100	56.7903	60.66986	0.020291	14.25178		
	120	63.35162	67.89484	0.022708	15.94898		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.56888E-05	
0.08							

Table G.2.1.4: P_{app} Calculations Negative Control AP-BL (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.129613629	4.63E-06
	20	0	0	0	0		
	40	15.27575	15.27575	0.005109	3.588383		
	60	23.58566	24.80772	0.008297	5.827511		
	80	37.33029	39.21714	0.013116	9.212387		
	100	48.35563	51.34205	0.017171	12.06062		
	120	56.91925	60.7877	0.020331	14.27947		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.56888E-05	
0.08							

Table G.2.1.5: P_{app} Calculations Negative Control AP-BL (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.150214236	5.36E-06
	20	0	0	0	0		
	40	20.97156	20.97156	0.007014	4.926369		
	60	31.35256	33.03028	0.011047	7.75905		
	80	45.72876	48.23696	0.016133	11.33121		
	100	57.73782	61.39612	0.020534	14.42239		
	120	64.72803	69.34706	0.023193	16.29012		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.56888E-05	
0.08							

Table G.2.1.6: P_{app} Calculations Negative Control AP-BL (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.125453308	4.48E-06
	20	0	0	0	0		
	40	19.83765	19.83765	0.006635	4.660006		
	60	29.01663	30.60364	0.010235	7.189014		
	80	40.69667	43.018	0.014387	10.10524		
	100	48.1682	51.42393	0.017199	12.07985		
	120	53.82738	57.68084	0.019291	13.54964		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.56888E-05	
0.08							

2.2 P_{app} Calculations Negative Control BL-AP (Indinavir)

Table G.2.2.1: P_{app} Calculations Negative Control BL-AP (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.24098	8.60028E-06
	20	25.34218	25.34218	0.006354	4.462715		
	40	51.61629	53.64366	0.01345	9.446559		
	60	80.22866	84.35796	0.02115	14.8553		
	80	109.2536	115.6719	0.029001	20.36963		
	100	123.6223	132.3626	0.033186	23.30883		
	120	153.5292	163.419	0.040973	28.77781		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.2.2: P_{app} Calculations Negative Control BL-AP (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.22714	8.10634E-06
	20	24.20396	24.20396	0.006068	4.262277		
	40	46.99126	48.92758	0.012267	8.616064		
	60	72.29725	76.05655	0.019069	13.39343		
	80	96.56702	102.3508	0.025662	18.0238		
	100	122.2149	129.9403	0.032579	22.88227		
	120	142.6954	152.4726	0.038228	26.85016		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.2.3: P_{app} Calculations Negative Control BL-AP (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.246498	8.7972E-06
	20	33.60902	33.60902	0.008427	5.918492		
	40	54.6963	57.38502	0.014388	10.10541		
	60	80.32896	84.70466	0.021237	14.91635		
	80	106.5272	112.9535	0.02832	19.89093		
	100	137.3093	145.8314	0.036563	25.68067		
	120	156.969	167.9538	0.04211	29.57637		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.2.4: P_{app} Calculations Negative Control BL-AP (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.235162	8.39264E-06
	20	29.34289	29.34289	0.007357	5.167234		
	40	47.20461	49.55204	0.012424	8.726031		
	60	75.55985	79.33622	0.019891	13.97098		
	80	101.8347	107.8794	0.027048	18.99739		
	100	125.8223	133.9691	0.033589	23.59173		
	120	149.364	160.0815	0.040136	28.19008		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.2.5: P_{app} Calculations Negative Control BL-AP (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.233402	8.32983E-06
	20	32.02072	32.02072	0.008028	5.638795		
	40	50.88618	53.44784	0.013401	9.412075		
	60	73.71586	77.78675	0.019503	13.69812		
	80	100.2322	106.1294	0.026609	18.68921		
	100	126.2996	134.3182	0.033676	23.65321		
	120	151.5464	161.6504	0.040529	28.46636		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.2.6: P_{app} Calculations Negative Control BL-AP (Indinavir)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.206123	7.35629E-06
	20	38.26573	38.26573	0.009594	6.738531		
	40	51.33164	54.3929	0.013637	9.578498		
	60	67.92234	72.02887	0.018059	12.68416		
	80	93.64745	99.08124	0.024842	17.44804		
	100	114.9919	122.4837	0.030709	21.56918		
	120	138.2528	147.4521	0.036969	25.96607		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.3 P_{app} Calculations Positive Control AP-BL (Indinavir Verapamil)

Table G.2.3.1: P_{app} Calculations Positive Control AP-BL (Indinavir Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.168154	6E-06
	20	23.35472	23.35472	0.005449	3.827241		
	40	46.99944	48.86782	0.011402	8.008186		
	60	64.09777	67.85773	0.015832	11.12015		
	80	89.10955	94.23737	0.021987	15.4431		
	100	101.7282	108.857	0.025398	17.83887		
	120	111.2783	119.4166	0.027862	19.56932		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.3.2: P_{app} Calculations Positive Control AP-BL (Indinavir Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.161226	5.75E-06
	20	21.9682	21.9682	0.005126	3.600026		
	40	41.07978	42.83724	0.009995	7.019928		
	60	60.8234	64.10978	0.014958	10.50595		
	80	77.76534	82.63121	0.019279	13.54114		
	100	97.39772	103.6189	0.024176	16.9805		
	120	108.1595	115.9513	0.027054	19.00146		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.3.3: P_{app} Calculations Positive Control AP-BL (Indinavir Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.171981	6.14E-06
	20	24.92701	24.92701	0.005816	4.0849		
	40	46.19153	48.18569	0.011243	7.896403		
	60	61.20034	64.89566	0.015141	10.63474		
	80	87.55485	92.45088	0.02157	15.15033		
	100	106.2418	113.2462	0.026422	18.55815		
	120	113.7669	122.2662	0.028527	20.03631		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.3.4: P_{app} Calculations Positive Control AP-BL (Indinavir Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.180499	6.44E-06
	20	20.93378	20.93378	0.004884	3.430511		
	40	46.53196	48.20666	0.011247	7.899839		
	60	75.25797	78.98053	0.018428	12.94289		
	80	93.76137	99.78201	0.023281	16.35172		
	100	105.3149	112.8158	0.026322	18.48763		
	120	118.7319	127.1571	0.029668	20.8378		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.3.5: P_{app} Calculations Positive Control AP-BL (Indinavir Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.181678	6.48E-06
	20	23.89345	23.89345	0.005575	3.915526		
	40	46.21334	48.12482	0.011228	7.886427		
	60	76.90903	80.6061	0.018807	13.20928		
	80	94.69801	100.8507	0.02353	16.52686		
	100	107.3073	114.8831	0.026804	18.82641		
	120	120.1267	128.7113	0.030031	21.09249		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.3.6: P_{app} Calculations Positive Control AP-BL (Indinavir Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.171814	6.13E-06
	20	22.80486	22.80486	0.005321	3.737133		
	40	51.04104	52.86543	0.012334	8.663292		
	60	74.06254	78.14582	0.018233	12.8061		
	80	87.21053	93.13553	0.02173	15.26253		
	100	104.7688	111.7457	0.026072	18.31226		
	120	114.6112	122.9927	0.028696	20.15536		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.4 P_{app} Calculations Negative Control BL-AP (Indinavir + Verapamil)

Table G.2.4.1: P_{app} Calculations Negative Control BL-AP (Indinavir + Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.188704	6.73E-06
	20	23.35472	23.35472	0.006115	4.29497		
	40	46.99944	48.86782	0.012795	8.986869		
	60	64.09777	67.85773	0.017767	12.47914		
	80	89.10955	94.23737	0.024674	17.3304		
	100	101.7282	108.857	0.028502	20.01897		
	120	111.2783	119.4166	0.031267	21.96089		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.4.2: P_{app} Calculations Negative Control BL-AP (Indinavir + Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.180929	6.46E-06
	20	21.9682	21.9682	0.005752	4.039987		
	40	41.07978	42.83724	0.011216	7.877836		
	60	60.8234	64.10978	0.016786	11.78989		
	80	77.76534	82.63121	0.021635	15.19601		
	100	97.39772	103.6189	0.027131	19.05569		
	120	108.1595	115.9513	0.03036	21.32363		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.4.3: P_{app} Calculations Negative Control BL-AP (Indinavir + Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.192999	6.89E-06
	20	24.92701	24.92701	0.006527	4.584117		
	40	46.19153	48.18569	0.012617	8.861425		
	60	61.20034	64.89566	0.016992	11.93442		
	80	87.55485	92.45088	0.024207	17.00186		
	100	106.2418	113.2462	0.029651	20.82615		
	120	113.7669	122.2662	0.032013	22.48496		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.4.4: P_{app} Calculations Negative Control BL-AP (Indinavir + Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.202558	7.23E-06
	20	20.93378	20.93378	0.005481	3.849755		
	40	46.53196	48.20666	0.012622	8.865282		
	60	75.25797	78.98053	0.02068	14.52464		
	80	93.76137	99.78201	0.026126	18.35007		
	100	105.3149	112.8158	0.029539	20.74701		
	120	118.7319	127.1571	0.033294	23.38439		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.4.5: P_{app} Calculations Negative Control BL-AP (Indinavir + Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.203881	7.28E-06
	20	23.89345	23.89345	0.006256	4.394043		
	40	46.21334	48.12482	0.012601	8.85023		
	60	76.90903	80.6061	0.021105	14.82359		
	80	94.69801	100.8507	0.026406	18.54661		
	100	107.3073	114.8831	0.03008	21.12719		
	120	120.1267	128.7113	0.033701	23.67021		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.4.6: P_{app} Calculations Negative Control BL-AP (Indinavir + Verapamil)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.192811	6.88E-06
	20	22.80486	22.80486	0.005971	4.19385		
	40	51.04104	52.86543	0.013842	9.722036		
	60	74.06254	78.14582	0.020461	14.37114		
	80	87.21053	93.13553	0.024386	17.12777		
	100	104.7688	111.7457	0.029259	20.55021		
	120	114.6112	122.9927	0.032203	22.61856		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.5 P_{app} Calculations Linctagon Forte® lowest conc AP-BL (Indinavir + Linctagon Forte®)

Table G.2.5.1: P_{app} Calculations Linctagon Forte® lowest conc AP-BL (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.089727	3.2E-06
	20	0	0	0	0		
	40	0	0	0	0		
	60	0	0	0	0		
	80	28.31642	28.31642	0.008056	5.658317		
	100	38.97005	41.23536	0.011732	8.23984		
	120	43.77214	46.88974	0.01334	9.369724		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.5.2: P_{app} Calculations Linctagon Forte® lowest conc AP-BL (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.096332	3.44E-06
	20	0	0	0	0		
	40	0	0	0	0		
	60	0	0	0	0		
	80	32.16438	32.16438	0.009151	6.427234		
	100	44.42713	47.00028	0.013372	9.391812		
	120	44.37989	47.93406	0.013637	9.578404		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.5.3: P_{app} Calculations Linctagon Forte® lowest conc AP-BL (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.086603	3.09E-06
	20	0	0	0	0		
	40	0	0	0	0		
	60	0	0	0	0		
	80	30.83522	30.83522	0.008773	6.161635		
	100	38.15279	40.61961	0.011556	8.116797		
	120	40.4897	43.54192	0.012388	8.700747		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.6 P_{app} Calculations Linctagon Forte® lowest conc BL-AP (Indinavir + Linctagon Forte®)

Table G.2.6.1: P_{app} Calculations Linctagon Forte® lowest conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.413604	1.48E-05
	20	23.82476	23.82476	0.010661	7.48799		
	40	48.20105	50.10703	0.022422	15.74836		
	60	71.63054	75.48662	0.033779	23.72503		
	80	102.4727	108.2031	0.048419	34.00765		
	100	120.4489	128.6467	0.057567	40.43294		
	120	146.7661	156.402	0.069987	49.15627		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.6.2: P_{app} Calculations Linctagon Forte® lowest conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.414873	1.48E-05
	20	32.72426	32.72426	0.014643	10.28505		
	40	46.98914	49.60708	0.022198	15.59123		
	60	73.9332	77.69233	0.034766	24.41827		
	80	105.4632	111.3779	0.049839	35.00544		
	100	127.4958	135.9328	0.060827	42.72294		
	120	146.8068	157.0065	0.070257	49.34627		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.6.3: P_{app} Calculations Linctagon Forte® lowest conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.430677	1.54E-05
	20	25.89587	25.89587	0.011588	8.138929		
	40	64.87762	66.94929	0.029958	21.04179		
	60	80.03666	85.22687	0.038137	26.78633		
	80	107.9084	114.3113	0.051152	35.92741		
	100	131.1907	139.8234	0.062568	43.94572		
	120	153.5549	164.0501	0.073409	51.56004		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.7 P_{app} Calculations Linctagon Forte[®] medium conc AP-BL (Indinavir + Linctagon Forte[®])

Table G.2.7.1: P_{app} Calculations Linctagon Forte[®] medium conc AP-BL (Indinavir + Linctagon Forte[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.099304	3.54E-06
	20	0	0	0	0		
	40	23.23974	23.23974	0.00601	4.220993		
	60	28.53213	30.39131	0.007859	5.51992		
	80	42.02222	44.30479	0.011457	8.047002		
	100	49.84908	53.21086	0.01376	9.664595		
	120	55.575	59.56293	0.015403	10.81831		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.7.2: P_{app} Calculations Linctagon Forte[®] medium conc AP-BL (Indinavir + Linctagon Forte[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.109193	3.9E-06
	20	0	0	0	0		
	40	21.8158	21.8158	0.005641	3.962366		
	60	45.00618	46.75144	0.01209	8.491383		
	80	46.92564	50.52613	0.013066	9.176974		
	100	52.82779	56.58184	0.014632	10.27686		
	120	60.70414	64.93036	0.016791	11.79319		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.7.3: P_{app} Calculations Linctagon Forte® medium conc AP-BL (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.091825	3.28E-06
	20	0	0	0	0		
	40	20.56826	20.56826	0.005319	3.735777		
	60	25.3826	27.02806	0.006989	4.909059		
	80	35.81628	37.84689	0.009787	6.874064		
	100	46.83107	49.69637	0.012851	9.026265		
	120	51.73569	55.48218	0.014347	10.07713		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.8 P_{app} Calculations Linctagon Forte® medium conc BL-AP (Indinavir + Linctagon Forte®)

Table G.2.8.1: P_{app} Calculations Linctagon Forte® medium conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.595446	2.13E-05
	20	22.5209	22.5209	0.012719	8.93369		
	40	53.77684	55.57851	0.03139	22.04713		
	60	86.68507	90.98722	0.051388	36.09321		
	80	109.7295	116.6643	0.06589	46.27891		
	100	141.7235	150.5019	0.085001	59.70175		
	120	163.1769	174.5148	0.098563	69.22731		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.8.2: P_{app} Calculations Linctagon Forte® medium conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.575796	2.05E-05
	20	29.46589	29.46589	0.016642	11.68866		
	40	53.02101	55.37828	0.031277	21.9677		
	60	78.73466	82.97634	0.046864	32.91542		
	80	111.7775	118.0763	0.066688	46.83903		
	100	139.7146	148.6568	0.083959	58.96982		
	120	159.4137	170.5908	0.096347	67.67073		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.8.3: P_{app} Calculations Linctagon Forte® medium conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.53559	1.91E-05
	20	26.80096	26.80096	0.015137	10.63152		
	40	51.26836	53.41244	0.030166	21.18788		
	60	73.74646	77.84793	0.043967	30.88106		
	80	107.6814	113.5811	0.064149	45.05585		
	100	130.903	139.5175	0.078797	55.34443		
	120	146.3582	156.8305	0.088575	62.2122		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.9 P_{app} Calculations Linctagon Forte[®] highest conc AP-BL (Indinavir + Linctagon Forte[®])

Table G.2.9.1: P_{app} Calculations Linctagon Forte[®] highest conc AP-BL (Indinavir + Linctagon Forte[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.095764	3.42E-06
	20	0	0	0	0		
	40	22.14948	22.14948	0.0047	3.30091		
	60	28.13445	29.90641	0.006346	4.456915		
	80	46.45749	48.70825	0.010335	7.25893		
	100	58.05892	61.77552	0.013108	9.206329		
	120	65.26804	69.91275	0.014834	10.41901		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.9.2: P_{app} Calculations Linctagon Forte[®] highest conc AP-BL (Indinavir + Linctagon Forte[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.058589	2.09E-06
	20	0	0	0	0		
	40	20.66137	20.66137	0.004384	3.079138		
	60	30.52895	32.18186	0.006828	4.796023		
	80	44.91498	47.3573	0.010048	7.057599		
	100	56.75654	60.54512	0.012847	9.022965		
	120	19.58279	24.12331	0.005119	3.595068		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.9.3: P_{app} Calculations Linctagon Forte® highest conc AP-BL (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.091007	3.25E-06
	20	0	0	0	0		
	40	21.94112	21.94112	0.004655	3.269858		
	60	32.92344	34.67873	0.007358	5.168128		
	80	46.52217	49.15605	0.01043	7.325665		
	100	55.81302	59.53479	0.012632	8.872397		
	120	60.76477	65.22981	0.013841	9.721118		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.10 P_{app} Calculations Linctagon Forte® high conc BL-AP (Indinavir + Linctagon Forte®)

Table G.2.10.1: P_{app} Calculations Linctagon Forte® high conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.275106	9.82E-06
	20	25.47846	25.47846	0.006082	4.271803		
	40	60.95113	62.98941	0.015036	10.56101		
	60	96.09933	100.9754	0.024104	16.92987		
	80	131.019	138.707	0.033111	23.25607		
	100	157.2107	167.6922	0.04003	28.11583		
	120	173.6625	186.2393	0.044458	31.2255		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.10.2: P_{app} Calculations Linctagon Forte® high conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.269371	9.61E-06
	20	27.82566	27.82566	0.006642	4.665342		
	40	58.7638	60.98985	0.014559	10.22576		
	60	92.94179	97.64289	0.023309	16.37113		
	80	129.7448	137.1801	0.032747	23.00007		
	100	153.8084	164.1879	0.039194	27.52829		
	120	171.2925	183.5972	0.043827	30.78251		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.10.3: P_{app} Calculations Linctagon Forte® high conc BL-AP (Indinavir + Linctagon Forte®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.265991	9.49E-06
	20	25.08987	25.08987	0.005989	4.206651		
	40	55.28008	57.28727	0.013675	9.604974		
	60	90.85912	95.28153	0.022745	15.97522		
	80	121.1289	128.3976	0.03065	21.52757		
	100	152.0003	161.6906	0.038598	27.10958		
	120	169.2087	181.3688	0.043295	30.40889		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.11 P_{app} Calculations Viral Choice[®] lowest conc AP-BL (Indinavir + Viral Choice[®])

Table G.2.11.1: P_{app} Calculations Viral Choice[®] lowest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.071916	2.57E-06
	20	0	0	0	0		
	40	0	0	0	0		
	60	17.5	17.5	0.004486	3.150693		
	80	29.3	30.7	0.007869	5.527216		
	100	32.5	34.844	0.008932	6.2733		
	120	38.5	41.1	0.010535	7.399628		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.11.2: P_{app} Calculations Viral Choice[®] lowest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.065008	2.32E-06
	20	0	0	0	0		
	40	0	0	0	0		
	60	15.7	15.7	0.004024	2.826622		
	80	28.6	29.856	0.007653	5.375263		
	100	30.3	32.588	0.008353	5.867131		
	120	33.3	35.724	0.009157	6.431735		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.11.3: P_{app} Calculations Viral Choice[®] lowest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.056886	2.03E-06
	20	0	0	0	0		
	40	0	0	0	0		
	60	21.6	21.6	0.005537	3.888856		
	80	27.2	28.928	0.007415	5.208186		
	100	26.5	28.676	0.007351	5.162816		
	120	28.1	30.22	0.007746	5.440797		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.12 P_{app} Calculations Viral Choice[®] lowest conc AP-BL (Indinavir + Viral Choice[®])

Table G.2.12.1: P_{app} Calculations Viral Choice[®] lowest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.254667	9.09E-06
	20	22.709	22.709	0.008147	5.722375		
	40	53.01558	54.8323	0.019672	13.81703		
	60	62.00115	66.2424	0.023766	16.69223		
	80	82.09888	87.05897	0.031234	21.93774		
	100	100.2725	106.8404	0.038331	26.92239		
	120	113.8002	121.822	0.043706	30.69757		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.12.2: P_{app} Calculations Viral Choice[®] lowest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.258168	9.21E-06
	20	27.19607	27.19607	0.009757	6.853058		
	40	42.1507	44.32639	0.015903	11.16968		
	60	55.59193	58.96399	0.021154	14.85816		
	80	81.61584	86.06319	0.030877	21.68681		
	100	98.86421	105.3935	0.037812	26.5578		
	120	117.2925	125.2016	0.044918	31.54919		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.12.3: P_{app} Calculations Viral Choice[®] lowest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.245814	8.77E-06
	20	25.20526	25.20526	0.009043	6.3514		
	40	39.28833	41.30475	0.014819	10.40826		
	60	55.2449	58.38797	0.020948	14.71301		
	80	78.84196	83.26155	0.029872	20.98084		
	100	92.03548	98.34284	0.035282	24.78112		
	120	111.9868	119.3497	0.042819	30.07457		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.13 P_{app} Calculations Viral Choice[®] medium conc AP-BL (Indinavir + Viral Choice[®])

Table G.2.12.1: P_{app} Calculations Viral Choice[®] medium conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.075918	2.71E-06
	20	0	0	0	0		
	40	21.22211	21.22211	0.004653	3.267882		
	60	30.43909	32.13686	0.007046	4.948587		
	80	39.32645	41.76158	0.009156	6.430647		
	100	43.30416	46.45028	0.010184	7.152635		
	120	50.75301	54.21734	0.011886	8.348645		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.12.2: P_{app} Calculations Viral Choice[®] medium conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.077267	2.76E-06
	20	0	0	0	0		
	40	19.95407	19.95407	0.004375	3.072623		
	60	33.42591	35.02224	0.007678	5.392891		
	80	40.43137	43.10544	0.00945	6.637581		
	100	45.55533	48.78984	0.010697	7.512892		
	120	49.77849	53.42292	0.011712	8.226315		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.12.3: P_{app} Calculations Viral Choice[®] medium conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.063772	2.28E-06
	20	0	0	0	0		
	40	20.34576	20.34576	0.004461	3.132937		
	60	29.85969	31.48735	0.006903	4.848572		
	80	36.37554	38.76432	0.008499	5.969114		
	100	37.17836	40.0884	0.008789	6.173003		
	120	41.46708	44.44135	0.009743	6.843291		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.14 P_{app} Calculations Viral choice[®] medium conc BL-AP (Indinavir + Viral Choice[®])

Table G.2.14.1: P_{app} Calculations Viral choice[®] medium conc BL-AP (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.253613	9.05E-06
	20	26.71871	26.71871	0.007503	5.269823		
	40	61.2279	63.3654	0.017794	12.49777		
	60	87.82613	92.72436	0.026038	18.28834		
	80	109.1475	116.1736	0.032623	22.91331		
	100	126.2106	134.9424	0.037894	26.61515		
	120	140.1769	150.2738	0.042199	29.63901		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.14.2: P_{app} Calculations Viral choice[®] medium conc BL-AP (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.244238	8.72E-06
	20	27.19011	27.19011	0.007635	5.362798		
	40	55.95016	58.12537	0.016322	11.46427		
	60	81.36806	85.84407	0.024106	16.93132		
	80	107.3596	113.869	0.031976	22.45878		
	100	121.485	130.0737	0.036526	25.65488		
	120	134.2642	143.983	0.040432	28.39826		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.14.3: P_{app} Calculations Viral choice[®] medium conc BL-AP (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.237268	8.47E-06
	20	23.62041	23.62041	0.006633	4.658734		
	40	48.16952	50.05915	0.014057	9.873338		
	60	63.7701	67.62366	0.01899	13.33765		
	80	85.88372	91.96372	0.025825	18.13832		
	100	100.9769	107.8476	0.030285	21.27115		
	120	146.3582	154.4364	0.043368	30.46001		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.15 P_{app} Calculations Viral Choice[®] highest conc AP-BL (Indinavir + Viral Choice[®])

Table G.2.15.1: P_{app} Calculations Viral Choice[®] highest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.07683	2.74E-06
	20	0	0	0	0		
	40	19.22077	19.22077	0.004154	2.917538		
	60	30.42227	31.95993	0.006907	4.851226		
	80	30.05371	32.48749	0.007021	4.931305		
	100	46.22829	48.63259	0.01051	7.381983		
	120	53.94081	57.63907	0.012457	8.749085		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.15.2: P_{app} Calculations Viral Choice[®] highest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.083459	2.98E-06
	20	0	0	0	0		
	40	18.21947	18.21947	0.003937	2.765549		
	60	35.26654	36.7241	0.007937	5.574383		
	80	37.77695	40.59827	0.008774	6.162447		
	100	48.39949	51.42165	0.011113	7.805337		
	120	57.022	60.89396	0.01316	9.243147		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.15.1: P_{app} Calculations Viral Choice[®] highest conc AP-BL (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.078548	2.8E-06
	20	0	0	0	0		
	40	19.84367	19.84367	0.004288	3.012088		
	60	32.55978	34.14727	0.00738	5.183245		
	80	43.48339	46.08817	0.00996	6.995764		
	100	46.43131	49.90998	0.010786	7.57588		
	120	50.85961	54.57411	0.011794	8.283853		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.16 P_{app} Calculations Viral Choice[®] highest conc BL-AP (Indinavir + Viral Choice[®])

Table G.2.216.1: P_{app} Calculations Viral Choice[®] highest conc BL-AP (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.22646	8.08E-06
	20	32.42379	32.42379	0.007973	5.600119		
	40	71.20107	73.79497	0.018147	12.7456		
	60	93.09063	98.78672	0.024292	17.06208		
	80	116.9054	124.3526	0.030579	21.47774		
	100	131.7691	141.1215	0.034703	24.37399		
	120	144.8915	155.4331	0.038222	26.84583		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.216.2: P_{app} Calculations Viral Choice[®] highest conc BL-AP (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.206218	7.36E-06
	20	30.85575	30.85575	0.007588	5.329293		
	40	59.42275	61.89121	0.015219	10.68962		
	60	87.64986	92.40368	0.022723	15.95963		
	80	78.72337	85.73536	0.021083	14.8079		
	100	125.3637	131.6616	0.032376	22.74011		
	120	137.6929	147.722	0.036326	25.51401		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.216.3: P_{app} Calculations Viral Choice[®] highest conc BL-AP (Indinavir + Viral Choice[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.182147	6.5E-06
	20	22.645	22.645	0.005569	3.911162		
	40	42.51518	44.32678	0.0109	7.65596		
	60	66.24806	69.64927	0.017127	12.02957		
	80	90.5164	95.81624	0.023562	16.54903		
	100	102.9127	110.1541	0.027088	19.02541		
	120	113.1234	121.3564	0.029842	20.96024		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.17 P_{app} Calculations Canova[®] lowest conc AP-BL (Indinavir + Canova[®])

Table G.2.17.1: P_{app} Calculations Canova[®] lowest conc AP-BL (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.073676	2.63E-06
	20	0	0	0	0		
	40	16.2	16.2	0.004067	2.856471		
	60	17.6	18.896	0.004744	3.331844		
	80	32.4	33.808	0.008487	5.961208		
	100	37.5	40.092	0.010065	7.069237		
	120	42.4	45.4	0.011397	8.005172		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.17.2: P_{app} Calculations Canova[®] lowest conc AP-BL (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.071974	2.57E-06
	20	0	0	0	0		
	40	18	18	0.004519	3.173857		
	60	18.5	19.94	0.005006	3.515928		
	80	30.5	31.98	0.008028	5.638886		
	100	35.1	37.54	0.009424	6.619255		
	120	43.7	46.508	0.011676	8.200541		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.17.3: P_{app} Calculations Canova® lowest conc AP-BL (Indinavir + Canova®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.072431	2.58E-06
	20	0	0	0	0		
	40	16.5	16.5	0.004142	2.909369		
	60	17.4	18.72	0.0047	3.300811		
	80	29.9	31.292	0.007856	5.517574		
	100	36.3	38.692	0.009713	6.822382		
	120	43.05	45.954	0.011537	8.102856		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.18 P_{app} Calculations Canova® lowest conc BL-AP (Indinavir + Canova®)

Table G.2.18.1: P_{app} Calculations Canova® lowest conc BL-AP (Indinavir + Canova®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.156233	5.58E-06
	20	16.26783	16.26783	0.004062	2.852975		
	40	34.79714	36.09857	0.009014	6.330795		
	60	58.5866	61.37037	0.015324	10.76284		
	80	77.26443	81.95136	0.020463	14.37224		
	100	78.0003	84.18145	0.021019	14.76334		
	120	99.49158	105.7316	0.0264	18.54271		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.18.2: P_{app} Calculations Canova® lowest conc BL-AP (Indinavir + Canova®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.14978	5.35E-06
	20	29.5594	29.5594	0.007381	5.183987		
	40	35.45055	37.8153	0.009442	6.631868		
	60	46.2527	49.08874	0.012257	8.608951		
	80	71.32678	75.027	0.018734	13.15788		
	100	81.8064	87.51254	0.021851	15.34753		
	120	101.8396	108.3841	0.027063	19.00788		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.18.3: P_{app} Calculations Canova® lowest conc BL-AP (Indinavir + Canova®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.153583	5.48E-06
	20	23.69262	23.69262	0.005916	4.155099		
	40	31.67142	33.56683	0.008381	5.886792		
	60	52.53985	55.07356	0.013751	9.658541		
	80	74.29561	78.4988	0.019601	13.76675		
	100	79.90335	85.847	0.021435	15.05544		
	120	100.6656	107.0579	0.026732	18.7753		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.19 P_{app} Calculations Canova[®] medium conc AP-BL (Indinavir + Canova[®])

Table G.2.19.1: P_{app} Calculations Canova[®] medium conc AP-BL (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.118164	4.22E-06
	20	49.78371	49.78371	0.008567	6.017138		
	40	79.6013	83.584	0.014383	10.10243		
	60	88.20818	94.57628	0.016275	11.43102		
	80	89.56463	96.62128	0.016627	11.67819		
	100	116.2841	123.4493	0.021244	14.92077		
	120	119.736	129.0387	0.022205	15.59635		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.19.2: P_{app} Calculations Canova[®] medium conc AP-BL (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.107295	3.83E-06
	20	68.70562	68.70562	0.011823	8.304146		
	40	77.25321	82.74966	0.01424	10.00159		
	60	84.11371	90.29397	0.015538	10.91343		
	80	96.70477	103.4339	0.017799	12.5016		
	100	105.9496	113.686	0.019563	13.74073		
	120	120.3511	128.8271	0.022169	15.57076		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.19.3: P_{app} Calculations Canova[®] medium conc AP-BL (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.102552	3.66E-06
	20	67.06209	67.06209	0.01154	8.1055		
	40	64.50522	69.87019	0.012024	8.444902		
	60	79.59742	84.75784	0.014585	10.24431		
	80	87.27142	94.05205	0.016185	11.36766		
	100	94.19501	101.1767	0.017411	12.22879		
	120	120.0436	127.5792	0.021954	15.41994		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.20 P_{app} Calculations Canova[®] medium conc BL-AP (Indinavir + Canova[®])

Table G.2.20.1: P_{app} Calculations Canova[®] medium conc BL-AP (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.200959	7.17E-06
	20	71.8595	71.8595	0.013369	9.389789		
	40	94.78262	100.5314	0.018703	13.13631		
	60	116.7524	124.335	0.023131	16.2467		
	80	147.3051	156.6453	0.029142	20.46864		
	100	169.5388	181.3232	0.033734	23.69327		
	120	181.8357	195.3988	0.036352	25.53251		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.20.2: P_{app} Calculations Canova[®] medium conc BL-AP (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.218569	7.8E-06
	20	62.84116	62.84116	0.011691	8.211374		
	40	115.1286	120.1559	0.022354	15.70062		
	60	118.2322	127.8447	0.023784	16.7053		
	80	148.4351	157.8936	0.029375	20.63176		
	100	189.5112	201.386	0.037466	26.31485		
	120	192.1335	207.2944	0.038565	27.0869		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.20.3: P_{app} Calculations Canova[®] medium conc BL-AP (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.180372	6.44E-06
	20	69.95936	69.95936	0.013015	9.1415		
	40	89.36466	94.96141	0.017667	12.40849		
	60	119.8312	126.9803	0.023624	16.59236		
	80	133.2576	142.8441	0.026575	18.66525		
	100	144.4555	155.1161	0.028858	20.26882		
	120	173.3817	184.9381	0.034406	24.16563		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.21 P_{app} Calculations Canova[®] highest conc AP-BL (Indinavir + Canova[®])

Table G.2.21.1: P_{app} Calculations Canova[®] highest conc AP-BL (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.121925	4.35E-06
	20	62.79243	62.79243	0.012255	8.607578		
	40	77.95115	82.97454	0.016194	11.37414		
	60	116.3977	122.6338	0.023934	16.81062		
	80	101.6035	110.9153	0.021647	15.20426		
	100	103.8667	111.995	0.021858	15.35226		
	120	115.6055	123.9148	0.024184	16.98623		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.21.2: P_{app} Calculations Canova[®] highest conc AP-BL (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.140006	5E-06
	20	22.66899	22.66899	0.004424	3.107462		
	40	68.24313	70.05665	0.013673	9.603357		
	60	74.71603	80.17548	0.015648	10.99044		
	80	93.33761	99.31489	0.019383	13.61407		
	100	108.8728	116.3398	0.022706	15.94785		
	120	109.7417	118.4515	0.023118	16.23732		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.21.3: P_{app} Calculations Canova[®] highest conc AP-BL (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.121793	4.35E-06
	20	69.03348	69.03348	0.013473	9.463101		
	40	72.50394	78.02662	0.015228	10.69588		
	60	83.3131	89.11342	0.017392	12.21566		
	80	105.4492	112.1142	0.021881	15.36861		
	100	110.5724	119.0083	0.023227	16.31364		
	120	112.3252	121.171	0.023649	16.61011		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

2.22 P_{app} Calculations Canova[®] highest conc BL-AP (Indinavir + Canova[®])

Table G.2.22.1: P_{app} Calculations Canova[®] highest conc BL-AP (Indinavir + Canova[®])

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.21318	7.61E-06
	20	75.93089	75.93089	0.014467	10.16123		
	40	108.5492	114.6237	0.021839	15.33918		
	60	125.8596	134.5435	0.025635	18.0049		
	80	143.0802	153.149	0.02918	20.49472		
	100	187.6995	199.146	0.037943	26.65013		
	120	187.3606	202.3765	0.038559	27.08245		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.22.2: P_{app} Calculations Canova® highest conc BL-AP (Indinavir + Canova®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.214474	7.65E-06
	20	58.93957	58.93957	0.01123	7.887416		
	40	92.09094	96.80611	0.018445	12.9548		
	60	120.5644	127.9316	0.024375	17.12008		
	80	145.7767	155.4218	0.029613	20.79888		
	100	174.1258	185.7879	0.035398	24.86253		
	120	181.1325	195.0625	0.037165	26.10367		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

Table G.2.22.3: P_{app} Calculations Canova® highest conc BL-AP (Indinavir + Canova®)

	Time	Reading	Correction	Conc (mg/ml)	%	Slope	Papp
	0	0	0	0	0	0.210792	7.52E-06
	20	66.40228	66.40228	0.012652	8.886092		
	40	85.72062	91.0328	0.017345	12.1822		
	60	119.2543	126.1119	0.024028	16.87656		
	80	145.9002	155.4405	0.029616	20.80137		
	100	180.4602	192.1322	0.036607	25.71154		
	120	174.3043	188.7411	0.035961	25.25773		
			C0			1/A.C0.60	
Factor for correction			0.142376			3.57E-05	
0.08							

3. Metabolism Results

Table G.3.1: M6/Indinavir Ratios

	M6/Indinavir ratio	Average	Standard Deviation
Indinavir	0.006278	0.010103	0.003413243
	0.011193		
	0.012839		
Indinavir + Ketoconazole	0.006523	0.005658	0.000837596
	0.005601		
	0.00485		
Linctagon Forte® Lowest conc	0.000912	0.003042	0.003706107
	0.000893		
	0.007322		
Linctagon Forte® Medium conc	0.001341	0.000992	0.000315027
	0.000729		
	0.000906		
Linctagon Forte® Highest conc	0.000496	0.000623	0.00017208
	0.000819		
	0.000555		
Viral Choice® Lowest conc	0.000948	0.00071	0.000214846
	0.000529		
	0.000654		
Viral Choice® Medium conc	0.001622	0.001674	4.82067E-05
	0.001683		
	0.001718		
Viral Choice® Highest conc	0.0033	0.003187	0.000258812
	0.003371		
	0.002891		
Canova® Lowest conc	0.000932	0.0008	0.000121558
	0.000776		
	0.000692		
Canova® Medium conc	0.000881	0.000786	8.83718E-05
	0.000773		
	0.000705		
Canova® Highest conc	0.000949	0.000852	0.000116394
	0.000884		
	0.000723		

APPENDIX H

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To	Carlemi Calitz
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