

EFFECT OF GRAPEFRUIT JUICE  
ON P-GLYCOPROTEIN ACTIVITY

By

WHOCELY VICTOR DE CASTRO

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To my parents, my wife and my daughter.

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## TABLE OF CONTENTS

	<u>page</u>
ACKNOWLEDGMENTS .....	iv
LIST OF TABLES .....	ix
LIST OF FIGURES .....	xi
ABSTRACT .....	xiii
CHAPTER	
1 INTRODUCTION .....	1
The Grapefruit ( <i>Citrus paradisi</i> ) .....	1
The Health Benefits of Polyphenolics .....	2
Grapefruit Juice-Drug Interactions .....	4
Phenolic Compounds in Grapefruit and Citrus with Potential for Drug Interactions...	9
Mechanism of Action .....	13
Cytochrome P450 Family .....	14
Permeability-glycoprotein: P-gp .....	20
Organic Anion Transporting Polypeptide: OATP .....	26
Oral Drug Absorption .....	28
Contribution of Small Intestine to Drug Absorption and Metabolism .....	29
Mechanisms of Transport of Molecules across Intestinal Epithelium.....	32
Passive Transcellular Diffusion.....	32
Paracellular Transport .....	34
Carrier-Mediated Transport.....	34
Transectosis.....	35
<i>In Vitro</i> Methods for Studying Drug Absorption .....	35
Talinolol: Model Compound for Drugs Subjected to Intestinal Efflux.....	37
Properties of Talinolol .....	38
Hypothesis and Objectives .....	39
Specific Aim 1: Contents of Flavonoids and Furanocoumarins in Grapefruit and Grapefruit Juice.....	41
Specific Aim 2: <i>In Vitro</i> Studies .....	41
Specific Aim 3: <i>In Vivo</i> Studies .....	41

2	VARIATION OF FLAVONOIDS AND FURANOCOUMARINS IN GRAPEFRUIT JUICES: A POTENTIAL SOURCE OF VARIABILITY IN GRAPEFRUIT JUICE-DRUG INTERACTION STUDIES.....	42
	Background.....	42
	Specific Aim.....	43
	Material and Methods.....	43
	Chemicals.....	43
	Sample Preparation.....	44
	Determination of Flavonoids (Naringin and Naringenin).....	44
	Extraction and Determination of Furanocoumarins (Bergamottin and 6',7'-Dihydroxybergamottin).....	45
	Stock and Work Solutions for the Preparation of Calibration Standards.....	46
	Naringin stock solution.....	46
	Naringenin stock solution.....	46
	Bergamottin stock solution.....	46
	6',7'-Dihydroxybergamottin stock solution.....	46
	Naringin work solution.....	47
	Naringenin work solution.....	47
	Furanocoumarins work solution.....	47
	Standard solutions of flavonoids.....	47
	Standard solutions of furanocoumarins.....	48
	Validation.....	49
	Statistical Analysis.....	50
	Results and Discussion.....	51
	Linearity.....	51
	Sensitivity.....	52
	Specificity.....	52
	Precision and Accuracy.....	52
	Recovery.....	57
	Stability.....	57
	Quantification of Naringin, Naringenin, Bergamottin, and 6',7'-Dihydroxybergamottin in Grapefruit Juices.....	58
	Localization of Naringin, Naringenin, Bergamottin, and 6',7'-Dihydroxybergamottin in Grapefruit.....	60
	Conclusions.....	64
3	GRAPEFRUIT JUICE AND ITS COMPONENTS INHIBIT P-GLYCOPROTEIN MEDIATED TRANSPORT OF TALINOLOL IN CACO-2 CELLS.....	65
	Background.....	65
	Specific Aim.....	66
	Material and Methods.....	66
	Chemicals.....	66
	Cell Culture.....	67
	Stock, Work Solutions, and Preparation of Calibration Standards.....	68

Transport Study .....	74
Sample Analysis .....	75
Data Analysis.....	77
Statistical Analysis .....	78
Results.....	78
Linearity .....	78
Specificity.....	79
Sensitivity.....	79
Precision and Accuracy .....	80
Stability.....	80
Concentration Dependence.....	81
Inhibition Studies.....	82
Dose-response Studies.....	85
Effects of NAR and DHB on the Secretary Transport of Talinolol .....	87
DISCUSSION.....	88
Conclusions.....	91
4 MODULATION OF P-GLYCOPROTEIN MEDIATED TRANSPORT OF TALINOLOL IN RATS BY GRAPEFRUIT JUICE, ORANGE JUICE, FLAVONOIDS AND FURANOCOUMARINS.....	92
Background.....	92
Specific Aim .....	93
Materials and Methods .....	93
Chemicals .....	93
Stock, Work Solutions, and Preparation of Calibration Standards .....	94
Talinolol test solutions .....	94
Talinolol stock solution I.....	94
Talinolol stock solution II.....	95
Talinolol stock solution III.....	95
Verapamil test solution I .....	95
Naringin test solutions.....	95
Naringenin stock solution.....	96
Naringenin test solution .....	96
Bergamottin test solutions.....	96
Talinolol standard solutions .....	97
Animals and Experimental Protocol.....	97
Sample Preparation.....	99
Sample Analysis .....	99
Validation .....	100
Pharmacokinetic Analysis .....	101
Statistical Analysis .....	102
Results.....	102
Validation of Analytical Method to Measure Talinolol in Rat Serum .....	102
Linearity .....	102
Specificity.....	103
Sensitivity.....	104

Precision, accuracy and recovery .....	104
Stability .....	105
Talinolol Pharmacokinetics .....	105
Discussion.....	108
Conclusions.....	111
5 CONCLUSIONS .....	113
LIST OF REFERENCES.....	116
BIOGRAPHICAL SKETCH .....	135

## LIST OF TABLES

<u>Table</u>	<u>page</u>
1-1 Pharmacokinetics parameters of talinolol after oral administration (50 mg) by health volunteers (192).....	39
2-1 Concentrations of the standard solutions used for the calibration curves and quality controls (QCs) of naringin (NAR) and naringenin (NAG).....	48
2-2 Concentrations of the standard solutions used for the calibration curves and quality controls (QCs) of bergamottin (BG) and 6',7'-dihydroxybergamottin (DHB).....	48
2-3 Intra-day (n = 3) and inter-day (n = 9) assay parameters of furanocoumarins (bergamottin and 6',7'-dihydroxybergamottin) and flavonoids (naringin and naringenin).....	55
2-4 Recovery values (means $\pm$ SD) of bergamottin, 6',7'-dihydroxybergamottin, naringin, and naringenin from the spiked orange juice.....	57
2-5 Naringin, bergamottin and 6',7'-dihydroxybergamottin content in different brands of grapefruit juice sold in Florida.....	63
3-1 Concentrations of the work solutions of talinolol (TAL) used during the transport studies.....	71
3-2 Concentrations of the standard solutions used for the calibration curves and quality controls (QCs) of talinolol.....	71
3-3 Concentrations of the inhibitory solutions of Verapamil hydrochloride (VER), naringin (NAR), naringenin, (NAG), bergamottin (BG), 6',7'-dihydroxybergamottin (DHB), and 6',7'-epoxybergamottin (EPBG).....	73
3-4 Concentrations of the inhibitory solution of naringin (NAR) plus 6',7'-dihydroxybergamottin (DHB) used during the transport studies.....	74
3-5 Intra-day (n = 3) and inter-day (n = 9) assay parameters of talinolol. Precision expressed as CV% and accuracy as % of the theoretical concentration.....	80
3-6 Concentration range that the grapefruit components are normally found in the juice.....	85

4-1	Concentrations of the standard solutions used for the calibration curves and quality controls (QCs) of talinolol. ....	97
4-2	Regression parameters obtained from calibration curves of talinolol in plasma in three different days.....	103
4-3	Intra-day (n = 3), inter-day (n = 9), and recovery (n = 3) assay parameters of talinolol in rat serum.. ....	104
4-4	Pharmacokinetics of talinolol in rats after a single dose oral dose (10 mg/kg).....	108

## LIST OF FIGURES

<u>Figure</u>	<u>page</u>
1-1 Chemical structures of phenolic compounds in citrus. ....	12
1-2 Mucosa of small intestine.....	31
1-3 Possible transport routes a drug can follow to cross the intestinal epithelium .....	32
1-4 Molecular structure of talinolol. (*) chiral center. ....	38
2-1 Mean calibration curves (n = 9) of naringin (A) and naringenin (B) in methanol/water (1:1), and of bergamottin (C) and 6',7'-dihydroxybergamottin (D) in DMSO/methanol (1:3).....	51
2-2 HPLC separation and absorbance-wavelength spectra of A - furanocoumarins: 6',7'-dihydroxybergamottin and bergamottin; B - flavonoids: naringin and naringenin.....	53
2-3 Chromatograms corresponding to the orange juice (OJ), standard solutions (Std) containing 6',7'-dihydroxybergamottin (DHB) plus bergamottin (BG) – (panel A), and naringin (NAR) plus naringenin (NAG) - (panel B).....	54
2-4 Absorbance-wavelength spectra of A – bergamottin; B - 6',7'-dihydroxybergamottin; C - naringin.....	54
2-5 Results of the stability test after 36 hours at room temperature (A) and after 4 freeze/thaw cycles at -20 °C (B).. ..	58
2-6 Distribution of naringin (A), bergamottin (B) and 6',7'-dihydroxybergamottin (C) in different tissues of white (open bars) and ruby red (shaded bar) grapefruits.....	62
3-1 Structures of selected flavonoids and furanocoumarins present in grapefruit juice and tested in this study. ....	66
3-2 Mean calibration curve (n = 9) of talinolol in mobile phase. Vertical bars represent the standard deviations (SD) of the means. ....	78
3-3 Chromatograms corresponding to the talinolol (100 ng/mL) in HBSS-MES. ....	79

3-4	Stability of talinolol (low, medium, and high QCs).....	81
3-5	Concentration dependent coefficient permeability ( $P_{app}$ ) corresponding to the AP to BL and BL to AP transport of talinolol across Caco-2 cells monolayers..	82
3-6	Coefficient of permeability ( $P_{app}$ ) of talinolol (100 $\mu$ M) across the Caco-2 cells monolayers. ....	84
3-7	Inhibitory dose-response effects.....	86
3-8	Effect of the combination NAR:DHB (50:1.2; 125:3, 625:15, 1250:30, and 2000:48 $\mu$ M) on the secretory transport (BL-AP) of talinolol (100 $\mu$ M) across the Caco-2 cells monolayers..	87
4-1	Comparison of chromatograms corresponding to the talinolol (I) and the internal standard propranolol hydrochloride (II) after extraction from rat serum (A) with that obtained after extraction of a blank sample (B). ....	103
4-2	Stability of talinolol (low, medium, and high QCs) in rat serum within 24 hours at room temperature (RT) (A) and during 3 weeks at -70 °C (B). Data represents means of three replicates and vertical bars the standard deviation of the means...	105
4-3	Mean serum concentration-time profiles (n = 8) of talinolol in rats after a single dose oral dose (10 mg/kg)..	107

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By

Whocely Victor de Castro

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Chair: Hartmut Derendorf

Cochair: Veronika Butterweck

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Grapefruit juice (GJF) interacts with several medications increasing their oral bioavailability and the risk of toxicity. The predominant mechanism is the inhibition of intestinal CYP3A4 by flavonoids and furanocoumarins present in the juice. Additionally, it has been suggested that GFJ modulates the activity of the permeability-glycoprotein (P-gp). In the intestine, this efflux transporter acts as a biological barrier pumping a variety of drugs back from the enterocytes to the intestinal lumen. Inhibition of this back-secretion may increase the initial amount of drug that enters the systemic circulation and the possibility of side effects.

The aim of this study was to compare the ability of GFJ and some of its specific flavonoids and furanocoumarins to interact with P-gp *in vitro* and *in vivo* in order to estimate the clinical relevance of this interaction. Additionally, the components of these interacting compounds in commercially available and fresh-squeezed GFJ as well as in

different tissues of grapefruit were quantified and compared in order to assess their potential influence on the data gained in drug interaction studies.

Flavonoids and furanocoumarins were quantified by high performance liquid chromatography (HPLC) and considerable variation was observed among the juices tested. White grapefruit showed the highest concentration of naringin and furanocoumarins located in albedo (inner peel) and flavedo (yellow layer of the peel) compared with red varieties. Findings from my study suggest that the concentration of potentially contributing compounds may crucially influence the magnitude of the interaction and invalidate direct comparisons of studies where different juices have been used.

The transport of talinolol, a non-metabolized P-gp substrate, across Caco-2 cell monolayers, was determined in the absence and presence of distinct concentrations of grapefruit juice and its constituents. A sigmoid dose-response model was used to fit the data and to estimate the potencies of the potential inhibitors. Results from this study show that isolated compounds of GFJ are able to inhibit the P-gp activity, but the overall effect seems not to be a simple additive effect.

The effects of different types of GFJ and its components on the oral pharmacokinetics of talinolol in rats suggest that this interaction is unlikely to be of clinical relevance.

## CHAPTER 1 INTRODUCTION

### **The Grapefruit (*Citrus paradisi*)**

The word citrus is the Latin name of “*Kedros*”, a Greek word to designate trees like cedar, cypress and pine. Because the scent of citrus leaves and fruits was suggestive of cedar, the name citrus has been linked to the citron (*Citrus medica*), the first citrus with which the Europeans got acquainted. Later, Linnaeus grouped all other citrus fruits known to him in the genus *Citrus* (1). However, the taxonomy and phylogeny have not been completely elucidated yet and are subject of controversy, mainly, due to sexual compatibility between citrus and related genera, the high frequency of mutations, long history of cultivation, and wide dispersion (2).

The grapefruit (*Citrus paradisi*), specifically, belongs to the Rutaceae family and received this denomination because the fruits grow in clusters similar to grapes (3). Studies using genetic markers, together with conventional and taxonomic approaches, strongly support that the grapefruit originated from an accidental hybrid between pummelo (*Citrus maxima*) and sweet orange (*Citrus sinensis*) (2-4).

Almost all of *Citrus* species and its wild relatives are supposed to have been originated in the tropical and subtropical regions of Southeast Asia (5). Conversely, it is suggested that grapefruit emerged from the island of Barbados in the Caribbean, where Griffith Hughes, the rector of St. Lucy’s parish of Barbados, earliest described it as the “forbidden fruit of Barbados” in 1750 (3). However, the first scientific description of

grapefruit is attributed to the botanist James Macfadyen in 1837, who named the forbidden fruit *Citrus paradisi* Macf. (3).

Grapefruit seeds were probably brought to Florida in the beginning of the eighteenth century by Counte Odette Phillippi and distributed to locate growers, where the settlers began to sow the seeds and acquired taste for the fruits (1, 6). Since that time, several cultivars have been created, especially in the Indian River citrus region stretching from Daytona Beach to central Palm Beach County. Today, in a typical season, Florida accounts for 2 million tons of grapefruit a year corresponding to about 80 percent of the U.S. total grapefruit production (7).

### **The Health Benefits of Polyphenolics**

The health benefits associated with the consumption of vegetables and fresh fruits are largely accepted and recognized by many (8-11). They are related to the presence of polyphenolic compounds, particularly, flavonoids described to act as antioxidants in different biological systems (12). In fact, Sun *et al.* (13) demonstrated a linear relationship between the total phenolic content and the antioxidant activity in phytochemical extracts of different fruits, including grapefruit ( $r^2 = 0.98$ ,  $p < 0.01$ ), indicating that phenols seem to be the major contributor to the total antioxidant activities in fruits. The antioxidant activity of flavonoids is principally based on their ability to capture electrons providing great stability to the flavonoid radical and preventing the propagating chain reactions of the oxygen-free radicals formed during the natural metabolism of any aerobic cells (12). Flavonoids may also act by chelating iron, which is thought to catalyze the process leading to the appearance of free radicals (14).

Included in the ordinary diet of many Americans, the grapefruit and the grapefruit juice are rich in flavonoids, such as narirutin, naringin, hesperidin, neohesperidin,

didymin, quercetin, poncirin and kaempferol. Their total concentration in the grapefruit juice (GFJ) range from 200 to 840 mg/L (15).

It has been demonstrated that flavonoids have a protective role against many degenerative disorders, such as cancer and cardiovascular diseases (11, 14, 16, 17). They can prevent or minimize the oxidation of low- and very low-density lipoproteins, disrupting a crucial step in the formation of the atherosclerosis lesion and then reducing thrombotic tendencies (14, 18). The low-density lipoprotein (LDL) can be normally oxidized or modified within sub-endothelial tissue by cells of the arterial wall including macrophages, vascular smooth cells, and endothelial cells (19). The modified LDL can trigger a receptor-mediated process, leading macrophages and smooth muscle cells to accumulate significant amounts of cholesteryl ester (CE). These CE-rich cells or foam cells are characteristic indicators of early atherosclerotic lesions. There is some evidence that after consumption of foods rich in flavonoids, such compounds were able to reach the sub-endothelial space of the arterial wall at concentrations enough to protect lipoproteins like LDL from oxidation (20).

Le Marchand *et al.* (21) found decreased rates of lung cancer among the Hawaiian population whose diet included a large amount of onions and apples (rich in the flavone quercetin) and white grapefruit (rich in the flavanone naringin). Additionally, Kawaii *et al.* (16) assessed the chemopreventive properties of pure flavonoids from citrus fruits against four human cancer cell lines (lung, melanoma, leukemia and gastric cancer). The anticarcinogenic properties of flavonoids are related to the suppression of metabolic activity; antimutagenic, antioxidative, and antiproliferative effects; detoxification of

carcinogens; inactivation of oncogenes; inhibition of DNA adduct formation and the carcinogenic cell invasion (11-14, 16, 18).

Cerda and co-workers (22) also have shown that dietary supplementation with grapefruit pectin resulted in the decrease of plasma cholesterol (7.6%), low density lipoprotein cholesterol (10.8%) and the ratio of low-density to high-density cholesterol (9.8%) in healthy subjects. Furthermore, orange juice, grapefruit juice, and naringin, a major grapefruit flavonoid, have been shown to have a beneficial effect on the blood lipid profile and antioxidant capacity in rats (23, 24).

A case-control study carried out in 299 women (154 had lung cancer and 145 were the control group) in Athens during a eighteen-month period did not find any correlation between intake of food with high content of flavonoids with the etiology of lung cancer (25). However, two distinct epidemiological studies involving around 11,000 volunteers conducted by different groups in Finland and Netherlands during 1966-1976 and 1985-1990, respectively, found that regular consumption of a diet rich in flavonoids was inversely related to coronary heart disease mortality; cerebrovascular disease, lung and prostate cancer, type 2 diabetes and asthma (26, 27).

Therefore, *in vitro*, pre-clinical, clinical, and epidemiological studies have suggested that ingestion of flavonoid-rich foods such as grapefruit juice and grapefruit juice provide some benefits against heart disease and cancer. However, since the early nineties these potential health benefits have been over shadowed by possible risks of interactions between drugs and grapefruit and grapefruit juice.

### **Grapefruit Juice-Drug Interactions**

The first report of grapefruit juice interacting with a drug, altering its bioavailability was published in 1991. This accidental discovery was made in a study on

ethanol drug-interactions where GFJ was used to mask the taste of the alcohol: the bioavailability of felodipine, a calcium channel blocker, was increased when subjects were consuming GFJ concomitantly with the drug. Additionally, the subjects showed a decreased diastolic blood pressure and an increased heart rate (28).

Subsequent research in the area of fruit-drug interactions focused on grapefruit and grapefruit compounds of which several were found to affect the absorption or metabolism of certain drugs. Grapefruit juice was shown to modify greatly the disposition of a variety of medications taken orally, including some 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (lovastatin, simvastatin) (29, 30), calcium channel blockers (felodipine, nicardipine, nisoldipine) (31-33), benzodiazepines (midazolam, diazepam) (34, 35), and HIV protease inhibitors (saquinavir) (36). Other fruits, vegetables and dietary supplements also have the potential to cause an adverse interaction with conventional drugs and are objects of concern (37).

Additionally, consumers have become more aware of the health benefits of antioxidants, which increased the availability and consumption of dietary supplements, phytochemical extracts, and foods rich in antioxidant flavonoids, especially, together with prescription drugs. In fact, over 16% of all prescription drug users reported that they concurrently take at least one plant-based dietary supplement including grapefruit and citrus products (38). This may increase the likelihood of an adverse interaction between foods and certain drugs. For example, the absorption and/or metabolism of a drug may be harmfully affected, shifting the administered dose outside of the therapeutic range, which may lead to a lower effectiveness of the drug or to an overdose associated with undesired or even dangerous side effects (39).

Byproducts from the citrus-processing industry such as grapefruit seed extract, flavonoids, essential oils from the peel, and pectins may be added to other food products in order to improve taste, consistency or overall quality. These byproducts also may be used in the production of dietary supplements (40). Consequently, consumption of food products containing such additives, which have a potential for an interaction with drugs, are not free of risk.

Based on the current knowledge, grapefruit compounds interact with drugs, which are metabolized by cytochrome P450 3A4 (CYP3A4) and also have a low or variable oral bioavailability. In general, the drugs subjected to GFJ interaction have their area under the concentration-time curve (AUC) and maximum plasma concentration ( $C_{max}$ ) increased from 1.2 to 15-fold after oral administration, and the magnitude of the effect maybe dependent on the brand and amount of GJF ingested, the timing of administration relative to the food intake and the intrinsic oral bioavailability of each drug (41-44).

The enhancement of the oral bioavailability of some drugs such as felodipine, nifedipine, and halofantrine after consumption of GFJ has been associated with higher incidence of dose-dependent side effects among the subjects (32, 45, 46). Indeed, one case-report study depicted a 59-year old patient receiving 60 mg of atorvastatin (Lipitor®) daily who developed rhabdomyolysis after drinking GFJ. Such condition was reverted and his cholesterol levels normalized after replacing the current drug by pravastatin, another HMG-CoA inhibitor but not but not metabolized by CYP3A4 (47).

Grapefruit and GFJ have a potential to interact with several oral medications when consumed in moderate amounts such as 1-2 servings (48). The concern, that the concomitant administration of grapefruit products with certain drugs may result in

toxicity has led to the recommendation to avoid consuming grapefruit products in combination with these medicines. The Food and Drug Administration (FDA) now requires drugs such as cyclosporine, sirolimus, simvastatin, lovastatin, and felodipine, to carry a warning label regarding the possibility of an interaction. For example, Neoral®, an immuno-suppressant drug, carries a label stating “...Grapefruit and GFJ affect metabolism, increasing blood concentrations of cyclosporine, thus should be avoided” (49). Procardia® is labeled “...Co-administration of nifedipine with GFJ resulted in approximately a 2-fold increase in nifedipine AUC and  $C_{max}$  with no change in half-life. The increased plasma concentrations are most likely due to inhibition of CYP3A4 related first-pass metabolism. Co-administration of nifedipine with GFJ is to be avoided” (50). Interactions with a number of medications also have been shown for Seville orange juice, although Seville oranges are usually not processed to juice (51, 52).

For most drugs in question, definitive recommendations regarding their concomitant administration with GFJ are not available, since conclusions concerning the clinical significance of the observed or predicted grapefruit-drug interactions are still limited and many data available are derived from *in vitro* experiments and not directly applied to human responses. Furthermore, the determination of the clinical relevance of observed interactions in human intervention trials is complicated by individual variability. Additionally, predicting the clinical significance of pharmacokinetic drug interactions is sometimes difficult especially for drugs where there are no robust methods to quantify effects or side effects. There has been recent effort in the United States by the FDA and the Pharmaceutical Research and Manufacturers of America (PhRMA) to establish some general guidelines to help drug companies, prescribers, and patients

interpret the clinical significance of drug interactions(53). These are the result of clinical experience gained from some well-known drug interactions, such as the inhibition of CYP3A4. For this interaction, specifically, it could be shown that the benzodiazepine midazolam is a reproducible probe that allows quantitative determination of a potential interaction with an inhibitor of the CYP3A4 enzyme. The degree of interaction can be measured based on the augmentation in the AUC of the midazolam serum concentrations. It was recently proposed to classify changes of midazolam AUC being less than 2-fold as “weak interaction”, which is the case observed on midazolam co-administration with ranitidine, relatively small volumes of GFJ, roxithromycin, fentanyl, or azithromycin(53). AUC changes that range from 2- to 4.9-fold, which occur on midazolam co-administration with erythromycin, diltiazem, fluconazole, verapamil, relatively large volumes of GFJ, and cimetidine are classified as “moderate interaction.” Changes that are 5-fold or higher in midazolam AUC are labeled as “strong interaction.” Examples of drugs demonstrating strong midazolam interactions include: ketoconazole, itraconazole, mibefradil, clarithromycin, and nefazodone. Strong drug interactions are considered clinically significant and result in contraindications or warnings on the product label. The clinical significance of moderate inhibitors may include decisions about dose adjustments that should be based on the concentration-effect relationship.

It should be noted, however, that almost all drugs, which show an interaction with grapefruit, can be replaced by alternative drugs belonging to the same therapeutic class but without a recognized potential for interaction.

The major drug classes for which grapefruit or other citrus interactions include antiallergics, antibiotics, anticoagulants, antimalaria drugs, antiparasitics, anxiolytics,

calcium channel blockers, HMG-CoA reductase inhibitors, anti-HIV drugs, hormones, immunosuppressants, anti-tumors, and beta blockers (40, 42, 43, 48, 54-57).

### **Phenolic Compounds in Grapefruit and Citrus with Potential for Drug Interactions**

Phenolic compounds represent the major group of citrus compounds. Included are hydroxycinnamic acids, flavonoids, such as flavanones, flavones, and flavonols, anthocyanins, and furanocoumarins (Figure 1-1) (58). As previously described, many of these components have antioxidant properties and may play an important role in preventing chronic diseases such as cancer, coronary heart disease, diabetes, among others (26, 27).

GFJ components such as the flavonoids naringin (NAR), naringenin (NAG), quercetin, kaempferol and the furanocoumarins bergamottin (BG), 6',7'-dihydroxybergamottin (DHB) and its dimers, bergapten, bergaptol, and 6',7'-epoxybergamottin (EPBG) (Figure 1-1) have been suggested to contribute to GFJ-drug interaction (54, 59-63).

*In vitro* and *in vivo* studies have demonstrated interactions between many drugs and the furanocoumarins, BG and DHB. These GFJ compounds decreased the hepatic metabolism of saquinavir *in vitro* and the concentrations necessary to reach half of the maximal effect ( $IC_{50}$ ) was shown to be 0.33  $\mu$ M and 0.74  $\mu$ M, respectively (64). Additionally, BG was able to augment the  $C_{max}$  and AUC of diazepam in dogs (65), of nifedipine in rats (66), and of felodipine in humans (67). Several human intervention studies also confirm the contribution of BG and DHB to drug-interactions with GFJ (41, 68-70).

GFJ components have been demonstrated to interact with susceptible drugs by different mechanisms besides the reduction of metabolism. For example, BG, DHB, bergaptol and bergapten were shown to elevate the steady state uptake of [<sup>3</sup>H]-vinblastine sulfate in Caco-2 cells (71), whereas BG and DHB caused a decrease in the organic anion transport peptide (OATP)-B-mediated uptake of estrone-3-sulfate into human embryonic kidney cells (72). Therefore, the effects of grapefruit and GFJ are not restricted on the activity of the enzyme CYP3A4 but also involve modulation of membrane influx and efflux transporters such as the permeability glycoprotein (P-gp) and the OATP, respectively.

Interactions with drugs also have been demonstrated for flavonoids from citrus. Naringenin and naringin have been shown to inhibit the OATPB-mediated uptake of estrone-3-sulfate in human embryonic kidney cells (72) and to interact with saquinavir (64) and simvastatin (73) *in vitro*. Compared to the control group, NAR increased by 2-fold the  $C_{max}$  and the AUC of diltiazem in rats, while there was no significant change in the time to reach the  $C_{max}$  ( $T_{max}$ ) and terminal plasma half-life ( $t_{1/2}$ ). NAR also increased the oral bioavailability of quinine in rats by 42% (74). However, this flavonoid did not show any effect on the pharmacokinetics and pharmacodynamics of nisoldipine in healthy volunteers (75).

Quercetin inhibited P-gp-mediated efflux of ritonavir in Caco-2 cells (76), increased the  $C_{max}$  and AUC of verapamil in rabbits by 2-fold (77), and inhibited the metabolism of midazolam and quinidine in human liver microsomes (78). It did not have an effect on CYP3A4-mediated metabolism and P-gp mediated transport of saquinavir

(64). Nobelitin and tangeretin inhibited OATP-B-mediated uptake of estrone-3-sulfate into human embryonic kidney cells (72).

For several phenolics from citrus such as eriocitrin, poncirin, and sinapic acid and anthocyanins, which occur in red grapefruit varieties and blood oranges, no interactions with CYP3A4 and P-gp have been reported yet.

*In vitro* studies of single compounds can help to understand the mechanism of action of each component as well as their relative contribution to the overall effect observed after ingestion of a food with potential for interacting with drugs. However, the clinical relevance of data obtained from *in vitro* studies with single compounds is questionable, because they have a limited predictability of the effects of the examined compounds *in vivo*. Moreover, some phenolics were shown to modify the absorption or metabolism of drugs only at very high concentrations, which are likely to exceed the expected *in vivo* concentration after the consumption of a moderate amount of a grapefruit/citrus product. Additionally, the overall effect of GFJ in drug interactions studies is probably the result of the mixture of several compounds present in the juice. Therefore, the existence of synergism, antagonism or additive effects needs to be investigated by testing, simultaneously, different combinations of phenolic compounds normally found in GFJ. Nevertheless, studies with single compounds have demonstrated that furanocoumarins and their dimers are primarily responsible for the interactions of GFJ and drugs.

In conclusion, grapefruit, sour orange (Seville), and limes, which contain BG (69) seem to have the highest potential among the citrus species for interacting with drugs, whereas other citrus varieties such as sweet orange seem to have an overall low potential

for interfering with medications. However, in studies using juices rather than single compounds, orange juice (*C. sinensis*), which does not contain NAR and only small amount of BG and DHB, has also been shown to interact with some drugs. A more recent study in rats demonstrated that orange juice (and apple juice) decreased the oral exposure of fexofenadine, possibly through an inhibition of the influx transporter OATP (79). The interaction of orange juice and fexofenadine has also been demonstrated in HeLa cells, where orange juice at 5% of the normal strength potentially reduced the uptake of fexofenadine, mediated through the human OATPA (80). In general, the effect of orange juice on CYP3A4 is negligible and in some GFJ-drug interaction studies orange juice was even used as control (81, 82).

Overall, it can be concluded that orange juice has a minor potential for drug interactions.

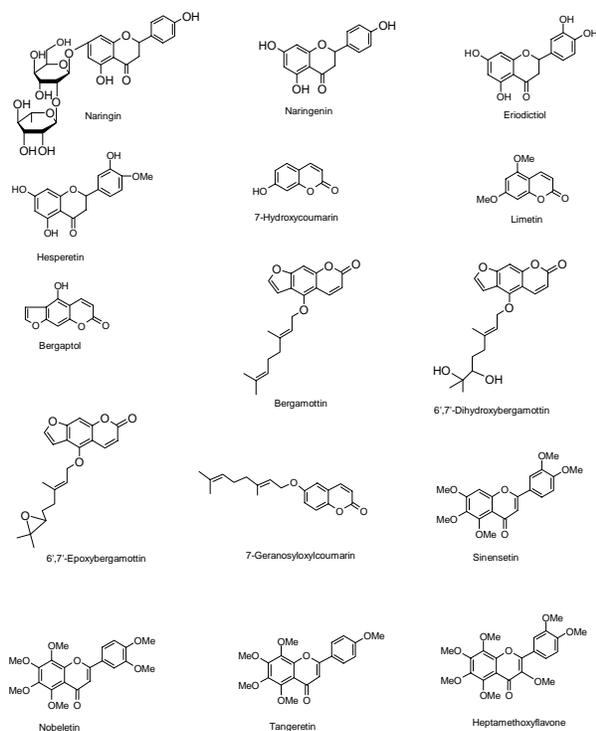


Figure 1-1. Chemical structures of phenolic compounds in citrus.

### **Mechanism of Action**

A food-drug interaction can be defined as the alteration of drug exposure and drug effects by food. The underlying mechanisms can be classified into two broad categories. The first category is pharmacokinetics, which includes alterations in absorption, distribution, metabolism, and excretion. The second category is pharmacodynamics, which describes alterations in the drug concentration-effect relationship (83). Changes in the pharmacokinetics of drugs are the more common consequences of citrus-drug interactions, which may shift the effect of the drug outside of its therapeutic window, possibly leading either to loss of effect or undesired side effects, or even toxicity (48).

Because the majority of drugs exhibiting an interaction with GFJ are metabolized mostly by CYP3A4, which is present not only in the hepatocytes, but also in the epithelial cells of the intestine, it has been suggested that the effect of GFJ may be due, primarily, to the inhibition of the activity of this drug-metabolizing enzyme (48, 57, 83). However, differently from the most potent CYP3A4 inhibitors such as ketoconazole, where the main site of action is the liver, GFJ when consumed in usual volumes, is an example of an inhibitor that appears to act preferentially in the enteric CYP3A4, resulting in a significant reduction of the presystemic metabolism of drugs without, therefore, modifying their elimination half-life (54, 56). For several drugs such as felodipine, cyclosporine, midazolam, and nifedipine, the gastrointestinal mucosa has been demonstrated to be a major metabolic organ, where an inhibition of CYP3A4 caused an increase in oral bioavailability (84). Additionally, GFJ intake did not change the pharmacokinetics and pharmacodynamics parameters of midazolam (85), and felodipine (45) after intravenous administration, but increased their systemic bioavailability from oral dosage forms,

supporting that inhibition of intestinal CYP3A4 by maybe particularly important when the drug is given orally together with the juice.

Additionally, it has been suggested that GFJ can delay the gastric emptying rate contributing, in part, to the double peak phenomenon observed after co-administration of nifedipine with the juice (86). In fact, the lower the pH of the gastric chyme that reaches the small intestine, the slower will be rate of gastric emptying (87). It is also suggested that GFJ influences not only the first-pass metabolism of drugs like nifedipine, but also its secondary oxidative steps (86).

In summary, the major mechanism leading to a grapefruit-drug interaction appears to be the reduction of the "first-pass" metabolism through the inhibition of intestinal CYP3A4 (57), although inhibition of secondary metabolic pathways and decrease of gastrointestinal motility cannot be excluded. In addition, GFJ also can modify the disposition of several drugs substrates of P-gp and recent reviews compile the effects and the clinical relevance of such interaction (43, 88). Moreover, GFJ and other fruit juices have been shown to inhibit *in vitro* and *in vivo* a number of organic anion-transporting polypeptides (OATPs) (72, 79, 80, 89, 90).

### **Cytochrome P450 Family**

The cytochrome P450 (CYP) enzyme family is the major catalyst of phase I drug biotransformation reactions and accounts for the metabolism, at least in part, of about 60% of all drugs subjected to oxidation in the body (91). CYP enzymes are bound to membranes of the endoplasmatic reticulum and are predominantly expressed in the liver, although they are also present in extra-hepatic tissues such as the gut mucosa (84). They can catalyze a notable number of metabolic processes including aliphatic oxidation, aromatic hydroxylation, *N*-dealkylation, *O*-demethylation, *S*-demethylation, oxidative

deamination, sulfoxide formation, *N*-oxidation, *N*-hydroxylation (83). Such biotransformation reactions usually lead to inactivation and elimination of most pharmaceuticals, therefore, ingestion of xenobiotics able to inhibit or activate the CYP enzymes can affect the disposition of many of CYP-drug substrates.

More than 50 different human CYPs have been recognized to date and their nomenclature is determined by resemblance of amino acid constitution (83). For instance, the isoform CYP3A4 belongs to family 3 (matching 40% of amino acid sequencing) and subfamily 3A (matching 55% of amino acid sequencing) (48). Among the CYP3A family, the CYP3A4 isoform is the most abundantly expressed and represents approximately 30% to 40% of the total CYP protein in human adult liver (92). It also can be found in the enterocytes of the small intestine at levels of 10 to 50% lower than those found in the liver (84). However, CYP3A4 concentrations equivalent or above of those found in liver have already been verified in some subjects (93-95), which can explain much of the inter-subject variability observed during clinical trials studying GFJ-drug interactions.

The relatively high expression levels in the intestinal mucosa and the broad substrate specificity may contribute to the high susceptibility of CYP3A4 for citrus-drug interactions (96). Many drugs for which interactions with citrus have been demonstrated are metabolized by CYP3A4 (48, 57, 83).

It is currently recognized that the major mechanism for grapefruit-drug interaction is the inhibition of the drug-metabolizing enzyme CYP3A4 in the small intestine resulting in a significant reduction of metabolism of drugs during their first passage from the intestinal lumen into the systemic circulation (54). Types of intestinal CYP3A4

inhibition by GFJ and its components comprise both competitive and mechanism-based, the latter resulting in accelerated degradation of the enzyme through suicide inhibition (97, 98). In fact, the amount of CYP3A4 in the small intestine of healthy volunteers has been shown to decrease by 50-60% after 4-6 h administration of 8 oz (~237 mL) GFJ, without any change in corresponding messenger RNA levels (60, 97).

Several drug classes such as dihydropyridine calcium antagonists and HMG-CoA-reductase inhibitors are affected by grapefruit-induced inhibition of CYP3A4. GFJ increased the AUC and maximal plasma concentration ( $C_{max}$ ) for these calcium antagonists within an approximated range of 1.5- to 4-fold on average of the values obtained with water (48).

For the HMG-CoA reductase inhibitor atorvastatin, double-strength GFJ increased the AUC 2.5-fold but not the  $C_{max}$ , when the GFJ was administered over three days and the drug was given on day three (99). In a very similar study design performed by the same group with simvastatin, double-strength GFJ increased the AUC 16-fold and  $C_{max}$  9-fold (100). Additionally, repetitive ingestion of high volumes of grapefruit juice has augmented the AUC values of lovastatin over 10- fold compared with water (29). Furthermore, a daily volume of 250 mL of grapefruit juice had a modest effect on lovastatin, however, in this study grapefruit juice had been taken about 12 h apart from statin (101). All drugs mentioned above are, to a certain extent, subject to CYP3A4 metabolism.

Lundhal *et al.* (102) compared the acute effect of GFJ intake (200 mL) on the pharmacokinetics and haemodynamics parameters of the CYP3A4 substrate felodipine with the interaction following 14-days intake of drug with juice. The AUC and  $C_{max}$  of

felodipine after acute treatment with GFJ and on day 14 were similar and around 57% to 73% and 114% to 138% higher than those from the control group, respectively.

Additionally, the authors reported a decrease of heart rate and increase of vascular adverse events during the GFJ period compared with the control. These results suggest that one glass of GFJ is enough to produce the maximum inhibitory effect on the intestinal CYP3A4 activity.

A moderate consumption does not appear to lead to an inhibition of hepatic CYP3A4 activity (103), however, the repeated consumption (200 mL three times a day) of double-strength GFJ induced a higher increase in triazolam concentrations and prolonged its half-life. The repeated consumption of GFJ may have caused an inhibition of hepatic CYP3A4 (104). In a three day study, GFJ increased the AUC of simvastatin 3.6-fold and that of its active metabolite, simvastatin acid, 3.3-fold.  $C_{max}$  of simvastatin and simvastatin acid were increased 3.9-fold and 4.3-fold, respectively, when the GFJ was administered for three days and simvastatin on day three (30). In an *in vitro* study performed with several grapefruit compounds, it was shown that BG, DHB, and the furanocoumarin dimers GF-I-1 and GF-I-4 inhibited CYP3A4-catalyzed nifedipine oxidation in a concentration- and time-dependent manner, which is consistent with the mechanism-based inhibition. DHB was more potent than BG, while the dimers were more potent than the monomers. Not only CYP3A4 but also CYP2C9, CYP2C19, and CYP2D6 seem to be affected by citrus compounds. In the same study, the inhibitory effect of BG was stronger on CYP1A2, CYP2C9, CYP2C19, and CYP2D6 than on CYP3A4 (105). In an intervention trial with healthy volunteers, GFJ (twice daily) decreased the activity of CYP1A2, as determined with caffeine as a probe (106). In

another human study, GFJ and naringenin caused a minor reduction of the activity of CYP1A2 (107). In addition, ingestion of 8 oz of regular-strength GFJ once or three times a day did not affect liver CYP3A4 activity, colon levels of CYP3A5, or small bowel concentrations of P-glycoprotein, villin, CYP1A1, and CYP2D6 (60, 97). Overall, the inhibition of CYP enzymes other than CYP3A4 does not appear to be of great magnitude and may clinically be relevant only for drugs with a narrow therapeutic range (96).

There is a lack of information available regarding the reversible and mechanism-based inhibition kinetics for grapefruit compounds. In a study conducted with human intestinal microsomes by Paine and coworkers (103), DHB induced a substrate-independent reversible and mechanism-based inhibition on CYP3A4. In contrast, BG, a more lipophilic compound, was a substrate-dependent reversible inhibitor and a substrate-independent mechanism-based inhibitor. For BG, the inhibition for testosterone was more potent than for midazolam, possibly due to the higher affinity of BG for the testosterone-binding site than for the midazolam-binding site. As mechanism-based inhibitors, BG and DHB are definitely substrates for CYP3A4, but the binding sites are not known. The authors suggested that both furanocoumarins inactivate CYP3A4 by the binding of the furanoepoxide to the apoprotein, presumably at or near their respective substrate domains. The same group determined the onset time of inhibition by both compounds (63). It was found that DHB inhibited 85% of CYP3A4 activity independent of substrate within 30 minutes, whereas the onset for BG-induced inhibition was much later (70% inhibition was reached after three hours). The substrate-dependent inhibition caused by BG was more than 50% after 0.5 to 3 hours for testosterone 6-hydroxylation, while midazolam 1'-hydroxylation was unaffected, or activated, within one hour. Both

furancoumarins caused 40% to 50% reduction of CYP3A4 protein, probably due to intracellular degradation of the enzyme caused by mechanism-based inactivation. These data imply that, after the consumption of GFJ, DHB causes the enzyme inhibition earlier than BG.

Lilja and coworkers (108) conducted a crossover study in which 10 healthy volunteers ingested 40 mg simvastatin with water (control), or with "high-dose" grapefruit juice (200 mL double-strength grapefruit juice three times a day for 3 days), or with water on days 1, 3, and 7 after ingestion of GFJ. When simvastatin was taken with GFJ, the mean  $C_{\max}$  and AUC of simvastatin were increased 12.0-fold and 13.5-fold, respectively, compared with control. The  $C_{\max}$  values when simvastatin was administered with water 24 hours, and 3 days after ingestion of the last dose of GFJ, were increased 2.4- and 1.5-fold, respectively, compared with the control. Similarly, the AUC values were 2.1- and 1.4-fold higher than those of the control group after 24 hours and 3 days of GFJ intake, correspondingly. Seven days after ingestion of GFJ, no difference in the  $C_{\max}$  or AUC of simvastatin were observed. Furthermore, Greenblatt *et al.* (109) determined, in a human intervention trial, the time of recovery of intestinal CYP3A4 after the consumption of 300 mL of regular-strength GFJ and a single dose of midazolam at 2, 26, 50, or 74 hours after administering the juice. After two hours, the AUC was 1.65-fold increased and after 26, 50, and 74 hours, the AUC was 1.29-, 1.29-, and 1.06-fold higher, respectively, in comparison to the control. The recovery half-life was estimated at 23 hours. These results indicate that the interaction of grapefruit juice with CYP3A4 substrates dissipates within 3 to 7 days after ingestion of the last dose of GFJ, consistent with a mechanism-based inhibition.

In summary, the presented *in vitro* studies confirm the inhibitory effects of grapefruit compounds on CYP-enzymes, with major effects on CYP3A4, involving both mechanism-based and reversible inhibition. DHB appears to be more potent than BG; however, furanocoumarin dimers seem to be more effective than monomers in the inhibition of CYP3A4. The human intervention trials examining the pharmacokinetic interaction of GFJ revealed a great inter-individual variability, where subjects with the highest content of CYP3A4 showed the largest reduction of this enzyme after the consumption of grapefruit.

### **Permeability-glycoprotein: P-gp**

The interest in transporters as mediators of interactions between grapefruit and drugs is increasing and one of the most studied drug transporters is the permeability-glycoprotein or P-gp. This efflux transporter consists of a 170KDa glycosylated plasma membrane protein that belongs to the ATP-binding cassette (ABC) transporters superfamily (110, 111).

In humans, the multidrug resistance genes *MDR1* and *MDR3* encode the P-gp whereas, three members of this family (*mdr1a*, *mdr1b*, and *mdr2*) are found in mice (88, 112). The P-glycoprotein encoded by human *MDR1* and mouse *mdr1a/1b* genes are drug transporters, while human *MDR3* and mouse *mdr2* P-glycoprotein are thought to be implicated mostly with the transport of phospholipids (113, 114). Structure-function analyses revealed that P-gp consists of two homologous halves, each containing six transmembrane domains where are located the drug-binding sites and a cytoplasmatic nucleotide-binding domain where the ATP hydrolysis takes place generating the driving force for the drug efflux (115, 116).

P-glycoprotein was first characterized in tumor cells contributing to multidrug resistance (MDR) (117), but it is also expressed constitutively at high levels on the apical membrane of luminal epithelial cells of different tissues, which are involved in drug absorption and disposition such as hepatocytes cannalicular membrane, renal proximal tubules, intestinal mucosa and capillaries in the brain (110, 118-120).

One of the most intriguing aspects of P-glycoprotein is its ability to recognize and transport many drugs with a wide array of chemical structures. So far, a clear structure activity relationship for predicting P-glycoprotein substrates has not been established and the only common feature is that most of P-gp substrates are hydrophobic in nature, suggesting that partitioning within the lipid membrane of cells is the first step for the interaction of substrate with the active sites of P-glycoprotein (110).

Several models have been proposed to describe the mechanism for drug transport activity. The more favorable model proposes that P-glycoprotein intercepts lipophilic drugs either from outer membrane of lipid bilayer, before they enter the cell cytosol, or from the inner membrane of the bilayer, and extrudes them into the extracellular medium (115, 116, 121).

The development of *mdr1a*, *mdr1b*, and *mdr1a/b* knockout mice was a landmark on P-glycoprotein research (122). In *mdr*-knockout mice one or both genes responsible for P-gp expression are deleted, allowing investigation of the function of P-gp in a very clear experimental design. For example, the AUC of paclitaxel was reported to be 2- and 6-fold higher in *mdr1a* (-/-) knockout mice than in the wild type animals after intravenous bolus (i.v.) and oral administration, respectively. The increased AUC of paclitaxel after i.v. administration in *mdr1a* (-/-) mice was attributed to the decrease in elimination clearance,

whereas the higher AUC after oral administration in *mdr1a* (-/-) mice resulted from combination of a decrease in the elimination clearance and enhancement of drug absorption. Based on the AUC values after i.v. and oral administration, the bioavailability of paclitaxel was calculated to be 11 and 35% for wild type and *mdr1a* (-/-) knockout mice, respectively (123).

Wetsphal *et al.* (124) demonstrated that rifampin (a P-gp inducer) was able to reduce the AUC of intravenously (30 mg) and orally administered (100 mg) P-gp substrate talinolol in healthy volunteers by 21% and 35%, respectively.

Experiments in mice with cannulated gallbladder revealed that within 90 minutes after intravenous bolus administration of [<sup>3</sup>H]digoxin approximately 16% of the dose was secreted into intestinal contents of wild-type mice, whereas in *mdr1a* (-/-) knockout animals this value was reduced to only 2% (125).

In summary, the intestinal P-glycoprotein hinder the absorption of distinct classes of drugs like anti-cancer, anti-HIV, cardiac glycosides, steroids,  $\beta$ -adrenergic agents, immuno-suppressants, and antibiotics by carrying them from the enterocytes back to the gut lumen (126, 127). Therefore, inhibition of the intestinal P-gp function may be expected to be followed by enhanced oral bioavailability of its substrates, increasing the risk of dose related side effects particularly for drugs with very low therapeutic index.

Due the remarkable overlaps in tissue distribution, substrates and inhibitors specificity between CYP3A4 and P-gp (128) it is not surprising that GFJ and its components can also interact with this membrane efflux transporter.

Among the many components of grapefruit juice (GFJ), the flavonoids (naringin, naringenin, kaempferol, quercetin), the furanocoumarin (bergamottin,

6',7'-dihydroxybergamottin, and 6',7'-epoxybergamottin), and some polymethoxylated flavones (tangeretin, nobelitin) derivatives (Figure 1-1) are able to modulate the activity of P-glycoprotein and have been suggested to contribute to grapefruit juice-drug interaction (56, 62, 71, 129-131).

In contrast to CYP3A4, the levels of P-gp in the small intestine are not affected by GFJ or its components. Ingestion of 8 oz of GFJ (three times per day for six days) did not alter the expression of intestinal P-gp in healthy volunteers (97).

Takanaga *et al.* (132) suggested that GFJ and its components were able to inhibit the P-gp activity in Caco-2 cells using vinblastine as probe. Since vinblastine also is a substrate of CYP3A4 (133), the GFJ effects regarding this inhibition were not conclusive. However, GFJ and phenolic compounds from orange, such as tangeretin, nobiletin, and heptamethoxyflavone, which have been demonstrated not to alter CYP3A4 activity, increased the net influx of vincristine into adriamycin-resistant human myelogenous leukemia cells conclusively, through the inhibition of P-gp (131). Several *in vitro* studies with different probes, such as vinblastine (71), talinolol (134), and digoxin (135) also confirm the findings that GFJ inhibits the efflux of P-gp substrates. In addition to GFJ, orange juice and pomelo juice also have been shown to inhibit the activity of P-gp *in vitro* (135). Flavones from orange juice have been described to be more potent than compounds from grapefruit in the inhibition of P-gp (132).

In contrast to these findings, Soldner *et al.* (136) suggested that GFJ significantly activates the P-gp-mediated efflux of drugs in Madin-Darby canine kidney (MDCK)-MDR1 cells model.

Five GFJ components (quercetin, naringin, naringenin, bergamottin and 6',7'-dihydroxybergamottin) were screened as modulators of P-gp activity by assessing the directional transport of saquinavir (a P-gp substrate) across Caco-2 cell monolayers (64). Only naringin and 6',7'-dihydroxybergamottin had any appreciable effect, reducing the net secretion of saquinavir transport from 25 to 7.6 and 7.1, respectively.

In order to evaluate the effect of grapefruit juice on P-glycoprotein-related transport processes, measurements of permeability through Caco-2 cells and *in vivo* drug absorption studies in Sprague-Dawley rats were performed using talinolol, which is a P-gp substrate but not metabolized by CYP3A4, as a model compound (134). Apical to basolateral talinolol transport across Caco-2 monolayers was increased 3-fold when GFJ was present. During *in vivo* study in rats, doubled maximum plasma concentrations, enhanced AUC values, and decreased apparent oral clearance were found for both talinolol enantiomers at the same order of magnitude, suggesting an inhibition of P-gp by GFJ.

A recent clinical trial, however, reported that a single glass of GFJ (300 mL) reduced the talinolol AUC, and  $C_{\max}$  to 56% and 57%, respectively, of those with water. Similar effect was observed after repeated ingestion of GFJ (300 mL three times a day for 6 days) (137). These results suggest that constituents in grapefruit juice preferentially inhibited an intestinal uptake process rather than P-glycoprotein, although it is not clear yet if talinolol is a substrate of any influx transporter, such as OATP. Therefore, activation of P-gp cannot be discharged as a grapefruit effect in this study.

Edwards *et al.* (130) evaluate the effect of GFJ and Seville orange juice on the disposition of cyclosporine, a P-gp substrate, in man. Compared with the control

experiment, the AUC and peak concentrations of cyclosporine were increased to 55% and 35%, respectively, by GFJ, whereas Seville orange juice did not have an influence on cyclosporine, while both juices reduced enterocyte concentrations of CYP3A4. The authors additionally demonstrated that DHB did not inhibit P-gp *in vitro*. In parallel, Malhotra *et al.* (138) through a randomized three-way crossover intervention trial studied the effect of Seville orange juice, diluted GFJ (normalized to contain equivalent total concentration of BG and DHB as in Seville orange juice), and sweet orange juice on the disposition of felodipine in healthy volunteers. Seville orange juice and GFJ increased the AUC of felodipine. Considering both studies, while Seville orange juice and GFJ probably interact with felodipine through inactivation of intestinal CYP3A4, the lack of interaction between Seville orange juice and cyclosporine indicates that grapefruit may cause interactions also through the inhibition of intestinal glycoprotein. In addition, these data imply that the inhibition of P-gp activity by other compounds in GFJ (different from those present in Seville orange) may be responsible for the increased bioavailability of cyclosporine.

When a tablet containing 0.5 mg of digoxin was co-administrated with GJF in healthy subjects the pharmacokinetics parameters were slightly increased (1.2- and 1.1-fold increase in AUC and  $C_{max}$ , respectively) (139), or not affected at all (140). However, in both studies it should not be concluded that there is no interaction between GFJ and P-gp since digoxin has a high oral bioavailability (70-80%) and even a total inhibition would be expected to enhance its oral bioavailability by only 1.2-1.3-fold (141). Similarly, the pharmacokinetics of indinavir, another known P-gp substrate was not altered by GFJ (52), suggesting that possible other unknown mechanisms and factors

such as strength of the administered juices and length of consumption are relevant for the interactions of citrus with drugs (142).

Overall, it can be stated that grapefruit and other citrus may interact with several drugs through the combined inhibition of CYP3A4 and P-gp. Additionally, there are significant differences among the published papers depending on the animal (rats, mice, dogs) or cell model (Caco-2 or MDCK cell lines) used as well as culture conditions, study design, and clinical relevance. Furthermore, the magnitude of interaction may strongly depend on the variations in the polyphenolic profile of grapefruit juice, which is related to the origin, quality of raw material, the manufacturing procedure, and storage conditions (40).

In conclusion, the modulation of P-gp activity by grapefruit juice or its components and the clinical relevance of such interaction is still unclear and is a controversial issue, since some authors have reported activation (136, 143), others inhibition of P-gp (61, 71, 144), or absence of effect (52, 139, 140). Therefore, additional studies are required in order to clarify the P-gp-related interactions between drugs and GFJ.

### **Organic Anion Transporting Polypeptide: OATP**

The organic anion-transporting polypeptides (OATPs) are sodium-independent influx membrane transporters belonging to the superfamily of solute carriers (SCL) (145). So far, 39 members of the OATP superfamily have been identified in mammalian species such as human, rat and mouse (146, 147) all of them with broad substrate specificity (148).

OATP transporters are composed by 12 transmembrane domains and a large extracellular region between the helices 9 and 10, which is believed to have a important role on solute transport (149). Most of OATP members are expressed in the basolateral

membrane of polarized cells of multiple tissues, exceptions are the OATP1B1 and OATP1B3 present predominantly on human hepatocytes (145, 150, 151) and the OATP2B1 that has been detected in the apical membrane of enterocytes (152).

The whole superfamily is classified into families and each family into subfamilies. The individual families contain proteins with not less than 40% in amino acid sequence identity and are indicated by numbers (e.g., OATP1, OATP2, OATP3, etc.). Subfamilies contain proteins with amino acid sequence identities equal or higher than 60% and are designated by letters (e.g., OATP1A, OATP1B, OATP1C, OATP2A, etc.). Individual paralogues within a subfamily are indicated by different final numbers (e.g., OATP1B1, OATP1B3) (152).

Considering the co-localization of these absorptive transporters and the secretory P-gp at the apical plasma membrane of the enterocytes, their potential significance in the oral absorption cannot be neglected and might be related with a number of drug-drug and food-drug interactions (120, 127).

GFJ and orange juice (OJ) have been reported to decrease the AUC and  $C_{\max}$  values of fexofenadine to 30 and 40%, respectively, of those when the drug was taken with water (80, 90). Similar results were also found when celiprolol was co-administered with GFJ or OJ (153, 154). Both drugs are substrates for P-gp and OATP, but not CYP3A4 (155, 156). If P-gp had played a major role in the observed interactions, the bioavailability would have been increased instead of decreased. This led to the conclusion GFJ and orange juice preferentially decreased the OATP-mediated transport of fexofenadine and celiprolol, than the transport carried-out by P-gp.

In a study with human embryonic kidney cells expressing OATP1B, different citrus juices were tested in their effect on the uptake of estrone-3-sulfate. GFJ, orange juice, BG, DHBG, quercetin, naringin, and naringenin significantly inhibited OATP1B-mediated uptake of estrone-3-sulfate. The citrus compounds DHB and tangeretin significantly inhibited OATP1B-mediated influx of the probe glibenclamide (72). The effects of fruit juices on the oral availability of fexofenadine also have been tested in rats (79). In this study, orange juice decreased the oral bioavailability of the drug to a lesser extent than that observed in humans. Overall, it has to be considered that genetic differences in the OATPs between humans and other species may contribute to differences in the susceptibility to grapefruit-induced inhibition, which also is true for other transporters and enzymes. In summary, OATPs appear to play an important role in the influx of a number of drugs into enterocytes and hepatocytes. The inhibition of OATP activity has been demonstrated for orange and GFJ in *in vitro* and *in vivo* experiments. The clinical relevance of this mechanism remains to be investigated in further human intervention trials.

### **Oral Drug Absorption**

Most of prescription drugs are administered orally due to convenience of administration, patient compliance, safety, ease of production and distribution. However, this administration route is frequently associated with issues of low bioavailability, defined as the rate and extent that a drug is absorbed from its dosage form and becomes available at the site of action (157, 158).

Among the factors with a potential to influence the bioavailability are the physical-chemical characteristics of the drug molecules (partition coefficient, ionization constant, solubility, polymorphism, particle size and particle shape distribution, stability at various

pH values and against gastrointestinal enzymes), the pH and physiological conditions of the absorption's site, gastric emptying, intestinal motility, absorption windows, transporters, biotransformation and the manufacturing factors as excipients and pharmaceutical technology (87, 157).

Additionally, the active ingredient of the dosage form should be appropriately released, solubilized in the GI fluids, and should cross the epithelial barriers in order to be absorbed throughout the gastrointestinal (GI) tract and reach the systemic circulation. In such scenario, the term "release" may include several processes like disintegration, deaggregation, diffusion, and dissolution of the drug in the GI fluids (87, 157, 159).

### **Contribution of Small Intestine to Drug Absorption and Metabolism**

In a very simplistic point of view, the human gastrointestinal tract can be functionally divided into a preparative and primary storage region (mouth and stomach), a secretory and absorptive region (small intestine), a water reclamation system (ascending colon), and finally, a waste-product storage system (the descending and sigmoid colon regions and the rectum) (160).

The small intestine, comprising of duodenum, jejunum and ileum, has two major functions: it can efficiently absorb nutrients, fluids, electrolytes, and many of the drugs already developed, and simultaneously act as a physical and metabolic barrier against of potentially toxic xenobiotics and pathogens (161). Such peculiarity can be explained by its morphology and physiology, which provide an enormous surface area with ability to absorb liquids and solutes selectively. The mucosa of small intestine is consisted of three groups of projections: circular folds, villi and microvilli (Figure 1-2). The absorptive cells or enterocytes are elongated, polarized and intimately connected by tight junctions, forming a physical barrier between the contents of GI tract and the intercellular space

(87). Another physical barrier located just above the enterocytes is the mucus, a viscous and elastic gel layer consisted of water, electrolytes, and high-molecular-weight ( $2 \times 10^6$  Da) glycoproteins, produced by the goblet cells (162, 163). The basolateral and apical membrane of the enterocytes are markedly distinct conferring a polarized feature due the presence of a striated end (brush border) at the top of the cells (apical side) towards to the interior of the tract (Figure 1-2). These microvilli multiply by a factor of 20 the surface area of the small intestine and are consisted, especially, of protein fibers of actin and myosin (87, 162).

Additionally, most of functionally active transporters protein systems (sugar transporters, P-gp, OATPs) and metabolizing enzymes (peptidases, esterases, CYP3A isoforms, N-acetyl transferases and glutathione transferases) are expressed in the mature enterocytes (163, 164) affecting, therefore, the fraction of the drug initially administrated available for absorption.

The cellular membrane contains fundamentally a phospholipid bilayer, in which glycoprotein molecules are located. The phospholipids are arranged such that the hydrophilic ends face the two membranes surfaces, inner and outer, and the hydrophobic tails are directed towards the membrane center (87, 162). Such characteristic makes lipophilic compounds be rapidly and completely absorbed, while slowly and incompletely passively absorbed drugs are, in general, hydrophilic and with poor distribution into the cell membranes (165).

Although the intrinsic selectiveness of the small intestinal may impair the bioavailability of many drugs, it is still the principal target for drug absorption following an oral administration due to its vast surface area. Physicochemical properties of drug

molecules and formulations parameters can be modified in order to obtain a better absorption profile. However, co-administration of two or more drugs subjects to similar metabolic pathways or sharing the same transporter systems increase the likelihood of drug-drug interaction. Similarly, the ingestion of dietary supplements and foods, like grapefruit juice, able to affect the normal physiology of the small intestine, the activity of the metabolizing enzymes, and carriers have a potential to modify markedly the disposition of drugs taken orally.

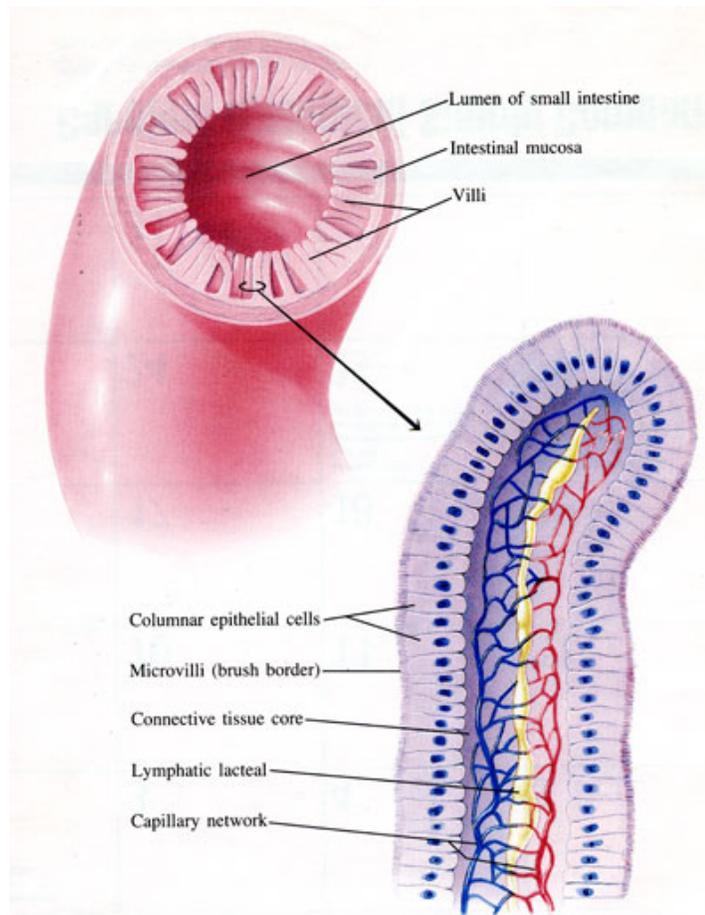


Figure 1-2. Schematic representation of the mucosa of small intestine consisted of circular folds, villi “finger-like” structures covered by a monolayer of epithelial cells separating the lumen from the blood capillary network, and microvilli the “brush board” projections located at the apical side of the enterocytes

Source: <http://instruct1.cit.cornell.edu/courses/biog105/pages/demos/105/unit6/media/villus.structure.jpg>.

### Mechanisms of Transport of Molecules across Intestinal Epithelium

Orally administered drugs can enter the systemic circulation by crossing the intestinal epithelium via two major routes, the paracellular or the transcellular pathways (164). Paracellular can be further divided in passive transcellular diffusion, carrier-mediated transport or transcytosis (Figure 1-3). The importance of each route for drug transport depends on the physicochemical properties of the compound being transported such as lipophilicity, size, pKa, hydrogen bond potential, affinity for transport proteins (166).

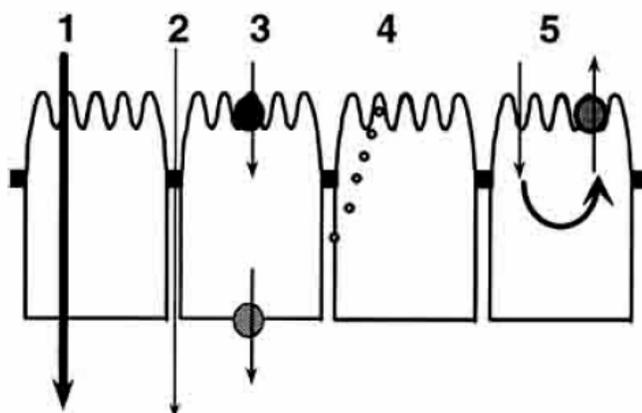


Figure 1-3. Possible transport routes a drug can follow to cross the intestinal epithelium: (1) passive transcellular transport, (2) passive paracellular transport, (3) active and/or carrier mediated transport, (4) transcytosis. In addition, drug transport can be modulated by active efflux carrier mechanisms such as P-gp (5) (167)

#### Passive Transcellular Diffusion

The majority of drugs given orally reaches the systemic circulation by passive transcellular diffusion (164). This process consists on permeation of the apical membrane of the enterocytes, movement across the cytoplasm, and permeation of the lateral or basolateral membranes (87). Since lipid barriers need to be crossed, passive diffusion of drugs through the small intestine depends to a large degree on their lipophilicity. The diffusion process can be mathematically described by the Fick's first law (equation 1-1):

$$J = P \cdot A \cdot (C_1 - C_2) \quad (\text{eq. 1-1})$$

where J is the flux or mass of transport over time, P is the permeability coefficient, A is the surface area of the absorptive membrane, and  $(C_1 - C_2)$  is the concentration gradient of the uncharged form of the solute over the membrane. Assuming that the drug does not accumulate in any region at the basolateral membrane of the enterocytes because the blood and lymph flow provide a sink condition, the  $C_2$  becomes insignificant and the flux can be expressed just in term of the apical concentration,  $C_1$ . Therefore, the driving force of diffusion is mainly controlled by the concentration of drug remaining to be transported from one to the other side of the enterocytes (87).

The permeability coefficient across the membrane, defined as the velocity of the drug transport (cm/sec) into the body (127), can be defined by the equation 2.

$$P = \frac{K \cdot D_m}{\lambda} \quad (\text{eq. 1-2})$$

where K is the partition coefficient between the water and the membrane,  $D_m$  is the membrane diffusion coefficient and  $\lambda$  is the thickness of the of the membrane (162).

Transport of ionized compounds rely on some the physical-chemistry properties such as the pKa of the drug molecule and pH of the absorption site, since these parameters will define the equilibrium of the charge-state of the compound (162).

In general, these principles are followed by many drugs with the exception of highly branched molecules, which are less permeable than expected due stereochemistry issue since branched molecules results in greater disturbance in the arrangement of the lipid layer (87).

### **Paracellular Transport**

This transport route takes place via the tight-junctions between the enterocytes and works as a semipermeable barrier, which protects against xenobiotics and controls the passage of ions, water and small hydrophilic molecules (168). The apically located tight-junctions restrict the paracellular transport of molecules that are larger than a certain threshold size (molecular weight ~ 350 Da) (164). Additionally, the surface area for the paracellular transport has been estimate to be about 1000-fold smaller than the total intestinal surface, suggesting a low efficiency of this pathway on drug transport (165). Such characteristic has stimulated investigations to enhance the permeability of this route and, therefore, increase the bioavailability of very hydrophilic drugs (165, 166, 169). The tight-junctions are also a fence separating lipids and proteins of the apical and basolateral domains of the enterocytes (168).

The tight-junctions become progressively tighter and the surface area decreases from the small intestine to the colon, which can explain why the permeability of hydrophilic compounds decrease in the same direction (170, 171).

Experiments using Caco-2 cells demonstrated that paracellular route can contribute significantly to the absorption of many drugs including: atenolol, pindolol, cimetidine, metformin, didanosine, and acyclovir (172). These molecules have a moderate molecular weight and are relative hydrophilic.

### **Carrier-Mediated Transport**

Generally, carrier-mediated transport processes consist of a reversible interaction between the solute and a membrane protein, which acts as a transporter. Such transport has unique characteristics such as specificity, competition, saturation kinetics, and energy requirement (87). Transporters that use the energy of ATP hydrolysis to maintain a

gradient across the membrane are named primary active transporters, whereas, secondary active transporters utilize the ion gradient across the membrane generated by the primary active carriers to transport their substrates.

These carriers can be responsible for the uptake (influx transporters) or secretion (efflux transporter) of many drug molecules across the intestinal epithelium and in both cases it may result in non-linear pharmacokinetics and dose-dependent absorption. The permeability-glycoprotein is a primary active efflux transporter, whereas the organic anion transport peptide (OATP) is an example of secondary active influx carrier.

The relevance of a carrier-mediated transport for drug absorption depends on the selectivity, capacity, direction, distribution, and expression of the transporters along the intestine (127).

### **Transcytosis**

Transcytosis is a specialized pathway where particles and macromolecules are entrapped in vesicles due to invagination of the apical membrane before to be released into the cytoplasm (87). Characteristics of this route include a very low capacity of transport and the large amount of proteolytic enzymes inside the vesicles, which can hydrolyze many of the solutes. The transport of vitamin B12 is the best example of transport mediated by transcytosis (165).

### ***In Vitro* Methods for Studying Drug Absorption**

Intestinal drug permeability is considered to be one of the two major barriers to intestinal drug absorption, solubility being the other. For assessment of the mechanistic aspects of intestinal drug permeability, several *in vitro* systems have been developed. Advantages of using *in vitro* models include small sample volumes, low variability between replicates, the fact that many external factors that influence transport can be

manipulated easily, and, in general, the outputs obtained by the *in vitro* assessment can be extrapolated to the *in vivo* scenario (165, 173, 174).

Epithelial cell culture models such as Caco-2 are commonly used (165). This human colon adenocarcinoma cell line undergoes enterocytic differentiation in culture allowing studies not only of passive diffusion processes but also, active drug uptake, efflux carrier systems and presystemic drug metabolism, since they are able to naturally express transporters like P-gp, sugar uptake transporters, multi drug resistance protein, and metabolizing enzymes such as CYP3A4, N-acetyl transferases, and glutathione transferase (89, 163, 167, 173, 175-177). The ability of the cells to form and maintain a confluent monolayer, with characteristics similar to the *in vivo* barrier being modeled, on some type of semipermeable support is a prerequisite for transport study.

Despite of the good versatility some potential weakness of the Caco-2 cell culture model should be considered. For instance, the Caco-2 form very tight monolayers compared to the human small intestine, which has been explained by the colonic origin of the cell line (165, 173). Also, a unexpectedly large percentage of the compounds are found to be substrates for P-gp, whereas the *in vivo* relevance of these findings remains unclear, probably because the higher P-gp expression observed in this cell line compared to the small intestine (163, 165).

The Madin Darby canine (MDCK) and porcine (LLC-PK1) kidney epithelial cell lines consist of fast and simple additional *in vitro* models mostly used for screening tests and measurement of passive diffusion, since they do not express transporters under the normal conditions. They also can be used to study the effect of one single transport system or the interplay of efflux and influx carriers, having a untransfected wild-type cell

line as control (62, 71, 76, 178). In contrast to Caco-2 cells, they do not need to be kept 3 weeks in culture before use. Limitations include the fact that MDCK and LLC-PK1 cells are non-intestinal and non-human cell models (163, 179).

Excised segments of human or rat intestines properly positioned in an Ussing chamber and the everted gut sac of rat small intestine placed in a oxygenated tissue culture media are sensitive detection methods to study mechanisms of drug absorption at different sites in the intestine, to evaluate intestinal metabolism of xenobiotics, to quantify the paracellular transport of hydrophilic molecules, and to estimate the effects of potent enhancers on their absorption (171, 180-183). Potential disadvantages of these approaches are the presence of muscularis mucosa which may lead to underestimation of compounds with propensity to bind to muscle cells, short duration of the experiments due to limited cell viability, and the availability of human tissue is scarce (163).

Therefore, distinct *in vitro* techniques are accessible for the prediction of the relevant parameters of drug absorption and their advantages, applicability, and limitations should be carefully considered.

#### **Talinolol: Model Compound for Drugs Subjected to Intestinal Efflux**

Talinolol, a highly selective  $\beta_1$ -adrenoceptor antagonist, was introduced into clinical practice in 1975 in the former German Democratic Republic by AWD-Pharma GmbH & Co. KG (Dresden, Germany) under the trade mark Cordanum®. It is used in the therapy of arterial hypertension, angina pectoris, ischemic heart disease, and rhythmic disturbances of the heart (184, 185).

Studies *in vitro* (186, 187) and *in vivo* (188-190) have demonstrated that talinolol is a substrate for the P-glycoprotein efflux system. Additionally, this drug is subjected to

minor metabolism in humans, dogs, and rats with less than 1% of the administered dose being excreted into urine in form of hydroxylated metabolites (124, 191-193). Due the remarkable overlaps in tissue distribution, substrates, inhibitors, and inducers specificity between CYP3A4 and P-gp (128), talinolol is considered a good probe for mechanistic studies involving interactions of drugs or food constituents, like grapefruit juice, with P-glycoprotein. The intestinal efflux of talinolol can be estimated without an interference of any biotransformation processes, and the variability of its oral pharmacokinetics should reflect the variability of the P-gp function (193).

Additionally, talinolol has a moderate passive membrane permeability, which prevents rapid passive absorption of the drug (187).

### Properties of Talinolol

The chemical structure of talinolol ((1-(4-cyclohexylureidophenoxy)-2-hydroxy-3-*tert*butylaminopropane) (molecular weight = 363.5 Da) and its pharmacokinetics parameters after oral administration are presented on Figure 1-4 and Table 1-1, respectively.

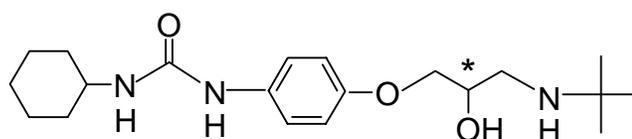


Figure 1-4. Molecular structure of talinolol. (\*) chiral center.

The basicity of the weak base talinolol ( $pK_a = 9.4$ ) refers to a secondary amino functional group located close to the chiral center. Since the nitrogen of this amino group can be protonated, talinolol features a pH-dependent solubility, which decreases with increasing pH values (162). The log P of 3.2 indicates a moderate lipophilicity, compared to other common  $\beta$ -adrenoceptor antagonists. It is less lipophilic than propranolol (log P: 4.6) and more lipophilic than atenolol (log P: 0.2) (194). Solubility in water (pH = 7.0)

have been reported to be 0.2 mg/mL and recommended daily doses range from 50 to 300 mg, in healthy volunteers safety of the drug was proven up to single doses of 400 mg (186, 192).

Table 1-1. Pharmacokinetics parameters of talinolol after oral administration (50 mg) by health volunteers (192).

Parameters	Values $\pm$ SD
AUC <sub>0-36</sub>	1180 $\pm$ 337 ng.h/mL
Vd <sub>ss</sub> *	3.3 $\pm$ 0.5 L/kg
Cl <sub>tot</sub>	11.9 $\pm$ 2.4 mL/min.kg
C <sub>max</sub>	168 $\pm$ 47 ng/mL
T <sub>max</sub>	3.2 $\pm$ 0.8 h
Elimination half life (t <sub>1/2</sub> )	11.9 $\pm$ 2.4 h
Biotransformation	< 1%
Renal Excretion	~ 60 % of the bioavailable dose
Extrarenal excretion	~ 40% of the bioavailable dose
Bioavailability	55 $\pm$ 22%; dose dependent

\* Vd<sub>ss</sub> was calculated after a constant rate intravenous infusion of 30 mg of talinolol over 30 min

### Hypothesis and Objectives

Grapefruit juice has been demonstrated to inhibit the metabolizing enzyme CYP3A4 in the small intestine via suicide inhibition mechanism. As the result of such inhibition, the oral bioavailability of many drugs of different therapeutic classes is increased and in some cases dose dependent side effects are observed.

Because CYP3A4 and P-glycoprotein share many substrates, inhibitors, and inducers, ingestion of GFJ is likely to modify the disposition of drugs transported by this efflux system. However, the influence of different GFJ compounds on P-gp is still unclear and is a controversial issue particularly, regarding the *in vivo* relevance of this potential interaction. Therefore, we will test the hypothesis that grapefruit juice and its constituents change significantly the disposition of talinolol, a substrate of P-glycoprotein.

Concentrations of potential compounds able to interact with P-gp will be determined in different brands and lots of grapefruit juice in order to assess the potential influence of the variability of these juice components on the variability of data gained in drug interaction studies.

Additionally, experiments using Caco-2 cells and talinolol as a probe compound will be performed with the purpose of study the mechanistic aspects of the interaction of grapefruit juice and its components with P-gp.

Furthermore, the effect of GFJ and some of its constituents on the pharmacokinetics of talinolol in male Sprague-Dawley will be evaluated and the data obtained from both *in vitro* and *in vivo* experiments will be correlated.

We expected that this study will aid to clarify the predominant mechanisms and the major compounds contributing to the overall effect of grapefruit juice on drug disposition.

Therefore, to test the hypothesis of this study the following specific aims were proposed:

**Specific Aim 1: Contents of Flavonoids and Furanocoumarins in Grapefruit and Grapefruit Juice**

1. To measure selectively, using validated and sensitive HPLC methods, the contents of the specific flavonoids (naringin and naringenin) and furanocoumarins (bergamottin and 6',7'-dihydroxybergamottin) in commercially available and fresh-squeezed GFJ as well as in different tissues of grapefruit.

**Specific Aim 2: *In Vitro* Studies**

1. To determine the permeability of talinolol across Caco-2 cell monolayer cultures in the absence and in the presence of different concentrations of grapefruit juice and several of its components.
2. To estimate the kinetic parameters  $K_m$  and  $P_{max}$  of P-glycoprotein regarding the transport of talinolol.
3. To calculate concentrations of grapefruit juice and its components able to produce a half maximum inhibition ( $IC_{50}$ ) of the P-gp-mediated transport of talinolol across the Caco-2 cell monolayers.

**Specific Aim 3: *In Vivo* Studies**

1. To evaluate the effect of grapefruit juice and its components on the pharmacokinetics of talinolol in male Sprague-Dawley rats.

CHAPTER 2  
VARIATION OF FLAVONOIDS AND FURANOCOUMARINS IN GRAPEFRUIT  
JUICES: A POTENTIAL SOURCE OF VARIABILITY IN GRAPEFRUIT JUICE-  
DRUG INTERACTION STUDIES

**Background**

The amount of active ingredients of grapefruit juice ingested may be an important factor influencing the mechanism, magnitude, and reproducibility of the grapefruit-drug interaction (48), compromising the comparison of data gained in different clinical studies. A clinical trial with healthy subjects has revealed that the AUC and the  $C_{\max}$  of felodipine were increased by about 73 and 138% compared with water, respectively, when a single oral dose (10 mg) of the drug was administered with 200 mL of GFJ (prepared by diluting 50 mL of frozen concentrate with 150 mL of tap water) (102). However, in a similar study co-administration of the same oral dose of felodipine and 250 mL of a commercial GFJ increased the AUC and  $C_{\max}$  values by 305 and 228%, respectively (68). Although the variability in magnitude of the grapefruit juice-drug interaction might be at least partially explained by inherent differences in enterocyte CYP3A4 content (68, 97), it is also possible that more concentrated or larger volumes of grapefruit juice caused a more pronounced interaction. Furthermore, differences in the concentrations of drug-interacting compounds in the juices used in both studies may have contributed to the discrepancy of results. This points out the potential role of the concentration of components causing drug interactions in juices used in clinical trials.

### **Specific Aim**

The objective of this study was to compare the contents of the specific flavonoids (naringin and naringenin) and furanocoumarins (bergamottin and 6',7'-dihydroxybergamottin) (Figure 1-1) in commercially available and fresh-squeezed GFJ as well as in different tissues of grapefruit in order to assess the potential influence of the variability of these juice components on the variability of data gained in drug interaction studies. These compounds have been already described as the most abundant present in GFJ and play a role in the GFJ-drug interaction (98, 195, 196).

### **Material and Methods**

#### **Chemicals**

Naringin (NAR) and naringenin (NAG), both > 95% pure, were from Roth GmbH & Co. (Karlsruhe, Germany), bergamottin (BG) (> 98% purity) was bought from Indofine Chemical Company, Inc. (Somerville, NJ, USA), 6',7'-dihydroxybergamottin (DHB), was kindly supplied by Dr. John Manthey at the U.S. Department of Agriculture (USDA), Citrus and Subtropical Products Laboratory, Agricultural Research Service, Winter Haven, FL. Upon isolation, the compound identification and purity (> 98%) was measured by analytical thin layer chromatography, HPLC-MS and melting point comparisons with authentic standard at the USDA laboratories. Ethyl acetate was bought from Sigma Chemical Company (St. Louis, MO, USA), methanol HPLC grade, dimethylsulfoxide, and orthophosphoric acid were acquired from Fisher Scientific (Fair Lawn, NJ, USA) and purified water was obtained using a NANOPure® system from Barnstead (Dubuque, IA, USA).

### **Sample Preparation**

Twenty-nine commercially available GFJ samples and two types of fresh grapefruit (white and red) were purchased at local supermarkets. Concentrations of flavonoids (naringin and naringenin) and furanocoumarins (bergamottin and 6',7'-dihydroxybergamottin) in fresh grapefruit were determined in the flavedo layer (0.1 g), pulp (0.1 g), albedo (1.0 g), seeds (1.0 g), and fresh-squeezed juice. Fruit tissues were first homogenized with 6 mL (flavedo and pulp) or 12 mL (albedo and seeds) of purified water using a PowerGen® homogenizer (Fisher Scientific, Pittsburgh, PA) prior to the extraction step. The freshly squeezed juices were obtained by using an HJ 29 handy juicer (Black & Decker, Towson, MD). Samples were analyzed by a Shimadzu VP series HPLC system (Kyoto, Japan) equipped with an SPD-M10Avp diode array detector, an LC-10ATvp solvent delivery unit, an SIL-10AF autosampler, a CTO-10Avp column oven, an SCL-10Avp system controller, a DGU-14A on-line degasser, an FCV-10ALvp low-pressure gradient unit, and Class VP 7.2 SP1 chromatographic software. Additionally, the peak purity software (Class VP 7.2 SP1 chromatographic software, Shimadzu) was applied to the diode array data to test for impurities in all of the chromatographic peaks of interest. The same software was used to determine the similarity of the ultraviolet (UV) spectra of the target compounds present in the grapefruit with those from the respective standard compounds.

### **Determination of Flavonoids (Naringin and Naringenin)**

The GFJ and the homogenate of tissues (200  $\mu$ L) were mixed with cold methanol (400  $\mu$ L), vortexed for 1 min, and centrifuged at 2500 g for 15 min, as previously described (197). After filtration through a 0.45  $\mu$ m PVDF membrane filter (Millipore

Corp., Bedford, MA), the supernatant (25  $\mu$ L) was injected and analyzed at 285 nm. The flow rate and the temperature were set to 0.5 mL/min and 35 °C, respectively. Mobile phases A and B consisted of water (pH 2.4) (adjusted with orthophosphoric acid) and water (pH 2.4) (adjusted with orthophosphoric acid)/ methanol (40:60), respectively. The 250 x 4.6 mm i.d., 5  $\mu$ m, Lichrospher RP-18 column and Lichrospher 100 RP-18 guard column (Merck KGaA, Darmstadt, Germany) were initially equilibrated during 30 min with solvent A. After sample injection, an initial isocratic run for 5 min was followed by a linear gradient from 100% of A at 5 min to 100% of B at 55 min. This condition was maintained until 70 min and then returned to 100% of A, which was kept constant during 5 min before proceeding to the next injection.

#### **Extraction and Determination of Furanocoumarins (Bergamottin and 6',7'-Dihydroxybergamottin)**

GFJ and the homogenate of tissues (3 mL) were mixed with ethyl acetate (2 mL). The extraction was performed by shaking the mixtures four times over 30 min. The mixture was centrifuged at 3200 g for 20 min; the organic phase was collected and evaporated under vacuum. The residue was reconstituted with 600  $\mu$ L of a DMSO/methanol solution (1:3 v/v). The reconstituted residues were filtered through a 0.45  $\mu$ m PVDF membrane filter (Millipore Corp.). Volumes of 25  $\mu$ L of each sample were injected and analyzed at 310 nm. The flow rate and the temperature were set to 1 mL/min and 35 °C, respectively. Solvents A and B consisted of water and methanol, respectively. The column and guard column (as used for flavonoids) were initially equilibrated with mobile phase consisting of a mixture of solvents A and B (45:55), respectively. Twenty minutes after injection, solvent B was increased linearly from 55 to 100% in 20 min. This condition was maintained for 5 min, after which the system

returned to the original mobile phase and was equilibrated for a further 5 min before the next injection.

### **Stock and Work Solutions for the Preparation of Calibration Standards**

All stock solutions were prepared in dimethylsulfoxide (DMSO) in order to obtain a final concentration of 50 mmol/L. The stock solutions were kept at -20 °C and used within 4 weeks.

#### **Naringin stock solution**

The amount of 58.5 mg of naringin (MW = 580.53 g/mol) was accurately weighed, and transferred quantitatively to a 2.0 mL volumetric flask. The standard was then dissolved in DMSO, the volume was completed with the same solvent, and the final solution mixed thoroughly.

#### **Naringenin stock solution**

The amount of 27.2 mg of naringenin (MW = 272.26 g/mol) was accurately weighed and transferred quantitatively to a 2.0 mL volumetric flask. The standard was then dissolved in DMSO, the volume was completed with the same solvent, and the final solution mixed thoroughly.

#### **Bergamottin stock solution**

The amount of 33.8 mg of bergamottin (MW = 338.42 g/mol) was accurately weighed and transferred quantitatively to a 2.0 mL volumetric flask. The standard was then dissolved in DMSO, the volume was completed with the same solvent, and the final solution mixed thoroughly.

#### **6',7'-Dihydroxybergamottin stock solution**

The amount of 37.2 mg of 6',7'-dihydroxybergamottin (MW = 372.42 g/mol) was accurately weighed and transferred quantitatively to a 2.0 mL volumetric flask. The

standard was then dissolved in DMSO, the volume was completed with the same solvent, and the final solution mixed thoroughly.

#### **Naringin work solution**

Volume of 1 mL from the naringin (NAR) stock solution was accurately transferred to a 5 mL volumetric flask, the volume was completed with a solution of methanol/water (1:1) and mixed thoroughly. The final concentration of NAR was 10 mmol/L.

#### **Naringenin work solution**

Volume of 100  $\mu$ L from the naringenin (NAG) stock solution was accurately transferred to a 5 mL volumetric flask, the volume was completed with a solution of methanol/water (1:1) and mixed thoroughly. The final concentration of NAG was 1 mmol/L.

#### **Furanocoumarins work solution**

Volumes of 250  $\mu$ L and 100  $\mu$ L from the bergamottin (BG) and 6',7'-dihydroxybergamottin (DHB) stock solutions, respectively, were accurately transferred to a 5 mL volumetric flask, the volume was completed with a solution of DMSO/methanol (1:3) and mixed thoroughly. The final concentrations of BG and DHB were 2.5 mmol/L and 1 mmol/L, respectively.

#### **Standard solutions of flavonoids**

From the NAR and NAG work solutions, five different concentrations of standard solutions of naringin and naringenin and three quality controls (QC) were prepared in methanol/water (1:1) according Table 2-1. All solutions were filtered through a 0.45  $\mu$ m PVDF membrane filter (Millipore Corp.) before analysis.

Table 2-1. Concentrations of the standard solutions used for the calibration curves and quality controls (QCs) of naringin (NAR) and naringenin (NAG).

Standard	NAR work solution ( $\mu\text{L}$ )	NAG work solution ( $\mu\text{L}$ )	methanol/water (1:1) q.s.p. (mL)	Concentration ( $\mu\text{mol/L}$ )	
				NAR	NAG
1	50	100	10	50	10
2	150	200	10	150	20
3	250	300	10	250	30
4	350	400	10	350	40
5	450	500	10	450	50
QC1	50	150	10	50	15
QC2	250	250	10	250	25
QC3	450	450	10	450	45

### Standard solutions of furanocoumarins

From the furanocoumarins work solution, six different concentrations of bergamottin and 6',7'-dihydroxybergamottin and three QCs were prepared in DMSO/methanol (1:3) according Table 2-2. All solutions were filtered through a 0.45  $\mu\text{m}$  PVDF membrane filter (Millipore Corp.) before analysis.

Table 2-2. Concentrations of the standard solutions used for the calibration curves and quality controls (QCs) of bergamottin (BG) and 6',7'-dihydroxybergamottin (DHB).

Standard	Furanocoumarins work solution( $\mu\text{L}$ )	DMSO/methanol (1:3) q.s.p. (mL)	Concentration ( $\mu\text{mol/L}$ )	
			BG	DHB
1	10	10	2.5	1
2	25	10	6.25	2.5
3	100	10	25	10
4	250	10	62.5	25
5	500	10	125	50
6	1000	10	250	100
QC1	50	10	12.5	5
QC2	125	10	31.25	12.5
QC3	750	10	187.5	75

## Validation

An external standardization method was validated over the range of concentration of the target compounds present in the grapefruit juice and tissues. The validation parameters of linearity, sensitivity, specificity, precision, accuracy, recovery and stability were determined.

For each level of concentration, standards solutions and QCs were injected in the HPLC in triplicate in three different days. GraphPad Prism® version 4.0 (GraphPad Software Inc, San Diego, CA, USA) was used to construct the calibration curves obtained by plotting the mean area *versus* the corresponding concentration of the each standard solution. The linearity of the standard curves was determined by least-squares linear regression method and expressed in terms of coefficient of determination ( $r^2$ ). The intra- and inter-day precision and accuracy of the quantification were measured by replicate analyses of three different concentration levels (low, medium and high QCs) on the same day and on alternate days. The precision was based on the calculation coefficient of variation (CV %), and the accuracy was expressed as percent of the found amount compared to the theoretical one. The calibration was considered suitable if not more than 1/3 of the quality controls showed a deviation from the theoretical values equal or greater than 20% at the lower limit of quantification (LLOQ) and within 15% at all other calibration levels.

The limit of detection (LOD) and LLOQ of NAR, NAG, BG, and DHB were expressed on the basis of the mean values of the intercept ( $Y_{bl}$ ), the standard deviation ( $S_{bl}$ ) of the blank responses, and the slope ( $b$ ) of the original calibration curve (equations 2-1 and 2-2) (198).

$$LOD = \frac{Y_{bl} + 10S_{bl}}{b} \quad (\text{eq. 2-1}) \quad LOQ = \frac{Y_{bl} + 3S_{bl}}{b} \quad (\text{eq. 2-2})$$

Briefly, a second standard curve for each grapefruit juice component was obtained, injecting each point in triplicate, but at lower concentrations than those of the used for quantification. The  $Y_{bl}$  values represent the intercepts of these new curves, corresponding to the output as the concentrations of the analytes are equal to zero. Similarly, the  $S_{bl}$  values were determined after plotting the standard deviation versus the correspondent concentration level.

The recovery of each compound was evaluated by adding known amounts of pure compounds to orange juice, from which they are naturally absent (199), and extracting them under the same conditions used in the grapefruit samples. The analytical results for extracted samples at three concentration levels were compared with unextracted standards that represent 100% recovery.

To assess the flavonoids and furanocoumarins stability at room temperature, three QCs (low, medium, and high) were prepared using fresh stock solutions and analyzed at 0 and 36 hours, as previously described. The stock solutions were then frozen at  $-20\text{ }^{\circ}\text{C}$  and freeze/thaw cycles were carried out once week up to 4 cycles. After each cycle, solutions of flavonoids and furanocoumarins corresponding to the medium QC level were prepared and analyzed by the validate HPLC method mentioned formerly. The areas were compared to those obtained from fresh solutions for each concentration level and results expressed as % of the control. Three replicates of each concentration level were prepared for each test performed.

### **Statistical Analysis**

Validation parameters were expressed as mean of three to nine determinations  $\pm$  SD. The concentration of each compound in the juice and tissues was expressed as the

mean of the three determinations  $\pm$  SEM. Asterisks indicate significant differences for white grapefruit compared to red grapefruit ( $p \leq 0.05$ ). Data were analyzed by one-way analysis of variance (ANOVA) with JMP5 software (SAS Institute Inc., Cary, NC, 1996). Mean separation was conducted using the Tukey-Cramer HSD comparison for all pairs ( $p \leq 0.05$ ). Values with different letters are significantly different ( $p \leq 0.05$ ).

## Results and Discussion

### Linearity

Calibration curves ( $n = 9$ ) for all four grapefruit juice constituents were linear within the tested range of concentration with  $r^2 > 0.999$  (Figure 2-1), indicating that both methods showed good linearity.

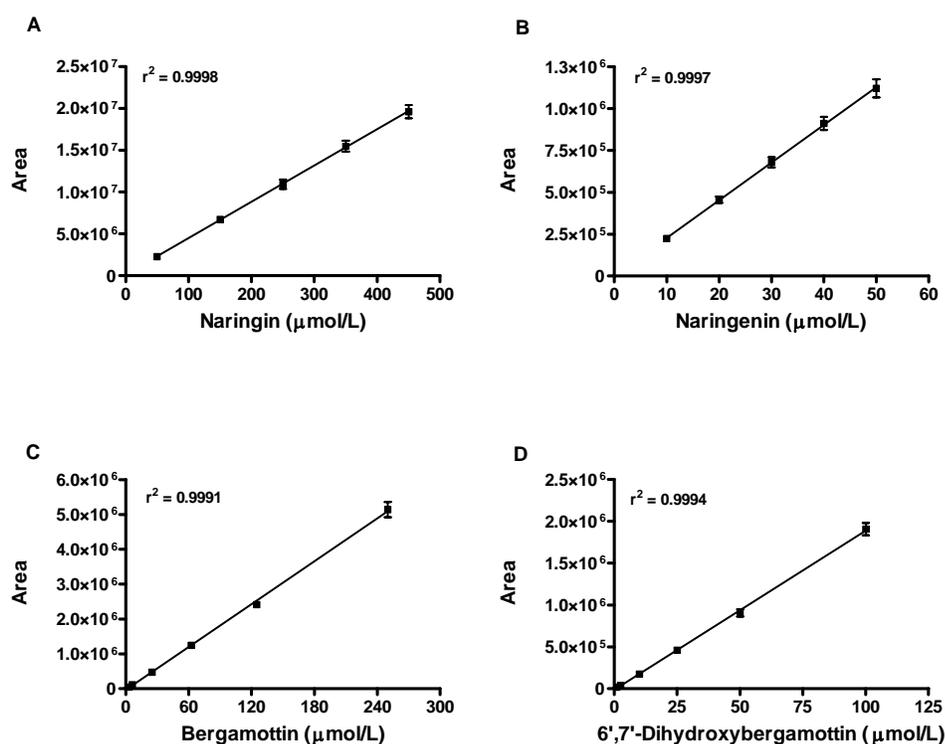


Figure 2-1. Mean calibration curves ( $n = 9$ ) of naringin (A) and naringenin (B) in methanol/water (1:1), and of bergamottin (C) and 6',7'-dihydroxybergamottin (D) in DMSO/methanol (1:3). Vertical bars represent the standard deviations (SD) of the means.

**Sensitivity**

The  $Y_{bl}$  values for the blank responses of NAR, NAG, BG, and DHB were 6170.9, 7462.9, 271.1, and 671.6, respectively. In the same way, the  $S_{bl}$  values were 5026, 1790, 6.2, 30.7. Therefore, the LODs values for naringin, naringenin, bergamottin, and 6',7'-dihydroxybergamottin were 0.47, 0.56, 0.01, and 0.04  $\mu\text{mol/L}$ , respectively, whereas the LLOQs were 1.25, 1.11, 0.02, and 0.05  $\mu\text{mol/L}$ .

**Specificity**

The methods provided good resolutions between naringin and naringenin as well as between bergamottin and 6',7'-dihydroxybergamottin. Peaks of the flavonoids and furanocoumarins had similar retention times and the UV spectra (200-400 nm) in all samples when compared to the standards. The wavelengths 285 and 310 nm used to quantify flavonoids and furanocoumarins at their maximum absorption, respectively, were confirmed by their UV spectra (Figure 2-2). There was no endogenous interference from GFJ or orange juice (Figure 2-3) in either assay, indicating specificity of both methods to the tested compounds. The results of the analysis of the peak purity software suggested the absence of impurities for all compounds (purity > 95% for each individual peak). Additionally, the UV spectra of all tested compounds showed more than 99% of similarity with those obtained using the respective standard compounds (Figure 2-4).

**Precision and Accuracy**

The precisions intra- and inter-day for both flavonoids and furanocoumarins were satisfactory with CV values between 0.55 and 6%. Similarly, the accuracy of the assay was between 93 and 114.8% for all compounds tested at three different concentrations. The results are summarized in Table 2-3.

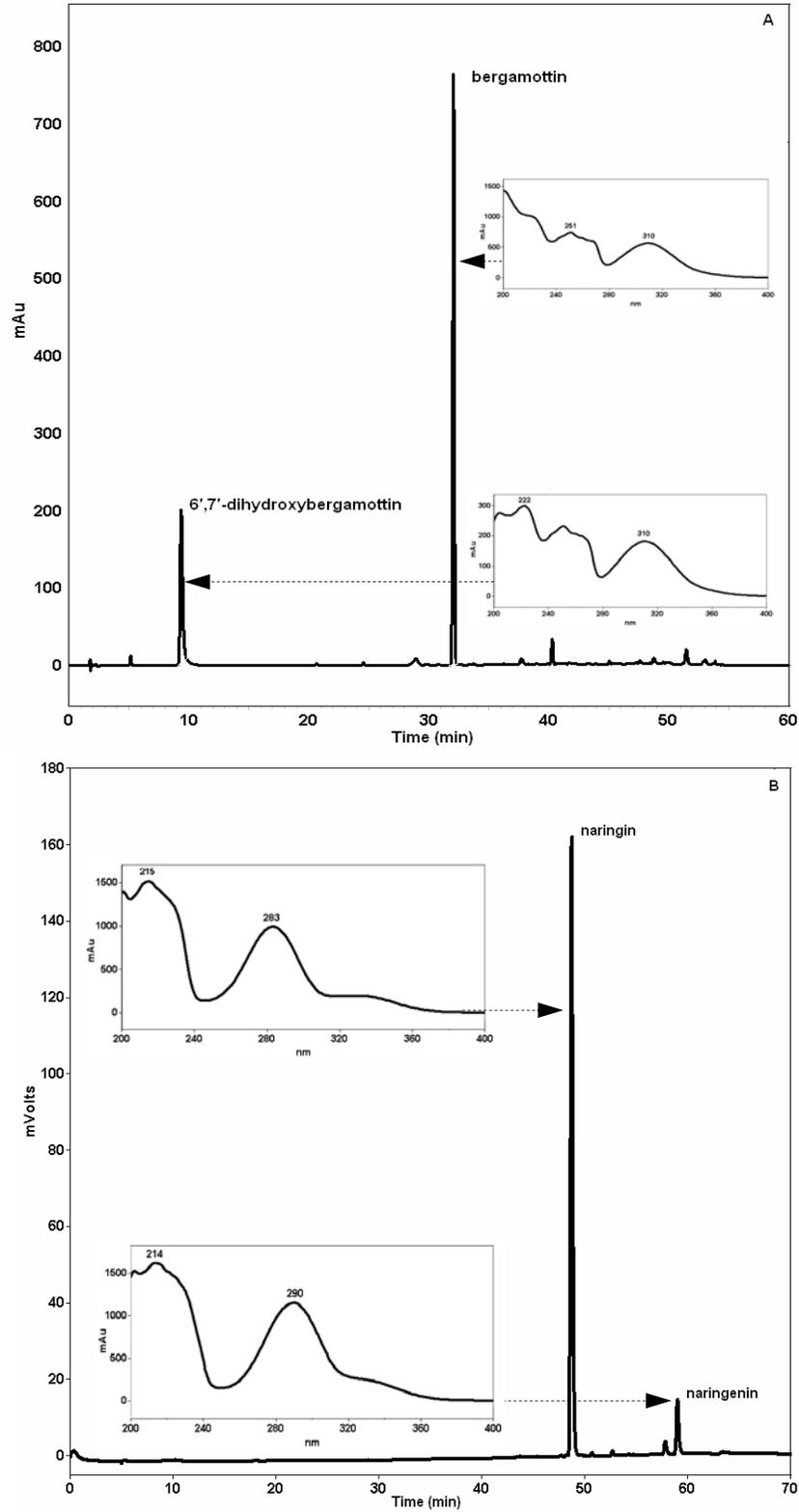


Figure 2-2. HPLC separation and absorbance-wavelength spectra of A - furanocoumarins: 6',7'-dihydroxybergamottin and bergamottin; B - flavonoids: naringin and naringenin.

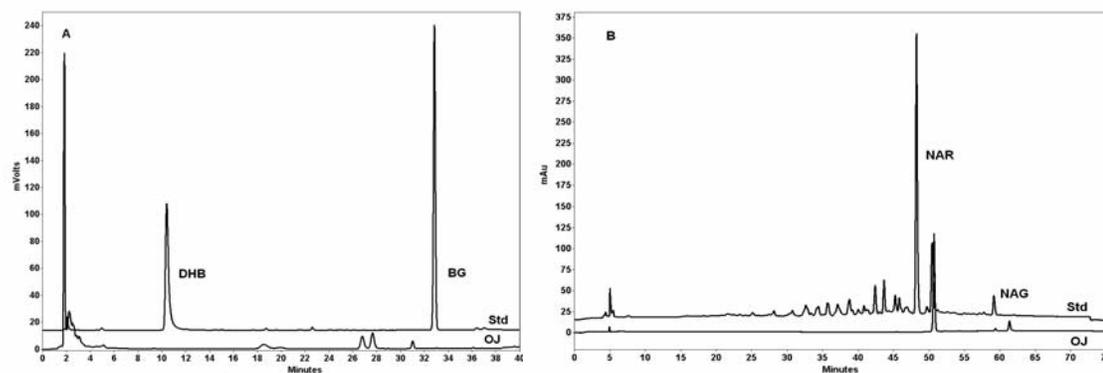


Figure 2-3. Chromatograms corresponding to the orange juice (OJ), standard solutions (Std) containing 6',7'-dihydroxybergamottin (DHB) plus bergamottin (BG) - (panel A), and naringin (NAR) plus naringenin (NAG) - (panel B).

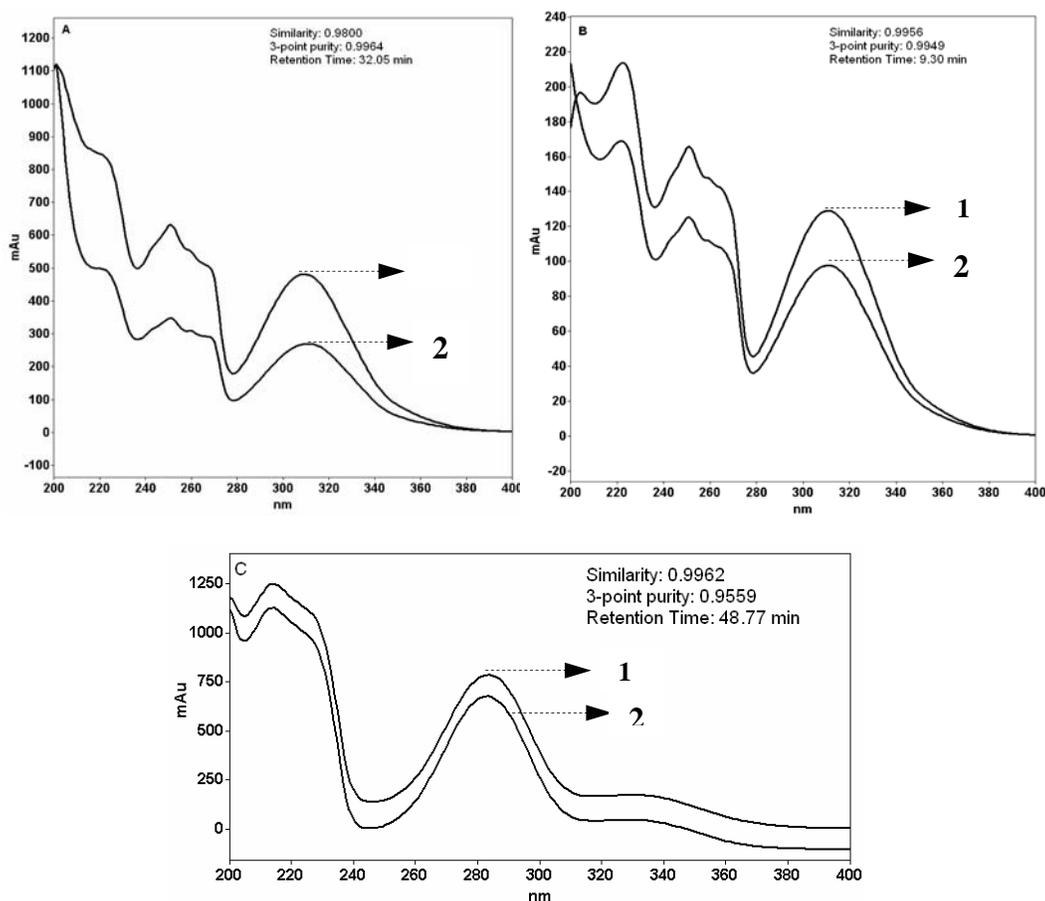


Figure 2-4. Absorbance-wavelength spectra of A - bergamottin; B - 6',7'-dihydroxybergamottin; C - naringin. (1) represents the spectra of the standard compound and (2) represents the spectra of the peak with same retention time of the corresponding standard but obtained after injection of the grapefruit sample.

Table 2-3. Intra-day (n = 3) and inter-day (n = 9) assay parameters of furanocoumarins (bergamottin and 6',7'-dihydroxybergamottin) and flavonoids (naringin and naringenin). Precision expressed as CV % and accuracy as % of the theoretical concentration.

Bergamottin									
	QC1-12.5 µmol/L			QC2-31.25 µmol/L			QC3-187.5 µmol/L		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Intra-day Precision	0.7	0.6	3.8	1.0	1.8	0.9	1.1	2.3	0.7
Accuracy	107.4	109.4	108.4	105.0	108.4	106.2	104.4	108.0	103.4
	QC1-12.5 µmol/L			QC2-31.25 µmol/L			QC3-187.5 µmol/L		
	Inter-day Precision			Inter-day Precision			Inter-day Precision		
	2.4			1.3			1.5		
Accuracy	108.6			106.5			105.3		
6',7'-Dihydroxybergamottin									
	QC1-5 µmol/L			QC2-12.5 µmol/L			QC3-75 µmol/L		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Intra-day Precision	1.5	1.2	1.9	1.2	4.9	1.7	5.2	0.8	0.9
Accuracy	110.3	114.8	114.2	106.0	107.0	99.0	94.6	107.4	105.0
	QC1-5 µmol/L			QC2-12.5 µmol/L			QC3-75 µmol/L		
	Inter-day Precision			Inter-day Precision			Inter-day Precision		
	2.9			4.1			4.03		
Accuracy	113.1			104.0			102.3		
Naringin									
	QC1-50 µmol/L			QC2-250 µmol/L			QC3-450 µmol/L		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Intra-day Precision	4.3	0.6	0.8	0.6	1.2	1.5	3.2	2.1	1.6
Accuracy	98.2	95.1	93.0	104.7	106.0	100.9	98.3	98.3	100.8
	QC1-50 µmol/L			QC2-250 µmol/L			QC3-450 µmol/L		
	Inter-day Precision			Inter-day Precision			Inter-day Precision		
	6.1			2.0			5.0		
Accuracy	95.4			103.9			99.1		

Table 2-3 Continued

Naringenin									
	QC1-15 $\mu\text{mol/L}$			QC2-25 $\mu\text{mol/L}$			QC3-45 $\mu\text{mol/L}$		
Intra-day	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Precision	2.4	0.8	3.6	2.3	1.2	0.7	1.3	0.6	5.9
Accuracy	100.9	96.6	96.6	106.1	103.1	96.2	100.8	99.5	99.8
Inter-day	QC1-45 $\mu\text{mol/L}$			QC2-25 $\mu\text{mol/L}$			QC3-15 $\mu\text{mol/L}$		
Precision	3.0			5.8			4.5		
Accuracy	98.1			101.8			102.7		

## Recovery

The recoveries from orange juice spiked samples were between 97.7 and 106% (Table 2-4). Concentrations were back calculated using the equation obtained from linear regression of the standard curves.

Table 2-4. Recovery values (means  $\pm$  SD) of bergamottin, 6',7'-dihydroxybergamottin, naringin, and naringenin from the spiked orange juice. Values in the parenthesis represent the mean % recovered from each added concentration.

GFJ components added to the orange juice	Theoretical Concentration ( $\mu\text{mol/L}$ )	Mean measured Concentration (n = 3)
Bergamottin	6.25	6.1 $\pm$ 0.3 (97.7)
	25	24.8 $\pm$ 1.7 (99.2)
	150	156.6 $\pm$ 12.4 (102.0)
6',7-Dihydroxybergamottin	5	5.2 $\pm$ 0.1 (103.7)
	25	24.9 $\pm$ 0.8 (99.8)
	100	101.3 $\pm$ 2.8 (101.3)
Naringin	50	52.2 $\pm$ 3.3 (103.4)
	250	265.9 $\pm$ 25.6 (106.2)
	450	475.5 $\pm$ 32.3 (105.9)
Naringenin	15	15.4 $\pm$ 0.4 (102.7)
	25	25.6 $\pm$ 1.5 (102.5)
	45	44.2 $\pm$ 1.2 (98.4)

## Stability

The standard solutions of flavonoid and furanocoumarins were found stable at room temperature within 36 hours, whereas the stock solutions showed adequate stability during at least 1 month after freeze/thaw cycles at -20 °C. In both cases, the shifting of the areas of each sample tested was less than 5% of those obtained from a fresh solution at the same level of concentration (Figure 2-5).

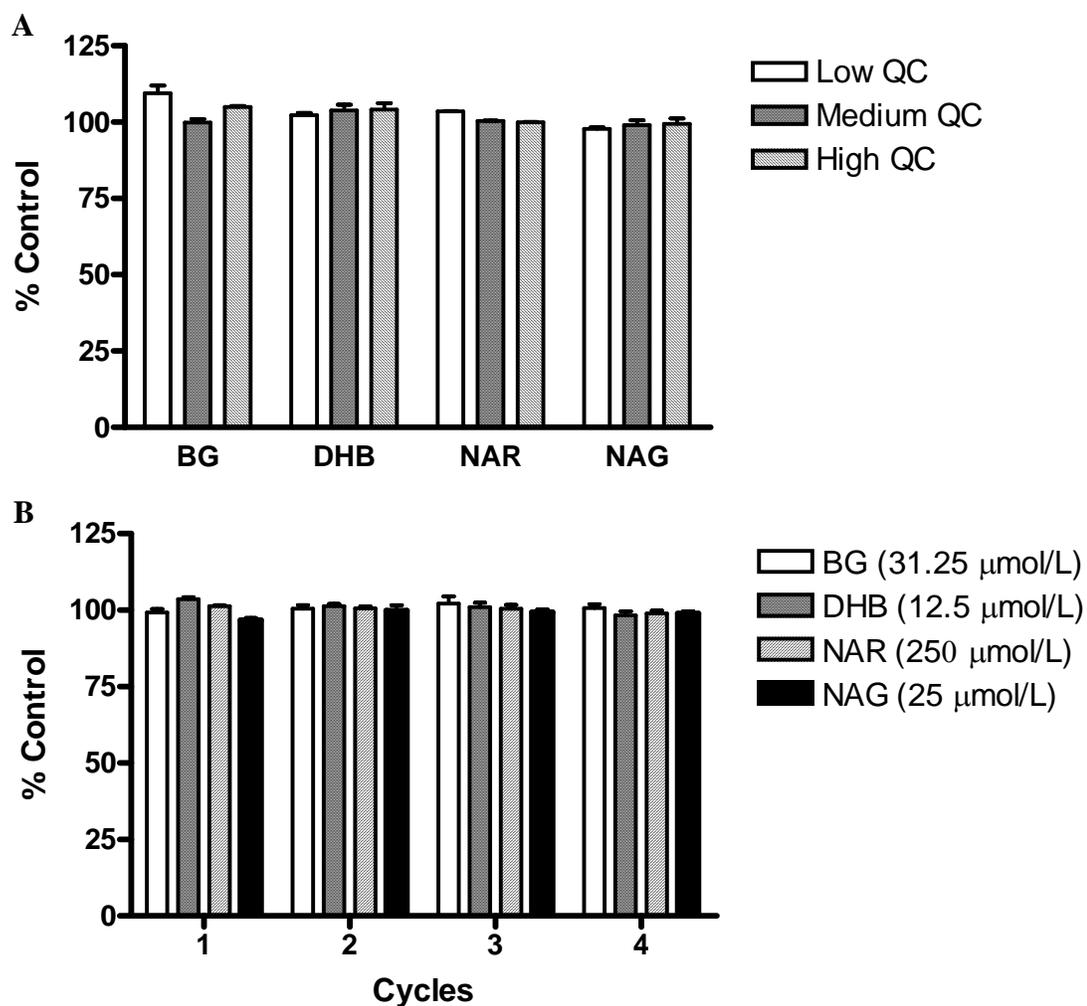


Figure 2-5. Results of the stability test after 36 hours at room temperature (A) and after 4 freeze/thaw cycles at  $-20^{\circ}\text{C}$  (B). Data represents means of three replicates and vertical bars the standard deviation of the means.

### Quantification of Naringin, Naringenin, Bergamottin, and 6',7'-Dihydroxybergamottin in Grapefruit Juices.

The values found for all compounds (Table 2-5) are in the same general range as those reported in previous publications except for naringenin, which was absent in all samples tested. In contrast to a previous study in which concentrations of naringenin were found from 19.5 to 595  $\mu\text{mol/L}$  in 8 different brands of GFJ (196), we did not detect this flavonoid in any of the 14 brands of juices analyzed, which was also in agreement with

other authors (41). However, a great variability of naringin (from 174 to 1492  $\mu\text{mol/L}$ ), bergamottin (from 1.0 to 36.6  $\mu\text{mol/L}$ ), and 6',7'-dihydroxybergamottin (from 0.22 to 52.5  $\mu\text{mol/L}$ ) contents was observed among all tested brands of GFJ. Although Bronner and Beecher (195) found similar concentrations of naringin (850  $\mu\text{mol/L}$ ) in two different brands of GFJ (Giant and Minute Maid), a large variability of naringin, bergamottin, and 6',7'-dihydroxybergamottin in GFJ has also been confirmed by different authors with ranges of 218-2062  $\mu\text{mol/L}$  (196), 2.0-28.3  $\mu\text{mol/L}$  (71), and 9.1-42  $\mu\text{mol/L}$  (71), respectively. In the fresh-squeezed GFJ the highest concentration of naringin (312.6  $\mu\text{mol/L}$ ) was measured in the white variety, whereas the red fruit provided the highest contents of bergamottin (3.1  $\mu\text{mol/L}$ ) and 6',7'-dihydroxybergamottin (10.3  $\mu\text{mol/L}$ ). In the pink GFJ (A-C) the concentrations of 6',7'-dihydroxybergamottin were not statistically different intra- and inter-lot ( $p > 0.05$ ) and brand B showed a higher content of naringin when compared with the other two brands, although the variability of this flavonoid between the lots was not significant. Additionally, the content of bergamottin in lot 2 of brand C was 2.4-fold higher than in lot 1. Intra-lot comparisons revealed that the content of naringin was significantly different ( $p < 0.05$ ) in all brands of white GFJ used in this study. The white brands D and E showed similar concentrations of bergamottin within lots. In red juices only brand H showed significant intra-lot variability of naringin and 6',7'-dihydroxybergamottin. The concentrations of bergamottin and 6',7'-dihydroxybergamottin in brand E (frozen from concentrate) were significantly higher when compared to all other juices. Although there is a lack of detailed information regarding the stability of furanocoumarins (bergamottin and 6',7'-dihydroxybergamottin) present in GFJ, brand E is sold as a frozen product, which may possibly have prevented

extensive degradation of furanocoumarins compared to juices stored at room temperature. In general, the white GFJ showed the highest levels of naringin, bergamottin, and 6',7'-dihydroxybergamottin with mean concentrations of  $1010 \pm 87$ ,  $24.5 \pm 2.3$ , and  $14.5 \pm 6.7$   $\mu\text{mol/L}$ , respectively. Additionally, the red variety showed higher amounts of bergamottin ( $9.5 \pm 1.8$   $\mu\text{mol/L}$ ) and 6',7'-dihydroxybergamottin ( $5.6 \pm 1.9$   $\mu\text{mol/L}$ ) compared to the pink variety, although the mean concentration of naringin was  $\sim 1.7$ - fold lower.

#### **Localization of Naringin, Naringenin, Bergamottin, and 6',7'-Dihydroxybergamottin in Grapefruit.**

A comparison of concentrations and distribution of the target compounds between fresh white and red grapefruit varieties (Figure 2-6) revealed that extracts from white grapefruit showed higher concentrations of naringin (3152  $\mu\text{g/g}$ ), bergamottin (19.8  $\mu\text{g/g}$ ), and 6',7'- dihydroxybergamottin (106  $\mu\text{g/g}$ ) located in the albedo and flavedo. The lowest concentrations were found in the seeds and pulp of red grapefruit. These results are in agreement with the values found in the commercial juices where, in general, the white grapefruit juices showed the highest contents of naringin, bergamottin, and 6',7'-dihydroxybergamottin compared to the pink or red varieties. A similar study was conducted to determine the content of the dimers of bergamottin and 6',7'-dihydroxybergamottin in the white and red grapefruits (200). Bergamottin and 6',7'-dihydroxybergamottin were able to inhibit the human microsomal CYP3A-mediated testosterone 6 $\beta$ -hydroxylation by half at 22 and 2  $\mu\text{mol/L}$ , respectively (98). With regard to their effects on P-gp activity, bergamottin (10  $\mu\text{mol/L}$ ) and 6',7'-dihydroxybergamottin (33  $\mu\text{mol/L}$ ) have been reported to reduce the activity of P-gp *in vitro* by 58 and 50%,

respectively (41, 80). In addition, 6',7'-dihydroxybergamottin has been shown to be a potent inhibitor of rat OATP3 ( $IC_{50} = 0.28 \mu\text{mol/L}$ ) (80). Although the flavonoid naringin has been excluded as the principal CYP3A4 inhibitor in GFJ (41, 75, 201), it promoted a significant inhibition on P-gp and OATP activity *in vitro* at concentrations of 1000 and 5  $\mu\text{mol/L}$ , respectively (80, 202). Naringin has also been shown to be hydrolyzed into the more potent CYP3A4 inhibitor naringenin by the gastrointestinal microflora located preferentially in the distal part of the small intestine and in the colon (203). The clinical relevance of this interaction is uncertain because most of the drug absorption takes place in the small intestine. Overall, the concentrations of naringin, bergamottin, and 6',7'-dihydroxybergamottin in the samples analyzed in this study appear to be high enough to considerably decrease CYP3A4, P-gp, and OATP activities. Other GFJ constituents (not tested in this study) have been shown to inhibit the CYP3A4 activity, such as the dimeric compounds ( $IC_{50} < 1 \mu\text{mol/L}$ ) (98) and epoxybergamottin ( $IC_{50} = 4.2 \mu\text{mol/L}$ ) (94). Additionally, the flavonoids quercetin and kaempferol have been shown to inhibit the organic cation transporters (OCT) by half at 32 and 38  $\mu\text{mol/L}$ , respectively (202). In general, the GFJ-drug interactions studies are characterized by a wide variability in the pharmacokinetics and pharmacodynamics data among patients from the same study, as well as between different studies. Variations in the intestinal concentrations of CYP3A4 among individuals seem to contribute to the differences in the intensity of this interaction (94). In addition, it was speculated that the high variability of components in GFJ may also increase the risk of an interaction in patients who drink GFJ habitually and appear to be equilibrated during a drug therapy, if the brand or even the lot is switched during drug therapy with a susceptible drug (43). However, further studies comparing the

effect of different brands on the magnitude of the GFJ-drug interaction are needed to clarify this issue. Additionally, the correlation between the content of the interacting compounds in a GFJ preparation and their inhibitory effects would help to estimate the role of each component on the overall GFJ-mediated inhibition and also to predict and circumvent such interactions (109).

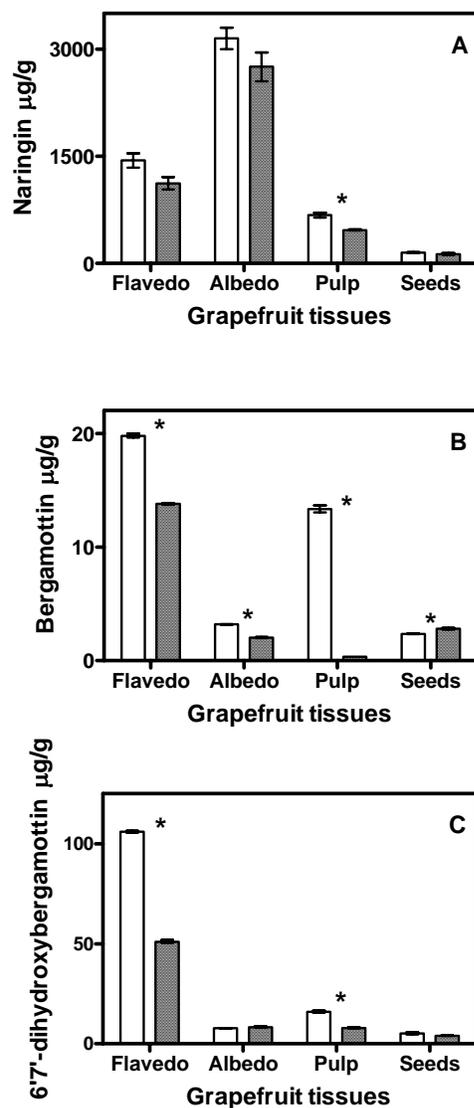


Figure 2-6. Distribution of naringin (A), bergamottin (B) and 6',7'-dihydroxybergamottin (C) in different tissues of white (open bars) and ruby red (shaded bar) grapefruits. Values are means  $\pm$  (SEM) ( $n = 3$ ). Asterisks indicate significant differences between values of white compared to red grapefruit.

Table 2-5. Naringin, bergamottin and 6',7'-dihydroxybergamottin content in different brands of grapefruit juice sold in Florida.

Grapefruit Juices	Variety	Lots	Concentration ( $\mu\text{mol/L}$ )		
			Naringin	Bergamottin	6',7'-Dihydroxy-Bergamottin
Brand A		L1	777.6 (4.3) <sup>a</sup> h-k <sup>b</sup>	9.76 (0.5) klm	0.44 (0.01) hij
		L2	772.3 (10.1) h-k	9.03 (0.2) lmn	0.44 (< 0.01) hij
Brand B*	Pink	L1	878.9 (32.4) gh	8.79 (1.0) lmn	0.65 (0.04) hij
		L2	939.3 (34.1) fgh	12.13 (0.6) jkl	1.07 (0.02) hij
Brand C		L1	668.5 (28.1) i-l	5.98 (0.02) mno	0.35 (< 0.01) hij
		L2	654.6 (6.3) i-l	14.24 (0.1) h-k	0.76 (0.02) hij
Brand B*		L1	1,262 (42.1) bc	18.81 (1.3) fgh	1.14 (0.1) hij
		L2	1,492 (44) a	25.86 (1.0) de	2.21 (0.1) hij
Brand C		L1	820.4 (43.9) ghi	21.95 (0.5) ef	1.03 (0.03) hij
		L2	589.9 (14) lm	28.04 (0.3) cd	2.29 (0.3) hij
Brand D*	White	L1	915.6 (8.8) fgh	15.54 (0.9) g-j	3.37 (0.2) g-j
		L2	651.8 (15.4) i-l	14.71 (0.7) g-j	1.34(0.2) hij
Brand E **		L1	810.9 (58.5) hij	31.77 (1.1) bc	45.04 (1.8) b
		L2	1,056 (22.6) def	34.37 (1.6) ab	49.01 (2.5) ab
		L3	1,318 (36.3) b	36.34 (1.6) a	52.51 (2.8) a
Brand F *		L1	1,216 (57) bcd	17.14 (0.4) ghi	0.73 (0.05) hij
		L2	980.6 (7.8) efg	25.42 (0.7) de	1.15 (0.03) hij
Brand C		L1	174.1 (2.8) o	2.95 (0.03) op	0.28 (< 0.01) ij
		L2	238.9 (3.8) o	5.06 (0.1) nop	0.28 (0.01) ij
Brand F *		L1	234.0 (3.1) o	3.59 (0.3) op	0.22 (< 0.01) j
		L2	303.6 (12.6) no	4.02 (0.1) op	0.25 (< 0.01) ij
Brand D *	Ruby Red	L1	667.5 (15.7) i-l	13.56 (0.2) g-j	14.57 (0.4) c
		L2	648.4 (14.8) jkl	14.16 (0.20) g-j	17.92 (0.7) c
Brand G		L1	303.9 (5.1) no	5.17 (0.3) m-p	0.25 (< 0.01) ij
		L2	291.5 (2.8) o	2.58 (0.2) op	0.24 (0.02) ij
Brand H		L1	469.4 (18) mn	12.19 (0.5) i-l	4.55 (0.05) j-i
		L2	1,147 (97) cde	12.73 (0.2) jkl	7.38 (0.02) efg
Brand I		L1	584.4 (10) lm	19.18 (1.3) fg	8.74 (0.4) def
		L2	613.4 (18.8) klm	18.92 (0.9) fg	12.10 (0.8) cd
Fresh squeezed	White		312.6 (9.5) no	0.97 (< 0.01) qr	4.71 (0.1) fgh
	Ruby Red		245.7 (28.3) o	3.10 (0.1) p	10.28 (0.6) cde

<sup>a</sup> Values are means (n=3) with SEM in parenthesis. <sup>b</sup> Values with same letter(s) are not significantly different (Tukey-Cramer multiple comparison,  $p \leq 0.05$ ). \* = from concentrate; \*\* = frozen from concentrate. Brands: A - Blue Bird®; B - Thirft Maid®; C - Ocean Spray®; D - Publix®; E - Minute Maid®; F - Win Dixie®; G - Sam's Choice®; H - Florida's Natural®; I - Indian River®.

## Conclusions

The analytical methods applied were suitable to quantify the predominant flavonoids and furanocoumarins in the juice and in different tissues of the grapefruit.

On the basis of the demonstrated variability and lack of information regarding the profile flavonoids and furanocoumarins of juices used for human trials, a direct comparison between different studies is difficult. Therefore, to minimize prevalent variability in GFJ-drug interaction trials, it is important to correlate measured endpoints to the concentrations of those grapefruit components that are considered to be relevant for the interaction with drugs. In future studies investigating grapefruit-drug interactions, concentrations of compounds with a suspected interaction should be considered. When possible, juices of very similar composition should be used for studies designed for direct comparison. In addition, for animal studies the concentrations of critical compounds may be adjusted by supplementation to normalize the administered juices.

CHAPTER 3  
GRAPEFRUIT JUICE AND ITS COMPONENTS INHIBIT P-GLYCOPROTEIN  
MEDIATED TRANSPORT OF TALINOLOL IN CACO-2 CELLS

**Background**

Due to the striking overlaps in tissue distribution, substrates and inhibitors specificity of CYP3A4 and permeability-glycoprotein (P-gp) (128) it is not surprising that grapefruit juice (GFJ) and its components can also interact with this membrane efflux transporter, which inhibition would be expected to be followed by augment of the oral bioavailability of its substrates. However, modulation of P-gp activity by GFJ and the clinical relevance of such interaction is still unclear and is discussed controversially, since some authors have reported activation (136, 143) and others inhibition of P-gp (61, 71, 144). Recently, the  $\beta$ -blocker talinolol, which is a non-CYP substrate, yet a P-gp substrate, showed an increase in bioavailability when associated with GFJ in both *in vitro* and *in vivo* models (134). However, a clinical trial had shown an opposite outcome, i.e. reduction of talinolol bioavailability (137) following its co-administration with GFJ in healthy subjects.

Many GFJ compounds have been proposed to interact with CYP enzymes and P-gp. These include both flavonoids (eg. naringenin, naringin, quercetin and kaempferol) and nonflavonoids (eg. bergamottin, 6',7'-dihydroxybergamottin). However, the influence of different GFJ constituents on P-gp is uncertain, particularly regarding the *in vivo* relevance of this potential interaction mechanism.

### Specific Aim

It was the aim of this study to systematically investigate *in vitro*, using a Caco-2 cell model and talinolol as a marker compound, the potential drug interaction between selected ingredients of GFJ (Figure 3-1) - alone and in combination- and the P-glycoprotein transporter system, which controls many barriers in the body. Additionally, the concentration dependent transport of talinolol was demonstrated and its kinetics parameters estimated.

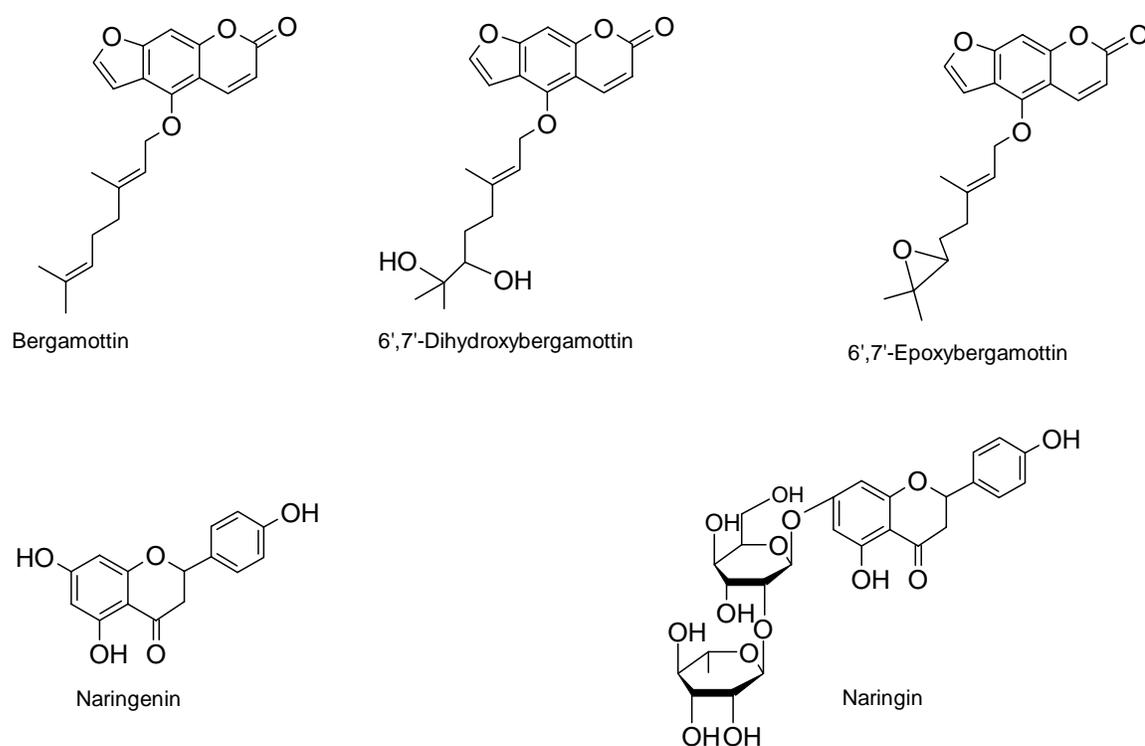


Figure 3-1. Structures of selected flavonoids and furanocoumarins present in grapefruit juice and tested in this study.

### Material and Methods

#### Chemicals

The following materials were used: (rac)-verapamil hydrochloride, Hank's balanced salt solution (HBSS), 2-(N-morpholino)ethanesulfonic acid solution (MES); sodium pyruvate solution (1 M), Lucifer yellow dipotassium salt, all of which were from

Sigma Chemical Company (St. Louis, MO, USA); Dulbecco's modified Eagle's medium (1X) high glucose without sodium pyruvate (DMEM), nonessential aminoacids, trypsin (0.05%)-EDTA (0.02%) solution, Fungizone® antimycotic solution containing 250 µg of amphotericin B and 205 µg of sodium deoxycholate per mL as solubilizer, penicillin G (10,000 units/mL) and streptomycin sulfate (10,000 µg/mL) solution, (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] buffer solution (1 M) (HEPES) were obtained from Gibco BRL Life Technology (Grand Island, NY, USA); fetal bovine serum heat inactivated and Dulbecco's phosphate buffered saline (DPBS) (pH 7.4) were purchased from Mediatech Inc. (Herdon, VA, USA); talinolol (99.9% purity) was gently provided by AWD-Pharma GmbH & Co. KG (Dresden, Germany), naringin (NAR) and naringenin (NAG), both > 95% pure, were from Roth GmbH & Co. (Karlsruhe, Germany), bergamottin (BG) (98% purity) was bought from Indofine Chemical Company, Inc. (Somerville, NJ, USA), 6',7'-dihydroxybergamottin (DHB), and 6',7'-epoxybergamottin (EPBG) were kindly supplied by Dr. John Manthey at the U.S. Department of Agriculture (USDA), Citrus and Subtropical Products Laboratory, Agricultural Research Service, Winter Haven, Florida. Upon isolation, the compounds identification and purity (> 98%) were measured by analytical thin layer chromatography, HPLC-MS and melting point comparisons with authentic standards at the USDA laboratories. Triethylammonium phosphate buffer (1 M) was obtained from Fluka (Buchs, Switzerland). All others reagent were analytical grade.

### **Cell Culture**

Caco-2 cells were obtained from ATCC (Rockville, MD, USA) and maintained as stock cells in T flasks of 150 cm<sup>2</sup> (Corning Costar Corp., Cambridge, MA, USA). The

growing medium consisted of DMEM containing 10% of fetal bovine serum, 1% of nonessential amino acids, 100U/mL penicillin G, 100 µg/ml streptomycin, 1.25 µg/mL amphotericin B, and 10 mM sodium pyruvate. The atmosphere was kept with 90-95% of relative humidity, 37 °C, 5% CO<sub>2</sub>, and 95% air. The cells were passaged upon reaching 80-90% confluence using trypsin-EDTA (~5mL) and plated at densities of 1:5 - 1:7 in new T flasks. After reaching passages 20-40 the Caco-2 cells were seeded at density of 60,000 cells/cm<sup>2</sup> onto each 12-mm transparent polyester cell culture insert - 12 well plates with area of 1.13 cm<sup>2</sup> and pore size of 0.4 µm (Transwell®, Corning Costar® Corporation, Cambridge, MA, USA). The DMEM medium was added in both apical (AP) and basolateral (BL) compartments and changed 4 days after seeding, and every 2 days thereafter. The cells were used on days 18 to 25 post-seeding to obtain differentiated monolayers and higher expression of P-glycoprotein (173, 174). Transepithelial electrical resistance values (TEER) were monitored with EndOhm Voltohmmeter equipped with a STX-2 “chopstick” electrode (World Precision Instruments Inc, Sarasota, FL, USA) in order to check cell confluence and integrity. Confluent Caco-2 monolayers with TEER values >350 Ω cm<sup>2</sup>, after correction for the resistance obtained in control blanks, were used in the transport experiment. All volumes amounted to 0.5 mL at the apical side and 1.5 mL at the basolateral side.

### **Stock, Work Solutions, and Preparation of Calibration Standards**

The stock solutions at 50 mmol/L of NAG, BG, and DHB were prepared in dimethylsulfoxide (DMSO) as previously described in Chapter 2. Talinolol stock solution I was prepared in ethanol, verapamil hydrochloride (HCl) in buffer pH 6.0, NAR and EPBG in DMSO.

**Talinolol stock solution I**

The amount of 36.3 mg of talinolol (MW = 363.5 g/mol) was accurately weighed, and transferred quantitatively to a 2.0 mL volumetric flask. The standard was then dissolved in ethanol, the volume was completed with the same solvent, and the final solution mixed thoroughly in order to obtain a final concentration of 50 mmol/L

**Talinolol stock solution II**

The amount of 25 mg of talinolol (TAL) was accurately weighed and transferred quantitatively to a 25 mL volumetric flask. The standard was then dissolved in ethanol, the volume was completed with the same solvent, and the final solution mixed thoroughly in order to obtain a final concentration of 1 mg/mL.

**Talinolol stock solution III**

Volume of 1 mL from the talinolol stock solution II was accurately transferred to a 100 mL volumetric flask, the volume was completed with a solution of 0.025 mol/L triethylammonium phosphate buffer (pH 3.0 - adjusted with 1 M NaOH) and acetonitrile (77:23) and mixed thoroughly. The final concentration of talinolol was 10 µg/mL.

**Talinolol stock solution IV**

Volume of 1 mL from the talinolol stock solution III was accurately transferred to a 10 mL volumetric flask, the volume was completed with a solution of 0.025 mol/L triethylammonium phosphate buffer (pH 3.0 - adjusted with 1 M NaOH) and acetonitrile (77:23) and mixed thoroughly. The final concentration of talinolol was 1000 ng/mL.

**Verapamil hydrochloride stock solution**

The amount of 49.1 mg verapamil HCl (MW = 491.05 g/mol) was accurately weighed, and transferred quantitatively to a 2.0 mL volumetric flask. The standard was then dissolved in buffer pH 6.0, the volume was completed with the same solvent, and the

final solution mixed thoroughly. The final concentration of verapamil HCl was 50 mmol/L.

#### **Naringin stock solution I**

The amount of 145.1 mg of naringin (MW = 580.53 g/mol) was accurately weighed, and transferred quantitatively to a 5.0 mL volumetric flask. The standard was then dissolved in 1 mL of DMSO, the volume was completed with HBSS-MES (pH 6.0) and mixed thoroughly in order to obtain a final concentration of 50 mmol/L.

#### **6',7'-Epoxybergamottin stock solution**

The amount of 35.4 mg of 6',7'-epoxybergamottin (MW = 354.41 g/mol) was accurately weighed, and transferred quantitatively to a 2.0 mL volumetric flask. The standard was then dissolved in DMSO, the volume was completed with the same solvent and solution mixed thoroughly in order to obtain a final concentration of 50 mmol/L.

#### **Talinolol work solutions**

For the inhibitory study, a dosing solution of talinolol (100  $\mu$ M) was prepared in HBSS-MES transport medium (HBSS buffered with 10mM MES and adjusted to pH 6 with 1 M NaOH) or in HBSS-HEPES transport medium (HBSS buffered with 10 mM HEPES and adjusted to pH 7.4 with 1 M NaOH). The concentration-dependent transport of talinolol was studied in the concentration range of 25 to 1000  $\mu$ M (25, 50, 200, 400, 600, 800 and 1000  $\mu$ M). Solutions were prepared according Table 3-1 using the talinolol stock solution I. In all cases the ethanol concentrations of the final solutions did not exceed 2 %. Yamashita *et al.* (204) already reported that ethanol (< 5 v/v %) did not affect the permeability of dexamethasone and the integrity of Caco-2 cells monolayers, suggesting its usefulness as a solubilizing agent of drugs during *in vitro* experiments.

Table 3-1. Concentrations of the work solutions of talinolol (TAL) used during the transport studies.

Solution	TAL stock solution I ( $\mu\text{L}$ )	Buffer pH 6.0 or pH 7.4 q.s.p. (mL)	Talinolol ( $\mu\text{mol/L}$ )
1	5	10	25
2	10	10	50
3	20	10	100
4	50	10	250
5	80	10	400
6	120	10	600
7	160	10	800
8	200	10	1000

### Talinolol standard solutions

From the talinolol stock solutions III and IV, nine different concentrations of standard solutions and three quality controls (QC) were prepared in 0.025 mol/L triethylammonium phosphate buffer (pH 3.0 - adjusted with 1 M NaOH) and acetonitrile (77:23) according to Table 3-2. All solutions were filtered through a 0.45  $\mu\text{m}$  PVDF membrane filter (Millipore Corp.) before analysis.

Table 3-2. Concentrations of the standard solutions used for the calibration curves and quality controls (QCs) of talinolol.

Standard	TAL stock solution III ( $\mu\text{L}$ )	TAL stock solution IV ( $\mu\text{L}$ )	Buffer/ACN (77:23) q.s.p. (mL)	Talinolol (ng/mL)
1		5	10	5
2		10	10	10
3		25	10	25
4		50	10	50
5	100		10	100
6	250		10	250
7	500		10	500
8	750		10	750
9	1000		10	1000
QC1		5	10	5
QC2	100		10	100
QC3	1000		10	1000

**Inhibitory solutions**

For the inhibitory experiments solutions of verapamil HCl (50, 100, 250, 500, and 1000  $\mu\text{M}$ ) (positive control) or NAR (100, 250, 500, 625, 1250, 2500  $\mu\text{M}$ ), NAG (10, 50, 100, 250, 500  $\mu\text{M}$ ), DHB (0.5, 10, 25, 50, 500  $\mu\text{M}$ ) and EPBG (0.5, 1, 5, 10, 50  $\mu\text{M}$ ) were prepared in HBSS-MES in the presence or absence of the marker compound talinolol (100  $\mu\text{M}$ ), according shown in Table 3-3. BG was tested only in two concentrations (1 and 10  $\mu\text{M}$ ) due to solubility issues. Similarly, solutions containing a fixed ratio of the flavonoid NAR to the furanocoumarin DHB (50:1.2, 125:3, 625:15, 1250:30, and 2000:48  $\mu\text{M}$ ) were prepared considering the range these compounds are found in the juice (Table 3-4). The effect of the combination of these major compounds present in the GFJ on the P-gp mediated transport of talinolol was evaluated. The white GFJ frozen concentrated (Minute Maid®) was bought in the local market and diluted with HBSS-MES to 0.5, 1, 5, 10, 25 and 50% in the presence or absence of talinolol (100  $\mu\text{M}$ ). The GFJ solutions were previously passed to a 45  $\mu\text{m}$  filter PVDF membrane filter (Millipore Corp., Bedford, MA) before addition of talinolol. Concentrations of NAR, NAG, BG, and DHB in the juice were 811  $\mu\text{M}$ , < 0.56  $\mu\text{M}$ , 31.8  $\mu\text{M}$ , 45  $\mu\text{M}$ , respectively, as previously reported (205). The concentration of DMSO in these inhibitory solutions was  $\leq 1\%$ .

Table 3-3. Concentrations of the inhibitory solutions of Verapamil hydrochloride (VER), naringin (NAR), naringenin, (NAG), bergamottin (BG), 6',7'-dihydroxybergamottin (DHB), and 6',7'-epoxybergamottin (EPBG) used during the transport studies.

VER	VER stock solution (μL)	Buffer pH 6.0 or pH 7.4 q.s.p. (mL)	VER (μmol/L)
1	25	25	50
2	50	25	100
3	125	25	250
4	250	25	500
5	500	25	1000
NAR	NAR stock solution (μL)	Buffer pH 6.0 or pH 7.4 q.s.p. (mL)	NAR (μmol/L)
1	50	25	100
2	125	25	250
3	250	25	500
4	312.5	25	625
5	625	25	1250
6	1250	25	2500
NAG	NAG stock solution (μL)	Buffer pH 6.0 or pH 7.4 q.s.p. (mL)	NAG (μmol/L)
1	5	25	10
2	25	25	50
3	50	25	100
4	125	25	250
5	250	25	500
BG	BG stock solution (μL)	Buffer pH 6.0 or pH 7.4 q.s.p. (mL)	BG (μmol/L)
1	1	50	1
2	10	50	10
DHB	DHB stock solution (μL)	Buffer pH 6.0 or pH 7.4 q.s.p. (mL)	DHB (μmol/L)
1	0.5	50	0.5
2	5	25	10
3	12.5	25	25
4	25	25	50
5	250	25	500
EPBG	EPBG stock solution (μL)	Buffer pH 6.0 or pH 7.4 q.s.p. (mL)	EPBG (μmol/L)
1	0.5	50	0.5
2	0.5	25	1
3	2.5	25	5
4	5	25	10
5	25	25	50

Table 3-4. Concentrations of the inhibitory solution of naringin (NAR) plus 6',7'-dihydroxybergamottin (DHB) used during the transport studies.

NAR:DHB inhibitory solution	NAR stock solution ( $\mu\text{L}$ )	DHB stock solution ( $\mu\text{L}$ )	Buffer pH 6.0 or pH 7.4 q.s.p. (mL)	Concentration ( $\mu\text{mol/L}$ )	
				NAR	DHB
1	25	0.6	25	50	1.2
2	62.5	1.5	25	125	3
3	312.5	7.5	25	625	15
4	625	15	25	1250	30
5	1000	24	25	2000	48

### Transport Study

On the study day, the DMEM was removed, the Caco-2 monolayers were rinsed with DPBS, and incubated for 30 min with the transport medium (TM) under the same atmospheric conditions described previously. The TM on the AP and BL side consisted of HBSS-MES and HBSS-HEPES, respectively.

After 30 min incubation, the drug-free transport medium on the AP side was replaced by the HBSS-MES medium (pH 6.0) containing talinolol or talinolol plus inhibitor in order to investigate the AP to BL transport. Alternatively, for the BL to AP transport assessment, the buffer of the donor chamber was replaced by a solution of talinolol in HBSS-HEPES medium (pH 7.4), additionally the volume of the acceptor chamber was replaced by HBSS-MES medium (pH 6.0) containing the inhibitor. For the control experiments only solutions of talinolol were used. Thereafter, sample aliquots (200  $\mu\text{L}$ ) were taken at 20, 40, 60, 90 and 120 min from the AP or BL side and the amount of talinolol transported at each time point was determined by HPLC. Following the transport experiment, the TEER values were measured again and an additional control of monolayer integrity was performed using Lucifer yellow (LY) (173)

### Sample Analysis

A reversed phase-HPLC method previously described (124) was optimized to enable talinolol to be quantified. Succinctly, samples from Caco-2 (25  $\mu$ L) were directed injected into a Shimadzu VP series HPLC system (Kyoto, Japan) equipped with a RF-10AXL fluorescence detector, a LC-10ATvp solvent delivery unit, an SIL-10AF autosampler, a CTO-10Avp column oven, a SCL-10Avp system controller, a DGU-14A on-line degasser, a FCV-10ALvp low-pressure gradient unit, and Class VP 7.2 SP1 chromatographic software. The excitation and emission wavelengths were set at 252 and 332 nm, respectively, the flow rate at 1 mL/min and the temperature at 40 °C. The analytical column was a Lichrospher 60RP-Select B column, 250 x 4.6 mm i.d., 5  $\mu$ m, preceded by a Lichrospher 60RP-Select B guard column (Merck KGaA, Darmstadt, Germany). Talinolol was eluted isocratically using a mobile phase consisted of 0.025 mol/L triethylammonium phosphate buffer (pH 3.0) and acetonitrile (77:23).

The validation parameters of linearity, specificity, sensitivity, precision, accuracy, and stability of this external standardization method for talinolol were determined. For each level of concentration, standards solutions and QCs were injected in the HPLC in triplicate in three different days. GraphPad Prism® version 4.0 (GraphPad Software Inc, San Diego, CA, USA) was used to construct the calibration curves obtained by plotting the mean area *versus* the corresponding concentration of the each standard solution. The linearity of the standard curves was determined by least-squares linear regression method and expressed in terms of coefficient of determination ( $r^2$ ). Specificity was determined by comparing the chromatogram of talinolol with that obtained from a blank solution containing only the inhibitory compounds. The intra- and inter-day precision and

accuracy of the quantification were measured by replicate analyses ( $n = 3$ ) of three different concentration levels (low, medium and high QCs) on the same day and on alternate days. The precision was based on the calculation coefficient of variation (CV%), and the accuracy was expressed as percent of the found amount compared to the theoretical one. The calibration model was accepted if the residuals (% difference of the back-calculated concentration from the nominal concentration) were within 20% at the lower limit of quantification (LLOQ) and within 15% at all calibration levels. Calibration curves were rejected if more than 1/3 of the QCs showed a deviation from the theoretical concentration equal or greater than 20%.

The lower limit of quantification (LLOQ) was established as the lowest concentration used in the calibration curve.

The stability at room temperature and at 37 °C of a solution of talinolol in HBSS-MES (pH 6.0) and HBSS-HEPES (pH 7.4) transport medium was assessed within 48 hours and 4 hours, respectively. The same samples were frozen at -20 °C during one week in order to check the freeze/thaw stability. Similarly, the stability of talinolol (low, medium, high QCs) in mobile phase was determined within 24 hours at room temperature. The samples were analyzed by the validate HPLC method mentioned formerly, the areas were compared to those obtained from fresh solutions for each concentration level, and results expressed as % of the control. Three replicates of each solution were prepared for each test performed.

An additional stability test was conducted during 3 hours in HBSS-MES at 37 °C for all grapefruit juice constituents tested in this study. Samples were analyzed as

described in Chapter 2. The 6',7'-epoxybergamottin was analyzed using the same method as described for furanocoumarins in Chapter 2.

### Data Analysis

The coefficients of permeability ( $P_{app}$ ) talinolol were calculated by using equation (1):

$$P_{app} = \frac{\Delta Q}{\Delta T \times A \times C_0} \quad (1)$$

where  $\Delta Q/\Delta T$  is the amount of drug (ng/min) appearing in the acceptor compartment as a function of time obtained from the slope of the linear portion of the amount transported vs time plot.,  $C_0$  is the initial concentration of talinolol in the donor compartment (ng/mL), and  $A$  the surface area of the semi-permeable membrane in  $\text{cm}^2$  (62, 135). All solutions were kept under 37 °C before use.

The passive and the Michaelis-Menten parameters of talinolol were estimated using Scientist v. 2.0 (MicroMath Inc., St. Louis, MO, USA). To apply the model the equation (2) was used:

$$P_{app (BL-AP)} = P_{app (BL-AP) passive} + \frac{P_{max} \times C_0}{K_M + C_0} \quad (2)$$

where the  $P_{app (BL-AP)}$  is the observed permeability (cm/sec) of talinolol from basolateral to apical side of Caco-2 monolayers. The  $P_{app (BL-AP) passive}$  is the permeability due only to diffusion,  $P_{max}$  (cm/sec) is the maximal permeability due to the active transport and the  $K_M$  ( $\mu\text{M}$ ) is the Michaelis-Menten constant.

The IC<sub>50</sub> values (concentration to reach half of the maximum inhibition) of verapamil, GFJ and its components were then determined using nonlinear regression according to the Hill equation with GraphPad Prism v.4.0 (GraphPad Software Inc, San Diego, CA, USA). For NAR and verapamil, the best fit was obtained according to the sigmoid dose-response model without the variable slope.

### Statistical Analysis

The mean of at least three experiments and their standard error (SEM) were used to express the values of P<sub>app</sub> and the kinetics parameters. The confidence interval of each IC<sub>50</sub> value was determined. Statistical analyses were performed with one-way ANOVA followed by Dunnet's multiple comparison tests as post-hoc analysis. A probability of less than 0.05 ( $p < 0.05$ ) was considered to be statistically significant.

## Results

### Linearity

The range of validation (5 to 1000 ng/mL) showed good linearity with  $r^2 > 0.998$  (Figure 3-2).

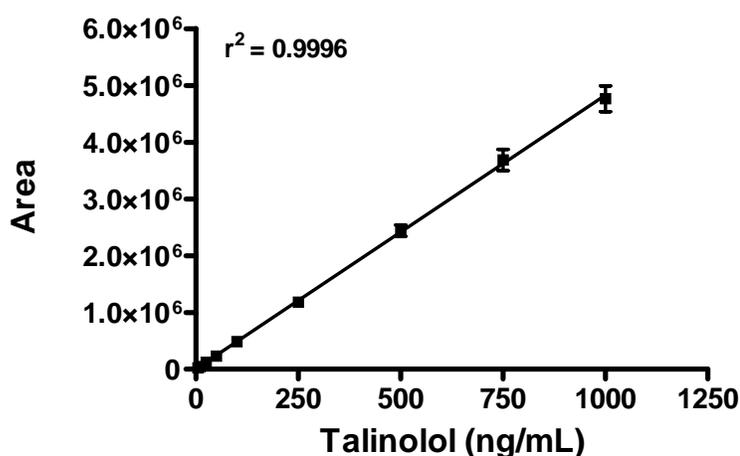


Figure 3-2. Mean calibration curve ( $n = 9$ ) of talinolol in mobile phase. Vertical bars represent the standard deviations (SD) of the means.

### Specificity

The method provided good specificity since the chromatograms of the blank solution did not show any peak with similar retention time as expected for talinolol (Figure 3-3)

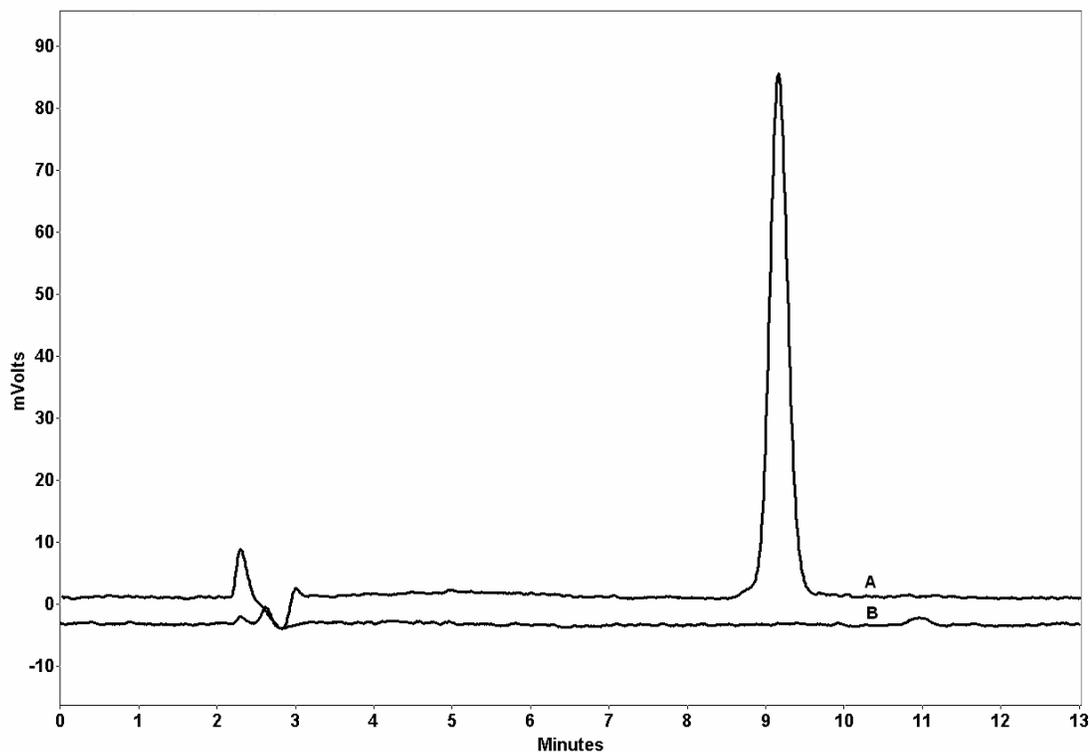


Figure 3-3. Chromatograms corresponding to the talinolol (100 ng/mL) in HBSS-MES with retention time about 9.3 min (A) and a blank solution (B) containing the inhibitory compounds NAR, NAG, BG, DHB, EPBG and VER all at 50  $\mu\text{mol/L}$  in HBSS-MES.

### Sensitivity

The LLOQ of 5 ng/mL was accepted as the lowest concentration of the calibration curve since their concentrations could be determined with acceptable precision (%CV < 20), and accuracy (%error < 20).

### Precision and Accuracy

The precisions intra- and inter-day for both flavonoids and furanocoumarins were satisfactory with CV values between 0.7 and 8%. Similarly, the accuracy of the assay was between 93 and 113% for all compounds tested at three different concentrations. The results are summarized in Table 3-5.

Table 3-5. Intra-day (n = 3) and inter-day (n = 9) assay parameters of talinolol. Precision expressed as CV% and accuracy as % of the theoretical concentration.

Intra-day	CQ – 5 ng/mL			CQ – 100 ng/mL			CQ – 1000 ng/mL		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Precision	0.8	0.7	7.9	4.7	4.2	4.8	4.2	0.8	2.9
Accuracy	109.9	105.6	113.1	93.0	93.3	98.3	100.4	100.1	98.9
Inter-day	CQ – 5 ng/mL			CQ – 100 ng/mL			CQ – 1000 ng/mL		
Precision	5.3			4.8			2.7		
Accuracy	109.9			94.9			99.8		

### Stability

The standard solutions of talinolol were found stable at room temperature within 48 hours and at 37 °C in both transport medium for 4 hours. Additionally, in general, all grapefruit components tested showed adequate stability under the test conditions for 3 hours. The 6',7'-epoxybergamottin showed a 20% of degradation and a peak at the same retention time as the DHB was observed concomitantly, indicating that EPBG was partially converted into DHB. For the others compounds, the shifting of the areas of each sample tested was less than 5% of those obtained from a fresh solution at the same level of concentration (Figure 3-4).

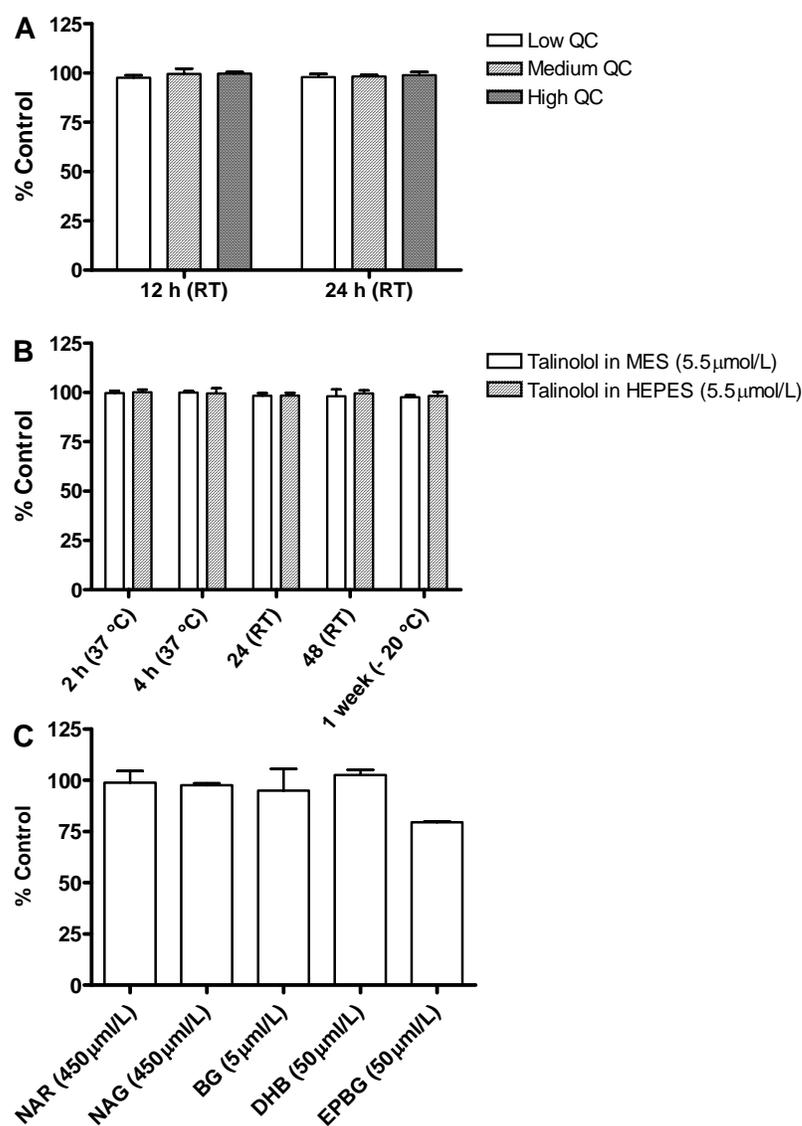


Figure 3-4. Stability of talinolol (low, medium, and high QCs) in mobile phase within 24 hours at room temperature (RT) (A). Stability of talinolol in transport medium within 4h at 37 °C, during 48 h at RT, and after one week at -20 °C (B). Stability of GFJ components in after 3h in HBSS-MES (pH 6) at 37 °C (C). Data represents means of three replicates and vertical bars the standard deviation of the means.

### Concentration Dependence

The absorptive permeability ( $P_{app}$  (AP-BL)) of talinolol was concentration independent and significantly lower than the permeability in the secretory direction ( $P_{app}$  (BL-AP)) at all concentrations (Figure 3-5). On the other hand, the  $P_{app}$  (BL-AP) decreased considerably as

the concentration of talinolol on the basolateral side of the Transwell® system was increased above 200  $\mu\text{M}$ . The apparent  $P_{\text{max}}$ ,  $P_{\text{app (BL-AP) passive}}$ , and  $K_M$  values and their standard deviations associated with the transport of talinolol in the secretory direction are  $(2.2 \pm 0.33) \times 10^{-6}$  cm/sec,  $(2.0 \pm 0.05) \times 10^{-6}$  cm/sec, and  $737 \pm 258$   $\mu\text{M}$ , respectively.

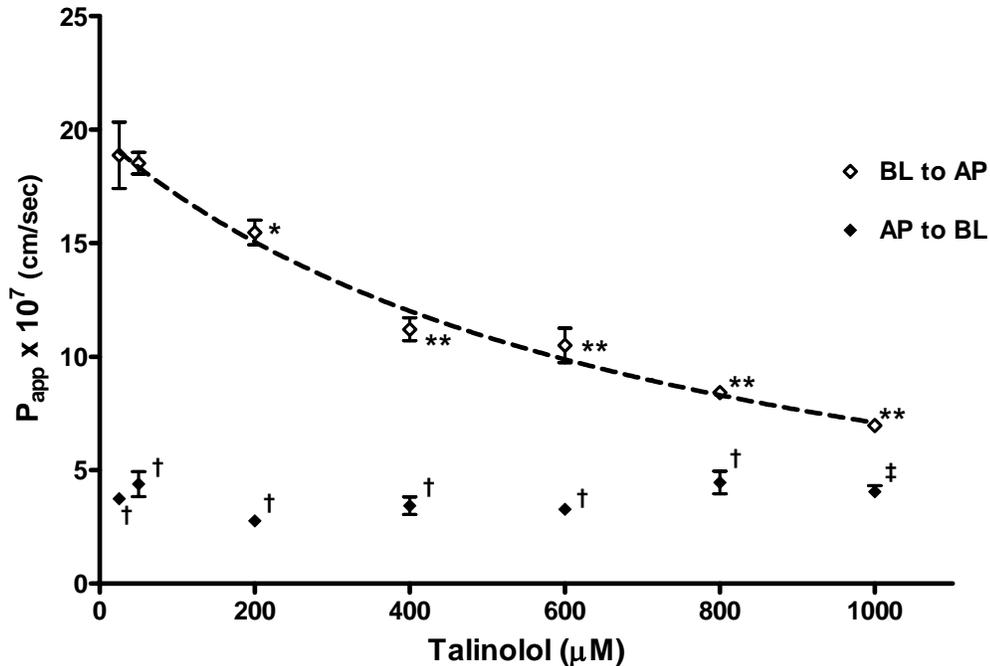


Figure 3-5. Concentration dependent coefficient permeability ( $P_{\text{app}}$ ) corresponding to the AP to BL and BL to AP transport of talinolol across Caco-2 cells monolayers. Data are expressed as mean  $\pm$  SEM ( $n = 3-6$ ). Fitting was performed using Scientist 2.0 as described in “Material and Methods”. All points of  $P_{\text{app (BL-AP)}}$  were compared with the talinolol 25  $\mu\text{M}$  and all data of  $P_{\text{app (AP-BL)}}$  were compared with the respective pair representing the secretory transport of talinolol. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; †  $p < 0.001$ ; ‡  $p < 0.01$ .

### Inhibition Studies

The Figure 3-6 shows the permeability of talinolol across the Caco-2 cells monolayers in the absence and presence of different concentrations of verapamil, GFJ, and its components. Verapamil, a known inhibitor of P-gp, decreased the ratio  $P_{\text{app (BL-AP)}}$  to  $P_{\text{app (AP-BL)}}$  of talinolol from 7.4 to 3. This effect was due to the attenuation of secretory ( $14 \times 10^{-7}$  to  $9.4 \times 10^{-7}$  cm/sec) and to the enhancement of absorptive ( $1.9 \times 10^{-7}$  to  $3.1 \times$

$10^{-7}$  cm/sec) transport of talinolol. However, compared with the respective controls reduction of  $P_{app(BL-AP)}$  was more significant than the enlargement of  $P_{app(AP-BL)}$ . Similarly, grapefruit juice (GFJ) reduced the  $P_{app(BL-AP)}$  of talinolol from  $22 \times 10^{-7}$  to  $4.5 \times 10^{-7}$  cm/sec but did not affect significantly its absorptive transport. Although NAR, the main constituent of GFJ, was found in the juice at relative high concentration (811  $\mu$ M), it showed a minor effect on the secretory transport of talinolol. Concentrations higher than 625  $\mu$ M decreased the  $P_{app(BL-AP)}$  to around 1.3-1.5 fold compared to the control. Likewise GFJ, the effect of NAR on the absorptive transport of talinolol was negligible. On the other hand, the aglycone NAG, which is not normally found in GFJ (205), decreased the BL to AP transport more than 2-fold compared to the control as its concentration was increased beyond 250  $\mu$ M. Analogous to 6',7'-dihydroxybergamottin, bergamottin at 1  $\mu$ M and 10  $\mu$ M did not change the permeability of talinolol however, *in vitro* studies of further concentrations were prejudiced because solubility issues. The furanocoumarins 6',7'-dihydroxybergamottin (DHB) at concentrations higher than 10  $\mu$ M and 6',7'-epoxybergamottin (EPBG) exerted a considerably reduction on the BL to AP transport of talinolol. In fact, the  $P_{app(BL-AP)}$  of talinolol in the presence of DHB (500  $\mu$ M) and EPBG (50  $\mu$ M) decreased 5- and 2.5- fold, respectively, when compared with the controls. The effective concentrations of these inhibitory compounds are in the same range as they are found in the juice (Table 3-2).

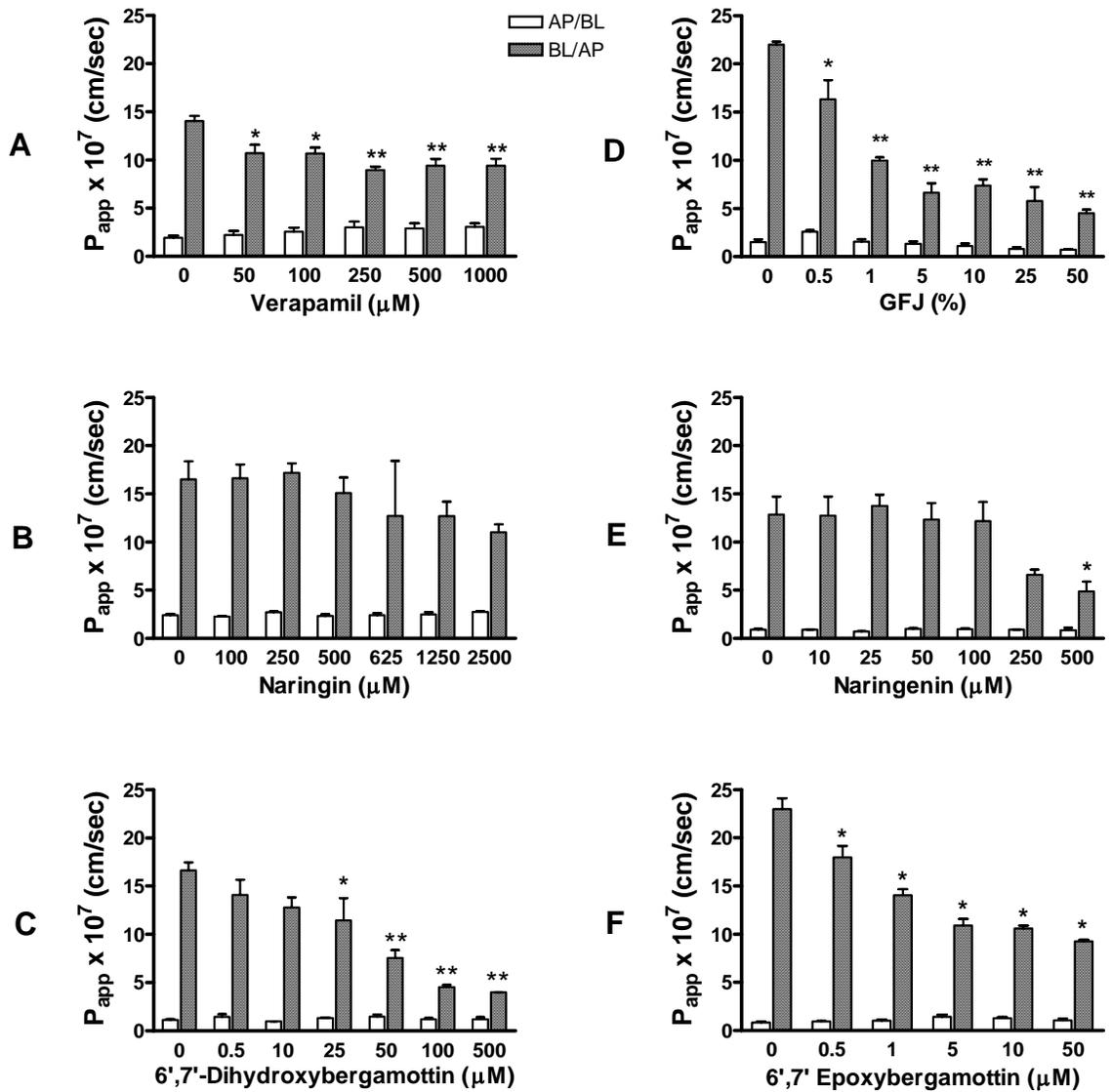


Figure 3-6. Coefficient of permeability ( $P_{\text{app}}$ ) of talinolol ( $100\mu\text{M}$ ) across the Caco-2 cells monolayers in the absence and presence of distinct concentrations of A: verapamil HCl (25, 50, 100, 250, and 500  $\mu\text{M}$ ); B: NAR (100, 250, 500, 625, 1250, 2500  $\mu\text{M}$ ); C: DHB (0.5, 10, 25, 50, 500  $\mu\text{M}$ ); D: GFJ (0.5, 1, 5, 10, 25, and 50 %); E: NAG (10, 50, 100, 250, 500  $\mu\text{M}$ ); F: EPB (0.5, 1, 5, 10, 50  $\mu\text{M}$ ). Data are expressed as mean  $\pm$  SEM (n = 3-6). \* p < 0.05; \*\* p < 0.01.

Table 3-6. Concentration range that the grapefruit components are normally found in the juice.

Inhibitor	Conc. in GFJ ( $\mu\text{M}$ )
Naringin	174 – 1492 <sup>(205)a</sup>
Naringenin	< 0.56 <sup>(205)</sup>
Bergamottin	1 – 36.6 <sup>(205)</sup>
6',7'-Dihydroxybergamottin	0.22 - 52.5 <sup>(205)</sup>
6',7'-Epoxybergamottin	0.1 - 7.4 <sup>(60)</sup>

<sup>a</sup> references are in parenthesis

### Dose-response Studies

The dose-response curves for the inhibitory effects and the  $\text{IC}_{50}$  values of verapamil, GFJ, and its components are presented in Figure 3-7, respectively. Except for NAR, the range of concentration tested was enough to obtain a sufficient estimation (narrow confidence interval range) of the potency and efficacy of all tested compounds and GFJ. The lower potency of NAR affected the accuracy of its  $\text{IC}_{50}$  value (2409  $\mu\text{M}$ ), since further concentrations ( $> 2500 \mu\text{M}$ ) could not be tested because of the negative impact on the integrity of the Caco-2 monolayers (decreased the TEER values and increased LY transport) and due to solubility problems. Verapamil, however, showed a relatively high potency (28  $\mu\text{M}$ ) but did not present a good efficacy reducing the activity of P-gp by only 35% compared to the control. GFJ ( $\text{IC}_{50} = 0.6\%$ ) diluted in HBSS-MES buffer solution (pH 6.0) decreased the secretory transport of talinolol to 27% of that without any inhibitor. Correspondingly, NAG was 10-fold more potent than its glycoside NAR reducing the P-gp mediated secretory transport of talinolol by 60% when compared to the control. Interestingly, the furanocoumarin EPBG was about 50-fold more potent than DHB with  $\text{IC}_{50}$  values of 0.7  $\mu\text{M}$  and 34  $\mu\text{M}$ , respectively. Additionally, compared

at the same concentration (50  $\mu\text{M}$ ), EPBG and DHB showed similar efficacy reducing the  $P_{\text{app(BL-AP)}}$  of talinolol by 55% and 60%, correspondingly.

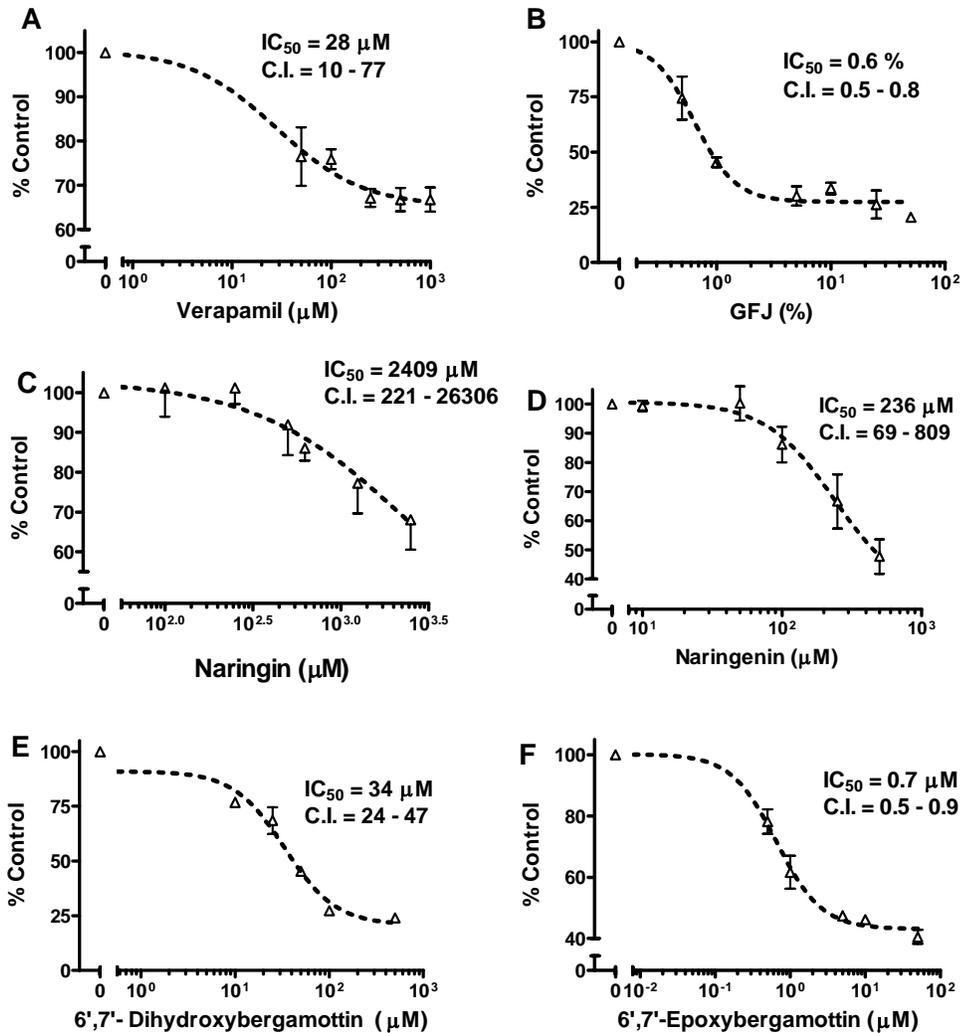


Figure 3-7. Inhibitory dose-response effects of A: verapamil HCl (50, 100, 250, 500, and 1000  $\mu\text{M}$ ); B: GFJ (0.5, 1, 5, 10, 25, and 50 %); C: NAR (100, 250, 500, 625, 1250, 2500  $\mu\text{M}$ ); D: NAG (10, 50, 100, 250, 500  $\mu\text{M}$ ); E: DHB (0.5, 10, 25, 50, 500  $\mu\text{M}$ ); F: EPBG (0.5, 1, 5, 10, 50  $\mu\text{M}$ ) on the transport of talinolol in the secretory (BL-AP) direction. Data are expressed as mean  $\pm$  SEM ( $n = 3-6$ ). The  $\text{IC}_{50}$  values of each compound and their respective 95% of confidence interval (C.I.) were estimated by nonlinear regression using GraphPad Prism 4.0 as described in "Material and Methods".

### Effects of NAR and DHB on the Secretory Transport of Talinolol

Although screening isolated compounds is an important tool to understand the mechanism of action and the relevance of each one on the interaction between GJF and P-gp, combinations of two or more compounds is a more realistic approach since the compounds are present all together in the juice and the overall outcome might be a result of an additive, or a synergistic or an antagonistic effect. Therefore, several combinations using a fixed NAR to DHB ratio of 41.7 were prepared and tested for their effect on the activity of P-gp. NAR is the main component present in the juice whereas, DHB has been shown to be a potent inhibitor of P-gp. Interestingly, the  $P_{app}$  (BL-AP) of talinolol went up considerably as the concentration of each compound was increased (Figure 3-8). In fact, the combinations NAR:DHB (1250:30  $\mu$ M and 2000:48  $\mu$ M) increased the secretion of talinolol about 2- and 2.7-fold, respectively, of that without inhibitor.

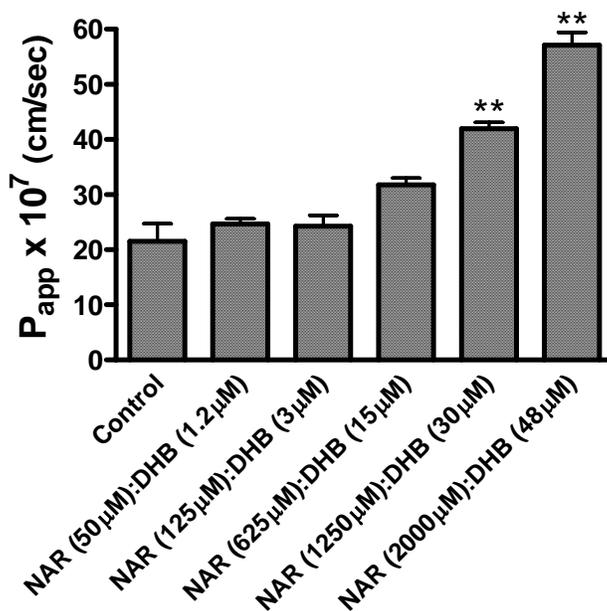


Figure 3-8. Effect of the combination NAR:DHB (50:1.2; 125:3, 625:15, 1250:30, and 2000:48  $\mu$ M) on the secretory transport (BL-AP) of talinolol (100 $\mu$ M) across the Caco-2 cells monolayers. Data are expressed as mean  $\pm$  SEM (n = 3). \* p < 0.05; \*\* p < 0.01.

## DISCUSSION

In the present study we systematically investigated *in vitro* the potential drug interaction between selected ingredients of GFJ - alone and in combination- and the P-glycoprotein transporter system, which controls many barriers in the body using talinolol as a probe substrate.

The stability studies demonstrated that any modification on talinolol concentration over the time on both apical and basolateral sides was regulated only by the transport phenomenon, which includes passive and active transport. Additionally, the GFJ components showed sufficient stability under the study conditions. However, since several metabolizing enzymes are present in Caco-2 the effect of GFJ metabolites can not be neglected and further studies are necessary to answer this issue.

The polarized transport of talinolol across the Caco-2 cell monolayers was noticeable with the BL-AP permeability exceeding significantly the AP-BL transport and exhibiting ratios higher than 5 (Figure 3-5 and Figure 3-6). Additionally, the decrease on the  $P_{app(BL\ to\ AP)}$  of talinolol was concentration-dependent demonstrating saturable efflux transport. Such behavior is consistent with the presence of the P-gp highly expressed in Caco-2 cells (173, 174). Similarly, it was demonstrated that the apparent oral clearance (CL/F) of talinolol in healthy volunteers decreased, approximately, from 1000 mL/min to 500 mL/min as the dose of talinolol increased from 25 mg to 400 mg. This phenomenon can be attributed to the saturation of the intestinal secretion of talinolol mediated by P-gp with consequent augment of fraction of the drug absorbed (186).

The  $P_{app(AP-BL)}$  of the marker compound remained unchanged at all concentrations (Figure 3-5). This asymmetry between the absorptive and secretory P-gp mediated transport was already described for talinolol (206) and other P-gp substrates like digoxin,

rhodamine 123 (207), fexofenadine (89), and taxol (175). It was suggested that P-gp was more rapidly saturated by substrates approaching from the BL than from AP side (207). Reasons for these observations were attributed to the differences in the lipid and protein composition between the AP and BL membranes (89). The apical membrane has about 2-fold more glycosphingolipids than the basolateral membrane. Such lipids have high capacity to form inter- and intra-molecular hydrogen bonds, which increase the microviscosity of the apical membrane (208).

Kinetic analyses revealed that the passive permeability of talinolol during the secretory transport was  $2.0 \times 10^{-6}$  cm/sec and similar of that obtained in previous studies  $3.6 \times 10^{-6}$  cm/sec (206). The  $P_{\max}$  value of  $2.2 \times 10^{-6}$  cm/sec, similar to the passive diffusion, supports the observation that P-gp-mediated transport exhibits a strong influence on the total permeability of talinolol over a wide range of concentrations (206)

In general, the  $P_{\text{app (AP-BL)}}$  of talinolol was not affected by the presence of any of the inhibitors tested (Figure 3-6). Verapamil up to 1000  $\mu\text{M}$  promoted a slight increase in the absorptive transport from  $(1.95 \pm 0.23) \times 10^{-7}$  cm/sec to  $(3.1 \pm 0.34) \times 10^{-7}$  cm/sec and the maximum effect on the secretory transport was reached at concentration of 500  $\mu\text{M}$  (Figure 3-6). However, verapamil ( $\text{IC}_{50} = 28 \mu\text{M}$ ) diminished the secretory activity of P-gp by only 35% (Figure 3-7). A complete inhibition would be characterized by total suppression of the polarized transport of talinolol. In contrast to others studies where verapamil was added on both the apical and basolateral side and sometimes pre-incubated for 15 to 30 min before addition of the substrate (186, 207, 209, 210), in the present study verapamil was present only in the apical side without previous incubation. Such approach

tried to simulate the effects of the several inhibitors tested when co-administrated with an oral dose of talinolol.

Although GFJ had a remarkable inhibitory effect on P-gp-mediated secretory transport of talinolol, and the half maximum effect was achieved at concentration of around 0.6%, its main constituent NAR had only a minor direct effect ( $IC_{50} = 2409 \mu\text{M}$ ). However, NAR may contribute indirectly to the overall inhibitory effect of GFJ, since this flavonoid can be converted by intestinal microflora to its aglycone NAG (211) which promotes a significant effect on P-gp activity ( $IC_{50} = 236 \mu\text{M}$ ) as presented in Figure 3-6 and Figure 3-7. Such observation supports that lipophilicity is an important parameter for P-gp substrates. Among all inhibitors tested, the furanocoumarin EPBG ( $IC_{50} = 0.7 \mu\text{M}$ ) seems to be the most potent compound against the P-gp mediated transport (Figure 3-7). Similarly, Dresser and co-workers (80) using vinblastine and digoxin as marker compounds, found that 6',7'-dihydroxybergamottin was the most potent inhibitor ( $IC_{50} = 33 \mu\text{M}$ ), bergamottin did not alter P-gp activity at concentrations up to  $50 \mu\text{M}$ , and naringin at  $3000 \mu\text{M}$  reduced P-glycoprotein to  $51\% \pm 9\%$  of control. Additionally, the components of grapefruit are present in the juice at enough concentration to promote a significant effect on P-gp activity (Table 3-2).

Interestingly, when combinations of NAR and DHB were tested a completely different profile was observed (Figure 3-8). The  $P_{\text{app (BL-AP)}}$  of talinolol increased from  $(21.5 \pm 1.85) \times 10^{-7} \text{ cm/sec}$  (control) to  $(57.1 \pm 2.33) \times 10^{-7} \text{ cm/sec}$ . So far, there is a lack of information regarding the simultaneous effect of multiple GFJ components on the activity of P-gp. The NAR to DHB ratio of 41.7 employed in this study differed from that found in the juice ( $\frac{NAR}{DHB} = 18$ ), suggesting that the relative concentrations of these

components may also play an important role on the mechanism and on the grapefruit juice-drug interaction outcomes. Since expression of others transporters such as OATP and organic cation transporters (OCT) in Caco-2 is uncertain (89), and talinolol is not substrate of multiple drug resistant protein (MRP) or breast cancer resistant protein (BCRP) which can be expressed in this cell line (89, 212, 213), we speculate that the effect observed was due to activation of P-gp activity or modification of membrane fluidity. Further *in vitro* and animal studies using different ratios of NAR and DHB, additional compounds, such as the dimers of furanocoumarins, and new combinations of GFJ components are necessary to clarify this issue.

### **Conclusions**

The results of this study support the importance of P-gp mediated transport on drug disposition. Modulation of P-gp activity by GFJ and its components may have some clinical relevance. Isolated compounds present in GFJ are able to inhibit directly or indirectly the P-gp activity. This study demonstrated that the modulation of P-gp by GFJ seems not to be a simple additive effect, instead a more complex association is likely, especially considering the variability of these components present in the grapefruit and in the juice. This may help to explain the controversial results previously reported for GFJ-Pgp interaction studies. Additionally, taking into account the lack of information about the effect on P-gp activity when two or more grapefruit juice constituents are tested simultaneously, further investigations are necessary to elucidate the mechanisms behind the GFJ-drug interactions and their clinical relevance.

CHAPTER 4  
MODULATION OF P-GLYCOPROTEIN MEDIATED TRANSPORT OF  
TALINOLOL IN RATS BY GRAPEFRUIT JUICE, ORANGE JUICE, FLAVONOIDS  
AND FURANOCOUMARINS

**Background**

There is a lack of information regarding the effect of different brands and strengths of grapefruit juices (GFJ) and its constituents on the disposition of drugs substrates for the permeability-glycoprotein (P-gp) intestinal efflux transporter. Most of the reported studies focus on evaluating the interaction between GFJ and its components with the drug metabolizing enzyme cytochrome P450 3A4 (CYP3A4) or the effect of only one juice on the activity of P-gp (40, 134, 137, 214). Reports of interaction between GFJ and talinolol, a well described non-metabolized P-gp substrate (215), in rats and humans have shown opposite results. Co-administration of white GFJ (Tropicana®) 50% diluted in saline pH 7.0 with talinolol (10mg/kg) was described to increase the maximum talinolol serum concentration ( $C_{max}$ ) and the area under the concentration-time profile curve (AUC) in rats by 2- and 1.4- fold, respectively (134). In contrast, the  $C_{max}$  and AUC of talinolol when taken orally with GFJ (Paradiso-Succo® - Germany) by healthy volunteers were reduced by half (137). The magnitude of the GFJ-drug interaction maybe dependent on the brand, the amount of GJF ingested, the timing of drug administration relative to the food intake, the intrinsic oral bioavailability of each drug, and the relative concentration of each grapefruit component in the juice (40, 128). Thus, the extent of which GFJ modifies P-gp activity remains unclear and more studies are desirable to clarify this issue.

### **Specific Aim**

It was the aim of this study to investigate the effect of different strengths and brands of GFJ and its components naringin (NAR), naringenin (NAG), bergamottin (BG) - alone and in combination - on the disposition of talinolol, in male Sprague-Dawley rats. These compounds together with 6',7'-dihydroxybergamottin (DHB), 6',7'-epoxybergamottin, and their dimers have been suggested to contribute to GFJ-drug interaction (68, 105). Additionally, the effects of both acute and one-week long of juice intake before drug administration were evaluated. The effect of orange juice on talinolol disposition was also estimated as preliminary data for futures studies.

We expect that this study will aid to clarify the predominant mechanism, the major compounds contributing to the overall effect of grapefruit juice on drug disposition and the *in vivo* relevance of this potential interaction mechanism.

### **Materials and Methods**

#### **Chemicals**

The following materials were used: (rac)-verapamil hydrochloride and halothane were obtained from Sigma Chemical Company (St. Louis, MO, USA); talinolol (99.9% purity) was a gently provided by AWD-Pharma GmbH & Co. KG (Dresden, Germany), naringin (NAR) and naringenin (NAG), both > 95% pure, were from Roth GmbH & Co. (Karlsruhe, Germany), bergamottin (BG) (98% purity) was bought from Indofine Chemical Company, Inc. (Somerville, NJ, USA); triethylammonium phosphate buffer (1M) was obtained from Fluka (Buchs, Switzerland); methanol HPLC grade was purchased from Fisher Scientific (Fair Lawn, NJ, USA). All others reagent were analytical grade.

**Stock, Work Solutions, and Preparation of Calibration Standards**

The stock solution of BG 50 mM was prepared in dimethylsulfoxide (DMSO) as previously described in Chapter 2 and NAG 2.5 mM in propylene glycol. Talinolol test solution I and II were prepared in propylene glycol:saline (20:80) and saline, respectively, whereas the talinolol stock solution I was obtained using ethanol. The final concentration of propylene glycol (PPG) was not higher than 20%.

**Talinolol test solutions**

The amount of 50 mg of talinolol (TAL) was accurately weighed and transferred quantitatively to a 25 mL volumetric flask. The standard was dissolved in 5 mL of propylene glycol and the volume completed with saline solution which pH has been previously adjusted to 3.0 in order to simulate the pH of the juice and guarantee a complete dissolution of the drug. The final dose solution containing 20% of PPG was mixed thoroughly to obtain a talinolol concentration of 2 mg/mL and was used as a control when evaluating the effects of GFJ constituents and verapamil on the disposition of talinolol in rats. Same solution was prepared in absence of propylene glycol and was used as a control when evaluating the effects of grapefruit juices and OJ on the disposition of talinolol in rats

**Talinolol stock solution I**

The amount of 25 mg of talinolol (TAL) was accurately weighed and transferred quantitatively to a 25 mL volumetric flask. The standard was then dissolved in ethanol, the volume was completed with the same solvent, and the final solution mixed thoroughly in order to obtain a talinolol concentration of 1 mg/mL.

**Talinolol stock solution II**

Volume of 100  $\mu\text{L}$  from the talinolol stock solution I was accurately transferred to a 10 mL volumetric flask, the volume was completed with a solution of 0.025 mol/L triethylammonium phosphate buffer (pH 3.0 - adjusted with 1 M NaOH) and acetonitrile (77:23) and mixed thoroughly. The final concentration of talinolol was 10  $\mu\text{g}/\text{mL}$ .

**Talinolol stock solution III**

Volume of 1 mL from the talinolol stock solution II was accurately transferred to a 10 mL volumetric flask, the volume was completed with a solution of 0.025 mol/L triethylammonium phosphate buffer (pH 3.0 - adjusted with 1 M NaOH) and acetonitrile (77:23) and mixed thoroughly. The final concentration of talinolol was 1000 ng/mL.

**Verapamil test solution I**

The amounts of 20 mg of verapamil hydrochloride and 50 mg of TAL were transferred quantitatively to a 25.0 mL volumetric flask. The compounds were dissolved in 5 mL of propylene glycol and the volume completed with saline solution which pH has been previously adjusted to 3.0. The solution was mixed thoroughly and the final concentrations of verapamil.HCl and talinolol were 0.8 mg/mL and 2 mg/mL, respectively.

**Naringin test solutions**

The amounts of 11.8 mg or 47 mg of naringin (MW = 580.53 g/mol) and 50 mg of TAL were transferred quantitatively to a 25.0 mL volumetric flask. The compounds were dissolved in 5 mL of propylene glycol and the volume completed with saline solution which pH has been previously adjusted to 3.0. The solutions were mixed thoroughly and the final concentrations of NAR and talinolol were 813  $\mu\text{M}$  or 3238  $\mu\text{M}$  and 2 mg/mL, respectively. The concentrations of NAR in these solutions approximately correspond to

that found in the white grapefruit juice normal strength (811  $\mu\text{M}$ ) or frozen concentrate (3240  $\mu\text{M}$ ) used in this study.

#### **Naringenin stock solution**

The amount of 17 mg of naringin (MW = 272.26 g/mol) was transferred quantitatively to a 25.0 mL volumetric flask. The compound was dissolved in 15 mL of propylene glycol, the volume was completed with the same solvent, and the final solution mixed thoroughly. The final concentration of NAG was 2.5 mM.

#### **Naringenin test solution**

The amount of 50 mg of talinolol was transferred quantitatively to a 25.0 mL volumetric flask containing 5 mL of the naringenin stock solution. Talinolol was then dissolved and the volume was completed by adding saline solution which pH has been previously adjusted to 3.0. The solution mixed thoroughly and the final concentrations of NAG and talinolol were 500  $\mu\text{M}$  and 2 mg/mL, respectively.

#### **Bergamottin test solutions**

From the bergamottin stock solution (50 mM), 16  $\mu\text{L}$  or 64  $\mu\text{L}$  were accurately transferred to a 25 mL volumetric flask containing 5 mL of propylene glycol. The amount of 50 mg of talinolol was transferred quantitatively to the same flask and dissolved. The volume was completed with saline solution which pH has been previously adjusted to 3.0 and mixed thoroughly. The final concentrations of BG and talinolol were 32  $\mu\text{M}$  or 128  $\mu\text{M}$  and 2 mg/mL, respectively. The concentrations of BG in these solutions correspond to that found in the white grapefruit juice normal strength or frozen concentrate used in this study.

### Talinolol standard solutions

From the talinolol stock solutions II (10 µg/mL) and III (1000 ng/mL), nine different concentrations of standard solutions and three quality controls (QC) were prepared in rat plasma (Biomeda, Foster City, CA) according Table 4-1.

Table 4-1. Concentrations of the standard solutions used for the calibration curves and quality controls (QCs) of talinolol.

Standard	TAL stock solution II (µL)	TAL stock solution III (µL)	Rat plasma q.s.p. (mL)	Talinolol (ng/mL)
1		5	1	5
2		10	1	10
3		25	1	25
4		50	1	50
5	10		1	100
6	25		1	250
7	50		1	500
8	75		1	750
9	100		1	1000
QC1		25	1	25
QC2	45		1	450
QC3	85		1	850

### Animals and Experimental Protocol

All animals were housed and all experiments performed according to the policies and guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of Florida, Gainesville, USA. Male Sprague-Dawley rats weighing 350 to 400 g were used in this study. The animals were not fasted and had free access to food and water during the experiment except for the first 2 hours after drug administration.

An oral dose of talinolol (10mg/kg; 5ml/kg) was prepared in the absence or presence of verapamil HCl (4mg/kg) (positive control), or NAG (500 µM), NAR (813 and 3238 µM) and BG (32 and 128 µM) in propylene glycol:saline solution (20:80). The pH of saline solution was previously adjusted to 3.0 with 1mM HCl in order to simulate

the juice pH and guarantee the talinolol dissolution. Furthermore, a solution containing the same dose of talinolol and a fixed ratio of the flavonoid NAR to the furanocoumarin BG (3238:128  $\mu\text{M}$ ) was prepared based on the concentration of these compounds in frozen concentrate GFJ (Minute Maid®). Similarly, the effects orange juice (OJ) (Tropicana®), Ruby Red GFJ (Florida Natural®) normal strength, White GFJ (Minute Maid®) concentrated (GFJC) and normal strength (GFJNS) (prepared by diluting one part of the concentrated juice into 3 parts of saline) on the P-gp mediated transport of talinolol were evaluated. In this scenario, the doses of talinolol were prepared directly in the juice or in pure saline pH 3.0 and administered by gavage. Ruby Red GFJ was also given twice daily (5ml/kg) during one week prior talinolol administration. Concentrations of NAR, NAG, BG and DHBG in GFJNS and Ruby Red GFJ were 811 and 469.4 $\mu\text{M}$ , < 0.56 $\mu\text{M}$ , 31.8 and 12.2 $\mu\text{M}$ , 45 and 0.28 $\mu\text{M}$ , respectively, as previously reported (205). Concentrations of components present in the orange juice with potential to interact with P-gp were not estimated in this study.

After drug administration, blood samples (500  $\mu\text{L}$ ) were collected from the sublingual vein of each rat at 0, 45 min, 1, 2, 3, 4, 5, and 6 hours which were separated into two different days apart by one week of washout period (4 blood collections per day per animal were performed). The variability of the weight of each animal on both periods was not higher than 20%. Prior to blood collection, the rats were anaesthetized with halothane and after each sampling, approximately 1000  $\mu\text{L}$  of isotonic saline were replaced by i.p. injection in order to maintain the blood fluid.

The samples were centrifuged at 2800 g for 15 min at room temperature and the serum separated. The collected samples were stored at -70 °C until analysis.

### **Sample Preparation**

Talinolol was extracted from the samples by using 96-well solid-phase extraction (SPE) disks (Polaris®, Varian, Lake Forest, CA, USA). Briefly, the disks were equilibrated with 400 µL of methanol and pre-conditioned by adding the same volume of 1mM HCl. Samples were diluted (1:2) with the conditioning solvent but containing the internal standard (propranolol hydrochloride at 500 ng/mL) and vortexed for 30 sec before they were applied to the SPE disks at a final volume of 750 µL. The interferents were eluted with 400 µL of methanol:water (20:80). Finally, the analytes were recovered rising the SPE disks twice with 200 µL of methanol:water (90:10). The solvent was evaporated to dryness and the residue redissolved in 125 µL of mobile phase before analysis.

### **Sample Analysis**

A reversed phase high-performance liquid chromatograph (HPLC) method with ultraviolet detection was applied for the quantification of talinolol in plasma. Samples (75µL) were injected into a Shimadzu LC2010C HPLC system (Kyoto, Japan) equipped with a 100µL loop and Class VP 7.2 SP1 chromatographic software. The wavelength was set at 241 nm, the flow rate at 1.00 mL/min and the temperature at 40 °C. The analytical column was a Lichrospher 60RP-Select B column, 250 x 4.6 mm i.d., 5 µm, preceded by a Lichrospher 60RP-Select B guard column (Merck GaA, Darmstadt, Germany). Talinolol was eluted isocratically using a mobile phase consisted of 0.025 mol/L triethylammonium phosphate buffer (pH 3.0) and acetonitrile (77:23).

## Validation

The validation parameters of linearity, specificity, sensitivity, precision, accuracy, recovery, and stability were determined. For each concentration, standard solutions and QCs were injected into the HPLC in triplicate on three different days. Calibration curves were obtained by plotting the mean standard/internal standard peak-area ratios *versus* the standard concentrations in Microsoft Excel® spreadsheets. The linearity of the standard curves was determined by least-squares linear regression method and expressed in terms of coefficient of determination ( $r^2$ ) using the Linest function. A weighing factor of  $1/\text{Conc}^2$  was chosen to achieve the homogeneity of variance. Specificity was determined by comparing the chromatogram of talinolol with that obtained from blank plasma. The intra- and inter-day precision and accuracy of the quantification were measured by replicate analyses of three different concentrations (low, medium and high QC) on the same day and on alternate days. The precision was based on the calculation coefficient of variation (CV%), and the accuracy was expressed as percent of the measured concentration compared to the theoretical one. The calibration model was accepted if the residuals (% difference of the back-calculated concentration from the nominal concentration) of the weighted curve were within 20% at the lower limit of quantification (LLOQ) and within 15% at all calibration levels. Calibration curves were rejected if more than 1/3 of the QCs showed a deviation from the theoretical concentration equal or greater than 20%. The LLOQ was established as the lowest concentration used in the calibration curve.

The recovery was evaluated by adding known amounts of three standard solutions of talinolol to blank rat serum to get final concentrations of 10, 250 and 500 ng/mL. Samples were extracted under the same conditions mentioned previously. A standard

curve was prepared in mobile phase considering the same range of concentration of that used for the rat samples. The analytical results for extracted samples at three concentration levels were then back-calculated using the equation obtained from the weighted linear regression of the standard curve and compared with their nominal values that represent 100%.

Stability of talinolol in rat serum was tested at room temperature and at  $-70\text{ }^{\circ}\text{C}$ . Three QCs (low, medium, and high) were prepared by spiking the blank serum with fresh talinolol stock solution II ( $10\text{ }\mu\text{g/mL}$ ) or III ( $1000\text{ ng/mL}$ ). Analyses of room temperature samples started immediately following the preparation of the test solutions and after 6, 12 and 24 hours. Another set of QCs in serum was prepared, stored at  $-70\text{ }^{\circ}\text{C}$  and analyzed once a week for 3 weeks. Samples were prepared following the steps mentioned previously and analyzed by the validated HPLC method already described. Results were expressed in terms of % of drug remaining compared to the original concentration.

On-instrument stability was verified by injecting another set of QCs (low, medium, and high) at the end of each run. The concentrations in these samples were compared to the initial injections of QCs and expressed in terms of % error. Three replicates of each concentration level were prepared for each test performed.

### **Pharmacokinetic Analysis**

The pharmacokinetics of talinolol was evaluated by noncompartmental method with WinNonlin Professional Edition (version 3.1; Pharsight Corp, Mountain View, CA, USA). The maximum talinolol serum concentration ( $C_{\text{max}}$ ) and the time to reach  $C_{\text{max}}$  ( $t_{\text{max}}$ ) were obtained directly from the concentration-time profile curves. The  $\text{AUC}_{(0-6)}$ , corresponding to the area under the concentration-time profile curve from time 0 to the

time of the last measurable concentration ( $C_{6h}$ ), was determined by the linear trapezoidal method. The terminal elimination rate constant ( $\lambda_z$ ) was determined by log-linear regression of at least the last 3 points. The terminal half-life ( $t_{1/2}$ ) was calculated as  $\ln 2/\lambda_z$ . The AUC from time 0 to infinity ( $AUC_{(0-\infty)}$ ) was the sum of  $AUC_{(0-6)}$  and  $C_{6h}/\lambda_z$ .

### **Statistical Analysis**

Descriptive and comparative statistics were calculated with the use of GraphPad Prism v.4.0 (GraphPad Software Inc, San Diego, CA, USA). Pharmacokinetic data are presented as mean values ( $n = 8$ )  $\pm$  standard error of the mean (SEM). Comparison among the groups initially used one-way ANOVA followed by Tukey's multiple comparison tests as post-hoc analysis. A probability of less than 0.05 ( $p < 0.05$ ) was considered to be statistically significant. Grubb's test was applied to detect possible outliers ( $\alpha = 0.05$ ) and Kolmogorov-Smirnov test was used to determine if the obtained data were normally distributed.

## **Results**

### **Validation of Analytical Method to Measure Talinolol in Rat Serum**

The validation parameters of the analytical method used to assess the concentration of talinolol in rat serum are now presented.

#### **Linearity**

The range of validation (5 to 1000 ng/mL) was linear with coefficients of correlation more than 0.995 (Table 4-2).

Table 4-2. Regression parameters obtained from calibration curves of talinolol in plasma in three different days.

Regression parameters			
Day 1	Slope (1/Conc <sup>2</sup> )	Intercept (1/Conc <sup>2</sup> )	r <sup>2</sup>
Rep1	0.003708	0.011549	0.9981
Rep2	0.002551	0.002643	0.9976
Rep3	0.003665	0.006256	0.9977
Day 2	Slope (1/Conc <sup>2</sup> )	Intercept (1/Conc <sup>2</sup> )	r <sup>2</sup>
Rep1	0.002939	0.016177	0.9982
Rep2	0.002805	0.017454	0.9977
Rep3	0.002749	0.023680	0.995
Day 3	Slope (1/Conc <sup>2</sup> )	Intercept (1/Conc <sup>2</sup> )	r <sup>2</sup>
Rep1	0.003111	0.011471	0.9958
Rep2	0.003152	0.011726	0.9998
Rep3	0.003206	0.010758	0.9982

### Specificity

The method provided good specificity since the chromatograms of the blank solution did not show any peak with similar retention time as expected for talinolol (Figure 4.1)

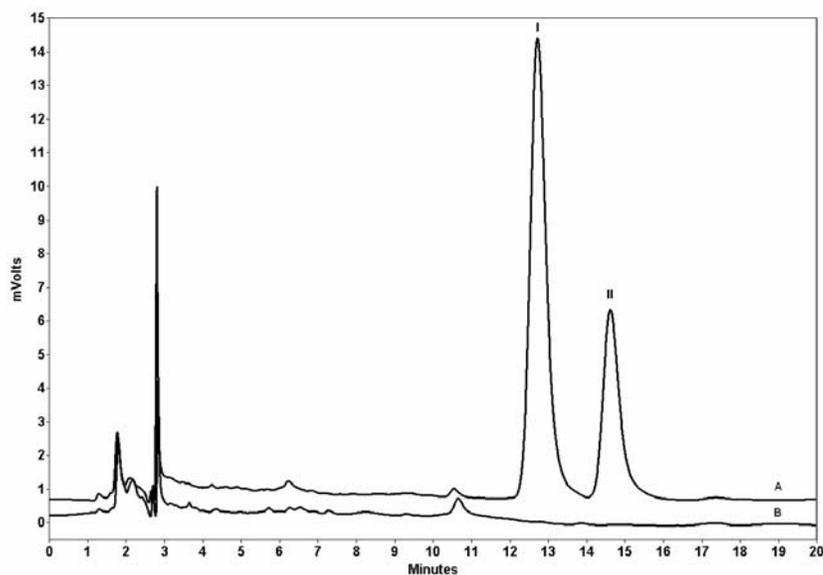


Figure 4-1. Comparison of chromatograms corresponding to the talinolol (I) and the internal standard propranolol hydrochloride (II) after extraction from rat serum (A) with that obtained after extraction of a blank sample (B).

### Sensitivity

The LLOQ of 5 ng/mL was accepted as the lowest concentration of the calibration curve since their concentrations could be determined with acceptable precision (%CV < 20), and accuracy (%error < 20).

### Precision, accuracy and recovery

The precisions intra- and inter-day for talinolol were satisfactory with CV values between 1.2 and 7.6%. Similarly, the accuracy of the assay obtained with quality control samples containing 25, 450, and 850 ng/mL talinolol was between 96.4 and 106.8% of the nominal values. The mean recovery assessed at three distinct levels of concentration (10, 250 and 500 ng/mL) ranged from 89.8 to 103.2% of the expected values. The results are summarized in Table 4-3.

Table 4-3. Intra-day (n = 3), inter-day (n = 9), and recovery (n = 3) assay parameters of talinolol in rat serum. Precision expressed as CV%, accuracy and recovery as % of the theoretical concentration.

Intra-day	CQ – 25 ng/mL			CQ – 400 ng/mL			CQ – 850 ng/mL		
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Precision	1.2	1.7	4.8	3.2	5.1	7.6	7.3	5.8	1.7
Accuracy	96.4	97.1	102.5	106.7	104.9	100.4	103.2	104.1	105.2
Inter-day	CQ – 25 ng/mL			CQ – 400 ng/mL			CQ – 850 ng/mL		
	Precision			Precision			Precision		
	3.9			5.0			5.7		
	Accuracy			Accuracy			Accuracy		
	98.4			104.2			104.7		
Recovery	CQ – 10 ng/mL			CQ – 250 ng/mL			CQ – 500 ng/mL		
	%			%			%		
	CV%			CV%			CV%		
	103.2			89.8			91.3		
	3.5			7.9			4.6		

## Stability

Results of stability tests in rat serum are presented in Figure 4-2. Talinolol was stable under the tested conditions. The mean % remaining in rat serum after 24 hours at room temperature was  $99.1 \pm 3.3$ ,  $97.8 \pm 1.4$ , and  $99.1 \pm 1.0$  for the low, medium and high concentrations, respectively. After 3 weeks at  $-70\text{ }^{\circ}\text{C}$  the mean % remaining was  $99.6 \pm 2.9$ ,  $101.3 \pm 3.7$ , and  $98.1 \pm 2.9$  for the low, medium and high concentrations, respectively.

No degradation of talinolol was observed in the on-instrument assay. The % error of all samples was  $< 5$ .

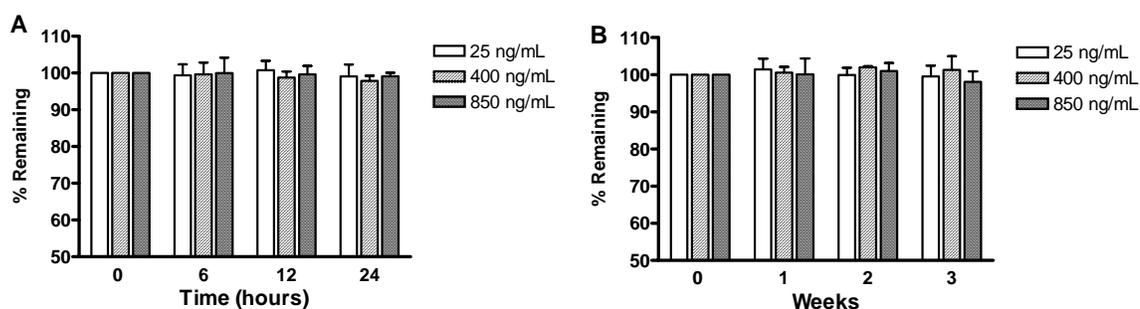


Figure 4-2. Stability of talinolol (low, medium, and high QCs) in rat serum within 24 hours at room temperature (RT) (A) and during 3 weeks at  $-70\text{ }^{\circ}\text{C}$  (B). Data represents means of three replicates and vertical bars the standard deviation of the means.

## Talinolol Pharmacokinetics

The concentration-time profiles and the pharmacokinetic parameters of talinolol are presented on Figure 4-3 and Table 4-3, respectively. According the Kolmogorov-Smirnov test the outcomes followed a normal distribution and any outlier was identified by Grubb's test. No difference was observed in the pharmacokinetic profiles when talinolol was given with or without propylene glycol (PPG). Verapamil (VER) (4 mg/kg), a known

inhibitor of P-gp, produced a slightly increase in  $C_{\max}$  and  $AUC_{(0-\infty)}$  of talinolol by 1.4- and 1.7-fold, correspondingly. Different from the other treatments, where no effect was observed, VER delayed the absorption ( $t_{\max}$ ) of talinolol from 2 to 2.8 hours. The elimination half-life ( $t_{1/2}$ ) remained practically unchanged among all treatments.

Orange juice, white GFJNS and GJFC had minor but distinct effects on talinolol pharmacokinetics. The GFNS provided a slight increase (1.3-fold) on  $C_{\max}$  and  $AUC_{(0-\infty)}$ , while GFJC or OJ reduced or had no effect on talinolol absorption, respectively.

Among the GFJ components, the furanocoumarin BG (128  $\mu\text{M}$ ) modified significantly the talinolol disposition, augmenting the  $C_{\max}$  and  $AUC_{(0-\infty)}$  by 2.4- and 1.8-fold, respectively. In general, the flavonoids NAG (500  $\mu\text{M}$ ) and NAR (813  $\mu\text{M}$  and 3238  $\mu\text{M}$ ) had a similar effect increasing the talinolol  $C_{\max}$  and  $AUC_{(0-\infty)}$  by 1.5- to 1.8-fold, respectively, compared to the control group. When the combination of BG (128  $\mu\text{M}$ ) and NAR (3238  $\mu\text{M}$ ) was tested, the  $C_{\max}$  and  $AUC_{(0-\infty)}$  of talinolol were reduced to around 63% of those obtained in the control group. This result was opposite of that when both compounds were given separately. Similarly, Ruby Red GFJ (acute) decreased the pharmacokinetic parameters of talinolol by half when contrasted with saline and by 2.7-fold when compared with the white GFJNS. However, a distinct result was observed after repeated ingestion of the same juice before talinolol administration. In this scenario, the  $C_{\max}$  of talinolol was increased by 1.6- and 3.6-fold and the  $AUC_{(0-\infty)}$  by 1.5- and 3-fold, when compared with the control or acute administration of Ruby Red GFJ, respectively.

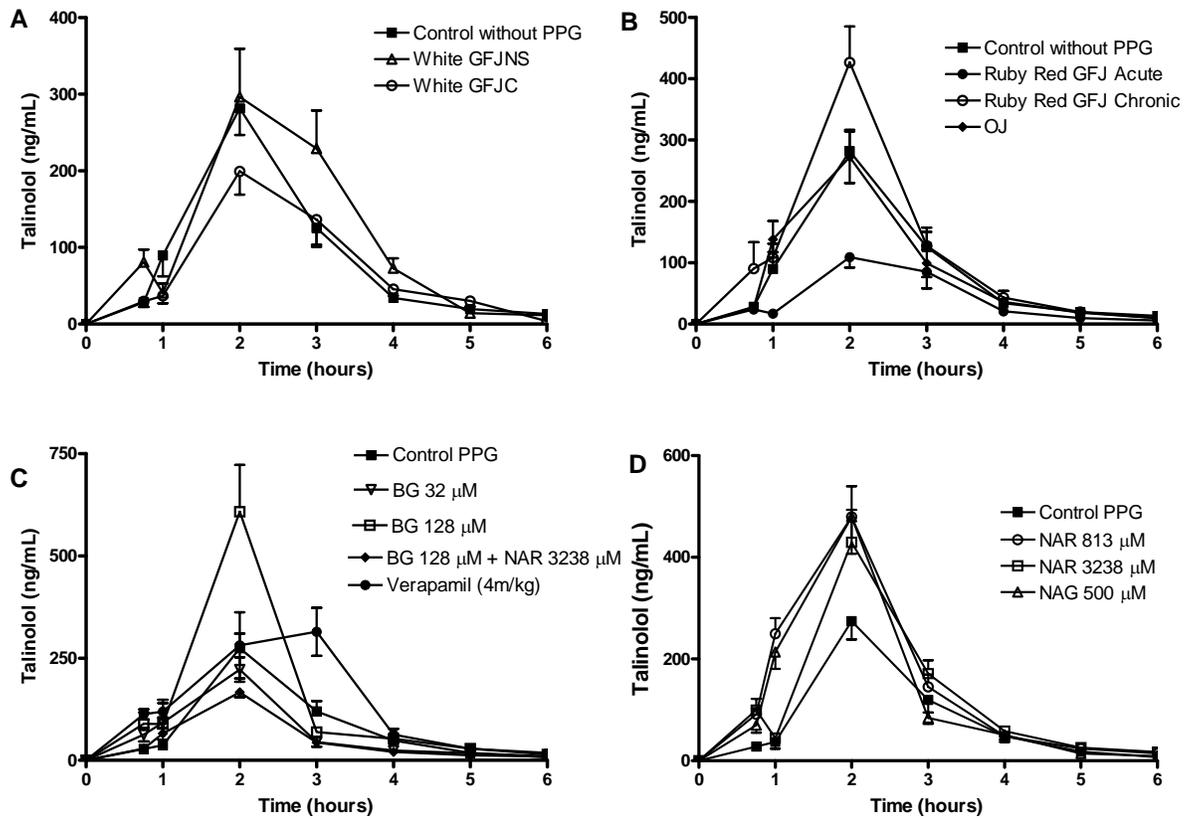


Figure 4-3. Mean serum concentration-time profiles ( $n = 8$ ) of talinolol in rats after a single dose oral dose (10 mg/kg) with: A - white GFJ normal strength (NS), or white GFJ concentrate (GFJC); B - Ruby Red GFJ acute or chronic treatment, or orange juice (OJ); C - bergamottin (BG) (32  $\mu\text{M}$  or 128  $\mu\text{M}$ ), BG (128  $\mu\text{M}$ ) plus naringin (NAR) (3238  $\mu\text{M}$ ), or verapamil; D - naringenin (NAG) 500  $\mu\text{M}$ , NAR (813  $\mu\text{M}$  or 3238  $\mu\text{M}$ ). Vertical bars represent the standard error of the means (SEM).

Table 4-4. Pharmacokinetics of talinolol in rats after a single dose oral dose (10 mg/kg) in saline (control) or with verapamil, white grapefruit juice concentrate (GFJC), white GFJ normal strength (NS), Ruby Red GFJ, naringenin (NAG) 500  $\mu$ M, naringin (NAR) 813 and 3238  $\mu$ M, bergamottin (BG) 32 and 128  $\mu$ M, and a solution containing NAR (3238  $\mu$ M) plus BG (128  $\mu$ M). Data are expressed as mean (n=8)  $\pm$  SEM.

Treatment	AUC(0-6) (ng.h/mL)	AUC(0- $\infty$ ) (ng.h/mL)	C <sub>max</sub> (ng/mL)	t <sub>max</sub> (h)	t <sub>1/2</sub> (h)
Control with PPG	501 (57)	512 (55)	274 (35)	2.0	0.8 (0.1)
Control without PPG	538 (36)	558 (36)	294 (29)	1.9 (0.2)	1.0 (0.1)
Verapamil (4mg/kg)	827 (133)	856 (132)	385 (84)	2.7 (0.2) b	1.0 (0.2)
White GFJC	451 (64)	486 (68)	206 (30)	2.1 (0.1)	1.0 (0.2)
WhiteGFJNS	684 (102) g	698 (102)g	337 (56)	2.1 (0.2)	0.7 (0.07)
Ruby Red GFJ (acute)	249 (43)	259 (43)	121 (21)	2.2 (0.2)	0.9 (0.2)
Ruby Red GFJ (chronic)*	735 (99) d	749 (98)d	442 (54) d	1.8 (0.2)	1.0 (0.1)
OJ	530 (70)	545 (68)	279 (42)	1.9 (0.1)	1.0 (0.2)
NAG (500 $\mu$ M)	801 (78)	812 (77)	493 (66)	1.7 (0.2)	0.8 (0.1)
NAR (813 $\mu$ M)	906 (72) a	937 (70) b	482 (59)	2.1 (0.1)	1.2 (0.3)
NAR (3238 $\mu$ M)	771 (81) e	797 (83) e	430 (64)	2.0	1.0 (0.1)
BG (32 $\mu$ M)	403 (42)	429 (45)	222 (30)	2.0	1.6 (0.2)
BG (128 $\mu$ M)	866 (108) f	896 (102) af	650 (91) cf	1.8 (0.2)	1.3 (0.3)
NAR (3238 $\mu$ M) + BG (128 $\mu$ M)	302 (17)	323 (17)	166 (12)	2.0	1.6 (0.1)

\* The juice was given twice daily during one week before drug administration. a p<0.05, b p<0.01, c p<0.001 when compared with the respective control; d p<0.01 when compared with Ruby Red GFJ (acute); e p<0.01 when compared with NAR (3238  $\mu$ M) + BG (128  $\mu$ M); f p<0.001 when compared with NAR (3238  $\mu$ M) + BG (128  $\mu$ M);. g p<0.01 when compared with Ruby Red GFJ (acute).

### Discussion

For the first time *in vivo* studies involving the effects of pure GFJ compounds and a comparison of different brands and strengths of GFJ on P-gp activity is reported. Most of reports in the literature have focused in the interaction between GFJ and its components with CYP3A4 or tested the effects of only one juice on the activity of P-gp (40, 134, 137, 214).

Similar as observed by other authors, the terminal half-lives of talinolol after oral administration in absence or presence of potential P-gp inhibitors was not affected, suggesting that any effect on talinolol pharmacokinetics parameters can be attributable to processes that occur in the gut rather than to modification of systemic clearance (134). Absence of significant effect of verapamil on talinolol disposition may be dose-related since this drug is also subjected to pre-systemic metabolism by intestinal CYP3A4 (216) and the augment on  $t_{\max}$  promoted by VER may be due to its inhibitory activity on the gastric emptying rate (217).

Orange juice has been described to be a potent inhibitor of P-gp *in vitro*, modifying the permeability of saquinavir (62). Additionally, Lilja and co-workers (154) have showed that OJ was able to reduce the  $C_{\max}$  and AUC of celiprolol in humans by 89% and 83%, respectively. According to the authors, modulation of the intestinal pH and the function of some transporters may be responsible for this interaction. However, we found that the effect of OJ on talinolol pharmacokinetics was negligible and that GFJNS promoted an increase on  $C_{\max}$  and  $AUC_{(0-\infty)}$  of talinolol, but in a lesser extent than previously reported (134). Differences in the concentrations of drug-interacting compounds in the juices, variability on P-gp expression in the intestine, presence of multiple binding sites on P-gp, and the interplay of distinct transporters present in the enterocytes may have contributed to this discrepancy of results. On the other hand, the decrease on  $C_{\max}$  and  $AUC_{(0-\infty)}$  values by GFJC compared to the normal strength may be accounted at least in part by the relative higher viscosity of the concentrated juice, which can form a physical barrier and impair the absorption of talinolol in the GI tract.

However, the effect observed with GFJC was not significantly different from the control group.

Different effects were obtained after co-administration of talinolol with Ruby Red GFJ compared with GJNS, suggesting that additional constituents seem to be involved in the interactions, affecting preferentially the intestinal uptake rather than P-gp (128). Recent studies also have demonstrated a remarkable decrease of the oral bioavailability of fexofenadine when co-administrated with GFJ. Because this drug is a substrate for both P-gp and OATP, these results indicate that the juice can inhibit preferentially the influx transporter rather than P-gp (79, 90).

Conversely, the continued intake of the juice significant increased the  $C_{\max}$  and  $AUC_{(0-\infty)}$  compared with the acute treatment. One hypothesis for this observation may be an over expression of some intestinal uptake transporter such as OATP. Contradictory results regarding the acute and repeated ingestion of GFJ for about one week on the disposition of P-gp substrates have been reported. One study using rat everted ileum sac suggested that co-treatment with GFJ inhibits while chronic administration activates P-gp (214). On the other hand, a recent human trial determined that the  $C_{\max}$  and AUC of talinolol were reduced in both cases by half indicating that this drug might also be a substrate for some influx transporters (137). Therefore, more information about which transporters are involved with the absorption of talinolol and which are subjected to interact with GFJ is required to better determine the mechanism and relevance of this interaction.

Regarding the flavonoids, it seems the maximum effect was reached with NAR (813  $\mu\text{M}$ ), which increased significantly the  $AUC_{(0-\infty)}$  from 512 to 937 ng . h/mL. This

NAR concentration is similar of that found in the GFJNS used in this study. Although the aglycone NAG is not present in the juice it may also contribute to the overall inhibitory effect of GFJ, since this flavonoid can be formed as a result of the metabolism of the glycoside NAR by intestinal microflora (211).

Among the components of GFJ tested, the furanocoumarin BG (128  $\mu\text{M}$ ) was the most potent inhibitor of P-gp activity. Studies *in vitro* have reported this compound as a potent inhibitor of P-gp activity ( $\text{IC}_{50} = 40 \mu\text{M}$ ) (61). Interestingly, the inhibitory effect of BG (128  $\mu\text{M}$ ) was not observed when it was combined with NAR (3238  $\mu\text{M}$ ). Although there is lack of information regarding the effect of multiple GFJ components on the activity of P-gp, this approach is an important tool to better understand such interactions allowing identify possible synergism or antagonism among GFJ components. The NAR to BG ratio of 25 employed in this study was the same found in the White GFJ but differed from that in the Ruby Red GFJ ( $\text{NAR}/\text{BG}=38$ ), suggesting that the relative concentrations of these components may also play an important role on the mechanism of grapefruit juice-drug interaction. Further investigations using different ratios, additional compounds, such as the dimers of furanocoumarins, and new combinations of GFJ components are necessary to clarify this issue.

### Conclusions

In this work we validated a sensitive, accurate and precise HPLC method with ultraviolet detection to quantify talinolol in rat serum. We demonstrated that outcomes obtained during this food-drug interaction study was dependent on the type of the juice, duration of juice intake and the relative concentration of the potential interacting compounds. Although some differences were observed on talinolol disposition among all

treatments, the overall effect of grapefruit juice or OJ was not statistically significant. Even in the cases when a statistical difference was detected the increase on the pharmacokinetic parameters of talinolol was around 2-fold compared with the control, which according to Bjornsson *et. al* (218) is considered a weak interaction and unlikely to be clinically relevant. However, this study only focused on the pharmacokinetic interaction rather than pharmacodynamics. Hence, physicians should be aware that ingestion of GFJ with other drugs substrates for P-gp with a narrow therapeutic index may not be free of side effects, especially considering that most of the drugs available are also metabolized by CYP3A4. Additionally patients should always report to their physician or pharmacist when they decide to take any drug with GFJ or whenever they experience any abnormality after concomitant drug intake with GJF.

## CHAPTER 5 CONCLUSIONS

Fresh fruits, juices and vegetables are considered part of a healthy diet. Their health benefits have been demonstrated by many studies and are mainly due the presence of polyphenolic compounds which act as antioxidant preventing the formation of oxygen radical species, lipid oxidation, or decreasing risk of cardiovascular disease and certain types of cancer. However, concomitant administration of drug and food is not free of risk since it creates an opportunity for interactions that may change the drug disposition resulting in undesired effects. In fact, it is well recognized that grapefruit juice (GFJ) can increase the oral bioavailability of distinct classes of drug. Although the main mechanism of GFJ-drug interaction is currently known to involve the inhibition of the intestinal drug metabolizing enzyme CYP3A4, other drug-absorption pathways, such as the intestinal P-glycoprotein (P-gp), have also been suggested to be affected by GFJ. However, the GFJ constituents and the clinical relevance of the interaction between GFJ and P-gp is still a controversial issue and may depend on the intrinsic bioavailability of the drug, on the expression of the intestinal CYP3A4 and P-gp among subjects, and on the amount and type of GFJ ingested together with the drug. Therefore, further investigations are required to clarify this issue.

The study presented here demonstrated a high variability regarding the concentration of the potential drug-interacting compounds present in different brands and lots of GFJ commercialized in our community, which may be responsible for the inconsistent results obtained in GFJ-drug interaction trials. The flavonoid naringin was,

by far, the most abundant compound found in all lots and brands of GFJ analyzed followed by the furanocoumarins bergamottin and 6',7'-dihydroxybergamottin.

On the basis of the demonstrated variability and lack of information regarding the profile of flavonoids and furanocoumarins on the juices used for human trials, a direct comparison between different studies is impaired. Therefore, to minimize prevalent variability in GFJ-drug interaction trials, it is important to correlate measured endpoints to the concentrations of those grapefruit components that are considered to be relevant for the interaction with drugs. In future studies investigating grapefruit-drug interactions, concentrations of compounds with a suspected interaction should be considered. When possible, juices of very similar composition should be used for studies designed for direct comparison. In addition, for animal studies the concentrations of critical compounds may be adjusted by supplementation to normalize the administered juices.

Our *in vitro* studies with Caco-2 cells as an intestinal barrier model and talinolol as a marker compound for P-gp transport suggest that among the GFJ constituents tested the furanocoumarins are much more potent P-gp inhibitors than the flavonoids. These compounds are present in the juice at high enough concentrations to significantly modify the activity of P-gp. Additionally, GFJ strongly inhibited the secretion of talinolol across the Caco-2 cells. In contrast, combination of both a flavonoid (naringin) and a furanocoumarin (6',7'-dihydroxybergamottin) increased the secretion of talinolol, suggesting an activation of P-gp activity or inhibition of uptake transporters. It seems that the relative concentrations of the interacting compounds may also play an important role on the outcome suggesting that the overall modulation of GFJ on P-gp activity is the result of a more complex interaction than a simple additive effect. Therefore, further

studies testing simultaneously of two or more grapefruit juice constituents are required to elucidate this question.

Regarding the *in vivo* studies, co-administration of talinolol (10mg/kg) with bergamottin (128  $\mu$ M) or naringin (813  $\mu$ M) increased significantly the AUC of the drug. However, the effects of GFJ and OJ were generally minor. Similar to the *in vitro* study, combination of one flavonoid (naringin) and one furanocoumarin (bergamottin) promoted an opposite effect suggesting an increase of the intestinal secretion of talinolol due activation of P-gp, or a decrease of its absorption due inhibition of some influx transporter.

Albeit Caco-2 is an established model for drug absorption, differences between this *in vitro* approach and *in vivo* studies are common and may be expected since the *in vitro* model does not account for the metabolism of grapefruit juice and its components, gastric emptying time, the presence of mucus layer, and other physiological variables.

In summary, our pharmacokinetic results suggest that the interaction of GFJ or OJ with intestinal P-gp is unlikely to be of clinical relevance. Studies combining the pharmacokinetic and pharmacodynamic profiles of drugs co-administered with GFJ will help to a better estimation of the magnitude and the clinical relevance of this interaction.

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### BIOGRAPHICAL SKETCH

Whocely Victor de Castro was born in July 10<sup>th</sup>, 1970, in Ipatinga, Minas Gerais, Brazil. He obtained his bachelor's degree in Pharmacy in 1992 and his Master of Sciences degree in Pharmaceutical Sciences in 2000 from Federal University of Minas Gerais. While doing his Master's, he became a substitute professor at the College of Pharmacy of the Federal University of Minas Gerais, teaching Biological Methods of Quality Control of Pharmaceutical Products and Cosmetics. During 6 years he was the Quality Control manager of the Ezequiel Dias Foundation, a governmental pharmaceutical company, in Belo Horizonte, Brazil. He started his PhD program in August 2002 in the Department of Pharmaceutics of the University of Florida under supervision of Dr. Hartmut Derendorf and Dr. Veronika Butterweck. Whocely received his PhD in pharmaceutics in December 2006.