Investigation of the pharmacokinetic interactions between *Hypoxis hemerocallidea* and indinavir

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It has been established that phytochemicals in herbal medicines can result in pharmacokinetic interactions when co-administrated with allopathic drugs. These herb-drug pharmacokinetic interactions may lead to an increase or decrease in drug bioavailability with consequences of adverse effects or toxicity due to increased drug plasma levels or treatment failure due to decreased plasma drug levels. The relevance of research concerning pharmacokinetic interactions between herbal medicines and anti-retroviral drugs is reflected by the fact that a relatively large portion of human immunodeficiency virus (HIV) infected patients with acquired immunodeficiency syndrome (AIDS) commonly use herbal medicines to complement their highly active anti-retroviral therapy. The aim of this study was to confirm pharmacokinetic interactions that different *Hypoxis hemerocallidea* (African potato) materials may have on indinavir by means of *in vitro* transport studies as well as by means of acute and chronic *in vivo* bioavailability studies.

The selected *H. hemerocallidea* test materials included a dried plant reference material, an aqueous extract and a solid oral commercial product. Bi-directional transport of indinavir across Caco-2 cell monolayers were determined in the absence and presence of the various *H. hemerocallidea* materials. Indinavir alone served as the negative control and the positive control consisted of verapamil, a known P-glycoprotein (P-gp) inhibitor. The transport samples obtained were analysed by a validated high performance liquid chromatography (HPLC) method. The apparent permeability coefficient ($P_{app}$) values in both transport directions and efflux ratio (ER) values were calculated.

*In vivo* studies were conducted in male Sprague-Dawley rats which were randomly selected and divided into 9 groups. The negative control consisted of indinavir alone. Verapamil served as the positive control for efflux inhibition and ketoconazole, a known CYP3A4 inhibitor, was used as positive control for metabolism inhibition. The *H. hemerocallidea* materials in combination with indinavir formed the experimental groups. The acute study consisted of a single administration and the chronic study entailed daily administrations over 14 days by means of oral gavage. A validated Liquid Chromatography tandem Mass Spectrometry (LC/MS/MS) method for the analysis of indinavir was used in order to analyse the plasma samples. Relevant pharmacokinetic parameters such as peak plasma concentration ($C_{max}$) and area under the curve (AUC) were determined.

The *H. hemerocallidea* test materials demonstrated an inhibition of efflux of indinavir in the Caco-2 cell model. In agreement with this finding and other published findings on metabolism inhibition, an increase in indinavir bioavailability in the presence of the selected test materials
was shown *in vivo* for both the acute and chronic studies. The commercial product had a similar increasing effect on indinavir bioavailability as the aqueous extract, while the reference plant material exhibited a higher effect on indinavir bioavailability enhancement. A higher effect on indinavir bioavailability was observed for two of the test materials during the chronic study when compared to the acute study.

The selected *H. hemerocalilidea* materials interfered with indinavir pharmacokinetics in both the *in vitro* and *in vivo* models and these effects may be attributed to inhibition of efflux transporters and enzymatic metabolism.

**Key words:** Herb-drug pharmacokinetic interactions, HIV, *Hypoxis hemerocalilidea*, indinavir, Caco-2, efflux, Sprague-Dawley
UITTREKSEL

Daar is vasgestel dat fitochemikalieë in kruiemedisyne kan lei tot farmakokinetiese interaksies wanneer dit saam met allopatiese medisyne geadministreer word. Hierdie plant-geneesmiddel farmakokinetiese interaksie kan lei tot 'n toename of afname in die geneesmiddel se biobesikbaarheid. Dit kan lei tot negatiewe gevolge of toksisiteit as gevolg van verhoogde geneesmiddelplasmavlakke of mislukte behandeling as gevolg van verminderde geneesmiddelplasmavlakke. Die noodsaaklikheid van navorsing met betrekking tot farmakokinetiese interaksies tussen kruiemedisyne en anti-retrovirale middels word weerspieël deur die feit dat 'n relatiewe groot deel van pasiënte wat besmet is met menslike immunititeitseveningsvirus (MIV) met verworwe immunititeitseveningsindroom (VIGS), gebruik kruiemedisyne algemeen saam met hulle hoogs aktiewe anti-retrovirale terapie. Die doel van hierdie studie is om die farmakokineteties interaksies te bevestig wat verskillende *Hypoxis hemerocallidea* (Afrika-aartappel) materiale op indinavir mag hê deur middel van in vitro transport studies sowel as deur middel van akute en chroniese *in vivo* biobesikbaarheid studies.

Die gekose *H. hemerocallidea* toetsmateriale bestaan uit 'n droë verwysingsplantmateriaal, 'n water-ekstrak en 'n solide orale kommersiële produk. Tweerigting beweging van indinavir oor Caco-2 selmonolae is bepaal in die afwesigheid en teenwoordigheid van die verskillende *H. hemerocallidea* materiale. Indinavir alleen dien as die negatiewe kontrole, terwyl die positiewe kontrole bestaan uit verapamil, wat 'n bekende P-glikoproteïen (P-gp) inhibeerder is. Die ingesamelde monsters is geanalyser deur middel van 'n gevaludeerde hoëdruk vloeistofchromatografiese (HDVC) metode. Die oënskynlike deurlaatbaarheidskoëffisiënt (P_{app}) waardes in beide vervoer rigtings en effluxverhouding (EV) waardes is bereken.

Die *in vivo* studies is in manlike Sprague-Dawley rotte uitgevoer wat lukraak gekies is en in 9 groepe verdeel is. Die negatiewe kontrole bestaan uit indinavir alleen. Verapamil dien as die positiewe kontrole vir efluxs inhibisie en ketokonasool, 'n bekende CYP3A4 inhibeerder, is gebruik as die positiewe kontrole vir metaboliëse inhibisie. Die *H. hemerocallidea* materiale in kombinasie met indinavir vorm die eksperimentele groepe. Die akute studie het bestaan uit 'n enkele toediening en die chroniese studie het daaglikske toedienings behels oor 14 dae by wyse van mondelinge intubering. 'n Gevaludeerde vloeistofchromatografie gekoppel aan 'n massaspektrometer (VC/MS/MS) metode vir die analise van indinavir is gebruik om die plasma monsters te ontleed. Relevante farmakokinetiese parameters soos piek plasmakonsentrasie ($C_{max}$) en area onder die kurwe (AOK) is vasgestel.
Die *H. hemerocallidea* toetsmateriale het efluks inhibisie van indinavir in die Caco-2 sel model gedemonstreer. In ooreenstemming met hierdie bevinding en ander gepubliseerde bevindinge op metaboliese inhibisie, het ’n toename in indinavir biobeskikbaarheid in die teenwoordigheid van die gekose toetsmateriale *in vivo* voorgekom vir beide die akute en chroniese studies. Die kommersiële produk het ’n soortgelyke toenemende effek op indinavir biobeskikbaarheid as die water-ekstrak gehad, terwyl die verwysing plantmateriaal ’n hoër uitwerking op indinavir biobeskikbaarheid verbetering getoon het. ’n Hoër uitwerking op indinavir biobeskikbaarheid was waargeneem vir twee van die toetsmateriale tydens die chroniese studie in vergelyking met die akute studie.

Die gekose *H. hemerocallidea* materiale het met die farmakokinetika van indinavir ingemeng in beide die *in vitro* en *in vivo* modelle en hierdie effekte kan aan inhibisie van efluks transporters en ensiematiese metabolisme toegeskryf word.

**Sleutel woorde:** Kruie-geneesmiddel farmakokinetiese interaksies, MIV, *Hypoxis hemerocallidea*, indinavir, Caco-2, efluks, Sprague-Dawley
CONGRESS PROCEEDINGS & ARTICLES

1.1 Congress proceedings

Pharmacokinetic interactions (in vitro and in vivo) between indinavir and Hypoxis hemerocallidea: comparing a commercial product with a crude extract and a dried plant material. Presented at the 37th Conference of the Academy of Pharmaceutical Sciences held from 5-8 October 2016 at Misty Hills Hotel and Conference Centre in Muldersdrift, Gauteng. The Congress was hosted by the Department of Pharmaceutical Sciences from the Tshwane University of Technology (on behalf of the Academy of Pharmaceutical Sciences South Africa) and the Department of Pharmacology and Therapeutics at the Sefako Makgatho Health Sciences University (on behalf of the South African Society for Basic and Clinical Pharmacology). (See Appendix A)

1.2 Articles

# TABLE OF CONTENTS

ACKNOWLEDGEMENTS........................................................................................................i

ABSTRACT..........................................................................................................................ii

UITTREKSEL....................................................................................................................iv

CONGRESS PROCEEDINGS & ARTICLES...........................................................................vi

1.1 Congress proceedings.........................................................................................vi

1.2 Articles...................................................................................................................vi

TABLE OF CONTENTS..................................................................................................vii

LIST OF TABLES..............................................................................................................xii

LIST OF FIGURES...........................................................................................................xvii

CHAPTER 1 INTRODUCTION..............................................................................................1

1.1 BACKGROUND..........................................................................................................1

1.1.1 Herb-drug pharmacokinetic interactions.........................................................1

1.1.2 African potato (Hypoxis hemerocallidea).........................................................2

1.1.3 Indinavir (Crixivan®).......................................................................................3

1.1.4 In vitro, ex vivo and in vivo models.................................................................4

1.1.4.1 In vitro cell cultures.....................................................................................4

1.1.4.2 Ex vivo (excised animal tissues)..................................................................5

1.1.4.3 In vivo.........................................................................................................5

1.2 RESEARCH PROBLEM............................................................................................5

1.3 AIM AND OBJECTIVES..........................................................................................5
1.4 STRUCTURE OF DISSERTATION ..................................................................6

CHAPTER 2 LITERATURE REVIEW ON THE PHARMACOKINETIC INTERACTIONS
BETWEEN HERBAL MEDICINES AND ANTI-RETROVIRAL DRUGS ......................................................7

2.1 INTRODUCTION ..................................................................................7

2.2 PHARMACOKINETIC HERB-DRUG INTERACTIONS .................................8

2.2.1 Efflux transporters .........................................................................9

2.2.1.1 P-gp .......................................................................................10

2.2.1.2 P-gp modulation ...................................................................12

2.2.1.3 Inhibition and induction of P-gp ............................................14

2.2.2 Interactions involving metabolism ..................................................15

2.2.2.1 Cytochrome P450 (CYP) superfamily of enzymes ...............16

2.2.2.2 Factors influencing cytochrome P450 enzyme expression and function .............................................17

2.2.2.3 Pre-systemic metabolism/first-pass effect .........................20

2.2.2.4 Inhibition and induction of metabolic enzymes .....................21

2.2.2.5 Herb-drug pharmacokinetic interactions involving anti-retroviral drugs ...............................................23

2.3 HYPOXIS HEMEROCALLIDEA (AFRICAN POTATO) ..............................24

2.3.1 Botany and uses ...........................................................................24

2.3.2 Biological activity ..........................................................................25

2.3.3 Phytochemistry ............................................................................26

2.3.4 Interactions ..................................................................................27

2.4 MODELS TO EVALUATE PHARMACOKINETIC INTERACTIONS ...........28

2.4.1 In vitro study models .....................................................................29
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.4.1.1 Caco-2 cell line</td>
<td>30</td>
</tr>
<tr>
<td>2.4.1.2 LS180 cell line</td>
<td>32</td>
</tr>
<tr>
<td>2.4.2 In vivo models</td>
<td>32</td>
</tr>
<tr>
<td>2.4.2.1 The rat model</td>
<td>33</td>
</tr>
<tr>
<td>2.4.2.1.1 Herb-drug interaction studies involving the rat model</td>
<td>34</td>
</tr>
<tr>
<td>2.5 SUMMARY</td>
<td>35</td>
</tr>
<tr>
<td>CHAPTER 3 METHODS AND MATERIALS</td>
<td>36</td>
</tr>
<tr>
<td>3.1 INTRODUCTION</td>
<td>36</td>
</tr>
<tr>
<td>3.2 MATERIALS</td>
<td>36</td>
</tr>
<tr>
<td>3.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYTICAL METHOD FOR</td>
<td>37</td>
</tr>
<tr>
<td>INDINAVIR SAMPLES FROM THE IN VITRO TRANSPORT STUDIES</td>
<td></td>
</tr>
<tr>
<td>3.3.1 Chromatographic conditions</td>
<td>37</td>
</tr>
<tr>
<td>3.3.2 Linearity</td>
<td>37</td>
</tr>
<tr>
<td>3.3.3 Accuracy</td>
<td>38</td>
</tr>
<tr>
<td>3.3.4 Precision</td>
<td>38</td>
</tr>
<tr>
<td>3.3.5 Ruggedness</td>
<td>39</td>
</tr>
<tr>
<td>3.4 LIQUID CHROMATOGRAPHY - TANDEM MASS SPECTROMETRY ANALYTICAL</td>
<td>39</td>
</tr>
<tr>
<td>METHOD FOR INDINAVIR SAMPLES FROM THE IN VIVO STUDY</td>
<td></td>
</tr>
<tr>
<td>3.4.1 Chromatography</td>
<td>39</td>
</tr>
<tr>
<td>3.4.2 Detection</td>
<td>40</td>
</tr>
<tr>
<td>3.4.3 Extraction from plasma</td>
<td>41</td>
</tr>
<tr>
<td>3.5 LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY ANALYTICAL METHOD FOR</td>
<td>41</td>
</tr>
<tr>
<td>PHYTOCHEMICAL CHARACTERISATION OF THE HYPOXIS HEMEROCALLIDEA MATERIALS</td>
<td></td>
</tr>
</tbody>
</table>
3.6 PREPARATION OF SOLUTIONS FOR THE IN VITRO TRANSPORT STUDY……..41

3.6.1 Preparation of the indinavir solution (negative control group)……………………41

3.6.2 Preparation of verapamil solution (positive control group)……………………42

3.6.3 Preparation of the test solutions for the transport studies……………………42

3.7 TRANSPORT STUDIES…………………………………………………………42

3.7.1 Caco-2 cell culturing…………………………………………………………42

3.7.2 Sub-culturing of the Caco-2 cells…………………………………………………43

3.7.3 Seeding of Caco-2 cells…………………………………………………………43

3.7.4 Bi-directional transport studies…………………………………………………44

3.7.4.1 Transport in the apical-to-basolateral direction……………………………44

3.7.4.2 Transport in the basolateral-to-apical direction……………………………45

3.8 IN VIVO PHARMACOKINETIC STUDY………………………………………………45

3.8.1 Animal selection and study design………………………………………………45

3.8.2 Administration of test solutions to rats…………………………………………46

3.8.3 Blood sampling…………………………………………………………………..47

3.9 DATA ANALYSIS………………………………………………………………….47

3.9.1 Transport data for indinavir……………………………………………………47

3.9.2 Pharmacokinetic data analysis for in vivo study model………………………47

3.9.3. Statistical Data Analysis………………………………………………………48

CHAPTER 4 RESULTS AND DISCUSSION………………………………………….49

4.1 INTRODUCTION……………………………………………………………………49

4.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYTICAL METHOD………49

4.2.1 Linearity……………………………………………………………………………49
4.2.2 Accuracy........................................................................................................50
4.2.3 Precision.......................................................................................................51
4.2.4 Ruggedness..................................................................................................52

4.3 LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY ANALYTICAL METHOD FOR
PHYTOCHEMICAL CHARACTERISATION OF THE HYPOXIS HEMEROCALLIDEA
MATERIALS..............................................................................................................52

4.4 BI-DIRECTIONAL TRANSPORT STUDIES......................................................57

4.5 IN VIVO PHARMACOKINETIC STUDIES......................................................63

4.6 CONCLUSIONS...............................................................................................66

CHAPTER 5 FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS ..........68

5.1 INTRODUCTION................................................................................................68

5.2 FINAL CONCLUSIONS....................................................................................69

5.3 FUTURE RECOMMENDATIONS......................................................................70

REFERENCES........................................................................................................71

APPENDIX A........................................................................................................80

APPENDIX B........................................................................................................89

APPENDIX C........................................................................................................94

APPENDIX D........................................................................................................97

APPENDIX E........................................................................................................132
LIST OF TABLES

Table 2.1: Examples of different drug classes that are P-gp substrates, inhibitors or inducers (Adapted from, Calitz, 2014; Hansten, 2001; Haritova, 2008; Hu et al., 2005; König et al., 2013; Mercer & Coop, 2011) .................................................................12

Table 2.2: Enzyme inhibition and induction of herbal medicines (Adapted from Liu et al., 2011) ........................................................................................................................................................................23

Table 2.3: Commonly used models to study metabolism and transport of drugs (Adapted from Alqahtani et al., 2013) ........................................................................................................................................................................29

Table 3.1: Ionisation source setting ........................................................................................................................................................................................................40

Table 3.2: MS/MS detector setting ........................................................................................................................................................................................................40

Table 4.1: Data obtained for accuracy of indinavir analysis with high performance liquid chromatography ........................................................................................................................................................................50

Table 4.2: Percentage recovery (%) obtained for inter-day precision of indinavir analysis by high performance liquid chromatography ........................................................................................................................................................................51

Table 4.3: Statistical analysis of data for inter-day precision ........................................................................................................................................................................................................52

Table 4.4: Quantity of hypoxoside in each of the selected Hypoxis hemerocallidea material ........................................................................................................................................................................................................57

Table 4.5: TEER values of Caco-2 cell monolayers taken at the beginning (0 min) and end (120 min) of the bi-directional transport study of indinavir in the presence of the selected H. hemerocallidea materials ........................................................................................................................................................................................................58

Table 4.6: Efflux ratio (ER) values for indinavir in the absence (negative control) and presence of the selected Hypoxis hemerocallidea materials as well as verapamil (positive control) ........................................................................................................................................................................................................62

Table 4.7: Biopharmaceutical parameters for indinavir administrated to rats in the absence and presence of the selected H. hemerocallidea materials ........................................................................................................................................................................................................65

Table D 1: TEER Negative control Indinavir AP-BL ........................................................................................................................................................................................................99

Table D 2: TEER Negative control Indinavir BL-AP ........................................................................................................................................................................................................99

Table D 3: TEER Positive Control Indinavir & Verapamil AP-BL ........................................................................................................................................................................................................99
Table D 4: TEER Positive Control Indinavir & Verapamil BL-AP ...............................................100
Table D 5: TEER Indinavir & Hypoxis hemerocallidea aqueous extract AP-BL ......................100
Table D 6: TEER Indinavir & Hypoxis hemerocallidea aqueous extract BL-AP ......................100
Table D 7: TEER Indinavir & Hypoxis hemerocallidea commercial product AP-BL ..............101
Table D 8: TEER Indinavir & Hypoxis hemerocallidea commercial product BL-AP ..............101
Table D 9: TEER Indinavir & Hypoxis hemerocallidea reference plant material AP-BL ..........101
Table D 10: TEER Indinavir & Hypoxis hemerocallidea reference plant material BL-AP .......102
Table D 11: Concentration and percentage transport for each sample of indinavir alone (AP-BL) over the pre-determined time intervals. n = 3 .................................................................103
Table D 12: $P_{app}$ values for each sample of indinavir alone (AP-BL) ........................................103
Table D 13: Average transport and standard deviation for each sample (indinavir alone AP-BL) over the pre-determined time intervals. n = 3 .................................................................104
Table D 14: Concentration and percentage transport for each sample of indinavir alone (BL-AP) over the pre-determined time intervals. n = 3 .................................................................105
Table D 15: $P_{app}$ values for each sample of indinavir alone (BL-AP) ........................................105
Table D 16: Average transport and standard deviation for each sample (indinavir alone BL-AP) over the pre-determined time intervals. n = 3 .................................................................106
Table D 17: Concentration and percentage transport for each sample of indinavir with verapamil (AP-BL) over the pre-determined time intervals. n = 3 .................................................................107
Table D 18: $P_{app}$ values for each sample of indinavir with verapamil (AP-BL) ......................107
Table D 19: Average transport and standard deviation for each sample (indinavir with verapamil AP-BL) over the pre-determined time intervals. n = 3 .................................................................108
Table D 20: Concentration and percentage transport for each sample of indinavir with verapamil (BL-AP) over the pre-determined time intervals. n = 3 .................................................................109
Table D 21: $P_{app}$ values for each sample of indinavir with verapamil (BL-AP) ......................109
Table D 22: Average transport and standard deviation for each sample (indinavir with verapamil BL-AP) over the pre-determined time intervals. n = 3……………………………………110

Table D 23: Concentration and percentage transport for each sample of indinavir with H. hemerocallidea aqueous extract (AP-BL) over the pre-determined time intervals. n = 3……………………………………………………………………………………..111

Table D 24: P_{app} values for each sample of indinavir with H. hemerocallidea aqueous extract (AP-BL)………………………………………………………………………………..111

Table D 25: Average transport and standard deviation for each sample (indinavir with H. hemerocallidea aqueous extract AP-BL) over the pre-determined time intervals. n = 3……………………………………………………………………………………………112

Table D 26: Concentration and percentage transport for each sample of indinavir with H. hemerocallidea aqueous extract (BL-AP) over the pre-determined time intervals. n = 3…………………………………………………………………………………………………113

Table D 27: P_{app} values for each sample of indinavir with H. hemerocallidea aqueous extract (BL-AP)………………………………………………………………………………..113

Table D 28: Average transport and standard deviation for each sample (indinavir with H. hemerocallidea aqueous extract BL-AP) over the pre-determined time intervals. n = 3……………………………………………………………………………………………114

Table D 29: Concentration and percentage transport for each sample of indinavir with H. hemerocallidea commercial product (AP-BL) over the pre-determined time intervals. n = 3…………………………………………………………………………………………………115

Table D 30: P_{app} values for each sample of indinavir with H. hemerocallidea commercial product (AP-BL)………………………………………………………………………………..115

Table D 31: Average transport and standard deviation for each sample (indinavir with H. hemerocallidea commercial product AP-BL) over the pre-determined time intervals. n = 3……………………………………………………………………………………………116

Table D 32: Concentration and percentage transport for each sample of indinavir with H. hemerocallidea commercial product (BL-AP) over the pre-determined time intervals. n = 3…………………………………………………………………………………………………117
Table D 33: \( P_{app} \) values for each sample of indinavir with *H. hemerocallidea* commercial product (BL-AP)...........................................................................................................117

Table D 34: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* commercial product BL-AP) over the pre-determined time intervals. \( n = 3 \)........................................................................................................................................118

Table D 35: Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* reference plant material (AP-BL) over the pre-determined time intervals. \( n = 3 \)........................................................................................................................................119

Table D 36: \( P_{app} \) values for each sample of indinavir with *H. hemerocallidea* reference plant material (AP-BL)...........................................................................................................119

Table D 37: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* reference plant material AP-BL) over the pre-determined time intervals. \( n = 3 \)........................................................................................................................................120

Table D 38: Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* reference plant material (BL-AP) over the pre-determined time intervals. \( n = 3 \)........................................................................................................................................121

Table D 39: \( P_{app} \) values for each sample of indinavir with *H. hemerocallidea* reference plant material (BL-AP)...........................................................................................................121

Table D 40: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* reference plant material BL-AP) over the pre-determined time intervals. \( n = 3 \)........................................................................................................................................122

Table D 41: Weight of rats used in the *in vivo* study......................................................................................................................123

Table D 42: Indinavir concentration for each rat (indinavir alone). \( n = 5 \)..................................................................................124

Table D 43: Indinavir concentration for each rat (indinavir with ketoconazole). \( n = 4 \).................................................125

Table D 44: Indinavir concentration for each rat (indinavir with verapamil). \( n = 5 \).................................................126

Table D 45: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* aqueous extract, acute). \( n = 5 \).........................................................................................................................127
Table D 46: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* aqueous extract, chronic). n = 5.

Table D 47: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* commercial product, acute). n = 5.

Table D 48: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* commercial product, chronic). n = 5.

Table D 49: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* reference plant material, acute). n = 5.

Table D 50: Indinavir concentration for each rat (indinavir with *H. hemerocallidea* reference plant material, chronic). n = 5.
LIST OF FIGURES

Figure 2.1: Schematic demonstration of the processes of pharmacokinetics and pharmacodynamics that may be involved in herb-drug interactions A: Absorption, D: Distribution, M: Metabolism, E: Elimination (Bounda & Feng, 2015)………………………….8

Figure 2.2: A schematic illustration of the structure of P-gp (Bansal et al., 2009)…………………11

Figure 2.3: Schematic illustration of the P-gp efflux mechanism (Gohel et al., 2011)……………..11

Figure 2.4: Schematic illustration of the effect of inhibition of P-gp on drug absorption (Hansten, 2001)…………………………………………………………………………………………14

Figure 2.5: Schematic illustration of the catalytic cycle of Cytochrome P450 (Singh, 2007)………………………………………………………………………………………………17

Figure 2.6: Fraction of clinical drugs metabolised by CYP450 isoforms and the factors influencing variability (Zanger & Schwab, 2013)………………………………………18

Figure 2.7: Schematic illustration of pre-systemic metabolism (Adapted from Dickens & Van de Waterbeemd, 2004)……………………………………………………………………20

Figure 2.8: Schematic illustration of cytochrome P450 inhibition and induction (Mukherjee et al., 2011)……………………………………………………………………………………22

Figure 2.9: Photograph of the African potato (Hypoxis hemerocallidea) plant showing its characteristic yellow flower (Germishuizen et al., 2003)……………………………..25

Figure 2.10: Chemical structures of hypoxoside, rooperol and β-sitosterol (Adapted from Owira & Ojewole, 2009)……………………………………………………………………27

Figure 2.11: Schematic illustration of a Caco-2 cell monolayer on a membrane in a Transwell plate (Li, 2001)………………………………………………………………………………31

Figure 3.1: Schematic illustration of the layout of the in vivo study design in Sprague-Dawley rats. Dosing concentrations were selected based on previous studies (Van Wauwe et al., 1990; Cools et al., 1992; Mogatle et al., 2008; Choi et al., 2009; Ho et al., 2009)………………………………………………………………………………...46
Figure 4.1: Calibration curve obtained for indinavir with high performance liquid chromatography where peak area is plotted as a function of concentration..............................................................................................................................50

Figure 4.2: TIC (A) and UV (B) chromatograms of Hypoxis hemerocallidea reference plant material..........................................................................................................................................................................................53

Figure 4.3: TIC (A) and UV (B) chromatograms of Hypoxis hemerocallidea aqueous extract..........................................................................................................................................................................................54

Figure 4.4: TIC (A) and UV (B) chromatograms of Hypoxis hemerocallidea commercial product..........................................................................................................................................................................................55

Figure 4.5: TIC (A) and UV (B) chromatograms of the marker molecule, hypoxoside (retention time = 4.37 min)..........................................................................................................................................................................................56

Figure 4.6: Percentage of indinavir transport across the monolayers of Caco-2 cells in the apical to basolateral (AP-BL) direction plotted as a function of time (n = 3, error bars indicate standard deviation)..........................................................................................................................................................................................59

Figure 4.7: Percentage of indinavir transport across the monolayers of Caco-2 cells in the basolateral to apical (BL-AP) direction plotted as a function of time (n = 3, error bars indicate standard deviation)..........................................................................................................................................................................................59

Figure 4.8: P_{app} values for indinavir in both directions across Caco-2 cell monolayers alone (negative control group) and in combination with the selected Hypoxis hemerocallidea materials as well as the positive control group (indinavir with verapamil). P_{app} bar graphs for A-B direction are indicated by dark colours and P_{app} bar graphs for B-A direction are indicated by light colours. (n = 3, error bars indicate standard deviation)..........................................................................................................................................................................................61

Figure 4.9: Plasma concentration time curves of indinavir in Sprague-Dawley rats in the absence and presence of the various Hypoxis hemerocallidea materials for the acute study (single administration). n = 5, error bars indicate standard deviation.................................................................................................................................................................................................................................63

Figure 4.10: Plasma concentration time curves of indinavir in Sprague-Dawley rats in the absence and presence of the various Hypoxis hemerocallidea materials for the chronic study (14 days). n = 5, error bars indicate standard deviation.................................................................................................................................................................................................................................64
Figure D 1: Percentage of indinavir transport for each of the samples (Indinavir alone AP-BL). n = 3…………………………………………………………………………………………104

Figure D 2: Percentage of indinavir transport for each of the samples (Indinavir alone BL-AP). n = 3…………………………………………………………………………………………106

Figure D 3: Percentage of indinavir transport for each of the samples (Indinavir with verapamil AP-BL). n = 3…………………………………………………………………………………………108

Figure D 4: Percentage of indinavir transport for each of the samples (Indinavir with verapamil BL-AP). n = 3…………………………………………………………………………………………110

Figure D 5: Percentage of indinavir transport for each of the samples (Indinavir with H. hemerocallidea aqueous extract AP-BL). n = 3…………………………………………………………………………………………112

Figure D 6: Percentage of indinavir transport for each of the samples (Indinavir with H. hemerocallidea aqueous extract BL-AP). n = 3…………………………………………………………………………………………114

Figure D 7: Percentage of indinavir transport for each of the samples (Indinavir with H. hemerocallidea commercial product AP-BL). n = 3…………………………………………………………………………………………116

Figure D 8: Percentage of indinavir transport for each of the samples (Indinavir with H. hemerocallidea commercial product BL-AP). n = 3…………………………………………………………………………………………118

Figure D 9: Percentage of indinavir transport for each of the samples (Indinavir with H. hemerocallidea reference plant material AP-BL). n = 3…………………………………………………………………………………………120

Figure D 10: Percentage of indinavir transport for each of the samples (Indinavir with H. hemerocallidea reference plant material BL-AP). n = 3…………………………………………………………………………………………122

Figure D 11: Indinavir concentration over time for each rat (indinavir alone). n = 5………………124

Figure D 12: Indinavir concentration over time for each rat (indinavir with ketoconazole). n = 4……………………………………………………………………………………………………125

Figure D 13: Indinavir concentration over time for each rat (indinavir with verapamil). n = 5……………………………………………………………………………………………………126

Figure D 14: Indinavir concentration over time for each rat (indinavir with H. hemerocallidea aqueous extract, acute). n = 5……………………………………………………………………………………………………127
Figure D 15: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* aqueous extract, chronic). n = 5.……………………………………………………….128

Figure D 16: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* commercial product, acute). n = 5………………………………………………………………………………129

Figure D 17: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* commercial product, chronic). n = 5………………………………………………………………………………130

Figure D 18: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* reference plant material, acute). n = 5………………………………………………………………………………131

Figure D 19: Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* reference plant material, chronic). n = 5………………………………………………………………………………132
CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

1.1.1 Herb-drug pharmacokinetic interactions

The use of herbal products as an alternative source of medicinal treatment has become more popular. More than 80% of the population in some developing countries, especially in Africa, use herbal medicines as part of their primary health care (Brijlal et al., 2011). A misinterpretation of safety regarding herbal treatment use is evident amongst the general public. Besides potential toxic effects, herbal medicines can cause alterations in drug transport and metabolism due to modulation of Cytochrome P450 (CYP) enzymes and efflux transporters such as P-glycoprotein (P-gp), thereby causing herb-drug pharmacokinetic interactions. These pharmacokinetic interactions involve changes in absorption, distribution, metabolism or elimination of the drug compound, which may result in an increase or decrease in drug plasma concentration (Cordier & Steenkamp, 2011; Lam & Ernst, 2006).

CYP enzymes are important metabolisers of xenobiotics, herbal and endogenous compounds as well as drugs. CYP iso-enzyme 3A4 (CYP3A4) is predominantly found in the liver and small intestinal epithelium and is responsible for about 30% (in the liver) and 70% (in the intestinal epithelium) of the total CYP450 activity. First-pass metabolism plays an important role in the poor and inconstant bioavailability of certain drugs, especially those that are CYP3A4 substrates (Li et al., 2002).

Drug absorption is influenced by the induction or inhibition of active transporters. Some drugs are pumped out of the epithelial cells back into the lumen of the gastrointestinal tract by means of active transporters, which is referred to as efflux. Efflux transporters such as P-gp are located on epithelial cell membranes. P-gp is susceptible to modulation, which includes activation, induction or inhibition by chemicals such as herbal medicines (van den Bout-van den Beukel et al., 2006). Reports have shown that P-gp and CYP3A4 have the possibility to share substrates and through interplay cause an enhanced effect on drug pharmacokinetics (Hellum et al., 2007). Intestinal P-gp efflux demonstrates a reduction in the bioavailability of numerous CYP3A4 substrates (Benet et al., 2004).

Some herbal products showed the ability to modulate CYP metabolizing enzymes, either through inhibition or induction, which may modify the bioavailability of drugs as a result. An
increase in plasma drug concentration levels is associated with inhibition of metabolic enzymes and/or efflux drug transporter activity. On the other hand, an increase in enzyme expression and/or efflux drug transporter activity reduces the drug plasma concentration (Liu et al., 2011).

The use of antiretroviral drugs together with herbal medicines may produce substantial interactions. Interactions with non-nucleoside reverse transcriptase inhibitors and protease inhibitors can clinically result in adverse effects (Mills et al., 2005). In vitro studies have suggested that *Echinacea purpurea* extracts can inhibit CYP3A4 and concurrent use with alprazolam, protease inhibitors and calcium channel blockers could cause an increase in plasma drug concentration levels (Scott & Elmer, 2002). Clinical studies have shown that Grapefruit (*Citrus paradisi*) juice is capable of inhibiting CYP3A4 and has reduced plasma concentrations of indinavir by 15%-30%, while an increase in saquinavir concentration was observed (Hernández et al., 2009). Chronic intakes of garlic supplements have shown a decrease in the plasma drug concentration of saquinavir (Piscitelli et al., 2002). In vitro studies showed that *Sutherlandia frutescens* extract inhibited CYP3A4 activity by 70-96% and *Hypoxis hemerocallidea* extract inhibited CYP3A4 activity by 31-79%. Both herbal medicines showed a significant activation of pregnane X receptor (PXR) which modulates expressions of both P-gp and CYP3A4. The concurrent use of these herbs with anti-retroviral drugs may result in adverse effects as a result of inhibition of drug metabolism and transport (Mills et al., 2005).

### 1.1.2 African potato (*Hypoxis hemerocallidea*).

*Hypoxis hemerocallidea* (also known as *H. rooperi*) is a popular traditional medicinal plant that is broadly distributed in southern Africa. It is characterized by its star-shaped flowers which are bright yellow in colour and its strap like leaves (Van Wyk et al., 2002). The plant has a potato shaped tuberous rootstock (the corm), which is referred to as the ‘African potato’. These corms are washed, chopped and then administered orally after boiling. ‘African potato’ powders, extracts, decoctions and infusions are popular amongst traditional healers. For centuries this plant has been used for the treatment or management of a number of human disorders (Owira & Ojewole, 2009). Many African countries’ Ministers of Health formulate policies promoting the usage of African traditional medicines for the control, management and treatment of HIV/AIDS related diseases as well as other chronic conditions (Morris, 2002). It has been used for certain types of cancers, heart failures, nervous disorders, immune-related illnesses and urinary tract infections (Singh, 1999). *H. hemerocallidea* extract which contains phytosterols, hypoxoside and its active form, rooperol, has also been used for the treatment of benign prostate hyperplasia, as an anti-inflammatory agent, anti-oxidant, anti-convulsant and as an anti-diabetic agent. Scientific evidence was found that it is active against cancerous and pre-malignant cancer cells (Drewes et al., 2008). The hypoxoside is considered to be the most important
phytochemical with regards to the African potato’s medicinal value (Nair et al., 2007). Although in vivo data is lacking, in vitro observations seemed to suggest the possibility of pharmacokinetic interactions between anti-retroviral drugs and African potato. Patients taking African potato extracts concurrently with anti-retroviral drugs may possibly develop adverse effects and/or may lead to viral resistance, treatment failure and drug toxicity (Owira & Ojewole, 2009).

Commercialised oral products containing H. hemerocallidea extracts or ground plant material are available. For example, Harzol® was released in 1974 and gained a widespread acceptance in Germany. This product contained β-sitosterols and its glucoside, which were originally obtained from H. hemerocallidea. Harzol® was used to treat benign prostate hypertrophy (Drewes & Khan, 2004). Moducare® is a commercially available herbal product that is used as an immune system enhancer and contains sitosterol and its glucoside, sitosterolin, in a ratio of 100:1 (Moducare, 2015). These phytochemical substances were originally isolated and prepared from H. hemerocallidea, but at a later stage manufactured synthetically or obtained from other plants (Drewes & Khan, 2004).

Another over-the-counter H. hemerocallidea product is Hypo-Plus™, which is marketed as an energy booster, immune modulator, food supplement and for improving other conditions such as diabetes, impotency, memory loss, gout, arthritis and HIV/AIDS. It is composed of amino acids, vitamins, an anti-oxidant component, plant sterols and ‘variable ratios of Mopanus vermus and Hypoxis’ (Drewes & Khan, 2004). Found on the KwaZulu- Natal north coast at Isithebe, the firm Impilo Drugs (Pty) Ltd has developed a factory production for indigenous products. ‘Impilo African Potato’ or ‘ilabatheka’ is one of the top sellers. The product is sold in a capsule or tablet form consisting of ground H. hemerocallidea plant material (Drewes & Khan, 2004).

1.1.3 Indinavir (Crixivan®)

Crixivan® (indinavir sulfate) is used for the treatment of human immunodeficiency virus (HIV) infection in combination with other anti-retroviral drugs. Indinavir is classified as an HIV protease inhibitor. During viral replication, cleavage of the viral polyproteins is inhibited by indinavir and immature non-infectious viral particles are formed. Hard gelatine capsules for oral administration are available in 200 mg and 400 mg dose strengths. The recommended dosage is 800 mg orally every 8 hours and should be taken without food for optimal absorption. Ideally the drug product should be taken 1 hour before or 2 hours after meals. Dose reduction of Crixivan® is considered with concomitant use of delavirdine, didanosine, itraconazole, ketoconazole and rifabutin. Patients with hepatic insufficiency as a result of cirrhosis should
decrease the dose to 600 mg every 8 hours (Merck & Co inc, 2013). Co-administration with hormonal contraceptives, anti-convulsants (e.g. carbamazepine, phenytoin) and anti-tuberculosis drugs are examples of drugs that produce an interaction with indinavir (Cohen et al., 2002).

Indinavir is a substrate for both CYP3A4 and P-gp (Hochman et al., 2001) and therefore undergoes metabolism by CYP3A4 and is effluxed by P-gp. Co-administration with drugs/herbs that inhibit CYP3A4 may decrease indinavir clearance, which may lead to an increased indinavir plasma concentration. Grapefruit juice and St. John’s Wort have, for example, exhibited pharmacokinetic herb-drug interactions if taken in conjunction with Crixivan® (Merck & Co inc, 2013).

1.1.4 In vitro, ex vivo and in vivo models

1.1.4.1 In vitro cell cultures

Cultured cells are commonly used to study the transport and metabolism of compounds. When cells are cultured as a monolayer, polarized behaviour is exhibited and therefore it represents the situation in the intestine (Tukker, 2000). The use of primary cells have been attempted for the intestinal epithelium, but poor viability was produced and it did not form a confluent monolayer where tight junctions were created (Barthe et al., 1999). Most cell culture models are based on immortalized cell lines that have been derived from normal cells, induced tumours or human colonic cancers. Human colonic cancer cell lines are widely used for drug absorption studies as they differentiate and readily form confluent and polarized monolayers. The Caco-2 cell line has originated from colon adenocarcinoma cells and is commonly used in pharmacokinetic studies. This cell line has the advantage of being able to form polarized cell monolayers on a porous membrane and differentiate into absorptive intestinal cells containing the typical enterocyte morphology, which includes some brush border enzyme activity and tight junctions. Caco-2 cells express active transport systems such as amino acid transporters, glucose, small peptide, bile acid and the P-gp efflux system (Tukker, 2000). Studies on Caco-2 cells have resulted in gaining valuable information on drug interactions involving both P-gp and CYP3A (Raessi, 1999). Another cultured cell line is the LS180 cell line, which is also derived from human colon adenocarcinoma. LS180 cells can be used to study induction of drug metabolising enzymes. Unlike Caco-2 cells, LS180 cells express PXR (Hartley et al., 2006).
1.1.4.2 Ex vivo (excised animal tissues)

Permeability screening for drug discovery purposes are usually conducted on different animals species' tissues as animal intestinal tissues are also made up of basically the same type of endothelial cells as in humans. Excised animal tissue models have been used since the 1950s to study the mechanism of intestinal absorption. However, the viability of the excised tissue is challenging to maintain as the tissues are devoid of direct blood supply and constant oxygenation is needed (Krishna & Yu, 2008).

1.1.4.3 In vivo

In vivo models refer to the use of living organisms such as vertebrates (e.g. mice or rats) or primates (e.g. vervet monkeys) or humans in pharmacodynamic and pharmacokinetic studies. During in vivo pharmacokinetic studies, a compound is administered extravascularly and its permeation into the systemic blood circulation is measured by means of blood sampling (Hidalgo, 2001).

1.2. RESEARCH PROBLEM

In vitro research has demonstrated that a potential herb-drug pharmacokinetic interaction exists between H. hemerocallidea and anti-retroviral drugs. The literature indicates alterations in the transport and metabolism of indinavir upon co-administration with extracts of H. hemerocallidea. For example, H. hemerocallidea extract has been observed to inhibit CYP3A4 enzyme activity and P-gp related efflux within in vitro models (Mills et al., 2005).

Research on pharmacokinetic interactions between H. hemerocallidea and anti-retroviral drugs has primarily been conducted by means of in vitro models using crude plant extracts or isolated phytochemicals. In vivo data is lacking in this regard (Cordier & Steenkamp, 2011) and this information is needed in order to determine the clinical significance of the pharmacokinetic interaction of commercially available H. hemerocallidea products on commercially available indinavir products (e.g. Crixivan®).

1.3. AIM AND OBJECTIVES

The aim of this study was to identify the pharmacokinetic interactions between H. hemerocallidea extracts as well as a commercial product and an indinavir commercial product (e.g. Crixivan®) by means of in vitro studies and to determine the significance of these interactions in vivo.
The objectives of the study were:

- To validate a high performance liquid chromatographic (HPLC) analysis method for indinavir for the *in vitro* transport study.
- To conduct bi-directional *in vitro* pharmacokinetic studies on indinavir (Crixivan®) in the presence of *H. hemerocallis*ea extracts and a product across Caco-2 cell monolayers.
- To conduct *in vivo* pharmacokinetic studies on indinavir (Crixivan®) in Sprague-Dawley rats. The acute effect will be measured after a single dose of each *H. hemerocallis*ea extract and a product, while the chronic effect will be measured after pre-treatment with each of the *H. hemerocallis*ea extracts and product for 2 weeks.
- To use a validated liquid chromatography linked to mass spectrometer (LC/MS/MS) method for analysis of indinavir and its metabolite (M6) in the plasma of the rats.

### 1.4 STRUCTURE OF DISSERTATION

This dissertation starts off with an introductory chapter (Chapter 1) that provides motivation and justification for the research study as well as the aim and objectives of the study. A literature overview follows in Chapter 2 that focuses on mechanisms of pharmacokinetic interactions, such as efflux transport modulation and alterations in the metabolism of drugs produced by co-administered drug compounds. The scientific methods performed during the *in vitro* transport study and *in vivo* pharmacokinetic study is described in Chapter 3. The results obtained from these study experiments are demonstrated and discussed in Chapter 4. The last chapter, Chapter 5, draws final conclusions from the results attained in this research study and suggests recommendations for future studies.
CHAPTER 2

LITERATURE REVIEW ON THE PHARMACOKINETIC INTERACTIONS BETWEEN HERBAL MEDICINES AND ANTI-RETROVIRAL DRUGS

2.1 INTRODUCTION

Herbal therapy has been used for thousands of years for a broad range of ailments and its use wasn’t unique to one specific civilisation, historical or cultural era (Venkataramanan et al., 2006). Although there is insufficient information available regarding the safety of some herbal medicines, their use as alternative or complementary medicinal treatment is popular around the world. About 40% of the American adult population makes use of herbal medicine and an exponential increase in the consumption rate has been observed in Canada, Australia and Europe. Medicinal herb consumption is also relatively high in Africa with 60 to 85% of native Africans estimated to use herbal medicines, typically in combination with prescribed medicines (Fasinu et al., 2012).

The use of complementary and alternative medicine is common amongst HIV-infected patients as surveys have shown that 67% of the patients receiving anti-retroviral drugs (ARV) were also using one or several supplementary natural health products (Gore-Felton et al., 2003). Reasons given for the use of complementary and alternative medicine include an increase in quality of life, perceived efficacy, a decrease in the adverse effects of ARVs and a sense of control experienced by the patients (Lee et al., 2006). A survey conducted in Massachusetts showed that 63% of physicians believed complementary and alternative medicine were helpful to patients infected with HIV (Rivera et al., 2005).

Studies have shown a habitual pattern concerning the concurrent use of herbal products together with prescription medication. A reported 14 - 16% of the American adult population use herbal medicines with their prescribed medicines (Kaufman et al., 2002). However, many patients do not disclose their use of herbal medicines to their health care providers, while many physicians are not aware of the possible risks of herb-drug interactions (Fasinu et al., 2012). Furthermore, there is a misconception regarding the safety of herbal medicines due to their “natural” origin. Herbal medicines are regarded as supplements or food products and are therefore normally not exposed to the same strict safety and efficacy trials and pre-marketing approval procedures that prescription drugs require (Tarirai et al., 2010).
2.2 PHARMACOKINETIC HERB-DRUG INTERACTIONS

Co-administration of herbal medicines with Western drugs may result in the reduction or increase of the effects of either compound, which represent herb-drug interactions that may in some cases be of clinical importance (Hu et al., 2005). The severity of the herb-drug interaction ranges from being minor to more serious effects that may result in prolonged morbidity, life threatening consequences and even death (Tarirai et al., 2010). Interactions between herbal medicines and drugs may involve pharmacokinetic and/or pharmacodynamic mechanisms. Pharmacokinetic interactions include alterations to absorption, metabolism, distribution or excretion of the affected herb or drug. Pharmacodynamic interactions involve alterations to the pharmacological response of the herb or drug (Lam & Ernst, 2006). The pharmacokinetic and pharmacodynamic processes where potential interactions can occur are schematically illustrated in Figure 2.1.

![Figure 2.1: Schematic demonstration of the processes of pharmacokinetics and pharmacodynamics that may be involved in herb-drug interactions A: Absorption, D: Distribution, M: Metabolism, E: Elimination (Bounda & Feng, 2015)](image)

A pharmacodynamic interaction occurs when the herb synergises, antagonises or enhances the biological activity of the drug (van den Bout-van den Beukel et al., 2006; Fasinu et al., 2012). Not all pharmacodynamic interactions produce an undesirable effect as some interactions may result in a beneficial effect by either increasing the efficacy of the drug or diminishing potential adverse effects (Shi & Klotz, 2012).
Clinical pharmacodynamic interactions between herbal medicines and ARV drugs have been observed, for example, some herbal medicines indirectly enhanced ARV therapy by stimulating the immune system (Lee et al., 2006). However, alteration of the pharmacokinetics (i.e. absorption, distribution, metabolism or elimination) of a drug can lead to a situation where the drug plasma concentration are shifted outside of its therapeutic limits, thus causing a potential for sub-therapeutic levels (low activity) or supra-therapeutic levels (toxicity) (Cordier & Steenkamp, 2011). The main mechanism underlying pharmacokinetic interactions is either the inhibition or induction of intestinal and hepatic metabolising enzymes (e.g. cytochrome P450 [CYP] enzymes). The effect on drug transporters such as efflux pumps, particularly intestinal p-glycoprotein (P-gp), also plays an important role (Fasinu et al., 2012). Pharmacokinetic interactions can become clinically significant when considerable alterations in pharmacokinetic parameters occur. Examples of these parameters include the area under the plasma concentration-time curve (AUC), the maximum plasma concentration (C\textsubscript{max}), the time of maximum plasma concentration (t\textsubscript{max}) and the elimination half-life (t\textsubscript{1/2}) of the drug (Shi & Klotz, 2012). Severe adverse effects and high risks associated with herb-drug pharmacokinetic interactions usually occur with drugs that exhibit narrow therapeutic indices such as phenytoin, digoxin and warfarin (Tarirai et al., 2010).

### 2.2.1 Efflux transporters

Drug transporters can be classified into those mediating the uptake of drugs into the cells and those mediating the export of drugs and its metabolites out of the cells. Uptake and efflux transporters are localised in organs such as the liver, small intestine and kidney, which are crucial for drug disposition (especially absorption and elimination). Induction or inhibition of transporters caused by co-administration of herbal medicines can possibly alter pharmacodynamics and pharmacokinetics of the drug of interest (König et al., 2013).

Studies have shown that efflux transporters, that act alone or together with drug metabolising enzymes, may play an important role in oral drug bioavailability. The ATP-binding cassette (ABC) transporters are a group of active transporters that have a significant influence in the absorption, distribution and elimination of some drugs (Fasinu et al., 2012). The ATP-binding cassette transporters mediate the efflux transporters that can actively transport drugs against a steep concentration gradient and are mostly found in the intestinal epithelium, the canaliculi membrane of the kidney, liver cell membranes and the endothelium of blood capillaries in the brain (Hellum & Nilsen, 2008; Tarirai et al., 2010). The efflux transporters limit the influx of xenobiotic compounds into cells and therefore prevent the intracellular accumulation of compounds that are substrates thereof (Chan et al., 2004).
Pharmacokinetic interactions can occur when herbal medicines inhibit or reduce the ordinary activity level of drug transporters through competitive or non-competitive mechanisms (Fasinu et al., 2012). Blood plasma concentrations that are potentially toxic may arise from an inhibited activity of the efflux transporters, while induction of efflux transporter can result in sub-therapeutic plasma drug levels (Tarirai et al., 2010).

2.2.1.1 P-gp

P-gp is the most studied member of the ABC family of transporters and functions as an efflux pump (Shi & Klotz, 2012). It is also known as multi-drug resistance protein 1 (MDR1) or as ABC subfamily B member 1 (ABCB1). Originally, P-gp was discovered in drug-resistant tumour cells and was only identified later in normal human tissues (Hansten, 2001). P-gp is a 170-kDa plasma glycoprotein consisting of 1 280 amino acids (schematically illustrated in Figure 2.2), which is constitutively expressed in body tissues such as in the apical epithelial surfaces of the liver’s bile canaliculi, pancreatic ductal cells, proximal tubules of the kidneys, small intestines columnar mucosal cells, the colon and adrenal glands (Fasinu et al., 2012). P-gp uses ATP as an energy source to actively pump compounds from the epithelial cells back to the intestinal lumen and from the brain’s capillary endothelial cells back into the blood (Tarirai et al., 2010).

P-gp plays a significant part in the regulation of absorption, distribution and re-absorption/elimination of many therapeutic compounds. P-gp decreases the bioavailability of a broad range of compounds from the gastro-intestinal lumen (Chan et al., 2004). The oral bioavailability of a drug may therefore be reduced as the net passage of orally administered drugs across the gastro-intestinal epithelium might be limited (Huisman et al., 2000). With regards to the blood-brain barrier, P-gp is important as a defense mechanism against the penetration of drugs and toxins from entering into the central nervous system (CNS) (König et al., 2013).

P-gp is involved in the manifestation of some cell drug resistance, which is mediated by a reduction in the accumulation of the therapeutic drug in the target cells. This P-gp mediated drug resistance has a negative effect on HIV and cancer therapies (Hansten, 2001).
Figure 2.2: A schematic illustration of the structure of P-gp (Bansal et al., 2009)

P-gp transports a wide range of moderately hydrophobic and amphipathic drugs out of cells (Huisman et al., 2000). As seen in Figure 2.2, P-gp’s single chain is divided into two homologous halves and each contains six trans-membrane domains. Furthermore, P-gp contains two ATP-binding regions that are divided by a flexible polypeptide linker (Ambudkar et al., 2006).

Figure 2.3: Schematic illustration of the P-gp efflux mechanism (Gohel et al., 2011)

The P-gp molecule is expressed over cell membranes and exhibits efflux action both inside and outside the cell. As illustrated in Figure 2.3, a pore or channel is found in the centre of the P-gp
structure and is responsible for the efflux of drugs out to the extracellular region. P-gp efflux action is energy dependent, ATP mediated (Gohel et al., 2011).

2.2.1.2 P-gp modulation

Modulation of P-gp by herbal medicines holds the potential for alterations involving the pharmacokinetic profile of drugs that are substrates. These pharmacokinetic changes can take place when herbal medicines inhibit the drug transporters through either a competitive or non-competitive mechanism. On the other hand, the induction of transporter proteins by means of an increase of the related proteins’ mRNA can also produce interactions (Fasinu et al., 2012).

P-gp has a broad substrate spectrum, which range from small molecules (200 Da) to peptides of a larger structure (4000 Da) (Estudante et al., 2013). P-gp substrates are generally hydrophobic but mycophenolic acid, a hydrophilic compound, has been shown to be transported by P-gp (Hansten, 2001). Various drugs that are substrates of P-gp are listed in Table 2.1. It is important to note that some drugs may be both substrates and inhibitors of P-gp.

Table 2.1: Examples of different drug classes that are P-gp substrates, inhibitors or inducers (Adapted from, Calitz, 2014; Hansten, 2001; Haritova, 2008; Hu et al., 2005; König et al., 2013; Mercer & Coop, 2011)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Macrolides (Erythromycin), Ketoconazole, Dicloxacillin</th>
<th>• Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluoroquinolones (Ciprofloxacin, Grepafloxacin, Levofloxacin and Sparfloxacin)</td>
<td>• Substrate  • Inhibitor</td>
</tr>
<tr>
<td>Anti-cancer drugs</td>
<td>Doxorubicin, Vinblastine, Topotecan, Docetaxel, Vincristine, Idarubicin, Paclitaxel, Bisantrene, Etoposide</td>
<td>• Substrate</td>
</tr>
<tr>
<td>Opioid Analgesics</td>
<td>Morphine, Loperamide</td>
<td>• Substrate</td>
</tr>
<tr>
<td>HIV protease inhibitors</td>
<td>Indinavir, Saquinavir</td>
<td>• Substrate  • Inhibitor</td>
</tr>
<tr>
<td>Category</td>
<td>Example Drugs</td>
<td>Role</td>
</tr>
<tr>
<td>----------------------------------</td>
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<tr>
<td>Nelfinavir, Rotinavir</td>
<td>Substrate Inhibitor</td>
<td></td>
</tr>
<tr>
<td>Antihistamines</td>
<td>Fexofenadine, Terfenadine</td>
<td>Substrate</td>
</tr>
<tr>
<td>H₂-receptor antagonists</td>
<td>Cimetidine</td>
<td>Substrate</td>
</tr>
<tr>
<td>Anti-epileptics</td>
<td>Felbamate, Topiramate</td>
<td>Substrate</td>
</tr>
<tr>
<td>Anti-arrhythmics</td>
<td>Quinidine</td>
<td>Substrate Inhibitor</td>
</tr>
<tr>
<td>Immunosuppressive drugs</td>
<td>Cyclosporine A</td>
<td>Substrate Inhibitor</td>
</tr>
<tr>
<td>Tacrolimus (FK506)</td>
<td></td>
<td>Substrate</td>
</tr>
<tr>
<td>HMG-CoA reductase inhibitors</td>
<td>Lovastatin</td>
<td>Substrate</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td></td>
<td>Substrate Inhibitor</td>
</tr>
<tr>
<td>Fluorescent compounds</td>
<td>Rhodamine 123, Calecin-AM</td>
<td>Substrate</td>
</tr>
<tr>
<td>Cardiac glycosides</td>
<td>Digoxin</td>
<td>Substrate</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>Nifedipine</td>
<td>Substrate</td>
</tr>
<tr>
<td>Verapamil</td>
<td></td>
<td>Substrate Inhibitor</td>
</tr>
<tr>
<td>Anti-hypertensives</td>
<td>Propanolol, Reserpine</td>
<td>Substrate Inhibitor</td>
</tr>
<tr>
<td>Anti-emetics</td>
<td>Ondansetron</td>
<td>Substrate</td>
</tr>
<tr>
<td>Anti-helminthics</td>
<td>Ivermectin</td>
<td>Substrate</td>
</tr>
<tr>
<td>Steroids</td>
<td>Cortisol Corticosterone</td>
<td>Substrate</td>
</tr>
<tr>
<td>Progesterone</td>
<td></td>
<td>Substrate Inhibitor</td>
</tr>
<tr>
<td>Anti-alcoholism drug</td>
<td>Disulfiram</td>
<td>Substrate</td>
</tr>
<tr>
<td>Psychotrophics</td>
<td>Carbamazepine,</td>
<td>Substrate</td>
</tr>
</tbody>
</table>
2.2.1.3 Inhibition and induction of P-gp

P-gp inhibition can either be competitive or non-competitive. Competitive inhibition takes place when two drug substrates are competing for the same drug-binding site. Non-competitive inhibition occurs when a drug substrate inhibits the ATP hydrolysis cycle and/or the conformation of the binding site changes by means of an allosteric mechanism (Fasinu et al., 2012; Marchetti et al., 2007).

As illustrated in Figure 2.4 a and b below, the P-gp transporter is situated on the apical membrane of polarised intestinal epithelial cells. P-gp substrates are pumped out of the cell through the apical membrane back into the intestinal lumen. The absorption of P-gp substrates is therefore reduced. When P-gp is inhibited, it allows for an increase in the movement of P-gp substrate molecules in the absorptive direction as the substrate molecules are now not pumped out of the cells in the secretive direction. This results in an increase in absorption from the intestinal lumen and a decrease in excretion (Hansten, 2001).

<table>
<thead>
<tr>
<th></th>
<th>Amitriptyline, Sertraline</th>
<th>• Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-gout drug</strong></td>
<td>Colchicine</td>
<td>• Substrate</td>
</tr>
<tr>
<td><strong>Pesticides</strong></td>
<td>Methylparathion, Endosulfan</td>
<td>• Substrate</td>
</tr>
</tbody>
</table>
Figure 2.4: Schematic illustration of the effect of inhibition of P-gp on drug absorption (Hansten, 2001)

Up-regulation of the MDR1/ABCB1 gene through pregnane X receptor (PXR) and constitutive androstone receptor (CAR), which act as drug substrate sensors, results in induction of P-gp. Activation creates PXR and CAR to dimerise with the retinoid-X-receptor (RXR) and a heterodimer is formed. This heterodimer is then bound to the response elements that are located on the MDR1/ABCB1 gene. The specific gene transcription is promoted and an increase in messenger ribonucleic acid (mRNA) is produced for the formation of protein (Pal & Mitra, 2006). Induction therefore causes higher expression of P-gp and thereby causes a reduced concentration of the drug reaching the systemic circulation and therefore a decrease in efficacy and oral bioavailability of the co-administrated drugs (Pal & Mitra, 2006).

Studies have shown that an extract of *Hypericum perforatum* (St John’s Wort) can induce P-gp and cause a reduction of bioavailability in vivo and in vitro of warfarin, theophylline, ARVs, oral contraceptives and anti-convulsants (Shi & Klotz, 2012; Tarirai et al., 2010). Co-administration of *H. perforatum* has also been reported to reduce digoxin plasma concentrations through an increase in P-gp activity (Fasinu et al., 2012).

Grapefruit juice is a well-known inhibitor of P-gp efflux. Cyclosporine is an immunosuppressant drug given during organ transplants to prevent organ rejection. Co-administration of grapefruit juice together with cyclosporine has resulted in a significant increase in cyclosporine bioavailability. On the other hand, *H. perforatum* co-administered with cyclosporine produced a decrease in cyclosporine plasma concentrations to sub-therapeutic levels, possibly resulting in the rejection of the transplanted organ (Hu et al., 2005). Other herbal products containing a P-gp modulator, flavonoid quercetin, such as *Ginkgo biloba* and *Sophora japonica*, have also shown the ability to reduce the bioavailability of cyclosporine (Tarirai et al., 2010).

2.2.2 Interactions involving metabolism

Drug metabolism entails the enzymatic conversion of a therapeutically active drug compound to a new molecule, which can be a pharmacologically active, but usually inactive metabolite. It often involves the conversion of lipophilic drug compounds to highly polar derivatives that are then excreted easily from the human body. The primary drug metabolism site is in the liver cells smooth endoplasmic reticulum. Other organs responsible for metabolism include the lungs, kidney, gastrointestinal tracts and the placenta (Taxak & Bharatam, 2014).

The most common cause of drug interactions of clinical significance is inhibition or induction of drug-metabolising enzymes (Lam & Ernst, 2006). Herb-drug metabolic interactions often occur
in the gastrointestinal epithelium and in the liver as CYP450 metabolic enzymes are present in high concentrations in these tissues but are also found in the placenta, lungs and kidneys. Metabolic enzymes mediate two categories of biotransformation reactions namely, Phase I biotransformation reactions (which include oxidation, reduction, hydration and hydrolysis) and Phase II biotransformation reactions (which include methylation, sulfation, acetylation, fatty acid conjugation, glutathione conjugation and glucuronidation) (Tarirai et al., 2010).

2.2.2.1 Cytochrome P450 (CYP) superfamily of enzymes

The cytochrome P450 (CYP) enzymes comprise of a super family of haematoprotein enzymes that are located on the membrane of the endoplasmic reticulum. They are responsible for catalysing the metabolism reactions of many exogenous and endogenous compounds (Singh, 2007). The CYP superfamily of enzymes is divided into families and sub-families based on their nucleotide sequence homology. A high degree of substrate specificity is present among the different families (Fasinu et al., 2012). CYP enzymes belonging to the families 1, 2 and 3 are primarily involved in xenobiotic metabolism, while other CYP enzymes have an essential role in the formation and elimination of endogenous compounds such as bile acids, hormones and fatty acids (Amacher, 2010). Only a portion of the total number of isoforms, belonging to families 1, 2 and 3, are responsible for the biotransformation of foreign compounds including 70-80% of all drugs currently in clinical use (Zanger & Schwab, 2013).

The highest expressed CYP iso-enzyme forms present in the liver include CYP3A4, CYP2C9, CYP2C8, CYP2E1 and CYP1A2, while CYP2J2, CYP1A1 and CYP1B1 are mostly expressed extra-hepatically. The expression of CYP enzymes is influenced by a number of factors and usually a combination of mechanisms including the induction by xenobiotics, genetic polymorphism, cytokine regulation, hormones, gender and age amongst others (Zanger & Schwab, 2013).

The oxidising site of CYP450 enzymes is located in the heme centre (i.e. an iron-porphyrin unit) and is responsible for catalising oxidation reactions. In a reduced state, the iron can be bound, with a high affinity, to carbon monoxide (CO). CYP enzymes catalyse the transfer of an oxygen atom to a substrate as schematically illustrated in Figure 2.5, resulting in an oxidised substrate together with a water molecule (Taxak & Bharatam, 2014).
The sub-family, CYP3A, constitutes over 40% of the total CYP present in the human body with CYP3A4 found to be the most abundant of all isoforms. CYP3A4 is highly expressed in the liver and intestines and contributes to the metabolism of approximately 50% of all drugs that are currently in use (Singh et al., 2011).

2.2.2.2 Factors influencing cytochrome P450 enzyme expression and function

Genetic polymorphism

Four phenotypic sub-populations based on drug metabolising enzymes exist. These phenotypic populations include poor, intermediate, extensive and ultra-rapid metabolisers (Satoh, 2007).

Factors that may influence some of the CYP450 enzymes are shown in Figure 2.6. CYP450 “loss-of-function” polymorphisms often affect expression and splicing rather than the protein structure or transcription. Variants of “gain-of-function” polymorphisms include copy number variants that involve an increase in the number of functioning gene copies in CYP2A6 and CYP2D6, promotor variants such as CYP2B6 and CYP2C19, and variants of amino acids with an increased substrate turnover. A few polymorphisms have an effect on substrate selectivity or drug metabolising CYP450 induction (Zanger & Schwab, 2013).
Figure 2.6: Fraction of clinical drugs metabolised by CYP450 isoforms and the factors influencing variability (Zanger & Schwab, 2013)

Variants of “loss-of-function” can result in a reduced drug clearance and an increase in drug plasma concentration, while variants of “gain-of-function” can result in an increased drug clearance and a lower drug plasma concentration (Zanger & Schwab, 2013).

**Epigenetic influence on drug metabolism**

Epigenetics describes the heritable changes that may occur in gene function that are not based on sequence variations in DNA. Epigenetics is caused by mechanisms such as DNA methylation and modification of histone protein, while gene regulatory mechanisms by microRNA also exist. Epigenetic patterns are primarily reversible and may possibly be tissue-specific and host factors such as sex and age play an important role. Environmental factors may also have an influence (Zanger & Schwab, 2013).

Studies on CYP1 genes have indicated that CYP1A1 promotor methylation in human lung tissue was the lowest amongst heavy tobacco smokers and the highest in non-smokers. This is an example of an environmental influence that can occur in DNA methylation patterns (Zanger & Schwab, 2013). Targets of microRNA include nuclear receptors and were also shown in the PXR, as this receptor has demonstrated to be under control of miR-148a. This can have an influence on CYP2B6 and CYP3A4 expression levels and xenobiotic drug substrate metabolism (Takagi et al., 2008).
**Non-genetic host factors**

Pharmacokinetic parameters such as body weight, blood flow, fat distribution and the expression of drug transporters and metabolising enzymes are influenced by gender (Gandhi et al., 2004). CYP450 sex-specific expression is typical in laboratory animals and was established to be controlled by different secretion profiles of growth hormone in male versus female animals (Waxman & Holloway, 2009). A genome-wide gene expression profiling investigation conducted in 112 female and 112 male livers recognised over 1300 genes whose mRNA expression was considerably affected by gender and 75% showed a higher expression in females (Zhang et al., 2011). Amongst these gene expression differences, there were 40 genes related to pharmacokinetics, which included CYP1A2, CYP3A4 and CYP1A7 displaying female bias, and a male bias displayed by CYP27B1, CYP3A5 and UGT2B15. Most clinical investigations have established that women metabolise drugs quicker than men and is particularly found in CYP3A4 substrates (Zanger & Schwab, 2013).

Age is a well-recognised and established factor that influences drug metabolism capacity. Extremes of life are usually involved in cases of a lower drug metabolism capacity. This is represented in neonates due to the fact that several enzymes are still undeveloped. In the elderly, the ability of drug clearance is decreased and therefore drugs with a narrow therapeutic index, including anti-depressants, anti-psychotics, beta-blockers and anti-coagulants, should be taken into consideration (Zanger & Schwab, 2013). It has been found in human liver studies that there is a modest increase in activity and expression of most CYPs during life, particularly for CYP2C9. The age influence on CYPs 1A2, 2A6, 2B6, 2C8 and 3A4 moderately interacted with sex (Yang et al., 2010). Limited drug clearance as a function of age can also be associated with the reduced renal function and liver blood flow in the elderly. Furthermore, age-associated modifications in gene expression involved in xenobiotic metabolism have been established in rats and mutant mice (Zanger & Schwab, 2013).

Disease conditions normally have a negative impact on drug metabolism. Liver cirrhosis represents liver architecture changes which results in a blood flow reduction, functional hepatocyte loss, and a reduction of drug metabolising enzymes. This all contributes to the decrease in drug metabolism capacity as well as a lower synthesis of serum proteins, leading to a decrease in drug clearance. During inflammation, infection and cancer, pro-inflammatory cytokines circulate and act as signalling molecules to stimulate marked changes in gene expression profiles of the liver. As a result, severe down regulation of numerous drug metabolising enzymes occurs (Zanger & Schwab, 2013).
2.2.2.3 Pre-systemic metabolism/first-pass effect

Orally administered drugs are subject to pre-systemic metabolism, also referred to as the first-pass metabolism effect, which can be defined as the metabolism of drug molecules between administration and appearance in the systemic circulation as schematically illustrated in Figure 2.7. The concentration of the drug that reaches the systemic circulation can be reduced by hepatic and intestinal metabolism (Pond & Tozer, 1984). The liver was thought to be fully responsible for the pre-systemic elimination until 1997, but it was found that CYP3A sub-family enzymes are expressed at high levels in the premature villous tips of enterocytes in the small intestine (Gavhane & Yadav, 2012).

Any molecule that has been absorbed from the gastrointestinal lumen passes through the gastrointestinal epithelial mucosa, intestinal capillaries and is then transferred to the liver via portal circulation before reaching the systemic circulation. It is transported in the blood to the rest of the organs after the systemic circulation has been reached (Gavhane & Yadav, 2012). Intestinal first-pass metabolism could result in a low oral drug bioavailability and provides a platform for drug-drug or herb-drug interactions (especially for CYP3A substrates) (Gertz et al., 2010).

![Schematic illustration of pre-systemic metabolism](image)

**Figure 2.7:** Schematic illustration of pre-systemic metabolism (Adapted from Dickens & Van de Waterbeemd, 2004)

Pre-systemic metabolism is clinically important when the fraction of administered drug dose that escapes this metabolism is small (Pond & Tozer, 1984).
2.2.2.4 Inhibition and induction of metabolic enzymes

Drug interactions involving the CYP enzymes are generally a result of either an induction or inhibition of these enzymes. The induction of CYP can lead to an increased metabolite production rate, which is associated with decreased plasma levels and drug response. CYP inhibition can be classified into reversible and irreversible inhibition. Reversible inhibition occurs when direct competition for the CYP enzyme binding site takes place between a substrate and an inhibitor. Irreversible inhibition is produced by reactive metabolites that are generated from CYP-catalysed reactions (Mukherjee et al., 2011). In the case of irreversible inhibition, the mechanism-based inhibitors become activated during metabolism and a complex with the CYP3A heme is formed, the metabolite intermediate complex, which result in a covalent modification of the enzyme. This results in irreversible enzyme activity loss. This irreversible mechanism seem to be the inhibition of CYPs that takes place after exposure to bergamottins (grapefruit juice), capsaicin (chili peppers), glabridin (licorice root), isothiocyanates (cruciferous vegetables), oleuropein (olive oil), resveratrol (red wine constituent) and diallyl sulfone (garlic). Irreversible inhibition can take one to two weeks to resolve after the inhibitor has been discontinued, as this is the time that it takes the CYP3A to restore to its pre-drug steady state (Lam & Ernst, 2006).

As demonstrated in Figure 2.8 bellow, CYP inhibition by herbal medicines may cause toxicity as a result of an increased plasma and tissue drug concentration. CYP induction may result in reduced drug concentrations and therefore causing a decrease in drug efficacy and potentially failure in treatment (Mukherjee et al., 2011). Induction may be a result of enhanced gene transcription rates, translational efficiency, increased stability of mRNA or protein stabilisation that have been induced by post-translational modifications or substrate binding. The most common induction mechanism is the binding and activation of nuclear factors that act in protein heteromer forms to increase gene transcription rates. A single nuclear factor has the potential to modulate the expression of several genes. The transcriptional regulation involving drug-metabolising enzymes is usually tissue selective. Tissues with a low concentration expression of nuclear factors do not show significant induction. Liver and intestines both express substantial concentrations of nuclear factors, such as PXR, and a profound induction are experienced (Lam & Ernst, 2006).
**Figure 2.8:** Schematic illustration of cytochrome P450 inhibition and induction (Mukherjee et al., 2011)

Examples of enzyme inhibition and induction by herbal products are listed in Table 2.2. Herbal medicines can cause CYP inhibition through competitive inhibition, non-competitive inhibition and mechanism-based inhibition. Induction is usually mediated through the ligand-binding domain of PXR. Studies have demonstrated that PXR is involved in both CYP3A and P-gp induction. The inductive effect caused by herbal medicines and drugs may be mediated through a few sub-mechanisms and not just one single mechanism (Tarirai et al., 2010).

**Table 2.2:** Enzyme inhibition and induction of herbal medicines (Adapted from Liu et al., 2011)

<table>
<thead>
<tr>
<th>Scientific plant name</th>
<th>Chemical Component</th>
<th>CYP substrate</th>
<th>Study method conducted</th>
<th>Effect on enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hypericum perforatum</em> (St. John’s wort)</td>
<td>Hypericin</td>
<td>CYP3A4</td>
<td><em>In vivo, in vitro</em></td>
<td>Induction</td>
</tr>
<tr>
<td><em>Ginkgo biloba</em></td>
<td>Quercetin, kaempferol, p-coumarin acid</td>
<td>CYP3A4</td>
<td><em>In vivo, in vitro</em></td>
<td>Inhibition</td>
</tr>
<tr>
<td><em>Angelica sinensis</em></td>
<td>Lactones of Artemisa, butane</td>
<td>CYP3A4</td>
<td><em>In vitro</em></td>
<td>Inhibition</td>
</tr>
</tbody>
</table>
2.2.2.5 Herb-drug pharmacokinetic interactions involving anti-retroviral drugs

Many herbal medications used by HIV infected patients may have an influence on the metabolism of their ARV drugs through interaction with CYP450 enzymes (Phase I reactions), uridine diphosphate (UDP)-glucuronosyltransferases (Phase II reactions) and P-gp (Tarirai et al., 2010). The ARV treatment therapy usually consists of either a protease inhibitor (PI) or non-nucleoside reverse transcriptase inhibitor (NNRTI) combined with nucleoside reverse transcriptase inhibitors (NRTI). NRTIs are primarily excreted through renal clearance and CYP based interactions are not frequently uncounted. PI and NNRTI are both mainly metabolised by CYP450 enzymes. Higher ARV plasma levels would be as a consequence of CYP inhibition by

<table>
<thead>
<tr>
<th>Herb (Herbal Medication)</th>
<th>Active Constituents</th>
<th>Enzymes and Reactions</th>
<th>In Vivo/In Vitro</th>
<th>Interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angelica sinensis</td>
<td>acid lactones, ferulic acid</td>
<td>CYP2E1, CYP3A4</td>
<td>In vivo, In vitro</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Glycyrrhiza uralensis (licorice)</td>
<td>Furanocoumarins, scopoletin, coumarin</td>
<td>CYP3A4</td>
<td>In vitro</td>
<td>Induction</td>
</tr>
<tr>
<td>Cortex acanthopanacis (Acanthopanax bark)</td>
<td>Isoflavones, glabridin</td>
<td>CYP2C9</td>
<td>In vivo</td>
<td>Induction</td>
</tr>
<tr>
<td>Panax ginseng</td>
<td>Ginsenoside Rd</td>
<td>CYP3A4, CYP2D6, CYP2C19, CYP2C9</td>
<td>In vitro</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Panax ginseng</td>
<td>Ginsenoside Re, Rf</td>
<td>CYP2C9, CYP3A4</td>
<td>In vitro</td>
<td>Induction</td>
</tr>
<tr>
<td>Panax ginseng (Red Ginseng)</td>
<td>Ginseng extract</td>
<td>CYP3A4</td>
<td>In vivo</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Panax ginseng (Red Ginseng)</td>
<td>Red ginseng extract</td>
<td>CYP2E1</td>
<td>In vivo</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Allium sativum (garlic extract)</td>
<td>Allicin, saponin</td>
<td>CYP3A4</td>
<td>In vivo</td>
<td>Induction</td>
</tr>
<tr>
<td>Salvia miltiorrhiza (red sage)</td>
<td>Salvia extract</td>
<td>CYP2C9</td>
<td>In vivo</td>
<td>Inhibition</td>
</tr>
</tbody>
</table>
herbal medicines and the patient would therefore be at a higher risk of experiencing side effects. CYP induction would result in sub-therapeutic plasma levels, causing therapeutic failure and a greater risk of developing ARV drug resistance (van den Bout-van den Beukel et al., 2006).

Garlic (*Allium sativum*) is popular amongst HIV-infected patients as a dietary supplement in order to improve health and fight opportunistic infections. Pharmacokinetic interactions between garlic and the ARVs saquinavir and ritonavir have been reported and studies have indicated the mechanism is most likely an induction of CYP3A4 and P-gp by garlic constituents (van-den Bout-van den Beukel et al., 2006).

Milk thistle (*Silybum marianum*) is administered by HIV-infected patients for the prevention of ARV treatment related hepatotoxicity and treatment of hepatitis (Lee et al., 2006). Co-administration with indinavir indicated a reduction in indinavir blood plasma concentration and *in vivo* data have suggested this is due to an induction of CYP3A4 or P-gp. Potential clinically important interactions of Milk thistle taken with PIs and NNRTs that are P-gp substrates have been observed (van den Bout-van den Beukel et al., 2006).

Clinically important interactions have been reported with St. John’s Wort and ARVs. Co-administration of indinavir and St John’s Wort has shown to decrease indinavir plasma concentrations. Concomitant use of St John’s Wort increased nevirapine clearance by 35% in HIV-infected patients (Lee et al., 2006; van den Bout-van den Beukel et al., 2006). This study will focus on the pharmacokinetic interactions that occur when *Hypoxis hemerocallidea* is co-administrated with indinavir.

**2.3 HYPOXIS HEMEROCALLIDEA (AFRICAN POTATO)**

The African potato, *Hypoxis hemerocallidea*, also known as *H. rooperi*, is a popular medicinal plant used as a traditional herbal medicine in southern Africa. It is found in the southern Africa sub-region where it usually grows in meanders, grasslands and mountain regions. It has the ability to survive well in high stressed environments (Cordier & Steenkamp, 2011; Owira & Ojewole, 2009).

**2.3.1 Botany and uses**

*H. hemerocallidea* is a plant of about 100 to 500 mm in height and is characterised by its strap-like deciduous leaves and star-shaped flowers that are bright yellow in colour as illustrated in Figure 2.9 (Van Wyk et al., 2002). The plant has an unbranched stem and a rootstock growing underground. This tuberous rootstock is also called the corm and has a potato-like shape. The
plant grows well in warm and cold sub-tropical areas as it prefers full sunlight with well-drained soil (Owira & Ojewole, 2009).

Traditionally, the corms of the plant are chopped into tiny pieces and boiled for approximately 20 min. The decoction is then administered orally. The daily oral dose administered is estimated to correspond to 250 ml derived from about 20 g of the corms (Nair et al., 2007). The corm and roots have been used to treat a wide range of diseases, which include colds and flu, hypertension, diabetes mellitus, psoriasis, urinary tract infections, testicular tumours, prostate hypertrophy and internal cancers, central nervous system disorders and HIV/AIDS. The South African Ministry of Health recommended that HIV-infected patients use this herbal plant because it contains immune system stimulating properties (van den Bout-van den Beukel et al., 2006). A recommended daily dose of 2 400 mg raw plant is claimed to be therapeutically effective (Mills et al., 2005).

![Figure 2.9: Photograph of the African potato (Hypoxis hemerocallidea) plant showing its characteristic yellow flower (Germishuizen et al., 2003)](image)

### 2.3.2 Biological activity

*In vivo* studies have indicated that aqueous and alcohol extracts of *H. hemerocallidea* hold pharmacological properties, which include anti-nociceptive, anti-inflammatory and anti-diabetic properties (Ojewole, 2006). The extracts could inhibit inflammatory cytokine synthesis, production and release mediators such as prostaglandins. Aqueous extracts of *H. hemerocallidea* corms have also shown anti-oxidant activity *in vitro* by scavenging free radicals
and both extracts, aqueous and alcohol, have indicated the ability to inhibit the cyclooxygenase (COX) enzyme that mediates the synthesis of prostaglandin (Owira & Ojewole, 2009).

Lectin-like proteins derived from *H. hemerocallidea* extracts have been reported to inhibit *Staphylococcus aureus* growth *in vitro*. Other laboratory reports have shown the extracts to inhibit *Escherichia coli* growth (Owira & Ojewole, 2009). This observation may explain the traditional use of the plant in the treatment of urinary tract infections and benign prostate hyperplasia. It has been suggested that the anti-bacterial properties of *H. hemerocallidea* might not be the only reason for the treatment of benign prostate hyperplasia as anti-inflammatory and anti-oxidant properties could also be responsible for treatment of this condition. This data supports the plant’s use in the treatment of certain cancers (Owira & Ojewole, 2009; Nair *et al*., 2007).

Certain healthcare providers (e.g. traditional healers) are using *H. hemerocallidea* extracts/plant materials in the treatment of patients infected with HIV/AIDS because it has been said to boost the human’s immune system. The plant’s usage has been extended to other immune-related conditions such as flu, the common cold and arthritis (Mills *et al*., 2005).

**2.3.3 Phytochemistry**

*H. hemerocallidea* has been chemically characterised in the attempt to determine which chemical constituents are responsible for its medicinal properties (Owira & Ojewole, 2009). A norlignan-diglucoside, hypoxoside, has been found to be one of the most important chemical constituents in terms of medicinal properties. Hypoxoside is a biologically inactive pro-drug, which has an aglycone structure that consists of a diphenyl-1-en-4-yne-pentane skeleton (Figure 2.10) (Nair *et al*., 2007). It is converted by β-glucosidase enzyme in the human gastrointestinal tract to a biologically active compound, rooperol (Figure 2.10) (Owira & Ojewole, 2009). Rooperol exhibited both anti-cancer and anti-inflammatory activity and most of *H. hemerocallidea*’s therapeutic properties have been scientifically, clinically and pharmacologically attributed to rooperol. Sterols and stanols, such as stigmastanol, stigmasterol and β–sitosterol (Figure 2.10), are also found in *H. hemerocallidea* extracts but their medicinal significance has not been proven yet (Cordier & Steenkamp, 2011).
Rooperol and hypoxoside have been found to undergo phase I hepatic metabolism by CYP450. A product containing rooperol from *H. hemerocallis* extract with the trade name, ‘Harzol™’, is registered in Germany for the treatment of prostate cancer. Many other commercial herbal medicines containing rooperol or *H. hemerocallis* extracts are found on the market (Nair *et al.*, 2007).

### 2.3.4 Interactions

Administration of *H. hemerocallis* extracts may possibly affect enzymes and transporters and therefore consequently have an effect on the metabolism and absorption of concurrently administered therapeutic drugs (Mogatle *et al.*, 2008). Possible herb-drug interactions between extracts of *H. hemerocallis* and ARV drugs have been identified (Mills *et al.*, 2005). Studies have shown that *H. hemerocallis* extract can inhibit CYP3A4 and P-gp. The extracts have also been claimed to activate PXR, which will modulate the expressions of P-gp and CYP3A4. As mentioned before, some ARV drugs are CYP3A4 substrates (Mills *et al.*, 2005; Tarirai *et al.*, 2010).

*H. hemerocallis* extracts are likely to affect cellular drug transport systems since an up-regulation of PXR has been reported (Tarirai *et al.*, 2010). From *in vitro* results it was postulated that patients administering *H. hemerocallis* extracts simultaneously with ARV...
treatment therapy may be at risk to experience pharmacokinetic interactions that may result in treatment failure, viral resistance and drug toxicity (Owira & Ojewole, 2009). Findings suggested co-administration may lead to early drug metabolism and transport inhibition (van-den Bout-van den Beukel et al., 2006).

*In vitro* studies in human liver microsomes have shown that *H. hemerocallidea* is capable of inhibiting CYP3A4 activity by 86%. *H. hemerocallidea* also showed a P-gp inhibition activity of 42% - 51% of that of verapamil, a known P-gp inhibitor. Exposure of *H. hemerocallidae* resulted in a two-fold activation of PXR (van-den Bout-van den Beukel et al., 2006).

Another *in vitro* study demonstrated that an aqueous extract of *H. hemerocallidea* presented a substantial inhibition (i.e. 33.9% – 85.6%) of CYP3A4 activity at an initial concentration of 100 mg/ml. An increase in inhibition to 56.1% - 79.4% was observed with the use of a methanol extract (Mills et al., 2005). A study revealed that stigmasterol and rooperol inhibited CYP3A4, CYP3A5 and CYP19 (Nair et al., 2007; Mogatle et al., 2008).

It is clear from previous studies that more research needs to be conducted in order to investigate the potential for clinically important interactions of *H. hemerocallidea* with drugs, especially ARVs (van-den Bout-van den Beukel et al., 2006).

### 2.4 MODELS TO EVALUATE PHARMACOKINETIC INTERACTIONS

Intestinal absorption and hepatic metabolism of orally administered drugs are essential factors in the determination of the drug’s systemic bioavailability and therapeutic effect. Pre-clinical examination of drug metabolism and transport plays an important role in the identification of potential bioavailability problems or pharmacokinetic interactions between drugs and other compounds. Several well-established cell-based and animal models are in use for the study of hepatic clearance and intestinal absorption (Alqahtani et al., 2013).

Permeability studies have shown that the *in vitro* Caco-2 cell culture model as well as the *in vitro* mucosal sheets of animals, such as rats and pigs, can predict human intestinal drug absorption to an acceptable extent (Versantvoort et al., 2000). Other cell culture models that are commonly used in drug transport and metabolism studies include the MDCK and LS180 cell lines. HepG2 is a well-known liver cell line used in metabolism studies, while microsomes and precision cut liver slices are also used for this application. A summary of commonly used models for drug permeation and metabolism studies is given in Table 2.3.
Table 2.3: Commonly used models to study metabolism and transport of drugs (Adapted from Alqahtani et al., 2013)

<table>
<thead>
<tr>
<th>INTESTINAL ABSORPTION MODELS</th>
<th>HEPATIC METABOLISM MODELS</th>
<th>SIMULATION MODELS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In vitro models</strong></td>
<td><strong>Primary hepatocytes model</strong></td>
<td><strong>Compartment models</strong></td>
</tr>
<tr>
<td>• Caco-2 cells</td>
<td>SCHs of human, rat and mice origin</td>
<td>• CAT model</td>
</tr>
<tr>
<td>• MDCK cells</td>
<td></td>
<td>• ACAT model</td>
</tr>
<tr>
<td><strong>Ex vivo models</strong></td>
<td><strong>Hepatoma cell lines</strong></td>
<td>• ADAN model</td>
</tr>
<tr>
<td>• Ussing chamber (rat/pig)</td>
<td>• HepG2 cells</td>
<td><strong>Dispersion model</strong></td>
</tr>
<tr>
<td>• Everted intestinal sac (rat/pig)</td>
<td>• Huh7 cells</td>
<td></td>
</tr>
<tr>
<td><strong>In situ perfusion models</strong></td>
<td>• HepaRG</td>
<td></td>
</tr>
<tr>
<td>• Small intestine segment of rats</td>
<td>• LS180 cells</td>
<td></td>
</tr>
<tr>
<td>• Human intestine</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>In vivo models</strong></td>
<td><strong>Microsomes</strong></td>
<td></td>
</tr>
<tr>
<td>• Animals</td>
<td><strong>Precision-cut liver slices</strong></td>
<td></td>
</tr>
</tbody>
</table>

2.4.1 In vitro study models

Most research studies on metabolic herb-drug interactions have been focused on in vitro models such as microsomal systems, cytosols, expressed enzymes or cell cultures. These cell culture systems include primary cultures of human hepatocytes, transfected cell lines and tumour derived cells (Venkataramanan et al., 2006). Immortalised cell cultures are continually being used to investigate drug permeability. The reason for this is because primary cultures of enterocyte cells are not able to form polarised monolayers containing specified apical and basolateral surfaces (Alqahtani et al., 2013). However, primary cultures of human hepatocytes can result in better predictions of metabolic herb-drug interactions. In addition, primary cultures of human hepatocytes are viable for a period of up to two weeks and maintain all the co-substrates and co-factors needed for Phase I and Phase II metabolic pathways as well as
transporter function. This makes these models useful to study inhibition and induction of drug metabolising enzymes (Venkataramanan et al., 2006).

*In vitro* models are popular as they have the potential for high throughput, cost-effectiveness and can display an adequate predictability of the *in vivo* situation. Furthermore, the use of human derived cell lines avoids problems that occur with animal tissues such as inter-species differences. Financial and ethical considerations are among many reasons why the cell culture model has been pursued (Sarmento et al., 2012).

However, the influence of physiological factors such as age, diseases, renal or hepatic dysfunctions and environment conditions cannot be included in the interpretation of the results from the *in vitro* model (Sarmento et al., 2012). The success of the *in vitro* model depends on how strictly the model mimics the conditions presented *in vivo* (Versantvoort et al., 2000).

### 2.4.1.1 Caco-2 cell line

Caco-2 cells are derived from human colon adenocarcinoma and are one of the most frequently used cell lines in the investigation of drug transport and permeation (Alqahtani et al., 2013). These cells hold many functional and morphological characteristics of normal, differentiated enterocytes when they are cultured as confluent monolayers. Caco-2 cells are cultured in cell-culture flasks and then seeded out onto filters in Transwell® plates, which have been designed specifically for drug uptake studies. Transwell® plates contain an inner well placed in a larger outer well. A semi-permeable membrane is found at the bottom of the inner well on which the cells are grown to confluence (Li, 2001).

Caco-2 cells grow to form a polarized monolayer on the porous membrane and tight junctions are formed and several enzymes and transporters are expressed. A well-defined brush border is formed on the apical surface which expresses digestive enzymes such as lactase, sucrase, isomaltase and alkaline phosphatase (Versantvoort et al., 2000). A schematic illustration of a Caco-2 cell monolayer is illustrated in Figure 2.11 bellow. Caco-2 cells express active transport systems which include carrier-mediated transport systems for peptides, glucose, vitamins, amino acids and bile acids (Alqahtani et al., 2013). Caco-2 cells also express efflux transporter systems (P-gp, MRP1-3), Phase I metabolic enzymes (CYP450) and Phase II conjugating enzymes (UDP-glucuronyltransferase, sulfotransferase, glutathione S-transferase). Caco-2 monolayers polarise more on permeable filter supports than on plastic when cultured for a period of 2-4 weeks and consequently provide a good *in vitro* study model for intestinal drug transport studies (Versantvoort et al., 2000).
Transport studies are conducted by placing the test compounds in the inner well (apical side) and observing the amount of test compound in the outer well (basolateral side) over a period of time (Li, 2001). There are two methods used to measure the epithelial tight junctions’ integrity before transport studies are conducted. First by measuring the transepithelial electrical resistance (TEER) (should be more than 150 ohm/cm²) or by making use of small hydrophilic molecules such as sucrose or mannitol, which can only pass through the tight junctions via paracellular transport. Caco-2 cells are also included as a model in several studies to test formulations as delivery systems (Alqahtani et al., 2013).

Caco-2 cells have potential for high-throughput screening as culturing the cells in large quantities is relatively simple and reproducible, and the permeability investigation results are also highly reproducible (Tukker, 2000).

The Caco-2 cell line also holds certain disadvantages. Because the cell line has been established on a colonic cell line that may differentiate into small intestinal-like epithelial tissue, it may have tight junctions tighter than that found in the small intestine. The under-prediction of hydrophilic compounds permeabilities with a low in vivo uptake could arise. Another disadvantage is that the expression of active transport systems is quantitatively different in numerous transporters when compared to excised intestinal tissue. The less expressed glucose transporter in cultured cells is an example. Expression variation between-laboratory or batch-to-batch can occur as the third disadvantage. A poor prediction of paracellular transport...
and carrier mediated compounds with limited cell usefulness thereof may be a consequence of the above (Tukker, 2000).

### 2.4.1.2 LS180 cell line

The LS180 cell line is also derived from human colon adenocarcinoma. The expression characterization of drug metabolising enzymes and drug transporters in LS180 cells has not been done as extensively as for Caco-2 cells. The level of expression of CYP3A4 and MDR1 is comparable in LS180 and Caco-2 cells and in both cell cultures CYP3A4 can be up-regulated by adding 1α, 25-dihydroxyvitamin D3 to the growth medium. However, the induction of MDR1 has been observed in LS180 cells because PXR is expressed in LS180 cells and not in Caco-2 cells (van de Kerkhof et al., 2007).

The LS180 cell line can be used to investigate the induction of drug metabolising enzymes. It has been shown to be responsive to 1α, 25-dihydroxyvitamin D3, dexamethasone, 3-methylcholantrene, rifampicin, 2,3,7,8-tetrachlorodibenzo-p-dioxin and phenobarbital (van de Kerkhof et al., 2007).

### 2.4.2 In vivo models

Ultimate information regarding oral bioavailability of a compound is derived from in vivo models. A wide range of methods is available for determining the bioavailability or intestinal absorption of compounds in both humans and experimental animals. The methods can be classified into three groups, namely intestinal perfusion (in humans), mass balance studies (in humans and animals) and blood kinetics (in humans and animals) (Versantvoort et al., 2000). In vivo studies conducted in an appropriate animal model can be of significant value when used in combination with in vitro models as it helps to verify in vivo relevance (Tang & Prueksaritanont, 2010).

After a drug has been orally administrated to an animal model, blood samples are withdrawn at pre-determined time intervals and then analysed to determine the drug concentration at each time point. The plasma level-time profile is then plotted and the area under the curve (AUC) is established as an indication of the bioavailability. In vivo animal models that are generally used for the prediction of drug pharmacokinetics include rats, monkeys, dogs and pigs (Alqahtani et al., 2013).

Concerning pharmacokinetics, a crucial consideration for the selection of an animal model is established on the extensive similarities to humans in important biochemical and physiological parameters that govern drug absorption, distribution, metabolism and elimination (ADME) processes (Tang & Prueksaritanont, 2010). The presence of the intact system including
lymphatic absorption, mesenteric blood circulation and intestinal membrane is the main advantage of the in vivo animal model as it results in a good prediction. Anatomical and physiological differences do, however exist between human and animal models, such as expression of metabolising enzymes, transport proteins, pH, gastrointestinal motility (GI) and gastric emptying rate. Other limitations of the animal in vivo model is the requirement of a large amount of resources, labour intensive, time-consuming, not practical for high-throughput screening and the difficulty studying individual drug absorption mechanisms (Alqahtani et al., 2013).

Ethical issues should be considered in terms of the animal sacrifice, pain and discomfort. The principle of 3Rs (Replacement, Reduction and Refinement) acknowledges these aspects. In essence this principle strives to replace animal usage with non-animal models where possible, reduce the amount of animals required by enhanced study designs and decrease pain and stress by refining methodologies (Sjögren et al., 2014).

### 2.4.2.1 The rat model

The rat is the most common species used for animal studies (Butterweck & Derendorf, 2008). There are differences between the rodent and human physiology, for instance rodents are nocturnal animals which warrants considerations concerning dose timing and the possibility of disrupting their natural circadian rhythm. Rodents are also susceptible to co-prophagy and so re-uptake of faecal excreted drugs can occur. A higher metabolism is generally observed in rodents when compared to humans (Sjögren et al., 2014).

The absorption rate of Biopharmaceutics Classification System (BCS) class 1 drugs in rats is similar to that in humans. The rat’s gastric pH is often higher than that of humans and has an increased gastric secretion. The small intestines pH increases from the duodenum to the terminal ileum within a range similar as found in humans (pH 4.5-7.5). As far as epithelial permeability is concerned in the small and large intestine, the rat has been used as a model in numerous in situ perfusion experimental studies and is considered to be the most appropriate animal model to predict human permeability and intestinal absorption. It has been observed that the jejunal permeability of the rat correlates strongly with human jejunal permeability (Sjögren et al., 2014).

GeneChip techniques have been used to determine rat and human jejunal permeability and examine expression levels of metabolic enzymes and transporters. The investigations have found a good correlation between the human and rat permeability ($R^2 = 0.8$), moderate correlation for expression levels of transporter in the duodenum ($R^2 > 0.56$), but no correlation
with regards to levels of metabolising enzymes (Sjögren et al., 2014). Based on data from the small and large intestine, it has also been reported that the Ussing chamber with rat tissue may be a useful model for the prediction of human intestinal absorption (Sjögren et al., 2014).

### 2.4.2.1.1 Herb-drug interaction studies involving the rat model

Grapefruit juice has been shown to inhibit CYP3A4 *in vitro* and this inhibition may be attributed to high concentrations of bioflavonoids and furanocoumarins. Data has suggested that cyclosporin-A metabolism can be inhibited by grapefruit juice. The main mechanism to identify grapefruit juice inhibitory compounds has been conducted in rat microsomes. Findings suggest that rats may be a good model for the investigation of herb-drug interactions (Mangano et al., 2001). Garlic oil and its organosulfur compounds, diallyl sulfide, diallyl disulfide and diallyl trisulfide, were demonstrated to potentially increase the protein content and levels of mRNA of CYP1A1, CYP2B1 and CYP3A1 in rats. Diallyl sulphide has the potential to inhibit the expression and activity of CYP2E1. Red garlic extracts have been shown, in rat liver slices, to cause a significant inhibition of saquinavir efflux and a substantial increase in the activity of darunavir efflux transporters (Shi & Klotz, 2012).

*Ginkgo biloba* extracts have been demonstrated in rats to inhibit or induce various CYP enzymes depending on the constituents or compositions of the extracts. At a concentration of 100-2500 ng/mL in rat primary hepatocytes, *G. biloba* extracts showed (in a dose-dependent manner) a significant induction of activity, protein expression and mRNA expression of CYP3A4 (Shi & Klotz, 2012). In the rat model, *Panax ginseng* (150 mg/kg/day) was administrated for 14 days and a decreased AUC of oral fexofenadine by 51% (p<0.005) was observed. The C<sub>max</sub> was decreased by 75% (p<0.001) and the ratio concentrations of brain to plasma were also significantly decreased (p<0.05). This indicated that *P. ginseng* administered over a long term may induce the expression of brain endothelium and intestinal P-gp (Zhang et al., 2009).

Pharmacokinetic interactions between indinavir and St John’s Wort that was given at a concentration of 150 or 300 mg/day for 15 days have been observed in rats. This was established to be as a result of CYP3A4 induction. Administration of St John’s Wort, 100 mg/kg/day for a period of 10 days, in rats demonstrated a substantial induction of CYP3A2 and CYP2D2, as well as CYP2C6 inhibition. St John’s Wort extracts administered in rats for 14 days displayed an increase in P-gp/MDRI expression 3.8-fold and a hepatic CYP3A2 2.5-fold expression (Shi & Klotz, 2012).
2.5 SUMMARY

The use of herbal medicines has gained popularity over the past few decades. Herbal medicine usage is common amongst HIV-infected patients who are receiving ARV therapy and often without the approval or knowledge of their health care provider. The interactions between herbal medicine and prescribed drugs remain a safety concern. Pharmacokinetic interactions include various mechanisms that have an influence on drug absorption and metabolism such as the inhibition or induction of CYP enzymes and drug transporters. Many herbs and drugs are substrates for both CYP3A4 and P-gp and their effect on these metabolic and efflux pathways have been studied. The possible pharmacokinetic interactions occurring as a result of the concomitant use of herbal medicines and prescribed drugs should be further investigated for clinical importance.
CHAPTER 3

METHODS AND MATERIALS

3.1 INTRODUCTION

The results obtained from in vitro studies can predict potential herb-drug interactions that will be experienced in vivo. Model systems that are commonly used to investigate metabolism include microsomes, recombinant enzymes and hepatocytes. Epithelial cell cultures such as Caco-2 or MDCK cell lines are typically used to investigate bi-directional transport activity (Brantley et al., 2014).

The Caco-2 cell line is derived from human colon adenocarcinoma, which has been the most popular model used to simulate intestinal epithelium monolayers and to predict drug transport. Caco-2 epithelial cells form tight junctions, differentiate to distinguish between the apical and basolateral sides and express several appropriate efflux transporters such as P-gp (Sarmento et al., 2012). The Caco-2 cell line was selected as the in vitro model for the transport experiments in this study.

In vivo models are essential to determine drug bioavailability and to demonstrate an estimate of exposure to metabolites after parent drug administration as well as the influence of co-administered compounds on drug bioavailability. Furthermore, the contribution of an enzymatic pathway to the overall elimination can be predicted (Brantley et al., 2014). In vivo animal models are typically used to predict drug pharmacokinetics. After oral administration, the plasma concentration-time profiles are plotted and the area under the curve (AUC) is calculated in order to indicate drug bioavailability. In vivo animal models that are commonly used for pharmacokinetic studies include dogs, monkeys, pigs and rats (Alqahtani et al., 2013). The Sprague-Dawley rat model was selected as the in vivo model for bioavailability experiments in this study.

3.2 MATERIALS

The materials used in this study were indinavir (Crixivan®, Merck, Kenilworth, NJ, USA) (negative control), and verapamil (donated by Novartis, Kemptonpark, South Africa) and ketoconazole (Aspen, Woodmead, South Africa) which served as the two positive control groups for the in vitro bi-directional transport study and pharmacokinetic in vivo study. The Hypoxis hemerocalleida test materials consisted of a solid oral commercial product (product name available on request) purchased from a local health shop (Potchefstroom, South Africa),
dried plant reference material (ChromaDex®, Cape Town, South Africa) and an aqueous extract. The aqueous extract was prepared by weighing approximately 5 g of dried *H. hemerocallis*dea plant material accurately in a 50 ml Erlenmeyer flask. A volume of 10 ml of deionized water was added and the mixture was sonicated at 45 °C for 30 min. After filtering the mixture through a filter paper (No 4, Whatman Ltd, England), the filtrate was kept aside and the residue returned to the flask. This process was repeated twice, where after the filtrates were combined and the residue discarded. The water filtrate was frozen and subsequently freeze-dried overnight and the extract (0.17 g) was stored in a desiccator.

**3.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYTICAL METHOD FOR INDINAVIR SAMPLES FROM THE IN VITRO TRANSPORT STUDIES**

A high performance liquid chromatography (HPLC) analysis method that has been previously developed was used to analyse indinavir concentrations in the *in vitro* transport samples (Roos, 2012; Calitz, 2014). Linearity, accuracy, precision and ruggedness were established to confirm that the method was valid, suitable and applicable for the current study.

**3.3.1 Chromatographic conditions**

The analysis of indinavir was performed on an HP1100n chromatograph which was equipped with a UV detector and Chemstation Rev.A.10.01 Agilent® Technologies data acquisition and analysis software (Hewllet-Packard Paulo Alto, California, USA). The indinavir separation was performed on a Venusil XBP C18(2) column, 150 x 4.6 mm, 5 μm, 100 Å (Agela technologies, Wilmington, Delaware, USA). A mixture of 50% acetonitrile and 0.1% ammonium formate (NH₄HCO₂) in water that has been adjusted to pH 7 was used as mobile phase in isocratic elution mode with an injection volume of 25 μL and a flow rate of 1 ml/min. The UV detector was set on a wavelength of 210 nm and the indinavir retention time was 4.6 min.

**3.3.2 Linearity**

Linearity is defined as the ability of the analytical method to produce test results (i.e. response values) that are directly proportional to the analyte concentration within a given range. The linear regression curve y-intercept and the correlation coefficient (R²) establish an indication of the acceptability of linearity. A value of R² that is higher than 0.998 is considered as evidence of an acceptable fit of the data to the line of regression (Shabir, 2003; Singh, 2013). A standard solution was prepared by dissolving 400 mg indinavir in 500 ml Dulbecco’s Modified Eagle’s Medium (DMEM) (800 μg/ml), which then was further diluted to samples of 80 μg/ml, 8 μg/ml and 0.8 μg/ml. Different volumes (5, 10, 15, 20, 25 μl) of the diluted sample solutions were
injected into the HPLC in order to construct a standard curve by plotting the peak area as a function of concentration.

### 3.3.3 Accuracy

Accuracy is defined as the closeness of the test result of the analytical method to the true value (Shabir, 2003). It is presented by an inverse relation to systematic as well as random errors, where a higher accuracy means a lower error (Singh, 2013). An assay method criteria for accuracy is that $100 \pm 2\%$ at each concentration over the range of $80 – 120\%$ of the target concentration will be the mean recovery (Shabir, 2003). A solution was made by dissolving 400 mg indinavir in 500 ml ultra-pure water. The standard solution consisted of 10 ml of this solution which was then diluted to 100 ml (80 µg/ml). Sample solutions were obtained by diluting 4 ml, 6 ml and 8 ml of the solution to a volume of 100 ml. Concentrations of 32 µg/ml, 48 µg/ml and 64 µg/ml were obtained. The solutions were withdrawn and transferred in vials and placed into the chromatograph auto sampler for analysis.

### 3.3.4 Precision

Precision can be defined as the measurement of the degree of repeatability of an analytical method conducted under normal operation conditions. Precision is usually expressed as the percentage relative standard deviation (%RSD) for a number of samples (Shabir, 2003). Precision is sub-divided into intra-day variation (the evaluation of the same batch on the same day) and inter-day variability. Intra-day precision, which can also be referred to as repeatability, is the results of the method operating under the same conditions over a relatively short period of time (Shabir, 2003; Singh, 2013). Inter-day precision is the results obtained within-laboratory variations which can be due to factors such as different day, equipment and analysts. The acceptance criteria for precision have limits of the RSD for intra-day repeatability of 5% and the inter-day precision of 10% (Shabir, 2003).

Intra-day precision was determined by producing a solution made of 400 mg indinavir dissolved in 500 ml ultra-pure water. The standard solution consisted of 10 ml of this solution which was then diluted to 100 ml (80 µg/ml). Sample solutions were obtained by diluting 4 ml, 6 ml and 8 ml of the solution to a volume of 100 ml. Concentrations of 32 µg/ml, 48 µg/ml and 64 µg/ml were obtained. The solutions were withdrawn at different time points on one day and transferred in vials and placed into the chromatograph auto sampler. Multiple injections were made on one day under the prescribed conditions. Inter-day precision was determined in the same way, but over three consecutive days. A standard solution (80 µg/ml) and three sample solutions (48 µg/ml) were prepared. Volumes of 5, 10, 15, 20 and 25 µl of the standard solution
were injected in duplicate to obtain a calibration curve. Precision was determined by conducting a HPLC analysis on the samples prepared over three consecutive days. A volume of 20 µl of the sample was injected eight times over one day and this procedure took place for three consecutive days.

3.3.5 Ruggedness

Ruggedness is defined by the USP (2015) as the degree of reproducibility of the results obtained by analysis of the same samples under diverse conditions such as different laboratories, analysts and instruments. Ruggedness is expressed as %RSD. The ruggedness acceptance criteria for stability are that sample solutions should not be used for a time period longer than it takes to degrade by 2%. The peak area and retention time with a %RSD of 2% or less is regarded as acceptable for system repeatability. A solution consisting of 400 mg indinavir dissolved in 500 ml ultra-pure water was made. The sample consisted of 5 ml of this solution which was then diluted to 50 ml. The sample was left on the autosampler tray and re-analysed over several time intervals (24 h) to determine the sample stability.

3.4 LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY ANALYTICAL METHOD FOR INDINAVIR SAMPLES FROM THE IN VIVO STUDY

A liquid chromatography tandem mass spectrometry (LC/MS/MS) method was used for the analysis of indinavir in the plasma samples. A stock solution of indinavir was prepared in dimethyl sulfoxide (DMSO) at a concentration of 1 mg/ml. Blank Sprague-Dawley plasma was spiked with the stock solution to attain standard 1 (STD 1) at a concentration of 2000 ng/ml. STD 2 (500 ng/ml), STD 3 (125 ng/ml), STD 4 (32 ng/ml), STD 5 (8 ng/ml) and STD 6 (2 ng/ml) were produced by diluting blank plasma. Quality control samples were prepared in the same pool of rat plasma at concentrations of 1600 ng/ml, 400 ng/ml, 50 ng/ml, 10 ng/ml and 2 ng/ml. The quality control samples and calibration standards were vortexed briefly, aliquotted into labelled polypropylene tubes and then stored at -80°C. Samples above the upper limit of quantification were diluted 4 times with blank plasma and re-analysed in a repeat batch.

3.4.1 Chromatography

The liquid chromatography was performed with a Kinetex F5 (4.6 x 100 mm, 2.6 µm) column using an Agilent 1100 series high performance liquid chromatograph. The mobile phase consisted of a mixture of A and B at 50:50 v/v; where mobile phase A was 0.1% v/v formic acid in water and B was 0.1% v/v formic acid in acetonitrile delivered at a flow rate of 500 µl/min. The column was kept in a column compartment at 40°C. An auto-sampler injected 10 µl onto
the HPLC column. The injection needle was rinsed with mobile phase before each injection for 30 s using the flush port wash station. The samples were cooled to 4 °C while awaiting injection.

3.4.2 Detection

The detection of indinavir and the internal standard (indinavir-d6) was performed on an AB Sciex API 3200 mass spectrometer (ESI in the positive ion mode, MRM) and the settings on the apparatus are summarised in Tables 3.1 and 3.2. 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 620.5 to the product ions at m/z 421.3 and m/z 421.2 for indinavir and the internal standard, respectively.

Table 3.1: Ionisation source setting

<table>
<thead>
<tr>
<th>Electro Spray Ionisation Settings</th>
<th>Value</th>
</tr>
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<tr>
<td>Nebulizer gas (Gas 1) (arbitrary unit)</td>
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</tr>
<tr>
<td>Turbo gas (Gas 2) (arbitrary unit)</td>
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</tr>
<tr>
<td>CUR (curtain gas) (arbitrary unit)</td>
<td>20</td>
</tr>
<tr>
<td>CAD (collision gas) (arbitrary unit)</td>
<td>3</td>
</tr>
<tr>
<td>TEM (source temperature) (°C)</td>
<td>500</td>
</tr>
<tr>
<td>IS (Ion Spray Voltage) (V)</td>
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</tbody>
</table>

Table 3.2: MS/MS detector setting

<table>
<thead>
<tr>
<th>MS/MS Settings</th>
<th>Indinavir</th>
<th>ISTD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protonated molecular mass (m/z)</td>
<td>614</td>
<td>620</td>
</tr>
<tr>
<td>Product ion molecular mass (m/z)</td>
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<td>421</td>
</tr>
<tr>
<td>Dwell time (ms)</td>
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<td>DP (declustering potential) (V)</td>
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</tr>
<tr>
<td>EP (entrance potential) (V)</td>
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<tr>
<td>CEP (collision cell entrance potential) (V)</td>
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</tr>
<tr>
<td>CE (collision energy) (eV)</td>
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<td>45</td>
</tr>
<tr>
<td>CXP (collision cell exit potential) (V)</td>
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<td>8</td>
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<tr>
<td>Scan Type</td>
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<td></td>
</tr>
<tr>
<td>Polarity</td>
<td>Positive</td>
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</tr>
</tbody>
</table>
3.4.3 Extraction from plasma

A volume of 200 µl of ice cold 0.1% v/v formic acid in methanol containing 20 ng/ml indinavir-d6 (ISTD) was added to a 20 µl of plasma sample and vortex mixed for 60 s. This was followed by ultra-sonication for 10 min and centrifugation at 10000 rpm for 10 min. Then 180 µl of the supernatant was transferred into a clean culture tube and evaporated to dryness under nitrogen gas at 40 °C. The residue was reconstituted with 100 µl of 0.1% v/v formic acid in water solution, vortex mixed for 60 s and transferred into a 96-well plate for injection. A volume of 10 µl of the reconstituted solutions were then injected onto the column.

3.5 LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY ANALYTICAL METHOD FOR PHYTOCHEMICAL CHARACTERISATION OF THE HYPOXIS HEMEROCALLIDEA MATERIALS

Ultra-Performance Liquid Chromatography (UPLC) analysis was used to determine the hypoxoside concentration in the selected Hypoxis hemerocallidea materials. The analysis was done with a Waters Acquity Chromatographic system with PDA detector (Waters, Milford, MA, USA). UPLC separation was achieved on an Acquity UPLC BEH C18 column (150 mm x 2.1 mm, i.d., 1.7 µm particle size, Waters) maintained at 40 °C. The mobile phase consisted of 0.1% v/v formic acid in water (solvent A) and acetonitrile (solvent B) at a flow rate of 0.3 ml/min. A gradient elution was used as follows: 85% A: 15% B to 65% A: 35% B in 7 min, changed to 50% A: 50% B in 1 min, keeping for 0.5 min and back to initial ratio in 0.5 min. The running time was 11 min. The standard and samples were injected in the mobile phase with an injection volume of 1.0 µl (full-loop injection). Mass spectrometry was operated in negative ion electrospray mode. Nitrogen (N₂) was used as the desolvation gas. The desolvation temperature was set to 350 °C at a flow rate of 500 L/Hr and the source temperature was 100 °C. The capillary and cone voltages were 2500 and 45 V, respectively. The data was collected between 100 and 1000 m/z.

3.6 PREPARATION OF SOLUTIONS FOR THE IN VITRO TRANSPORT STUDY

3.6.1 Preparation of the indinavir solution (negative control group)

The negative control group for the transport studies consisted of a 200 µM indinavir solution. In order to prepare this solution for the transport study, a Crixivan® capsule (containing 400 mg indinavir) was opened and 2.02 mg of the capsule contents was weighed and added to 10 ml
DMEM (for the permeation study in the A-B direction) and to 10 ml DMEM and HEPES solution (for the permeation study in the B-A direction).

3.6.2 Preparation of verapamil solution (positive control group)

Verapamil is a known P-gp inhibitor and was therefore used as the positive control group for the transport studies. A 100 μM verapamil solution was prepared by dissolving 0.45 mg verapamil in 5 ml DMEM containing 200 μM indinavir for the permeation study in the A-B direction (and 5 ml DMEM and HEPES solution containing 200 μM indinavir for the permeation study in the B-A direction).

3.6.3 Preparation of the test solutions for the transport studies

A final concentration of 500 μg/ml of each of the selected *H. hemerocallidea* materials were used for the transport studies (Fasinu *et al.*, 2013). A double strength of this concentration was prepared as it was diluted when combined with the indinavir solution. For the commercial product, this was obtained by weighing 6.729 mg of the capsule contents (to compensate for additives in the capsule formulation according to the label) and adding it to 5 ml DMEM for the permeation study in the A-B direction and to 5 ml DMEM and HEPES solution for the permeation study in the B-A direction. For the *H. hemerocallidea* aqueous extract and dried plant reference material, 5 mg of each of these materials were added to 5 ml DMEM for the permeation study in the A-B direction and 5 mg were added to 5 ml DMEM and HEPES solution for the permeation study in the B-A direction. These materials were dissolved in combination with 200 μM indinavir which was applied to the donor chamber of the Transwell® plates containing Caco-2 cell monolayers during the bi-directional transport study.

3.7 TRANSPORT STUDIES

3.7.1 Caco-2 cell culturing

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC), by Sigma Aldrich, Johannesburg, South Africa. The Caco-2 cells were subsequently cultured in high-glucose Dulbecco’s Modified Eagles Medium (DMEM) (Separations, Randburg, South Africa) supplemented with 10% v/v foetal bovine serum (The Scientific Group, Johannesburg, South Africa), 1% v/v non-essential amino acids (NEAA) (Whitehead Scientific, Cape Town, South Africa), 1% v/v penicillin/streptomycin (Separations, Johannesburg, South Africa), 1% v/v of 2 mM L-glutamine (Whitehead Scientific, Cape Town, South Africa) and 1% v/v amphotericin B (250 μg/ml) (The Scientific Group, Randburg, South Africa).
The Caco-2 cells were cultured at a temperature of 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 under sterile conditions in a laminar flow hood. The cells were examined by means of a light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) prior to exchange of the growth medium. The percentage confluency was estimated and the absence of any contamination was ensured by macroscopic visual evaluation. The growth medium was decanted and removed from the cell culture flask and thereafter replaced with 10 ml pre-warmed growth medium by means of a serological pipette and pipettor.

3.7.2 Sub-culturing of the Caco-2 cells

The Caco-2 cells were sub-cultured by means of trypsinisation upon reaching 50% confluency. The growth medium and phosphate buffered saline (PBS) (Sigma Aldrich, Johannesburg, South Africa) solutions 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 by using a pipettor and serological pipettes. A volume of 3 ml Trypsin-Versene mixture (Whitehead scientific, Cape Town, South Africa) was supplemented to the cell culturing flask and the mixture was evenly distributed on the cell layer. The cell flask was placed in the CO₂ incubator and incubated for 5 min at 37°C. After the 5 min incubation time, the flask was removed and tapped. After ensuring 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 means of a pipette to ensure that all the cells were rinsed from the bottom of the flask. The cell suspension was then divided to a ratio of 1:4 and hereafter 10 ml pre-warmed growth medium was added to each flask. The flasks were returned back to the CO₂ incubator to grow under normal cell culturing conditions.

3.7.3 Seeding of Caco-2 cells

Caco-2 cells were seeded onto Transwell® 6-well membrane filters (Corning Costar® Corporation, Tewksbury, USA) with a surface area of 4.67 cm² and a pore diameter of 0.4 µm. As described previously, the cell suspension was obtained by trypsinisation with Trypsin-Versene. After cell detachment, 6 ml of pre-warmed growth medium was added to the flask. The cell suspension was extensively agitated with a pipette to ensure complete cell detachment and de-agglomeration in order to form a suspension consisting of single cells. This single cell suspension was then transferred to a 50 ml tube. A Pasteur pipette was used to agitate the cell suspension to make sure that a homogenous cell distribution is present. A haemocytometer was used to count the cells in the suspension after Trypan blue was added. Using a pipette,
10 μl cell suspension together with 15 μl PBS and 25 μl Trypan blue (Sigma Aldrich, Johannesburg, South-Africa) were mixed in a 2.5 ml Eppendorf tube in order to count the cells. The mixture was left for 3 min and thereafter 10 μl of the mixture was placed on either side of the cover slip of the haemocytometer. A light microscope (Nikon Eclipse TS100/TS100F, Nikon Instruments, Tokyo, Japan) was used to count the cells. The 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 and this number was then multiplied with the dilution factor (5 x 10⁴). This 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 for seeding onto the Transwell® filter membranes.

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

3.7.4 Bi-directional transport studies

The transepithelial electrical resistance (TEER) of each cell monolayer was measured with a Millcell ERS II meter (Millipore, Billerica, Massachusetts, USA) at the start of the transport study, which served as an indication of the integrity of the cell monolayers. A TEER reading higher than 250 Ω (equivalent to 1167.5 Ω/cm²) was required before conducting the transport study.

3.7.4.1 Transport in the apical-to-basolateral direction

For the transport study in the apical to basolateral (AP-BL) direction, the growth medium was removed from the basolateral chambers of the Transwell® plates using an aspirator. A volume of 2.5 ml pre-heated transport medium (DMEM) buffered with 25 mM 2-4-2-hydroxyethyl piperazin-1-yl-ethanesulfonic acid (HEPES) was added to the basolateral compartments and then the plate was placed in the CO₂ incubator at 37°C for 30 min. The growth medium was hereafter removed from the apical chambers and replaced with 2.5 ml test solutions (i.e. indinavir with or without the selected H. hemerocallidea material) in DMEM. The negative control group consisted of indinavir alone (200 μM) and indinavir combined with verapamil (100 μM) (a known P-gp inhibitor) as the positive control group. A sample volume of 200 μl of each test solution was withdrawn at time 0 min. Samples (200 μl) were withdrawn from the basolateral chambers at time intervals of 20, 40, 60, 80, 100, and 120 min for the transport study in the A-B direction. Each sample withdrawn was replaced with an equal volume of pre-
warmed DMEM buffered with HEPES. The plates were incubated in a CO₂ incubator at 37°C between the withdrawals. The TEER was measured again at the end of the AP-BL transport study to confirm that the cell monolayer integrity was still intact after the cells have been exposed to the test and control solutions. Samples were analysed by a validated high performance liquid chromatography (HPLC) analysis method to determine the indinavir concentration.

3.7.4.2 Transport in the basolateral-to-apical direction

For the transport in the basolateral to apical (BL-AP) direction, the growth medium was removed from the apical chambers of the Transwell® plates using an aspirator. A volume of 2.5 ml pre-warmed DMEM was added to the apical chambers of the Transwell® plates and the plate was thereafter incubated in a CO₂ incubator for 30 min at 37°C. The growth medium was removed from the basolateral chambers and replaced with 2.5 ml of each test solution (i.e. indinavir with or without the selected *H. hemerocalleida* material) in DMEM buffered with HEPES. Indinavir alone served as the negative control group, while indinavir combined with verapamil served as the positive control group. A test solution sample (200 µl) was withdrawn at time 0 min, while samples (200 µl) were withdrawn from the apical chambers at the predetermined time intervals of 20, 40, 60, 80, 100, 120 min for the transport study in the B-A direction and immediately replaced with an equal volume of pre-warmed DMEM. The TEER was measured again at the end of the BL-AP transport study, which provided an indication of the cell monolayers integrity. The samples were analysed by a validated high performance liquid chromatography (HPLC) analysis method to determine the indinavir concentration.

3.8 *IN VIVO* PHARMACOKINETIC STUDY

3.8.1 Animal selection and study design

A total of 45 male Sprague-Dawley rats weighing 250 - 300 g were randomly selected and divided into 9 different groups (i.e. 3 control groups, 3 acute experimental groups and 3 chronic experimental groups), which consisted of 5 animals per group (as schematically illustrated in Figure 3.1). During the acute herb-drug pharmacokinetic interaction study, a single administration of each test solution was given to each rat in concentrations outlined in Figure 3.1 and described in section 3.8.2 below. During the chronic herb-drug pharmacokinetic interaction study, daily administrations were given over a total period of 14 days in concentrations outlined in Figure 3.1 and described in section 3.8.2 below (de Peyster *et al.*, 2003; Husna *et al.*, 2013; Robertsa *et al.*, 2016).
This *in vivo* study in Sprague-Dawley rats was approved by the Animal Ethics Committee of the Faculty of Health Sciences of the University of Cape Town (FHC AEC Ref no: 015/041).

**Figure 3.1:** Schematic illustration of the layout of the *in vivo* study design in Sprague-Dawley rats. Dosing concentrations were selected based on previous studies (Van Wauwe *et al*., 1990; Cools *et al*., 1992; Mogatle *et al*., 2008; Choi *et al*., 2009; Ho *et al*., 2009)

### 3.8.2 Administration of test solutions to rats

The test solutions were administered by means of an oral gavage technique at a volume of 500 µl per animal. The test solutions consisted of *H. hemerocallidea* materials (i.e. *H. hemerocallidea* reference plant material, an aqueous extract and commercial oral product) at a concentration of 15 mg/kg (Mogatle *et al*., 2008), together with indinavir (40 mg/kg) in distilled water. All *H. hemerocallidea* materials were administrated in the same concentration to the animals in order to measure the effects of the difference in chemical composition (e.g. hypoxoside content) of the selected materials on indinavir bioavailability. Indinavir (40 mg/kg) administered alone served as the negative control (Ho *et al*., 2009). The positive control for metabolism inhibition consisted of indinavir (40 mg/kg) with ketoconazole (40 mg/kg) (Van...
Wauwe et al., 1990; Cools et al., 1992) and the positive control for transport (efflux inhibition) was indinavir (40 mg/kg) with verapamil (9 mg/kg) (Choi et al., 2009).

3.8.3 Blood sampling

Blood samples (200 µl) were collected from the tail veins of the animals at the predetermined time intervals of 0, 0.5, 1, 2, 4, 8 and 24 h after each experimental and control test solution. Sample tubes were sprayed with heparin beforehand, which served as an anti-coagulant. The blood samples were then centrifuged for 8 min at 14 000 rpm, after which the plasma was recovered from each blood sample. The plasma samples were kept at -80°C until the indinavir analysis was done with LC/MS/MS (as describe in section 3.3).

3.9 DATA ANALYSIS

3.9.1 Transport data for indinavir

The apparent permeability coefficient \( P_{app} \times 10^{-6} \text{cm/s} \) values for indinavir transport in the AP-BL and BL-AP direction across the Caco-2 monolayers were calculated according to the following equation (Tarirai et al., 2012).

\[
P_{app} = \frac{dQ}{dt} \left( \frac{1}{A \cdot C_0 \cdot 60} \right)
\]  
(Eq. 1)

Where \( P_{app} \) is the apparent permeability coefficient \( \text{(cm.s}^{-1}\text{)} \), \( dQ/dt \) is the permeability rate (amount permeated per minute), \( A \) is the diffusion area of the membrane \( \text{(cm}^2 \text{)} \) and \( C_0 \) is the initial concentration of the model drug.

The efflux ratio (ER) values demonstrate any asymmetry regarding indinavir directional transport in combination with the \( H. \) hemerocallidea materials. The ER was calculated according to the following equation (Tarirai et al., 2012):

\[
ER = \frac{P_{app}(BL-AP)}{P_{app}(AP-BL)}
\]  
(Eq. 2)

Where \( P_{app} \) (BL-AP) is the permeability coefficient for the permeation in the basolateral to the apical direction and \( P_{app} \) (AP-BL) is the permeability coefficient for the permeation in the apical to basolateral direction.

3.9.2 Pharmacokinetic data analysis for \textit{in vivo} study model

The bioavailability profiles were created by using WinNonlin software (Pharsight Corporation, California USA) to gather the pharmacokinetic parameters of relevance (i.e. peak plasma
concentration ($C_{\text{max}}$) and area under the curve (AUC)). The relative bioavailability ($F_{\text{rel}}$) of indinavir was calculated by the following equation:

$$F_{\text{rel}} = \frac{[\text{AUC}]A}{[\text{AUC}]B}$$  

Eq. (4)

Where $[\text{AUC}]A$ represents the area under the curve for indinavir in the presence of the experimental material (i.e. *H. hemerocallidea* materials) and $[\text{AUC}]B$ represents the area under the curve for indinavir alone.

### 3.9.3. Statistical Data Analysis

Data analyses were performed by using STATISTICA Ver 12. Analysis of variances (ANOVA's) were conducted with Tukey's Honest significant post-hoc tests and statistically significant differences were accepted when $p < 0.05$. All results obtained were verified with nonparametric Kruskall-Wallis and Dunn's post-hoc tests.
CHAPTER 4
RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this study, both in vitro and in vivo pharmacokinetic herb-drug interactions between selected Hypoxis hemerocallidea materials (i.e. an aqueous extract, dried plant reference material and commercial oral product) and indinavir were investigated. The Caco-2 cell line was employed as the in vitro model to investigate the potential modulation of indinavir transport by the selected H. hemerocallidea materials, specifically efflux modulation concerning P-glycoprotein (P-gp) inhibition. The Sprague-Dawley rat model was employed as the in vivo model to study the possible influence of the selected H. hemerocallidea materials on the bioavailability of indinavir after oral administration.

The in vivo bioavailability study was divided into acute and chronic sub-studies. During the acute study, each rat received a single administration of each H. hemerocallidea experimental test solution together with indinavir, while multiple administrations were given daily over a total period of 14 days during the chronic study.

METHOD VALIDATION RESULTS

4.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Linearity, accuracy, precision and ruggedness for the indinavir analysis method were confirmed even though a previously validated HPLC analytical method was used.

4.2.1 Linearity

Linearity is defined as the method’s ability to produce a response that is directly proportional to the analyte concentration within a given range (Shabir, 2003; Singh, 2013). A correlation coefficient ($R^2$) that is higher than 0.998 is indicated as an acceptable fit of the data to the line of response plotted as a function of concentration. An $R^2$ value of 0.999 was obtained for the calibration graph (Figure 4.1) that was constructed for indinavir with the HPLC method employed in this study. It can be concluded that the HPLC method used for analysis of indinavir in the in vitro transport samples complies with the criteria for linearity.
Figure 4.1: Calibration curve obtained for indinavir with high performance liquid chromatography where peak area is plotted as a function of concentration

4.2.2 Accuracy

Accuracy is defined as the closeness of the obtained test results by the analytical method to the true value (Shabir, 2003). Recovery must be between 98% to 102% and over the range of 80% to 120% of the sample concentration. The HPLC method employed for indinavir analysis yielded a mean recovery of 101.4% with a %RSD of 1.2% as indicated in Table 4.1.

Table 4.1: Data obtained for accuracy of indinavir analysis with high performance liquid chromatography

<table>
<thead>
<tr>
<th>Concentration spiked (µg/ml)</th>
<th>Area 1 (µg/ml)</th>
<th>Area 2 (µg/ml)</th>
<th>Mean area (µg/ml)</th>
<th>Recovery (µg/ml)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32.0</td>
<td>1490.8</td>
<td>1489.4</td>
<td>1490.1</td>
<td>31.9</td>
<td>99.8</td>
</tr>
<tr>
<td>32.0</td>
<td>1487.6</td>
<td>1490.0</td>
<td>1488.8</td>
<td>31.9</td>
<td>99.7</td>
</tr>
<tr>
<td>32.0</td>
<td>1485.7</td>
<td>1487.0</td>
<td>1486.4</td>
<td>31.9</td>
<td>99.6</td>
</tr>
</tbody>
</table>
4.2.3 Precision

Precision can be defined as the measurement of the degree of repeatability of an analytical method conducted under normal operation and is usually expressed in terms of the %RSD (Shabir, 2003). The acceptance criterion for inter-day precision is given by the USP (2015) as a % RSD ≤ 5%.

**Table 4.2:** Percentage recovery (%) obtained for inter-day precision of indinavir analysis by high performance liquid chromatography

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Between days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>101.77</td>
<td>99.50</td>
<td>99.27</td>
<td>100.18</td>
</tr>
<tr>
<td>SD</td>
<td>0.10</td>
<td>0.07</td>
<td>0.12</td>
<td>1.13</td>
</tr>
</tbody>
</table>

*SD refers to the standard deviation, **% RSD refers to the percentage relative standard deviation
Table 4.3: Statistical analysis of data for inter-day precision

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between days</td>
<td>11.503</td>
<td>2.0</td>
<td>5.751</td>
<td>405.843</td>
<td>8.552E-05</td>
</tr>
<tr>
<td>Within days</td>
<td>0.085</td>
<td>6.0</td>
<td>0.014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>11.588</td>
<td>8.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The % RSD of the inter-day analysis of indinavir was 1.13%, which means the repeatability is within the specified limits. Furthermore, the statistical analysis of the inter-day precision data revealed that there is no statistical significant difference between the values obtained on different days.

4.2.4 Ruggedness

Ruggedness is defined by the USP (2015) as the degree of reproducibility of the results obtained by analysis of the same samples under diverse conditions such as different laboratories, analysts and instruments. Ruggedness is expressed in terms of %RSD, which should be ≤ 2%, between measurements made under diverse conditions. The ruggedness acceptance criteria for stability are that sample solutions should not be used for a time period longer than it takes to degrade by 2%. The sample solution proved to be stable over a period of 24 h with a %RSD value of 1.57 % when measured under different conditions.

4.3 LIQUID CHROMATOGRAPHY - MASS SPECTROMETRY ANALYTICAL METHOD FOR PHYTOCHEMICAL CHARACTERISATION OF THE HYPOXIS HEMEROCALLIDEA MATERIALS

TIC and UV chromatograms obtained for the various selected H. hemerocallidea materials are shown in the Figures 4.2 to 4.5 below.
Figure 4.2: TIC (A) and UV (B) chromatograms of *Hypoxis hemerocallidea* reference plant material

Hypoxoside peak
**Figure 4.3**: TIC (A) and UV (B) chromatograms of *Hypoxis hemerocallis*de* a*queous extract
Figure 4.4: TIC (A) and UV (B) chromatograms of *Hypoxis hemerocallidea* commercial product.
Figure 4.5: TIC (A) and UV (B) chromatograms of the marker molecule, hypoxoside (retention time = 4.37 min)

The quantities of the marker molecule, hypoxoside, as determined by UHPLC analysis in each of the selected *H. hemerocallidea* materials are shown in Table 4.4.
Table 4.4: Quantity of hypoxoside in each of the selected *Hypoxis hemerocallidea* materials

<table>
<thead>
<tr>
<th>Selected <em>H. hemerocallidea</em> material</th>
<th>Hypoxoside (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference plant material</td>
<td>13.3</td>
</tr>
<tr>
<td>Commercial product</td>
<td>0.6</td>
</tr>
<tr>
<td>Aqueous extract</td>
<td>151.7</td>
</tr>
</tbody>
</table>

UHPLC analysis demonstrated that all three the selected *H. hemerocallidea* materials contained the marker molecule, hypoxoside, although in different quantities. As expected, the aqueous extract contained a higher quantity of hypoxoside compared to that of the dried plant reference material and commercial product. The relatively low hypoxoside content of the commercial product can possibly be explained by a low content in the original plant material used or degradation of this phytochemical during production and/or storage of the product.

**EXPERIMENTAL RESULTS**

**4.4 BI-DIRECTIONAL TRANSPORT STUDIES**

The TEER of Caco-2 cell monolayers on Transwell® 6-well plates was measured at the beginning, before commencing the transport study, and a TEER value higher than 250 Ω (i.e. equal to 1167.5 Ω/cm²) served as an indication of cell-monolayer integrity. The average TEER values were all above this prescribed value, which can be seen in Table 4.5. The TEER values obtained at the end of the transport study (120 min) were in some instances slightly lower, but remained constant or even increased slightly. These changes in TEER values could be attributed to possible modulation effects on the tight junctions between adjacent epithelial cells when the Caco-2 cell monolayers were exposed to the test solutions.
Table 4.5: TEER values of Caco-2 cell monolayers taken at the beginning (0 min) and end (120 min) of the bi-directional transport study of indinavir in the presence of the selected *H. hemerocallidea* materials

<table>
<thead>
<tr>
<th>Experimental groups</th>
<th>Average TEER value (Ω)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP-BL direction</td>
</tr>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>Indinavir alone (negative control)</td>
<td>252.7</td>
</tr>
<tr>
<td>Indinavir with verapamil (positive control)</td>
<td>279.0</td>
</tr>
<tr>
<td>Indinavir with <em>H. hemerocallidea</em> commercial product</td>
<td>258.0</td>
</tr>
<tr>
<td>Indinavir with <em>H. hemerocallidea</em> aqueous extract</td>
<td>261.7</td>
</tr>
<tr>
<td>Indinavir with <em>H. hemerocallidea</em> plant reference material</td>
<td>284.0</td>
</tr>
</tbody>
</table>

The percentage transport of indinavir was calculated in both directions, apical to basolateral (AP-BL) and basolateral to apical (BL-AP) and these values were plotted as a function of time as shown in Figures 4.6 and 4.7. This percentage transport is an indication of the amount of indinavir crossing the cell monolayer into the acceptor chamber in relation to the indinavir concentration applied to the donor chamber. Active efflux of the investigated compound is demonstrated when the transport percentage is higher in the BL-AP direction compared to that in the AP-BL direction.
Figure 4.6: Percentage of indinavir transport across the monolayers of Caco-2 cells in the apical to basolateral (AP-BL) direction plotted as a function of time (n = 3, error bars indicate standard deviation)

Figure 4.7: Percentage of indinavir transport across the monolayers of Caco-2 cells in the basolateral to apical (BL-AP) direction plotted as a function of time (n = 3, error bars indicate standard deviation)
It is clear from Figures 4.6 and 4.7 that verapamil (positive control group), increased the uptake of indinavir in the AP-BL direction compared to the indinavir alone (negative control group) and resulted in the highest percentage indinavir transport in the AP-BL direction. Verapamil is a known P-gp inhibitor and therefore decreased the efflux of indinavir in the BL-AP direction. Furthermore, the transport results in the AP-BL direction demonstrated an overall increase in the uptake of indinavir in the presence of the *H. hemerocallidea* materials (i.e. oral commercial product, an aqueous extract and dried plant reference material) when compared to that of the negative control group. Out of the selected *H. hemerocallidea* materials, the commercial product produced the most pronounced effect regarding indinavir uptake. The transport results in the BL-AP direction (Figure 4.7) demonstrated a prominent decrease of efflux of indinavir in the presence of all the *H. hemerocallidea* materials. The commercial product produced the most pronounced effect on indinavir efflux, followed by the reference plant material and aqueous extract.

The $P_{app}$ values for indinavir in both directions across Caco-2 cell monolayers in the presence of each selected *H. hemerocallidea* material as well as the control groups are demonstrated in Figure 4.8.
Figure 4.8: $P_{\text{app}}$ values for indinavir in both directions across Caco-2 cell monolayers alone (negative control group) and in combination with the selected Hypoxis hemerocallidea materials as well as the positive control group (indinavir with verapamil). $P_{\text{app}}$ bar graphs for A-B direction are indicated by dark colours and $P_{\text{app}}$ bar graphs for B-A direction are indicated by light colours. ($n = 3$, error bars indicate standard deviation)

The $P_{\text{app}}$ values represent the transport (diffusion rate, cm/s) of the compound across the Caco-2 cell monolayer normalised for absorption surface and initial concentration applied (Tarirai et al., 2012). Figure 4.8 confirmed an increase in the uptake of indinavir in the presence of all the H. hemerocallidea materials with the commercial product exhibiting the highest effect. An obvious decrease in the $P_{\text{app}}$ value of indinavir in the BL-AP direction demonstrates inhibition of efflux produced by the positive control group (i.e. verapamil), as well as the experimental groups (i.e. selected H. hemerocallidea materials).

The efflux ratio (ER) values for indinavir for all the experimental and control groups are shown in Table 4.6.
Table 4.6: Efflux ratio (ER) values for indinavir in the absence (negative control) and presence of the selected *Hypoxis hemerocallidea* materials as well as verapamil (positive control)

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>ER ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indinavir alone (negative control)</td>
<td>6.27 ± 4.75</td>
</tr>
<tr>
<td>Indinavir with verapamil (positive control)</td>
<td>1.10 ± 0.40</td>
</tr>
<tr>
<td>Indinavir with <em>H. hemerocallidea</em> commercial product</td>
<td>1.42 ± 0.33</td>
</tr>
<tr>
<td>Indinavir with <em>H. hemerocallidea</em> aqueous extract</td>
<td>2.37 ± 1.70</td>
</tr>
<tr>
<td>Indinavir with <em>H. hemerocallidea</em> reference material</td>
<td>2.61 ± 1.02</td>
</tr>
</tbody>
</table>

*SD = standard deviation

The ER values establish any asymmetry regarding indinavir directional transport in combination with the *H. hemerocallidea* materials. An ER value >> 1 indicates that a compound undergoes active efflux transport in the secretory direction. It is clear from Table 4.6 that indinavir is a substrate for P-gp related efflux due to the relatively high ER value (6.27) obtained for the negative control group, which was reduced to just above unity (1.10) by verapamil, a known P-gp inhibitor. Higher uptake transport of a drug is expected when the efflux of a drug compound from the epithelium is decreased. A higher drug bioavailability is consequently expected as a higher drug concentration will most probably reach the systemic circulation due to the result of efflux inhibition.

The pronounced reduction in ER values clearly indicate that active efflux transport of indinavir was inhibited by the selected *H. hemerocallidea* materials, although not to the same extent of efflux inhibition observed for verapamil.

From the *in vitro* transport results obtained in this current study and metabolism results obtained in previous *in vitro* studies, it can be expected that co-administration of indinavir (as well as other anti-retroviral drugs that are substrates for P-gp) with *H. hemerocallidea* extracts and plant materials will most probably lead to enhanced blood plasma levels due to efflux inhibition.


### 4.5 IN VIVO PHARMACOKINETIC STUDIES

The plasma concentration time curves for indinavir in Sprague-Dawley rats in the absence (control group) and presence of the selected *H. hemerocalidea* materials administered after a single administration (acute study) and after multiple administrations over 14 days (chronic study) are shown in Figures 4.9 and 4.10, respectively.

**Figure 4.9:** Plasma concentration time curves of indinavir in Sprague-Dawley rats in the absence and presence of the various *Hypoxis hemerocalidea* materials for the acute study (single administration). $n = 5$, error bars indicate standard deviation.
Figure 4.10: Plasma concentration time curves of indinavir in Sprague-Dawley rats in the absence and presence of the various *Hypoxis hemerocallidea* materials for the chronic study (14 days). \( n = 5 \), error bars indicate standard deviation.

The bioavailability parameters for indinavir in the absence and presence of the selected *H. hemerocallidea* test materials after a single administration (acute study) and multiple administrations over 14 days (chronic study) are listed in Table 4.7. The bioavailability parameters include the area under the curve extrapolated to infinity (AUC\(_{0-\infty}\)) as well as maximum plasma concentration (C\(_{\text{max}}\)) and relative bioavailability (F\(_{\text{rel}}\)) values.
Table 4.7: Biopharmaceutical parameters for indinavir administrated to rats in the absence and presence of the selected *H. hemerocallidea* materials

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>AUC$_{0-\infty}$ (ng.min/ml)</th>
<th>C$_{\text{max}}$ (ng/ml)</th>
<th>F$_{\text{rel}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indinavir alone (negative control)</td>
<td>1371.0 ± 389.8</td>
<td>1108.6 ± 484.3</td>
<td>1.00</td>
</tr>
<tr>
<td>Indinavir with ketoconazole (positive control)</td>
<td>1428.6 ± 303.4$^*$</td>
<td>689.0 ± 354.0$^*$</td>
<td>1.04</td>
</tr>
<tr>
<td>Indinavir with verapamil (positive control)</td>
<td>1501.3 ± 462.2</td>
<td>644.1 ± 488.8</td>
<td>1.10</td>
</tr>
<tr>
<td>Indinavir with aqueous extract (acute)</td>
<td>1458.3 ± 512.6</td>
<td>813.6 ± 563.5</td>
<td>1.06</td>
</tr>
<tr>
<td>Indinavir with aqueous extract (chronic)</td>
<td>1847.2 ± 517.2</td>
<td>1483 ± 555</td>
<td>1.35</td>
</tr>
<tr>
<td>Indinavir with commercial product (acute)</td>
<td>1564.4 ± 638.2</td>
<td>928 ± 493</td>
<td>1.14</td>
</tr>
<tr>
<td>Indinavir with commercial product (chronic)</td>
<td>1530.7 ± 375.7</td>
<td>1333 ± 492</td>
<td>1.12</td>
</tr>
<tr>
<td>Indinavir with reference plant material (acute)</td>
<td>1456.6 ± 316.3</td>
<td>1022 ± 412</td>
<td>1.06</td>
</tr>
<tr>
<td>Indinavir with reference plant material (chronic)</td>
<td>2226.3 ± 930.6</td>
<td>2001 ± 955</td>
<td>1.62</td>
</tr>
</tbody>
</table>

*average and standard deviation values after removal of an outlier

From Table 4.7, it is clear that co-administration of the selected *H. hemerocallidea* test materials increased the bioavailability (AUC$_{0-\infty}$) of indinavir in rats in both the acute and chronic studies compared to the negative control group (indinavir alone), albeit not statistically significantly ($p \geq 0.05$). In accordance with the *in vitro* transport results, where the *H. hemerocallidea* commercial product increased the AP-BL transport the highest of all three the selected materials, it also exhibited the highest enhancement of indinavir bioavailability (AUC$_{0-\infty}$) in the rats during the acute study when compared to the other two *H. Hemerocallisidea* materials. This acute effect correlates well with the *in vitro* transport results in terms of the relatively high efflux inhibition as expressed by the efflux ratio results (i.e. ER = 1.42 for the commercial product compared to ER = 2.37 and ER = 2.61 for the aqueous extract and reference material, respectively). On the
other hand, the aqueous extract and reference plant material showed higher bioavailability enhancement effects during the chronic study when compared to that of the commercial product during the chronic study.

In addition, a higher effect on indinavir bioavailability enhancement was obtained during the chronic study for the aqueous extract and reference plant material compared to the bioavailability enhancement effect during the acute study, which was not observed for the commercial product. This can possibly be explained by the hypoxoside content of the different materials investigated in this study (Table 4.4). CYP3A4 can be inhibited by both hypoxoside and rooperol \textit{in vitro}, but since only rooperol is absorbed into the systemic circulation it is this compound that will cause enzyme inhibition \textit{in vivo} rather than hypoxoside. Hypoxoside is converted to rooperol in the gastrointestinal tract after oral ingestion (Mogatle et al., 2008). Multiple administrations during the chronic study of the aqueous extract and reference plant material provided potentially higher cumulative levels of rooperol compared to that after a single administration during the acute study, which led to an increased effect on indinavir bioavailability. Since the commercial product contained a relatively low concentration of hypoxoside (0.6 mg/g), a relatively low level of hypoxoside was available for conversion to rooperol. This low level may be eliminated before the next dose and therefore would not lead to a cumulative increase during the chronic study, which may explain why there is a negligible difference between the acute and chronic study in terms of the effect on indinavir bioavailability. These results therefore indicate that longer term exposure to \textit{H. hemerocallidea} materials may have a higher effect on the pharmacokinetics of indinavir and also potentially on other drugs that are substrates of CYP450 enzymes and P-gp efflux transporters, depending on the concentration of hypoxoside in the product that is consumed concomitantly with the drug. On the other hand, \textit{H. hemerocallidea} contains other bioactive chemical compounds such as phytosterols and sterolins which could have affected the outcome (Owira & Ojewole, 2009).

The expression of CYP450 enzymes is influenced by a combination of different factors and mechanisms. A change in CYP450 enzyme expression would possibly alter the metabolism of drug compounds (Zanger & Schwab, 2013). Long term exposure to CYP450 inhibition (as in this study) could lead to changes in the expression of these enzymes therefore influencing metabolism.

### 4.6 CONCLUSIONS

The selected \textit{H. hemerocallidea} materials inhibited \textit{in vitro} efflux of indinavir and enhanced \textit{in vivo} bioavailability of indinavir. The effect \textit{in vivo} was more prominent after multiple administrations (i.e. chronic study) compared to a single administration (i.e. acute study) for the
*H. hemerocallidea* materials. This could possibly be related to the differences found in hypoxoside concentration of the *H. hemerocallidea* materials. Concomitant administration of *H. hemerocallidea* with indinavir causes pharmacokinetic interactions, especially during long term use depending on the concentration of hypoxoside in the material.
CHAPTER 5

FINAL CONCLUSIONS AND FUTURE RECOMMENDATIONS

5.1 INTRODUCTION

Concomitant use of herbal medicines and other drugs have the potential to produce herb-drug pharmacokinetic and pharmacodynamic interactions. Phytochemicals in herbal medicines can cause these pharmacokinetic interactions with other drugs when co-administrated. Pharmacokinetic interactions occur by means of induction and/or inhibition of intestinal efflux proteins such as P-gp or multiple resistance proteins, as well as modulation of intestinal and hepatic metabolising enzymes, specifically the CYP450 super family. These pharmacokinetic interactions may lead to increased or decreased bioavailability of the drug. Consequences of these bioavailability changes may either be adverse effects due to increased drug plasma levels or lack of pharmacological responses due to decreased drug plasma levels.

_Hypoxis hemerocallidea_ is a popular traditional herbal medicine in the southern Africa region. Traditionally, the corms of this plant were administered orally after boiling it in water. For many years, this plant has been used for the treatment or management of HIV/AIDS related diseases as well as for the treatment of cancers, heart failures, nervous disorders, immune-related illnesses and urinary tract infections. Furthermore, _H. hemerocallidea_ has also been used for the treatment of benign prostate hyperplasia, as an anti-inflammatory agent, anti-oxidant, anti-convulsant and as an anti-diabetic agent. Hypoxoside is considered to be one of the most important phytochemicals with regards to the medicinal value of this plant. Although _in vivo_ data is lacking, _in vitro_ observations seemed to suggest the possibility of pharmacokinetic interactions between anti-retroviral drugs and African potato.

Obtaining new knowledge with respect to pharmacokinetic interactions between _H. hemerocallidea_ and anti-retroviral drugs can contribute to better patient management and to give the correct advice to patients that use herbal medicines together with prescribed chronic medication. This study aimed at identifying pharmacokinetic interactions between different _H. hemerocallidea_ materials and indinavir in both _in vitro_ and _in vivo_ studies. The study specifically measured the effect of a commercial product containing _H. hemerocallidea_ plant material on indinavir pharmacokinetics, which was compared to the effects of an aqueous extract and a reference dried plant material.
5.2 FINAL CONCLUSIONS

The results from this study indicate that the selected *H. hemerocallidea* materials caused inhibition of efflux of indinavir *in vitro*, with the commercial product demonstrating the most pronounced effect (although it contained the lowest concentration of hypoxoside). This finding indicated that other components present in the commercial product may also have contributed to efflux inhibition of indinavir in the Caco-2 cell model. The *in vitro* result of a potential pharmacokinetic interaction with respect to efflux inhibition, indicated an increase in the bioavailability of indinavir can be expected when co-administrated with *H. hemerocallidea* materials. In addition, previous *in vitro* metabolism studies have also demonstrated the possibility of *H. hemerocallidea* inhibiting CYP450 enzymes (Nair et al., 2007).

Indeed, as predicted from the results of the *in vitro* study, an increase in indinavir bioavailability in the presence of the selected *H. hemerocallidea* materials was observed *in vivo* in Sprague-Dawley rats for both the acute and chronic studies. The commercial product exhibited the highest enhancement of indinavir bioavailability in the rats during the acute study when compared to the other two *H. Hemerocallidea* materials. This acute effect correlates well with the results obtained during the *in vitro* bi-directional transport study. On the other hand, the aqueous extract and reference plant material displayed higher bioavailability enhancement effects of indinavir during the chronic study when compared to the commercial product. This can possibly be explained by the hypoxoside content of the different materials investigated in this study. Hypoxoside is converted to rooperol in the gastrointestinal tract after oral ingestion and absorbed into the system circulation, which possibly causes CYP3A4 enzyme inhibition. Multiple administrations during the chronic study of the aqueous extract and reference plant material provided potentially higher cumulative levels of rooperol compared to that after a single administration during the acute study. Furthermore, longer exposure of the active ingredients at higher levels most probably modulated the metabolism to a larger extent. This resulted in an increased effect on indinavir bioavailability. The commercial product contained a relatively low hypoxoside concentration, which resulted in a lower level of hypoxoside converted to rooperol.

The *in vivo* results indicated that a longer term exposure to *H. hemerocallidea* materials may have a higher effect on the pharmacokinetics of indinavir (which also depended on the hypoxoside concentration). This pharmacokinetic interaction may also potentially be relevant to other drugs that are substrates of CYP450 enzymes and P-gp efflux transporters. Patients are therefore advised to avoid long term use of this herbal medicine together with anti-retroviral treatment.
5.3 FUTURE RECOMMENDATIONS

This study mainly focused on the hypoxoside concentration present in the selected *H. hemerocallidea* materials. *H. hemerocallidea* contains other bioactive chemical compounds and it is suggested that these other chemical compounds be extracted to evaluate the effect of each of these compounds individually on indinavir pharmacokinetics. More *H. hemerocallidea* materials should also be investigated, such as a methanol extract as well as other commercial products available on the market that may have different chemical compositions when compared to those investigated in this study. It is further recommended that the half-life of hypoxoside should be taken into account when investigating its cumulative effect during chronic administration. Chronic studies that are longer than 14 days can be conducted. *In vitro* metabolism studies involving the various *H. hemerocallidea* materials can also be examined.

The pharmacokinetic interactions should also be investigated on other ARV drugs that are substrates for P-gp and CYP3A4 than the protease inhibitor, indinavir. This could lead to the identification of other probable herb-drug interactions. Inter-individual variability regarding to polymorphisms of CYP3A4 and P-gp, in various ethnic groups, could be studied in order to determine how these factors influence the metabolism of indinavir and other ARVs during the co-administration of *H. hemerocallidea* materials.

Numerous herbal medicines are available commercially and by investigating possible herb-drug interactions, awareness will be raised and knowledge broadened with regards to the issue. The overall treatment and welfare of HIV/AIDS infected patients can be improved when these herb-drug pharmacokinetic interactions are acknowledged.
REFERENCES


with *in vitro* methods (IVIVC), and applications for formulation/API/excipient characterization including food effects. *European Journal of Pharmaceutical Sciences*, 57:99–151.


APPENDIX A

CONGRESS PROCEEDINGS & ARTICLES
Pharmacokinetic interactions (*in vitro* and *in vivo*) between indinavir and *Hypoxis hemerocallidea*: comparing a commercial product with a crude extract and a dried plant material

Kaylee Havenga¹, Josias Hamman¹, Dewald Steyn¹, Carlemi Calitz¹, Efrem Abay², Lubbe Wiesner² and Alvaro Viljoen³.
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²Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, Observatory, South Africa.
³Department of Pharmaceutical Sciences, Tshwane University of Technology, Private Bag X680, Pretoria, 0001, South Africa

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**Purpose:** It is known that phytochemicals in herbal medicines can cause pharmacokinetic interactions with allopathic drugs when taken concomitantly. These interactions may lead to an increase or decrease in drug bioavailability with consequences of adverse effects or treatment failure. The relevance of research concerning pharmacokinetic interactions between herbal medicines and anti-retroviral drugs is reflected by the fact that a relatively large portion of human immunodeficiency virus infected patients with acquired immunodeficiency syndrome commonly use herbal medicines to complement their anti-retroviral therapy.

**Methods:** *Hypoxis hemerocallidea* (African potato) test materials investigated in this study included a commercial solid oral product, a dried reference plant material and an aqueous extract. Bi-directional transport studies of indinavir across Caco-2 cell monolayers were conducted in the absence and presence of the selected African potato test materials. The bioavailability of indinavir in the absence and presence of the selected African potato test materials was determined in Sprague-Dawley rats in acute (single administration) and chronic (daily administrations over 14 days) studies. Ketoconazole was used as positive control for metabolism inhibition and verapamil as positive control for efflux inhibition.

**Results:** The selected African potato test materials demonstrated an inhibition of efflux of indinavir in the Caco-2 cell model. In agreement with this finding and other published
findings on metabolism inhibition, an increase in indinavir bioavailability in the presence of the selected African potato test materials was shown in vivo for both the acute and chronic studies. The commercial product had a similar increasing effect on indinavir bioavailability as the aqueous extract, while the reference plant material exhibited a higher effect on indinavir bioavailability enhancement. A higher effect on indinavir bioavailability was observed for two of the test materials during the chronic study when compared to the acute study.

**Conclusion:** All the selected African potato test materials interfered with indinavir pharmacokinetics in both the in vitro and in vivo models and these effects may be attributed to inhibition of efflux transporters and enzymatic metabolism.
Pharmacokinetic interactions (*in vitro* and *in vivo*) between indinavir and *Hypoxis hemerocallidea*: comparing a commercial product with a crude extract and a dried plant material

Kaylee Havenga¹, Josias Hamman¹, Dewald Steyn¹, Carlemi Calitz¹, Efrem Abay², Lubbe Wiesner² and Alvaro Viljoen³

¹Centre of Excellence for Pharmaceutical Sciences, Faculty of Health Sciences, North-West University, Potchefstroom, South Africa.
²Division of Clinical Pharmacology, Department of Medicine, University of Cape Town, Observatory, South Africa.
³Department of Pharmaceutical Sciences, Tshwane University of Technology, Private Bag X680, Pretoria, 0001, South Africa

1. Background

The use of herbal medicines is popular as an alternative treatment source and in some developing countries, especially in Africa, more than 80% of the population still use traditional medicines on a regular basis (Brijlal et al, 2011). A misinterpretation of safety regarding herbal treatment use is evident amongst the general public. Besides potential toxic effects, herbal medicines can cause alterations in drug transport and metabolism due to modulation of Cytochrome P450 (CYP) enzymes and efflux transporters such as P-glycoprotein (P-gp), causing herb-drug pharmacokinetic interactions (Cordier & Steenkamp, 2011).

*In vitro* research has demonstrated that a potential herb-drug pharmacokinetic interaction exists between *Hypoxis hemerocallidea* Fisch., C.A.Mey. & Avé-Lall. (African potato) and anti-retroviral drugs. Literature indicates alterations in the transport and metabolism of indinavir upon co-administration with extracts of *H. hemerocallidea* such as inhibition of CYP3A4 enzyme activity and P-gp related efflux within *in vitro* models (Mills et al., 2005).

2. Aim

The aim of this study was to confirm pharmacokinetic interactions that different *H. hemerocallidea* materials including a dried plant reference material, an aqueous extract as well as a solid oral commercial product (Afrigetics®) may have on indinavir by means of *in vitro* bidirectional transport studies as well as by means of acute and chronic *in vivo* bioavailability studies.
3. Methods

3.1 Chemical composition of the selected *H. hemerocallisida* materials

The concentration of hypoxoside, a marker molecule, in all the selected *H. hemerocallisida* materials was determined by means of liquid chromatography linked to mass spectrometry (LC/MS).

3.2 *In vitro* bi-directional transport studies

Caco-2 cells were seeded and grown onto Transwell® 6-well membranes with a surface area of 4.67 cm² for a period of 21-24 days. Indinavir alone (200 µM) served as the negative control group, while indinavir in combination with 100 µM verapamil served as the positive control group. A final concentration of 500 µg/ml of each selected *H. hemerocallisida* material in combination with indinavir formed the experimental groups. For the transport studies in the apical-to-basolateral (AP-BL) direction, the growth medium in the apical chambers was replaced with test solutions in DMEM, while the growth medium in the basolateral chambers was replaced with test solutions in DMEM and HEPES for the transport studies in the basolateral-to-apical (BL-AP) direction. Samples (200 µl) were withdrawn from the acceptor chambers at time intervals of 20, 40, 60, 80, 100, 120 min and analysed by means of a validated high performance liquid chromatograph (HPLC) method.

The apparent permeability coefficient (*P*<sub>app</sub>) values as well as efflux ratio (ER) values were calculated as previously described (Calitz *et al.*, 2015).

3.3 *In vivo* pharmacokinetic study

A total of 45 male Sprague-Dawley rats weighing 250-300 g were randomly selected and divided into 9 groups (i.e. 3 control groups, 3 acute experimental groups and 3 chronic experimental groups) which consisted of 5 animals per group. The acute study consisted of a single administration per rat and the chronic study entailed daily administrations over 14 days by means of oral gavage at a volume of 500 µl per animal (Table 1).

Blood samples (200 µl) were collected from the tail veins of the animals at pre-determined time intervals of 0, 0.5, 1, 2, 4, 8 and 24 h after administration of each experimental test solution. A validated Liquid Chromatography tandem Mass Spectrometry (LC/MS/MS) method for the analysis of indinavir was used in order to analyse the plasma samples.
Table 1: Experimental groups for the *in vivo* studies (both acute and chronic)

<table>
<thead>
<tr>
<th>Composition of the experimental solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1:</strong> Indinavir alone at a concentration of 40 mg/kg. (IND alone) (Negative control)</td>
</tr>
<tr>
<td><strong>Group 2:</strong> Indinavir (40 mg/kg) with ketoconazole (40 mg/kg) in water. (IND with ketoconazole) (Positive control for enzyme inhibition)</td>
</tr>
<tr>
<td><strong>Group 3:</strong> Indinavir (40 mg/kg) with verapamil (9 mg/kg) in water. (IND with verapamil) (Positive control for efflux inhibition)</td>
</tr>
<tr>
<td><strong>Group 4 &amp; 5:</strong> Indinavir (40 mg/kg) with <em>H. hemerocallidea</em> aqueous extract at a concentration of 15 mg/kg. 4: acute, 5: chronic. (IND with HH aq extract)</td>
</tr>
<tr>
<td><strong>Group 6 &amp; 7:</strong> Indinavir (40 mg/kg) with <em>H. hemerocallidea</em> commercial product at a concentration of 15 mg/kg. 6: acute, 7: chronic. (IND with HH CP)</td>
</tr>
<tr>
<td><strong>Group 8 &amp; 9:</strong> Indinavir (40 mg/kg) with <em>H. hemerocallidea</em> reference dried plant material at a concentration of 15 mg/kg. 8: acute, 9: chronic. (IND with HH RM)</td>
</tr>
</tbody>
</table>

4. **Results and discussion**

4.1 Chemical composition of selected *H. hemerocallidea* materials

The hypoxoside contained in each of the selected *H. hemerocallidea* materials is presented in Table 2.

**Table 2:** Hypoxoside contained in the *H. hemerocallidea* test materials

<table>
<thead>
<tr>
<th>Test Material</th>
<th>Hypoxoside</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference dried plant material (RM HH)</td>
<td>13.3 mg/g</td>
</tr>
<tr>
<td>Commercial product (HH CP)</td>
<td>0.6 mg/g</td>
</tr>
<tr>
<td>Aqueous extract (HH aq extract)</td>
<td>151.7 mg/g</td>
</tr>
</tbody>
</table>

4.2 *In vitro* transport study

The overall transport of indinavir in the AP-BL direction increased when applied with the selected *H. hemerocallidea* materials, while the transport decreased in the BL-AP direction.
This indicates that the selected *H. hemerocallidea* test materials caused inhibition of efflux of indinavir in the Caco-2 cell model.

**Figure 1:** Bi-directional P\text{app} values for indinavir obtained from the experimental and control groups. n = 3, error bars indicate standard deviation.

4.3 *In vivo* pharmacokinetic study

An increase in indinavir bioavailability in the presence of the selected *H. hemerocallidea* test materials was observed *in vivo* for both the acute and chronic studies (Table 3). Of the selected *H. hemerocallidea* materials investigated, the dried plant material exhibited the highest enhancement of indinavir bioavailability. In general, a higher effect on indinavir bioavailability enhancement was observed during the chronic study.

**Table 3:** Area under the curve (AUC\text{0–∞}) and maximum plasma concentration (C\text{max}) values for indinavir administrated to rats with and without selected *H. hemerocallidea* materials

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>AUC\text{0–∞} (min.μmol/L)</th>
<th>C\text{max} (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IND alone (negative control)</td>
<td>1371.0 ± 389.8</td>
<td>1109 ± 484</td>
</tr>
<tr>
<td>IND with ketoconazole (positive control)</td>
<td>1428.6 ± 303.4*</td>
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<td>2226.3 ± 930.6</td>
<td>2001 ± 955</td>
</tr>
</tbody>
</table>

* average and standard deviation after removal of an outlier

5. Conclusions

The *in vitro* permeation studies showed that the selected *H. hemerocallidea* test materials inhibited efflux of indinavir, while the *in vivo* studies indicated enhanced bioavailability of indinavir. Concurrent administration of *H. hemerocallidea* with indinavir may result in possible adverse effects such as drug toxicity, which can possibly be overcome by taking them at different time periods (Owira et al., 2009).

6. References


ARTICLE

Effect of *Hypoxis hemerocallidea* on indinavir pharmacokinetics (*in vitro* and *in vivo*): comparing a commercial product with plant extract and reference material

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(In process of submission to The Journal of Ethnopharmacology)
SCHEDULING STATUS 3A
PREPARED NALX 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.

PHARMACOLOGICAL CLASSIFICATION
AZT/3C inhibitors

MECHANISM OF ACTION
Indinavir is an enzyme inhibitor for the protease of the viral gag polyproteins and thus reduces the activity of the viral enzymes, thereby inhibiting the intracellular accumulation of viral RNA and DNA. Indinavir is active against multi-drug-resistant strains of HIV-1, including strains resistant to other protease inhibitors (PIs) and nucleoside reverse transcriptase inhibitors (NRTIs).

Elimination
Over the 200 to 1,000 mg dose range, administration of both CRIXIVAN 200 mg and CRIXIVAN 400 mg capsules is recommended. In volunteers, the intracellular accumulation of indinavir is higher than the intracellular accumulation of other protease inhibitors. The recommended dose range of intracellular accumulation is 20-100 mg/mL. Elimination of indinavir is not affected by changes in the above-mentioned parameters. The long half-life of indinavir makes it well suited for once-daily dosage administration.

Characteristics in Patients
Patients with mild to moderate hepatic insufficiency and clinical evidence of chronic heart failure or overt congestive heart failure may experience a higher incidence of adverse effects. The recommended dose of indinavir is not to be increased in these patients.

PHARMACODYNAMICS
The combination of indinavir and one or more nucleoside analogues was associated with a higher median CD4 cell count and a lower risk of treatment failure than indinavir plus NRTIs. The effects of indinavir were also more pronounced in patients with lower CD4 cell counts. The median CD4 cell count at 48 weeks was 119 cells/mm³ with indinavir plus NRTIs and 126 cells/mm³ with indinavir plus NRTIs. The median time to AIDS event was 42 weeks with indinavir plus NRTIs and 37 weeks with indinavir plus NRTIs. The recommended dose of indinavir is 800 mg once daily.

WANING
Indinavir may be effective for up to 3 days in patients with CD4 cell counts of less than 200 cells/mm³ and CD4 cell counts of less than 100 cells/mm³. The recommended dose of indinavir is 800 mg once daily. Indinavir is not indicated for treatment of opportunistic infections, nor is it recommended for treatment of HIV-1-infected patients with AIDS or AIDS-related complex (ARC).

ADVICE
The concomitant use of indinavir and other antiretroviral drugs may reduce the activity of the viral enzymes and thus increase the risk of treatment failure. The recommended dose of indinavir is 800 mg once daily. Indinavir is not indicated for treatment of opportunistic infections, nor is it recommended for treatment of HIV-1-infected patients with AIDS or AIDS-related complex (ARC).
Side effects occurring less frequently (less than 2 %) of drug receiving CRWAN include unusual taste sensations and concomitant allergic reaction to issues related to treatment and other related symptoms are listed below by body system:

**Body as a Whole**

- Abnormal/edema: stool, hair loss, fatigue, Flank pain, diarrhea, Fossa ulceration, Fungus infection

**Cardiovascular System**

- Cardiac arrhythmias, Palpitations

**Digestive System**

- Acute pancreatitis, Anorexia

**Hypersensitive reaction**

- Angioneurotic edema, Rash, increased intraocular pressure

**Respiratory System**

- Cough, Dyspnea, Hallucinations

**Skin and Skin Appendage**

- Acne, Erythema, Dermatitis, Dry skin

**Special Senses**

- Anosmia, blurred vision

**Urinary System**

- Dysuria, Hematuria, Hypertension

**Laboratory Test Findings**

- Transaminases, alkaline phosphatase, blood urea nitrogen, creatinine, sodium, potassium, calcium, phosphorus, carbon dioxide, chloride, bicarbonate, magnesium, uric acid, albumin, globulin, hematocrit, hemoglobin, white blood cell count, red blood cell count, platelet count, prothrombin time, partial thromboplastin time, activated partial thromboplastin time, international normalized ratio, prothrombin time, partial thromboplastin time, activated partial thromboplastin time, international normalized ratio

**Post-transection Experience**

- The following additional laboratory experience have been reported and the frequencies are unknown:
  - Increased serum alkaline phosphatase
  - Increased serum creatinine

**Special Precautions**

- Hypotension

- Hypoglycemia, occurring with CRWAN. In some cases, hypotension has been associated with renal insufficiency and acute renal failure is not reversible. Signs and symptoms of hypotension, including fluid, pain, or weakness, have been reported in patients receiving the recommended dose of CRWAN (see WARNINGS AND DISEASE AND DIRECTIONS FOR USE). During post-marketing surveillance of patients treated with CRWAN, care reports of initial hypotension with initial weight loss and central nervous system symptoms have been observed in patients with symptomatic hypotension (greater than 100 mmHg), partial dysfunction in patients with symptomatic hypotension. Further evaluation may be necessary.

**Acute Haemolytic Anemia**

- Acute haemolytic anemia has been reported in patients with CRWAN. The majority of these patients had constricting medical conditions and were receiving concomitant treatments. As a result relationship between CRWAN and these events has not been established.

**Bacteremia and Pyelonephritis**

- There have been reports of new onset diabetes mellitus or pyelonephritis, or of exacerbations of pre-existing diabetes mellitus occurring in HLT-treated patients receiving prophylaxis for diabetic therapy. Some patients require admission or induction of insulin to treat these events. In some cases diabetic control has been lost.

In majority of cases, treatment with prophylactic infections was continued, while some cases treatment was discontinued. In some cases, prophylaxis after the prophylactic infection was withdrawn, whether or not diabetes was improved. A causal relationship between these prophylactic infections and these events have not been established.

**Immunological Reactions**

- Immune reactions are known to occur in patients treated with CRWAN. During the initial phase of treatment, a patient whose immune system responds to CRWAN may experience an inflammatory response to a drug or an allergic reaction. In patients with a history of immune reactions, which may require further evaluation and treatment.

**Concomitant use of CRWAN and HMG-CoA Reductase Inhibitors**

- See INTERACTIONS.

Concomitant use of CRWAN with a statin or another HMG-CoA reductase inhibitors that are metabolized by the CYSP3A pathways. The risk of myopathy, including rhabdomyolysis, may be increased when CRWAN is used in combination with these agents.

**Fat redistribution**

- Redistribution/circumference of body fat, including central obesity, weight loss, and peripheral edema, may be associated with increased CRWAN dose. In patients receiving CRWAN for weight loss, peripheral edema, and/or hand swelling, the occurrence of edema has been noted. In patients receiving CRWAN without dose reduction, and in patients treated with CRWAN for weight loss, peripheral edema and/or hand swelling may reduce body fat in addition to the control of peripheral edema.

**Patients with Concomitant Conditions**

- There have been reports of pancreatitis with CRWAN in patients with concomitant bleeding or coexisting pancreatic diseases. In some patients, additional factors have been noted. In many of the reported cases, treatment with CRWAN has been continued to the time of presentation. A causal relationship between pancreatitis and these diseases has not been established (see Dose Examples and Post-Marked Experience).

**Pregnancy**

- CRWAN is classified as a Class C drug in the United Kingdom. Use in pregnant women has not been evaluated. There are reports of the safety of CRWAN in pregnant women, but there is no evidence that CRWAN is a teratogenic or abortifacient agent.

**Identification**

- CRWAN 200mg is a white semi-solvent capsule with CRWAN 400mg is a white semi-solvent capsule with CRWAN 400mg is a white semi-solvent capsule.

**Presentation**

- CRWAN 200mg is available in plastic bottles containing 30 capsules. CRWAN 400mg is available in plastic bottles containing 10, 40, or 60 capsules.

**Storage Instructions**

- Store in well-closed containers at room temperature, 20-30 °C. Protect from moisture.

**Registration Number**

- CRWAN 200mg: 01/20/20/19
- CRWAN 400mg: 01/20/20/19

**Name and Business Address of the Holder of the Certificate of Registration**

- MSD P-Ltd. 10th Floor, HALIFAX HOUSE 1005

**Date of Publication of this Package Insert**

- 10 August 2007
The risk of side effects, including nephropathy, may be increased when this drug is used in combination with diuretics. Cholestyramine (240 mg every 8 hours) should be administered to patients taking this drug.

The drug should be taken with or without food, but the patient must be instructed to take the entire 8-hour dose. The patient must be monitored for bile duct obstruction, as well as for signs of liver disease. The patient should be instructed to report any jaundice, dark stools, or yellowing of the skin or eyes.

The drug should be discontinued if there is evidence of bile duct obstruction or if liver function tests are abnormal.

The drug should not be used in patients with severe liver disease or those who have had a liver transplant.

The drug should be used cautiously in patients with a history of gastrointestinal bleeding.

The drug should be used with caution in patients with a history of cardiac disease.

The drug should be used with caution in patients with a history of cancer.

The drug should be used with caution in patients with a history of kidney disease.

The drug should be used with caution in patients with a history of liver disease.

The drug should be used with caution in patients with a history of pancreatitis.

The drug should be used with caution in patients with a history of peptic ulcer disease.

The drug should be used with caution in patients with a history of pulmonary embolism.

The drug should be used with caution in patients with a history of renal disease.

The drug should be used with caution in patients with a history of rheumatoid arthritis.

The drug should be used with caution in patients with a history of scleroderma.

The drug should be used with caution in patients with a history of skin lesions.

The drug should be used with caution in patients with a history of upper respiratory tract infections.

The drug should be used with caution in patients with a history of upper respiratory tract infections.

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The drug should be used with caution in patients with a history of upper respiratory tract infections.
CONCENTRATION CALCULATIONS OF TRANSPORT EXPERIMENTAL GROUPS

Indinavir

Indinavir was provided in the form of Crixivan® capsules (400 mg/capsule). Each capsule contains 658 mg powder in total, of which 400 mg is pure indinavir.

\[
\text{indinavir concentration} = \frac{10 \times 0.12276}{1000}
\]

\[
\text{indinavir concentration} = 0.0012276 \text{ g in 10 ml}
\]

\[
\text{indinavir concentration} = 1.2276 \text{ mg in 10 ml}
\]

400 mg indinavir powder in every 658 mg powder

\[
\frac{1.23 \text{ mg indinavir}}{10 \text{ ml}} = \frac{x}{10 \text{ ml}}
\]

\[x = 2.02 \text{ mg powder}\]

2.02 mg powder was needed in 10 ml. The transport study required a double concentration so therefore 4.04 mg powder in 10 ml (2.02 mg powder in 5 ml).
Hypoxis hemerocallidea commercial product

Each capsule contained 403.74 mg powder, of which 300 mg Hypoxis tuber.

A concentration of 500 ug was required for the transport studies:

\[
\frac{0.5 mg}{300 mg \text{ Hypoxis tuber}} = \frac{x}{403.74 mg \text{ capsule powder}}
\]

\[x = 0.6729 mg\]

A double concentration was needed, therefore 1.3458 mg per 1 ml (13.458 mg in 10 ml and 6.729 mg in 5 ml)

Hypoxis hemerocallidea aqueous extract and reference material

For the transport study, a final concentration of 500 ug/ml was required, 0.5 mg H. hemerocallidea material in 1 ml. A double concentration needed to be prepared which entailed 1 mg in 1 ml, so 5 mg in 5 ml.

Verapamil

Verapamil has a molar mass of 454.602 g/mol.

1 M verapamil: 454.602 g in 1000 ml

1 mM verapamil: 0.454602 g in 1000 ml

1 uM verapamil: 0.000454602 g in 1000 ml

100 uM verapamil: 0.0454602 g in 1000 ml

\[x = \frac{10 \times 0.0454602}{1000}\]

\[x = 0.00045602 g\]

\[x = 0.454602 mg\] verapamil in 10 ml

Double concentration: 0.454602 x 2 = 0.909204 mg in 10 ml. (0.45 mg in 5 ml)
APPENDIX D

RAW DATA
IN VITRO TRANSPORT STUDY

1. Transepithelial electrical resistance (TEER) values

**Table D 1:** TEER Negative control Indinavir AP-BL

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>223</td>
<td>253</td>
<td></td>
</tr>
<tr>
<td>268</td>
<td>258</td>
<td></td>
</tr>
<tr>
<td>267</td>
<td>244</td>
<td></td>
</tr>
</tbody>
</table>

**Table D 2:** TEER Negative control Indinavir BL-AP

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>274</td>
<td>281</td>
<td></td>
</tr>
<tr>
<td>262</td>
<td>275</td>
<td></td>
</tr>
<tr>
<td>205</td>
<td>201</td>
<td></td>
</tr>
</tbody>
</table>

**Table D 3:** TEER Positive Control Indinavir & Verapamil AP-BL

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>272</td>
<td>244</td>
<td></td>
</tr>
<tr>
<td>281</td>
<td>280</td>
<td></td>
</tr>
<tr>
<td>284</td>
<td>236</td>
<td></td>
</tr>
</tbody>
</table>
**Table D 4:** TEER Positive Control Indinavir & Verapamil BL-AP

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>287</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>294</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>255</td>
<td>247</td>
</tr>
</tbody>
</table>

**Table D 5:** TEER Indinavir & *Hypoxis hemerocallidea* aqueous extract AP-BL

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>235</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>277</td>
<td>268</td>
</tr>
<tr>
<td></td>
<td>273</td>
<td>258</td>
</tr>
</tbody>
</table>

**Table D 6:** TEER Indinavir & *Hypoxis hemerocallidea* aqueous extract BL-AP

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
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<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>231</td>
<td>269</td>
</tr>
<tr>
<td></td>
<td>280</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>285</td>
<td>261</td>
</tr>
</tbody>
</table>
### Table D 7: TEER Indinavir & *Hypoxis hemerocallidea* commercial product AP-BL

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>260</td>
<td></td>
<td>284</td>
</tr>
<tr>
<td>285</td>
<td></td>
<td>233</td>
</tr>
<tr>
<td>229</td>
<td></td>
<td>325</td>
</tr>
</tbody>
</table>

### Table D 8: TEER Indinavir & *Hypoxis hemerocallidea* commercial product BL-AP

<table>
<thead>
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<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
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<td>380</td>
</tr>
<tr>
<td>317</td>
<td></td>
<td>399</td>
</tr>
<tr>
<td>350</td>
<td></td>
<td>380</td>
</tr>
</tbody>
</table>

### Table D 9: TEER Indinavir & *Hypoxis hemerocallidea* reference plant material AP-BL

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>280</td>
<td></td>
<td>241</td>
</tr>
<tr>
<td>274</td>
<td></td>
<td>278</td>
</tr>
<tr>
<td>298</td>
<td></td>
<td>241</td>
</tr>
</tbody>
</table>
**Table D 10:** TEER Indinavir & *Hypoxis hemerocallidea* reference plant material BL-AP

<table>
<thead>
<tr>
<th>TEER value (Ω)</th>
<th>0 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>292</td>
<td>252</td>
</tr>
<tr>
<td></td>
<td>276</td>
<td>258</td>
</tr>
<tr>
<td></td>
<td>275</td>
<td>282</td>
</tr>
</tbody>
</table>
2. Transport Calculations

2.1 Indinavir alone AP-BL

**Table D 11**: Concentration and percentage transport for each sample of indinavir alone (AP-BL) over the pre-determined time intervals. \(n = 3\).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>3146.90</td>
<td>3146.90</td>
<td>3146.90</td>
<td>52.19</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>14.63</td>
<td>14.63</td>
<td>14.63</td>
<td>0.24</td>
<td>0.46</td>
</tr>
<tr>
<td>40</td>
<td>25</td>
<td>72.45</td>
<td>73.62</td>
<td>80.02</td>
<td>1.33</td>
<td>2.54</td>
</tr>
<tr>
<td>60</td>
<td>25</td>
<td>78.80</td>
<td>84.60</td>
<td>91.95</td>
<td>1.52</td>
<td>2.92</td>
</tr>
<tr>
<td>80</td>
<td>25</td>
<td>99.27</td>
<td>105.57</td>
<td>114.75</td>
<td>1.90</td>
<td>3.65</td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td>132.25</td>
<td>140.19</td>
<td>152.38</td>
<td>2.53</td>
<td>4.84</td>
</tr>
<tr>
<td>120</td>
<td>25</td>
<td>135.97</td>
<td>146.55</td>
<td>159.29</td>
<td>2.64</td>
<td>5.06</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>2970.85</td>
<td>2970.85</td>
<td>2970.85</td>
<td>49.27</td>
<td>100.00</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>14.35</td>
<td>14.35</td>
<td>14.35</td>
<td>0.24</td>
<td>0.46</td>
</tr>
<tr>
<td>40</td>
<td>25</td>
<td>63.34</td>
<td>64.49</td>
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<td>1.16</td>
<td>2.36</td>
</tr>
<tr>
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<td>25</td>
<td>89.59</td>
<td>94.65</td>
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<td>1.71</td>
<td>3.46</td>
</tr>
<tr>
<td>80</td>
<td>25</td>
<td>113.09</td>
<td>120.26</td>
<td>130.71</td>
<td>2.17</td>
<td>4.40</td>
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<tr>
<td>100</td>
<td>25</td>
<td>136.34</td>
<td>145.39</td>
<td>158.03</td>
<td>2.62</td>
<td>5.32</td>
</tr>
<tr>
<td>120</td>
<td>25</td>
<td>147.37</td>
<td>158.28</td>
<td>172.05</td>
<td>2.85</td>
<td>5.79</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>25</td>
<td>2378.57</td>
<td>2378.57</td>
<td>2378.57</td>
<td>39.45</td>
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<td>25</td>
<td>43.47</td>
<td>43.47</td>
<td>43.47</td>
<td>0.72</td>
<td>1.83</td>
</tr>
<tr>
<td>40</td>
<td>25</td>
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<td>97.41</td>
<td>1.62</td>
<td>4.10</td>
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<td>60</td>
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<td>134.07</td>
<td>2.22</td>
<td>5.64</td>
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<tr>
<td>80</td>
<td>25</td>
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<td>158.78</td>
<td>2.63</td>
<td>6.68</td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td>160.85</td>
<td>171.79</td>
<td>186.73</td>
<td>3.10</td>
<td>7.85</td>
</tr>
<tr>
<td>120</td>
<td>25</td>
<td>190.32</td>
<td>193.19</td>
<td>209.95</td>
<td>3.48</td>
<td>8.83</td>
</tr>
</tbody>
</table>

**Table D 12**: \(P_{app}\) values for each sample of indinavir alone (AP-BL)

<table>
<thead>
<tr>
<th>Slope</th>
<th>1/A.60.C0</th>
<th>(P_{app})</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.044721483</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M2</td>
<td>0.051940303</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M3</td>
<td>0.073413403</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STDEV.P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure D 1:** Percentage of indinavir transport for each of the samples (Indinavir alone AP-BL). $n = 3$.

**Table D 13:** Average transport and standard deviation for each sample (indinavir alone AP-BL) over the pre-determined time intervals. $n = 3$.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average transport</th>
<th>STDEV.P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0.464870825</td>
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<td>0.925097599</td>
<td>0.638112998</td>
</tr>
<tr>
<td>40</td>
<td>2.542869712</td>
<td>2.359528696</td>
<td>4.095235665</td>
<td>2.999211358</td>
<td>0.778612203</td>
</tr>
<tr>
<td>60</td>
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<td>4.00731525</td>
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<tr>
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</tr>
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<td>6.560440499</td>
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</table>


2.2 Indinavir alone BL-AP

Table D 14: Concentration and percentage transport for each sample of indinavir alone (BL-AP) over the pre-determined time intervals. n = 3.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 1</td>
<td>25</td>
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<td>2555.28</td>
<td>2555.28</td>
<td>42.38</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
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<td>0</td>
<td>0.00</td>
<td>0.00</td>
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<td>4.22</td>
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<td>11.52</td>
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</tr>
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<td>25</td>
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<td>875.13</td>
<td>951.23</td>
<td>15.78</td>
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</tr>
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<td>960.00</td>
<td>1025.26</td>
<td>1114.41</td>
<td>18.48</td>
<td>43.61</td>
</tr>
</tbody>
</table>

Table D 15: \( \text{P}_{\text{app}} \) values for each sample of indinavir alone (BL-AP)

<table>
<thead>
<tr>
<th></th>
<th>Slope</th>
<th>( 1/A.60.C0 )</th>
<th>( \text{P}_{\text{app}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.356938082</td>
<td>3.56888E-05</td>
<td>1.27387E-05</td>
</tr>
<tr>
<td>M2</td>
<td>0.42522683</td>
<td>3.56888E-05</td>
<td>1.51758E-05</td>
</tr>
<tr>
<td>M3</td>
<td>0.283362783</td>
<td>3.56888E-05</td>
<td>1.01129E-05</td>
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<tr>
<td>Average</td>
<td></td>
<td></td>
<td>1.26758E-05</td>
</tr>
<tr>
<td>STDEV.P</td>
<td></td>
<td></td>
<td>2.06742E-06</td>
</tr>
</tbody>
</table>
Figure D 2: Percentage of indinavir transport for each of the samples (Indinavir alone BL-AP). n = 3.

Table D 16: Average transport and standard deviation for each sample (indinavir alone BL-AP) over the pre-determined time intervals. n = 3.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average Transport</th>
<th>STDEV.P</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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<td>9.605022072</td>
<td>5.626736</td>
<td>8.395358932</td>
<td>1.962899</td>
</tr>
<tr>
<td>60</td>
<td>27.19638945</td>
<td>28.12729553</td>
<td>16.9732</td>
<td>24.09896172</td>
<td>5.052986</td>
</tr>
<tr>
<td>80</td>
<td>33.61582612</td>
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<td>5.760397</td>
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<tr>
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<td>27.67133</td>
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<td>7.492197</td>
</tr>
<tr>
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<td>33.6759</td>
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</tbody>
</table>
### 2.3 Indinavir & Verapamil AP-BL

**Table D 17:** Concentration and percentage transport for each sample of indinavir with verapamil (AP-BL) over the pre-determined time intervals. $n = 3$.

<table>
<thead>
<tr>
<th>Time</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
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<td>3077.05</td>
<td>3077.05</td>
<td>48.61</td>
<td>100</td>
</tr>
<tr>
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<td>25</td>
<td>65.95</td>
<td>65.95</td>
<td>65.95</td>
<td>1.04</td>
<td>2.14</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>192.50</td>
<td>197.77</td>
<td>214.97</td>
<td>3.40</td>
<td>6.99</td>
</tr>
<tr>
<td>60</td>
<td>25</td>
<td>388.35</td>
<td>410.85</td>
<td>471.05</td>
<td>7.06</td>
<td>13.29</td>
</tr>
<tr>
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<td>25</td>
<td>541.76</td>
<td>575.32</td>
<td>628.35</td>
<td>9.88</td>
<td>20.32</td>
</tr>
<tr>
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<td>25</td>
<td>695.71</td>
<td>639.05</td>
<td>694.82</td>
<td>10.97</td>
<td>22.57</td>
</tr>
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<td>75.42</td>
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<td>216.93</td>
<td>3.43</td>
<td>6.45</td>
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<td>280.68</td>
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<td>10.59</td>
</tr>
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<td>658.43</td>
<td>10.40</td>
<td>19.59</td>
</tr>
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<td>656.51</td>
<td>690.86</td>
<td>751.93</td>
<td>11.86</td>
<td>28.33</td>
</tr>
<tr>
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<td>2650.26</td>
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<td>100.23</td>
<td>100.23</td>
<td>1.58</td>
<td>3.78</td>
</tr>
<tr>
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<td>25</td>
<td>250.21</td>
<td>258.23</td>
<td>280.68</td>
<td>4.43</td>
<td>10.59</td>
</tr>
<tr>
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<td>276.39</td>
<td>300.42</td>
<td>4.75</td>
<td>11.34</td>
</tr>
<tr>
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<td>429.32</td>
<td>449.83</td>
<td>488.95</td>
<td>7.72</td>
<td>18.45</td>
</tr>
<tr>
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<td>656.51</td>
<td>690.86</td>
<td>751.93</td>
<td>11.86</td>
<td>28.33</td>
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<td>746.05</td>
<td>11.79</td>
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</table>

**Table D 18:** $P_{app}$ values for each sample of indinavir with verapamil (AP-BL)

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<th>Slope</th>
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<th>Papp</th>
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</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.20134436</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M2</td>
<td>0.168812683</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M3</td>
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<td>3.56888E-05</td>
</tr>
<tr>
<td>Average</td>
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<td>STDEV.P</td>
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<td>1.2297E-06</td>
</tr>
</tbody>
</table>
Figure D 3: Percentage of indinavir transport for each of the samples (Indinavir with verapamil AP-BL). n = 3.

Table D 19: Average transport and standard deviation for each sample (indinavir with verapamil AP-BL) over the pre-determined time intervals. n = 3.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average transport</th>
<th>STDEV.P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
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</tr>
<tr>
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<td>6.986269316</td>
<td>6.453222099</td>
<td>10.59076</td>
<td>8.010082768</td>
<td>1.83774</td>
</tr>
<tr>
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<td>10.99386492</td>
<td>9.179230468</td>
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<td>10.50287292</td>
<td>0.94629</td>
</tr>
<tr>
<td>80</td>
<td>15.65683467</td>
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<td>18.44906</td>
<td>15.79701091</td>
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<td>21.79054385</td>
<td>4.85602</td>
</tr>
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<td>22.57430684</td>
<td>19.58733019</td>
<td>28.15005</td>
<td>23.43723061</td>
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</tr>
</tbody>
</table>
2.4 Indinavir & Verapamil BL-AP

Table D 20: Concentration and percentage transport for each sample of indinavir with verapamil (BL-AP) over the pre-determined time intervals. n = 3.

<table>
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<tr>
<th>Time</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
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<td>2321.72</td>
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<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
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<td>104.86</td>
<td>104.86</td>
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</tr>
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<td>25</td>
<td>277.98</td>
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<td>311.27</td>
<td>4.92</td>
<td>13.41</td>
</tr>
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<td>372.23</td>
<td>5.88</td>
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</tr>
<tr>
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<td>578.01</td>
<td>9.13</td>
<td>24.90</td>
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<tr>
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<td>694.76</td>
<td>10.98</td>
<td>29.92</td>
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Table D 21: P_{app} values for each sample of indinavir with verapamil (BL-AP)

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<th>Papp</th>
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</thead>
<tbody>
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</tr>
<tr>
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<td>7.88556E-06</td>
</tr>
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<td>7.72673E-06</td>
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<td></td>
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</tbody>
</table>
**Figure D 4:** Percentage of indinavir transport for each of the samples (Indinavir with verapamil BL-AP). \( n = 3 \).

**Table D 22:** Average transport and standard deviation for each sample (indinavir with verapamil BL-AP) over the pre-determined time intervals. \( n = 3 \).

<table>
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<th>Time (min)</th>
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<th>M2</th>
<th>M3</th>
<th>Average transport</th>
<th>STDEV.P</th>
</tr>
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</table>
### 2.5 Indinavir & Hypoxis hemerocallidea aqueous extract AP-BL

**Table D 23:** Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* aqueous extract (AP-BL) over the pre-determined time intervals. \( n = 3 \).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inj vol (µl)</th>
<th>Peak area (µg/ml)</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
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<td>3.13</td>
</tr>
<tr>
<td>60</td>
<td>25</td>
<td>240.65</td>
<td>255.87</td>
<td>278.12</td>
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<td>4.11</td>
</tr>
<tr>
<td>80</td>
<td>25</td>
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<td>332.02</td>
<td>360.90</td>
<td>5.98</td>
<td>5.33</td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td>436.74</td>
<td>461.76</td>
<td>501.91</td>
<td>8.32</td>
<td>7.41</td>
</tr>
<tr>
<td>120</td>
<td>25</td>
<td>530.01</td>
<td>564.95</td>
<td>614.07</td>
<td>10.18</td>
<td>9.07</td>
</tr>
</tbody>
</table>

**Table D 24:** \( P_{\text{app}} \) values for each sample of indinavir with *H. hemerocallidea* aqueous extract (AP-BL)

<table>
<thead>
<tr>
<th>Slope</th>
<th>1/A.60.C0</th>
<th>Papp</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.075748536</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M2</td>
<td>0.067218765</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M3</td>
<td>0.076004596</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STDEV.P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure D 5: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* aqueous extract AP-BL). \( n = 3 \).

Table D 25: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* aqueous extract AP-BL) over the pre-determined time intervals. \( n = 3 \).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average transport</th>
<th>STDEV.P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>0.378903921</td>
<td>0.609853379</td>
<td>0.836756</td>
<td>0.608504587</td>
<td>0.18692</td>
</tr>
<tr>
<td>40</td>
<td>2.00558918</td>
<td>2.432873175</td>
<td>3.126826</td>
<td>2.521762865</td>
<td>0.4620384</td>
</tr>
<tr>
<td>60</td>
<td>3.530349119</td>
<td>3.380251856</td>
<td>4.10749</td>
<td>3.672696893</td>
<td>0.313492</td>
</tr>
<tr>
<td>80</td>
<td>4.271569464</td>
<td>4.186960717</td>
<td>5.330011</td>
<td>4.596180496</td>
<td>0.5200451</td>
</tr>
<tr>
<td>100</td>
<td>6.281446336</td>
<td>6.060804027</td>
<td>7.412714</td>
<td>6.584988005</td>
<td>0.5921813</td>
</tr>
<tr>
<td>120</td>
<td>9.449371628</td>
<td>8.328839933</td>
<td>9.069158</td>
<td>8.949123247</td>
<td>0.4652627</td>
</tr>
</tbody>
</table>
2.6 Indinavir & *Hypoxis hemerocallidea* aqueous extract BL-AP

**Table D 26:** Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* aqueous extract (BL-AP) over the pre-determined time intervals. \( n = 3 \).

<table>
<thead>
<tr>
<th>Time</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 1</td>
<td>25</td>
<td>7310.22</td>
<td>7310.22</td>
<td>7310.22</td>
<td>121.23</td>
<td>100</td>
</tr>
<tr>
<td>0</td>
<td>25</td>
<td>0</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>265.10</td>
<td>265.10</td>
<td>265.10</td>
<td>4.40</td>
<td>3.63</td>
</tr>
<tr>
<td>40</td>
<td>25</td>
<td>452.55</td>
<td>473.76</td>
<td>514.96</td>
<td>8.54</td>
<td>7.04</td>
</tr>
<tr>
<td>60</td>
<td>25</td>
<td>719.28</td>
<td>755.48</td>
<td>821.17</td>
<td>13.62</td>
<td>11.23</td>
</tr>
<tr>
<td>80</td>
<td>25</td>
<td>943.52</td>
<td>1001.06</td>
<td>1088.11</td>
<td>18.04</td>
<td>14.88</td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td>1196.51</td>
<td>1271.99</td>
<td>1382.60</td>
<td>22.93</td>
<td>18.91</td>
</tr>
<tr>
<td>120</td>
<td>25</td>
<td>1336.06</td>
<td>1431.80</td>
<td>1556.31</td>
<td>25.81</td>
<td>21.29</td>
</tr>
</tbody>
</table>

| M 2  | 25      | 8040.00   | 8040.00      | 8040.00      | 133.33               | 100.00      |
| 0    | 25      | 0.00      | 0.00         | 0.00         | 0.00                 | 0.00        |
| 20   | 25      | 182.45    | 182.45       | 182.45       | 3.03                 | 2.27        |
| 40   | 25      | 478.24    | 492.83       | 535.69       | 8.88                 | 6.66        |
| 60   | 25      | 641.98    | 680.24       | 739.39       | 12.26                | 9.20        |
| 80   | 25      | 863.17    | 914.52       | 994.05       | 16.49                | 12.36       |
| 100  | 25      | 1228.16   | 1297.21      | 1410.01      | 23.38                | 17.54       |
| 120  | 25      | 1300.14   | 1398.39      | 1519.99      | 25.21                | 18.91       |

| M 3  | 25      | 7279.41   | 7279.41      | 7279.41      | 120.72               | 100.00      |
| 0    | 25      | 0.00      | 0.00         | 0.00         | 0.00                 | 0.00        |
| 20   | 25      | 286.19    | 286.19       | 286.19       | 4.75                 | 3.93        |
| 40   | 25      | 427.56    | 450.46       | 489.63       | 8.12                 | 6.73        |
| 60   | 25      | 580.14    | 614.35       | 667.77       | 11.07                | 9.17        |
| 80   | 25      | 842.12    | 888.53       | 965.80       | 16.02                | 13.27       |
| 100  | 25      | 1153.05   | 1220.42      | 1326.55      | 22.00                | 18.22       |
| 120  | 25      | 1264.70   | 1356.95      | 1474.94      | 24.46                | 20.26       |

**Table D 27:** \( P_{app} \) values for each sample of indinavir with *H. hemerocallidea* aqueous extract (BL-AP)

<table>
<thead>
<tr>
<th>Slope</th>
<th>1/A.60.C0</th>
<th>Papp</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.182647539</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M2</td>
<td>0.16598791</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M3</td>
<td>0.171268391</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STDEV.P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure D 6: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* aqueous extract BL-AP). n = 3.

Table D 28: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* aqueous extract BL-AP) over the pre-determined time intervals. n = 3.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average transport</th>
<th>STDEV.P</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>3.626366101</td>
<td>2.269271891</td>
<td>3.931539</td>
<td>3.275725787</td>
<td>0.7224933</td>
</tr>
<tr>
<td>40</td>
<td>7.044361349</td>
<td>6.662804142</td>
<td>6.72618</td>
<td>6.811115052</td>
<td>0.1669471</td>
</tr>
<tr>
<td>60</td>
<td>11.23322601</td>
<td>9.196387319</td>
<td>9.173397</td>
<td>9.867670255</td>
<td>0.9656393</td>
</tr>
<tr>
<td>100</td>
<td>18.91324069</td>
<td>17.53744488</td>
<td>18.22328</td>
<td>18.2246559</td>
<td>0.5616671</td>
</tr>
<tr>
<td>120</td>
<td>21.28948538</td>
<td>18.90530404</td>
<td>20.26183</td>
<td>20.15220648</td>
<td>0.9764197</td>
</tr>
</tbody>
</table>
2.7 Indinavir & *Hypoxis hemerocallidea* commercial product AP-BL

**Table D 29:** Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* commercial product (AP-BL) over the pre-determined time intervals. n = 3.

<table>
<thead>
<tr>
<th>Time</th>
<th>ln vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 1</td>
<td>25</td>
<td>3397.05</td>
<td>3397.05</td>
<td>3397.05</td>
<td>58.75</td>
<td>100</td>
</tr>
<tr>
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<td>0</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>61.08</td>
<td>61.08</td>
<td>61.08</td>
<td>1.06</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>129.13</td>
<td>134.01</td>
<td>145.66</td>
<td>2.52</td>
<td>4.29</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>268.51</td>
<td>278.84</td>
<td>303.08</td>
<td>5.24</td>
<td>8.92</td>
</tr>
<tr>
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<td>80</td>
<td>364.21</td>
<td>385.69</td>
<td>419.23</td>
<td>7.25</td>
<td>12.34</td>
</tr>
<tr>
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<td>100</td>
<td>404.44</td>
<td>433.57</td>
<td>471.27</td>
<td>8.15</td>
<td>13.87</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>511.40</td>
<td>543.76</td>
<td>591.04</td>
<td>10.22</td>
<td>17.40</td>
</tr>
</tbody>
</table>

| M 2  | 25     | 6277.95   | 6277.95      | 6277.95      | 108.58                | 100.00      |
|      | 0      | 0         | 0.00         | 0.00         | 0.00                  | 0.00        |
|      | 25     | 49.24     | 49.24        | 49.24        | 0.85                  | 0.78        |
|      | 40     | 172.48    | 176.42       | 191.76       | 3.32                  | 3.05        |
|      | 60     | 339.25    | 353.05       | 383.75       | 6.64                  | 6.11        |
|      | 80     | 440.72    | 467.86       | 508.54       | 8.80                  | 8.10        |
|      | 100    | 501.72    | 536.98       | 583.68       | 10.09                 | 9.30        |
|      | 120    | 595.39    | 635.52       | 690.79       | 11.95                 | 11.00       |

| M 3  | 25     | 6449.86   | 6449.86      | 6449.86      | 111.55                | 100.00      |
|      | 0      | 0         | 0.00         | 0.00         | 0.00                  | 0.00        |
|      | 25     | 95.90     | 95.90        | 95.90        | 1.66                  | 1.49        |
|      | 40     | 185.41    | 193.08       | 209.87       | 3.63                  | 3.25        |
|      | 60     | 334.13    | 348.97       | 379.31       | 6.56                  | 5.88        |
|      | 80     | 487.86    | 514.59       | 559.34       | 9.67                  | 8.67        |
|      | 100    | 581.56    | 620.59       | 674.55       | 11.67                 | 10.46       |
|      | 120    | 643.14    | 689.67       | 749.64       | 12.97                 | 11.62       |

**Table D 30:** $P_{app}$ values for each sample of indinavir with *H. hemerocallidea* commercial product (AP-BL)

<table>
<thead>
<tr>
<th>Slope</th>
<th>1/A.60.C0</th>
<th>Papp</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>0.15071257</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M2</td>
<td>0.098360386</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M3</td>
<td>0.103980277</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>Average</td>
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<td>4.20001E-06</td>
</tr>
<tr>
<td>STDEV.P</td>
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<td>8.37504E-07</td>
</tr>
</tbody>
</table>
Figure D 7: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* commercial product AP-BL). $n = 3$.

Table D 31: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* commercial product AP-BL) over the pre-determined time intervals. $n = 3$.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average Transport</th>
<th>STDEV.P</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>1.356394722</td>
<td>0.42394603</td>
</tr>
<tr>
<td>40</td>
<td>4.287978124</td>
<td>3.054485196</td>
<td>3.253814943</td>
<td>3.532092754</td>
<td>0.540650908</td>
</tr>
<tr>
<td>60</td>
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<td>6.112595917</td>
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</tr>
<tr>
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<td>12.34099535</td>
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<td>9.704517555</td>
<td>1.878821663</td>
</tr>
<tr>
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<tr>
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<td>11.00337206</td>
<td>11.62254791</td>
<td>13.34150164</td>
<td>2.87990607</td>
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</table>
2.8 Indinavir & *Hypoxis hemerocallidea* commercial product BL-AP

**Table D 32:** Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* commercial product (BL-AP) over the pre-determined time intervals. \( n = 3 \).

<table>
<thead>
<tr>
<th>Time</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>M 1</td>
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<td></td>
<td></td>
</tr>
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<td>25</td>
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<td>7144.77</td>
<td>7144.77</td>
<td>123.57</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>25</td>
<td>186.48</td>
<td>186.48</td>
<td>186.48</td>
<td>3.23</td>
<td>2.61</td>
</tr>
<tr>
<td>40</td>
<td>25</td>
<td>439.95</td>
<td>454.86</td>
<td>494.42</td>
<td>8.55</td>
<td>6.92</td>
</tr>
<tr>
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<td>25</td>
<td>713.97</td>
<td>749.16</td>
<td>814.31</td>
<td>14.08</td>
<td>11.40</td>
</tr>
<tr>
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<td>894.37</td>
<td>972.14</td>
<td>16.81</td>
<td>13.61</td>
</tr>
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<td>1239.92</td>
<td>21.44</td>
<td>17.35</td>
</tr>
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</tr>
<tr>
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<td></td>
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<td>M 2</td>
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</tr>
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<td>7154.33</td>
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<td>212.81</td>
<td>212.81</td>
<td>3.68</td>
<td>2.97</td>
</tr>
<tr>
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</tr>
<tr>
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<td>25</td>
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<td>13.02</td>
</tr>
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<td>1123.18</td>
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<td>25</td>
<td>1144.02</td>
<td>1221.58</td>
<td>1327.80</td>
<td>22.96</td>
<td>18.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>M 3</td>
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<td>7180.93</td>
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<td>155.27</td>
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<td>25</td>
<td>611.83</td>
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<td>1005.43</td>
<td>17.39</td>
<td>14.00</td>
</tr>
<tr>
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<td>1102.69</td>
<td>1198.57</td>
<td>20.73</td>
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</tr>
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<td>1263.46</td>
<td>1373.33</td>
<td>23.75</td>
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</table>

**Table D 33:** \( P_{app} \) values for each sample of indinavir with *H. hemerocallidea* commercial product (BL-AP)

<table>
<thead>
<tr>
<th>Slope</th>
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<th>Papp</th>
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<tr>
<td>M2</td>
<td>0.157140322</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M3</td>
<td>0.167724091</td>
<td>3.56888E-05</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>STDEV.P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure D 8:** Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* commercial product BL-AP). *n* = 3.

**Table D 34:** Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* commercial product BL-AP) over the pre-determined time intervals. *n* = 3.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average Transport</th>
<th>STDEV.P</th>
</tr>
</thead>
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<td>0</td>
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<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>20</td>
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<td>2.974560344</td>
<td>2.162214</td>
<td>2.582255851</td>
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</tr>
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<td>40</td>
<td>6.920004053</td>
<td>6.152245018</td>
<td>6.507567</td>
<td>6.526605358</td>
<td>0.313725</td>
</tr>
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<td>60</td>
<td>11.39722897</td>
<td>11.62399036</td>
<td>9.766667</td>
<td>10.92929543</td>
<td>0.827298</td>
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<td>13.0231168</td>
<td>14.00138</td>
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<td>0.401831</td>
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<td>16.69105</td>
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<td>0.677966</td>
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<td>0.95316</td>
</tr>
</tbody>
</table>
2.9 Indinavir & Hypoxis hemerocallidea reference plant material AP-BL

**Table D 35:** Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* reference plant material (AP-BL) over the pre-determined time intervals. \( n = 3 \).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
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<tbody>
<tr>
<td><strong>M 1</strong></td>
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<td>7423.62</td>
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<td>0</td>
<td>117.28</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0</td>
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<tr>
<td>20</td>
<td>25</td>
<td>42.33</td>
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<td>42.33</td>
<td>0.67</td>
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</tr>
<tr>
<td>40</td>
<td>25</td>
<td>199.50</td>
<td>202.89</td>
<td>220.53</td>
<td>3.48</td>
<td>2.97</td>
</tr>
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<td>25</td>
<td>297.72</td>
<td>313.68</td>
<td>340.96</td>
<td>5.39</td>
<td>4.59</td>
</tr>
<tr>
<td>80</td>
<td>25</td>
<td>376.61</td>
<td>400.43</td>
<td>435.25</td>
<td>6.88</td>
<td>5.86</td>
</tr>
<tr>
<td>100</td>
<td>25</td>
<td>636.63</td>
<td>666.75</td>
<td>724.73</td>
<td>11.45</td>
<td>9.76</td>
</tr>
<tr>
<td>120</td>
<td>25</td>
<td>650.60</td>
<td>701.53</td>
<td>762.53</td>
<td>12.05</td>
<td>10.27</td>
</tr>
<tr>
<td><strong>M 2</strong></td>
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<td>0.00</td>
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<td>106.36</td>
<td>1.68</td>
<td>1.41</td>
</tr>
<tr>
<td>40</td>
<td>25</td>
<td>277.74</td>
<td>286.25</td>
<td>311.14</td>
<td>4.92</td>
<td>4.12</td>
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<tr>
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<td>25</td>
<td>376.57</td>
<td>398.79</td>
<td>433.47</td>
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<td>5.74</td>
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<td>25</td>
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<td>507.95</td>
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<td>8.72</td>
<td>7.31</td>
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<tr>
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<td>25</td>
<td>569.91</td>
<td>608.14</td>
<td>661.02</td>
<td>10.44</td>
<td>8.75</td>
</tr>
<tr>
<td>120</td>
<td>25</td>
<td>679.47</td>
<td>725.07</td>
<td>788.12</td>
<td>12.45</td>
<td>10.44</td>
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<tr>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>90.36</td>
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<td>238.51</td>
<td>3.77</td>
<td>3.20</td>
</tr>
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<td>25</td>
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<td>462.12</td>
<td>502.31</td>
<td>7.94</td>
<td>6.74</td>
</tr>
<tr>
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<td>548.19</td>
<td>595.86</td>
<td>9.41</td>
<td>8.00</td>
</tr>
</tbody>
</table>

**Table D 36:** \( P_{app} \) values for each sample of indinavir with *H. hemerocallidea* reference plant material (AP-BL)

<table>
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<tr>
<th>Slope</th>
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<th>Papp</th>
</tr>
</thead>
<tbody>
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<td><strong>M1</strong></td>
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</tr>
<tr>
<td><strong>M2</strong></td>
<td>0.08785424</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td><strong>M3</strong></td>
<td>0.06825082</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td><strong>Average</strong></td>
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</table>
Figure D 9: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* reference plant material AP-BL). \( n = 3 \).

Table D 37: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* reference plant material AP-BL) over the pre-determined time intervals. \( n = 3 \).

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average transport</th>
<th>STDEV.P</th>
</tr>
</thead>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
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<td>0.35816112</td>
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<td>3.430885873</td>
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</table>
2.10 Indinavir & *Hypoxis hemerocallidea* reference plant material BL-AP

**Table D 38:** Concentration and percentage transport for each sample of indinavir with *H. hemerocallidea* reference plant material (BL-AP) over the pre-determined time intervals.  \( n = 3. \)

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Inj vol</th>
<th>Peak area</th>
<th>Correction 1</th>
<th>Correction 2</th>
<th>Concentration (µg/ml)</th>
<th>% Transport</th>
</tr>
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<td>16.77</td>
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</tr>
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<td>25</td>
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<td>1338.30</td>
<td>21.14</td>
<td>18.40</td>
</tr>
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<td>25</td>
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<td>1660.45</td>
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</tr>
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<td>0.00</td>
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</table>

**Table D 39:** \( P_{app} \) values for each sample of indinavir with *H. hemerocallidea* reference plant material (BL-AP)

<table>
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<tr>
<th>Slope</th>
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<th>( P_{app} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
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<td>3.56888E-05</td>
</tr>
<tr>
<td>M2</td>
<td>0.203373943</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>M3</td>
<td>0.230004179</td>
<td>3.56888E-05</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STDEV.P</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure D 10: Percentage of indinavir transport for each of the samples (Indinavir with *H. hemerocallidea* reference plant material BL-AP). *n* = 3.

Table D 40: Average transport and standard deviation for each sample (indinavir with *H. hemerocallidea* reference plant material BL-AP) over the pre-determined time intervals. *n* = 3.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>Average transport</th>
<th>STDEV.P</th>
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<tr>
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</table>
**IN VIVO bioavailability study**

**Table D 41:** Weight of rats used in the *in vivo* study

<table>
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<th>Rat</th>
<th>Weight (g) of rats used in Indinavir PK-study (a=Acute; C=Chronic)</th>
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<tr>
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<td>R 3</td>
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<tr>
<td>R 4</td>
<td>294</td>
</tr>
<tr>
<td>R 5</td>
<td>260</td>
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</table>
Indinavir alone (Negative control)

Table D 42: Indinavir concentration for each rat (indinavir alone). n = 5.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Conc. (ng/ml)</th>
<th>R 1</th>
<th>R 2</th>
<th>R 3</th>
<th>R 4</th>
<th>R 5</th>
<th>Mean</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
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<td>32.62</td>
<td>29.93</td>
<td>34.48</td>
<td>5.43</td>
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<td>28.02</td>
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<td>18.35</td>
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<td>4.95</td>
<td>2.02</td>
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</table>

Figure D 11: Indinavir concentration over time for each rat (indinavir alone). n = 5.
**Indinavir with ketoconazole (Positive control)**

**Table D 43:** Indinavir concentration for each rat (indinavir with ketoconazole). \( n = 4 \).

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Conc. (ng/ml)</th>
<th>R 1</th>
<th>R 2</th>
<th>R 3</th>
<th>R 4</th>
<th>R 5</th>
<th>Mean</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
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<td>0</td>
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<td>1006.0</td>
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<td>97</td>
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<td></td>
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<tr>
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<td>70</td>
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<td>27</td>
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<td></td>
</tr>
</tbody>
</table>

**Figure D 12:** Indinavir concentration over time for each rat (indinavir with ketoconazole). \( n = 4 \).
Indinavir with verapamil (Positive control)

Table D 44: Indinavir concentration for each rat (indinavir with verapamil). n = 5.

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
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<td>39.440</td>
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</table>

Figure D 13: Indinavir concentration over time for each rat (indinavir with verapamil). n = 5.
Indinavir with *H. hemerocallidea* aqueous extract (acute)

**Table D 45:** Indinavir concentration for each rat (indinavir with *H. hemerocallidea* aqueous extract, acute). n = 5.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Conc. (ng/ml)</th>
<th>R 1</th>
<th>R 2</th>
<th>R 3</th>
<th>R 4</th>
<th>R 5</th>
<th>Mean</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
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<td>640.9</td>
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<td>192.7</td>
<td>143.7</td>
<td>214.0</td>
<td>61.77</td>
<td>25.21</td>
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<td>26.47</td>
<td>45.97</td>
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<td>74.95</td>
<td>91.68</td>
<td>37.42</td>
</tr>
</tbody>
</table>

**Figure D 14:** Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* aqueous extract, acute). n = 5.
Indinavir with *H. hemerocallidea* aqueous extract (chronic)

**Table D 46:** Indinavir concentration for each rat (indinavir with *H. hemerocallidea* aqueous extract, chronic). n = 5.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Conc. (ng/ml)</th>
<th>R 1</th>
<th>R 2</th>
<th>R 3</th>
<th>R 4</th>
<th>R 5</th>
<th>Mean</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
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</tr>
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</table>

**Figure D 15:** Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* aqueous extract, chronic). n = 5.
**Indinavir with *H. hemerocallidea* commercial product (acute)**

**Table D 47:** Indinavir concentration for each rat (indinavir with *H. hemerocallidea* commercial product, acute). *n* = 5.

<table>
<thead>
<tr>
<th>Time (h)</th>
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<th></th>
<th></th>
<th></th>
<th>Mean</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R 1</td>
<td>R 2</td>
<td>R 3</td>
<td>R 4</td>
<td>R 5</td>
<td></td>
<td></td>
</tr>
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<td>133.9</td>
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<td>101.6</td>
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</table>

**Figure D 16:** Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* commercial product, acute). *n* = 5.
Indinavir with *H. hemerocallidea* commercial product (chronic)

**Table D 48:** Indinavir concentration for each rat (indinavir with *H. hemerocallidea* commercial product, chronic). n = 5.

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<th>R 2</th>
<th>R 3</th>
<th>R 4</th>
<th>R 5</th>
<th>Mean</th>
<th>STDEV</th>
<th>SEM</th>
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</table>

**Figure D 17:** Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* commercial product, chronic). n = 5.
**Indinavir with H. hemerocallidea reference plant material (acute)**

**Table D 49:** Indinavir concentration for each rat (indinavir with H. hemerocallidea reference plant material, acute) . \( n = 5 \).

<table>
<thead>
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<th>Time (h)</th>
<th>Conc. (ng/ml)</th>
<th>R 1</th>
<th>R 2</th>
<th>R 3</th>
<th>R 4</th>
<th>R 5</th>
<th>Mean</th>
<th>STDEV</th>
<th>SEM</th>
</tr>
</thead>
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<td>50.19</td>
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</tbody>
</table>

**Figure D 18:** Indinavir concentration over time for each rat (indinavir with H. hemerocallidea reference plant material, acute) . \( n = 5 \).
Indinavir with *H. hemerocallidea* reference plant material (chronic)

**Table D 50:** Indinavir concentration for each rat (indinavir with *H. hemerocallidea* reference plant material, chronic). \( n = 5. \)

<table>
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<th>Time (h)</th>
<th>Conc. (ng/ml)</th>
<th>R 1</th>
<th>R 2</th>
<th>R 3</th>
<th>R 4</th>
<th>R 5</th>
<th>Mean</th>
<th>STDEV</th>
<th>SEM</th>
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<td>87.67</td>
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<td>51.84</td>
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<td>11.75</td>
<td>4.795</td>
</tr>
</tbody>
</table>

**Figure D 19:** Indinavir concentration over time for each rat (indinavir with *H. hemerocallidea* reference plant material, chronic). \( n = 5. \)
UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Animal Ethics Committee

Room E52-24 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone (021) 404 7682
Email: nswl.tsarna@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/animalethics/forms

17 March 2016

Dr L Wiesner
Pharmacology
Old Main Building

Dear Dr Wiesner

PROTOCOL TITLE: PHARMACOKINETIC INTERACTIONS BETWEEN HYPOXIS HEMEROCALLIDEA AND INDINAR IN SPRAGUE DAWLEY RATS

FHS AEC REF NO: 015/041

Thank you for submitting your protocol to the Faculty of Health Sciences (FHS) Animal Ethics Committee (AEC) for review.

I am pleased to inform you that the FHS AEC has approved your protocol, which will terminate on 30 March 2019

Number of animals & species: 66 Rats

Please quote the FHS AEC REF NO (above) in all future correspondence.

Please note that the approval of this protocol imposes the following obligations on the principal investigator (PI):

1. To submit an annual mandatory progress report. The first annual report for this protocol is due on 28 February 2017. The forms can be accessed from http://www.health.uct.ac.za/fhs/research/animalethics/forms
2. To submit a final mandatory report on the 28 February 2019, please access the final report form from: http://www.health.uct.ac.za/fhs/research/animalethics/forms
3. Ensuring that all study participants perform within the confines of the procedures and experimental design of the protocol as approved, or as amended.
4. Ensuring that all study participants comply with all applicable national legislation, UCT policies, FHS AEC policies and standard operating procedures (SOPs) and national standards (SANS 10386: 2008).

AEC REF# 015/041

133
5. Ensuring that you as the PI immediately alert the FHS AEC to any event involving the welfare of the animals which has occurred during the course of the study, as well as the actions that were taken to respond to these events.

6. Ensuring that you as the PI alert the FHS AEC to any new or unexpected ethical issues that arose during the course of the study, and how these issues were addressed.

7. Ensuring that all study participants are registered with or have been authorised by the South African Veterinary Council (SAVC) to perform the procedures on animals, or will be performing the procedures under the direct and continuous supervision of SAVC-registered veterinary professionals or SAVC-registered para-veterinary professionals.

8. If the PI or any study participant is in any way uncertain how to respond to any of these obligations or deal with any of the issues referred to above, they must consult with FHS AEC.

9. All animals found dead must be reported to the RAF on the appropriate form: http://www.health.uct.ac.za/fhs/research/animalethics/forms

10. All animals found in distress must be reported to the RAF on the appropriate form.

My best wishes for a successful research and/or teaching endeavour.

Yours sincerely

[Signature]

PROF P.J. COMMERFORD
CHAIR, FHS AEC

AEC REF# 015/441